VICTORIA UNIVERSITY OF TECHNOLOGY

The Isolation, Modification and Evaluation of Field Pea Proteins and Their Applications in Foods

A Thesis Submitted

for the Degree of

Doctor of Philosophy

by

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Declaration

I hereby declare that all work carried out in this project was performed while I was enrolled as a Ph.D. student in the School of Life Sciences and Technology, Victoria University of Technology, Werribee Campus. To the best of my knowledge, this work has not been submitted in whole or part for any other degree or diploma in any University and no material contained in this thesis has been previously written or published by another person, except where due reference is made in the text.

Shaojun Tian

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Publications

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2. Tian, S.J., Small, D.M., Kyle, W.S.A. and Popovik, N. (1996). Isolation, fractionation and characterisation of field pea proteins. Proceedings of the *46th Australian Cereal Chemistry Conference* (Sydney), Pp.298-301. C.W.Wrigley (Ed.), Royal Aust. Chem. Instit., Melbourne, Australia.

3. Tian, S.J., Kyle, W.S.A. and Small, D.M. (1997). The uses of protein isolates from field peas in food processing. Proceedings of the *47th Australian Cereal Chemistry Conference* (Perth), Pp.344-349. A.W.Tarr, A.S.Ross and C.W.Wrigley (Eds.), Royal Aust. Chem. Instit., Melbourne, Australia.

4. Tian, S.J., Kyle, W.S.A. and Small, D.M. (1997). The electrophoretic and solubility characteristics of modified field pea protein isolates. Handbook of the *International Food Legume Research Conference III* (Adelaide), P.149.

5. Tian, S.J., Kyle, W.S.A. and Small, D.M. (1997). The functional properties of field pea protein isolates before and after modification. Handbook of the *International Food Legume Research Conference III* (Adelaide), P.150.

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Abstract

Field pea (*Pisum sativum* L.) is a well established crop around the world and over the last ten years, the production of this grain has been increasing in Australia. As with other grain legumes, field pea proteins contain high levels of lysine which may be important in balancing the deficiencies of this essential amino acid in cereal-based diets. In recent years, interest in plant proteins for feed and food led to the evaluation of field peas as an economical and nutritional source of proteins. Although a few research work has been done on the production of field pea protein, starch and fiber as food ingredients in Europe and Canada, none of these attempts have resulted in a commercial scale applications of pea proteins in the food market. Currently field peas are still mainly used for animal feeding. The primary limitation to the development of pea proteins as food ingredients is the lack of information on the technology and characterisation of the resulting products, in comparison with well-established soy proteins. More importantly, data on the assessment of the functional properties of field pea proteins and modification of the protein structure for improving the functional behaviours are lacking. Accordingly, the major purpose of this project has been focused on the isolation and characterisation of field pea proteins, both on a laboratory scale and on a pilot scale, as well as the functional properties evaluation of the resultant products for appropriate food applications. The feasibility of chemical modification in order to enhance the functional properties of the proteins is also included.

Field pea protein isolates were extracted with different solutions including acidic, neutral, alkaline and salt solutions. Alkaline extraction and salt (0.5M NaCl) extraction provide better potential in the large scale production of pea protein isolates in terms of higher recovery and better physico-chemical properties. Osborne fractions (albumin, globulin, prolamin and glutelin) were also separated by using different buffer solutions and solvents. The recovery of these fractions showed some variation depending on the extraction conditions used. Albumin fraction represented a larger proportion of the soluble proteins than previously reported and accounted the major composition of total proteins along with globulin fraction. The major subunit of albumins had a molecular

weight of 27-28 kDa and this result was confirmed by using column chromatograph and two-dimensional electrophoresis, as well as preparative electrophoresis. Pea protein isolate, globulin and albumin fractions demonstrated different solubility characteristics and showed some variations in scanning electron micrograph patterns. This indicated that different protein fractions may find a variety of food applications depending on the different functional properties required.

With respect to the isolation of field proteins on a pilot scale, the use of salt solution with the combination of ultrafiltration and diafiltration processes was studied in addition to the traditional wet method of alkaline extraction and iso-electric precipitation. Both spray-drying and freeze-drying were employed to dry the products. The results showed that the protein isolates produced by different extraction procedures and drying methods exhibited little variation in electrophoretic patterns and solubility characteristics. However, freeze drying is time-consuming and the resultant product is of a dark colour and non-uniform particle size. Compared with alkaline extracted proteins, salt extracted protein isolate demonstrated better physico-chemical properties including colour, particle size, protein-water interactions and foaming properties.

Functional properties of the proteins were affected by their intrinsic structural and surface properties including hydrophobicity, type of the proteins, as well as by many extrinsic factors including the method of isolation and environmental conditions of the measurement. Field pea proteins extracted in the pilot scale showed good solubility, emulsifying and foaming behaviours but the other functional properties including oil absorption, viscosity and gelation had lower potential without further modifications. The change of pH, salt (NaCl) addition and temperature were shown to have a great influence on the functional properties of the proteins. The information obtained will be useful for the prediction of functional behaviours of the proteins in complicated food systems. The application of the proteins in model food systems indicated that field pea proteins are a good substitute for eggs in sponge cakes and mayonnaise.

In order to further improve the functional properties of the proteins, acetic anhydride, succinic anhydride and phosphorus oxychloride were used to modify field pea isolate

extracted in the pilot scale. The results showed that solubility, viscosity, emulsifying and foaming properties of the proteins were significantly enhanced by succinylation and acetylation. However, the level of enhancement was related to chemical used and the extent of modification. Phosphorus oxychloride showed little potential in modifying field pea proteins since it did not result in any significant improvement in the functional properties of the proteins. The change of functional behaviour of the modified proteins under different conditions including variations in pH, temperature and salt addition was different from that of the native proteins. This may have been due to the different groups introduced and some structural changes as indicated in gel electrophoresis patterns. *In vitro* digestibility analysis indicated that the nutritional value of the proteins were not reduced by modification and the amino acid profiles remained the similar before and after modifications. This information indicated that acetylated and succinylated field pea proteins provide good potential as a protein ingredient for a variety of food applications due to the enhanced functional characteristics.

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List of Abbreviations (arranged in alphabetical order)

a**(+)	redness
a**(-)	greenness
AA	acetic anhydride
AACC	American Association of Cereal Chemists
Afisc	Australia Food Industry Science Centre
API	field pea protein isolate extracted with alkaline solution on a pilot scale
APS	ammonium persulphate
***b** (+)**	yellowness
***b** (-)**	blueness
BHA	butylated hydroxyanisole
BSA	bovine serum albumin
cP	centipoise
cm	centimetre
2-D Electrophoresis	two-dimensional electrophoresis
DEAE	diethylaminoethyl
DSC	differential scanning calorimetry
DTT	dithiothreitol
EDTA-Na_2	ethylenediaminetetra-acetic acid- disodium salt
EMC	equilibrium moisture content
ERH	equilibrium relative humidity
FMOC	9-fluorenylmethyl chloroformate
g	gram
g	acceleration due to gravity
µg	microgram
ΔH	enthalpy of denaturation
HPLC	high-performance liquid chromatography
hr	hour
IPI-2, IPI-7, IPI-9	isoelectric protein isolate extracted at pH 2, 7, 9, respectively
J	Joule
kDa	kiloDalton

kg	kilogram
kN	kiloNewton
L	litre
µL	microlitre
L*	whiteness/brightness
M	moles per litre
mA	milliampères
2-ME	2-mecaptoethanol
mg	milligram
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mmHg	gas pressure expressed in mm of mercury
MPI	micelle protein isolate
MW	molecular weight
N	Newton
nm	nanometre
NSI	nitrogen solubility index
pI	protein iso-electric point
PO	phosphorus oxychloride ($POCl_3$)
rpm	revolutions per minute
SS	succinic anhydride
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
sec	second
SEM	scanning electron microscopy
So	surface hydrophobicity
SPI	field pea protein isolate extracted with salt solution on a pilot scale
STMP	sodium trimetaphosphate
T_1, T_2	transition peak temperatures for DSC peaks 1 and 2, respectively
Td	denaturation temperature

TEMED	N,N,N'N'-Tetra-methylethylenediamine
Tris	Tris- (hydroxymethyl) aminomethane
TNBS	trinitrobenzene sulphonic acid
ULA	ultra low adaptor
UV	ultraviolet
Ve	elution volume
Vo	void volume
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

General Introduction

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1.1. The Importance of Food Proteins

Proteins are a vital part of living muscle tissue and are one of the most important nutrients in the human diet. They have been called the building blocks of nutrition because they are broken down by digestive enzymes to provide amino acids for the building and repair of tissues (Ory, 1985). Hence the primary nutritional importance of protein is as a source of amino acids. There are twenty-two amino acids which are generally found as constituents of most proteins. Of these, the human body can synthesise fourteen amino acids, provided that adequate levels of the necessary precursors are available in the diet. However, eight amino acids cannot be synthesised: iso-leucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. Therefore these eight are considered as essential amino acids and must be supplied in the diet to maintain growth and health. Hence ideal food proteins would produce food products which supply and maintain a good balance of amino acids in forms that are also readily digestible and easily absorbed into the body.

In addition to this nutritional requirement, proteins as food components have other important functions, namely, those relating to the physico-chemical properties essential for maintaining good product quality (Nakai, 1996). Good taste, aroma, texture and colour are very important in determining the usefulness of particular food proteins in food systems. Moreover, different food applications require different characteristics, e.g. in a beverage, the protein should be soluble; in a comminuted meat, it should absorb moisture and form a gel upon heating; in a whipped topping, it should have the property of producing a thermostable foam. Traditionally, proteins from particular raw materials have been used to give the different functional properties required in different applications. Accordingly, where foaming properties are important to the texture of a

food, egg white proteins have commonly been used; whereas if emulsification is required, proteins from egg yolk have been utilised.

1.2. Sources of Food Proteins

1.2.1. Animal Proteins

Milk, eggs and muscle (including fish) are the three main sources of proteins from animals. The main edible portions of animal tissues consist substantially of muscle which contains proteins of high nutritional value. Sufficient amounts of the essential amino acids, lysine, methionine and tryptophan are consumed from meats and fish proteins. Different protein components are found in the various types of meat and these are affected in different ways during processing. As a result, specific products have a characteristic texture and "bite".

Egg and milk proteins also represent a very valuable source of proteins due to the high content of essential amino acids (Robinson, 1987). They have often been considered for nutritional purposes as reference proteins. In recent years, egg and milk proteins have not only been consumed as traditional types of food, but have also been subjected to fractionation and modification, so that a variety of egg and dairy fractions have become available commercially (Pomeranz, 1991a). For example, separated egg white and yolk are now utilised in a variety of foods including baked goods, noodles, ice cream and salad dressing. These applications utilise the different physico-chemical properties of the proteins, including colour and aroma, thermal coagulation, foaming ability and emulsifying properties. Dairy materials have also been converted to milk fat, casein, protein hydrolysates, lactose and whey fractions. These developments in fractionation provide ingredients having a range of functional characteristics useful in different food applications.

1.2.2. Single-Cell Proteins

Single cell protein refers to the dried biomass produced from microorganisms grown in culture systems. A wide variety of sources for nitrogen and energy can be used. Among such potential novel sources of protein are bacteria, yeasts and algae. The potential uses of single cell protein are as a food or as a food ingredient for humans and animals, but commercial production of single cell protein is currently limited. This is due to the high capital and operating costs, as well as to the high cost of nutritional and toxicological assessments. Nevertheless, single cell proteins have a number of advantages over plant and animal protein sources, e.g. the short generation time and high protein content obtained. In addition, the raw materials are readily available and include wastes from other industries (Sadler, 1994).

1.2.3. Plant Proteins

Plant sources of proteins include those derived from cereal grains, oilseeds, legumes and leaf tissues. On a worldwide basis, especially in developing countries, approximately 88% of the energy requirements and 90% of the protein intake in human diets come from plant sources (Salunkhe and Deshpande, 1991).

Cereals have been important crops for thousand of years. The chemical composition of cereals is characterised by a high content of starch, a moderate protein level (8-14%), and low lipid content. However, most wheat, rice, rye, sorghum and millet are used for food and these contribute an important source of protein in diets all around the world. On the other hand, maize, barley and triticale are commonly used in animal feed, especially in developed countries.

Oilseeds, such as peanut, sunflower, canola and cottonseed, owing to their increasing use as a source of vegetable oil also offer a viable source of protein. Limitations to the uses of these proteins may result from protein denaturation during the defatting

procedures, as well as the presence of antinutritional components including glucosinolates, phenolics, phytate and trypsin inhibitor.

Leaf protein could have great potential in the long term. Although the levels of protein in leaves is low (4-5% of dry mass), large quantities of protein could be made available from prolific plants grown in tropical regions (Douillard and de Mathan, 1994). Furthermore, the process for the preparation of concentrates by crushing of green leaves is relatively simple and the energy requirements are small.

A number of legume seeds, such as soybean, lupin, peas and beans have been evaluated as high protein crops over many years. Most legume proteins contain relatively high levels of lysine, which is the limiting amino acid in cereal grains; whereas the amount of methionine, cysteine and tryptophan are relatively low. Supplementation of cereal products with legume flours can assist in balancing the nutritional intake of essential amino acids.

1.3. Development of Legume Proteins

The terms legume grains and pulses are both widely used and are used interchangeably in this thesis to refer to the seeds from crops belonging to the botanical family Leguminosae. Interest in protein sources from legume grains, or pulses has been growing steadily during the past two decades because of the expanding world population, varying levels of income, religious beliefs and health concerns. In many parts of the world, legumes are a major contributor to both energy and protein intakes. The advantages of using legumes are many: they can be stored for long periods, even under adverse environmental conditions; they are easily transported; and they require minimum equipment for production and processing.

Soybeans have been for many decades the only leguminous crop on which intensive research has been undertaken. Their high protein and oil content make them a valuable commodity, both from an economic and nutritional standpoint. The major uses of

soybeans have been not only in traditional beancurd "Tofu" or "Miso" but also in processed products, either as oil or as protein concentrates and isolates added to a variety of foods. Concerning other legume grains, such as peas, beans and lupins, relatively little has been done compared with soybean to improve their production, yield and quality, and especially to develop industrial processes for human food applications.

Field peas, both yellow and green, are grown in Canada, Northwest United States, Northern Europe and also Australia. Field pea production in Australia has been increasing steadily in recent years and it is currently the second major grain legume after lupins. However, currently the field peas are mainly used for stockfeed or for export. An example of a field pea crop grown in Australia and the dry seeds (yellow and green) of field peas are shown in Fig. 1.1 and 1.2, respectively.

It is now recognised that the value of the field pea crop can be increased by processing and greater use for human food applications. The pea proteins can offer immense possibilities in the development of new classes of formulated foods; they have potential to be processed into meat analogues or high protein snack foods, and due to their functional properties, they can also be used as ingredients in various other preparations. Nevertheless, the field pea proteins have not been thoroughly studied, especially the extraction, fractionation and modification for improving the functional properties. Accordingly, the aims of this project have been proposed as following.

1.4. Objectives of the Current Project

1.4.1. General Aims of the Project

The general aim of the project has been to investigate the physico-chemical characteristics of the main protein fractions of field pea seed, to study the modification of the proteins and also to assess the potential of the large scale isolation of the proteins in providing novel products for human food applications.

Figure 1.1 Field pea (*Pisum sativum* L.) crop grown in South Australia.

Figure 1.2 Dry seeds from field peas. Left: green peas; right: dehulled yellow peas.

1.4.2. Experimental Aims of the Project

The specific objectives have been to:

1. Investigate the protein isolation and fractionation techniques and to characterise the major proteins extracted;
2. Assess the production of protein isolates on a pilot scale;
3. Modify these proteins by chemical means and study the structural changes during modification procedures;
4. Evaluate the functional properties of the pea proteins before and after modification; and
5. Investigate the behaviour of these proteins in model food systems.

CHAPTER 2

Literature Review- Field Pea and Other Pulse Proteins

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Field pea (*Pisum sativum* L.) is one of the most important cool season food legumes in dry-land regions around the world. The total production of field peas was 11.7 million metric tonnes in 1997 and this accounted for 21% of the production of major food legumes (FAO, 1998) (Table 2.1). In Australia, field pea is a major grain legume second only to lupin and the production of field peas has been increasing during recent decades. The annual production reached a peak in 1993 for Australia and the World with production showing some declines in subsequent years. This has been partly due to less favourable climate conditions. There remains considerable potential for increased production if new applications are developed for this crop.

Table 2.1 Production of Field Pea and all Pulse Grains in Australia and the World (all values expressed in tonnes)

Year	Field Pea		All Pulses	
	Australia	World	Australia	World
1988	517,334	15,129,270	1,622,327	54,908,460
1989	398,416	15,148,030	1,373,792	54,940,470
1990	318,068	16,613,830	1,353,670	58,235,900
1991	472,571	12,239,200	1,862,182	54,085,420
1992	456,311	13,373,070	2,009,701	50,874,840
1993	558,453	14,791,740	2,443,475	55,025,840
1994	240,407	14,309,080	1,262,800	56,147,840
1995	529,919	11,292,460	2,448,628	54,586,090
1996	466,000	10,698,720	2,560,000	54,188,110
1997	303,000	11,651,920	2,120,000	55,009,260
1998*	340,000	11,904,940	2,269,000	55,249,930

Source: all data obtained from FAO (1998), *data for 1998 are provisional estimates

In most countries, the primary interest in peas has traditionally been in the immature, green vegetable, which is either consumed fresh, canned or frozen, rather than in the mature dry seed. Thus, much of the genetic research and breeding efforts have been directed towards improving appearance, yield, disease resistance, canning and freezing quality (Klein and Raidl, 1985). In the past decades, advances in processing technology have made it possible to produce protein concentrates or isolates from dry peas and more importantly, field peas are being evaluated as a high protein crop for food and feed in some areas where soybeans cannot be grown (Bramsnaes and Olsen, 1979). This chapter provides a review of current knowledge on the chemical composition and physico-chemical properties of field pea proteins, as well as processing and utilisation of the proteins from dry peas.

2.1. Chemistry of Field Pea Proteins

2.1.1. Composition and Protein Content

The major constituents of field peas are starch and protein. The composition of the field pea depends on the cultivar that is being processed (Ali-Khan and Youngs, 1973). Variations exist among cultivars (e.g., Trapper, Century) in protein, fat, carbohydrate (crude fiber and starch), and ash contents, as shown in Table 2.2.

Table 2.2 Proximate Composition of Peas (g per 100g)

	Protein	**Fat**	**Carbohydrate**		**Ash**
Dry seeds	24.1	1.3	60.3		2.6
			Starch	Fibre	
cv Trapper	14.5	4.1	59.8	4.3	3.3
	18.3	3.7	56.7	3.7	3.0
	24.2	3.3	53.8	3.5	2.7
	28.5	3.0	49.7	3.1	2.8
cv Century	23.3	1.2	54.0	7.6	2.5

Source: Klein and Raidl, 1985

The reported protein contents of field peas range from 13.3 to 39.7%. The great variability of the protein content and composition has been highlighted by many authors, both between genotypes and also due to environmental effects within genotypes (Gueguen and Barbot, 1988; Schroeder, 1982). Environmental factors which affect protein content of field pea include nitrogen fertiliser, maturation, soil P and K content and temperature (Klein and Raidl, 1985). Selection of high protein content and high yielding genotypes are primary goals of plant breeders (Slinkard, 1980). Despite the widely held belief that yield and protein contents of grains are negatively correlated, various investigations have shown this not to be the case for peas (Cousin, 1983; Matthews and Arthur, 1985). Consequently, it is possible by breeding to increase the protein content without adversely affecting yield (Gueguen and Cerletti, 1994).

2.1.2. Major Protein Fractions of Field Peas

Most laboratory procedures developed for characterising protein fractions are derived from that of Osborne (Osborne, 1924), which is based upon the solubility characteristics of these proteins. The albumin fraction is defined as the water-soluble fraction whereas the globulins are extracted in salt solutions. The albumin and globulin fractions constitute the major protein classes in legume seeds (Table 2.3).

Table 2.3 Protein Fractions in Peas and Soybeans (g per 100g)

Protein fraction	**Albumins**	**Globulins**	**Glutelins**
Extracting solution	Water	Salt solution	Dilute acid or base
Peas	21	66	12
Soybean	10	90	0

Source: Klein and Raidl, 1985

Albumins

The water-soluble proteins of field peas, the albumin fraction, have not been studied as comprehensively as the globulins (Owusu-Ansah and McCurdy, 1991). This fraction generally accounts for 20-35% of the total extractable cotyledon proteins (Schroeder, 1984), although Gueguen and Barbot (1988) reported a variation of between 12% and 38% for 34 pea cultivars. Albumins include most of the enzymatic and metabolic proteins which represent the functional proteins of the seed. Examples are the glycosidases and as well as the proteases, which are involved in protein degradation at germination. Some others may play an important role in plant defence, such as trypsin inhibitors and lectins (Gueguen and Cerletti, 1994). Schroeder (1984) has identified two major albumins, with molecular weights of 8 kDa and 22 kDa, which make up 34% of the total albumin fraction. One of these was determined to be a storage protein.

Globulins

The main storage proteins in field peas are two globulins, vicilin and legumin, which are similar to the 7S and 11S fractions of soy protein. These account for 65-80% of proteins present in peas. Extensive studies have shown that legumin and vicilin sedimented at 12.64S and 8.10S, had molecular weights of 331 kDa and 186 kDa and isoelectric points of 4.8 and 5.5, respectively (Gueguen, 1991). A third globulin, convicilin, having subunits of molecular weight of 71 kDa, is also present in small quantities (Gueguen *et al.*, 1984; Croy *et al.*, 1980). During seed development, vicilin synthesis commences first, but the rate and extent of legumin biosynthesis is often higher than vicilin, so legumin is frequently present in greater quantity in the mature seed (Mossé and Pernollet, 1983).

Legumin is a larger molecule than vicilin, and appears to have a more compact structure than the 11S soybean fraction (Klein and Raidl, 1985). The two pea globulins differ in their properties. Compared to vicilin, legumin is less soluble in salt solutions, coagulates less easily at 95°C, and has larger amounts of nitrogen and sulphur (Mossé and Pernollet, 1983). Legumin maintains its native structure only in the pH range 7 to 9 and is largely dissociated at extreme pH values. Vicilin is soluble at pH 4.8, while

legumin is not; and vicilin contains significant amounts of covalently linked sugar, which is different from legumin and convicilin (Casey *et al.*, 1982).

2.1.3. Amino Acid Composition of Field Peas

The amino acid composition of peas (whole seeds) is characterised by a high lysine content and an especially low methionine, cystine and tryptophan content (Holt and Sosulski, 1979; Leterme *et al.*, 1990). Globulins are characterised by a high level of arginine and, like most of the seed storage proteins, by a large amount of aspartic and glutamic acids (Table 2.4).

Table 2.4 Amino Acid Composition of Pea Proteins (g/100g protein)

	Flour	Albumin	Globulin
ASP	12.25	11.90	12.99
THR	3.65	5.66	3.34
SER	4.79	5.03	5.30
GLU	17.41	14.95	18.66
PRO	3.91	4.46	4.36
GLY	4.29	5.97	3.89
ALA	4.06	5.85	3.97
CYS	1.39	3.15	0.80
VAL	4.69	4.41	4.73
MET	0.99	1.34	0.70
ILE	4.23	3.86	4.59
LEU	7.20	4.87	8.23
TYR	3.19	4.71	3.37
PHE	4.75	4.52	5.40
TRP	0.95	1.47	0.67
LYS	6.92	9.34	6.41
HIST	2.30	2.63	2.55
ARG	8.28	5.67	8.00

Source: Gueguen, 1991

Albumin has a higher content of sulphur amino acids and lysine. In fact, the nutritional quality of pea protein has been positively correlated with the albumin fraction content of the peas. Breeding to increase the levels of the albumin fraction has been suggested as a method to increase pea protein nutritional value (Schroeder, 1982).

2.1.4. Anti-Nutrients and Undesirable Components in Field Peas

Biological effects of legume proteins on human health have attracted wide attention because of the presence of various anti-nutrients including trypsin inhibitors, lectins and some other components, e.g. lipoxygenase. Even though adequate cooking and/or processing inactivates these materials and can improve the quality of plant food (Ory, 1985), effects due to anti-nutrients has been one of the major limitations on the use of legume proteins in food applications. However, in comparison to other legumes, peas have a relatively low content of anti-nutritive substances (Gwiazda *et al.*, 1979).

Lectins, earlier characterised as proteins able to agglutinate blood cells, were recently defined as carbohydrate-binding. Their presence in vegetables and particularly in the seeds of leguminous plants has been known for decades (Gueguen and Cerletti, 1994). The lectin content is generally determined by the agglutination test and the haemagglutinating activities established with rabbit trypsinised red blood cells were considerably lower for pea, as compared with crude soybean (Table 2.5). It can be seen that the level of haemagglutinating activities from peas is in the same range as toasted soybean meal. Some lectins have been associated with growth depression in experimental animals. However, isolated pea lectin does not produce any toxic effects when fed to rats at a 1% level in the diet (Liener, 1983).

A large number of legumes contain proteins which have the ability to inhibit the proteolytic activity of certain digestive enzymes, such as trypsin. Dry peas have been shown to contain trypsin inhibitor, 90% of which was found in the cotyledon and 10% in the hull, in direct proportion with the weight distribution of these fractions in the

Table 2.5 Trypsin Inhibitor and Haemagglutinating Activities of Some Legume Flours

Flour source	Trypsin inhibitor activity (units/mg dry matter)	Haemagglutinating activity (units/mg dry matter)
Fababean (*Vicia faba*)	5.6-11.8	25-100
Pea (*Pisum sativum*)	4.4-9.3	100-400
Lupin (*Lupinus albus*)	<1	0.1
Soybean (*Glycine max*)		
Raw flour	70	
Defatted flour	85	1600-3200
Industrial meal (toasted)	5.5-6.3	25-200

Source: Gueguen and Cerletti, 1994

whole seed (Owusu-Ansah and McCurdy, 1991). However, the protease inhibitor content is considerably lower in peas compared with crude soybean (Table 2.5). On the other hand, Liener (1983) demonstrated that the significance of trypsin inhibitor content to human nutrition is probably not very great, even in soybean where the level is much higher.

Flavour is one of the major characteristics that restricts the use of legume flours and proteins in foods. The presence of lipoxygenase in raw legumes is associated with the development of off-flavours during storage and processing. Many of the objectionable flavours such as grassy, beany and rancid odours come from oxidative deterioration of the unsaturated lipids. Lipoxygenase catalyses the hydroperoxidation of unsatured fatty acid, followed by their degradation to volatile and non-volatile compounds (Klein and Raidl, 1985). Relationships between lipoxygenase activity and off-flavour development are well documented for soybeans and fresh peas (Cowan *et al.*, 1973; Kinsella, 1979; Sosulski and Mahmoud, 1979). However, lipoxygenase contributes some desirable effects in foods as well. For example, carotene oxidation is a secondary reaction associated with lipoxygenase, which results in the bleaching action in a flour-water

system. This oxidative improvement of dough that contains enzyme-active flours has been recognised in the baking industry for many years (Rackis, 1977).

2.2. Processes for Extracting Pulse Proteins

2.2.1. Dry Processes

Dry processing of legumes, which have relatively low oil contents, such as field peas, uses pin milling and air classification techniques (Sosulski, 1982). Whole or dehulled field pea seeds are pin milled to yield flours with a specific particle size and density. Such flours can be further separated into protein (fine fraction) and starch (coarse fractions) using an air classifier. As can be seen from Fig. 2.1, by using pilot plant equipment, Youngs (1975) separated field pea flour in to fines (PI) containing 60% protein and a coarse fraction (SI) containing only 8% protein and with over 85% of nitrogen-free extract. To release more of the adhering protein, the course fraction (SI) was remilled and reclassified to give additional fines (PII) with 46% protein and a final starch fraction (SII) containing 2-3% protein. Many investigators have found air classification effective in separating starch and protein-rich fractions in other starchy grain legumes as well as in field peas (Sosulski and McCurdy, 1987; Sosulski, 1982; Vose, 1980; Han and Khan, 1990). Much of the technology for producing pea protein concentrate by dry milling and air classification was pioneered at the Prairie Regional Laboratory (PRL), now known as the Plant Biotechnology Institute (PBI) of the National Research Council in Saskatoon, Saskatchewan.

The composition of protein and starch fractions produced from pin milling and air classification are related to a number of parameters, variability in composition of field pea cultivars, number of passes through pin mill and classifier, and seed moisture (Tyler *et al.*, 1981). Smooth, round-seeded peas have generally been found suitable for pin milling and classification, but wrinkled peas often show very low protein and starch separation efficiencies. This is due to the high lipid content (4.5% compared to 2.0% for smooth) and the broad size distribution of the starch granules in wrinkled

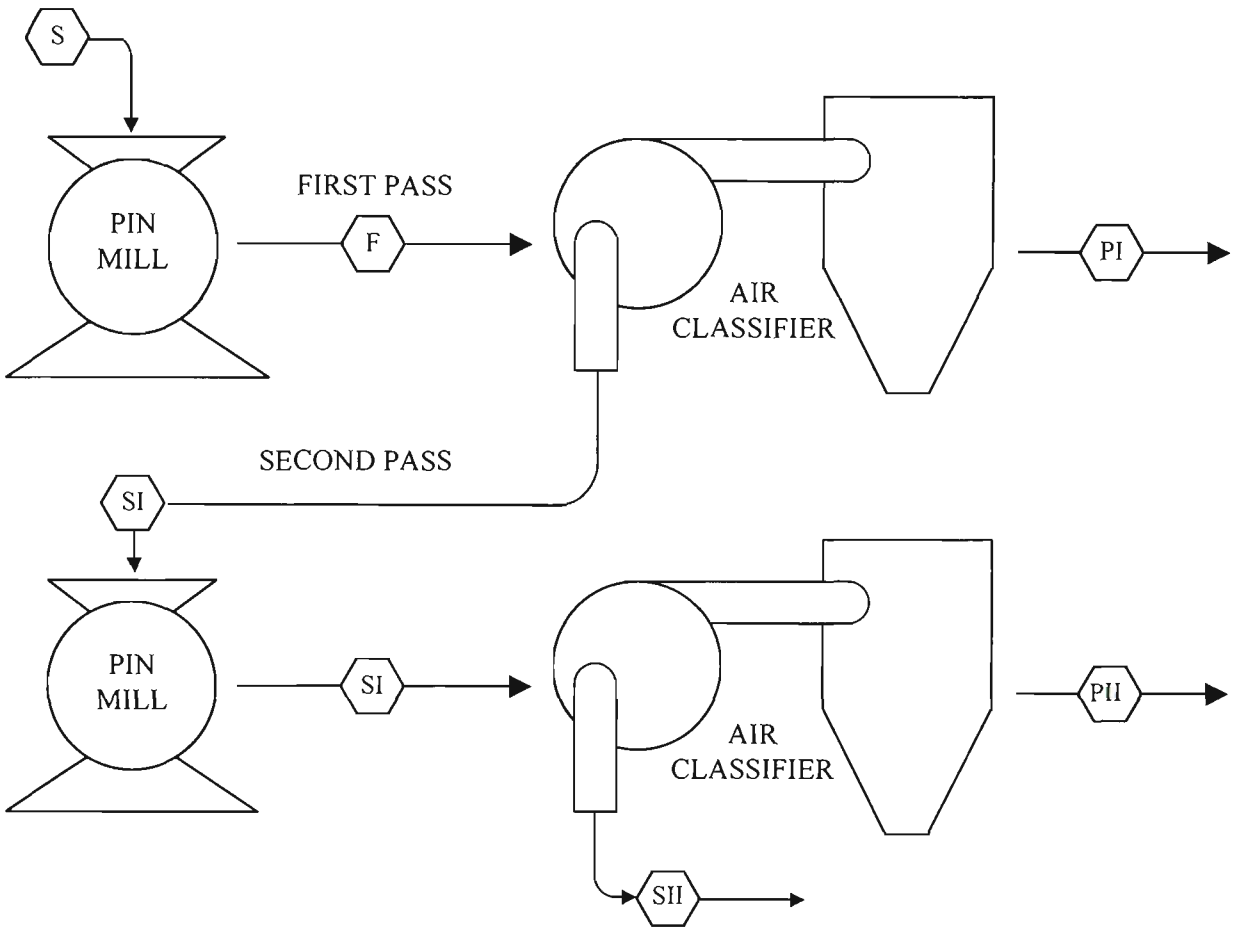


Figure 2.1 Schematic flowsheet for the pin milling and air classification of field peas by the single- and double-pass procedures.

S- seeds; F- flour; PI- first pass protein-rich fraction; SI- first pass starch-rich fraction; PII- second pass protein rich fraction; SII- second pass starch fraction.

peas (Owusu-Ansah and McCurdy, 1991). Lower moisture contents have been found to improve protein yield and protein separation efficiency, but decrease starch separation efficiency (increased starch content of protein fraction) (Klein and Raidl, 1985). This is probably due to increased seed brittleness. It has been found that the optimum moisture levels for separation of legume proteins by air classification are between 7% and 9% (Sosulski, 1982).

For large scale isolation, this physical separation of the protein-rich fraction would be more convenient because it eliminates the handling of large volumes of slurries. However, air classification does not yield proteins as pure as those produced by aqueous extraction. Although the air classification process did not markedly decrease the nitrogen solubility which occurred during the protein separation (Sosulski and McCurdy, 1987), it is generally believed that the functional properties of the protein concentrate obtained by physical processes are poorer than for the product obtained by wet processes (Gueguen, 1991).

2.2.2. Wet Processes

Enriched protein products, protein concentrates (ca. 70% protein) and protein isolates (ca. 90% protein), can also be prepared by wet processes. These processes were initially developed for processing of soybean (Gueguen and Cerletti, 1994).

Isoelectric Precipitation Process

In order to prepare protein isolates, the most widely used process is that patented by Anson and Pader (1957). After an alkaline, water or acid solubilisation of the proteins, the insoluble material is removed by centrifugation. By adding acid to the supernatant, the protein isolate is precipitated isoelectrically. Fig. 2.2 shows the flow chart for preparing protein isolate from legume seeds, such as chickpeas (Liu *et al.* 1994).

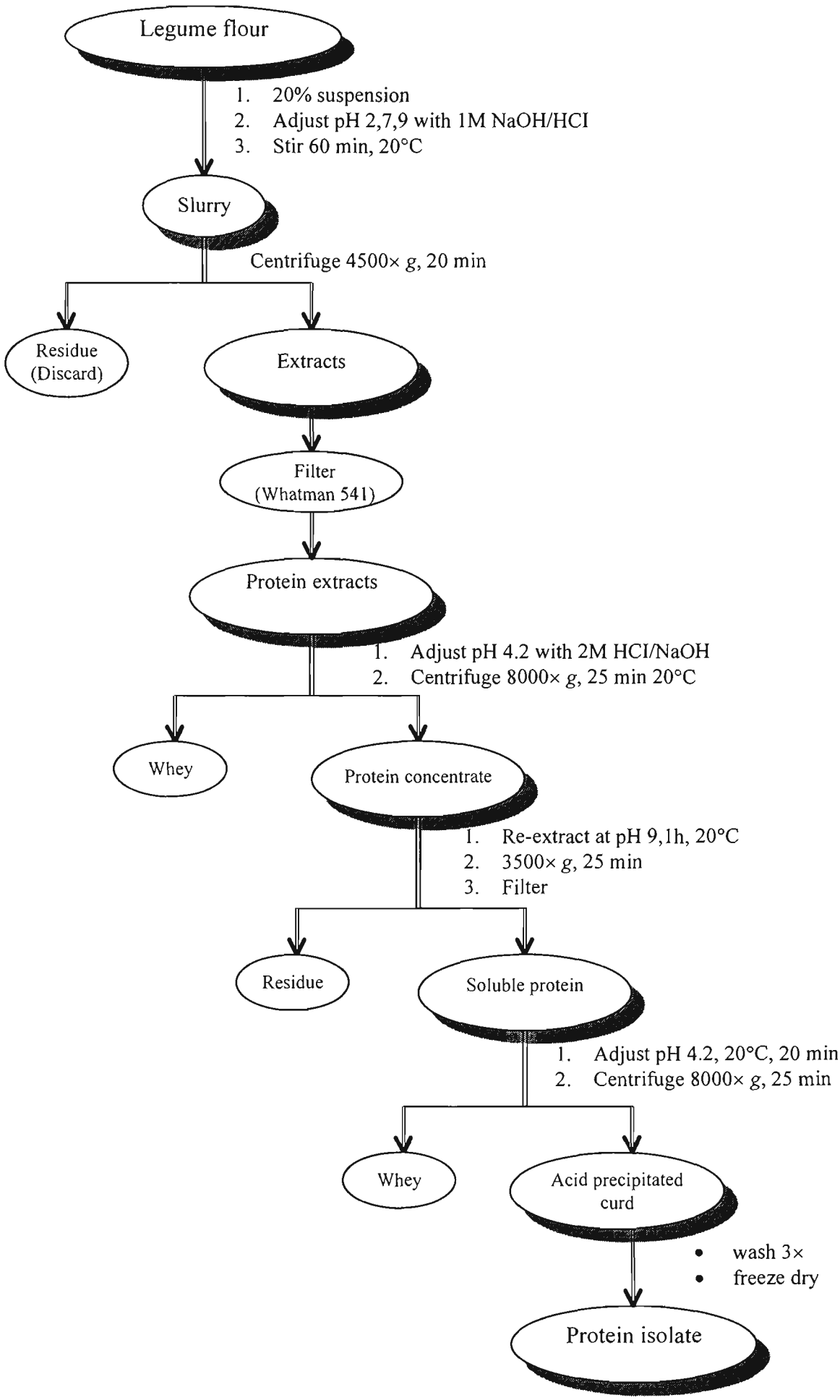


Figure 2.2 Process for legume protein isolate preparation.

Several factors, such as particle size of the flour, the type of the solubilising agent, and the pH of solubilisation and precipitation, affect the yield of the protein isolate prepared by isoelectric precipitation (Liu, 1996; Gueguen, 1983). Larger particle sizes have been shown to decrease the protein yield compared with the smaller particle sizes. When potassium and sodium hydroxide are used as protein solubilising agent, similar protein yields are obtained, but calcium hydroxide has been found to solubilise less than 10% of pea protein, apparently due to the "salting-out" effect of the calcium ions (Gueguen, 1983). In the food industry, sodium hydroxide is the most common reagent used to solubilise vegetable proteins.

The protein product can be dried using either a spray, drum or freeze drying method. Spray-dried isolate has been reported to have the lightest colour and taste, while freeze-dried and drum-dried isolates are darker. Oxidation of polyphenols causes the darkening of freeze-dried product while the Maillard reaction from heat processing creates a darker product in drum-dried isolates (Sumner *et al.*, 1981).

Ultrafiltration Process

Another wet process involves using ultrafiltration and reverse osmosis instead of acidification to recover the extracted proteins. This process has been used in vegetable protein preparation from soybean (Deeslie and Cheryan, 1991; Nichols and Cheryan, 1981), rapeseed (Diosday *et al.*, 1984) and fababean (Bérod *et al.*, 1987). The yields for the ultrafiltration process have been found to be similar to the isoelectric process. For example, the overall recovery for fababean is 62% and the protein content of the spray-dried isolate is 90% (Gueguen, 1991). The main problem preventing the use of ultrafiltration in recent years has been the low flow rate and the plugging of the membrane when the protein concentration increases (Gueguen, 1983; Deeslie and Cheryan, 1991).

Hydrophobic-Out Process

A Canadian Patent involving a process referred to as "hydrophobic-out", describes a wet method for the preparation of protein isolates from legume seeds. The flours are

extracted with a salt solution of at least 0.2 ionic strength and the insoluble material is removed by centrifugation (Murray *et al.*, 1978). The proteins are then precipitated as micelles when the ionic strength is decreased by dilution with water. It is claimed that an isolate with a very high protein content (95%) can be obtained from some legume seeds by using this method (Abdel-Aal *et al.*, 1986).

Salting-Out Process

The salting-out process is widely used in biochemical laboratories. Following the extraction of proteins with a solution of relatively low salt concentration, the total extract is subjected to fractional salting out using ammonium sulphate (Owusu-Ansah and McCurdy, 1991). In the first stage ammonium sulphate is added to 35% saturation to precipitate some of the proteins along with nucleic acids. The precipitate is removed by centrifugation and the proteins in the remaining solution are further salted out at 65-100% saturation of ammonium sulphate. The sequential "salting out" is said to be a successful way of separating the main and secondary proteins from each other and from nucleic acids. However, this process has not been commonly used to isolate proteins for food applications because of safety considerations and cost of the reagent.

2.2.3. Fractionation of Pulse Proteins

The fractionation of grain proteins is based on the classical work of Osborne (1924) who pioneered the use of different solvents. Albumin, globulin, prolamin and glutelin are the main fractions from cereal and grain legume proteins. However, unlike the cereal proteins, in which the major storage proteins are alcohol-water-soluble prolamins, the major storage proteins from legume seed are globulins. Albumin is the second major portion which is also important in terms of functional and nutritional properties. A typical procedure leading to the separation of albumin and globulin fractions from legume flours is shown in Fig. 2.3.

There have been few published reports on the fractionation of pea proteins (Gueguen and Barbot, 1988; Gueguen *et al.*, 1984) and these have been on a laboratory scale. The

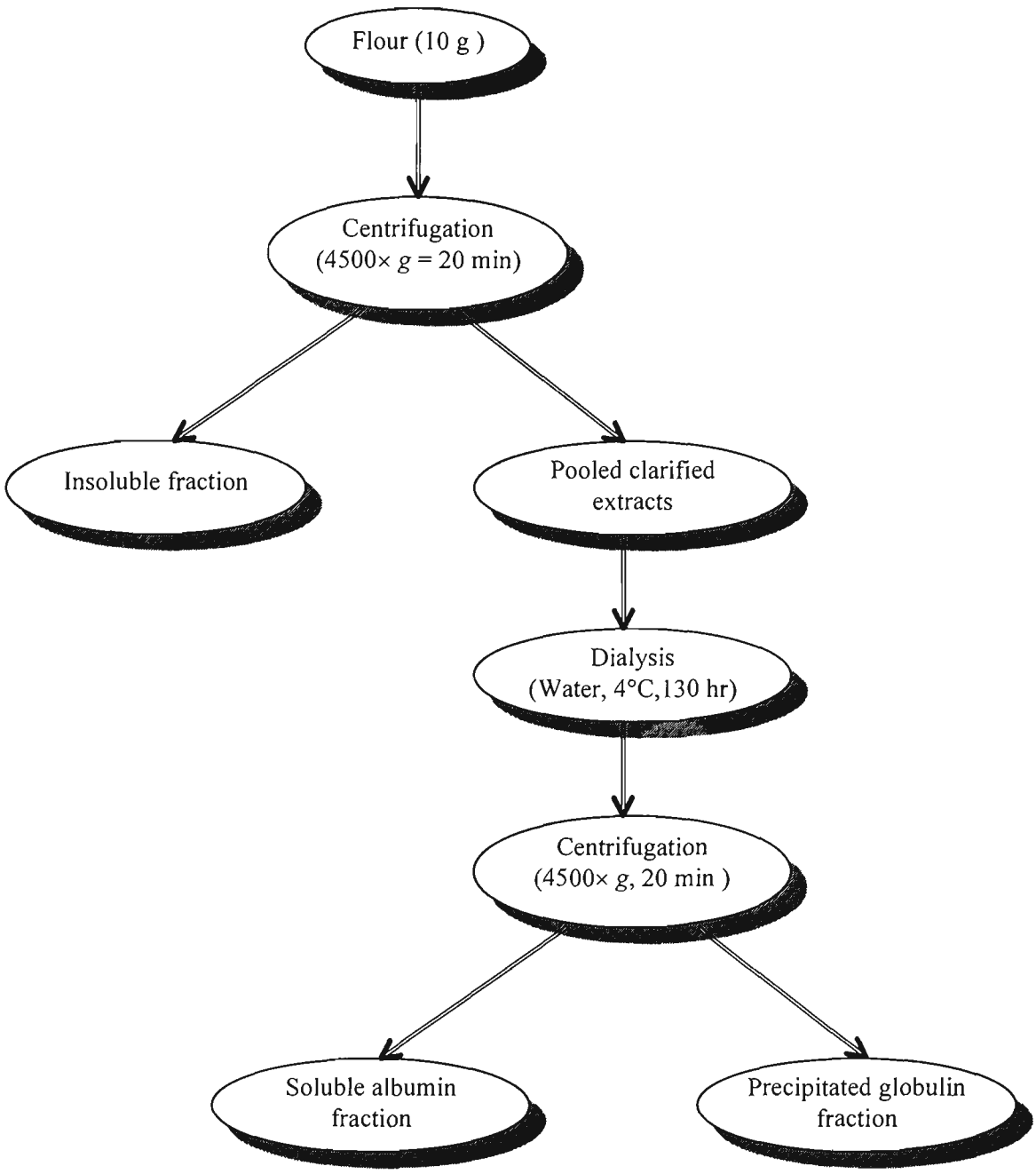


Figure 2.3 Experimental procedure for separation of albumin and globulin fractions from leguminous flours.

reports available are mainly concerned with purification and characterisation of pea globulins, composed of vicilin and legumin, by gel chromatography.

More extensive studies have been published on the fractionation of proteins from other grain legumes, such as great northern bean and fenugreek (Sathe and Salunkhe, 1981b; Sauvaire *et al.*, 1984; Taylor *et al.*, 1984). The flours were first extracted with salt solution (NaCl or phosphate buffer) and then centrifuged. The solutions obtained were dialysed for several days prior to separation of the supernatant (albumin fraction) and pellet (globulin fraction). The solids obtained from the centrifuge were re-extracted with alcohol and the supernatant was dialysed to recover the prolamin fraction. The residue after the prolamin extraction was solubilised with dilute alkali (NaOH or $Na_2B_4O_7$) (Rosa *et al.*, 1992) to collect the glutelin fraction. To facilitate the prolamin and glutelin extraction and characterisation, a small amount of sodium dodecyl sulphate (SDS) or /and reducing reagent, 2-mercaptoethnol (2-ME) can be used in this procedure. In combination with protein fractionation, the most common way to characterise these proteins is by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

As the potential uses of field pea proteins are linked to their functional properties (Vose, 1980; Sumner *et al.*, 1981), it is necessary to understand the relations between the physico-chemical and the functional properties of the major individual proteins (Gueguen *et al.*, 1984) as well as the total protein isolates.

2.3. Functional Properties of Pulse Proteins

Among the most important aspects of plant proteins are their functional properties. These will determine the field of application as well as whether a new protein ingredient will be competitive on the market. Functional properties of food proteins can be defined as physico-chemical properties which give information on how a protein will behave in a food system (Hermansson, 1979a). Several other definitions for functional properties of food proteins have been proposed but the meanings are similar.

For example, Kinsella (1976) has defined functional properties of proteins as "those physical and chemical properties which affect the behaviour of proteins in food systems during processing, storage, preparation and consumption." Pour-EI (1981) defined functional properties as " any property of a food or food ingredient except its nutritional ones, which affects its use." Properties of proteins such as solubility, swelling, viscosity, texture, water- and fat-binding, emulsion, foam and gel characteristics are of particular interest. The functional properties which are important for food proteins are summarised in Table 2.6.

When newly developed sources of proteins are used to replace traditional proteins in conventional foods, it is imperative to study the functional properties of the prepared protein concentrates or isolates in order to determine which food systems they can be incorporated in and be utilised effectively. Therefore, certain functional properties of proteins which are important for food applications are now discussed.

Table 2.6 Summary of Functional Properties of Proteins Important in Foods

Property	**Functional criteria**
Organoleptic	Colour, flavour, odour, texture, mouthfeel, smoothness, grittiness
Hydration	Solubility, swelling, wettability, water absorption, thickening, gelling, syneresis
Surface	Emulsification, foaming (aeration-whipping), protein-lipid, film formation, lipid binding, flavour binding
Structural/rheological	Elasticity, grittiness, cohesiveness, chewiness, viscosity, adhesion, network cross-binding, aggregation, stickiness, gelation, dough formation, texturisability, fibre formation, extrudability
Other	Compatibility with additives, enzymatic antioxidant

Source: Kinsella, 1979

2.3.1. Solubility of Pulse Proteins

Good solubility is often considered to be a prerequisite for the performance of a protein in various food applications (Kinsella, 1976). For food systems such as beverages, soups and salad dressings, the solubility of the proteins is probably the most important criterion. In addition, to obtain optimum functionality in foods that require gelation, emulsification and foaming properties, a relatively soluble protein is also desirable (Damodaran, 1996).

The solubility of proteins is markedly affected by pH, ionic strength, ion types, temperature, solvent polarity, and processing conditions. Methods for assessing solubility may vary but the nitrogen solubility index (NSI) (AACC, 1983) and variations of it are most commonly used. Solubility of proteins is highly dependent on the pH. Hence, in the case of protein isolates extracted from legumes, most of the studies available and reported are on the neutralised proteins which exhibit more desirable functional properties than the corresponding isoelectric product.

The functional properties of field pea proteins have been studied as pea isolates by some researchers (Naczk *et al.* 1986; Vose, 1980). The field pea protein preparations had higher solubility in water than the other products studied (e.g. soy-protein isolate, wheat gluten). They are highly soluble at acidic pH (pH 2), and rapidly become less soluble as pH increases to a range of minimum solubility (isoelectric point, pH 4-6). Then the proteins are highly soluble again at alkaline pH values. However, the actual solubility of pea protein materials at a given pH in the pH region of 5 to 9 can vary widely depending on the specific nature of the proteins and the method of preparation. Koyoro and Powers (1987) noted that vicilin is more soluble than legumin at pH 7. Sosulski and McCurdy (1987) observed that protein solubility at pH 6.6 is lower in air-classified concentrates as compared to flours, perhaps due to poor dispersibility or denaturation during grinding. Drum-dried protein preparations generally had poorer solubility than those which were spray or freeze dried (Sumner *et al.*, 1981).

In view of the importance of the solubility of food proteins in many food applications, it is pertinent to understand the factors, both intrinsic as well as extrinsic, that affect protein solubility (Damodaran, 1996). For the water-soluble proteins, generally most of the charged and hydrophilic residues are located at the surface, while hydrophobic residues are buried in the interior. The solvents and processing conditions affect the solubility of proteins principally by causing alterations in the ionic, hydrophilic, and hydrophobic interactions at the protein surface. However, the fundamental relationship between the conformational, hydrodynamic, and surface properties of legume proteins and their functional behaviour in food systems is poorly understood. Furthermore, very little research has been found to explain the relationship between the solubility and other functional properties of the proteins, such as emulsifying and foaming (Paulson and Tung, 1987; Schaffner and Beuchat, 1986), which are good indices for evaluating potential applications of legume proteins.

2.3.2. Water Binding of Pulse Proteins

Water binding or absorption is commonly measured as the amount of water retained by a sample of protein powder after mixing with water, centrifuging, and decanting off the excess water. The water absorption capacity of field pea proteins was found to be lower than that of soy-protein products but much higher than for gluten-protein preparation (Naczk *et al.*, 1986). Pea protein materials appear to absorb between 1 to 3.3 times their weight of water. Sosulski and McCurdy (1987) observed that water absorption of pea protein isolates increases with increasing protein content. Water binding ability of proteins is significant in food products including meat, sausages and doughs where there is insufficient water to allow the protein to dissolve, but where the hydrated protein imparts structure (swelling, gelation) and viscosity to the food (Owusu-Ansah and McCurdy, 1991).

2.3.3. Emulsifying Properties of Pulse Proteins

Emulsifying properties of proteins relate to the ability to stabilise an emulsion of oil and water. This property is critical for many food applications such as finely comminuted meats, soups, cakes, mayonnaise and salad dressings (Jackman *et al.*, 1989). Emulsifying properties are commonly discussed in terms of emulsifying capacity, emulsifying stability and emulsifying activity (Pearce and Kinsella, 1978).

Emulsifying capacity is generally defined as the quantity (volume) of oil that can be emulsified by a standard weight of protein before the emulsion collapses. Emulsion stability and emulsion activity refer to the ability of a protein to form an emulsion that remains unchanged for a particular time interval under specified conditions (Kinsella, 1976). Protein concentration, solubility, pH of the medium, and salt affect the emulsifying capacity of vegetable proteins (Wang and Kinsella, 1976). Other factors such as equipment design, shape of container, rate of oil addition, type of oil used and nature of proteins also have great effects (Christian and Saffle, 1967; Kinsella, 1976). Thus emulsifying properties are not solely a property of the protein under test but rather, a property of the emulsion system, the equipment and method used to produce the emulsion (Tornberg and Hermansson, 1977).

Compared with commercial soybean protein isolate, Vose (1980) found pea protein isolate showed similar emulsifying activity. Emulsifying capacity of field pea and faba bean increases with increased processing to obtain more concentrated protein materials (Sosulski and McCurdy, 1987). Hsu *et al.* (1982) noted that emulsifying capacity of field pea protein increased when peas were germinated prior to protein processing. Dagorn-Scaviner *et al.* (1987) studied the emulsifying properties of purified pea globulins and of vicilin-legumin mixtures. They found that vicilin, which was shown to be more surface active at two phase interfaces (air/water and dodecane/water), led to better emulsifying properties than those shown by legumin. On the contrary, Koyoro and Powers (1987) found the emulsion capacity of legumin to be superior to that of vicilin at pH values of 3 and 7. In fact, the relationship between structure and surface

behaviour of proteins is poorly understood (Gueguen and Cerletti, 1994). Hence it is difficult to compare results especially those obtained under different fractionation procedures and testing systems.

2.3.4. Foaming Properties of Pulse Proteins

Protein foams are important for various types of foods including meringues, souffles, whipped toppings, chiffon desserts and leavened bakery products (Townsend and Nakai, 1983). Foaming or whipping properties refer to the ability of a dispersion of protein material to form a stable foam when air is incorporated by beating. Foam ability of protein materials is generally measured by the increase in volume attained by a protein dispersion after incorporation. Foam stability is the ability of a foam to retain volume over time and is usually measured as the rate of fluid leak from the foam (Owusu-Ansah and McCurdy, 1991).

Due to the importance of protein foams in the food industry, a large number of empirical studies have been carried out to examine the foaming behaviour of proteins under a variety of conditions (Cherry and McWatters, 1981; Kinsella, 1976). Several other studies have demonstrated that many factors including pH, temperature, the presence of salts and sugars, affect the foaming behaviour of proteins for great northern bean (*Phaseolus vulgaris* L.) and lupin seed (Sathe and Salunkhe, 1981a; Sathe *et al.*, 1982). However, there is relatively little literature data on foaming properties of field pea proteins. A few studies (Vose, 1980; Sumner *et al.*, 1981) found that spray-dried pea protein isolates produce a higher foam volume than soy protein isolates. Air-classified pea protein concentrate has been found to have good foam expansion but poor stability (Sosulski and Youngs, 1979). Hsu *et al.* (1982) demonstrated that germinated peas produced an isolate with better foaming properties. It has also been found that the foaming capacity of legumin is superior to that of vicilin, but vicilin provides a more stable foam (Koyoro and Powers, 1987).

2.3.5. Viscosity and Gelation Properties of Pulse Proteins

Viscosity is one of the most salient physical and sensory characteristics of semisolid and liquid foods (Christensen, 1987). It describes the resistance to flow demonstrated by a solution and is defined as $\eta = \tau/\gamma$. For a Newtonian fluid, shear rate γ (expressed as sec^{-1}) is exactly proportional to shear stress τ (expressed as $dynes/cm^{-2}$) and thus, the viscosity η (usually expressed as centipoise (cP)) is independent of the rate at which the solution is sheared. However, liquid and semi-solid foods usually exhibit very complex flow properties and most show non-Newtonian flow characteristics.

Gueguen and Cerletti (1994) found that the protein isolate dispersions from some legume seeds exhibited non-Newtonian, time-dependent behaviour. However, data on viscosity or flow properties of field pea proteins are scanty. Gueguen and Lefebvre (1983) reported that pea isolates had similar thickening characteristics as a soy isolate (Supro 620), but to a lesser degree. Hsu *et al.* (1982) noted that the viscosity of pea protein isolate dispersions to be 1/12 of that of a soy protein isolate (Promine D). Several other researchers studied the effects of solutes on the viscosity of soy protein dispersions (Hermansson, 1975; Urbanski *et al.*, 1982; Babajimopoulos *et al.*, 1983). It was found that addition of salt (sodium chloride) or carbohydrate (glucose or sucrose) reduced the viscosity of the protein slurries.

The formation of gels (gelation) is also important in many foods. Protein gels may be visualised as three-dimensional matrices or networks of intertwined, partially associated polypeptides in which water is entrapped (Pomeranz, 1991b). The gels have relatively high viscosity, plasticity, and elasticity (Kinsella, 1976). Soybean tofu is a good example of a protein gel from a legume source. Gebre-Egziabher and Sumner (1983) investigated the yield and quality of protein curd from field peas compared with soybean curd. They found that the curd yield from pea flour was lower than soy curd but this could be improved by using the more expensive pea protein concentrate. Although the sensory properties of pea curd were judged inferior to tofu, they were generally acceptable. Addition of gluten improved the colour and texture of pea curd

and may also improve the nutritional quality. However, Hsu *et al.* (1982) found that the pea protein isolate did not form a gel, but had rather a pastelike consistency when a 10% slurry was first heated and then cooled. Sosulski and Youngs (1979) reported that a 15% slurry of pea flour was intermediate in gelation as compared to nine other legume flours. It is noted that in the heat-induced gelation system, the protein slurry is heated above the denaturation temperature, which results in the formation of a high-viscosity progel. Upon cooling, the proteins in their unfolded conformation form the gel through disulphide, hydrogen and hydrophobic interactions (Gueguen and Cerletti, 1994). As a result, in addition to the nature of the proteins, several other environmental factors, such as pH, heating temperature, the rate of heating and cooling, salt (e.g. the coagulant added to make the curd), also affect the gel formation. Data on these factors which impact upon the gelation properties of field pea proteins are lacking.

2.3.6. Flavour and Colour of Pulse Proteins

Effective utilisation of protein materials in human foods depends to a large degree upon consumer acceptance, and good flavour and colour are the essential requirements (Owusu-Ansah and McCurdy, 1991). Price *et al.* (1985) identified soya saponin I, a saponin also found in soybean flour, as a source of bitterness and astringency in pea flour. Murray *et al.* (1976) isolated three methoxypyrazines which might be of major significance in pea flavour. Klein and Raidl (1985) suggested that when unheated legume flours are used as a supplement in doughs, the resulting flavour characteristics could be a result of enzymatic activity, particularly that of lipoxygenase. The volatile carbonyl compounds produced by protein supplements in yeast bread are shown in Table 2.7. Commercial steaming could reduce the bitter flavour and taste form legumes (Owusu-Ansah and McCurdy, 1991). Sumner *et al.* (1981) observed that spray drying was more effective than drum or freeze drying for eliminating legume and other objectionable flavours when the protein isolates were produced.

Table 2.7 Carbonyl Compounds in Yeast Bread (mg/100g dry weight)

	Wheat	Soy[a]	Field pea[a]
Ethanal	166	325	457
Propanal	25	56	37
2-Propanone	982	1332	1155
Butanal	25	38	37
2-Butanone	306	358	300
2-Methyl Butanal	107	840	616
Unknown	47	151	599
Hexanal	139	1096	520
Furfural + HMF[b]	600	1470	1730

a: Wheat flour/protein supplement/vital gluten = 83:15:2

b: Hydroxymethyl furfural

Source: Sosulski and Mahmoud (1979)

If a protein material is to be incorporated into food products, a colour as close to white as possible is considered most acceptable (Blouin *et al.*, 1981). Pea protein isolates range from cream to beige colour, depending on the processing method (Sumner *et al.*, 1981). They found that spray drying produces lighter colour isolates than drum or freeze drying, and that sodium proteinates were lighter in colour than isoeletric precipitates. Isolation and identification of the pigments producing colour from peas has not been reported. In addition, descriptions of colour contributions from peas to the final food product have not been found.

2.4. Modification of Proteins

Food proteins have been subjected to modifications empirically since 5000 B.C., mainly for the purpose of enhancing the palatability and stability of products. An example is the enzymatic modification of milk proteins in yogurt and cheese (Howell, 1996). More recently, however, intentional modification has been used as a tool for improving

the functional properties and studying the structure-function relationships of food proteins. Few proteins, especially plant proteins in the native state have optimum functional properties. Hence, modification of native proteins is potentially an effective way to impart the required functional attributes to an already available raw material and minimise the costs of producing novel foods (Pomeranz, 1991b).

Factors which affect the functional properties of food proteins are described in Table 2.8 (Sathe *et al.*, 1984). Most of the research work in this area is focused on the influence of the processing parameters and chemical or enzymatic treatment on functionality of food proteins (Johnson and Brekke, 1983; Schwenke and Rauschal, 1980).

Table 2.8 Factors Governing the Functional Properties of Food Proteins

Intrinsic factors	**Process/Treatments**	**Environmental factors or food system components**
Composition of protein(s)	Heating	Water
Conformation of protein(s)	pH	Carbohydrates
Mono- or multicomponent	Ionic strength	Lipids
Homogeneity/heterogeneity	Reducing agent	Salts
Components bound to protein	Storage conditions	Surfactants
	Drying	Flavours
	Physical modification	Redox potential
	Chemical modification	pH
	Enzymatic modification	Chelating agents
		Presence/absence of antinutritional factors

Source: Sathe *et al.*, 1984

2.4.1. Physical Modification of Pulse Proteins

Changing the processing parameters including temperature and pH are examples of the physical modification of proteins. These generally involve the use of heat (dry or moist) to bring about partial denaturation of proteins. Legume proteins are known to be

compact and, hence, resistant to proteolytic enzyme attack in the human body. This leads to incomplete digestion and underutilisation of these proteins. It is believed that denaturation results in a partial opening of the close-packed structure of the globulins or in the controlled unfolding of their polypeptides. This should improve the protein functionality by increasing the accessibility of buried reactive areas of the molecules (Sathe *et al.*, 1984). Schwenke *et al.* (1990) have found that heat treatment led to a higher water adsorption capacity and better thickening properties of faba bean isolates. They also improved the functionality of faba bean proteins by controlled denaturation in acidic condition (Schwenke, 1988). Gueguen *et al.* (1988) investigated the effect of pH and ionic strength on the association and dissociation phenomena of pea 11S type globulin (legumin). They found that most of the legumin molecules were dissociated under extreme acidic conditions. At low ionic strength aggregation usually occurred. Such knowledge could be of great interest for technological applications and lead to a better understanding of protein structure. Nevertheless, data on this area is limited.

2.4.2. Chemical Modification of Pulse Proteins

Chemical modification of proteins includes the derivatisation of the amino acid side chains of proteins as well as hydrolysis of the peptide bonds (Howell, 1996). The chemical modification of proteins can be achieved through treatment with various agents including alkalis and acids, acetylation, alkylation, esterification, oxidation and reduction. Table 2.9 shows a number of functional groups on the amino acid side chains which are available for chemical derivatisation. However, many of the methods for chemical modification are unsuitable for food uses. Concerns surrounding chemical modification of food proteins include toxicity, deterioration of organoleptic properties, loss of nutritional value, interaction with other food consumed, and reversibility of modification. In addition, possible barriers to the use of chemically modified proteins entail aesthetic, cultural, legal, and economic aspects (Feeney and Whitaker, 1985). Nevertheless, chemical modification is one of the most efficient tools for improving the functional properties of plant proteins as well as other food proteins (Gueguen and Cerletti, 1994).

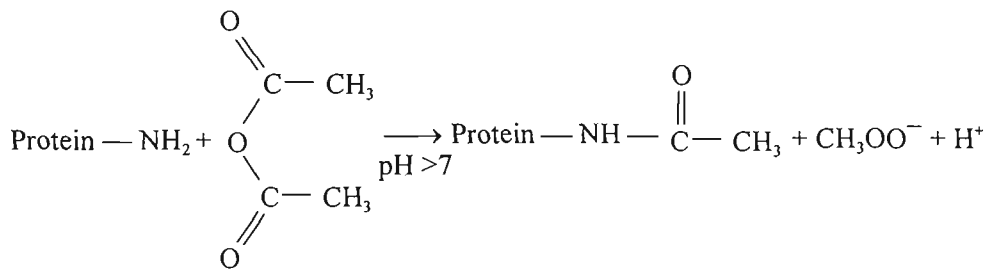
Table 2.9 Amino Acid Side Chains Involved in Chemical Modification

Side Chain	Chemical Modification
Amino	Acylation, alkylation
Carboxyl	Esterification, amidation
Disulphide	Oxidation, reduction
Sulphhydryl	Oxidation, alkylation
Thioether	Oxidation, alkylation
Phenolic	Acylation, electrophilic substitution
Imidazole	Oxidation, alkylation
Indole	Oxidation, alkylation

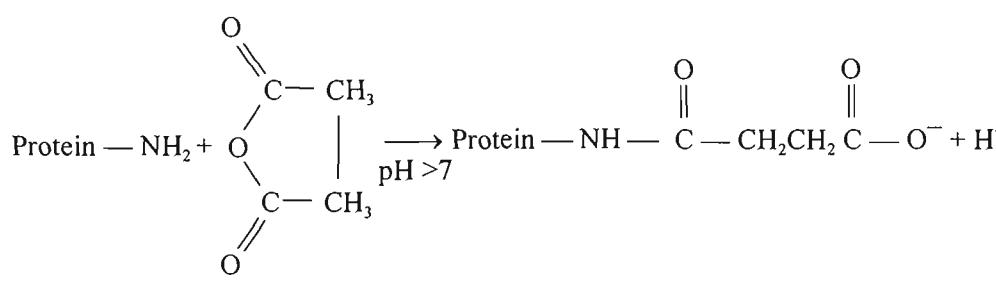
Source: Howell, 1996

Acetylation and Succinylation

A common modification of food proteins is acylation with acetic and succinic anhydrides. The reagents react with the ε-amino groups of lysine significantly (Fig. 2.4) and with tyrosine phenolic groups to a limited extent (Howell, 1996). Acetylation has been applied to various plant proteins including wheat (Grant *et al.*, 1973; Barber and Warthesen, 1982), soybean (Franzen and Kinsella, 1976a; Kim *et al.*, 1988), cottonseed (Childs and Park, 1976; Rahma and Rao, 1983), peanut (Beuchat, 1977; Shyamasundar and Rajagopal Rao, 1978), and rapeseed (Schwenke *et al.*, 1991a; Paulson and Tung, 1988a,b). More recently, acetylated and succinylated fababean proteins have been extensively studied by the Potsdam (GDR) group (Gueguen, 1991). Depending on the degree of acetylation or succinylation the proteins may be more highly charged and more disordered, inducing a shift of the solubility curve to lower pH values, an increase of the shear modulus and the net density of the resulting gels, and also enhanced foaming and emulsifying properties. However virtually little information is available on modification of field pea proteins (Johnson and Brekke, 1983). Schwenke *et al.* (1993) investigated some physico-chemical properties of succinylated legumin from peas. They found that high degrees of succinylation resulted



Protein + acetic anhydride ⟶ acetylated protein



Protein + succinic anhydride ⟶ succinylated protein

Figure 2.4 Modification of amino groups by acetylation and succinylation.

in dissociation of pea legumin and shifted the iso-electric point (pI) from 4.75 to 3.5.

Phosphorylation

Phosphate groups can be covalently attached to proteins to increase the negative charge (Matheis and Whitaker, 1984). Inorganic phosphate can be bound to proteins either by the O- or N- esterification reaction (Shih, 1992). The most widely used phosphorylation method is the reaction with phosphorus oxychloride (Howell, 1996):

$$\text{protein-NH}_2 + \text{POCl}_3 \xrightarrow[\text{CHCl}_3]{\text{H}_2\text{O}} \text{protein- NH- PO(O}_2\text{H)}^-$$

Phosphorylation of proteins enhances gel forming properties, particularly in the presence of Ca^{2+} (Matheis and Whitaker, 1984). Huang and Kinsella (1986a) found that the functional properties of yeast proteins, such as solubility, viscosity, and water absorption were greatly improved by phosphorylation. They suggested that phosphorylated yeast proteins would be useful thickening agents in foods. No data has been found in relation to modification of plant proteins with $POCl_3$.

An alternative phosphorylating agent, sodium trimetaphosphate (STMP), has been used in the modification of soy proteins (Sung *et al.*, 1983; Kim *et al.*, 1988). They found that the STMP modified soy protein isolate exhibited enhanced functional properties in terms of aqueous solubility, water-holding capacity, emulsification ability and whippability. *In vitro* and *in vivo* digestibility studies of phosphorylated proteins indicated that the nutritional value of the protein was not reduced to a significant extent by the phosphorylation (Matheis and Whitaker, 1984).

Other Chemical Modifications

Acid and alkali have been used extensively for protein extraction and to cause increased solubility, lower viscosity, and fiber formation (Meyer and Williams, 1977). Peptide bond hydrolysis by acid or alkali results in low molecular weight products with increased ionisable groups that are more soluble. The reactions of alkaline hydrolysis

proceeds faster than with acid. Alkali treatment has also been used in the production of texturised soy proteins for use as meat analogues and in the manufacture of gelatin from collagen (Howell, 1996). However, severe treatment of proteins with alkali can lead to the destruction of amino acids including lysine, cystine, and serine; as well as formation of nondigestible products due to racemisation.

Oxidation and reduction of proteins is another possible mode for modifying food proteins (Sathe *et al.*, 1984). Oxidation, for example, may improve the food protein colour through oxidation of the pigments (which are normally associated with proteins that are concentrated from legumes). Use of reducing agents such as sulphite during preparation of bean protein concentrates and isolates may also be advantageous, in that it will assist in retarding the growth of microorganisms during wet extraction of proteins and may inhibit the aggregation of proteins which, in turn, may render the isolated proteins more soluble and reduce the viscosity of protein solutions. These reactions have not been studied from the viewpoint of improved functionality of proteins of other legume seeds except that limited work has been reported for soybean proteins (Meyer and Williams, 1977).

2.4.3. Enzymatic Modification

Enzymatic modification processes, as compared with conventional chemical reactions, are generally performed under milder experimental conditions, occurring at moderate temperatures and atmospheric pressure. It has an advantage of potential stereochemical specificity, facilitates process control and causes fewer side reactions (Adler-Nissen, 1986; Dickinson and Stainsby, 1987).

Enzymes generally modify food proteins through hydrolysis of peptide bonds. Since enzymes are expensive, this approach is not widely applied at present. Immobilisation of enzymes may permit repeated use of enzymes and thus reduce operational costs (Lee and Lopez, 1984). Currently, the proteases, papain, chymotrypsin and trypsin are the preferred enzymes used to improve the general functional properties and nutritional

value of proteins (Phillips *et al.*, 1994a). The optimum degree of hydrolysis depends on the products required and partial hydrolysis appears to improve the functional properties, such as emulsifying and whipping abilities of food proteins. Also, by limiting the extent of hydrolysis it is possible to avoid the bitter taste of protein hydrolysates in which extensive hydrolysis has occurred (Adler-Nissen, 1986). Several other reports have been found studying the effect of limited proteolysis on the functional properties of plant proteins, e.g. cottonseed flour (Rahma and Rao, 1983), soybean (Deeslie and Cheryan, 1988), sunflower (Parrado *et al.*, 1993).

In addition to the hydrolysis of peptide bonds, enzymes can also promote inter-molecular crossing-linking, deamidation and the attachment of specific functional groups to proteins of biological interest (Arai and Watanbe, 1988). For example, transglutaminase has been shown to catalyse the cross-linking of a number of proteins, including casein (Neidle *et al.*, 1958), and soybean proteins (Motoki *et al.*, 1984). These groups found that the solubility, the emulsifying activity, and especially the hydration properties of polymerised proteins were greatly modified. Such products might be interesting for the production of intermediate moisture protein foods (Larré *et al.*, 1993). In recent years, some authors have studied the action of transglutaminase on pea legumin (Larré *et al.*, 1992; Colas *et al.*, 1993), and they suggested that the introduction of covalent peptide bonds into processing gels or films might be useful to obtain novel textural properties. However, further investigations are needed before these can be put to practical use.

Enzymatic modification of food proteins can also be achieved by fungal and bacterial proteases. Don *et al.* (1991) used a neutral fungal protease from *Aspergillus oryzae* and a *Bacillus subtilis* protease to modify soy protein concentrate obtained from toasted flour. They found that the solubility, foaming capacity and foam stability of denatured soy proteins were improved by proteolysis with these fungal and bacterial proteases. However, no data has been found to describe the enzymatic modification of other legume seed proteins with fungal or bacterial proteases.

In general, physical, chemical or enzymatic modification of food proteins has the potential to control the functionality, microbiological stability, nutritional value and acceptability of foods. One of the major obstacles in the commercial production of modified proteins is the expensive, time-consuming process of safety evaluation of these valuable products (Howell, 1996). In addition, there is a need for continuing effort in the area of elucidating the relationship between protein structure, conformation and functional properties (Phillips *et al.*, 1994a).

2.5. Utilisation of Field Pea Proteins

2.5.1. Utilisation for Animal Feeding

Before considering use of pea proteins in human food it is worth reporting briefly on some results of studies on the digestibility and utilisation of the proteins in animal feeds. Because of the agronomic development of peas, most of the studies devoted to the nutritive value of the proteins have been carried out on whole seeds, especially for pig and poultry nutrition (Gueguen, 1991). The essential amino acid compositions of field pea protein show a lower sulphur amino acid content and a deficiency in tryptophan compared with soybean protein. However, because peas do not generally represent the whole protein content of the diet, these deficiencies can be complemented by the use of other protein sources, like cereals. According to Henry and Bourdon (1977), peas can be included at levels of up to 30% in the growing and finishing diets of pigs, without affecting the performance, if the diets are well balanced in essential amino acids. Supplementation with alfalfa protein concentrate was shown to be a very suitable source of tryptophan and other essential amino acids.

Besides the amino acid composition of the proteins, their digestibility has also to be considered (Gueguen and Cerletti, 1994). Average crude protein and amino acid digestibility coefficients of grain legumes have been published for pigs and poultry (Rhone Poulenc Animal Nutrition, 1989). As can be seen from Table 2.10, pea is

characterised by a satisfactory digestibility of lysine and threonine, but a relatively poor digestibility of sulphur amino acids compared with soybean. Little is known about the digestibility *in vivo* of each of the proteins of legume seeds. Aubry and Boucrot (1986) compared the hydrolysis of pea legumin and vicilin with that of radiolabelled casein in rats. From their basic study it seems that the pea globulin fraction is efficiently degraded but is less readily absorbed than casein (Gueguen and Cerletti, 1994).

Table 2.10 Average Crude Protein and Amino Acid Digestibility Coefficients for Pigs[a]

	Crude Protein	Amino Acid Lys	Met	Cys	Thr
Spring peas	0.743	0.815	0.767	0.621	0.710
Field bean	0.763	0.837	0.741	0.771	0.741
Lupin	0.708	0.656	0.536	0.695	0.693
Soybean meal	0.798	0.847	0.862	0.762	0.786
Wheat	0.801	0.679	0.844	0.816	0.677

a: Excreta were collected for 48 h from ileorectal-anastomised growing pigs after a 5 h/day adaptation period

Source: Rhone Poulenc Animal Nutrition, 1989

2.5.2. Utilisation for Human Food

Although the main uses of field pea are still in animal feeding, there is considerable interest in this crop as a potential source for the production of new protein-rich products for the food industry. Like soybeans, field pea flour or proteins can be incorporated into cereal and bakery products, meat products, milk products, textured protein, as well as other applications.

Cereal and Bakery Products

Pea flours have been used for protein enrichment of a number of cereal-based products, such as pasta, bread and biscuits (Fleming and Sosulski, 1977; McWatters, 1980). According to Klein and Raidl (1985), substitution of wheat flour with pea flour or concentrate up to 15% in yeast bread results in generally acceptable products. However, the use of pea flours is limited by some of the less desirable effects. At low levels of fortification (1%-3%), unheated pea flour is an effective dough improver, improving mixing time and tolerance, and providing bleaching action through lipoxygenase activity. But at levels above 8%, changes in crumb quality appear; volume, flavour, aroma and overall acceptability are altered. Heating pea flour or concentrates improves flavour characteristics, but the heated product may not retain the desirable functional properties. However, it has been shown that the nutritional quality of wheat protein has been improved by addition of pea flour or protein concentrate (Sumner, 1980).

As with baked goods, pasta products made from wheat alone do not provide complete protein quality. Pasta supplemented with pea protein has been successfully produced (Nielsen *et al.*, 1980). The supplemented pasta cooks faster, has a better protein score, and is slightly firmer than pasta prepared from wheat alone. Other properties, such as chewiness, adhesiveness, and gumminess are similar. The flavour of the supplemented pasta is inferior to an all-wheat product and the colour is yellow which might be considered objectionable by some consumers, but it has been shown that the colour bleaches upon cooking.

As an alternative approach in testing the performance of pea protein in bread, McWatters (1980) substituted pea protein for milk protein in preparing baking powder biscuits. Doughs containing either heated or unheated pea flour or pea protein concentrate were found to be less sticky than those with no pea products and could be easily handled. The appearance, colour, and textural properties of the biscuits containing pea protein were not significantly different from the reference biscuit

containing milk. However, the flavour of the pea supplemented products was found to be objectionable when assessed by a sensory panel.

Meat Products

The use of plant proteins as extenders in meat products is widely practiced in the food industry. However, over a long period, only soybean and wheat proteins have been used in producing various comminuted meat products on the market. The functional requirements for a plant protein to be useful for meat systems include good fat and water absorption, emulsification capacity and stability, gelation, texturisability, and sensory attributes. There have been a limited number of studies on the use of pea protein as meat extenders, restricted to comminuted products such as meat patties, hamburgers, and sausages (McWatters, 1977).

Studies mainly conducted in the Soviet Union have shown that incorporation of pea protein in the formulation of liver sausage at the 5% level enhances the sausage quality and facilitates the exclusion of some conventional additives. The use of pea flour at a level greater than 10% has been found to produce a strong pea flavour, but the nutritional value of the products has been improved compared to unsupplemented sausages. It has been estimated that the use of pea flour in the meat industry in the Soviet Union would lower the industrial expenditure for sausage production by 20-25% (Owusu-Ansah and McCurdy, 1991). In North America, some attempts have been made in incorporating pea protein into meat patties (Vaisey *et al.*, 1975; McWatters and Heaton, 1979; McWatters, 1977). In these studies pea protein performed well in all quality attributes, except that the aroma and flavour of the products were judged to be inferior to their respective control samples.

Imitation Milk and Milk Replacement Products

Few studies have been carried out on the use of pea proteins for preparing milk substitutes or milk replacers directly for human use or as an ingredient in foods. Attempts to produce imitation milk from pea and other legumes were made by Sosulski *et al.* (1978). Taste panel evaluation of the imitation milk from all the legumes rated

poorly in taste and odour compared to cow's milk. The milk prepared from field peas was the second least preferred milk among the ten legumes evaluated. In addition, care must be exercised in the use of vegetable milks as they are noted to be low in calcium and zinc (Caygill *et al.*, 1981).

Patel *et al.* (1981) evaluated the potential of pea protein concentrate-whey blend as a nonfat dry milk substitute for baking. They found that incorporation of 6% of this pea protein-whey blend into bread did not significantly change the quality of the bread, as compared to breads prepared from nonfat dry milk and soy flour-whey blends.

Textured Products

Texturisation of plant proteins is accomplished by processes in which the structures of the proteins are altered to reticulate forms (Owusu-Ansah and McCurdy, 1991). The texturisation process that has been applied to pea protein is wet spinning (Gallant *et al.*, 1984; Culioli *et al.*, 1986). In this procedure, which is commonly applied to soybeans, a protein concentrate is solubilised in an alkaline solution and then pumped through a spinneret (50-250 μm in diameter) into a coagulation bath containing acid and salt (2-20% NaCl) to yield insoluble fibers. The fibers are then washed, neutralised and spin-dried. They can also be immersed in binders (e.g. gluten, hydrocolloids) and coagulated by heat setting, drying, or pH change. These may be used as simulated meat products. Currently this process has had limited commercial exploitation due to high operational costs and the attendant effluent disposal problems.

Miscellaneous Products

Field pea flour and protein have been studied for potential use in other food applications, including protein curd, soup and snack foods. Gebre-Egziabher and Sumner (1983) prepared and evaluated a simulated tofu product from field peas. They found that the pea curd was softer than that of the soybean and the yield was lower. However, both had comparable amino acid composition and the flavour was also rated as similar. Pea soup is a common dish in various homes and lunch cafeterias (Owusu-Ansah and McCurdy, 1991). The nutritional value and overall acceptability of the pea

soup has been found to be enhanced by supplementation with 5% sunflower protein. For snack food manufacture, pea flour or concentrate (with or without additional ingredients) was prepared into a thick slurry (Panchuk *et al.*, 1975; Panchuk *et al.*, 1979). The slurry was dried and allowed to build up into a multi-laminar sheet. As the sheet was dried and the starch in it was gelatinised, the sheet was cut into chip sizes and fried into puffed, well-textured chips. The flavour of the product could be tailored by the addition of external flavours. Despite these earlier studies, the utilisation of field pea proteins in these ways has not been extensively studied.

2.6. Chapter Review and Summary

Field pea is a well established crop around the world and the production has been increasing in Australia during the recent decades. Field pea proteins contain high levels of lysine which is potentially significant in balancing the deficiencies of this essential amino acid in cereal-based diets. In recent years, interest in plant proteins for feed and food has led to the evaluation of field peas as a high protein crop. Some research work has been undertaken on the production of field pea proteins, starch and fiber as food ingredients in Europe and Canada. However, none of these attempts have resulted in a commercial scale application of pea ingredients in the food industry. Currently field peas are still mainly used for animal feeding.

The major drawback of pea supplemented products is the poor acceptability which results from the unsuitable functional properties of the proteins in some applications. Compared with soybean, which has dominated the food ingredient market for a long time, field pea protein has not been investigated systematically in terms of its fractionation, modification and application into the food industry. Few reports are available on the large scale isolation and characterisation of proteins from field peas. Limited research has been carried out on some of the physico-chemical properties of field pea proteins and their potential food applications. However, most of the data has been obtained from isolates produced on a very small scale and some results are conflicting and require confirmation. There is very little data which could illustrate the

factors affecting the functional properties of pea proteins in food system. In addition, there is a lack of fundamental understanding of the structure-functionality relationships of plant proteins. Accordingly, the purpose of this project has been to investigate the large scale isolation of field pea proteins and to thoroughly assess the functional properties of the isolates for food applications. The characterisation of the proteins and the use of chemical modification for enhancing the functional behaviour of the proteins have also been the goals of the current study. These should provide detailed information on the chemistry, nutrition and technology of field pea proteins which will have considerable potential for scientific and economic significance.

CHAPTER 3

Materials and Methods

CHAPTER 3

Materials and Methods

3.1. Materials and Proximate Analyses

3.1.1. Materials

Dehulled, split yellow peas (Dunn type - approx 250 kg) were obtained in Victoria, Australia and a flour was produced by grinding the seeds in a local commercial plant. The mean particle diameter of the flour was 21.4 μm and all of the particles were less than 600 μm in diameter. The procedure for particle size analysis is described in section 3.3.2.2 and the results of the particle sizes of the flour are given in Table 5.3. The flour has been used to isolate field pea proteins on a laboratory scale as well as on a pilot scale.

3.1.2. Proximate Analyses

All procedures were performed in triplicate and mean results are presented. Proximate analyses for chemical composition were carried out according to AACC (1983) procedures for moisture (air-oven method 44-15A), crude protein (method 46-13), crude fat (method 30-25), crude fibre (method 32-10), total ash (method 08-03).

The protein content was determined using a Tecator Kjeltec System consisting of the digester (Model 1015 for 20 tubes) and the distillation unit (Model 1002) including an alkali tank. 0.5 g field pea flour or 0.2 g field pea protein isolate were weighed into the tubes and digested with concentrated sulphuric acid in which process the organic nitrogen was converted to ammonium ions. In the steam distillation procedures, the liberated ammonia was released by the addition of 4 M NaOH solution and trapped into 4% boric acid. The excess boric acid solution was then titrated against a 0.1 M standard HCl solution. The protein content was calculated as N × 6.25.

For moisture analysis, an accurately weighed sample (2 g) was dried at 130°C for 1 hour and cooled before weighing to constant weight. Analysis of fat content was based on 5 g samples which were extracted with petroleum ether in a Soxhlet type flask. The ash content was measured by weighing 2 g sample into a crucible and placing in a muffle furnace at 600°C for 2 hour. The crucible was cooled in a desiccator and weighed to obtain the ash content. Crude fibre determined as the loss on ignition of dried residue remaining after digestion of 2 g sample with 1.25% H_2SO_4 and 1.25% NaOH. Carbohydrate content was calculated by difference.

3.2. Laboratory Preparation of Field Pea Protein Isolates and Fractions

3.2.1. Isolation of Field Pea Proteins

3.2.1.1. Extraction at Different pH and Recovery by Iso-electric Precipitation

Field pea flour (100 g) was suspended in distilled water (1:5 w/v), adjusted to pH 2, 7, or 9 by using 1 M NaOH or 1 M HCl and stirred for 1 hr. The slurry was centrifuged at 8 000× *g*, 20°C for 25 min (Centrifuge Model J2-HS, Rotor JA-14, Beckman, U.S.A.). The solution was then filtered through Whatman paper (No. 541) and the pH adjusted to 4.5. The white coloured precipitate was centrifuged again and the protein obtained was neutralised and freeze dried (Freeze Drier DynaVac. Model FD 300, Australia). The dried samples were ground by using a coffee grinder (Kenwood, Model A979) and the resultant flour passed through a 600 μm screen (Test Sieve No.30, Endecotts, England).

3.2.1.2. Micellisation Procedures

Field pea flour (50 g) was dissolved in 0.5 M NaCl solution (1:10 w/v), adjusted to pH 7.0 (the pH of the slurry was 6.6 without adjusting) by using 1 M HCl and stirred for 1 hr. The mixture was centrifuged at 8 000× *g*, 20°C for 25 min (Model J2HS, Beckman). After filtering with Whatman (No. 541) paper, the solution was passed through a Minitan ultrafiltration system (Minitan Membranes, 4 plates, MW cut-off at 5 kDa, type NMWL Low Binding Regenerated Cellulose, Millipore Corp., Bedford, U.S.A.). When the

protein solution was concentrated to approximately half of the original volume (about 5 hr), the content was transferred into dialysis tubes (Spectra/Por Membranes, No.1, MW cut off at 6 -8 kDa, U.S.A.) and dialysed at 4°C against deionised water for 72 hr. As the ionic strength was reduced, the protein in the solution was precipitated as a micellar structure. The content in the dialysis tube was then centrifuged at 10 000× *g* and the pellet protein obtained was freeze-dried.

3.2.2. Fractionation of Field Pea Proteins by Osborne Procedures

3.2.2.1. Globulins and Albumins

Several buffers were used to extract globulins and albumins, based on published procedures (Rose *et al.*, 1992; Taylor *et al.*, 1984; Sauvaire *et al.*, 1984). Field pea flour (20 g) was suspended 1:10 (w/v) in salt solutions (0.5 M NaCl, 0.86 M NaCl, 1 M NaCl at pH 7) or buffer solutions (0.2 M NaH_2PO_4+ 0.2 M Na_2HPO_4, 0.1 M NaH_2PO_4 + 5% K_2SO_4, 0.1 M Citric acid + 0.2 M Na_2HPO_4 at pH 7) and stirred for 1 hr at room temperature and centrifuged at 10 000× *g* for 20 min. A second extraction was carried out with the same agent and the supernatant collected and dialysed at 4°C against deionised water for 5 days; dialysate was changed every day. The contents of dialysis tubes (Spectra/Por Membranes, No.1, MW cut off at 6 -8 kDa, U.S.A.) were centrifuged at 10 000× *g* for 20 min and the pellet (globulin fraction) was freeze-dried. The supernatant (albumin fraction) was concentrated by passing through ultrafiltration membranes (4 plates, MW cut-off at 5 kDa, Millipore Corp., Bedford, U.S.A.) followed by freeze drying.

3.2.2.2. Prolamins and Glutelins

After albumins and globulins were extracted by using 0.5 M NaCl, the subsample from the centrifuge was extracted (1:10 w/v): under magnetic stirring for 1 hr for prolamin fractions. Four different reagents were used (a) 70% ethanol; (b) 70% ethanol+0.1% dithiothreitol (DTT); (c) propan-2-ol+0.06% DTT; (d) butan-1-ol+0.05% DTT. The mixture was centrifuged at 12 000× *g* for 20 min and the extraction procedure was

repeated. The combined supernatants were dialysed against deoinised water for 5 days at 4°C and then freeze-dried.

For glutelin extraction, the residue from the prolamin-alcohol solutions was re-extracted twice with 0.1 M NaOH. The combined supernatants following centrifugation were adjusted to pH 4.5. The resulting micellar system was centrifuged at 12 000× *g* for 20 min and the pellet obtained was neutralised and freeze-dried to give the glutelin fraction.

3.2.3. Characterisation of Protein Fractions

3.2.3.1. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the procedure of Laemmli (1970) using a Bio-Rad Mini-Protein II Dual slab cell (Bio-Rad, U.S.A.). Protein samples (1 mg/mL) were dissolved in 0.1 M Tris-HCl, pH 6.8 containing 2% (w/v) SDS (Sodium Dodecyl Sulphate). Reduction of disulphide bridges was carried out using 2-mercaptoethanol (2-ME) (5% v/v) and heating for 2 min at 100°C. The separating gel and stacking gel were 12.5% and 4% polyacrylamide, respectively. The formulations for separating and stacking gels are shown in Table 3.1.

Table 3.1 Formulation for Separating and Stacking Gels (for One Mini-Protein Gel)

Reagents	Separating Gel (12.5%)	Stacking Gel (4%)
Stock 30% Acrylamide	2.08 mL	0.33 mL
1.875M Tris-HCl, pH 8.8	1.00 mL	--
1.25M Tris-HCl, pH 6.8	--	0.25 mL
Distilled Water	1.82 mL	1.90 mL
10% SDS	75 µL	25 µL
10% APS*	25 µL	5 µL
TEMED	5 µL	2.5 µL

*APS- Ammonia persulphate

A ten well comb was used and 10 μL each of the samples was applied to the corresponding wells by using a Hamilton syringe. The standard proteins used (Sigma Chemical Co., St. Louis, Mo) were: phosphorylase b (MW 94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Electrophoresis was conducted at a constant voltage of 130 volts with a Bio-Rad Power Supply (Model 200/2.0) for approximately 1.2 hr.

The gels were stained with Coomassie Blue R250 (0.5%) in a solution of ethanol/acetic acid/water (3.3/1/5.7, v/v/v) for 30 min and then destained with a solution of ethanol/acetic acid/water (3.5/1/5.5, v/v/v) until the background of the gel was clear. The gels were either photographed or preserved with the Gel Bond PAG film (Pharmacia LKB, Uppsala, Sweden) for densitometric scanning of the gel at a later stage.

3.2.3.2. Two-Dimensional Gel Electrophoresis

Iso-Electric Focusing

This was performed by the Pharmacia method (Pharmacia LKB Biotechnology, Notes for Immobiline DryStrip Kit for 2-D Electrophoresis). Protein samples (1 mg/mL) were first subjected to iso-electric focusing with the Immobiline DryStrips in the pH range of 4.0-7.0. The sample solution was composed of 48% (w/v) urea, 2% (v/v) 2-ME, 2% (v/v) Pharmalyte 3-10, 0.5% (v/v) Triton X-100 and a few grains of Bromophenol blue. 40 μL samples (unheated) were applied to each of the sample cups of the isoelectric focusing unit (Muitiphor II Electrophoresis Unit, LKB Bromma Model 2117, Sweden). For running 8 strips, the power and total running time were set into three stages (Bio-Rad Computer Controlled Electrophoresis Power Supply Model 3000Xi): 1, 300 Volts, 8 mA and 3 hr; 2, 2 000 Volts, 8 mA and 5 hr; 3, 3 000 Volts, 8 mA and 16 hr. The temperature was maintained at 10°C during electrophoresis by using a water circulator (Grant, Cambridge, England).

SDS-PAGE

When the iso-electric focusing run was complete, The sample strips were equilibrated with 0.05 M Tris/HCl (pH 6.8, with 36% w/v Urea, 30% v/v Glycerol and 1% w/v SDS) twice (each for 10 min). For the second dimension, SDS-PAGE was performed (Bio-Rad, Protein II xi cell, CA 94547) and the gel gradient was prepared at polyacrylamide concentrations of 8-18%. The recipe for stacking gel (4%) was the same as shown in Table 3.1. A one well comb was used and one equilibrated strip was placed into the well after the stacking gel was set up. Therefore for running 8 sample strips, 8 SDS-PAGE gels were needed in the second dimension. Electrophoresis was carried out at a constant current of 20 mA for each gel for 3-4 hr. The temperature was maintained at 10°C during the process. A 2-D SDS-PAGE standard was first applied to iso-electric focusing and then run on the second dimension for estimating the pI ranges of the samples. The pI values for the standard proteins (Sigma Chemical Co., St. Louis, Mo) are: soybean trypsin 4.5, bovine serum albumin 5.07, bovine muscle actin 5.5, bovine carbonic anhydrase 6.0, conalbumin 6.6, equine myoglobin 7.0. The staining and destaining procedures used were those described in section 3.2.3.1.

3.2.3.3. Gel Scanning

An Enhanced Laser Densitometer (LKB Ultroscan XL) was used to scan the gels and estimate the ratio of one particular band to the total protein subunits and the molecular weight of different protein bands. A standard curve was established by scanning the lane of low range molecular markers (section 3.2.3.1) in the gel. The MW of the major bands of the samples were calculated by the computer software based on the standard curve.

3.2.3.4. Amino Acid Composition

Hydrolysis of the Protein Samples

Freeze-dried protein fractions (30 mg per sample) were weighed into hydrolysis tubes (Kontes Hydrol Tube SZ 8, 19×100, New Jersey, U.S.A.). 10 mL 6 M HCl was added followed by degassing for 30 min using a vacuum pump. The dissolved gases were removed by evaporating the contents of the tubes under vacuum by freeze-thawing twice in an ice bath containing dry ice and ethanol. This involved first placing the tubes in a

container which was filled with ethanol. When the dry ice was added in, the contents of the tubes were frozen. After dissolution, the tubes were shaken lightly and the air was again removed under vacuum. The tubes were then sealed under vacuum and placed in a 110°C oven for 24 hr. When the hydrolysis was completed, the hydrolysate was filtered through a glass filter (0.2 μm, Gelman Sciences Acrodisc 32) from a 10 mL syringe. The filtered hydrolysate was collected into an eggplant-shape flask and evaporated to dryness in a rotary evaporator, under vacuum (15-20 mmHg, 50-60°C). 10 mL of water was added and re-evaporated to dryness repeatedly to remove the HCl. The residue was dissolved in 5.0 mL deionised water for amino acid analysis via high-performance liquid chromatography (HPLC).

The hydrolysis method described above cannot be applied for the analysis of tryptophan and sulphur amino acids, i.e. methionine, cystine and cysteine, due to their instability during acid hydrolysis. Tryptophan was not determined in the current study. For analysis of sulphur amino acids, the sample was oxidised with performic acid prior to the HCl hydrolysis in order to convert methionine into methionine sulphone, and both cystine and cysteine into cysteic acid quantitatively in the form of their residues in polypeptide chains. The procedure of sample preparation and oxidation with performic acid was that of Spindler *et al.* (1984). The excess of performic acid was destroyed by conversion to acetic acid using addition of 0.7 mL 48% hydrobromic acid. After the mixture was stirred for approximately 0.5 hr, the residue was evaporated to dryness using a rotary evaporator under vacuum (15-20 mmHg, 50-60°C). The oxidised sample was then hydrolysed with 6 M HCl as described earlier.

Amino Acid Analysis by High-Performance Liquid Chromatography (HPLC)

Standard amino acids were prepared separately as stock solutions in 100 mL volumetric flasks and the concentration was 6×10^{-3}M. Preliminary studies showed that field pea proteins do not contain glutamine (an amino acid rarely found in plants), therefore glutamine was chosen as the internal standard when the amino acid composition of the sample was analysed. Hydrolysed sample or each amino acid standard was diluted with

0.1 M $NaHCO_3$ buffer (sample or standard: internal standard: buffer = 1: 1: 9, v/v/v) before the sample vials were placed into the auto-sampler (Varian Instrument 9100).

Amino acid composition was analysed by HPLC (Varian Instrument 9010) on a C-18 column (Phenomenex, Spherex 5 C18, 250×4.60 mm). 9-Fluorenylmethyl chloroformate (FMOC) (Sigma Chemical Co.) was used as a precolumn derivatising agent for amino acid analysis since the reaction is complete in minutes and provides a hundred-fold increase in sensitivity over colorimetric determination. The FMOC solution was prepared fresh at 4 mM in dry acetone. The detection of FMOC derivatives was carried out using a Fluorescence Detector (Varian 9070). The excitation and emission wavelengths were 264 nm and 340 nm, respectively. The mobile phase was the gradient of three solutions: A= 0.02 M sodium citrate plus 0.005 M tetramethyl ammonium chloride (pH 2.85, adjusted with H_3PO_4), B= 80% solvent A plus 20% methnol (pH 4.5, adjusted with H_3PO_4), C= acetonitrile. Solvents A and B were filtered through Nylon 66 membranes (0.45 μm, Alltech) and degassed under vacuum for 30 min prior to their introduction to the HPLC column. The running conditions followed the instruction for Varian Liquid Chromatography and the temperature was maintained at 32°C with a column temperature controller. The mobile flow rate was 1.4 mL/min and the running time for each sample was 45 min.

Prior to the injection of the samples, 20 μL diluted sample solution or standard was mixed with 20 μL FMOC. Pentane reagent (60 μL) was added in order to concentrate derivatised sample in the aqueous layer since pentane would extract 60-70% of the acetone present in the aqueous sample layer. 25 μL of derivatised sample solution was taken from the auto-sampler and a 10 μL loop was used so that 10 μL of each sample was injected into the column. All of the determinations were carried out in duplicate and the calculation of the amino acid concentration was based on the ratio of the internal standard to each standard amino acid and the ratio of the internal standard to each of the amino acids contained in the hydrolysed protein solutions.

3.2.3.5. Scanning Electron Microscopy (SEM)

Microscopy studies of the field pea protein samples were carried out on a Cambridge Instruments Stereoscan 90 scanning electron microscope. Specimens were prepared by placing some of the dry sample onto double-sided carbon tape and a thin layer of gold particles was deposited using a Magnetron Sputter Coater Model SC100M (Dynavac). Micrographs were stored on a compact disk.

3.2.4. Further Purification of Albumins and Globulins

3.2.4.1. Ion Exchange Chromatography and Gel Filtration

The procedure was performed according to Gueguen *et al.* (1984). Field pea flour (50 g) was suspended (1:10 w/v) in phosphate-citrate buffer (pH 7, 0.1 M sodium phosphate) and stirred for 1 hr. The mixture was centrifuged at 8 000× *g*, 20°C for 20 min. A DEAE Sepharose column (Fast Flow, 100×2.5 cm) was pre-equilibrated with extracting buffer. DEAE-Sepharose was a product of Pharmacia Fine Chemicals. The flow rate of the liquid through the column was controlled at 60 mL/hr with a Bio-Rad variable speed pump. The supernatant of the sample solution after centrifugation was applied and then eluted with the equilibrating buffer to eliminate the non-bound material. For further elution, a series of sodium chloride solutions was used sequentially (0.05 M, 0.25 M, 0.5 M, 1.0 M). A Bio-Rad Econo UV Monitor was used to detect the separation of the protein fractions with the absorbance wavelength at 280 nm. The chart was recorded by a Bio-Rad Econo Recorder (Model 1325) at the speed of 2 cm/hr. The pattern of the ion exchange chromatography is shown in Fig. 4.13. The different fractions collected were then pooled, concentrated or freeze-dried for protein electrophoresis analysis using a SDS-PAGE system. The procedure for SDS-PAGE has been described in section 3.2.3.1. The gel patterns of these different fractions from DEAE-Sepharose column are shown in Fig. 4.14.

For further fractionation of albumin and globulins, a gel filtration chromatography (Sephacryl 200S, Pharmacia Fine Chemicals) was used. The protein fractions collected from the DEAE-Sepharose ion exchange chromatography were pooled and concentrated

by ultrafiltration (MW cut-off at 5 kDa). Either a Millipore apparatus or Sartorius Disc Ultrafilters (50 mm holder) was used depending on the volume to be treated. 4 mL concentrated fractions (0.5 mg protein/mL) were chromatographed on the Sephacryl 200S column (100×2.5 cm) which was pre-equilibrated with Tris-HCl buffer (50 mM, pH 7.5), containing 0.1M KCl. Prior to the running of the samples, the column was calibrated with the standard proteins of Cytochrome C (MW 12.4 kDa, red in colour), Carbonic Anhydrase (29 kDa), Bovine Serum Albumin (BSA 66 kDa) and Alcohol Dehydrogensase (150 kDa) (Sigma Co.). The void volume (Vo) of the column was determined by the elution of Blue Dextran (MW 2 000 kDa). The elution volume (Ve) of each standard protein was recorded and a standard curve was plotted for Log of MW vs. Ve/Vo on semi Log paper. The elution buffer was Tris-HCl (50 mM, pH 7.5), containing 0.1 M KCl and the flow rate was maintained at 20 mL/hr.

When the unknown sample was applied to the gel filtration column, the elution volume (Ve) for different fractions was determined and the respective Ve/Vo was calculated. The MW of each respective fraction was determined from the calibration curve. For further characterisation, the different fractions collected from the gel filtration column were also pooled, concentrated or freeze-dried for protein electrophoresis analysis using SDS-PAGE, which has been described in section 3.2.3.1.

3.2.4.2. Preparative Electrophoresis of Albumin Fractions

Preparative Electrophoresis

Since the separation of albumin fractions by Sephacryl 200S gel filtration chromatography was not very effective, a preparative electrophoresis apparatus (Prep-cell Model 491, Bio-Rad) was applied. On this apparatus, proteins are electrophoresed vertically through a cylindrical sieving gel. As individual bands migrate off the bottom of the gel, they pass directly into an elution chamber consisting of a thin frit. A dialysis membrane (MW cut-off 6 kDa), directly underneath the elution frit, traps proteins within the chamber. Elution buffer enters the chamber around the perimeter of a specially designed gasket which results in an even flow of buffer into the elution frit. Buffer is drawn radially inward to an elution tube in the center of the cooling core, and out to

accessory equipment including peristaltic pump, ultraviolet (UV) monitor and fraction collector.

Approximately 2.0 mL of albumin fraction resulting from ion exchange chromatography after concentration (to 0.5 mg protein/mL) was taken. Sucrose (0.2 g) was added and dissolved, followed by addition of 80 μL SDS-PAGE loading buffer (section 3.2.3.1). The sample was applied to the Prep-cell apparatus and run overnight to collect the purified fractions (Bio-Rad, Prep Cell Instruction Manual). The separating gel (30 mL) and stacking gel (10 mL) were 7.5% and 4% polyacrylamide, respectively. The resultant fractions were collected and further characterised by SDS-PAGE (Bio-Rad Mini-Protein II Dual slab cell).

SDS-PAGE

A comb with 15 wells was used and the gel was stained with silver solutions. The procedure for SDS-PAGE was described in section 3.2.3.1. The SDS-PAGE pattern of albumin fractions from preparative electrophoresis shown in Fig. 4.16.

Silver Staining

For silver staining, the procedure of fixation, incubation, washing, silver reaction and developing was that described by Pharmacia (Instruction for Immobiline DryStrip Kit for 2-D Electrophoresis, Pharmacia LKB Biotechnology). First the mini-slab gels were immersed in fixation solution (ethanol/acetic/water = 4/1/5, v/v/v) for 30 min. Then the gels were placed incubation solution (75 mL ethanol, 1.3 mL glutardialdehyde, 17.0 g sodium acetate trihydrate, 0.50 g sodium thiosulphate, made up to 250 mL with distilled water) for another 30 min. After the gels were washed with distilled water three times (each time 5 min), silver solution (0.25 g silver nitrate, 50 μL formaldehyde, made up to 250 mL) were poured in. When the reaction was completed (40 min), the gels were left in developing solution (6.25 g sodium carbonate and 25 μL formaldehyde, made up to 250 mL) until the bands were clear. The gels were immediately placed into the stop solution (3.65 g EDTA-Na_2 dihydrate, made up to 250 mL) for 5-10 min. The gels were either photographed or preserved with the Gel Bond PAG film after washing with distilled

water for 10 min.

3.3. Pilot Plant Preparation of Protein Isolates

3.3.1. Processing Procedures

The pilot scale isolation of field pea proteins was conducted in the pilot scale facilities of the Australian Food Industry Science Centre (Afisc), Werribee, Victoria. The flowcharts of the processes to prepare the protein isolates extracted with alkaline solution and salt solution are presented in Fig. 5.1 and Fig. 5.2, respectively.

3.3.1.1. Extraction with Alkaline Solution

Field pea flour (190 kg) was mixed with 950 L filtered water (MEMCOR Self cleaning Crossflow Microfiltration System, Model 910064-000, MEMTEC Limited) at 40°C in a 1 000 L vat with vigorous agitation. The pH was adjusted to 9.0 using 4 M NaOH. After 1 hour, the mixture was pumped to a decanter centrifuge (Westfalia Separator AG, Model D-59302 Oelde, F.R. Germany) where most of the carbohydrate solid was separated from the protein solutions. In order to further purify the proteins, the solution was then passed through a clarifier centrifuge (Westfalia Separator AG, Model D-4740 Oelde, F.R. Germany) to remove the fine particles of the carbohydrates. For checking the solids content in the solution, 50 mL liquid was taken in a graduated centrifuge tube and centrifuged at 2 000× *g* for 5 min. The solid content was estimated approximately from the proportions of the liquid layer and solid layer in the centrifuge tube. This procedure was important in the determination of the conditions for use of the clarifier as well as the standardisation of parameters for the separation procedures. The control of the running time for the clarifier centrifuge in the current study was: separation- 2.00 min, preflushing- 0.3 sec, partial ejection- 3.5 sec, post flushing-14 sec. The flow rate was 200 L/hr.

The extract of the proteins was then adjusted to pH 4.5 by using 4 M HCl and mixed for 0.5 hr. The acid precipitated curd was concentrated by passing the mixture to the

clarifier and the whey discarded. The curd was washed with 200 L filtered water and reclarified. The product was then neutralised to pH 7 using 4 M NaOH with mild agitation and dried either by spray drier (NIRO FSD-4, Australia) or by freeze drier (DynaVac. Model FD 300, Australia). Prior to drying, the solids content of the protein slurry was 8%. The major parameters for Niro spray drier were: *atomisation,* centrigugal at 22 500 rpm, distribution disk hole aperture 1.2 mm; *materials temperature*, in balance tank 20°C, preheat prior to drying 60°C; *air temperature*, inlet 185°C, outlet 80°C. The power was collected primarily at the base of the drier and some fines were obtained from the cyclone. Following freeze drying, the protein isolates were reground in a hammer mill (Cereal Mill 6000, Newport Scientific Pty Ltd., Sydney, Australia) to pass a 400 micron screen.

3.3.1.2. Extraction with Salt Solution

For protein isolation with salt solution, 60 kg field pea flour was mixed with 600 L 0.5 M sodium chloride solution and the pH was adjusted to pH 7. After 1 hr agitation, the slurry was passed through the decanter to separate the protein extracts from the carbohydrate. The protein solution was purified by clarification and then passed through the ultrafiltration and continuous diafiltration system (DDS Model 37, 6.6 m^2, Pasilac-Danish Turnkey Dairies Ultrafiltration Plants, Denmark) to remove salt and concentrate the proteins. The operation was conducted at 48°C. The membrane used was plate and frame, polysulphone GR 61 PP with a molecular weight cut-off at 20 kDa. The product was either spray dried or freeze dried. The determination of salt content in the product followed the procedure of Dixon (1965). The principle is the titration of Cl^- in the sample solution with $AgNO_3$. The preparation of the indicator involved dissolving potassium chromate and potassium dichromate in water ($K_2CrO4/K_2Cr_2O_7/H_2O$ = 6/1/93, g/g/g).

3.3.2. Analysis of Physical Properties of Protein Isolates

3.3.2.1. Colour

Colour of flours was measured with a Minolta Chromameter (Model CR-300, Minolta Camera Co., Osaka, Japan) on the flours. Colour is expressed as L^* =

whiteness/brightness, $a^*(+)$ = redness, $a^*(-)$ = greenness, b^* (+) = yellowness, b^* (-) = blueness. The instrument was calibrated using a standard white tile with the values L*= 98.03, a *(-) = 0.01 and b *(+) = 1.65. The final result for colour was the mean of eight readings of each sample.

3.3.2.2. Particle Size Analysis

Particle size measurement was performed on a Malvern Mastersizer-X particle size analyser (Malvern Instruments Ltd., Worcester, UK). The sample dispersant was butan-2-ol and the results are volume based.

3.3.2.3. Scanning Electron Microscopy (SEM)

Microscopy of the pilot scale pea protein samples involved procedures similar to those described in section 3.2.3.5.

3.3.2.4. Differential Scanning Calorimetry (DSC)

Field pea protein samples (approximately 500 mg) were accurately weighed into plastic vials. 2.0 mL buffer (0.05 M phosphate, pH 7, plus 0.1 M NaCl) was mixed into the protein samples with a spatula. The mixtures were held at 4°C for 1 hr, then removed from the chiller and accurately weighed (approximately 450 mg) into the stainless DSC vessels. The mixtures were placed in the DSC (SETARAM, M DSC3) and allowed to equilibrate to 25°C prior to heating. Buffer was used as a reference. At least two repeats for each sample were analysed. These samples were heated from 25 to 110°C at 1°C/min and then cooled to 25°C at 3^0C/min. A re-cycling of each sample was undertaken (reheating to 110°C at 1°C/min).

Denaturation profiles were integrated by computer software. Selection of the baseline so that the area above the minima could be integrated was subjective, in particular, where the baseline was not a straight and/or horizontal line. The enthalpy data (ΔH) for individual peaks was calculated by separating dual minima with a straight vertical line from the central maximum.

3.4. Determination of Functional Properties of Protein Isolates

All of the measurements of the functional properties of the proteins were performed at least in duplicate determinations. In addition to the assessment of field pea protein samples, the functionality of a commercial soy product (Supro 500E, Supro Co., U.S.A.) with a protein content 87.4% was also determined for comparison purposes.

3.4.1. Determination of the Protein Solubilities

Protein samples (20 mL, 0.4%, w/v) were suspended in a 25 mL beaker under pH 2, 3, 4, 5, 6, 7, 8, 9, 10 at room temperature and stirred for 30 min. The respective slurries were then centrifuged at 4 000× *g* for 20 min. The soluble protein contents were determined by the method of Lowry *et al.* (1951) following appropriate modifications (Britz, 1978). 50 μL of protein solution was diluted to 500 μL with deionised water. 0.5 mL of reagent A (0.1 mL of 5%, w/v, $CuSO_4$, 0.9 mL of 1%, w/v, potassium tartrate, 10 mL of 10%, w/v, Na_2CO_3 in 0.5 M NaOH) was added in. After 10 min at 37°C, 1.5 mL of solution B (1 mL Folin-Ciocalteu reagent plus 10 mL deionised water) was added and the solution immediately mixed by vortex. Absorbance at 680 nm against reagent blank was recorded after incubation at 52°C for 20 min. Reagents A and B were prepared immediately before use.

Standards containing 0 to 100 μg of protein were prepared from 0.1% BSA solution and the standard curve was plotted on graph paper as μg BSA vs absorbance at 680 nm. The soluble protein concentration of the unknown samples was calculated from the standard curve and the appropriate dilution factor.

3.4.2.Water Absorption

3.4.2.1. Relative Humidity Method

The equilibrium moisture contents were determined by using constant relative humidity environments similar to those described by Schaffner and Beuchat (1986). Saturated salt solutions were placed in desiccators and allowed to equilibrate at 21°C; equilibrium relative humidity (ERH) values were estimated from those reported by Rockland (1960). The salts and their ERH values used were CH_3COOK (23%), K_2CO_3 (44%), $CuCl_2$ (68%), KCl (85%) and K_2SO_4 (97%). Duplicate 1g samples in small petri dishes were weighed and placed in desiccators. The desiccators were evacuated for 10 min to facilitate equilibration. At the end of 10 days, samples were removed and immediately weighed. The samples were then placed in a drying oven under 50 mmHg vacuum at 65°C for 48 hr. The equilibrium moisture content was calculated from the weight of the dried samples and the weight gain of the samples after equilibration.

3.4.2.2. Excess Water Method

250 mg samples were combined with 5 mL distilled water in 10 mL centrifuge tubes. Slurries were thoroughly mixed and kept for 30 min at room temperature and then centrifuged at 4 000× *g* for 20 min. The supernatant water was removed and the tubes were reweighed. The water absorption capacity is expressed as grams of water bound per gram of sample.

3.4.3. Oil Absorption

The method was similar to that of water absorption (excess water method) except that 5 mL canola oil was used instead of distilled water. The oil absorption capacity is expressed as grams of oil bound per gram of sample.

3.4.4. Emulsifying Properties

3.4.4.1. Emulsifying Capacity

The emulsifying capacity of protein samples was determined by a modified method of Webb *et al.* (1970). 50 mL 0.2% (w/v) protein dispersion was thoroughly mixed

for 20 min. Canola oil (Crisco brand) was delivered from a 250 mL separating funnel by Silicone tubing to a peristaltic pump (Pharmacia, Model p-3) set at a flow rate of 30 mL/min. The oil was emulsified with protein solution by using a Polytron (Kinematica AG PT2000, Switzerland) set at medium speed (2.5 on a scale of 5). The end point was detected by a sudden increase in electrical resistance of the emulsion using an ohmeter (Multitester YF-206) which was attached to electrodes suspended in the emulsion mixture. The result was expressed as mg of oil emulsified per 100 mg of protein.

To study the effect of pH on emulsification, the 0.2% protein solution (20°C) was adjusted to the desired pH value (pH 2, 4, 6, 8, or 10) using 1 M HCl or 1 M NaOH prior to preparing the emulsions.

To study the effect of Salt (NaCl) on emulsifying capacity, 0.2% protein solution (pH 7, 20°C) with different NaCl concentration (1, 2, 3, 4, 5, 10%, w/v) was used to prepare the emulsion.

The effect of temperature on emulsifying capacity was also studied. The 0.2% protein solution (pH 7) was kept in a water bath (20, 40, 60, 80, or 95°C) for 30 min and cooled to room temperature prior to preparation of the emulsions.

3.4.4.2. Emulsifying Stability

Emulsion stability was determined as the amount of water released from the emulsions following centrifugation (modified method of Johnson and Brekke, 1983). 10 mL 0.2% (w/v) protein solutions and Canola oil (80% emulsifying capacity, approximately 22 mL oil, Crisco brand) were emulsified by using a Polytron for 1 min at medium speed (2.5 on a scale of 5). Each emulsion was placed in a 50 mL centrifuge tube and stressed by centrifugation at 100× *g* (GS-15R Centrifuge, Beckman) for 2 min at 20°C. Emulsion stability was expressed as the percent water retained by the emulsion after centrifugation:

$$\%\text{Water retained} = \frac{(\text{Total mL water in emulsion} - \text{mL water released}) \times 100}{\text{Total mL water in emulsion}}$$

3.4.5. Foaming capacity and stability

Foaming characteristics were determined by whipping the protein solutions in a Kenwood chef mixer at max speed for 5 min. Prior to whipping, 200 mL protein solution (1.0%, w/v) was mixed by stirring for 30 min. The solution was then adjusted to pH 7.0 with either 1 M NaOH or 1 M HCl and transferred to a stainless Kenwood mixing bowl (4 L). The sample was whipped with a wire whisk at maximum speed for 5 min. The foams and the residue of the liquid were then immediately transferred to 1 000 mL graduated cylinders and the total volume at time intervals of 0.0, 0.25, 0.50, 1.0, 2.0 and 3.0 hr was noted. The effect of pH on foaming properties was determined by adjusting the 1.0% protein solution to the desired pH value (2, 4, 6, 8, or 10) at room temperature prior to whipping. To study the effect of the temperature, the protein samples (1.0%, pH 7.0) were kept in a water bath at 20, 40, 60, 80, or 95°C for 30 min and cooled to room temperature prior to whipping. To study the salt effect on the foaming properties, protein samples (1.0% solution, pH 7.0, 20°C) with different NaCl concentrations (0.25, 0.5, 1.0, 2.0, 4.0%, w/v) were used. The foam capacity was expressed as the volume increase (%) calculated from the following equation:

$$\text{Volume increase}(\%) = \frac{(\text{Volume after whipping in mL} - \text{Volume before whipping in mL}) \times 100}{\text{Volume before whipping in mL}}$$

3.4.6. Protein (Surface) Hydrophobicity

Protein hydrophobicity (So) was determined by the method of Kato and Nakai (1980) after modifications. 10 mg *cis*-parinaric acid (Molecular Probes, special packaging, U.S.A.) was dissolved in absolute ethanol to a concentration of 3.6 mM. During this

step, purging with nitrogen was carried out. Butylated hydroxyanisole (BHA) was added into the solution (10 μg/mL) to prevent oxidation. 10 mL 0.2% (w/v) protein solution dissolved in 0.05 M phosphate buffer, pH 7.4, containing 0.002% SDS was diluted with the same buffer to obtain protein concentrations ranging from 0.0025 to 0.1% (w/v). *cis*-Parinaric acid (20μL) was added to one set of test tubes which contained 4 mL each of diluted protein solutions. Another set of tubes included the respective diluted protein solutions but without the addition of *cis*-parinaric acid. An Epson Perkin-Elmer Luminescence spectrometer (Model LS 50, UK) was used to measure the relative fluorescence intensities of the *cis*-parinaric acid-protein conjugates using a slit width of 5.0 nm. An excitation wavelength of 325 nm and an emission wavelength of 420 nm were selected. The fluorometer was standardised by adjusting the reading to 50% relative fluorescent intensity when 20 μL of *cis*-parinaric acid solution was added to 4 mL of n-Decane (Sigma Chemical Co.). The net fluorescence intensity was determined by subtracting the fluorescent reading of each sample without *cis*-parinaric acid from the reading with *cis*-parinaric acid. The So value was calculated as the initial slope of the curve of fluorescence intensity vs protein concentration plot. One example of these curves with the initial slope is shown in Fig. 3.1 for the field pea protein sample extracted with alkali on the pilot scale.

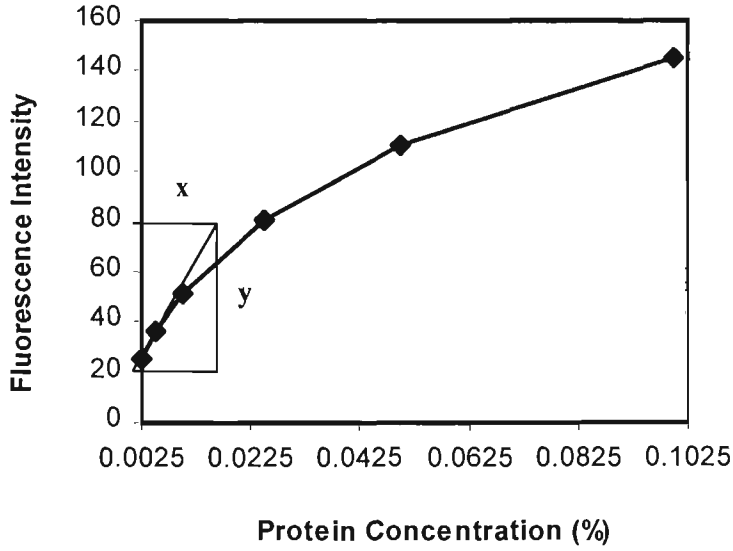


Figure 3.1 Example of the curve for calculation of hydrophobicity (So). So= y/x.

To study the effect of heating on surface hydrophobicity, the protein solutions were kept in water bath at different temperatures (20, 40, 60, 80, or 95°C) for 30 min and cooled to room temperature prior to dilution. So values were determined as outlined above.

3.4.7. Surface Tension

Surface tension of protein solutions was measured based on the Ring Method by using a Cambridge Tensiometer (Cat. No. 32231/D). The pointer reading was taken when the ring suddenly left the liquid. Duplicate readings of 0.2% (w/v) protein solutions were recorded.

3.4.8. Viscosity Measurement

Viscosity of protein solutions was measured using a Brookfield Digital Viscometer (Model DVII, Brookfield Engineering Laboratories, Inc. U.S.A.). All measurements were carried out by using a ULA (Ultra Low Adaptor). For studying the viscosity of protein solution at different concentrations, 20 mL of protein solution was prepared at 2, 4, 6, 8, 10, 15% (w/v) respectively in a 25 mL beaker and mixed for 20 min by using a stirrer. The protein solution was then transferred into the ULA and the viscosity was measured in units of centipoise (cP). The readings were taken after the spindle spined for 2 min at a range of speeds (0.6, 1.5, 3, 6, 12, 30, 60 rpm). The respective shear rate (sec^{-1}) was calculated as 1.224×N where N is the speed in rpm. In order to study the change in viscosity with time (thixotropic behaviour- a decrease in viscosity with time or rheopectic behaviour- an increase in viscosity with time), the viscosity of the fluids at a certain protein concentration was also recorded at a range of times (2 min to 30 min) at a constant shear rate.

To study the effect of pH on viscosity behaviours, 8% (w/v) protein solution (20°C) was adjusted to the desired pH value (pH 2, 4, 6, 8, 10) by 1 M HCl or 1 M NaOH prior to the viscosity measurement (shear rate at 73.44 sec^{-1}).

To study the effect of Salt (NaCl) on the change of viscosity, 8% (w/v) protein solution (pH 7, 20°C) with different NaCl concentration (0.5, 1, 2, 3, 4, 5, w/v) was used for the viscosity measurements (shear rate at 73.44 sec^{-1}).

The effect of temperature on viscosity characteristics of the protein solutions was also studied. Protein solution (8%, pH 7) was kept at temperatures of 20, 40, 60, 80°C in the ULA which was connected to a water circulator (Grant, Cambridge, England). The viscosity was recorded after the sample solution was equilibrated for 20 min at the shear rate of 73.44 sec^{-1}.

3.4.9. Gelation Properties

3.4.9.1. Gel Formation

The method of studying gelation properties of proteins was followed that of Boye *et al.* (1997) after modification. 20 mL field pea protein solutions (12.5, 15.0, 17.5%, w/v) were prepared in 25 mL beakers by dispersing appropriate amounts of protein in 0.1M phosphate buffer (NaH_2PO_4 and Na_2HPO_4, pH 7). The dispersions were thoroughly mixed by a glass rod and equilibrated for 30 min. The beakers were covered with aluminum foil to prevent moisture loss and were heated in a water bath at 97°C for 30 min. The heated samples were immediately put in a ice-box and left in a 4°C cool room for 24 hr. For studying the effect of pH on gel formation, samples at 15.0% protein concentrations in 0.1 M phosphate buffer (pH 7) were adjusted to pH 3, 4, 5, 6, 7, 8, 9 by using 1 M H_3PO_4 or 1 M NaOH prior to heating. In order to study the effect of salt on gel formation, appropriate concentrations of NaCl (1, 2, 3, 4, 5%, w/v) were added to the protein dispersions (15.0% protein concentration in 0.1M phosphate buffer, pH 7) and mixed before heat treatment.

3.4.9.2. Gel Strength Measurements

After the gels were cooled at 4°C for 24 hr, they were allowed to equilibrate at room temperature (approximately 20°C) for 1 hr prior to compression testing. Samples (30 mm

in diameter and 20 mm in length) were uniaxially compressed to 70% deformation using an Instron Universal Testing Machine (Series IX, Model 4465, England) with a 5 kN load cell. The gels were not taken out of the beakers since the gels were very weak and prone to collapse. The diameter of the plunger was 12.7 mm and the speed of the plunger was set at 40 mm/min. Gel strength reported in this study corresponded to the peak force during the first compression cycle (Bourne, 1978) and results are expressed in units of Newtowns (N).

3.5. Modification of Field Pea Proteins

3.5.1. Material

Field pea protein isolate that had been extracted with alkaline solution (pH 9) on a pilot scale (section 3.3.1.1) was used. The protein content of this product was 77.1% on a dry weight basis. This protein material was selected to undergo the chemical modifications with acetic anhydride (AA), succinic anhydride (SA) and $POCl_3$.

3.5.2. Modification Procedures

3.5.2.1. Acetylation and Succinylation

Field pea protein isolate (50 g) was suspended in distilled water (15%, w/v) at room temperature and stirred for 30 min. Acetic anhydride (density: 1.08 g/mL, Sigma) or succinic anhydride (Sigma) was added over a 1 hr period and the final treatment levels used were 0.1, 0.2, 0.4, 0.6, 0.8 g/g protein. These operations were performed in a fumehood. The pH of the slurry was maintained in the range of 7.5-8.5 by the addition of 4 M NaOH. The slurry was left for 2 hr and dialysed against distilled water at 4°C for 42 hr to remove the excess reagent. The chemically modified proteins were then recovered by freeze-drying. The dried samples were ground and the resultant flours were sieved through a 600 μm screen (Test Sieve No.30, Endecotts, England).

3.5.2.2. Phosphorylation

Field pea protein solution (25 g, 5%, w/v) was prepared and phosphorus oxychloride ($POCl_3$) (density: 1.645 g/mL, Sigma) was added dropwise into the solution at levels of 0.1, 0.2, 0.4, 0.6, 0.8 g/g protein. This operation was also carried out in a fumehood and an ice bath was used to prevent over-heating due to the reactions. The pH was maintained between 8.0-8.5 by using 4 M NaOH and the slurry was left for 1 hr. The pH was then adjusted to 4.2 to precipitate the proteins and the slurry was centrifuged at 8 000× *g* for 20 min. The phosphorylated protein was redissolved in distilled water, dialysed against water at 4°C for 24 hr and then freeze dried.

3.5.3. Characterisation of Modified Proteins

3.5.3.1. Extent of Modification

The modified trinitrobenzene sulphonic acid (TNBS) method of Habeeb (1966) was used to determine the extent of modification of field pea proteins. Sample (2 mg) was dissolved in 2 mL 0.1M sodium borate buffer (pH 9), and then 1 mL 0.025% TNBS was added into the solution and kept in a 52°C water bath for 2 hr. After cooling to room temperature, 1 mL of 10% SDS and 0.5 mL 1M HCl were added and the absorbance of the solutions was read at 335 nm against a reagent blank (Model 4054 UV/Visible Spectrophotometer, LKB Biochrom, UK). The absorbance of the control protein was set equal to 100% free amino groups. The degree of modification was calculated based on the decrease in absorbance and expressed as a percentage.

3.5.3.2. SDS Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the modified proteins was carried out according to the procedure described in 3.2.3.1. Polyacrylamide slab gels (12.5%) were prepared and the gels were stained with 0.5% Coomassie Blue R250. The standard proteins used (Sigma Chemical Co., St. Louis, Mo) were: phosphorylase b (MW 94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

3.5.3.3. Amino Acid Analysis

The procedure for the hydrolysis of the modified protein samples followed that described in section 3.2.3.4. The amino acid composition was also analysed by HPLC with FMOC as the precolumn derivatising agent (section 3.2.3.4).

3.5.3.4. *In vitro* Digestibility

The multienzyme method developed by Hsu *et al.* (1977) and Sathe *et al.* (1982) was used to determine the *in vitro* digestibility of the native and modified protein samples after slight modifications. 20 mL of aqueous suspension of sample (6.25 mg protein/mL) was adjusted to pH 8.0 with 0.1 M NaOH or 0.1 M HCl. The slurry was then incubated in a water bath at 37°C for 15 min. The multienzyme solution consisting of 1.50 mg trypsin (15 200 unit/mg, Sigma Chemical Co.), 3.58 mg chymotrysin (52 unit/mg, Sigma) and 0.51 mg peptidase (102 unit/g, Sigma) was prepared fresh and maintained in an ice bath. The pH of the enzyme solution was adjusted to 8.0 with 0.1 M NaOH or 0.1 M HCl. 2 mL of this solution was added to the sample suspension with constant shaking at 37°C. The pH change of the suspension was recorded at times of 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 min after the addition of the enzyme solution. The hydrolysis curve was plotted as the change of pH vs time (min). The resultant curves are shown in Chapter 7 (Fig. 7.3).

The *in vitro* digestibility was calculated according to the regression equation of Hsu *et al.* (1977): Y= 210.464 - 18.103X. Where, Y= *In vitro* digestibility (%), X= pH of the sample suspension after 10 min digestion with multienzyme solution.

3.5.4. Functional Properties of Modified Proteins

In order to study the effect of the extent of modifications on the functional properties of modified field pea proteins, acetylated, succinylated and phosphorylated samples at different levels of treatments (from 0.1 g to 0.8 g chemicals/g proteins) were prepared. The functional properties including solubility, water and oil absorption, emulsifying and foaming properties, viscosity and gelation characteristics were assessed and compared

with the native proteins. The procedures for determination of these functional properties were the same as those described in section 3.4.

In order to study the effects of pH, NaCl concentration and temperature on the functional properties of the modified proteins, samples which had been modified with 0.4 g succinic anhydride/g protein and 0.2 g acetic anhydride/g protein were selected. In addition, the protein concentration used for viscosity measurement was 4% for acetylated and succinylated samples instead of the value of 8% used for native proteins. This is due to the significant increase of the viscosities of the modified proteins and readings of some samples under particular conditions were beyond the range of measurement capacity of the ULA attachment of the viscometer if the protein concentration was at 8%.

3.6. Applications of Field Pea Protein Isolates in Food Systems

3.6.1. Sponge Cakes

3.6.1.1. Preparation of Cakes

The basic recipe used was wheat flour 100 g, peanut oil 100 mL, fresh eggs 140 g, caster sugar 100 g, mono-and di-glyceride emulsifier 8 g, and baking power 4 g. The procedure involved the mixing of eggs, sugar and emulsifier in a Kenwood Chef mixing bowl for 2 min at maximum speed until the eggs became creamy. Wheat flour and baking power were then added and mixed for 2 min at minimum speed. Peanut oil was slowly poured in and the dough was mixed using a wood spoon. The contents were immediately transferred into a rectangular baking pan and baking was performed using a hot air oven (Combi-Steamer at Hot Air Media, Germany) at 180°C for 21 min.

In order to study the application of field pea proteins in cakes, salt extracted and alkaline extracted pea proteins on the pilot scale were used to replace egg (protein) by up to 10, 25, 50, 75, 100%. Field pea protein isolate was dissolved in an appropriate amount of water (to compensate the moisture content in eggs) in the whipping bowl before the sugar, emulsifier and remainder of the eggs were added. The process

followed was the same as described above. In order to evaluate the effects of modified pea proteins on cake quality, protein samples modified with succinic anhydride (0.4 g/g protein) and acetic anhydride (0.2 g/g protein) were incorporated into cakes replacing egg proteins at levels of 25 and 50%.

3.6.1.2. Assessment of Cake Quality

Cake volumes were determined by displacement of rapeseed in a container which was large enough to accommodate the product. This was performed after the cakes were taken out from the oven and cooled at room temperature for 30 min. Texture (crumb firmness) was assessed by an Instron Universal Testing Machine (Model 4465) with a 5 kN load cell and a compression anvil attachment (diameter 35 mm). Samples for Instron testing were prepared from the core of the cake and cut into a rectangular prism (35×35 mm long and 30 mm high). The crosshead probe moved at a speed of 40 mm/min to produce 50% deformation from the height of the cake. The firmness of the cake was taken as the peak force (N) required for the deformation of the product. Colour was determined using a Minota Chromameter (CR-300) (see section 3.3.2.1). Cake samples for colour measurement were taken from different positions within the cake slices and eight readings were recorded and averaged.

3.6.2. Mayonnaise

The basic recipe used was 2 egg yolks (34 g), salt 2.5 g, pepper 0.3 g, sugar 2.5 g, white vinegar 17 mL and vegetable oil (Crisco Brand) 70 mL. First egg yolks, salt, pepper and 1 teaspoon vinegar were placed into a Kenwood mixing bowl. While beating continued at the maximum speed with a electric whisk, oil was added drop by drop. As mixture became thick the remaining oil was added in a thin stream while beating continually at a medium speed. When all the oil was added the remaining vinegar was stirred in using a wooden spoon. The product was stored in a cool place for 2 hr before sensory evaluation was carried out. For studying the application of field pea proteins in mayonnaise, pea proteins extracted with salt and alkali on the pilot scale were used to replaced egg yolk (protein) at levels of 10, 25, 50, 75, and 100%.

3.6.3. Sensory Evaluation of Food Products

Sensory evaluation of the cakes and mayonnaise involved a panel of 12 participants. Products containing modified pea proteins were not included. Overall acceptance was assessed using a 1-9 hedonic scale in terms of colour, texture and flavour. The panellists were also asked to give additional comments on the texture and flavour of the products. Examples of the forms for sensory evaluation for sponge cake and mayonnaise are given in Appendices I and II, respectively.

CHAPTER 4

Isolation, Fractionation and Characterisation of Pea Proteins

CHAPTER 4

Isolation, Fractionation and Characterisation of Pea Proteins

Field pea (*Pisum sativum* L.) is a potential source of novel proteins. However, the successful utilisation of these proteins will depend on their physico-chemical properties, which in turn, will be dependent on processing conditions and the nature of the proteins. Hence, isolation techniques and the characterisation of different protein fractions are very important for effective utilisation of field pea proteins.

In this chapter, protein isolates and Osborne protein fractions (albumins, globulins, prolamins and glutelins) were prepared from dry field pea seeds on a laboratory scale. The effects of various parameters were studied and the protein contents, recovery rates, and solubilities of the resultant fractions were compared. Among the parameters investigated were pH, the use of various buffers and extracting solutions as well as the use of reducing agents during extraction. Gel filtration and electrophoretic techniques were used to purify and characterise the extracted protein fractions. Amino acid analysis and scanning electron microscopy were also used for further characterisation. Results from this work gave detailed information on pea storage proteins and their extraction that will facilitate an assessment of their potential as novel food ingredients.

4.1. Protein Isolation from Field Peas

As for other legume seeds, field pea proteins can be extracted from the seeds by wet methods as well as air classification techniques. However, air classification yields proteins which are not as pure as those produced by aqueous extraction. Also, it has been reported that functional properties of the proteins obtained by dry processing are poorer than those of the product obtained by wet processes (Gueguen, 1991). Hence wet processes were chosen for this study and two different approaches were used to isolate field pea protein. One process involved solubilisation of proteins at different pH values

(pH 2, 7 and 9) and removal of insoluble material by centrifugation. Then the proteins were recovered by adding acid until the isoelectric point was reached. Another process was to use neutral salt solution (0.5M NaCl) to extract the proteins and the product was precipitated by dilution in cold water. This process is termed "micellisation" because the protein produced in this way has a micellar structure before being dried (Murray *et al.*, 1981). The purpose was to study the effect of pH and the extracting agents on recovery and physico-chemical properties of the proteins, which, in turn, might provide information for optimising conditions for isolation of field pea proteins in pilot scale operations.

4.1.1. Composition of Field Pea Proteins and Protein Recovery during Isolation

The total protein content of the field pea flour sample constituted 28.8% (on a dry weight basis) as determined by direct Kjeldahl analysis. This therefore represents the maximum level of extractable protein in the sample. The results of the proximate composition of the raw material and the protein isolates are shown in Table 4.1. The micelle protein isolate

Table 4.1 Proximate Composition of Field Pea Flour (Dehulled) and Protein Isolates[a]

Component	Flour	IPI-2[b]	IPI-7[c]	IPI-9[d]	MPI[e]
Moisture (%)	8.6	3.6	3.9	3.7	3.4
Protein (N×6.25) (%)	28.8	93.3	90.8	91.6	95.4
Crude fat (%)	2.68	2.35	2.58	2.33	1.91
Crude Fibre (%)	1.10	0.05	0.07	0.02	0.01
Ash (%)	2.67	2.47	1.98	2.72	1.08
Carbohydrate[f] (%)	64.7	1.8	4.6	3.3	1.6

a: Moisture values expressed "as is", others on a dry weight basis; Mean of triplicate determinations
b: Isoelectric protein isolate, extracted at pH 2
c: Isoelectric protein isolate, extracted at pH 7
d: Isoelectric protein isolate, extracted at pH 9
e: Micelle protein isolate, extracted with 0.5M NaCl
f: Carbohydrate calculated by difference

gave a significantly higher protein content than the isoelectric protein isolates, while little difference in the fat, ash and crude fiber contents was found between these isolates. Similar results were obtained for chickpeas (Paredes-López *et al.*, 1991) and faba beans (Abdel-Aal *et al.*, 1986). For the micellisation technique, the protein-protein association was favoured when ionic strength of the extracted sample was reduced and this was probably the main reason that the isolate had higher protein content (Murray *et al.*, 1981; Paredes-López *et al.*, 1988). However, the micellisation procedure would not be practical in a large-scale extraction, because the precipitation of proteins was achieved by dilution of the extracts with a large volume of cold water, or through dialysis over a long period to reduce the ionic strength. The procedure involving dialysis resulted in the significant loss of albumin fractions, which were removed with whey after centrifugation to recover the protein precipitates. Handling large amounts of slurry would add to these problems, especially in pilot scale operations.

No attempt was made to remove the crude fat because the fat content in field pea is relatively low (2.68% on a dry basis) and the additional step to extract the oil would be time consuming and increase production costs. Desolventing procedures following fat extraction may also result in the denaturation of proteins and thus influence the extractability and the functional properties of the proteins.

Protein recoveries in isolates extracted at pH 2, 7, and 9 were 43.9%, 36.6% and 58.8% respectively. The micellisation procedure resulted in the lowest recovery rate of 32.3%. Ionic strength and ion types greatly affect the solubility of proteins by causing alterations in the ionic, hydrophilic, and hydrophobic interactions at the protein surface (Damodaran, 1996). Shen (1981) studied the solubilities of soy protein in various sodium salt solutions and found that at concentrations above 0.15M, the anions chloride, bromide and iodide increased the solubilities in neutral conditions. Similar results were observed for gluten in various salt solutions (Preston, 1981). Thus it was expected that the extractability of pea proteins would be higher for 0.5M NaCl solution than for neutral water. The low recovery might again result from the micellisation procedures. In order to achieve a micelle arrangement, a specific intermolecular hydrophilic-hydrophobic balance is

required. Slight environmental modifications may alter this balance so that micelle formation no longer occurs (Tanford, 1973; Ismond *et al.*, 1990). Thus it is very difficult to manipulate the conditions such that proteins are fully precipitated. Furthermore, the low recovery of the proteins is also attributable to the loss of albumin during the micellisation process.

However, if an alternative method, such as ultrafiltration, could be used to recover proteins instead of micellisation, the use of salt extraction remains a feasible approach. Especially this offers potential advantages if performed under neutral conditions such that the physico-chemical properties of the proteins would not be impaired. In the current study, a Minitan ultrafiltration system (Millipore Corp. Belford, MA) with 4 filter plates was used to concentrate the protein extracts to half volume before micellisation. However, it was very difficult to achieve further concentration because of the limitation of the equipment. The solute-solute and membrane-solute interactions became significant with increasing retentate concentration. In an effort to reduce these interactions, a retentate "washing" step was used, i.e. the diafiltration mode of operation (Nichols and Cheryan, 1981). Water was added to the concentrated retentate and the ultrafiltration step repeated. However, because of the type and size of the equipment used, this process was very time consuming and found to be impractical. Nevertheless, industrial ulitrafiltration membranes have been successfully used in protein isolation from soybeans (Lawhon *et al.*, 1979; Deeslie and Cheryan, 1991), as well as rapeseed (Diosday *et al.*, 1984), peanut and cottonseed (Manak *et al.*, 1980). As a result, the feasibility of recovering pea protein isolates by membrane processing is quite promising, provided that a suitable ultrafiltration system is available.

4.1.2. Solubility of Field Pea Protein Isolates

The solubility profiles of field pea protein isolates are shown in Fig. 4.1. This includes those extracted at pH 2, pH 7 and pH 9 (IPI-2, IPI-7, IPI-9). All three isolates gave typical U shape solubility curves with the minimum solubility at pH values in the range of pH 4 to pH 6, as found for proteins from other grain legumes. However, protein

isolates extracted at pH 7 had higher solubilities than the proteins extracted at either pH 2 or pH 9, especially in the neutral pH range. The partial denaturation of the proteins during processing at extreme pH conditions could explain the lower solubilities of the protein isolates extracted at pH 2 or pH 9. However, both pH and ionic strength influence solubility of proteins by their effects on electrostatic forces (Damodaran, 1996). Hence the three protein isolates (extracted at pH 2, pH 7 and pH 9) had similar solubilities in the lower pH range (pH<3) and higher pH range (pH>9). This results from the increased net charge on proteins as the pH is further from the pI. For a large-scale isolation of total proteins, the alkaline solution (pH 9) is recommended, because of the high recovery rate and the solubility of the proteins obtained in this laboratory study.

The solubility of micelle protein isolate (MPI) is shown in Fig. 4.2. The solubilities of IPI-9 and soy protein isolate (commercial product, Supro-500E) are presented in this figure for comparison. As expected, MPI had a higher solubility than IPI because of the mild processing conditions. The isoelectric point of MPI is slightly higher (pH 5.3) than IPI-9 (pH 4.9). The significance of protein solubilities is further addressed in a later chapter in which functionality is considered. The insolubility of most proteins at their pI is due to neutralisation of charge repulsion between protein molecules (Damodaran, 1996). Differences in surface charge of the proteins may have resulted from the various isolation steps and might explain the differences of the isoelectric points between MPI and IPI. As can be seen from the graph in Fig. 4.2, both of the field pea protein isolates presented much higher nitrogen solubility than the soy protein isolate at all the pH ranges. Similar results have been reported by Naczk *et al.* (1986). However, the types of the materials used and the processing conditions greatly affect solubility of proteins. The partial denaturation of soy proteins may occur during commercial preparation. For example, moist heat treatment, which is necessary to inactivate lipoxygenase and inhibitors of proteases, rapidly insolubilises soy proteins (Smith and Circle, 1978). Also, in the case of soybean, the initial fat content is relatively high (approximately 20%) and solvent extraction and subsequent removal of solvent are required. These steps and the involvement of heat in processing reduce solubility. These problems do not occur in the case of field peas.

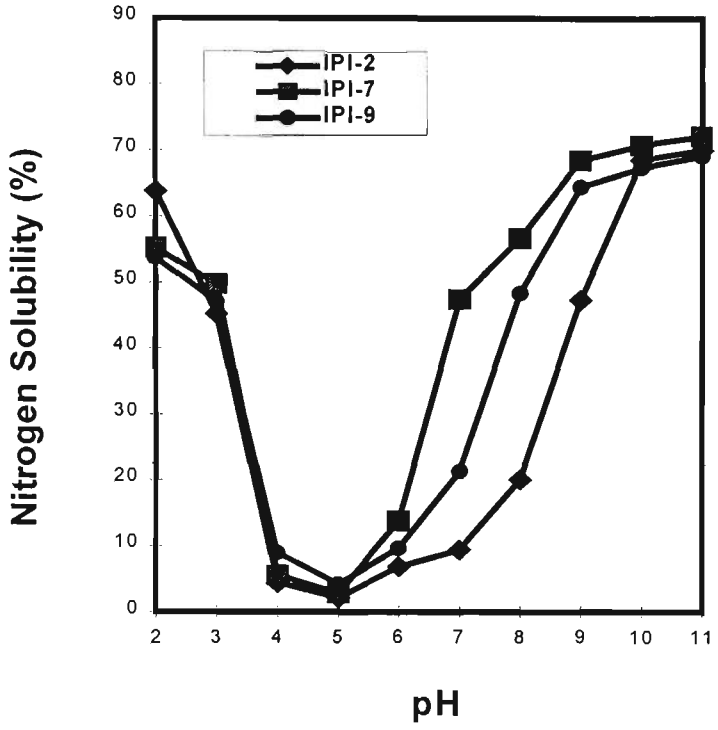


Figure 4.1 Solubility profiles of field pea protein isolates prepared at pH 2 (IPI-2), pH 7 (IPI-7) and pH 9 (IPI-9).

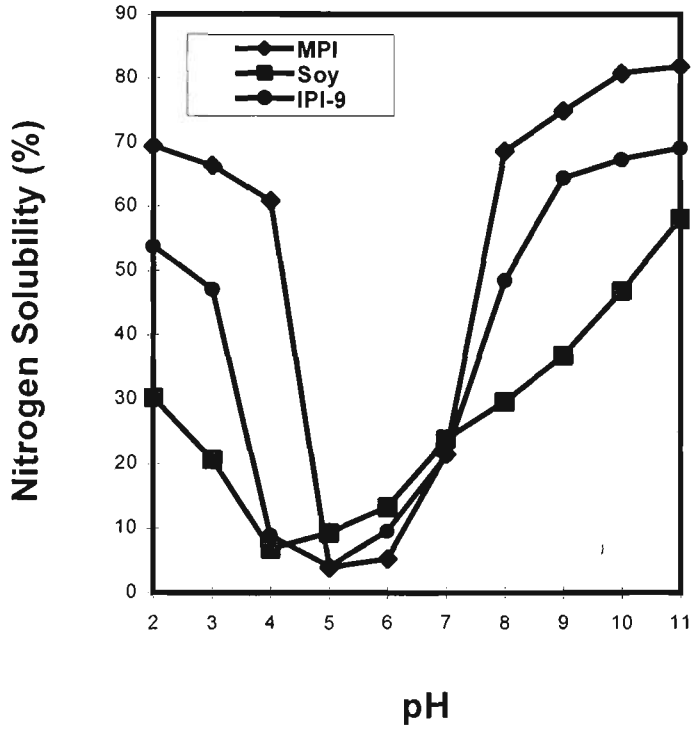


Figure 4.2 Solubility profiles of micelle protein isolate (MPI) compared with IPI-9 and a commercial soy protein isolate (Supro-500E).

4.1.3. SDS Polyacrylamide Gel Electrophoresis of Field Pea Protein Isolates

In order to further characterise the various protein fractions isolated from field peas, electrophoresis was carried out using a SDS-PAGE system. The method followed was that of Laemmli (1970) and the gels were stained with Coomassie brilliant blue. Details of the procedure are described in Chapter 3 (3.2.3.1). SDS-PAGE resolution of the protein isolates is shown in Fig. 4.3. The results indicate that most components were common for the protein isolates extracted using different conditions. The Enhanced Laser Densitometer (LKB Ultroscan XL) was used to scan gels and estimate the molecular weights of selected protein bands, and to determine the relative quantities of protein in these bands. Protein bands ranged in size from 14.6 kDa to 88.7 kDa, the dominant bands being 22.0 kDa, 34.2 kDa, 42.3 kDa, 45.1 kDa, 71.4 kDa, accounting for 23.8%, 10.9%, 14.8%, 13.5% and 10.7% of total protein, respectively.

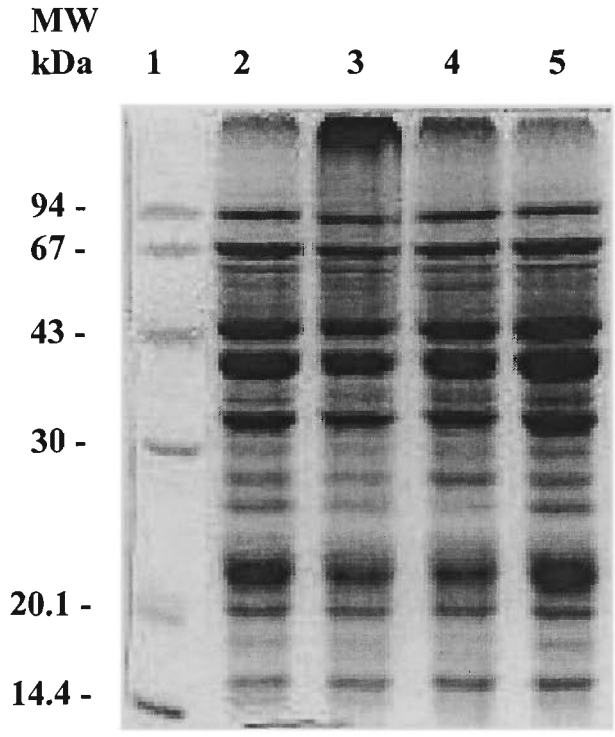


Figure 4.3 SDS-PAGE gel of field pea protein isolates. 1, protein markers; 2, MPI; 3, IPI-2; 4, IPI-7; 5, IPI-9.

4.1.4. Amino Acid Composition of Field Pea Protein Isolates

The amino acid composition of the micelle protein isolate and isoelectric protein isolates extracted at pH 2, 7 and 9 were measured using HPLC and the results are presented in Table 4.2. These show that there was little variation in amino acid composition among the four isolates. Arginine, aspartic acid and glutamic acid were found in greatest amounts, but relatively large amounts of lysine and leucine were also present. These results were in agreement with the values obtained for Century field peas, as determined

Table 4.2 Amino Acid Composition of Field Pea Protein Isolates (g/100g protein)[a]

Amino acid	MPI[b]	IPI-2[c]	IPI-7[d]	IPI-9[e]
Essential				
Lysine	6.54	6.25	6.82	6.38
Threonine	3.23	3.51	3.42	3.13
Valine	4.65	4.42	4.08	4.53
Methionine	0.79	0.83	1.01	0.91
Cysteine	0.92	0.97	0.87	0.82
Isoleucine	4.98	4.54	4.42	4.62
Leucine	8.21	8.68	8.45	8.59
Phenylalanine	5.67	5.38	5.52	5.23
Tyrosine	2.42	2.61	2.23	2.36
Histidine	2.72	2.41	2.56	2.63
Subtotal	40.1	39.6	39.4	39.2
Nonessential				
Arginine	9.08	8.78	8.62	8.37
Aspartic acid	10.32	10.54	10.89	11.03
Serine	5.37	5.21	5.45	5.19
Glutamic acid	17.06	17.45	17.63	17.82
Proline	5.82	4.93	4.81	4.79
Glycine	3.65	4.73	4.54	4.66
Alanine	3.62	3.87	4.05	3.92

a: Mean of duplicate determinations. Tryptophan not determined
b: Micelle protein isolate, extracted with 0.5M NaCl
c: Isoelectric protein isolate, extracted at pH 2
d: Isoelectric protein isolate, extracted at pH 7
e: Isoelectric protein isolate, extracted at pH 9

by Holt and Sosulski (1979). However, for the field pea used in this study, serine and proline content were slightly higher, whilst glycine and alanine contents were lower than previously reported for Century field peas. These differences may result from the different genetic varieties of field peas. Dry dehulled seeds of Dunn type were used in this study.

The total amount of essential amino acids in protein isolates extracted at pH 2, 7 and 9 accounted for 39.6, 39.4, 39.2 g/100g protein, respectively. For the micelle protein isolate, the amount of total essential amino acid was 40.1 g/100g protein. Compared with values recommended by FAO/WHO/UNU (1985) (Table 4.3), the results indicate that field pea protein isolates contained adequate amounts of most essential amino acids except methionine for children and adults.

Table 4.3 Essential Amino Acids Recommended by FAO/WHO/UNU

	FAO/WHO/UNU Reference protein		
Essential amino acid	**Infant**	**Child**	**Adult**
Lysine	6.6	5.8	1.6
Threonine	4.3	3.4	0.9
Valine	5.5	3.5	1.3
Methionine	4.2	2.5	1.7
Cysteine*			
Isoleucine	4.6	2.8	1.3
Leucine	9.3	6.6	1.9
Phenylalanine	7.2	6.3	1.9
Tyrosine*			
Histidine	2.6	1.9	1.6

* no recommendations made

4.2. Osborne Fractionation of Field Pea Proteins - Albumin, Globulin, Prolamin and Glutelin

The classification of seed proteins according to their solubility was developed by Osborne (1924), distinguishing four different fractions: albumins (water soluble),

globulins (soluble in salt solution), prolamins (alcohol soluble), and glutelins (partially soluble in dilute NaOH). While this classification is very suitable in the case of cereals, it has not been applied extensively to legume seed proteins. In this study, albumins, globulins, prolamins and glutelins were extracted from field peas using various buffer solutions and solvents, based on the principles of Osborne classification. The protein solubility and amino acid composition were analysed; SDS gel electrophoresis and scanning electron microscopy were also used to characterise these protein fractions.

4.2.1. Fractionation with Different Extracting Solutions

Albumins, globulins, prolamins and glutelins from field peas accounted for approximately 40%, 50%, 1% and 9% of the total proteins, respectively. For the extraction of albumins and globulins, the results of trials using different solutions are summarised in Table 4.4.

Table 4.4 Effects of Extraction Conditions on the Recovery of Albumins and Globulins

	Recovery rate (%)	**Protein content (%)**	**Albumin/globulin ratio**
0.5M NaCl	54.2	Albumins 70.3 Globulins 97.4	0.58
1M NaCl	52.8	Albumins 74.8 Globulins 87.5	0.73
0.2M NaH_2PO_4 0.2M Na_2HPO_4	48.6	Albumins 74.6 Globulins 88.7	0.90
0.1M NaH_2PO_4 5% K_2SO_4	53.1	Albumins 73.3 Globulins 92.6	0.66
0.1M Citric acid 0.2M Na_2HPO_4	38.2	Albumins 72.7 Globulins 84.5	0.87

There was some difference in protein recovery rate, protein content and albumin/globulin ratio when different extracting agents were used. With phosphate buffer extraction (0.2M, pH 7), the quantity of albumin fraction was nearly the same as for the globulin fraction. Even with other buffer extractions, the albumin fraction was higher than the 20-25% of

the total proteins reported in the review by Gueguen (1991). Proteins extracted with buffer solutions were not superior to the proteins extracted with salt (NaCl) in terms of yield or protein content. Generally speaking, the globulin fractions had a higher protein content than albumins. This may have been due to the presence of some carbohydrate remaining in the albumin fractions after the centrifugation to collect the globulins following dialysis.

As is the case for most legume grains (Sauvaire *et al.*, 1984), the prolamin content in field pea was very low. Table 4.5 shows prolamin extractability under various solvent systems with and without the addition of the reducing agent DDT. The use of DDT resulted in a slightly higher recovery of prolamin. However, because of the tiny amount of prolamin obtained in the laboratory, it was very difficult to analyse the proximate composition of the prolamins extracted under different conditions. Even though the fat content in raw materials was very low and a significant amount of fat was removed in the earlier fractionation steps, a small amount of oil may have remained in the organic solvent together with the prolamins. It was decided that it was not worthwhile to attempt further purification of this fraction because its protein content is so low and will be of limited or no value in food applications.

Table 4.5 Solvent Systems Used to Extract Prolamins from Peas

Solvent	DTT (%)	Amount of protein extracted (%)
70% Ethanol/Water	0	0.9
70% Ethanol/Water	0.10	1.2
100% Propan-2-ol	0.06	1.3
100% Butan-1-ol	0.05	1.0

After the albumin, globulin and prolamin fractionation, the subsamples were used to extract glutelins with 0.1 M NaOH. The glutelins accounted for about 9% of the total proteins extracted and the protein content in this fraction was ca. 66%. A reasonable amount of carbohydrate remained in the glutelin product, because the solid and liquid

could not be separated efficiently by centrifugation. After the salt and alcohol extraction, the subsamples were not sufficiently coherent and thus some of the fine particles of the carbohydrates stayed in the alkaline solution that contained the glutelin fraction, and was recovered together with the proteins in the isoelectric precipitation procedures.

4.2.2. Solubility Characteristics of Albumin and Globulin Fractions

Solubility of proteins is often considered to be a prerequisite for good performance in food applications (Kinsella, 1976); many functional properties of proteins, such as emulsifying, foaming and gel properties, depend upon the initial capacity to dissolve. As about 90% of proteins in peas are albumins and globulins, the solubility of these fractions is important.

The solubility curves obtained for globulins and albumins extracted with 0.5M NaCl is shown in Fig. 4.4, in comparison with the field pea protein isolate extracted at pH 9 (IPI-9). It can be seen that these fractions have different pI values and solubility patterns. The pI for albumin, globulin and protein isolate IPI-9 were 4.0, 5.5 and 5.0, respectively. The solubility of the albumin fraction was very high even in the range of the isoelectric point (pH 4-6). This feature might be very useful in the food industry, especially in the acid-based food formulas, e.g. protein beverages.

The solubilities of the albumin and globulin fractions extracted with different buffer solutions at neutral pH have also been determined (Table 4.6). There were only minor differences in the solubilities between these fractions. A number of criteria need to be considered in the selection of a suitable extracting solution for the large scale isolation of proteins from field peas. Safety and cost factors are the primary considerations. It is therefore concluded from this study that salt solution (NaCl) offers greater potential for commercial extraction of albumins and globulins from legume seeds, because alternative solutions have resulted in no enhancement to protein characteristics.

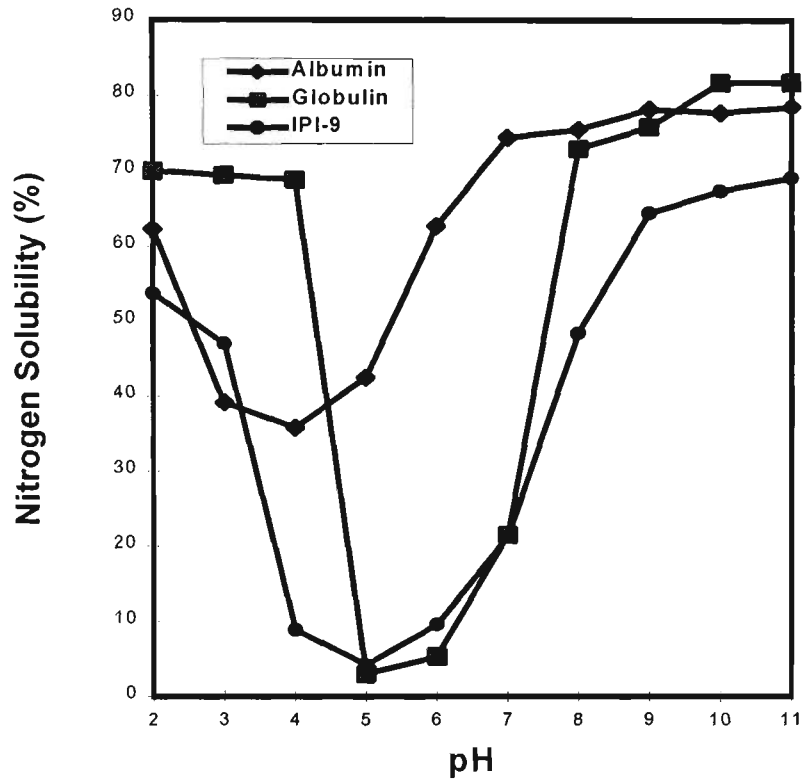


Figure 4.4 Solubility profiles of albumins, globulins and protein isolate (extracted at pH 9) from field peas.

Table 4.6 Solubilities of Albumins and Globulins Extracted Using Different Buffer Systems (pH 7)

	Nitrogen Solubility (%)	
Extracting Agent	**Albumins**	**Globulins**
0.5M NaCl	78.5	34.5
1M NaCl	82.3	32.7
0.2M NaH_2PO_4 0.2M Na_2HPO_4	80.6	35.8
0.1M NaH_2PO_4 5% K_2SO_4	77.1	36.2
0.1M Citric acid 0.2M Na_2HPO_4	81.0	34.3

4.2.3. SDS Polyacrylamide Gel Electrophoresis of Osborne Fractions

The SDS-PAGE banding patterns for albumin fractions were quite similar regardless of the agents used for extraction (Fig. 4.5). Similarly, the globulin patterns were unaffected by extraction agent used. SDS-PAGE was also performed on prolamin and glutelin fractions (Fig. 4.6). The prolamin bands on SDS-PAGE are weak and diffuse except for those extracted by butan-1-ol+ 0.05% DTT. This may have resulted from the poor solvent extractability of proteins and the presence of fat in the fractions. The bands of glutelins are quite clear but indicate that there are residual globulin and albumin components remaining in this fraction.

The electrophoretic patterns of the different fractions were also characterised by scanning densitometry. The profiles of albumin, globulin, prolamin and glutelin fractions from field peas compared with the known MW protein markers are shown in Fig. 4.7. The major protein components in the prolamin fractions have estimated MW of 21.9 kDa, 27.4 kDa, 38.3 kDa, 45.1 kDa, 53.8 kDa, accounting for 17.4%, 10.9%, 11.4%, 29.3%, 14.8% respectively. Glutelins contain six major polypeptides of 21.8 kDa, 38.0 kDa, 42.4 kDa, 45.6 kDa, 71.6 kDa, 83.7 kDa, and four minor components at 14.6 kDa, 19.3 kDa, 27.4 kDa, 62.4 kDa. The major bands of globulin fractions were also present in the glutelin fractions. Of these, the most significant polypeptides in globulins were 22.0 and 42.1 kDa, constituting 24.1 and 20.5% respectively. The band at 71.5 kDa also accounted for a reasonable quantity of the total globulins.

It has been reported that pea legumin has polypeptides of approximately 40 kDa and 20 kDa, while a band at approximately 70 kDa corresponds to convicilin (Casey, 1979; Davey and Dudman 1979; Hurkman and Beevers, 1980). Another major storage protein also found in pea globulins, is vicilin. Major polypeptides of 50 kDa, 30-35 kDa, and 19 kDa along with minor lower MW polypeptides have been reported for pea vicilin (Davey and Dudman 1979, Gatehouse *et al.*, 1981; Koyoro and Powers, 1987). However, these results are based on more highly purified legumin, vicilin, and convicilin fractions. Results following further purification and characterisation of the globulin fractions from

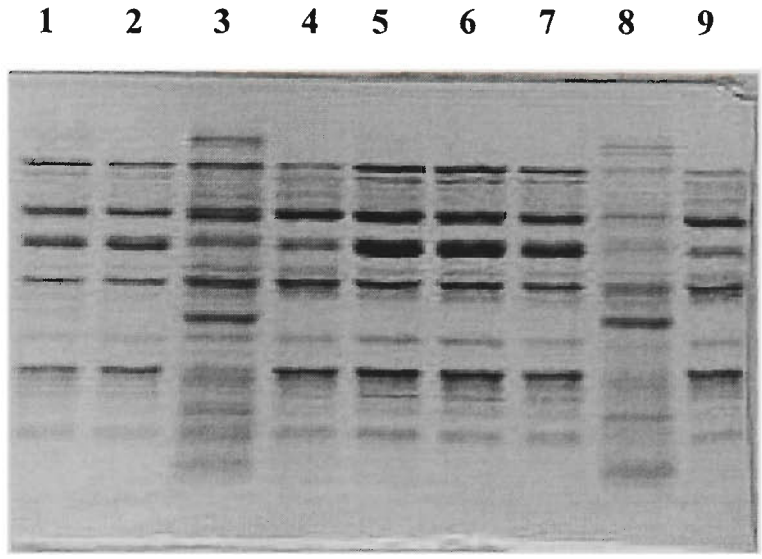


Figure 4.5 SDS-PAGE of albumins and globulins extracted from field peas. 1 and 7, globulins extracted with 0.5M NaCl; 2, globulins extracted with 0.2M NaH_2PO_4-Na_2HPO_4 buffer; 3, albumins extracted with 0.2M NaH_2PO_4-Na_2HPO_4 buffer; 4, globulins extracted with 0.1M NaH_2PO_4+5% K_2SO_4; 5, globulins extracted with 0.1M citric acid-0.2M Na_2HPO_4; 6, globulins extracted with 1M NaCl; 8, albumins extracted with 0.5M NaCl; 9, protein isolate extracted at pH 9.

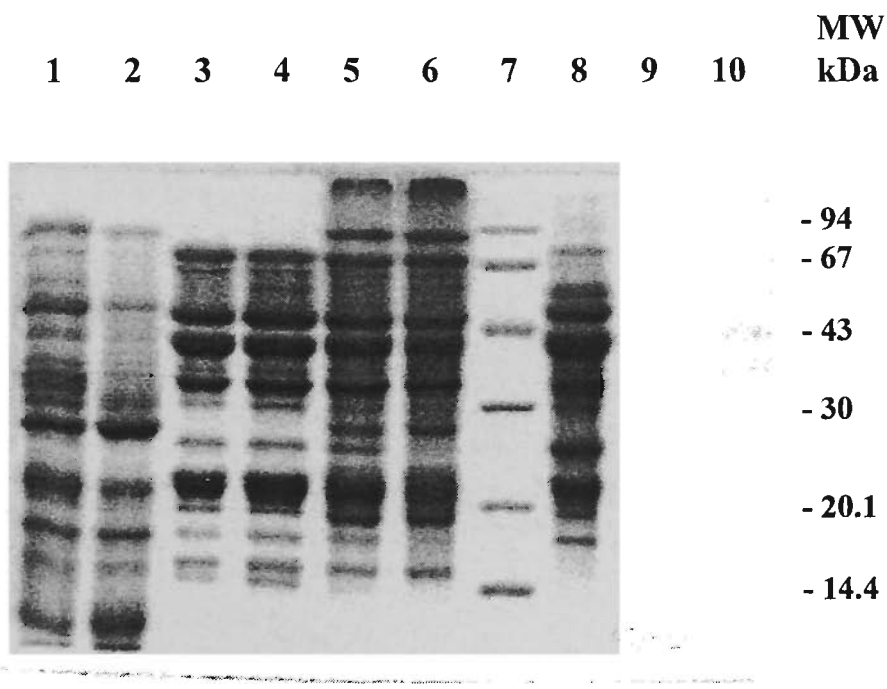


Figure 4.6 SDS-PAGE of albumins, globulins, prolamins and glutelins from field peas. 1 and 2, albumins extracted with 0.5M and 1M NaCl; 3 and 4, globulins extracted with 0.5M and 1M NaCl; 5 and 6, glutelins extracted with 0.1M NaCl; 7, standard proteins; 8, prolamins extracted with butan-1-ol + 0.05% DTT; 9 and 10, prolamins extracted with 70% ethanol and 2-propanol+0.06% DTT, respectively.

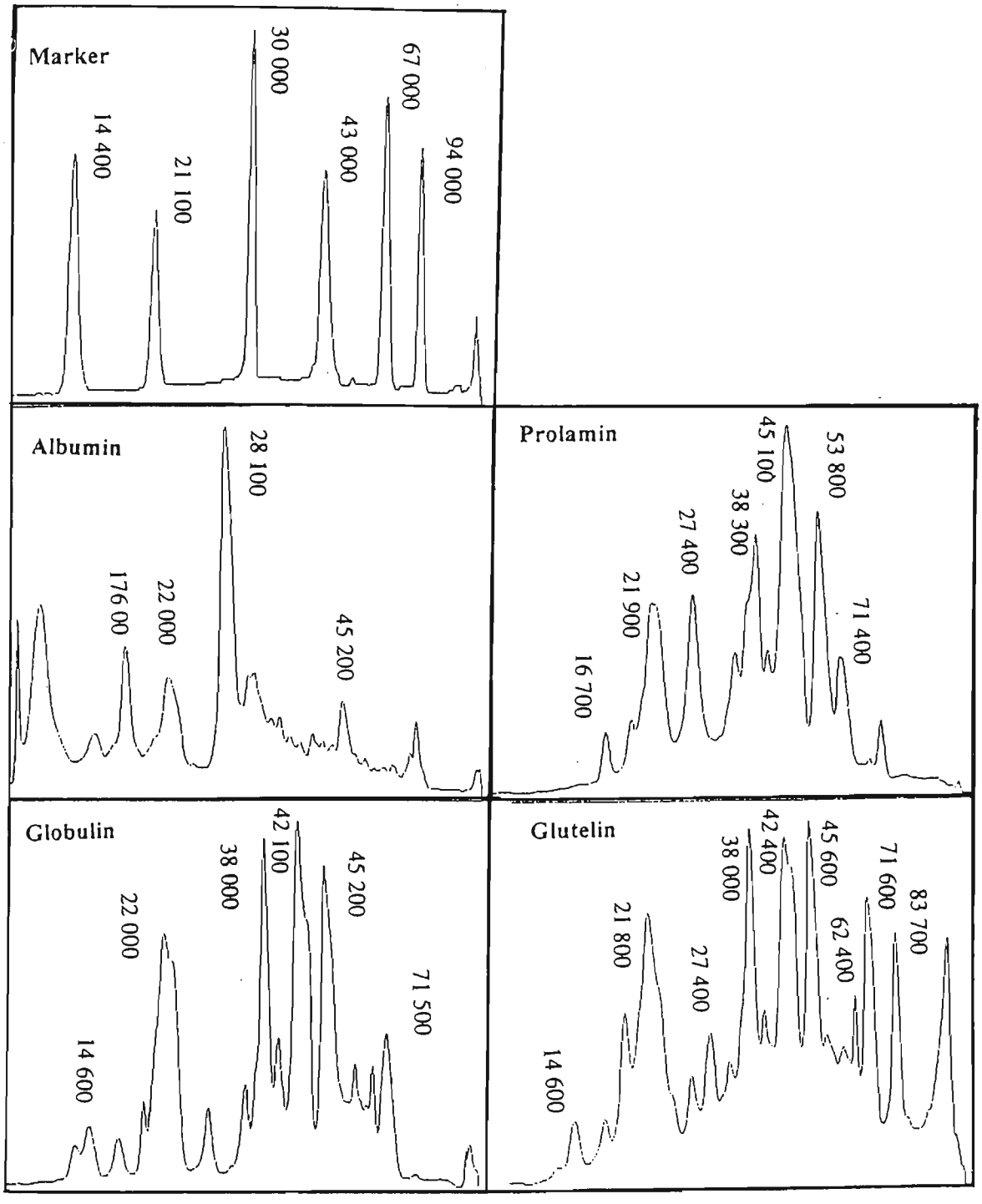


Figure 4.7 Densitometric scanning profiles of albumin, globulin, prolamin and glutelin fractions from field peas. Marker, albumin, globulin, prolamin and glutelin corresponded with lane 7, lane 2, lane 3, lane 8 and lane 5 of Fig. 4.6, respectively.

field pea proteins will be presented in the next section. Nevertheless, the results in Fig. 4.6 and 4.7 indicate that the mixed globulin fraction contains the components of legumin, vicilin and convicilin. Albumins consisted mainly of small polypeptides; 31.6% had a molecular weight of 28.1 kDa. This result was confirmed by two-dimensional electrophoresis.

4.2.4. Two-Dimensional Gel Electrophoresis of Globulin and Albumin Fractions

The two-dimensional electrophoresis for analysing mixtures of polypeptides separates proteins in the first dimension on the basis of charge by isoelectric focusing and then in the second dimension on the basis of molecular mass by SDS-PAGE (Rickwood *et al.*, 1990). From Fig. 4.8, it can be seen that the globulin fraction was concentrated in three major polypeptides of approximately 40 kDa, 50 kDa and 70 kDa, which corresponded to legumin, vicilin and convicilin components respectively. The albumin fraction exhibited a clear band with a molecular weight of approximately 27-28 kDa. Globulin fractions had a higher pI range (5.2-5.5) than the albumin fractions, a finding that is consistent with and confirms the results obtained from solubility determinations in the current study (Fig. 4.4).

4.2.5. Amino Acid Analysis of Osborne Fractions

Amino acid profiles of different protein fractions from peas are given in Table 4.7. Here the albumin and globulin fractions used for amino acid analysis were extracted with 0.5M NaCl, while the prolamin fraction was extracted with butan-1-ol+0.05% DTT.

Compared with globulin and other protein fractions, albumin contains higher levels of lysine, methionine and cysteine. These results are in agreement with the work of Gwiazda *et al.* (1980). However, this group claimed that all essential amino acids including lysine and sulphur-containing amino acids, in albumins were present at twice or three times the concentration of other fractions including mixed globulin, legumin and vicilin. This was not the case for the field pea albumin fraction examined for this thesis. Nevertheless, the

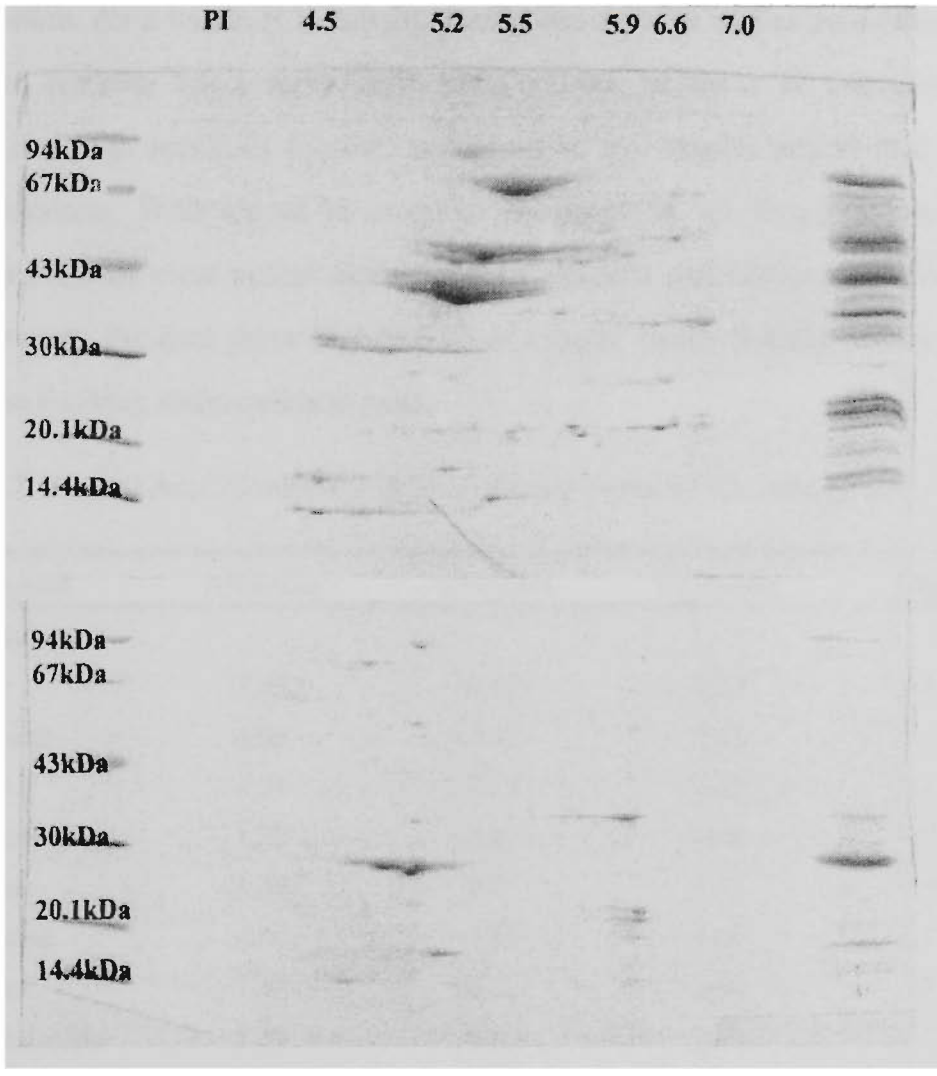


Figure 4.8 Two-dimensional electrophoresis of albumin and globulin fractions extracted with 0.5M NaCl. Top: globulins; Bottom: albumins. The bands on the right hand side of each gel represents sample which was only subjected to the second electrophoresis procedure for comparative purposes.

higher levels of sulphur-containing amino acids in the water-soluble fractions have also been reported by Smith and Circle (1978) in the case of soybean, and by Singh and Jambunathan (1982) for chickpea. Bhatty (1982) studied albumin proteins of eight edible grain legumes and found that they contained more tryptophan, lysine, threonine, valine, and methionine. As a result, it is usually considered that the amino acid composition of the albumin fraction has a more favourable balance in terms of nutritional quality. Prolamins had high levels of leucine, and glutelins had similar amino acid profiles to globulin fractions. With regard to essential amino acids, all four fractions exhibited adequate amount of most amino acids for both children and adults (FAO/WHO/UNU, 1985). However, the data show that like other legume seeds, sulphur containing amino acids are the limiting amino acids in peas.

Table 4.7 Amino Acid Composition of Different Protein Fractions (g/100g protein)[a]

Amino acid	Albumin	Globulin	Prolamin	Glutelin
Essential				
Lysine	7.85	6.52	6.27	6.36
Threonine	4.02	3.31	3.12	3.55
Valine	4.31	4.54	4.22	4.65
Methionine	1.22	0.85	1.03	0.79
Cysteine	1.94	0.91	1.17	0.94
Isoleucine	4.41	4.87	4.38	4.96
Leucine	7.32	8.27	9.48	8.71
Phenylalanine	5.28	5.63	5.03	5.42
Tyrosine	2.32	2.40	2.68	2.54
Histidine	2.86	2.63	3.72	2.71
Subtotal	41.5	39.9	41.1	40.6
Nonessential				
Arginine	6.62	8.86	8.45	8.17
Aspartic acid	9.83	10.41	10.23	10.56
Serine	4.73	5.45	5.02	5.21
Glutamic acid	15.87	17.12	16.49	16.92
Proline	6.28	5.14	5.13	5.08
Glycine	4.72	3.83	3.67	3.35
Alanine	4.62	3.04	3.27	4.24

a: Mean of duplicate determinations. Tryptophan not determined

4.2.6. Scanning Electron Microscopy (SEM) of Field Pea Flour and Proteins

The advent of SEM has given food scientists a powerful tool for investigating surface microstructure. The basis of SEM involves scanning a high energy beam of primary electrons across the surface of a bulk sample, which excites the release of secondary electrons. These are captured and electronically formed into an image of surface topography, which can be displayed via a cathode ray tube in raster form (Friberg and El-Nokaly, 1983). SEM has been used to study the internal microstructure of meat products (Jones *et al.*, 1976; Józsa *et al.*, 1980), milk products (Gastaldi *et al.*, 1996; Eino *et al.*, 1976) and soybean foods (Saio 1981; Wolf and Baker 1980). For foods in powder form, the dimensions, shapes, and characteristic surface features have been studied in addition to internal microstructure (Moss *et al.*, 1980, Smith 1979). However, SEM has not been widely used in the study of physical properties of legume proteins. Accordingly, it has been applied in the current study to provide further information to explain the functional properties of different proteins present in field peas.

The scanning electron micrographs of field pea flour, protein isolate (IPI-9), albumin and globulin are shown in Fig. 4.9 - 4.12, respectively. Dehulled pea flour is made up of a series of larger spherical particles of starch granules, with a number of small particle matrices attached to them. Most of the attachments are protein bodies; the others may include non-starch carbohydrates or other components. Similar observations have been made by Reichert and Youngs (1977), who compared pea flour, starch, protein fractions separated by air classification using scanning electron microscopy.

Albumins showed leaf, or rod-like structures with relatively smooth surface topography (Fig. 4.11). This pattern is much like the albumin shapes of the Great Northern Bean (Sathe and Salunkhe, 1981b). The thin wafers having large surface area could partially explain the high solubility of albumins in neutral conditions, giving greater accessibility to water molecules. Both protein isolate (extracted at pH 9) and globulins (Fig 4.10 and 4.12 respectively) presented irregular, rectangular-shaped particles, which were

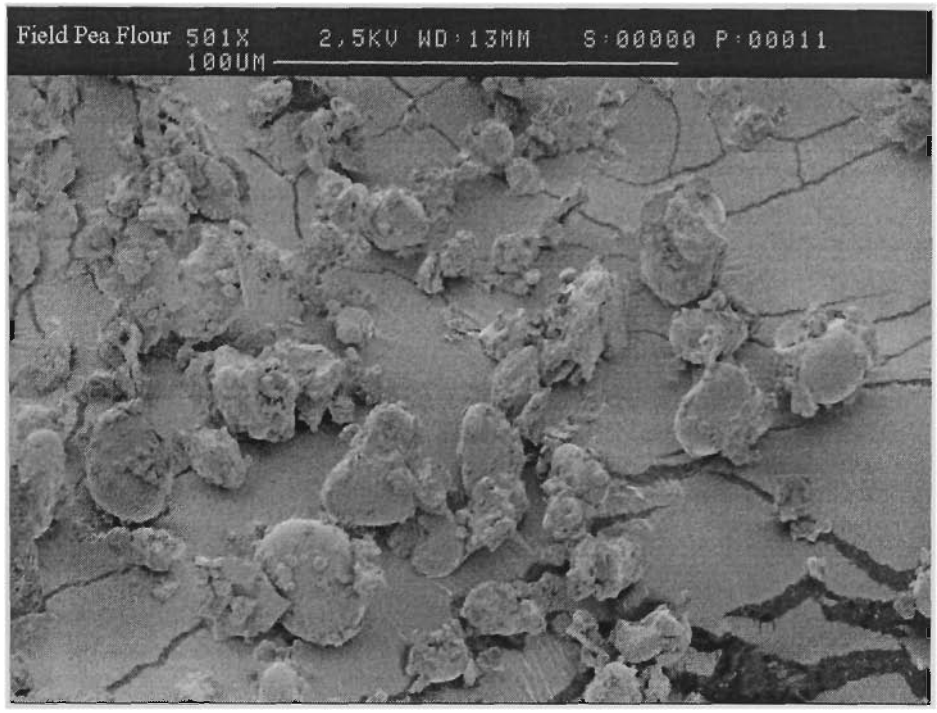


Figure 4.9 Scanning electron micrograph of field pea flour.

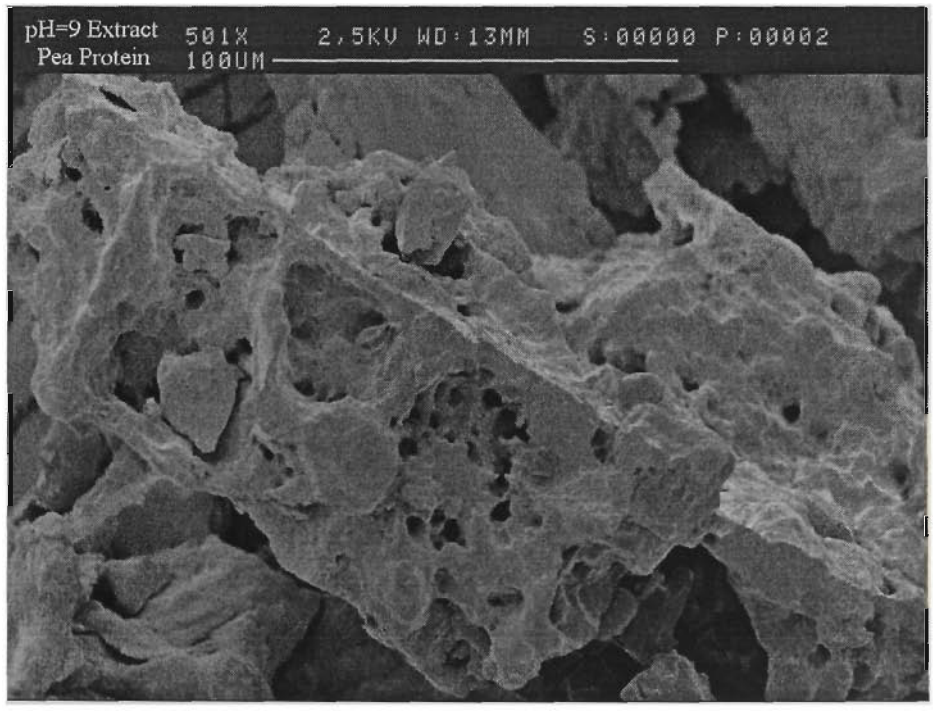


Figure 4.10 Scanning electron micrograph of field pea proteins extracted at pH 9 (IPI-9).

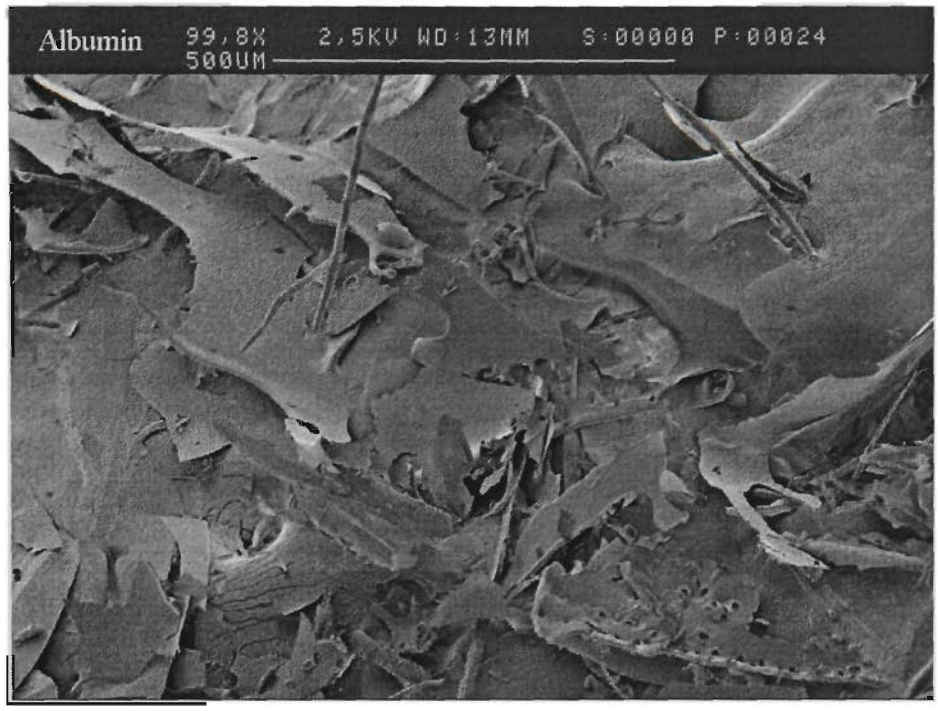


Figure 4.11 Scanning electron micrograph of field pea albumins.

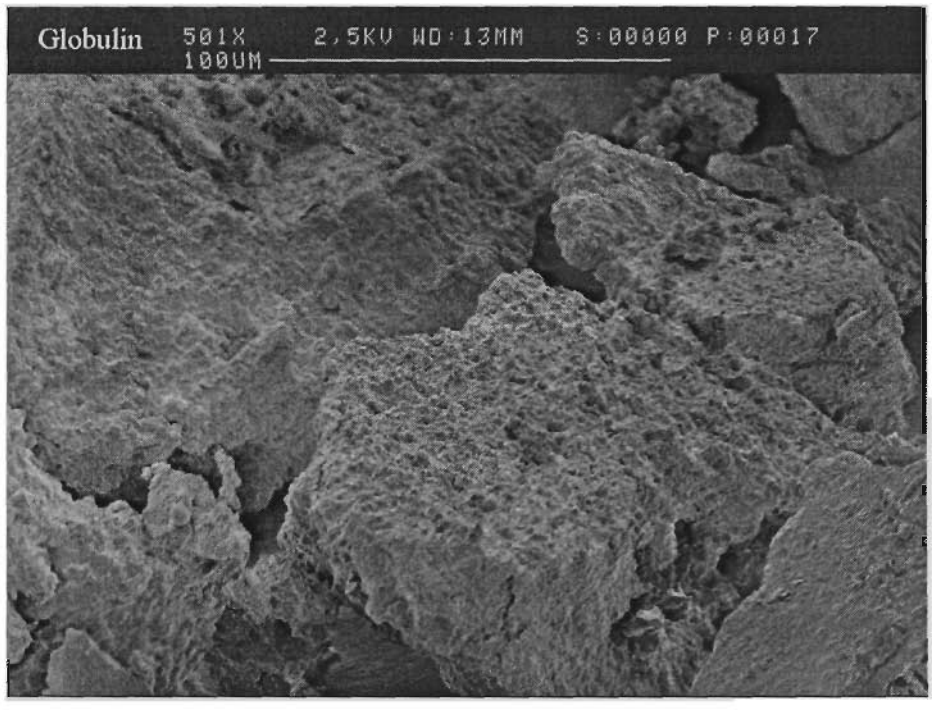


Figure 4.12 Scanning electron micrograph of field pea globulins.

agglomerated and had a dense mass with few pores. The similarity between the particles in these micrographs is consistent with loss of albumin proteins from the isolate extracted at pH 9. This isolate is expected to possess most of the characteristics of globulins.

4.3. Further Purification and Characterisation of Albumins and Globulins from Field Pea Proteins

4.3.1. Ion Exchange Chromatography and Gel Filtration

According to Gueguen *et al.* (1984), ion exchange chromatography in combination with gel filtration is the most suitable method to purify two major pea globulins, legumin and vicilin. The purpose of this study was also to isolate the major components of albumin fractions using this method. Field pea extract (dissolved in 0.1M phosphate-citrate buffer, pH 7) were applied into a DEAE Sepharose column. The non-bound materials were eluted with extracting buffer and a series of sodium chloride solutions (0.05M, 0.25M, 0.5M, 1.0M) were used to elute the adsorbed proteins.

From the DEAE ion exchange column, proteins were eluted as three major fractions (Fig. 4.13). About 50% of the total protein was eluted without retention. This first fraction was mainly composed of albumins. This result confirmed the report of Gueguen *et al.* (1984). SDS-PAGE banding patterns of the fractions from the DEAE ion exchange chromatography are shown in Fig. 4.14. The first peak of the eluate (lanes 3-5) was found to comprise mainly albumin bands (contaminated with a small proportion of the second peak). The second part of the eluate (lanes 6-7) is vicilin, which is one of the major componemnts of globulins. The other major fraction of globulin, legumin, was eluted as the last peak from the column. This was concentrated in two major electrophoretic bands (lanes 8-10). All three fractions presented very distinct gel patterns. Fig. 4.15 shows the densitometric scanning profiles of the original mixed proteins in comparison with the peaks separated chromatographically (albumins, vicilin and legumin fractions). Legumins constituted of 35% of the proteins at 22.1 kDa, 45% at 42.4 kDa. The enriched vicilin

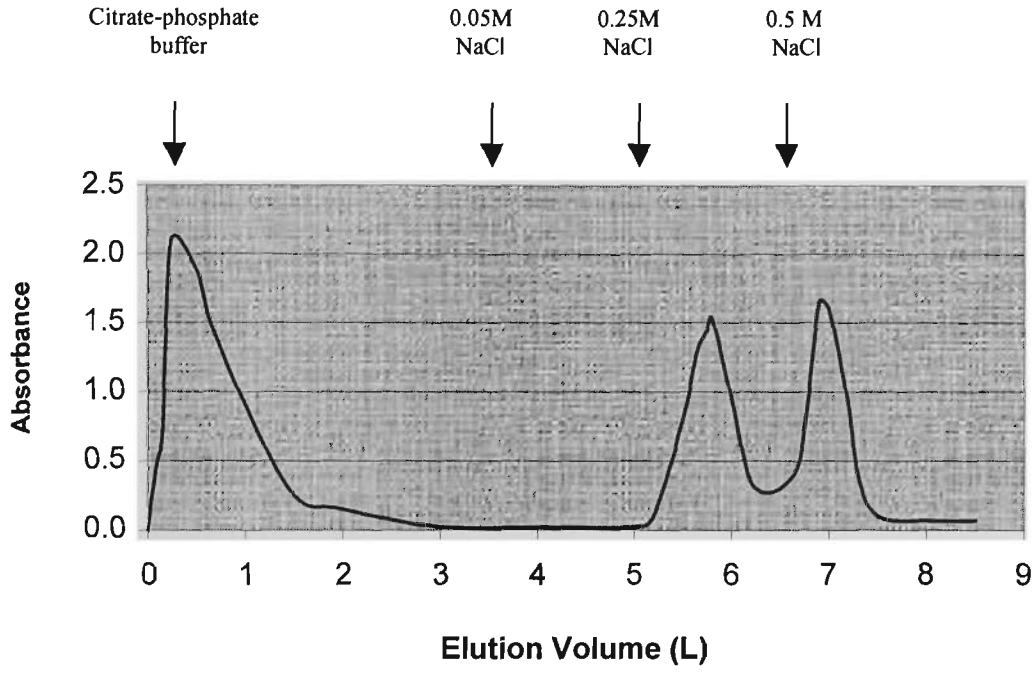


Figure 4.13 Elution profile of pea protein extracts obtained from ion exchange chromatography on a column of DEAE Sepharose

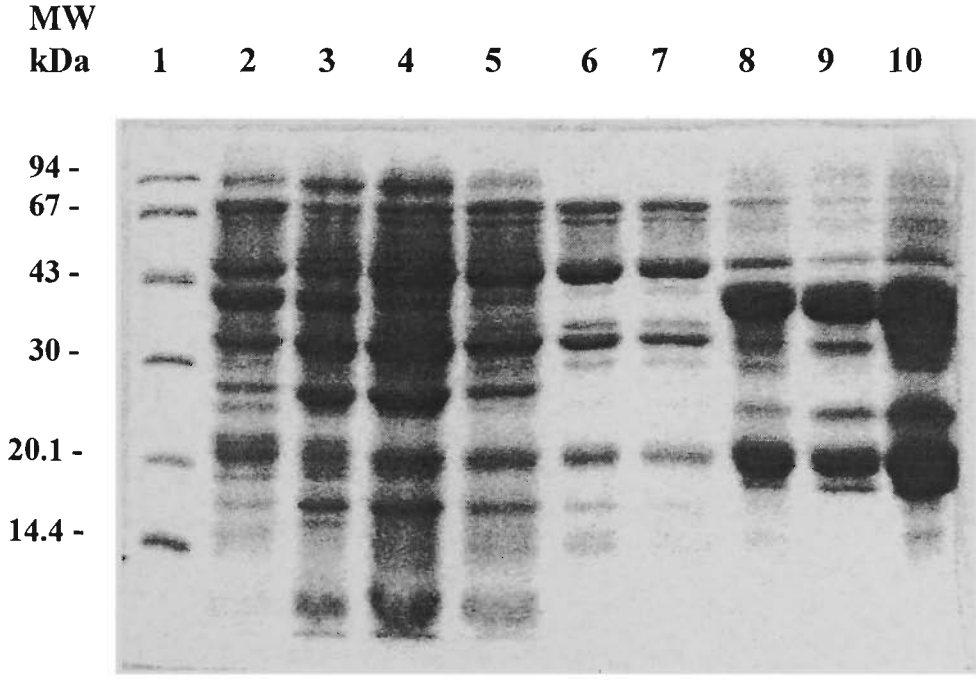


Figure 4.14 SDS-PAGE gel of protein fractions from DEAE ion exchange chromatography. 1, standard marker proteins; 2, initial protein extracts; 3-5, fractions of the first part from the column-albumins; 6-7, fractions of the second part from the column-vicilin; 8-10, fractions of the third part from the column-legumin.

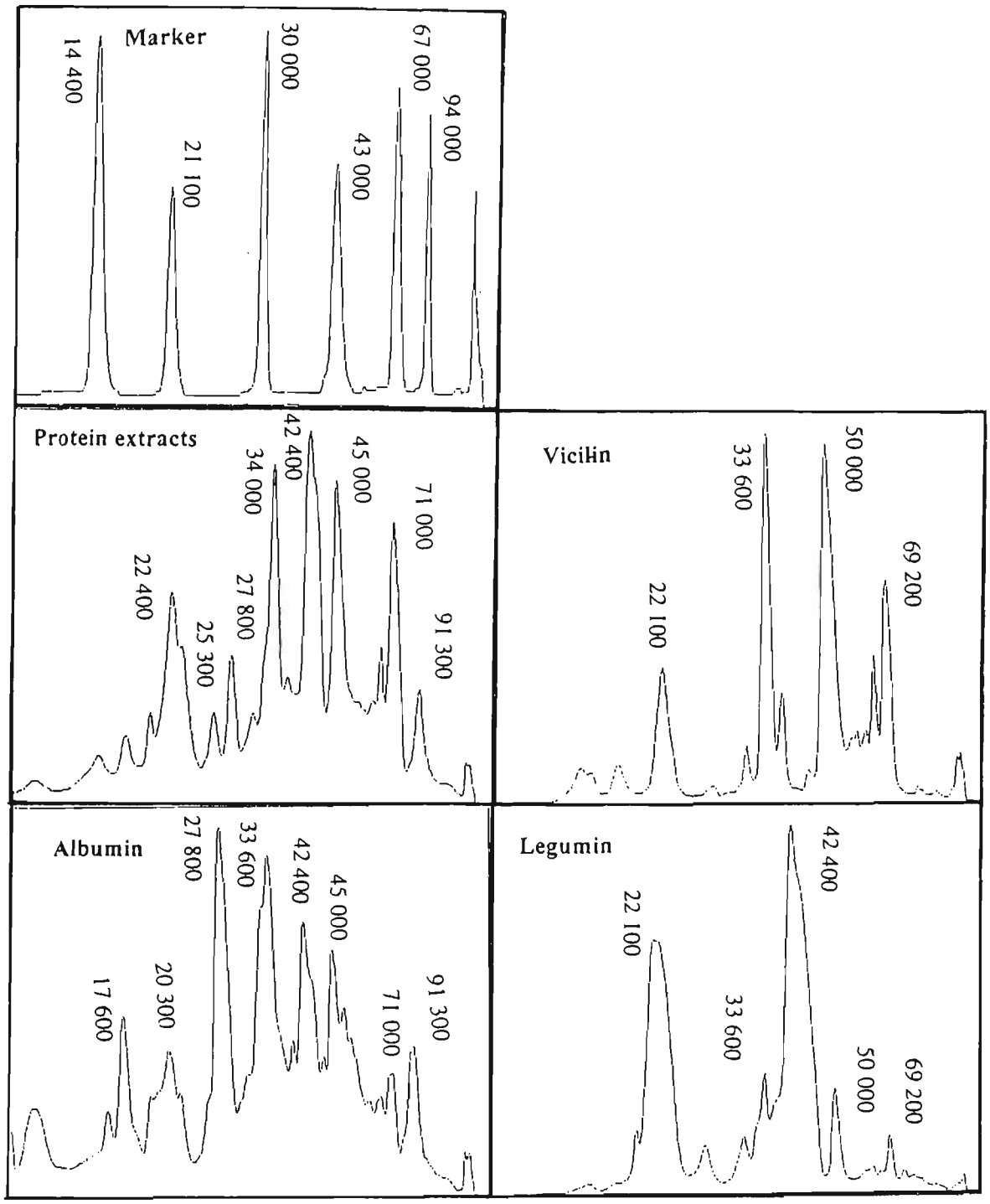


Figure 4.15 Densitometric scanning profiles of protein extracts, albumin, vicilin and legumin fractions from ion exchange column. The mixed proteins corresponded with lane 2; albumin to lane 3; vicilin to lane 6; legumin to lane 8 and marker to lane 1 of Fig. 4.14.

fraction corresponded to four major subunits at MW values of approximately 69.2 kDa, 50.0 kDa, 33.6 kDa and 22.1 kDa. However, albumin cannot be purified as readily as legumin and vicilin fractions from the ion exchange column. The albumin fraction contained most of the components of the starting protein materials as well as vicilin and legumin. On the other hand, it was interesting to find that a band at MW ~27-28 kDa was clearly evident in albumin fractions; this band was very faint in the original material and was not present in the legumin and vicilin fractions.

The albumin fraction from ion exchange was again applied to a Sephacryl 200S column for further characterisation. However, the separation was not as efficient as expected. Theoretically, in gel filtration, molecules are separated according to size in a bed packed with a porous medium. Molecules larger than the largest pores in the swollen gel beads cannot enter the gel and are eluted first. Smaller molecules which enter the gel beads to varying extents, depending on their size and shape, are slowed on their passage through the bed and eluted at a rate that is inversely proportional to their size. However, Sephacryl gel filtration media is usually suitable for globular proteins. The poor resolution found here for pea albumins using Sephacryl gel filtration, may be related to the irregular, thin layer, rod shape of the proteins (Fig. 4.11). The other possible reason could be the mixed type of proteins in this fractions, for example, the globulin proteins may be bound to the albumins intimately, made them difficult to separate on the Sephacryl gel bed. Also, albumins may have interacted with the Sephacryl resulting in the poor resolution of the proteins. As a result, preparative electrophoresis was used instead of gel filtration for further purification of albumin fractions after the ion exchange chromatography.

4.3.2. Preparative Electrophoresis of Albumin Fractions

The Prep Cell (Bio-Rad, Model 491) is a preparative electrophoresis apparatus that is used to purify proteins from complex mixtures by continuous-elution electrophoresis. Fractions that passed into the elution chamber were collected, concentrated and applied to an SDS-PAGE gel to for characterisations. The SDS-PAGE banding patterns of albumins

further purified by preparative electrophoresis were shown in Fig. 4.16. These results indicate that the main band of the albumin fraction is 27-28 kDa. This further confirmed the earlier results (Fig. 4.6 and Fig. 4.8), in which it was found that a major component in albumin fractions is a polypeptide with molecular weight of approximately 28 kDa. However, this result differs from that of Schroeder (1984), who found that the albumin fraction of the cotyledons of *Pisum sativum* L. contains two major polypeptides at MW~8 kDa and ~22 kDa. However, fresh, green seeds were used in Schroeder's work whilst the dried, yellow peas were used in the current study. This indicates that the cross-linking of protein polypeptides may occur during the later stage of maturation of the seeds.

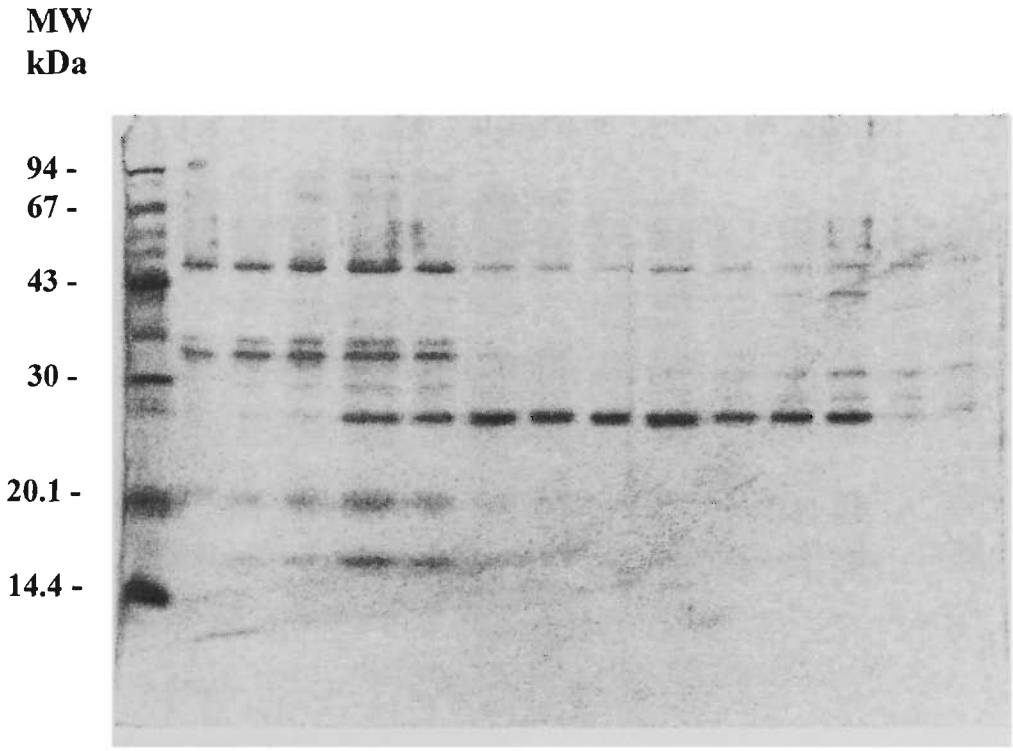


Figure 4.16 SDS-PAGE analysis of albumin fractions from preparative electrophoresis. This gel was stained with silver solution.

4.4. Conclusions

In this chapter, total protein isolation and fractionation of field pea proteins on the basis of solubility in various solutions has been extensively studied. The results reported here indicate that for the future production of total protein isolate on the pilot scale, the use of alkaline solution (pH 9) is recommended in terms of the highest recovery rate. The use of neutral salt solution (0.5M NaCl) is also a feasible way to obtain the protein isolates. The quantities of globulin and albumin fractions showed considerable variation depending upon the extraction conditions used. The albumin fraction represents a larger proportion of the soluble proteins than previously reported. Prolamin content is very low in pea proteins and butan-1-ol is a suitable solvent to extract this fraction. SDS-PAGE studies show that this fraction has quite different subunits than the globulins and albumins. The recovery of glutelin was 9% and the isolate is most likely to have been contaminated with carbohydrates.

When albumin fractions were prepared using column chromatography and preparative electrophoresis, the major protein subunit was of MW 27-28 kDa. Albumin fractions isolated using a variety of extracting solutions showed similar patterns on SDS-PAGE. These fractions had solubility characteristics and isoelectric points different from those of the protein isolate and the globulin fraction. The scanning electron microscopic observations indicated that pea albumins and globulins had different internal microstructure and surface features. This information suggests that specific fractions of field pea proteins may well have different functional properties and hence find a variety of distinct applications in food processing.

CHAPTER 5

Pilot Scale Preparation of Pea Protein Isolates

CHAPTER 5

Pilot Scale Preparation of Pea Protein Isolates

In recent years, research efforts on plant proteins have often been aimed at effective utilisation of inexpensive proteins for nutritional and functional purposes. However, most of the research has been conducted on a relatively small scale under laboratory conditions. There is little documentation on the large-scale isolation of legume proteins which might relate to potential commercial production.

Based on the information obtained from preliminary studies reported in Chapter 4, the pilot scale extraction of field pea proteins has been undertaken. Two different approaches have been used and the recoveries of protein compared. The first involved extraction with a salt solution followed by decantation and clarification to remove solids. The solution was then further concentrated and salt removed by ultrafiltration and diafiltration. The second procedure was based upon alkaline extraction followed by decantation and recovery by isoelectric precipitation and neutralisation. Both spray drying and freeze drying methods were employed. The protein isolates have been compared and characterised with respect to solubility, chemical composition and to electrophoretic patterns. The physical properties of the protein isolates, such as colour, particle size, thermal properties (by differential scanning calorimetry) and characteristic surface features (via SEM) have also been evaluated. These properties are relevant to each stage of utilisation encompassing product handling, processing to consumer acceptance, as well as the functional properties which the products will demonstrate in food applications.

5.1. Pilot Plant Preparation of Protein Isolates

The outlines of the processes to prepare the field pea protein isolates extracted with alkaline solution (API) and salt solution (SPI) are summarised in Fig. 5.1 and Fig. 5.2.,

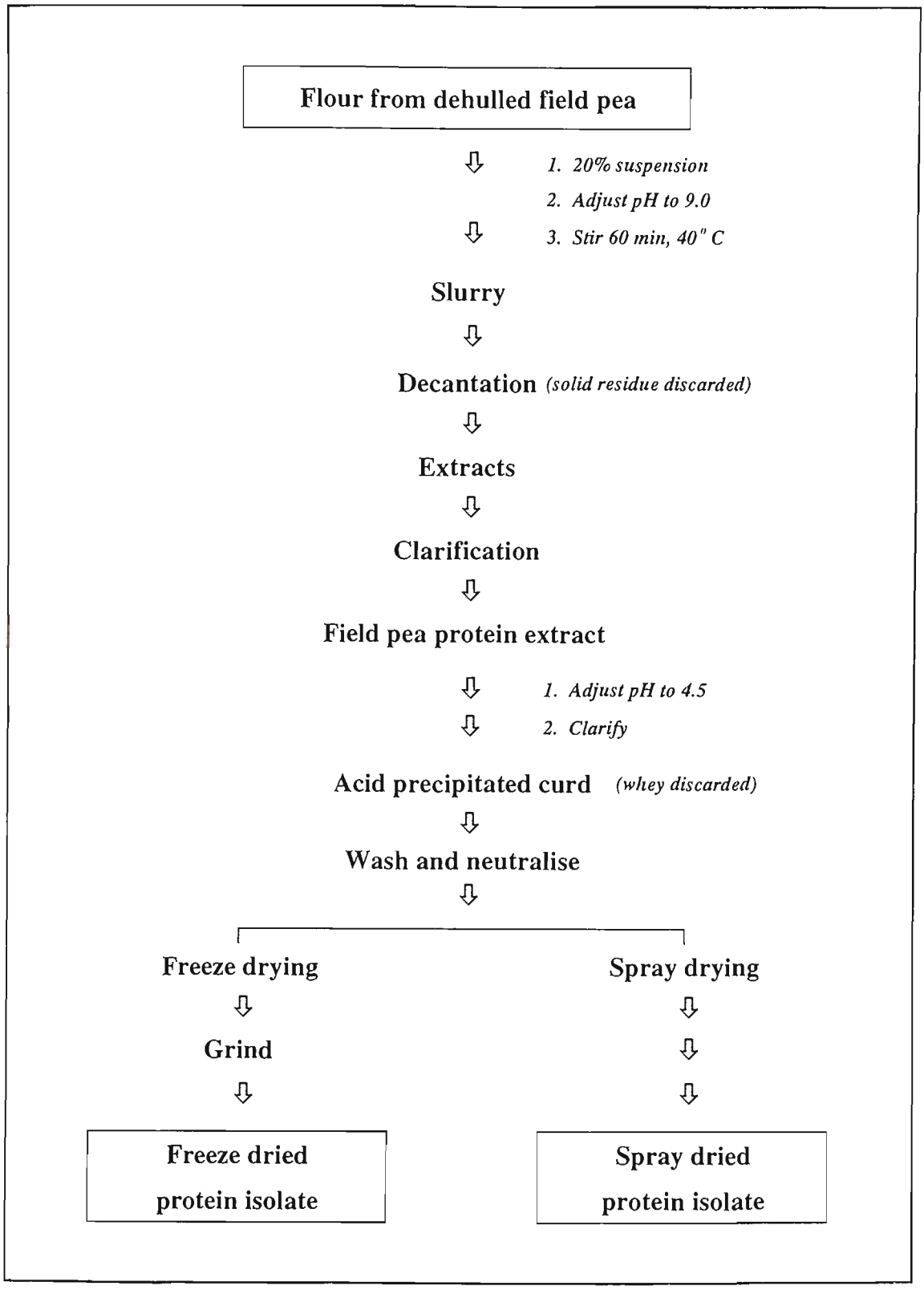


Figure 5.1 Flowchart of the process to prepare field pea protein isolate with alkaline solution.

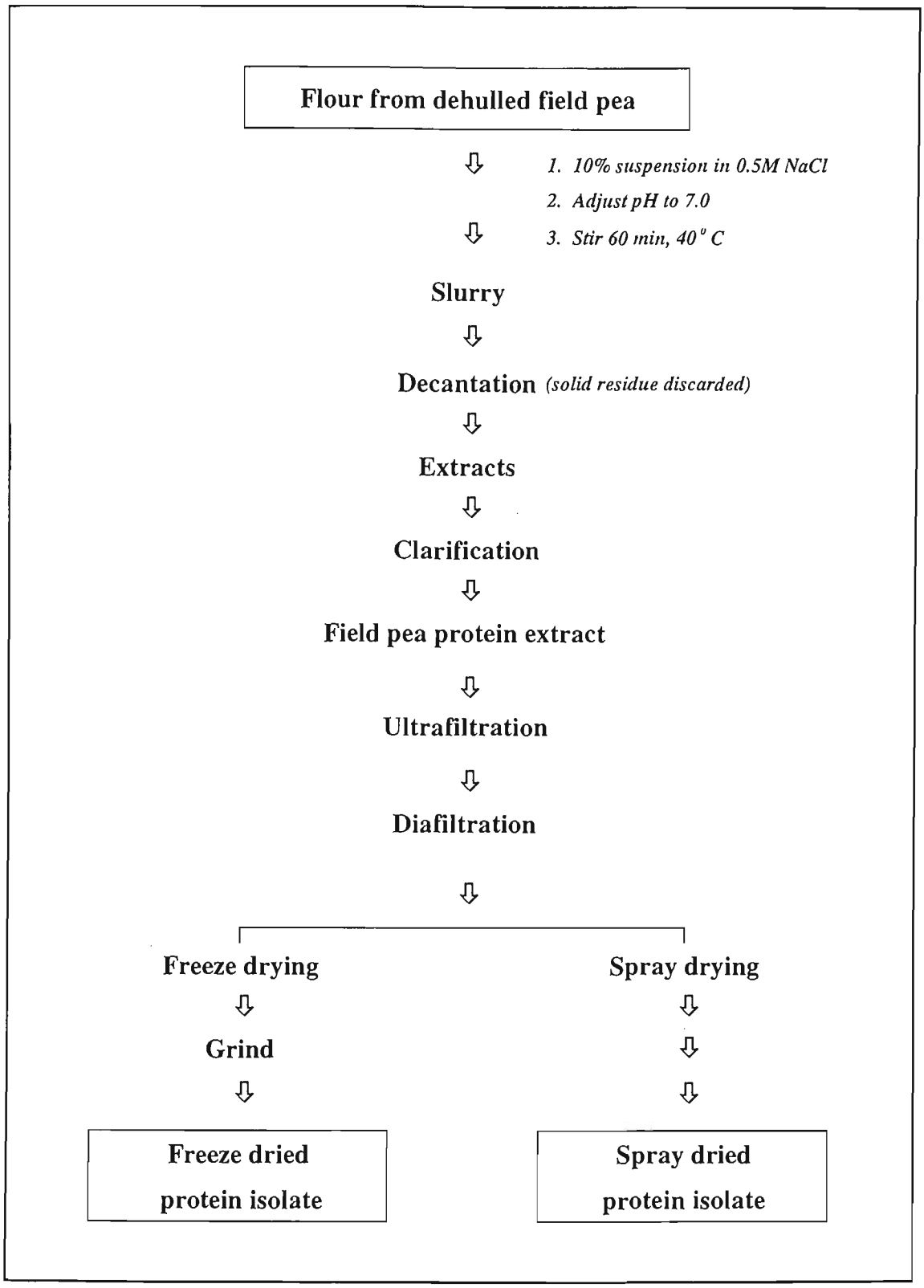


Figure 5.2 Flowchart of the process to prepare field pea protein isolate with salt solution.

respectively. Details of the processes have been described in Chapter 3 (3.3.1). Fig. 5.3-5.5 show some of the equipment used in the pilot factory, including the decanter centrifuge and clarifier centrifuge (for separating the solids and solutions), ultrafiltration membranes, and spray drier. The intermediate protein isolate which was extracted with salt solution, is shown in Fig. 5.6 at the stage prior to drying.

In this study, 190 kg and 60 kg dehulled field pea flour have been used for the extraction of the proteins with alkaline solution (pH 9, 20% suspension) and salt solution (pH 7, 10% suspension in 0.5M NaCl), respectively. The pH value of the starting material in the neutral water was around 6.5-6.6. Thus with both procedures, the pH has to be carefully adjusted to the desired range before extraction commenced. It has been found that variations of temperatures (15°C- 45°C) did not result in any significant differences in the recovery of some legume proteins including those of chickpeas (Liu, 1996). On the other hand, the most suitable temperature range for the process of ultrafiltration and diafilitration was 40°C- 50°C (Bérod *et al.*, 1987), since the relatively higher temperature could help to prevent membrane adsorption. Therefore in order to consistently control the conditions during processing, 40°C filtered water was used in the extraction step, as well as in the ultrafiltration and diafiltration systems in the current study.

5.2. Composition of Field Pea Protein Isolates and Protein Recovery

Results of the proximate analyses of the original grain flour and the protein isolates are listed in Table 5.1. The protein recovery rates for alkaline and salt extractions were 59%, 40%, respectively. Isolate extracted with salt (SPI) contained more protein than the alkaline extract (API) and both had higher ash contents than the original flour. This reflects a small amount of salt remaining after processing as well as the salt produced due to the food-grade acid and alkali used for protein precipitation and neutralisation (Sosulski & McCurdy, 1987).

It is likely that the further application of ultrafiltration and diafiltration would have increased the protein content and decreased the salt content of the dried product.

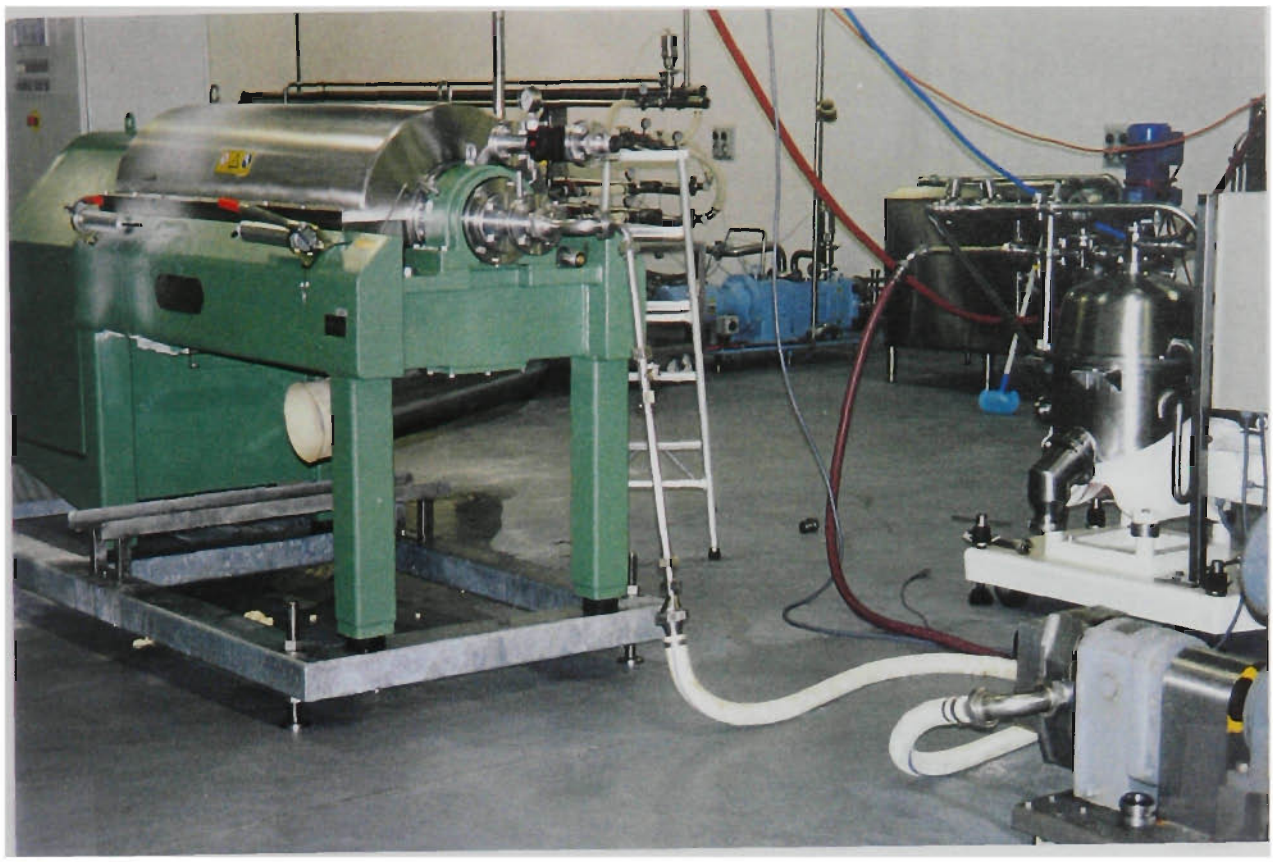


Figure 5.3 Decanter (left) and clarifier (right) centrifuge used in pilot processing.

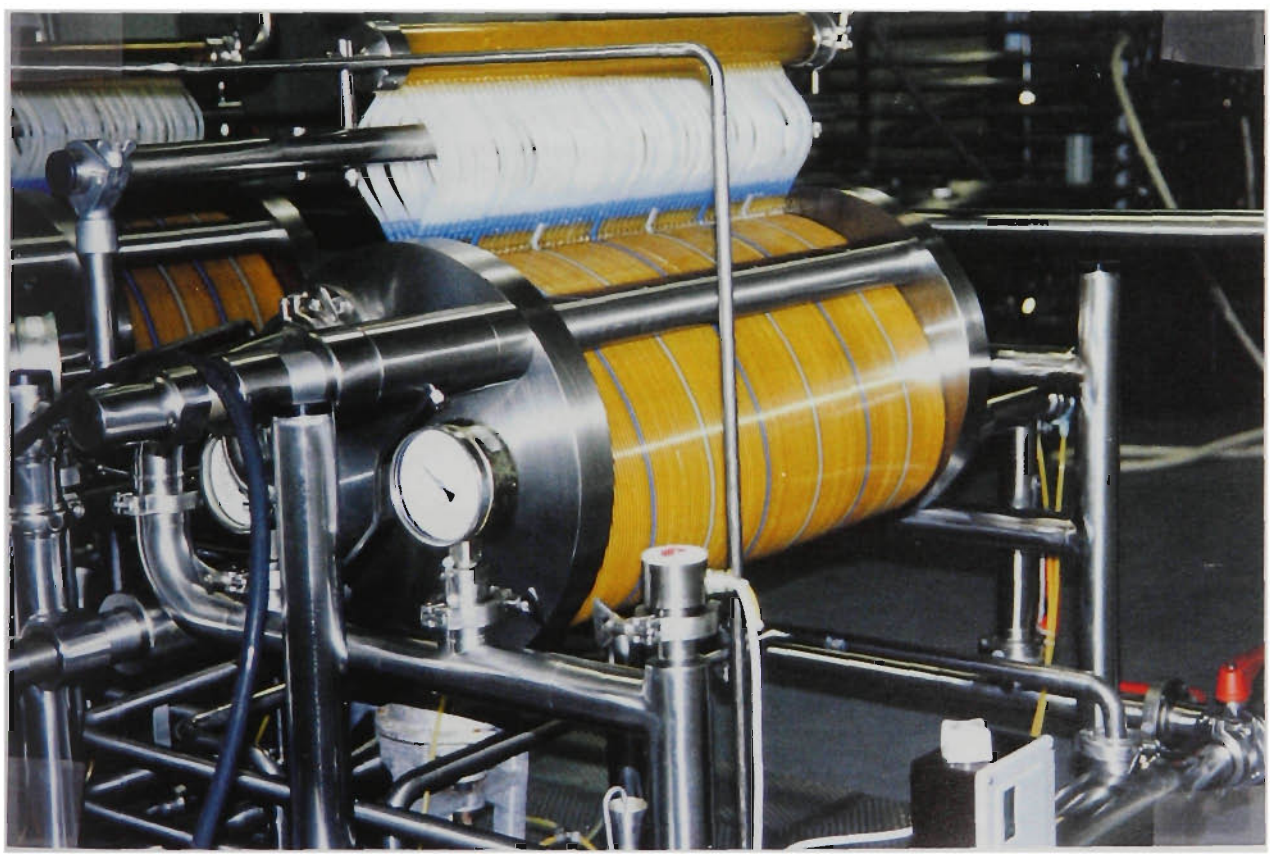


Figure 5.4 Ultrafiltration membranes used in pilot processing.

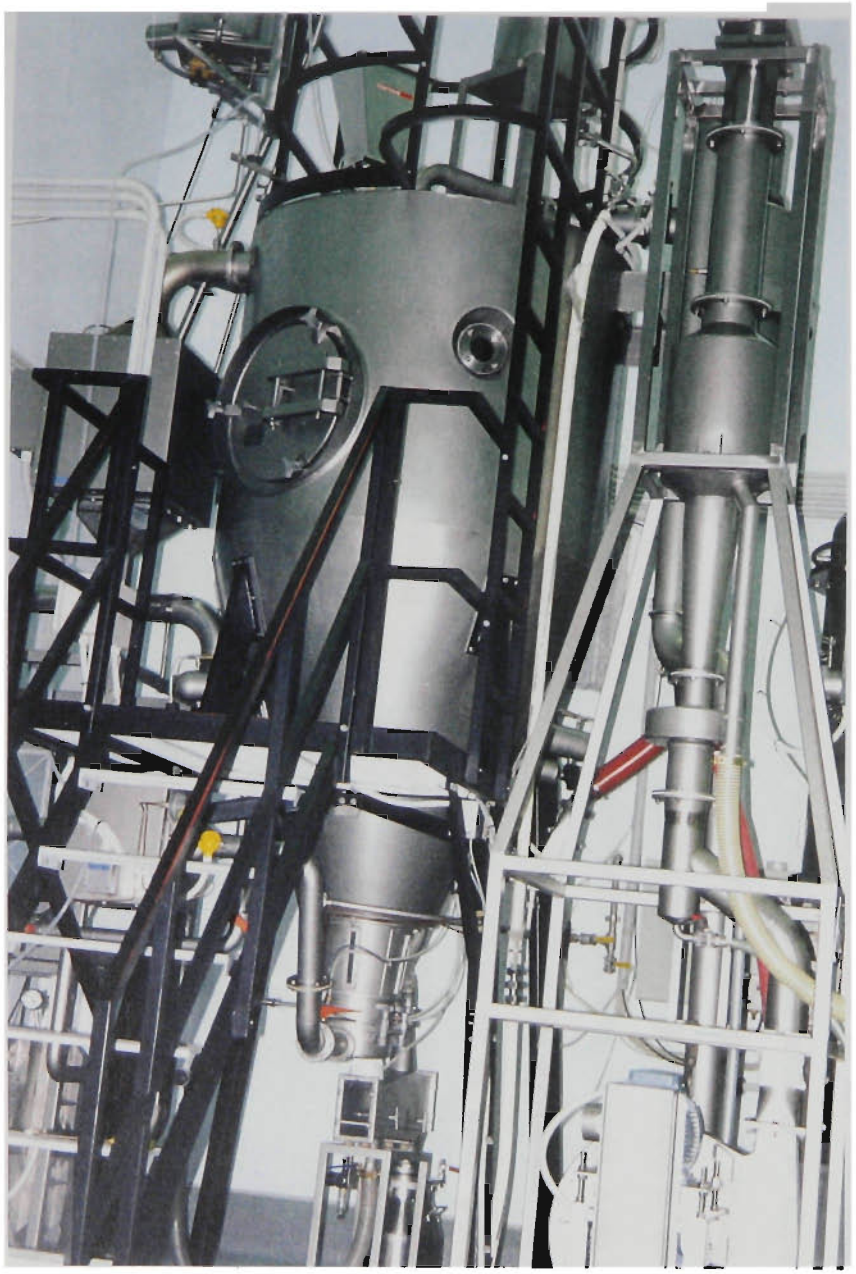


Figure 5.5 Spray drier used in pilot processing.

Figure 5.6 Salt-extracted protein solution prior to drying.

Table 5.1 Proximate Composition of Field Pea Flour (dehulled) and Protein Isolates Produced on the Pilot Scale[a]

Component	Flour	SPI[b]	API[c]	Residue[d]
Moisture (%)	8.6	3.9	3.7	58.3
Protein (N×6.25) (%)	28.8	81.1	77.1	1.84
Crude fat (%)	2.68	2.54	2.43	0.65
Crude Fibre (%)	1.10	0.07	0.02	1.23
Ash (%)	2.67	5.56	5.97	1.29
Carbohydrate[e] (%)	64.8	10.7	14.5	95.0
Salt (NaCl) (%)	--	5.36	5.23	--

a: Moisture values expressed "as is", others on a dry weight basis
b: Pilot scale salt (0.5M NaCl) extracted protein isolate
c: Pilot scale alkaline (pH 9) extracted protein isolate
d: Residue from alkaline extraction
e: Carbohydrate calculated by difference

However the resulting recovery of the protein would have been lower. Another consideration is that continuous diafiltration systems add water to the retentate as permeate is removed (Nichols & Cheryan, 1981). Accordingly the addition of steps to remove salt would result in problems being encountered during subsequent drying of the isolate. If more water were used to wash the protein extracts, the concentration of the proteins would not be high enough (less than 8-10%) for the spray drying. Also for the freeze drying, more time and energy would be needed to remove the excess water. However, the primary advantage of continuous diafiltration is to keep protein concentration low during processing which reduces yield losses due to membrane adsorption.

For the other process where alkaline solution was the extracting agent, use of water to wash the isoelectric precipitated proteins could increase the protein content by 6%, but the recovery rate would be decreased by 7%. This result was obtained during the first trial, in which a relatively small amount of protein extract was separated and used for a second washing step for comparative purposes. In addition, the clarification process

was found time consuming and hence only one washing process was used in processing of the protein isolate during other trials. Generally speaking, the protein contents of the isolates produced in pilot scale were lower than for the proteins extracted in the laboratory (cf Table 4.1). This is believed to result mainly from the differences of equipment used for removing solids from the liquids. The speed of the centrifuge in the laboratory is high enough to separate the solids and liquids very effectively, especially where only a few millilitres of solution are applied. However, on the pilot scale, hundreds of litres of the extracted mixture were continuously passed through the decanter, which has different separating and discharge systems compared with the single step centrifuge system used in the laboratory. The smaller particles of the solids cannot be removed using this procedure. Even with the clarifier, which is more efficient in separating the fine particles from the liquids, a small amount of carbohydrate still remains with the protein extracts after this separation step.

5.3. Solubility of Field Pea Protein Isolates

5.3.1. Solubility Profiles of Field Pea Proteins

The solubility curves for the pilot scale isolates were determined, as shown in Fig. 5.7 and Fig. 5.8. These indicate that the salt extracted proteins had a higher pI (5.5) than those extracted with alkali (4.5). Salt extracted and alkaline extracted proteins have similar solubility profiles, but the former exhibited more soluble properties at its pI than the latter. This is because of the relatively mild conditions for extraction and preparation of the proteins with salt solutions. In comparison to the pea proteins extracted on the laboratory scale (Fig. 4.2), pilot scale production did not result in any significant deterioration of the nitrogen solubilities.

5.3.2. Effect of Drying Method on the Solubility of Field Pea Proteins

Compared with the spray dried products, the solubilities of freeze-dried proteins were not superior. Generally, freeze drying minimises the physical changes that normally

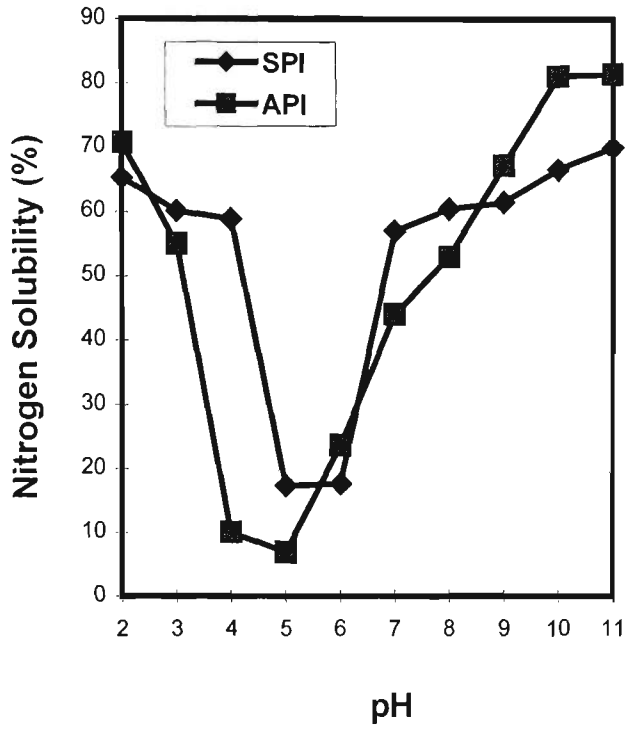


Figure 5.7 Solubility profiles of field pea protein isolates by spray drying. SPI, prepared with salt (0.5M NaCl) solution; API, prepared with alkaline solution.

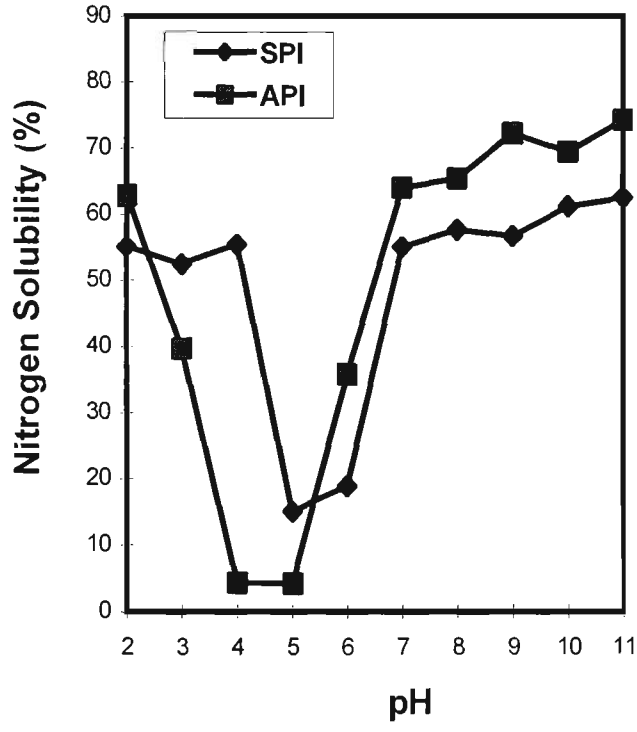


Figure 5.8 Solubility profiles of field pea protein isolates by freeze drying. SPI, prepared with salt (0.5M NaCl) solution; API, prepared with alkaline solution.

accompany drying, with a good retention of aroma, flavour and nutrients (Snowman, 1997). The products usually can be readily rehydrated in subsequent use. However, spray drying is now regarded as a mature technology, often combining atomisation, fluidisation and agglomeration in a single system to meet particular end-product quality specifications (Masters, 1997). The atomisation stage creates a very large wet surface area in the form of millions of droplets which, when exposed to the hot drying air, results in very high rates of heat and mass transfer. Drying times are very short and are carefully controlled to protect protein products from thermal denaturation. Hence the nitrogen solubilities of the products produced by freeze drying and spray drying did not show any major differences. Moreover, to obtain 30 kg protein isolates, it required between 3-4 days to freeze dry the 300 L protein slurries from an initial concentration of approximately 10% (w/v). On the other hand, a period of only 6-7 hours was necessary in order to supply the same amount of spray dried product. As a result, for producing powdered legume protein isolates on a pilot scale, spray drying has a clear advantage over freeze drying in terms of both operation cost and product quality. However, spray drying has not been commonly used on a laboratory scale. Thus sometimes it is difficult to compare the results obtained from laboratory and pilot factory, particularly because the products were not produced under identical conditions. For this reason both freeze drying and spray drying have been used as drying methods to produce pea proteins in the pilot scale in this study.

5.4. SDS Polyacrylamide Gel Electrophoresis of Pea Protein Isolates

The structural characteristics of the proteins have been investigated using SDS-PAGE. The patterns of the pilot scale isolates have been compared with those of fractions isolated in the laboratory by the traditional Osborne procedures (Fig. 5.9). For further comparison, a laboratory extraction using the same alkaline solution was also prepared and subjected to electrophoresis (lane 10). This total protein isolate showed a similar pattern to the pilot scale isolates.

The four different laboratory fractions (albumin, globulin, prolamin and glutelin) extracted from field peas have distinct patterns of subunits. In contrast, each of the protein isolates prepared on a pilot scale showed electrophoretic patterns which were indistinguishable from each other. The use of the two different extracting solutions did not appear to result in any molecular changes which would be reflected in changes to the electrophoretic pattern. Furthermore, the application of freeze drying or spray drying did not cause any differences to the patterns of the isolates. As might be expected, both albumins and globulins are present in each of the pilot scale isolates. However, the major bands of the albumins which were present in the albumin fraction extracted in the laboratory (lane 2), are relatively faint in the pilot scale isolates, indicating that part of the albumin fraction was lost during processing. This may have occurred as whey was discarded when the bulk of the protein was precipitated during the purification of the isolate extracted with the alkaline solution. In the case of the

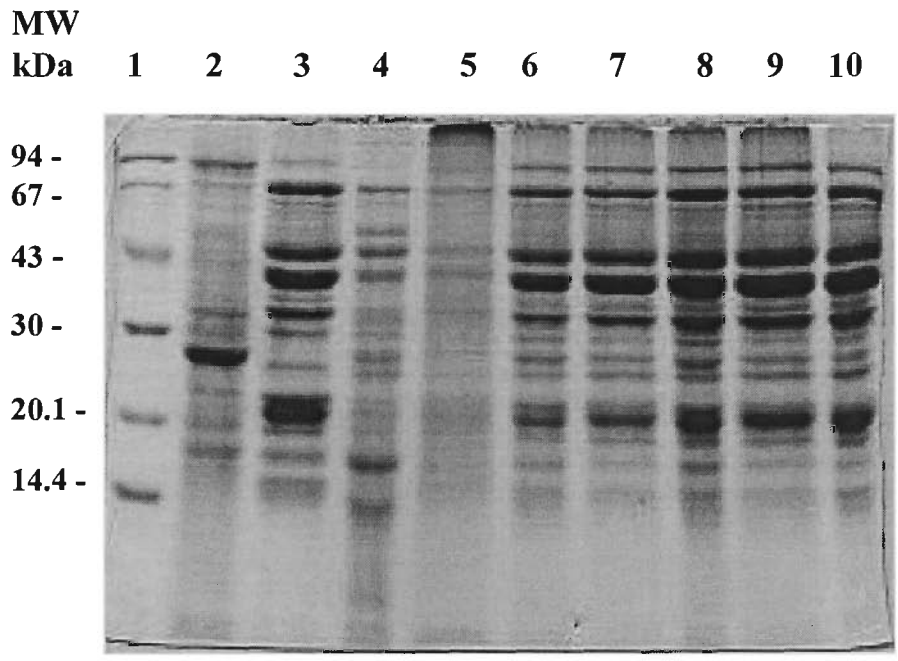


Figure 5.9 Electrophoretic patterns (SDS-PAGE) of field pea protein isolates and fractions. 1, standard proteins; 2, albumin; 3, globulin; 4, prolamin; 5, glutelin; 6 and 7, protein isolates prepared with alkaline solution by spray drying and freeze drying on the pilot scale, respectively; 8 and 9, protein isolates prepared with salt solution by spray drying and freeze drying on the pilot scale, respectively; 10, protein isolate prepared with alkaline solution on the laboratory scale.

other isolate, losses of albumins may have occurred at the concentration stage where ultrafiltration and diafiltration were applied. It might be hypothesised that the properties of the pilot scale isolates would more closely reflect those previously reported for globulins isolated in the laboratory studies (Chapter 4).

5.5. Physical Properties of Field Pea Protein Isolates

5.5.1. Colour Characteristics

The colour parameters of the flour and the protein isolates are presented in Table 5.2. The field pea flour was white-yellow in colour with the highest L^* value of 88.95, whereas the pea protein isolates were creamy to beige in colour. Fig. 5.10 shows the appearances of pea protein isolates produced in the pilot studies. As can be seen from this picture, salt extracted proteins had a lighter colour than the alkaline extracted proteins and this was confirmed by Minolta Chromameter measurement (Table 5.2). Sosulski and McCurdy (1987) also found that during the process of acid or alkali extraction, isoelectric precipitation caused more darkening of field pea and faba bean compared with air-classified fractions. Data has not been found on isolation and identification of the pigments producing colour in dry peas. However, clearly colour

Table 5.2 Colour Parameters of Field Pea Flour and Protein Isolates

Colour	Flour	SPI.1[a]	SPI.2[b]	API.1[c]	API.2[d]
L^*	88.95	74.42	83.66	65.86	79.29
a^*	-5.55	-2.35	-4.35	-1.07	-3.31
b^*	+22.97	+25.69	+21.12	+22.90	+23.96

a: Pilot scale salt (0.5M NaCl) extracted protein isolate by freeze drying
b: Pilot scale salt (0.5M NaCl) extracted protein isolate by spray drying
c: Pilot scale alkaline (pH 9) extracted protein isolate by freeze drying
d: Pilot scale alkaline (pH 9) extracted protein isolate by spray drying

L^* =whiteness/brightness, a^*(+) =redness, a^*(-) =greenness, b^* (+) =yellowness, b^* (-) =blueness

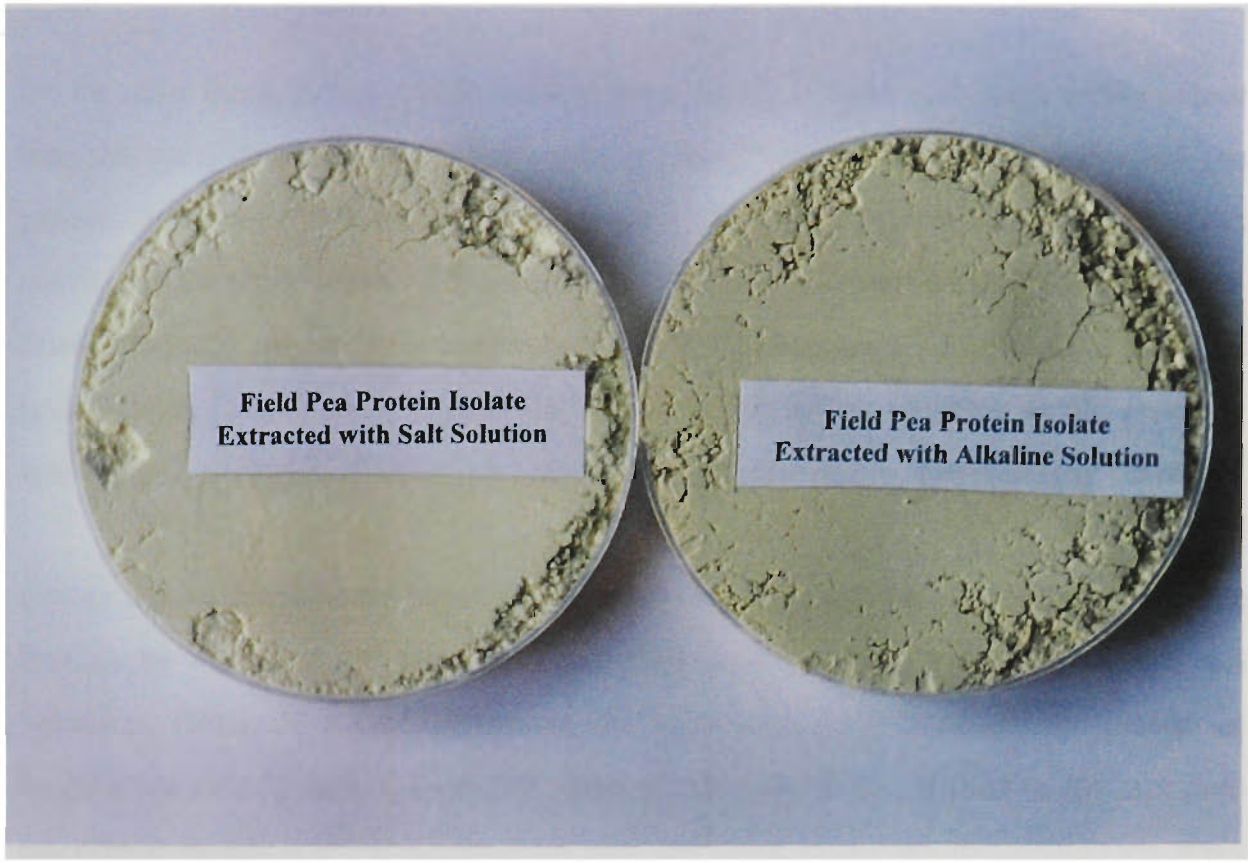


Figure 5.10 Field pea protein isolates produced on the pilot scale.

development is favoured by the alkaline conditions. Even with the isoelectric precipitation procedures, the protein curd presented white in colour prior to neutralisation. Plant phenols are usually the major contributors to the colour problem of these protein materials, as oxidative reactions of phenolics quite often lead to attachment of these coloured components to protein and polysaccharide (Blouin *et al.*, 1981). Thus the dark colour of pea proteins extracted under alkaline conditions are probably due to oxidative products of alkaline stable phenolic components.

On the other hand, it was also found that freeze-dried samples had much darker colour than the spray-dried products. For example, the L^* values of alkaline extracted pea proteins by freeze frying and spray drying are 65.86 and 79.29, respectively. This result confirmed the observation of Sumner *et al.* (1981) who hypothesised that the freeze-dried products might have been darkened by oxidation of components such as polyphenols. During spray drying, the hot air in the drying chamber would probably inactive polyphenol oxidase which usually promotes the enzymatic browning reactions.

Colour and appearance are major quality attributes of foods, since these factors are the first to be evaluated by the consumer when they purchase foods (von Elbe and Schwartz, 1996). As a food ingredient, the legume protein isolates should present as bright a colour as possible. From this point of view, the better method to produce field pea protein isolate is to extract the flour with salt solution and dry the product via spray drying.

5.5.2. Particle Size Characterisation

Fig. 5.11 shows the particle size profiles of field pea flour, salt extracted protein isolate by spray drying, alkaline extracted protein isolate by freeze drying and spray drying. The results of particle size analyses are further demonstrated in Table 5.3. The salt extracted product after spray-drying gave a relatively even size with most particles between 10 μm and 100 μm diameter. On the other hand, for spray dried isolate extracted with alkaline solution, there were some very fine particles having a diameter

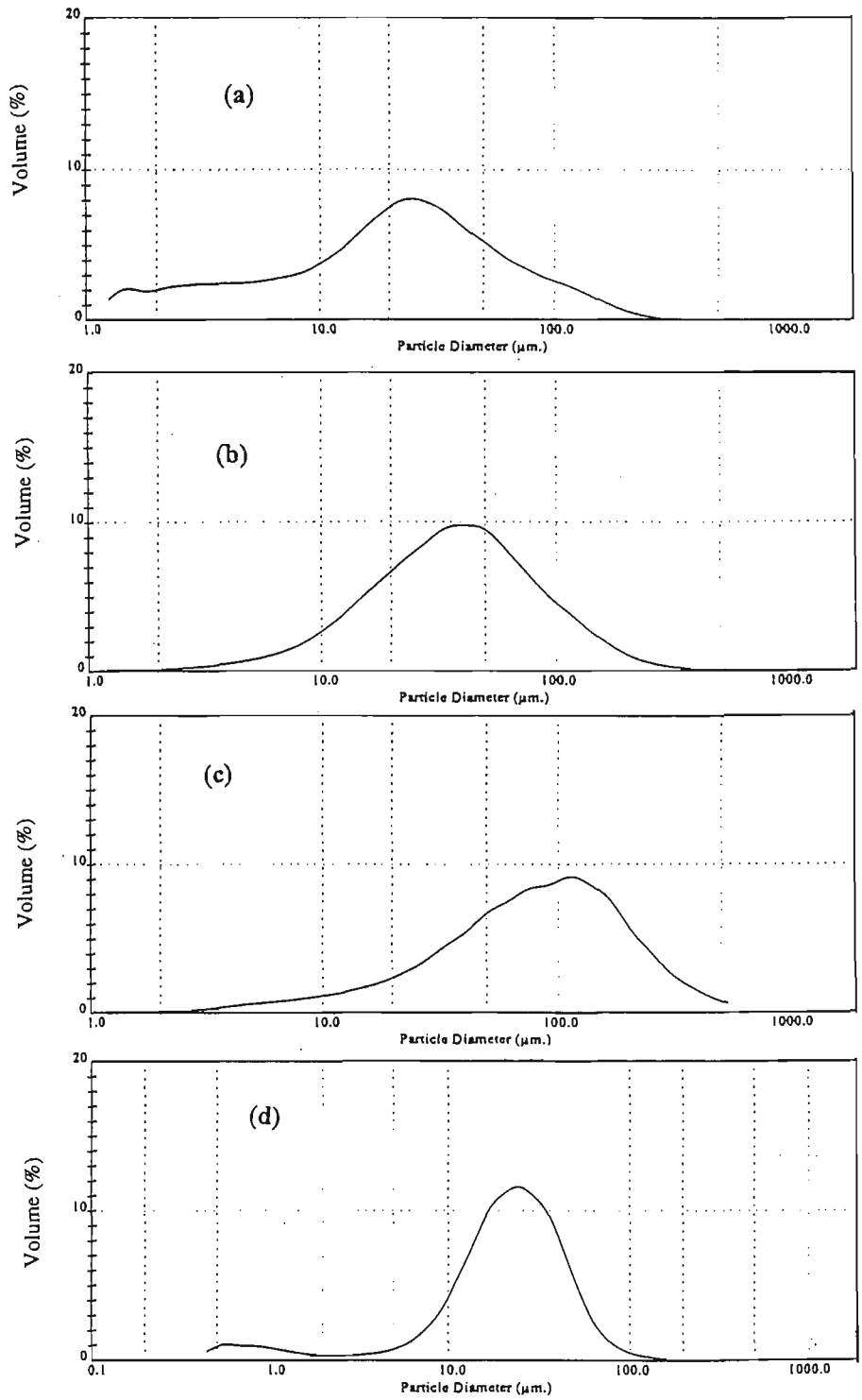


Figure 5.11 Particle size profiles of field pea flour and protein isolates. (a) flour, (b) salt extracted isolate by spray drying, (c) alkaline extracted isolate by freeze drying (d) alkaline extracted isolate by spray drying. Note that the scale on the horizontal axis of the profile (d) is different from the other profiles.

Table 5.3 A Comparison of the Particle Sizes of Field Pea Flour and Protein Isolates

	Flour	SPI[a]	API.2[b]	API.1[c]
Mean particle diameter (μm)	21.4	37.4	83.1	21.7
Proportion of particles (%) with diameter less than--[d]				
1 μm	2.3	0.1	0.1	5.1
5 μm	17.3	2.1	1.7	9.1
10 μm	27.9	7.6	5.0	16.1
50 μm	81.4	64.3	31.3	92.8
100 μm	93.3	88.3	58.2	99.1
400 μm	99.9	99.8	98.0	100.0
600 μm	100.0	100.0	100.0	100.0

a: Pilot scale salt (0.5M NaCl) extracted protein isolate by spray drying
b: Pilot scale alkaline (pH 9) extracted protein isolate by freeze drying
c: Pilot scale alkaline (pH 9) extracted protein isolate by spray drying
d: expressed as percentage of particles having a diameter less than that indicated

≤ 1.0 μm. Freeze-dried products showed a larger particle size between 100 μm and 400 μm and this could be reduced by regrinding. However this is time consuming and the heat generated in the grinding procedure may cause partial denaturation of the protein molecules.

5.5.3. Microstructure and Surface Features by Scanning Electron Microscopy

Even where the particle sizes are similar, shape variations in food powders are enormous and these are mainly determined by the material from which they are made and the process by which they are formed (Peleg, 1983). Fig. 5.12 and 5.13 shows the scanning electron micrographs of spray-dried field pea protein isolates extracted with salt and alkaline solution, respectively. The differences of microstructure of the protein powders resulting from freeze drying are demonstrated in Fig. 5.14. As can be seen from the micrographs, spray drying gave a spherical shape, regardless of whether the

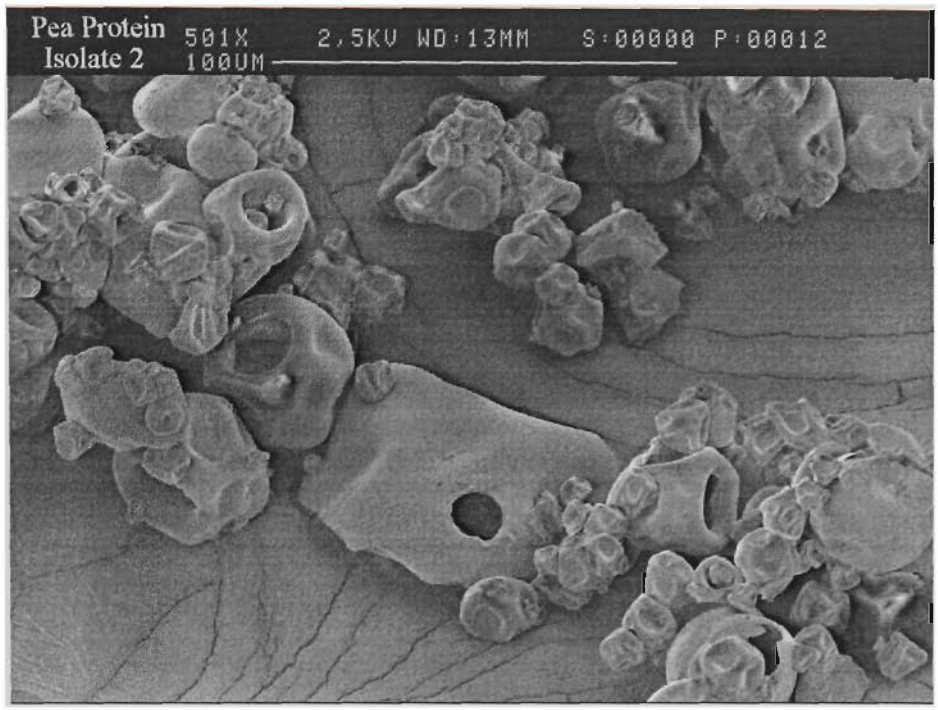


Figure 5.12 Scanning electron micrograph of spray-dried field pea proteins extracted with salt solution on pilot scale.

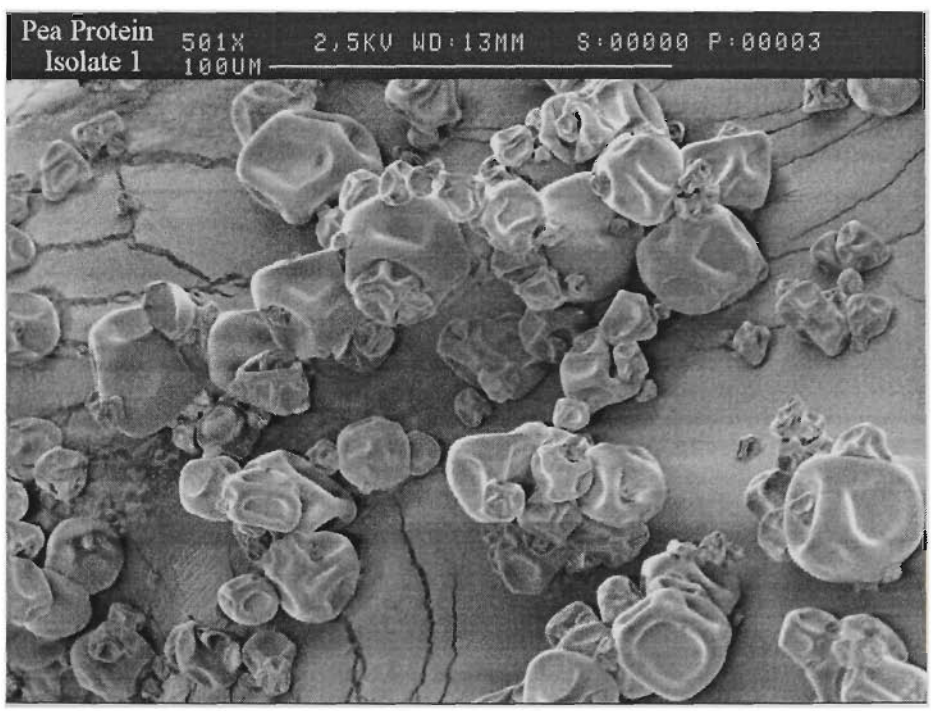


Figure 5.13 Scanning electron micrograph of spray-dried field pea proteins extracted with alkaline solution on pilot scale.

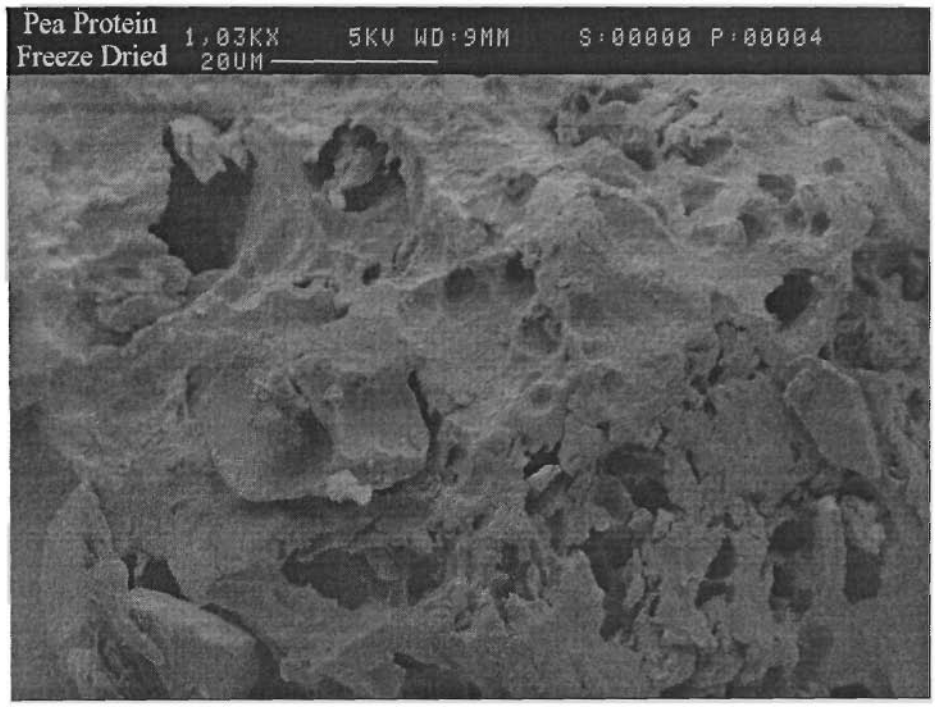


Figure 5.14 Scanning electron micrograph of freeze-dried field pea proteins extracted with alkaline solution on pilot scale.

protein isolates were extracted with salt or alkaline solution. On the other hand, freeze drying resulted in totally different surface features, which presented a irregular shape, denser mass with some pores inside when compared to spray-dried proteins. This characteristic resembles the surface morphology of the pea protein isolate extracted in the laboratory and subjected to freeze drying (Fig. 4.10).

In order to fully understand and control the physical characteristics of a food requires a good knowledge of what happens to the microstructure during processing (de Man, 1983). Initial studies on the microstructure of legume products by SEM have particularly included those from soybeans (Saio and Watanabe, 1966; Wolf, 1970; Wolf and Baker, 1972). These provide the basic information on the nature of the protein bodies in soybeans, as well as the differences of the microstructure resulting from various processing conditions, such as heat, pressure, freezing or texturisation. Micrographs of spray-dried soy proteins (Peleg, 1983) show a very similar globular pattern to spray-dried pea proteins obtained in this study. Thus the variations in

processes appear to have a greater effect on the microstructure of plant proteins than the source of the proteins. Hence it is likely that field pea proteins, like soybeans, could also provide protein foods with more interesting physical characteristics, such as textured products, if dedicated effort was applied to research in this area.

5.5.4. Thermal Properties Measured by Differential Scanning Calorimetry (DSC)

DSC offers considerable potential for studying physico-chemical changes that occur in foods. It has been used especially to study the state of water and ice in foods, denaturation of proteins, and gelatinisation of starches (Lund, 1983). Thermal denaturation of food proteins, including plant proteins, could result in changes in the secondary, tertiary or quaternary structure of the protein molecules. These changes will affect the functionality and thus their application in food systems (Arntfield and Murray, 1981). For example, the loss of native structure or denaturation is critical to the functional properties of proteins, such as gelation, emulsification and foaming (Kinsella, 1976).

DSC is used to assess the thermal properties of proteins and can supply both kinetic and thermodynamic data, including temperature of denaturation (Td) and the enthalpy change associated with transition (ΔH) (Murray *et al.*, 1985). The enthalpy changes are measured as differential heat flow between sample and reference and recorded as a peak. The peak analysis enables determination of Td and ΔH from maximum peak temperature and area of the peak respectively (Harwalkar and Ma, 1987). The sharpness of the peak also indicates the cooperative nature of the transition from native to denatured state. If the rupture of intramolecular bonds occur within a very narrow range of temperature (very sharp peak) the transition is considered highly cooperative. The broader the peak the less cooperative is the transition (Wright *et al.*, 1977).

DSC has been used to study thermal denaturation of some food proteins such as muscle proteins (Wright *et al.*, 1977), egg albumin (Raeker and Johnson, 1995; Donovan *et al.*, 1975), soybean proteins (Hermansson, 1978; 1979b), oat proteins (Ma and Harwalkar,

1984) and fababean proteins (Arntfield and Murray, 1981). Factors such as salt or alkali which affect the thermal properties of oat or fababean proteins have also been investigated (Ma *et al.*, 1990; Arntfield *et al.*, 1986). However, limited data has been found with respect to the thermal properties of field pea proteins (Bacon *et al.*, 1990).
In order to study the effect of processing conditions on the thermal denaturation of field pea proteins, the isolates were examined using DSC. The thermograms of the proteins extracted with salt and alkaline solution in the pilot scale, are compared in Fig. 5.15. In addition, the thermogram for the globulin fraction, which was isolated with NaCl solution in the laboratory, was also evaluated. Table 5.4 shows the results of the protein denaturation profiles analysed by DSC.

With regard to the field pea isolates produced on the pilot scale, two distinct peaks were identified in the thermograms (Fig. 5.15a and b). These peaks represented two structurally distinct proteins. One showed the lower temperature endotherm with the Td at 82.4- 85.3°C, and the other gave the higher temperature endotherm with the Td at 97.0- 98.7°C. These results are comparable with those attributed to the denaturation of vicilin and legumin by Casey *et al.* (1982), who studied the vicilin- legumin ratios by using different techniques including differential scanning calorimetry, analytical ultracentrifugation and crossed immuno-electrophoresis. However, the effects of extracting agents and processing conditions were not considered in their studies. For example, from their results, the higher value of denaturation enthalpies (ΔH) was found with legumin (which showed a higher Td) in comparison with vicilin. The extracting buffer used by Casey and coworkers was 0.2 M NaCl + 0.05M NaH_2PO_4 (pH 7). In another study, when the proteins were prepared by ammonium sulphate extraction, a higher value of ΔH has been found for vicilin (Bacon *et al.*,1990). The results in the current study show that the API has a relatively high ΔH value for legumin (T_2), while the salt extracted protein isolate presents a much higher peak area for vicilin (T_1). This indicates that DSC is not an accurate method of quantitating storage proteins from legumes, based on the ratio of ΔH values obtained from the areas of the different peaks.

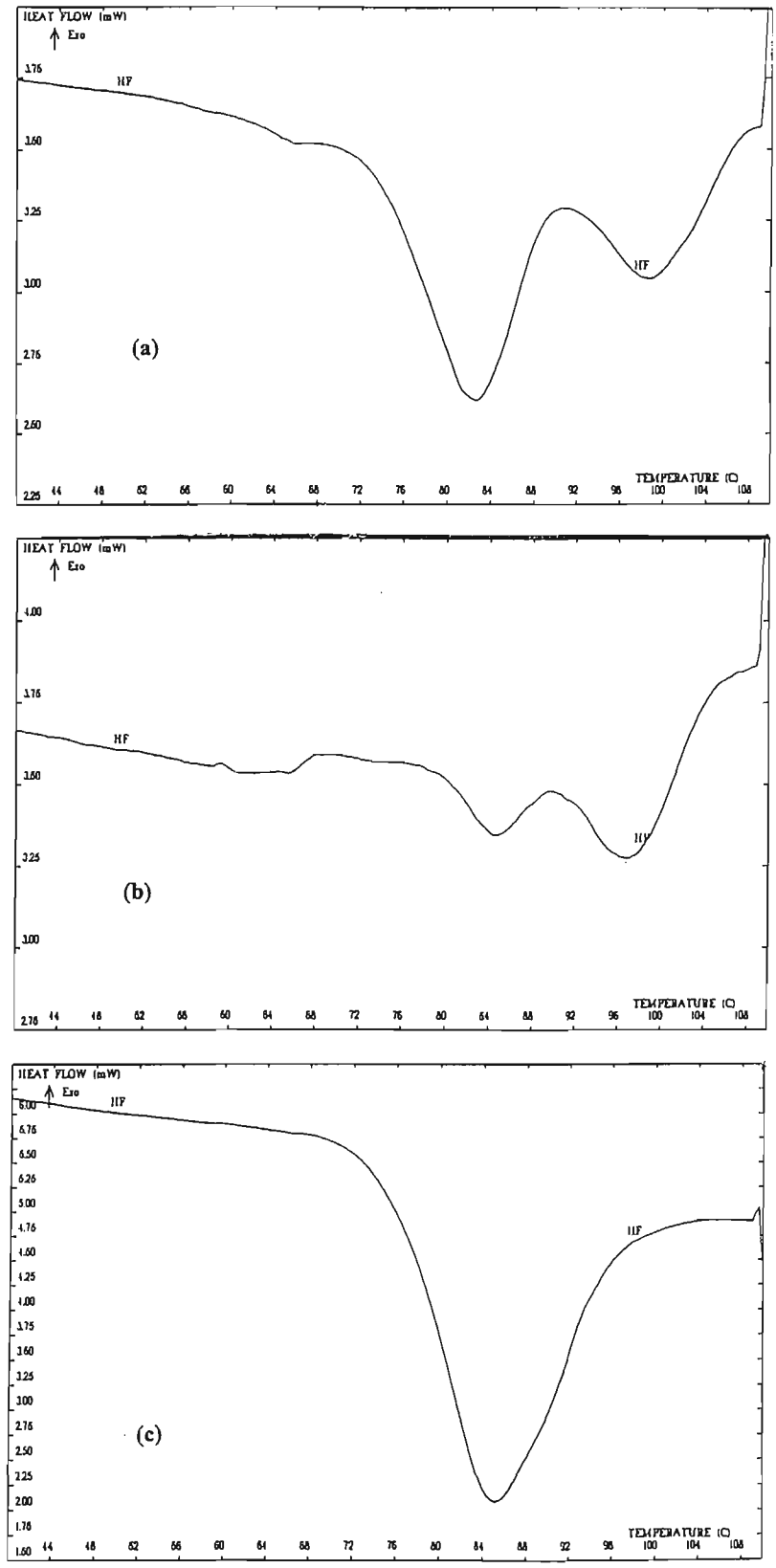


Figure 5.15 DSC thermograms of field pea proteins. (a) isolate extracted with salt solution, (b) isolate extracted with alkaline solution, (c) micellar-globulin.

Table 5.4 Thermal Denaturation Profiles of Field Pea Proteins[a]

Sample	T_1 (°C)	T_2 (°C)	ΔH of T_1 (J/g)	ΔH of T_2 (J/g)	Whole peak ΔH (J/g)
SPI[b]	82.4 ± 0.2	98.7 ± 0.3	1.202 ± 0.076	0.736 ± 0.014	1.938 ± 0.089
API[c]	85.3 ± 0.5	97.0 ± 0.2	0.257± 0.027	0.595 ± 0.034	0.852 ± 0.061
Globulin	85.0 ± 0.2				5.543 ± 0.517

a: All figures are shown ± one standard deviation. T_1 and T_2= denaturation temperatures for the first and second peaks respectively, ΔH= enthalpy required to achieve denaturation
b: Pilot scale salt (0.5M NaCl) extracted protein isolate
c: Pilot scale alkaline (pH 9) extracted protein isolate

Apart from the effect of different salts and processing conditions on denaturation enthalpies (ΔH), they could also affect the denaturation temperatures (Td) greatly. As can be seen from the thermogram of globulin fractions, only one transition was found between 74 and 96°C with a maximum at 85°C. This was essentially the same temperature (86.2°C) previously reported by Bora *et al.* (1994) with mixed pea globulins. A similar pattern had also been found even earlier by Arntfield and Murray (1981). It should be pointed out that all of these proteins, including the globulins in the current study, were isolated by micellisation procedures. Thus it is hypothesised that changes of ionic strength during the long-term dialysis procedure, particularly at low temperature (4°C) may result in the changes of thermal properties of the proteins. Particularly with legumin, the process could destabilise its thermal properties and shift the Td to a lower temperature range. Thus this transition might merge with that of vicilin, to give the one big peak found in thermogram. The effects of salt on the thermal stability of storage proteins from fababean have been studied by Arntfield *et al.* (1986). They claimed that both the type and concentration of the salts greatly affect the Td values of the proteins. They also found that the changes in Td values differed for vicilin and legumin, and these differences were mainly attributed to the structural variations in the two proteins.

In the current study, the micellar globulin protein has a much higher thermal enthalpy (ΔH= 5.543 J/g) compared with the salt extracted protein isolate (whole peak ΔH= 1.938 J/g), and alkaline extracted proteins (whole peak ΔH= 0.852 J/g). This reflected the partial denaturation of pea proteins during the pilot scale processing, since partially unfolded proteins require less heat energy (seen as a lower ΔH) to complete denaturation. Arntfield and Murray (1981) also demonstrated that commercial soybean protein isolate lacks the endotherm compared to micelle isolate, indicating that the commercial isolate is completely denatured. Meanwhile, as can be seen from Table 5.4, the higher thermal enthalpy transition has been found with salt extracted proteins compared with alkaline extracted proteins. The same is true with individual transition peaks (ΔH of T_1 and ΔH of T_2). The effect of pH on the degree of denaturation with fababean proteins, which was monitored by DSC, has been studied by Arntfield and Murray (1981). They found that both ΔH and Td decrease markedly in the acid and alkaline regions. Especially with increasing pH during protein extraction, a gradual decrease in the size of the endotherm and therefore ΔH was observed. Ma *et al.* (1990) reported that alkaline conditions also resulted in the decline of enthalpy value for oat proteins. They attributed this change to partial denaturation and alteration in the oligomeric structure (both degradation and aggregation) of the proteins, as well as the excess of repulsive negative charges. However, as can be seen from Fig. 5.9, the similar SDS-PAGE patterns of the two different pea isolates demonstrates that the use of the salt or alkaline solutions did not appear to result in any degradation or aggregation, which would be reflected in changes to the electrophoretic pattern. Thus the thermal destabilisation of alkaline extracted pea proteins could be mainly due to the combination of charge effect and endothermic reactions, such as the breakage of hydrogen bonds as well as exothermic reactions, such as the disruption of hydrophobic interactions. In the case of vital wheat gluten, Arntfield and Murray (1981) found no recognisable endotherm in DSC studies. They suggested that the thermal denaturation of gluten involves the disruption of a sufficient number of hydrophobic interactions to cancel the endothermicity of a polar interaction breakup.

In summary, the thermal behaviour of food proteins is affected by the source of proteins, the processing and handling conditions, and the specific conditions under which testing is performed. In terms of food applications, this has some interesting implications. For example, the lower thermal enthalpy of the pea proteins produced in pilot scale, especially that extracted with alkaline solution, indicated the partial denaturation of the proteins. However, this property may be useful in some food applications such as baking and emulsification in meat products, where heat-induced gelation is desirable. On the other hand, some food formulations, such as protein-fortified beverages, require high thermal stability of proteins, since pasteurisation may cause denaturation and precipitation of unstable proteins during processing.

5.6. Conclusions

For large-scale extraction of field pea proteins, the use of 0.5M NaCl solution is recommended. Ultrafiltration is a feasible way to concentrate proteins. Overall, the salt extracted proteins exhibited better physical properties than alkaline extracted proteins in terms of colour and particle size. The solubilities showed little variation and the electrophoretic patterns were similar.

From the technological point of view, freeze drying is relatively simple to control, but is time-consuming and the resultant product is of a dark colour and non-uniform particle size. Freeze drying also resulted in the proteins with different surface microstructure, which showed a denser mass, compared with the spherical shape of spray-dried proteins.

The thermal denaturation properties of pea proteins have been studied by differential scanning calorimetry. The lower transition enthalpies of the pilot-scale isolates indicated the partial denaturation of the proteins. However, this property might be useful in some food applications.

CHAPTER 6

Functional Properties of Field Pea Proteins and Their Applications in Foods

CHAPTER 6

Functional Properties of Field Pea Proteins and Their Applications in Foods

In previous chapters, it has been suggested that field pea proteins offer good potential for food processing. However, the technological use of legume proteins depends largely on the functional properties which are necessary for their successful incorporation into food systems. Functional properties of proteins are affected by their intrinsic physico-chemical and structural properties as well as by environmental and processing conditions commonly referred to extrinsic factors. The method of isolation, the effect of pH, temperature and ionic strength are important extrinsic factors (Damodaran, 1996).

In an earlier phase of the current study, field pea proteins have been isolated by different procedures on a pilot scale (Chapter 5). One process used traditional alkaline extraction and precipitation at isoelectric pH; the other involved extracting the proteins by salt and membrane processing to concentrate the proteins. For better utilisation of these products in food processing, it is very important to evaluate the functional properties of these proteins and understand the fundamental relationship between the conformational, hydrodynamic and surface properties of these proteins and their functional behaviour in food systems. In addition to soybean, functional properties of proteins from other grain legumes including chickpea, winged bean, lupin and great northern bean have previously been studied (Paredes-López *et al.*, 1991; King *et al.*, 1985; Sathe and Salunkhe, 1981a; Okezie and Bello, 1988). Some of the functional properties of field pea proteins, especially those produced by air-classification, have been evaluated (Naczk *et al.*, 1986; Sumner *et al.*, 1981). However, few of these attempts have been concerned with the factors which could particularly affect the functional properties of proteins, such as the intrinsic molecular factors as well as the extrinsic factors. Furthermore,

the variations in the sample size and methodologies between laboratories make it very difficult to compare these results. Accordingly, the purpose of the present investigation was to study the functional properties of field pea proteins including protein-water interactions, emulsifying and foaming properties, viscosity, and gelation properties for the pilot scale protein isolates. The methodologies and procedures have been carefully chosen and modified where necessary, in order to control the experimental conditions. The effect of NaCl concentration, pH and temperature on the functional properties has also been investigated. Hydrophobicity (So) and surface tension have been measured in order to characterise their relationship to the emulsifying and foaming properties and also to provide some information for understanding the structure-function relationship of legume proteins. Based on the results obtained from the functionality assessment, food applications of these proteins have been studied in two model food systems, sponge cakes and mayonnaise.

6.1. Functional Properties of Field Pea Proteins

6.1.1. Protein-Water Interactions

6.1.1.1. Solubility Characteristics of Field Pea Proteins

In a previous phase of this study (Chapter 5) it has been shown that the salt extracted field pea protein isolate (SPI) had a similar nitrogen solubility pattern to that of the iso-electric-protein isolate (API) (Fig. 5.1). Generally speaking, the protein solubility of field pea proteins is higher than that of soy-protein isolate (Fig. 4.2). Similar results have been previously reported for field pea proteins by Naczk *et al.* (1986). Solubility characteristics are one of the most important indices for evaluating the potential applications of proteins; good solubility can markedly expand potential utilisation of proteins (Kinsella, 1976). Thus the relatively high solubility of field pea proteins is consistent with the pea protein preparations being considered as useful protein ingredients in food formulations.

6.1.1.2. Water Absorption Characteristics of Field Pea Proteins

The terms water absorption, water hydration capacity, water binding, and water holding ability are used interchangeably in the literature to denote the maximum amount of water that a protein material can take up and retain in food formulations (Quinn and Paton, 1979). However, these terms often lead to confusion in the interpretation of results, since no standard methods exist for the evaluation of this functional property of proteins (Hutton and Campbell, 1981). One of the most popular techniques is the "excess water method", which involves equilibration of the sample with excess water and application of mild stress to separate the retained water from the free water. In practice, the protein sample is mixed with a several–fold excess of water and the dispersion in then centrifuged at low gravity. The supernatant is decanted and the absorbed water is calculated by measuring either weight differences or volume differences (Quinn and Paton, 1979). The second approach used to estimate water absorption of a sample is referred to as the "swelling method". A system to measure swelling was devised by Hermansson (1972). In this method, a small amount of sample is dusted on to a wetted filter paper fastened on a glass filter. The filter is fitted on top of a thermostated funnel filled with water and connected to a circular capillary. The amount of water absorbed by the sample can be followed by observing the capillary. However, neither of these methods accounts for the portion of the protein that is solubilised by the procedure. In the excess water-centrifugation method, soluble proteins are decanted with the supernatant, and in the swelling method, they diffuse into the water reservoir (Quinn and Paton, 1979). Thus samples containing different proportions of soluble to insoluble protein cannot be accurately compared as to water absorption of the proteins. Quinn and Paton (1979) developed a technique called the "water saturation method" to solve the problem. In this method, only enough water is added to saturate the sample. This water is entirely retained upon centrifugation, ie, there is no supernatant. However, certain drawbacks of the method are evident. It is difficult and time-consuming to adjust the moisture content until the water saturation point just appears. For a range of samples, it is

hard to control the process, since the time of exposure of proteins to water may be different. In addition, the centrifugation step may have to be repeated several times. All of these factors could cause errors in the measurement of water absorption between different samples.

The relative humidity method has also been used to study the water binding of dry protein powders from legumes and oilseeds, including cowpea and peanut (Schaffner and Beuchat, 1986; Beuchat, 1977). The results obtained by this procedure are commonly referred to as water adsorption values whereas data obtained by centrifugation methods are described as water absorption. Water adsorption is defined as the water absorbed by a dried protein powder in equilibration with water vapour at a known relative humidity. This technique eliminates the problem of solubilised proteins and thus makes it possible to give results which may be readily compared. In the current study, the water absorption of field pea proteins has been measured in terms of equilibrium moisture contents (EMC) at equilibrium relative humidities (ERH) in the range of 23-97%.

The ability of field pea protein isolates to adsorb water at different relative humidities is demonstrated in Fig. 6.1. Salt-extracted proteins exhibited a slightly higher ability to adsorb water especially under higher equilibrium relative humidities. According to Damodaran (1996), the sharp increase in water uptake at ERH 70-95% is due to hydrodynamic hydration of the protein, which refers to formation of multilayer water associated with proteins. At this level, the intrinsic properties of proteins including size, shape, amino acid composition seem to have very little effect on the hydration capacity. However, several extrinsic factors including pH, ionic strength, temperature, particle size of protein powders markedly influence the water-binding capacity of proteins (Berlin, 1981; Berlin and Anderson, 1975). Thus the higher water-binding capacity of salt extracted pea proteins may result from the charge effect of the remaining salt, which could enhance hydrophilic interaction between protein and water molecules. Hsu *et al.* (1982) studied the water adsorption at 85% relative humidity for soybean, yellow

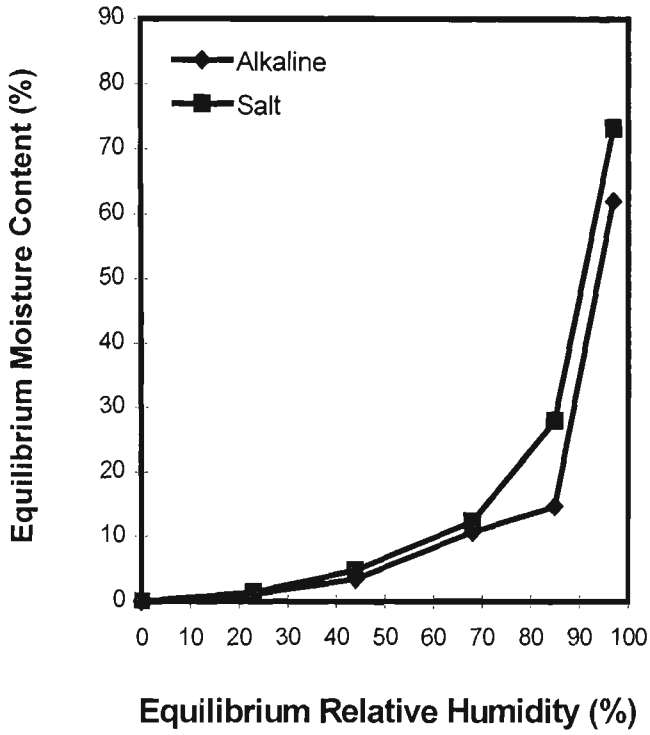


Figure 6.1 Water adsorption isotherms of field pea protein isolates. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

pea, fababean and lentil proteins from ungerminated and germinated seeds. They found that the ungerminated soybean and field pea protein adsorbed 0.24, 0.20 grams of water per gram of protein respectively. The results of fababean and lentil were similar to those of field peas. In another study, Schaffner and Beuchat (1986) found that at 85% relative humidity, the EMC of cowpea, peanut and soybean proteins were approximately 26, 15, 37% respectively. The results in the current study show that under the condition of 85% relative humidity, the EMC of field pea proteins extracted with salt and alkaline solution were 28, 15% respectively; and the commercial soybean protein (Supro-500E) showed an EMC of 34%. The differences found in the water adsorption by different researchers for protein isolates from a particular legume source may have resulted from differences in sample preparation. Generally, the results show that the water-binding capacity of field pea proteins is lower than that of soy proteins, but is comparable to the other legume proteins such as fababean and cowpeas. Hsu *et al.* (1982) reported that soy

protein isolate is more hygroscopic than other legume protein isolates and thus is readily hydrated in water. However, despite the higher water binding capacity shown by soy isolate, earlier results in the current study have shown that soy protein isolate presented lower solubility characteristics than field pea proteins. Hence the water adsorption ability is not always directly related to the solubilities of the proteins. Hermansson (1979a) also stated that solubility measurements are not a reliable indicator as to whether or not a protein ingredient will bind water.

Nevertheless, field pea proteins, especially the isolate extracted by salt solution, demonstrated a reasonably high ability to absorb water. This property would be useful in food applications. For instance, the rheological properties of wheat dough and the tenderness of meat and meat analogues are affected by water-binding capacity (Slade *et al.*, 1989; Hermansson, 1975). The addition of legume proteins with good water hydration abilities could give a food product having enhanced textural and mouth-feel qualities.

6.1.2. Oil Absorption of Field Pea Proteins

Oil absorption of food products is also an important functional property because it improves mouth-feel and flavor retention (Kinsella, 1976). However, fewer researchers have studied the oil absorption characteristics of protein isolates in comparison with water absorption. Where this property has been investigated there has been little variation in the procedure applied (Hutton and Campbell, 1981). It is usually measured by adding excess liquid oil to a protein powder, mixing and holding, centrifuging, and determining the amount of absorbed oil (Lin *et al.*, 1974).

The oil absorption capacities of field pea protein isolates obtained by pilot scale extractions have been measured. The values are compared with that obtained for a commercial soy protein in Table 6.1. The results are comparable with those

Table 6.1 Oil Absorption Capacity of Field Pea Protein Isolates

	Pea protein (API)[a]	Pea protein (SPI)[b]	Soy protein (Supro-500E)
Oil Absorbed (g/g)	1.44	1.68	3.89

a: Protein isolate extracted with alkaline solutions (pH 9), recovered by isoelectric precipitation and dried by spray drying
b: Protein isolate extracted with NaCl solutions (0.5M), recovered by ultrafiltration and dried by spray drying

reported by Naczk *et al.* (1986) for pea protein preparations. They also found that the oil absorption of pea proteins is similar to that of gluten but substantially lower than that of soy-protein products. Rapeseed protein isolates and meals had an oil absorption up to 4 times higher than pea protein products (Sosulski *et al.*, 1976). Lin *et al.* (1974) observed that sunflower proteins had a higher oil absorption compared with soy proteins. It was suggested that the sunflower isolate contained more non-polar side chains which retained oil by associative binding. Thus it has been hypothesised that the low oil absorption of pea proteins could result from the presence of a larger proportion of hydrophilic than hydrophobic groups on the surface of the protein molecules (Naczk *et al.*, 1986). On the other hand, Sumner *et al.* (1981) reported that oil absorption of pea proteins depended on the drying method employed. They found that drum and freeze drying increased the oil absorption of the products (double of that for spray drying). However, in the current study, it has been found that the drying method had only a minor effect on the oil absorption capacity of pea proteins. For example, the freeze-dried pea proteins extracted by alkaline solution showed an oil absorption capacity of 1.65g oil/g protein. In comparison, spray-dried protein isolates showed the oil absorption of 1.44g oil/g protein. The mechanism of oil or fat absorption by proteins is not fully understood, but it appears to be affected by lipid-protein complexes and protein content (Kinsella, 1979). The availability of lipophilic groups may also have an important role in contributing to higher absorption of fat (Lin *et al.*, 1974).

6.1.3. Emulsifying Capacity and Stability of Field Pea Proteins

An emulsion is a two-phase liquid system in which one of the liquids is dispersed as droplets in the other liquid (Damodaran, 1996). The most common types of emulsions are oil-in-water systems such as mayonnaise and milk, in which an oil is dispersed in an aqueous continuous phase; and water-in-oil types such as butter or margarine, in which water is dispersed in an oil continuous phase of oil (McWatters and Cherry, 1981). Since the interfacial tension between water and oil is quite high, emulsions are thermodynamically unstable and phase separation occurs over time. The stability of emulsions can be improved by adding amphiphilic surface-active molecules that adsorb at the oil-water interface and reduce the interfacial tension. Proteins, which contain both hydrophobic and hydrophilic amino acid residues, are well suited to act as macromolecular surfactants. They enter the interface of the emulsion, and cover the interface, thereby lowering the energy of the system (Damodaran, 1996; Tornberg *et al.*, 1997).

In various food systems emulsification is usually achieved by use of approved food emulsifiers. These are typically lower in molecular weight and examples include mono-glycerides and di-glycerides. In comparison with these, the emulsifying properties of food proteins are influenced by many factors such as temperature, pH, salt concentration, as well as the characteristics of the oil involved (Wang and Kinsella, 1976). It has also been observed that proteins from different sources vary widely in emulsifying properties (Saffle, 1968; Kinsella, 1976). Many other factors influence the measurement of emulsification including the method used, equipment design and rate of oil addition. In the current study, emulsification capacity of field pea proteins was measured as the maximum quantity of oil emulsified by the protein solution and the end point was determined by the change of electric resistance (Webb *et al.*, 1970). Emulsifying stability was determined by the amount of water released from the emulsions following centrifugation (Johnson and Brekker, 1983). The effects of various conditions including pH, temperature

and salt (NaCl) concentration on the emulsifying properties have also been studied. In addition, the relationship between emulsifying properties and surface hydrophobicity (So) has been evaluated.

6.1.3.1. Effect of pH on Emulsifying Capacity and Stability

The results of the effects of pH on the emulsifying capacities and stabilities of field pea protein isolates are presented in Fig. 6.2. It can be seen that the patterns of the emulsifying properties of salt extracted proteins are similar to those of alkaline extracted proteins. Both emulsifying capacity and stability were pH dependent, with the stability being particularly enhanced at higher pH values. The results indicate that solubility is an important factor controlling the emulsion properties, with the lower capacity and stability occurring where the solubilities are lowest (Fig. 6.2 and Fig. 5.1).

Hsu *et al.* (1982) also found that the emulsifying capacities of legume proteins including field pea and fababean were relatively poor at pH 6.5 but were improved at pH 7.5. They hypothesised that this is due to increased protein solubility as the pH was raised above the apparent isoelectric range of the legume proteins. In other studies, King *et al.* (1985) and Sathe *et al.* (1982) reported that the emulsifying capacities of lupin proteins followed their pH-solubility profiles. They explained that the dependence of emulsifying capacity on pH was expected, since emulsion capacity of soluble proteins depends upon the hydrophilic-lipophilic balance which is influenced by pH. However, as can seen from Fig. 6.2, the acid conditions slightly reduced emulsion capacities and stabilities of field pea proteins. Similar observations have been reported by Schaffner and Beuchat (1986) on several extracts of legume and oilseeds including cowpea, peanut and soybean. Hence solubility is not the only factor controlling the emulsifying properties of legume proteins. McWatters and Holmes (1979a) showed that large concentrations of soluble nitrogen from peanut flour were not necessarily related to maximum emulsifying capacities. Thus the emulsification properties of the proteins are influenced by the type of the seed as well as the pH to which the flour is exposed

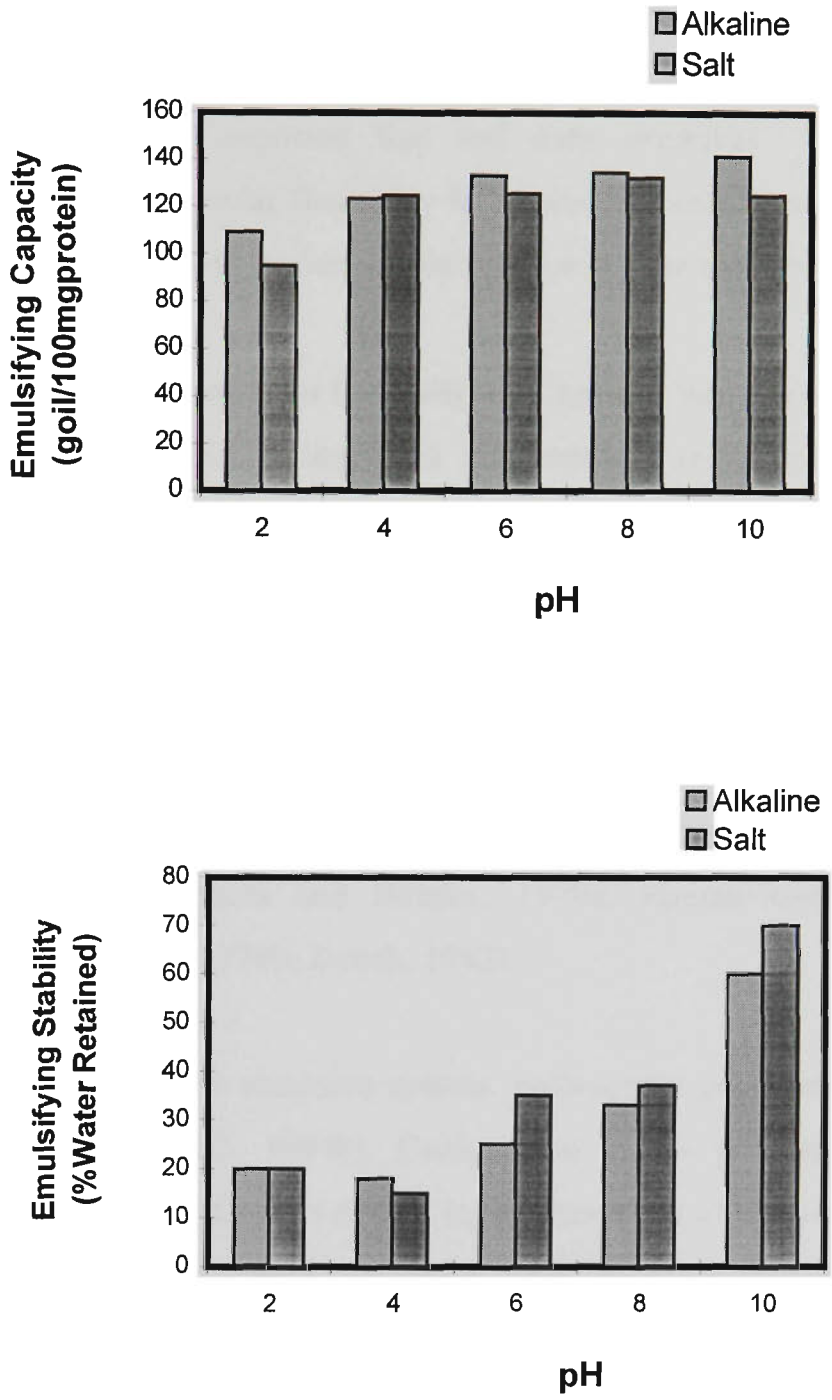


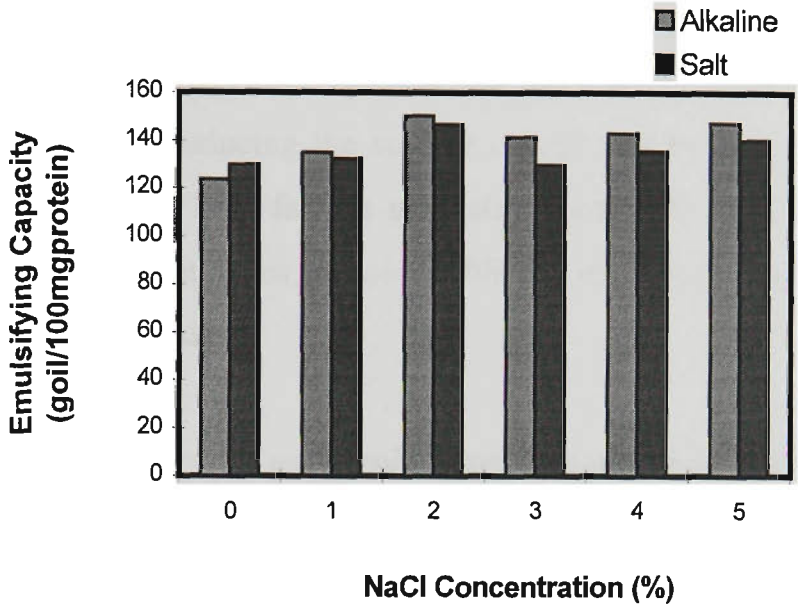
Figure 6.2 Effect of pH on the emulsifying capacity and stability of field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

(McWatters and Cherry, 1977). In the current study the emulsifying properties of a commercial soy protein isolate were found to be similar to those of field pea proteins under neutral conditions, even though the solubility of pea proteins is superior. Nakai (1983) reported that not only solubility, but also surface hydrophobicity and molecular flexibility influence the emulsification behaviour of globular proteins. This will be further discussed in a later section.

6.1.3.2. Effect of Salt (NaCl) on Emulsifying Capacity and Stability

The addition of salt (NaCl) improved the emulsifying capacities but greatly decreased the emulsifying stabilities with increasing NaCl concentration (Fig. 6.3). The improvement of the emulsifying capacities may result from the increased solubilities of the proteins since neutral salts are known to exert strong effects on solubility (Adeyeye *et al.*, 1994). It has been reported that the addition of salt increased solubilities of plant proteins including those of soybean, peanut and winged bean and this property resulted in an increase of the emulsifying capacities of the proteins (McWatters and Holmes, 1979a; Ramanatham *et al.*, 1978; McWatters and Holmes, 1979b; Dench, 1982).

In order to obtain a stable emulsion system, coalescence and creaming should be prevented (Phillips *et al.*, 1994b). Coalescence is the process by which the collision of two or more droplets results in the formation of one larger drop and it is the primary cause of emulsion breakdown. This process is irreversible because it essentially involves dissolution of the interfacial film (Phillips *et al.*, 1994b). Various factors, such as the solubility of the emulsifier, pH, salts, protein concentration, temperature and the properties of the interfacial film itself, all affect the coalescence stability of emulsions (Das and Kinsella, 1989). Under certain conditions such as the neutral pH and room temperature in the current study, the change of ionic environment due to salt addition may have a significant influence on emulsion stability. Adsorbed protein affects a number of attractive and repulsive forces between emulsion droplets, most notably van der Waals attractive forces, electrostatic and steric repulsive interactions and hydration forces (Phillips



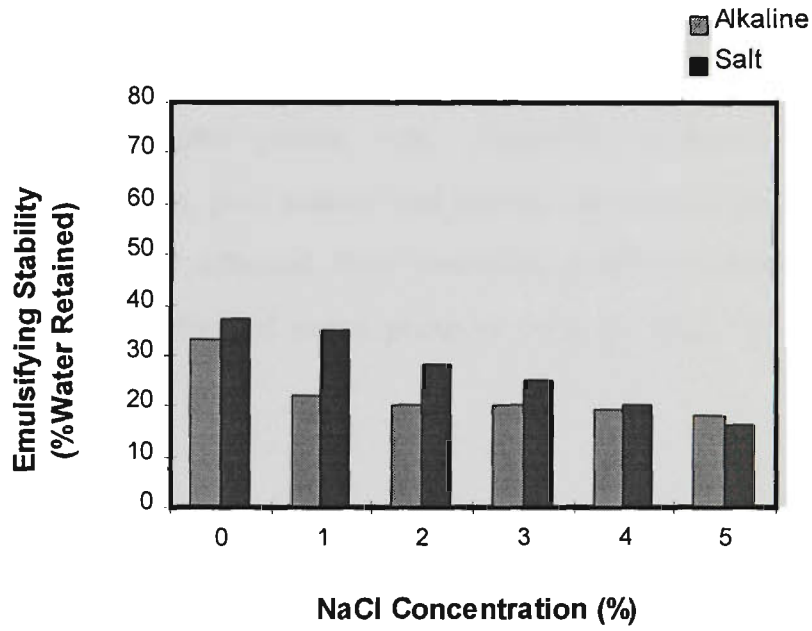


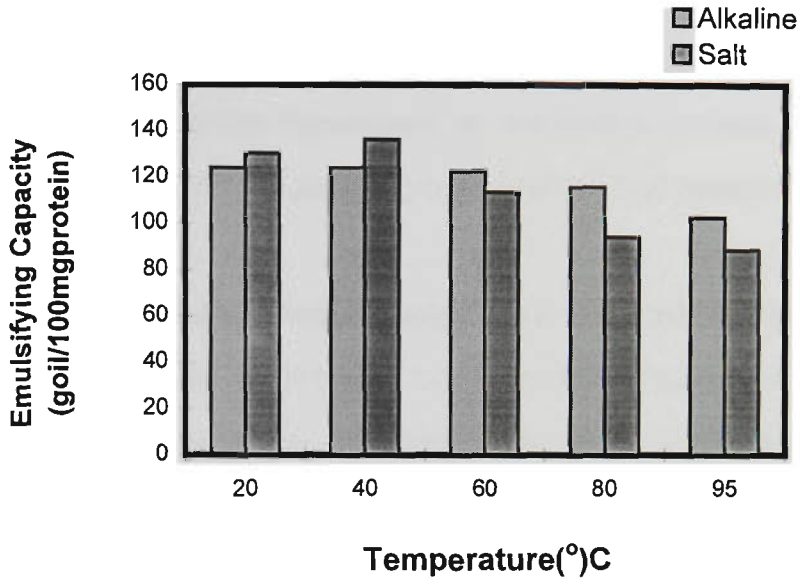
Figure 6.3 Effect of salt (NaCl) on the emulsifying capacity and stability of field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

et al., 1994b). The balances between these forces in the emulsion system would probably be disrupted by the increased ionic strength and thus coalescence could occur. Voutsinas *et al.* (1983) also reported that NaCl might exert negative effects on emulsion stability by reducing the surface charge and by withdrawing surface water from oil droplets. These factors may also account for the decrease of the emulsifying stability of field pea proteins with the increase of salt concentration observed in the current study.

6.1.3.3. Effect of Temperature on Emulsifying Capacity and Stability

Temperature also has a strong effect on the emulsifying properties of field pea proteins (Fig. 6.4). With the increase of temperature, both the emulsifying capacity and stability have decreased. Voutsinas *et al.* (1983) have studied the effect of heating on the emulsifying properties of a number of proteins including BSA, β-lactoglobulin, gluten, whey protein, casein, gelatin, ovalbumin, as well as soy, canola and pea protein. They found that heating had varying effects on emulsifying properties when different proteins were heated. For example, the emulsifying properties of ovalbumin and gelatin were markedly improved upon heating, whereas for β-lactoglobulin, pea, canola and casein, the emulsifying properties of the proteins were adversely affected. Heat treatment could also have minor effects on the emulsifying properties of some proteins such as BSA, gluten and whey protein.

Protein denaturation upon heating is usually associated with aggregation and the decrease of solubility and these are primarily responsible for the loss of the emulsifying properties (McWatters and Holmes, 1979c). In addition, heat treatment of globular proteins invariably causes polymerisation via sulphydryl-disulphide interchange reactions and this may also affect the emulsifying properties of the proteins (Kinsella *et al.*, 1985; Damodaran, 1996). On the other hand, for some proteins, the emulsifying properties may be improved upon heating because of the increased protein hydrophobicity (Kato and Nakai, 1980; Kato *et al.*, 1981). This is due to the protein unfolding and the gradual exposure of hydrophobic amino acid



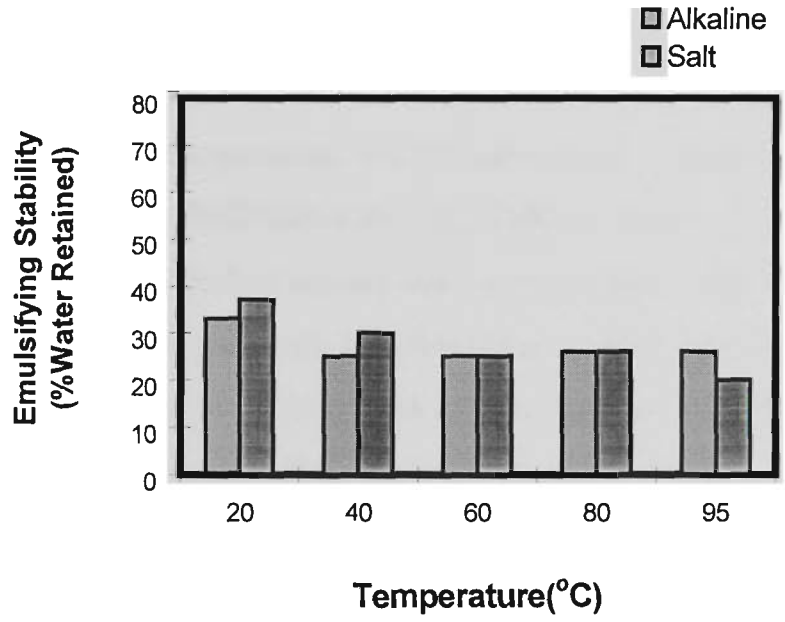


Figure 6.4 Effect of temperature on the emulsifying capacity and stability of field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

residues of the proteins which are usually buried in the interior of the molecules (Tanford, 1973; Voutsinas *et al.*, 1983). Consequently the protein molecules become more amphiphilic and capable of orienting at the oil-water interface (Morr, 1979). Hence in addition to the importance of solubility, surface properties could also have a great influence on the emulsifying properties of proteins.

6.1.3.4. Relationship between Emulsifying Properties and Surface Properties

Surface hydrophobicity (So) of proteins has been receiving much attention since the hydrophobic interactions are considered to play important roles in the functional properties of food proteins (Gueguen, 1989; Nakai, 1983; Nakai *et al.*, 1986; Li Chan *et al.*, 1984). A variety of methods for the determination of protein hydrophobicity have been reviewed by Nakai (1983), and these included reverse-phase chromatography, binding of hydrocarbons to proteins, hydrophobic partition between phases containing dextrans with polyethylene glycol as well as fluorescence probe methods. In the current study, *cis*-parinaric acid, which fluoresces under a hydrophobic environment, was used as a probe to measure the hydrophobicity of field pea proteins. *cis*-Parinaric acid, which has the formula $CH_3CH_2CH{=}CH\text{-}CH{=}CHCH{=}CHCH{=}CH(CH_2)_7COOH$, is an natural polyene fatty acid and thus can readily simulate natural lipid-protein interacting systems (Nakai, 1983). Compared with other methods, this fluorescent probe technique is relatively simple with great detection sensitivity and a large number of parameters can be monitored continuously on a rapid time scale (Sklar *et al.*, 1976).

In the current study, the spectra of *cis*-parinaric acid in absolute ethanol at 20°C were determined (Fig. 6.5). The reagent has absorption maxima at 313 nm and 328 nm and a broad emission maximum near 420 nm. Similar spectral patterns have been reported for some linear polyenes including α-parinaric acid, β- parinaric acid and α-eleostearic acid (Sklar *et al.*, 1976). Thus an excitation wavelength of 325 nm and an emission wavelength of 420 nm were chosen for the measurement of the relative fluorescence intensities of the *cis*-parinaric-protein conjugates. So values were calculated based on the initial slope of the curve obtained when fluorescence

intensity was plotted against protein concentration. An example of the curve is shown in Chapter 3 (Fig. 3.1).

Furthermore, surface tension of protein solutions has also been found to have a significant effect on the stability of oil-in-water emulsions (Acton and Saffle, 1970). Therefore in the current study, a surface tensiometer has been used to measure the surface tension of the protein solutions in order to assess the correlation of protein hydrophobicity with surface tension. This instrument operates on the DuNouy principle, in which a platinum-iridium ring is suspended from the torsion balance, and the force (in dynes per centimetre) necessary to pull the ring free from the surface film is measured (Handbook of Cambridge Instrument Company Limited, Cat. No. 32231/D).

The relationships of hydrophobicity, surface tension and solubility of field pea proteins with their emulsifying properties are demonstrated in Table 6.2. Although the correlation between hydrophobicity and emulsifying properties of proteins have been observed in several cases (Kato and Nakai, 1980; Kato *et al.*, 1981), which showed improvements of emulsifying properties with the increase of protein hydrophobicity upon heating, some evidence suggests that this relationship is not an absolute one (Shimizu *et al.*, 1985). This is confirmed by the current results on field pea protein isolates. As can be seen from Table 6.2, the higher the hydrophobicity, the lower the surface tension, with the increase of temperature. However, the improvement of the emulsifying capacities and stabilities has not been observed. When Voutsinas *et al.* (1983) studied the emulsifying properties of different types of proteins upon heat denaturation, a similar result was also found with field pea proteins. In their study, the pea proteins were heated at 80°C from 1 to 7 min. With the increase of heating time, a gradual increase of So was observed, but at the same time, the emulsifying activity of the proteins dropped. Hence the origin of the protein, rather than the hydrophobicity, greatly affects the emulsifying properties of proteins.

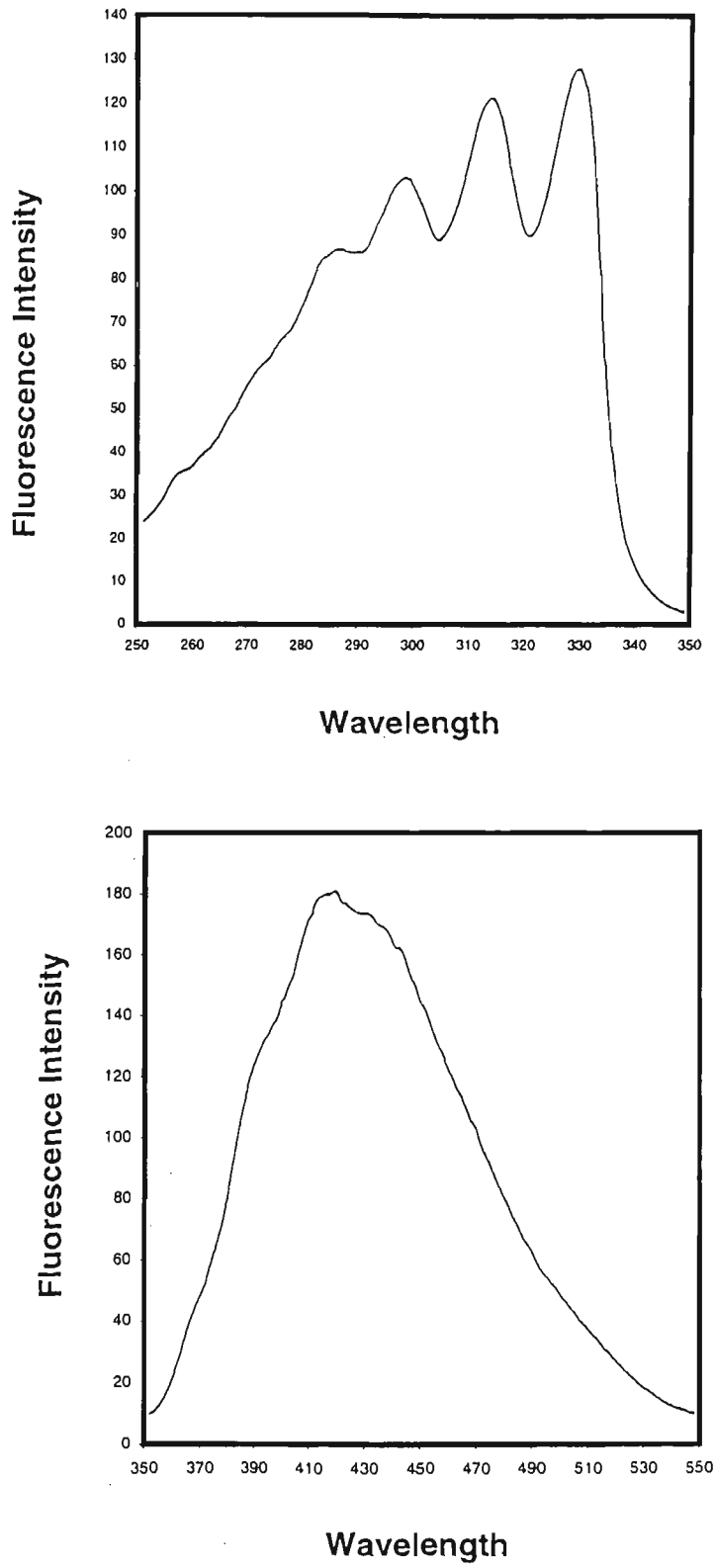


Figure 6.5 The spectra of *cis*-parinaric acid in absolute ethanol at 20°C. Top: absorption; Bottom: emission.

Table 6.2 Relationships between Protein Solubility, Hydrophobicity, Surface Tension and Emulsifying Properties of Field Pea Protein Isolates

Protein	Temperature (°C)	Solubility (%)	Hydrophobicity (So)	Surface tension (Dynes/cm)	Emulsify capacity (g oil/100mg)	Emulsion stability (% water retained)
API[a]	20	63.8	1024	57.0	123.7	33
	40	62.6	1160	59.3	123.9	25
	60	73.3	2420	56.1	122.3	25
	80	76.8	4820	54.8	115.3	26
	95	79.9	5062	51.7	102.6	26
SPI[b]	20	68.3	768	52.0	129.8	37
	40	69.6	746	56.5	135.9	30
	60	68.8	1810	53.3	113.0	25
	80	70.7	3576	53.5	93.7	26
	95	70.2	3870	52.1	88.4	20

a: Protein isolate extracted with alkaline solutions (pH 9) and recovered by isoelectric precipitation

b: Protein isolate extracted with NaCl solutions (0.5M) and recovered by ultrafiltration

Meanwhile, heat treatment did not result in any decrease of solubility of field pea proteins (Table 6.2). Heating is not always accompanied by the loss of solubility of the proteins (Hermansson, 1979b). For example, gelatin is completely solubilised upon heating due to the rupture of hydrogen bonds which are responsible for its insolubility (Blanshard, 1970). However, although solubility is important, no positive correlation exists between solubility and emulsifying properties (Aoki *et al.*, 1981; McWatters and Holmes, 1979c). Hence the combined influences of hydrophobicity and solubility cannot fully explain the emulsifying properties of some proteins including those of field peas. Apart from the type of seed, this result also suggests that molecular factors such as the conformational rearrangement at the interface rather than surface hydrophobicity may be important in the expression of emulsifying properties of proteins (Damodaran, 1996).

The effects of salt (NaCl) concentration on the surface tension and hydrophobicity of field pea proteins are shown in Fig. 6.6 and 6.7, respectively. It can be seen that with the addition of salt, the surface tension and So did not change greatly. This result again confirms that the emulsifying properties of field pea proteins do not seem to be correlated with the surface hydrophobicity and surface tension, since the emulsion stability decreased with the increase of salt concentration (Fig. 6.3). The effect of pH on the surface properties including surface tension and So has also been studied (data not shown). It seems that the comparison of the data determined at different pH is not accurate, since the measurement of So and surface tension is based on the soluble proteins, and the readings are protein concentration dependent. Meanwhile, it is obvious that the amount of solubilised proteins is significantly different at various pH values. However, the published result has not taken this into consideration when the results of So determinations were compared at pH 3 and 7 (Koyoro and Powers, 1987). As a result, to explain the emulsifying properties of proteins at different pH, the use of So and surface tension probably has low significance unless the solubilities of the proteins are similar.

In summary, the emulsifying capacity and stability of field pea proteins are affected by pH, temperature and salt addition. These properties, on the other hand, are influenced by solubility as well as surface properties including surface tension and protein hydrophobicity. Surface tension is well correlated with hydrophobicity of the proteins. However, when both are considered, solubility and hydrophobicity cannot fully explain the emulsifying properties of field pea proteins especially when the proteins are subjected to different conditions such as heating or change of the pH or ionic strength. Many other factors, such as type of the proteins, molecular size, molecular flexibility and charge may also be important in determining the emulsifying properties of the proteins. This is in agreement with the conclusions of Voutsinas *et al.* (1983) and Damodaran (1996).

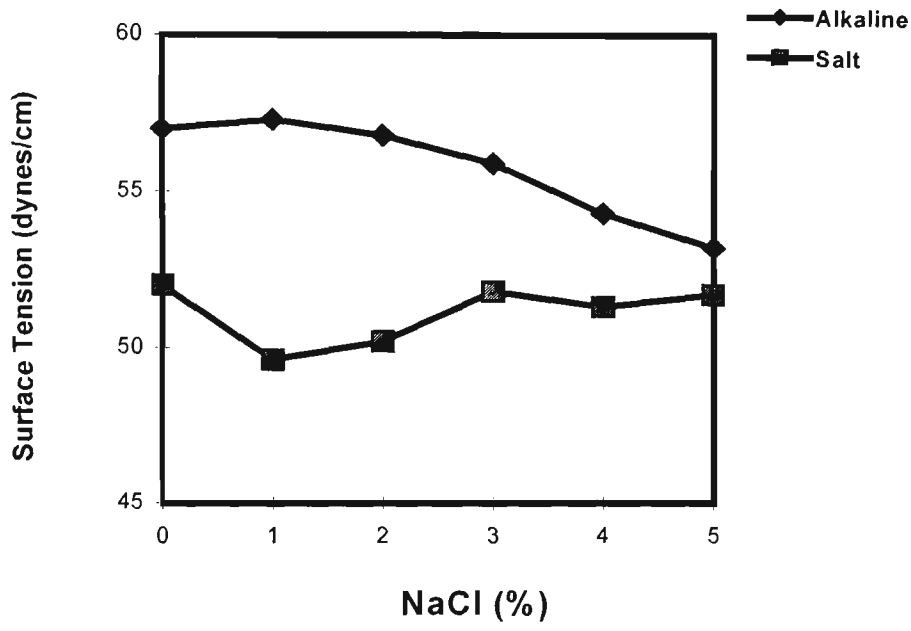


Figure 6.6 Effect of NaCl on the surface tension of field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

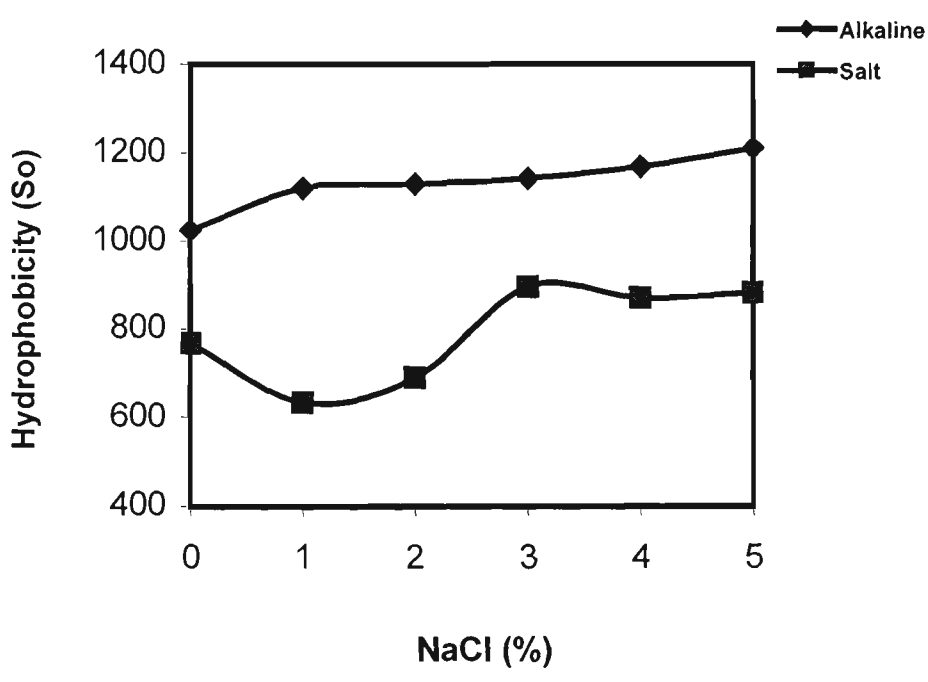


Figure 6.7 Effect of NaCl on the surface hydrophobicity of field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

6.1.4. Foaming Capacity and Stability of Field Pea Proteins

A foam is also a two-phase system, which consists of air cells separated by a thin liquid lamellar phase (Britten and Lavoie, 1992). Because of its large liquid-gas interfacial area, a foam can only be formed if energy is expanded and, once formed, is fundamentally unstable (Halling, 1981). Various proteins can be used as foaming agents. Through rapid adsorption, they form a stabilising film around bubbles which promotes foam development (Britten and Lavoie, 1992). Protein foams are important in many processes in the beverage and food industries and they are used to improve texture, consistency, and appearance of foods (Vani and Zayas, 1995). Foams in food systems are found commonly in baked, confectionery, and other goods (Kitabatake and Doi, 1982). However, numerous factors including pH, temperature, the presence of salts, sugars and lipids and the protein source, affect the foaming behaviour of proteins (Townsend and Nakai, 1983). On the other hand, Nakai (1983) proposed that protein surface properties are primarily responsible for foam development. The purpose of the current study has been to investigate the effects of pH, temperature and salt concentration on the foaming properties of field pea proteins in order to assess the potential of field pea as a protein source to replace egg in foods. The relationship between the foaming properties and surface characteristics including hydrophobicity and surface tension has also been evaluated.

6.1.4.1. Effect of pH on Foaming Properties

The results for the foaming capacities of field pea proteins at different pH values are shown in Fig. 6.8. At neutral pH, salt-extracted field pea protein isolate has better foaming capacity and stability than alkaline extracted pea protein isolates (Fig. 6.9). It was also found that the foaming properties of field pea proteins are much better than soy protein (Supro-500E) which showed virtually no entrapment of air bubbles under neutral conditions. Proteins of different origins vary greatly in foaming properties, reflecting differences in amino acid sequence and disposition; molecular size, shape, conformation and flexibility; surface polarity; charge and hydrophobicity (Vani and Zayas, 1995), as well as processing conditions.

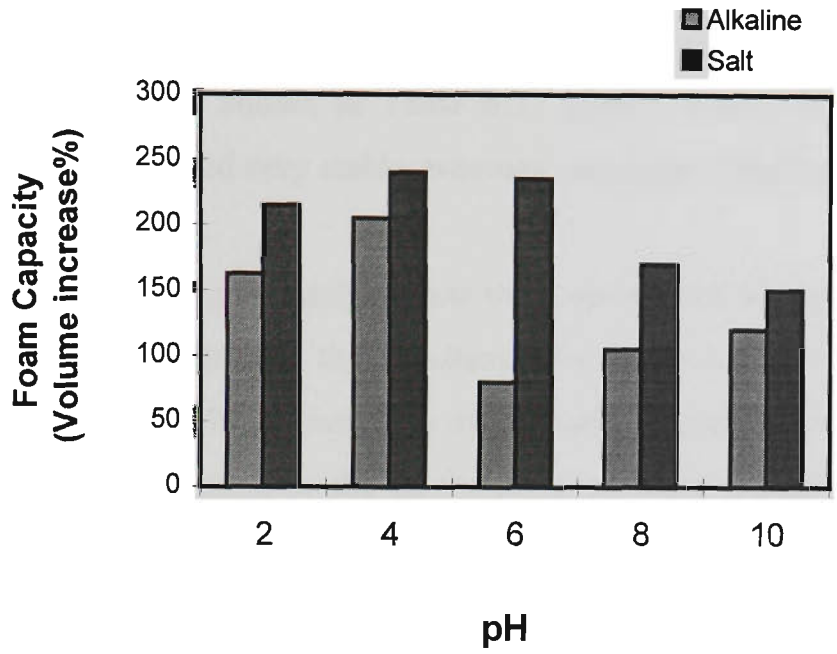


Figure 6.8 Effect of pH on the foaming capacities of field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

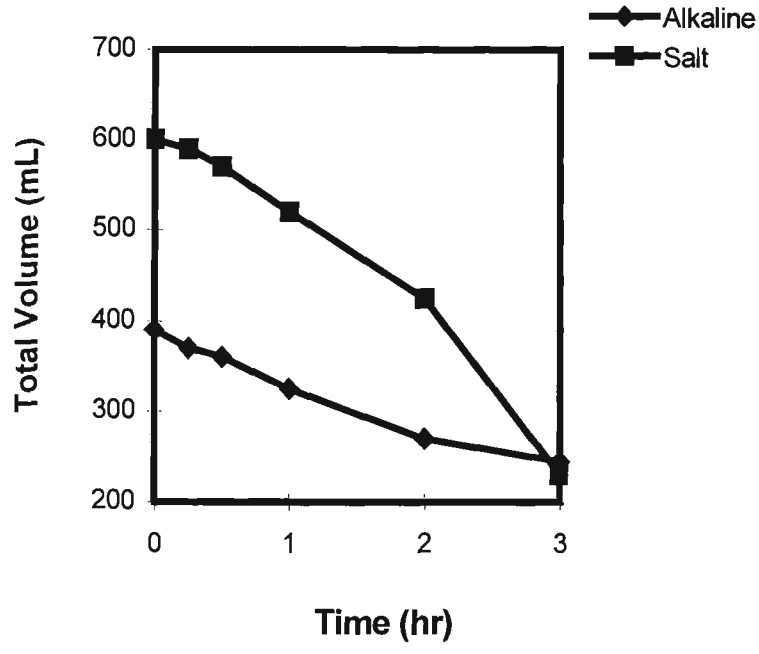


Figure 6.9 Foaming capacity and stability of field pea proteins at pH 7. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

From Fig. 6.8, it can be seen that in the acid pH range, the foam capacity of field pea proteins was enhanced. The foam stability was also greatly increased in the isoelectric pH range, as shown in Table 6.3. After 3 hours, the protein foams formed at pH 4-6 remained very stable, whereas very little remained at pH 8-10.

The pH of the solution significantly affects the properties of foams by affecting the net charge of the protein and the resultant film formation and film properties (German and Phillips, 1994). Generally more rapidly formed, stronger films are obtained at pH values close to the pI for most proteins including BSA and β-lactoglobulin (Halling, 1981; Kim and Kinsella, 1985; Waniska and Kinsella, 1985). A pronounced enhancement in foam stability has also been reported in the pI range for many proteins (German *et al.*, 1985; Phillips *et al.*, 1990). Sathe *et al.* (1982) also reported high stability of foams in the acid pH range for lupin proteins. They hypothesised that this may have been due to the formation of stable molecular layers in the air-water interface, which impart texture, stability and elasticity to the foams. Hence it seems that the solubility of proteins is not necessarily related to the foaming properties of the proteins, since the solubility of most proteins are at a minimum at their pI. This is due to neutralisation of charge repulsion among the protein molecules and consequently aggregation of the proteins is more likely to occur. However, the reduced electrostatic repulsion allows greater protein adsorption at the interface and this increases film thickness and improves the rheological –mechanical properties of the film (Mita *et al.*, 1977; Graham and Phillips, 1980). This viscous and elastic film dramatically retards liquid drainage by hydrostatic and gravitational forces (Damodaran, 1994). Thus molecular flexibility and rigidity are probably the more important factors which affect the foaming properties of proteins. The results for the acid-stable foams of field pea proteins in the current study indicate that pea proteins have potential in many food applications where the formulation is acid-based.

Table 6.3 The Effect of pH on the Foam Capacity and Stability of Field Pea Protein Isolates

Sample	pH	Volume before whipping (mL)	Volume after whipping (mL)	Volume increase (%)	Volume (mL) at room temperature (21°C) after time (hr)				
					0.25	0.5	1	2	3
API[a]	2	200	525	163	515	510	465	330	220
	4	200	610	205	610	605	605	595	580
	6	200	360	80	360	360	355	355	335
	8	200	410	105	405	400	350	300	260
	10	200	440	120	435	425	400	320	290
SPI[b]	2	200	630	215	630	625	620	555	520
	4	200	660	230	655	635	600	560	540
	6	200	750	275	750	745	725	710	650
	8	200	540	170	535	530	410	250	210
	10	200	500	150	500	480	360	240	220

a: Protein extracted with alkaline solutions (pH 9) and then recovered by isoelectric precipitation on pilot scale
b: Protein extracted with salt solutions (0.5M NaCl) and then recovered by ultrafiltration on pilot scale

6.1.4.2. Effect of Salt (NaCl) on Foaming Properties

The effects of salt concentration on the foaming properties of field pea proteins are shown in Fig. 6.10 and Table 6.4. It can be seen that addition of salt improved the foaming capacity of pea proteins (Fig. 6.10). However, the maximum improvement was observed at a salt concentration of 0.5% (w/v). Beyond these levels, the increases of foaming capacity gradually dropped as salt concentration was increased. Similar results were found for the stability of the foams (Table 6.4). After 3 hours, a reasonably stable foam was still observed with the addition of 0.5% (w/v) NaCl, especially for the protein isolate extracted with salt solution on the pilot scale. However, at salt concentrations approaching 4% (w/v), a low volume of foam remained which was similar to that of the control (without salt addition). Sathe *et al.* (1982) also found that the addition of salt enhanced foaming capacity of lupin protein concentrate and the improvement was found to be at a maximum at a salt concentration of 0.6% (w/v) in the slurry. It has been suggested that foam capacity may increase because salt improves protein solubility at the interface of the colloidal suspension during foam formation (Cherry and McWatters, 1981).

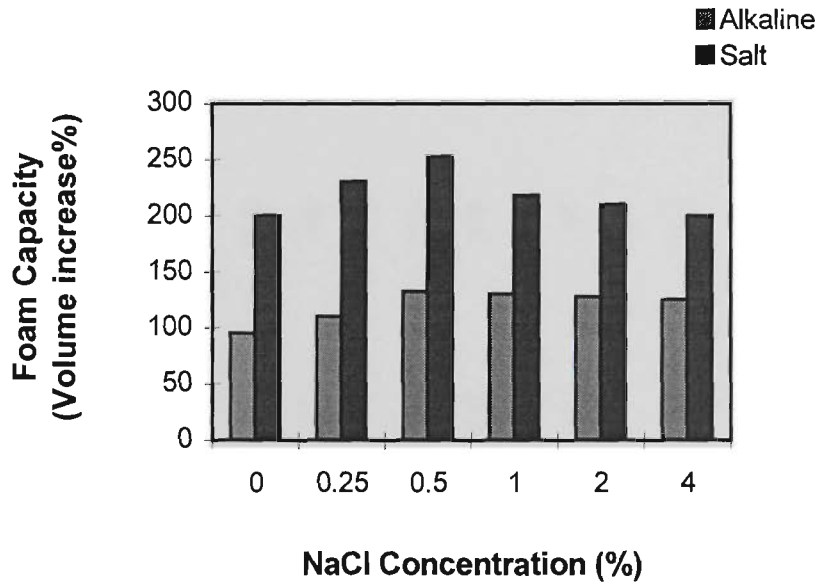


Figure 6.10 Effect of salt (NaCl) on the foaming capacities of field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

Table 6.4 The Effect of NaCl on the Foam Capacity and Stability of Field Pea Protein Isolates

Sample	NaCl (%)	Volume before whipping (mL)	Volume after whipping (mL)	Volume increase (%)	Volume (mL) at room temperature (21°C) after time (hr)				
					0.25	0.5	1	2	3
API[a]	0	200	390	95	370	360	325	270	245
	0.25	200	420	110	420	420	415	370	350
	0.5	200	465	133	460	460	445	420	405
	1.0	200	460	130	455	455	440	410	390
	2.0	200	455	128	445	435	415	375	305
	4.0	200	450	125	448	445	425	330	260
SPI[b]	0	200	600	200	590	570	520	425	230
	0.25	200	660	230	660	650	610	470	360
	0.5	200	705	253	705	690	655	595	545
	1.0	200	635	218	635	620	575	430	300
	2.0	200	620	210	615	610	590	505	360
	4.0	200	600	200	595	580	550	360	250

a: Protein extracted with alkaline solutions (pH 9) and then recovered by isoelectric precipitation on pilot scale
b: Protein extracted with salt solutions (0.5M NaCl) and then recovered by ultrafiltration on pilot scale

However, neutral salts affect the physico-chemical properties and interactions between proteins either by ionic strength effects, binding to the charged groups on the protein, or at high concentration by altering water structure with subsequent changes in hydrophobic effects (Damodaran and Kinsella, 1982). Thus the effect of salt on the molecular flexibility of proteins is probably more important in influencing the foaming properties of the proteins. Meanwhile, the effects on foaming properties varied with ion species and concentration (German and Phillips, 1994). Sucrose, is though to enhance adsorption of certain proteins at air-water interfaces, and it may minimise surface denaturation thereby enhancing film strength and viscoelasticity (MacRitchie, 1978). Sodium chloride may have similar effects to certain proteins including field peas. However, with the increase of salt concentration, the charge effect due to the change of ionic environment would become significant. Foam formation and foam stability could be inhibited because of the excess electrostatic repulsion at the interface.

6.1.4.3. Effect of Temperature on Foaming Properties

Heat treatment of field pea proteins results in an improvement in the foam propertie, as shown in Fig. 6.11 and Table 6.5. This enhancement is partly attributable to the increase of the surface hydrophobicity, which decreases the energy barrier for adsorption at the air-water interface (Damodaran, 1996). The improvement of foaming properties of food proteins upon heating have also been reported by several other researchers (de Wit *et al.*, 1986; deVilbiss *et al.*, 1974; Haggett, 1976). Upon heating of whey protein concentrate dispersions at 65-85°C for 30 minutes, improved foamability was observed in comparison to the unheated control (Graham and Phillips, 1980), whereas heating above 80°C caused a decrease in foamability. This suggests that above a critical level, the insoluble protein particles and the high molecular weight polymers which resulted from heat denaturation may adversely affect foamability of the proteins (Damodaran, 1996). It was reported that when heat-coagulable whey proteins were removed from milk, the remaining solution showed excellent foaming properties (Jelen, 1973).

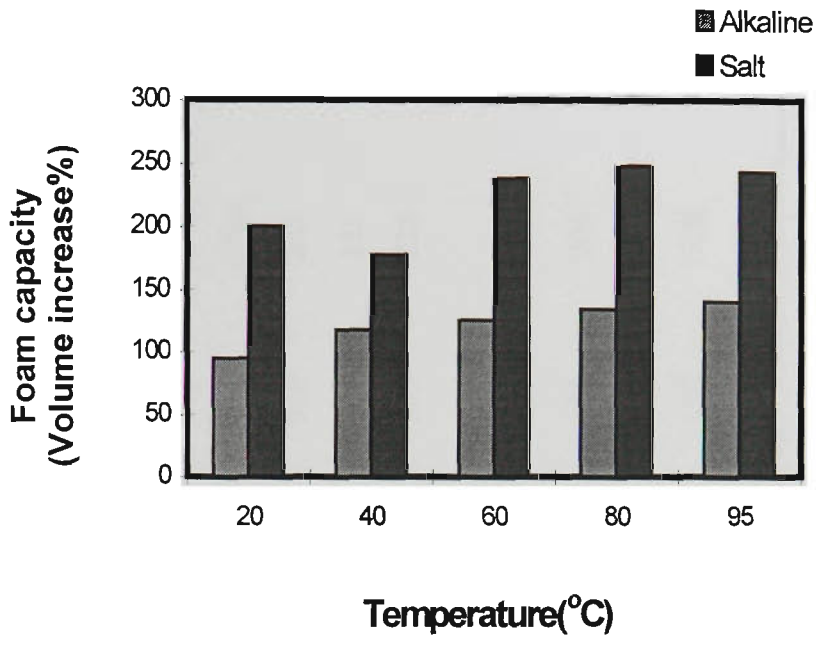


Figure 6.11 Effect of temperature on the foaming capacities of field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

However, the critical ratio of undenatured to denatured proteins that imparts better foamability may not be the same for all proteins (Townsend and Nakai, 1983). For the field pea proteins in the current study, with the increase of the temperature from 20°C to 95°C, the solubility of the proteins did not show any significant difference (Table 6.2). This indicated that a large portion of the proteins remains undenatured and heating did not result in any heat-coagulation of pea proteins which could decrease the foaming properties. Thus field pea proteins, especially those extracted with salt solution and recovered by ultrafiltration, exhibited good foaming properties upon heat treatment. This property would be useful in food applications where heat processing is required, including baked food products.

Table 6.5 The Effect of Temperature on the Foam Capacity and Stability of Field Pea Protein Isolates

Sample	Temperature (°C)	Volume before whipping (mL)	Volume after whipping (mL)	Volume increase (%)	Volume (mL) at room temperature (21°C) after time (hr)				
					0.25	0.5	1	2	3
API[a]	20	200	390	95	370	360	325	270	245
	40	200	435	118	435	410	335	270	235
	60	200	450	125	445	420	330	245	225
	80	200	468	134	455	410	315	225	210
	95	200	480	140	470	350	305	225	210
SPI[b]	20	200	600	200	590	570	520	425	230
	40	200	555	178	495	400	300	205	200
	60	200	675	238	670	650	580	450	290
	80	200	695	248	688	665	610	520	445
	95	200	685	243	685	675	650	590	540

a: Protein extracted with alkaline solutions (pH 9) and then recovered by isoelectric precipitation on pilot scale
b: Protein extracted with salt solutions (0.5M NaCl) and then recovered by ultrafiltration on pilot scale

6.1.4.4. Relationship between Foaming Properties and Surface Hydrophobicity

The results of the current study show that the emulsifying properties of field pea proteins are not correlated with the surface properties of the proteins (refer to Table 6.2). However, the higher the temperature, the lower the surface tension, and the higher was the hydrophobicity observed. This seems to relate well to the foaming characteristics of the pea proteins. As shown in Table 6.5, with the increase in temperature, the foaming capacity and stability were both improved. Townsend and Nakai (1983) also reported that hydrophobicity measured fluorometrically for food proteins had significant correlations to foaming capacity when the proteins in solution were unfolded by heating. The process of partial denaturation of proteins results in the extensive unfolding of the proteins and thus the gradual exposure of hydrophobic groups of native proteins which are usually buried in the interior of the molecules (Tanford, 1973). Hence the air –water interfacial tension is reduced and the interfacial area of the foam is expanded due to the exposure of most of the nonpolar residues at the interface. However, it is clear that the increase of interfacial area (i.e., foamability) is limited not only by the surface hydrobicity but also by the total number of hydrophobic groups in the protein (Damodaran, 1996).

While the positive correlation between hydrophobicity and foaming properties has been observed previously (Kato *et al.*, 1981; Townsend and Nakai, 1983), as well as in the current study, it is not the only factor which influences the foaming properties of proteins. For example, when salt (NaCl) was added into field pea protein solutions at different concentration, the hydrophobicity and surface tension did not show significant differences (Fig. 6.6 and 6.7). However, the foaming properties were improved to a maximum amount and then dropped with further increases in the salt concentration (Fig. 6.10 and Table 6.4). Damodaran (1996) suggested that the foaming properties of proteins depend on an optimum balance of hydrophobicity and charge density, as well as other noncovalent interactions. Along with the inherent physico-chemical properties of proteins, the additional external factors including protein concentration, ionic strength, pH, temperature,

and the presence of other food constituents all affect the foaming properties of food proteins.

6.1.5. Viscosity Characteristics of Field Pea Proteins

Viscosity is the measure of the internal friction of a fluid. This friction becomes apparent when a layer of fluid is made to move in relation to another layer (Handbook of Brookfield Viscometer). The greater the friction, the greater the amount of force required to cause this movement, which is called "shear". Shearing occurs whenever the fluid is physically moved or distributed, as in pouring, spreading, spraying, mixing, etc. Therefore, highly viscous fluids require more force, if such movement is to occur, than for less viscous materials. However, apart from the shear rate, many other factors including temperature, sample preparation, viscometer model, time, composition and additives in the material, all affect the viscosity measurements. The knowledge of the viscosity of protein dispersions is of practical significance in relation to processing, process design, mouthfeel of viscous fluid products, and new product development (Hermansson, 1975). In this section, the results of viscosity behaviour of field pea proteins are presented. The effects of protein concentration, temperature, pH and salt concentration on the viscosities of the protein solution are also evaluated.

6.1.5.1. Effect of Concentration on Viscosity

The effects of protein concentration on the apparent viscosity of field pea proteins are shown in Fig. 6.12. The apparent viscosity of pea protein dispersions increased progressively with the increase of protein concentration, especially at higher concentrations of 8% to 15%. Similar trends have been reported for other food proteins including those from soy (Hermansson, 1975), chickpea (Liu and Hung, 1998a), fababean (Schmidt *et al.*, 1986), oat (Ma, 1993) and yeast (Huang and Kinsella, 1986a). However, compared with soy protein (Supro-500E), the viscosity of field pea proteins is relatively low (Table 6.6). Hsu *et al.* (1982) also reported that the viscosity of soy protein was about 12 times greater than that of fababean,

yellow pea, and lentil proteins. This result is similar to that in the current study, although slight differences were found between the viscosity readings of different field pea isolates. The differences most likely resulted from the different protein concentrations and different models of viscometer used for the measurements. Meanwhile, from Fig. 6.12 and Table 6.6, it can be seen that the salt extracted field pea proteins exhibited lower viscosities than that of alkaline extracted proteins. Different processing conditions may result in the different physical properties of these proteins including the particle sizes, as well as the differences in the ionic strength of the protein solutions. Such factors could have a great influence on protein-protein interactions in solution and thus cause the differences in the flow behaviour of the protein dispersions. The higher viscosity of alkaline extracted proteins may also result from alkali-induced unfolding of the protein molecules.

The effect of shear rate on the apparent viscosity of pea proteins at different concentrations was also investigated (Fig. 6.13). At protein concentrations below 10%, the viscosity of the solutions remains unchanged at different shear rates, indicating Newtonian or near Newtonian behaviour of protein dispersions. However, at higher concentrations (above 10%), field pea protein dispersions showed non-Newtonian behaviour, exhibiting shear thinning over a range of shear rates. Accordingly, it is appropriate that the measured viscosity of field pea protein isolate as non-Newtonian fluid should be referred to as apparent viscosity. This shear thinning phenomenon is also known as pseudoplastic flow behaviour and has been found with a number of other food proteins including those from oat (Ma, 1993), soy (Hermansson, 1975), canola (Paulson and Tung, 1988b), chickpea (Liu and Hung, 1998a) and yeast (Huang and Kinsella, 1986a). In very dilute protein dispersions, the apparent viscosity reflects the individual contributions of each dispersed protein molecule. As the concentration is increased, the disturbances of flow caused by the dispersed protein molecules are no longer independent. The protein-protein interactions become dominant and more water molecules are immobilised, resulting in the additional increase of apparent viscosity (Frisch and Sinha, 1956).

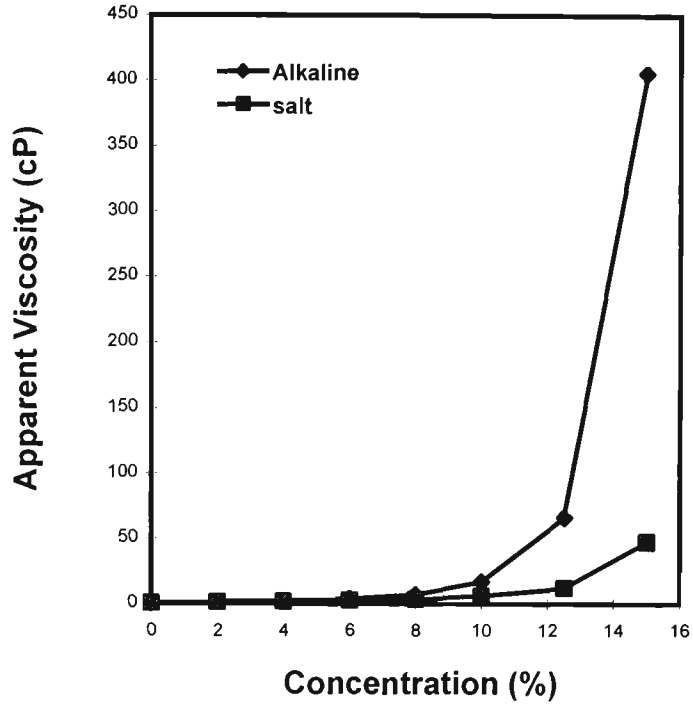
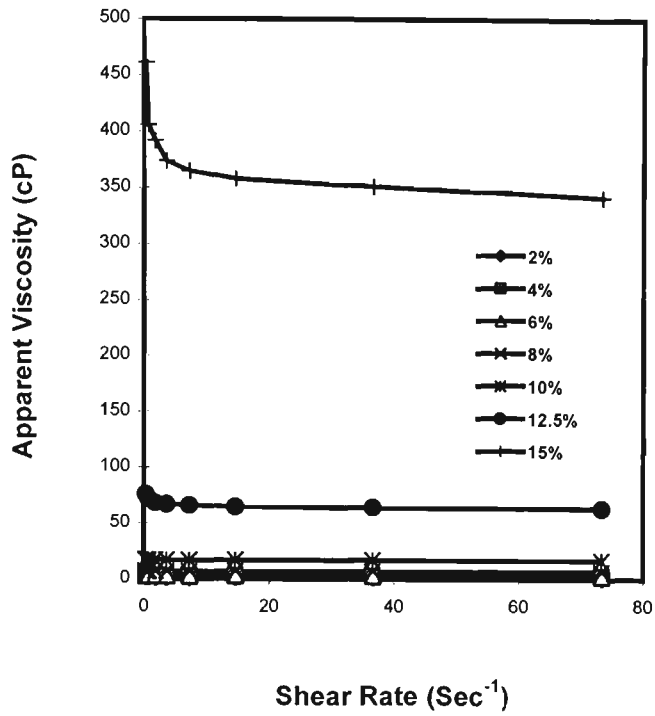


Figure 6.12 Effect of protein concentration on the apparent viscosity of field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

Table 6.6 Viscosities of Field Pea Proteins and Soy Protein

	Viscosity (cP) of dispersions[a]	
Sample[b]	**4%**	**8%**
API[c]	2.25	7.06
SPI[d]	1.69	3.49
Soy Protein (Supro-500E)	17.4	184

a: Viscosity measured as cP: centipoise
b: Percentage values indicate amount of protein isolate in solution (w/v)
c: Pilot scale alkaline extracted protein isolate
d: Pilot scale salt (0.5M NaCl) extracted protein isolate



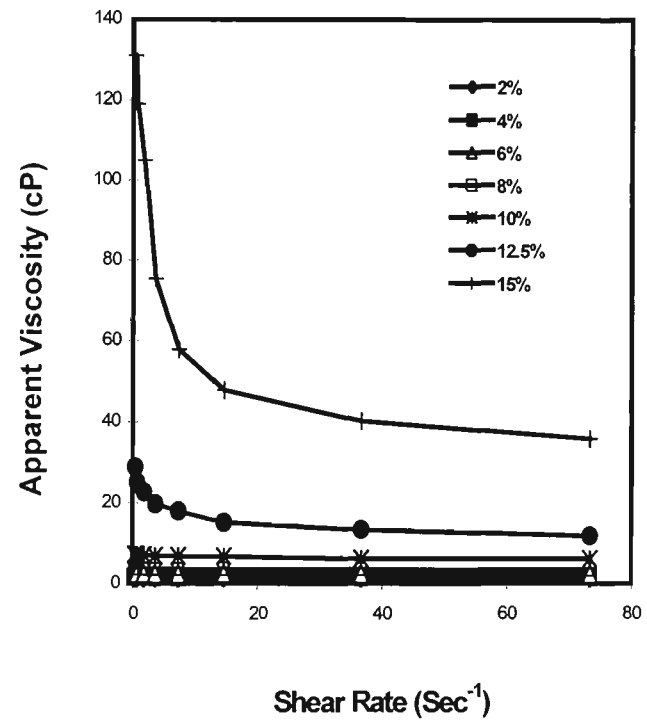


Figure 6.13 Effect of shear rate on the apparent viscosity of field pea protein dispersions at different concentrations. Top: pea proteins extracted with alkaline solution; Bottom: pea proteins extracted with salt solution.

However, as the shear rate increases, the water layers could be progressively removed, resulting in reduction of the size of hydrated aggregates with a concomitant decrease in the apparent viscosity (Tung, 1978). Hence shear thinning behaviour has been observed with protein dispersions at high concentrations. On the other hand, Gueguen and Lefebvre (1983) reported that pea protein isolates exhibited thickening behaviour which showed the same general characteristics as Supro-620 soy isolate, but to a much lesser degree. The result of this previous work is at variance from those obtained in the current study. The differences may result from different type of viscometers used, as well as different sample preparation procedures and thus different physico-chemical properties of the proteins such as the solubility. As "thickening" means increasing viscosity with an increase in shear rate and is frequently observed in fluids containing high levels of deflocculated solids (Handbook of Brookfield Viscometer), the solubility of the protein sample prepared by Gueguen and coworker was probably low although the data was not presented.

In the current study, the field pea protein dispersions also displayed thixotropic properties in which there was a decrease in viscosity with time under constant shear rate. This behaviour is more pronounced with the pea protein extracted by salt solution on the pilot scale. This suggests that pea proteins possessed relatively unstable structures that were unable to resist the constant shear force over a long period of time.

6.1.5.2. Effect of pH on Viscosity

The effects of pH on the apparent viscosity of field pea proteins are shown in Fig. 6.14. The viscosity-pH curve resembles the solubility curves of field pea proteins, especially for the protein extracted by alkaline solution on the pilot scale (Fig. 5.1). Minimum viscosity was observed at the isoelectric range (pH 4-6) where the minimum solubility occurs. As the pH is adjusted to values further from the pI range, especially at the alkaline conditions (pH>8), the viscosity of the proteins increased markedly. The positive correlation between viscosity and solubility has also been found with some other proteins including soy protein (Shen, 1981), yeast protein (Huang and Kinsella, 1986a), canola protein (Paulson and Tung, 1988b) and chickpea protein (Liu and Hung,

1998a). However, apart from the effect of solubility, many other factors including conformation, hydration, exposure of hydrophobic groups, and charge distribution also contribute to the intermolecular interactions that result in different viscosity characteristics (Shen, 1981). The higher viscosity under alkaline conditions may result from the combined effects of increased hydration with the increased charge density and possibly greater electrostatic repulsion between molecules (Huang and Kinsella, 1986a), as well as alkali-induced protein unfolding and increased solubility.

6.1.5.3. Effect of Salt (NaCl) on Viscosity

The effects of salt concentration on the apparent viscosity of field pea proteins are demonstrated in Fig. 6.15. For the protein isolate extracted with salt solution on the pilot scale, there are only minor differences between the viscosities of protein dispersions with or without salt addition. This indicates that the proteins extracted with salt solution might have a more rigid structure than those extracted with alkaline solution. In the latter case there may have been some alkali-induced unfolding of the protein molecules during processing. In terms of viscosity determinations, the salt extracted proteins may not be as sensitive as the alkaline extracted proteins to the change of ionic environment since the protein molecules have already been subjected to varying salt levels and the resultant effects on the structure and protein-protein interactions during processing.

On the other hand, as can be seen from Fig. 6.15, there was an initial drop in apparent viscosity of alkaline extracted pea proteins at the lower concentrations of salt up to 1%. The viscosities gradually increased with the increase of salt concentration from 2-5%. Similar patterns of viscosities for oat protein have also been found at salt concentrations from 0-4% (Ma, 1993). A decrease in apparent viscosity of soy protein dispersions up to 0.5M (ca. 2.8%) salt concentration has also been observed, followed by a reversal between 0.5 and 1.0M (Hermansson, 1975). This reversal in viscosities has been attributed to the critical salt concentration for the solubilisation of proteins (Megen-van, 1974). However, although solubility is an important factor which affects the flow behaviour of food proteins, the effects are not always uniform. For example,

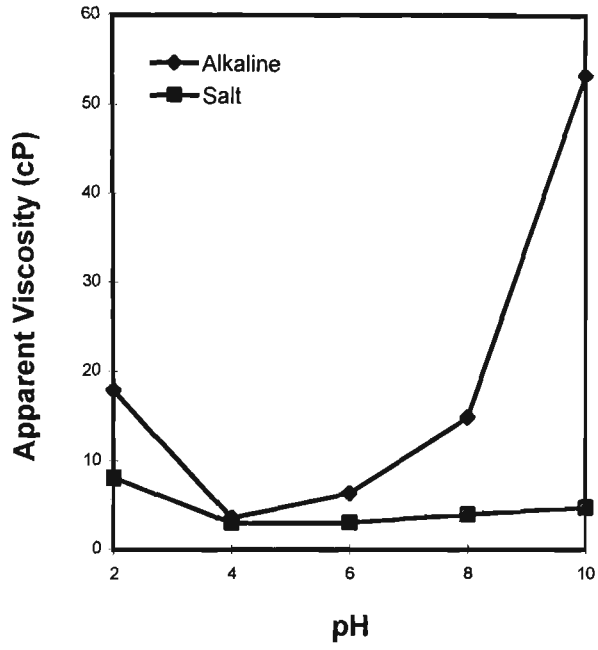


Figure 6.14 Effect of pH on the apparent viscosity of field pea proteins (at 20°C, 8% dispersion). Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

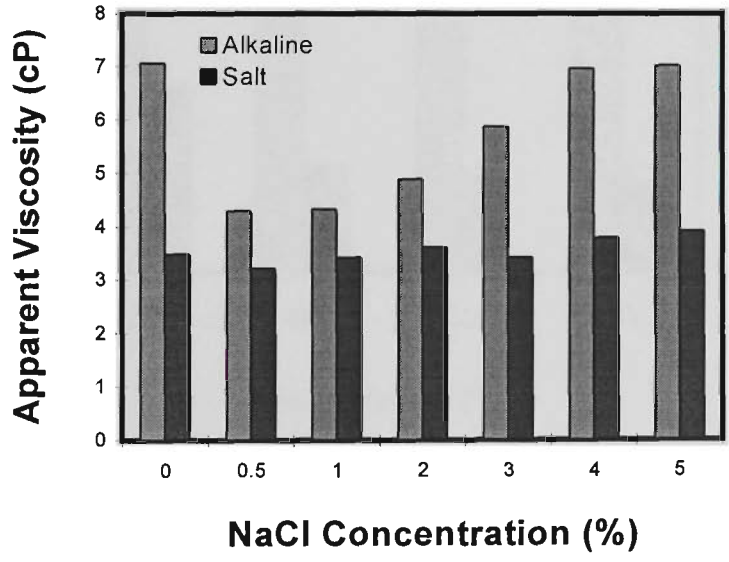


Figure 6.15 Effect of NaCl on the apparent viscosity of field pea proteins (at pH7, 20°C, 8% dispersion). Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

highly soluble proteins such as whey protein concentrate have low viscosity, whereas soy protein (Promine-D), with its lower solubility, exhibited high viscosity at relatively low concentrations (Ma, 1993).

6.1.5.4. Effect of Temperature on Viscosity

Generally speaking, increasing temperatures resulted in decreased viscosity of field pea proteins, as shown in Fig. 6.16. Higher temperature induces decreased viscosity probably by destabilising both protein-protein and protein-water interactions (Huang and Kinsella, 1986a). Similar results have been reported by Mita and Matsumoto (1980), who found that the apparent viscosities of 12% gluten and gluten methyl ester dispersions decrease as the temperature is increased from 20 to 50°C.

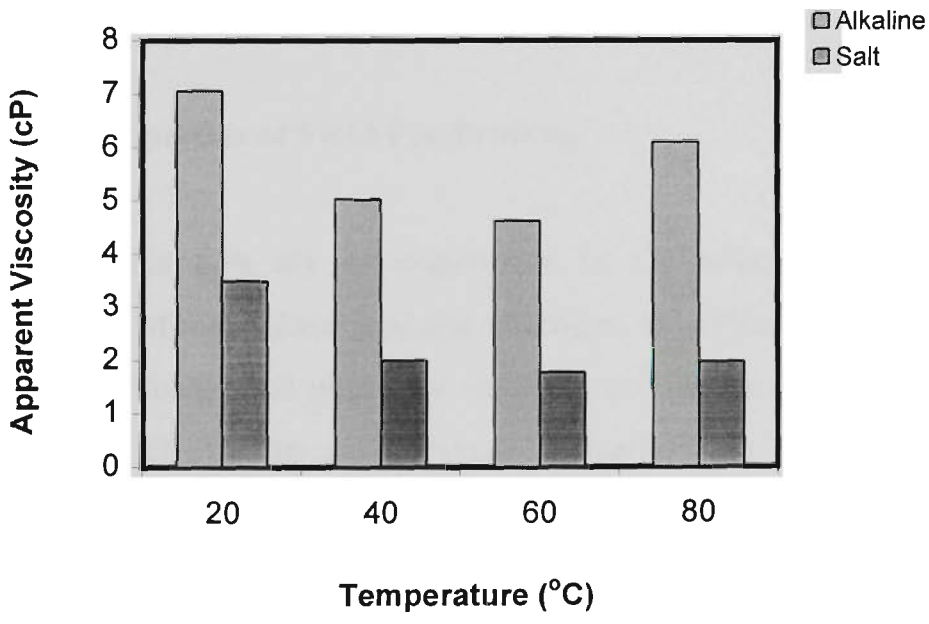


Figure 6.16 Effect of temperature on the apparent viscosity of field pea proteins (at pH7, 8% dispersion). Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

However, as for the thermal effects on the emulsifying properties of proteins, heating dose not produce consistent effects on the viscosity behaviour of proteins. For example, increases in viscosities upon heating have been reported with blood plasma protein (Howell and Lawrie, 1987), faba bean (Schwenke *et al.*, 1990) and oat protein (Ma, 1993). Partial denaturation of proteins due to thermal treatment possibly induces protein unfolding and thus enhances the viscosity of the proteins (Lee and Rha, 1979; Ma, 1993). Hence the thermal effects on viscosity behaviours also depend on the type of proteins, as well as the protein flexibility, which may reflect the sensitivity of protein dispersions to thermal treatment. In the case of field pea proteins, the exhibited high solubility upon heating might minimise noncovalent associations between protein molecules and inhibit coagulation, and at the same time, no increase is seen in viscosity. From these results it may also be predicted that field pea protein solution may not show strong gel formation properties which also depend upon the heat denaturation and coagulation of the protein molecules.

6.1.6. Gelation Properties of Field Pea Proteins

Heat-induced protein gels are of importance to the structural formation and physical properties of many food products (Zheng et al., 1991). The formation of a gel is a complex process but generally involves two major steps. The first step includes either a change in conformation (usually heat induced) or partial denaturation of the protein molecules (Phillips *et al.*, 1994c). As denaturation proceeds, the viscosity of the dispersion increases due to an increase in molecular dimensions of the unfolding proteins. This is followed by the second step which is a gradual association or aggregation of the denatured proteins (Ferry, 1948). For the formation of a highly ordered gel matrix, it is imperative that the aggregation step proceed at a slower rate than the unfolding step (Hermansson, 1978). In addition, the type and properties of gels are sensitive to many other factors, including protein concentration, pH, type of salt and salt concentration as well as interactions with other food components including sugars (Kinsella et al., 1985;

Smith, 1994). Since the capacity of gels to act as a matrix for holding water, lipids, sugars, flavours and other ingredients is useful in food applications and for development of new products (Kinsella, 1979), gel forming ability is potentially an important functional property in food systems. However, with respect to vegetable proteins, thermal gelation studies have been focused on soybeans (Utsumi and Kinsella, 1985; Wang and Damodaran, 1991; Nakamura *et al.*, 1986). The purpose of this section is to examine and discuss thermal gelation properties of field pea protein isolates under a variety of conditions.

6.1.6.1. Selection of Heating Temperature and Heating Time

In an earlier phase of this study the thermal properties of the field pea protein isolates were investigated using DSC (Chapter 5). The results indicated two transitions between 82-85°C and 96-98°C (Fig. 5.15). Since heating to temperatures above the minimum denaturation temperature of the proteins is generally required for gel formation (Phillips *et al.*, 1994c), a 97°C water bath was selected and used to heat the samples in order to study the gelation properties in the current research. Although heating at a higher temperature generally produces a stronger gel, excessive heating causes thermal scission of peptide bonds, which prevents gel network formation (Furukawa, *et al.*, 1979). Several studies have indicated that the optimum heating temperature for gelation is just above the thermal transition temperature of the protein. For instance, soy protein exhibits highest gel strength when heated at 80-90°C, which is close to the thermal transition temperature of 84.6°C for the 11S globulin (Damodaran, 1988). Similarly, the optimum heating temperature for gelation of myosin is 60-70°C (Hermansson and Lucisano, 1982), which is just above its thermal transition temperature of 57°C (Samejima *et al.*, 1983). Thus 97°C was considered a suitable temperature for studying the heat-induced gelation of field pea proteins. Preliminary studies also indicated that heating times from 15 to 45 min did not result in significant differences in gel hardness for the 15% pea protein slurries. Since a longer time is usually required for gel formation at a lower concentration (Zheng *et al.*, 1991), 30 min was

adopted as the standard heating time to study the gelation properties when other conditions were varied.

6.1.6.2. Effect of Protein Concentration on Gelation

The effects of protein concentration on the gel hardness of pea proteins are shown in Fig. 6.17. With increasing concentration, the gel strength gradually increased. Similar trends have also been reported for gel formation from soy proteins (Wang and Damodaran, 1991) and whey proteins (Boye et al., 1997). However, for each particular type of protein, a critical concentration is required for the formation of a gel and the type of gel varies with the protein concentration (Schmidt, 1981). For example, gelatin and polysaccharide solutions will form gels at relatively low concentrations of the gelling materials. Considerably higher protein concentrations are usually required for the gelation of globular proteins. With regard to the field pea proteins in the current study, no gels were formed below a concentration of 12.5%. Catsimpoolas and Meyer (1970) reported that the minimum protein concentration of soy proteins needed to form gels was 8%. Hence field pea proteins did not produce better gelation properties than soy. As can be seen from Fig. 6.17, the peak force values for the gels were very low (< 1.3 Newtons) even at a concentration of 17.5%. From visual assessment, the gels

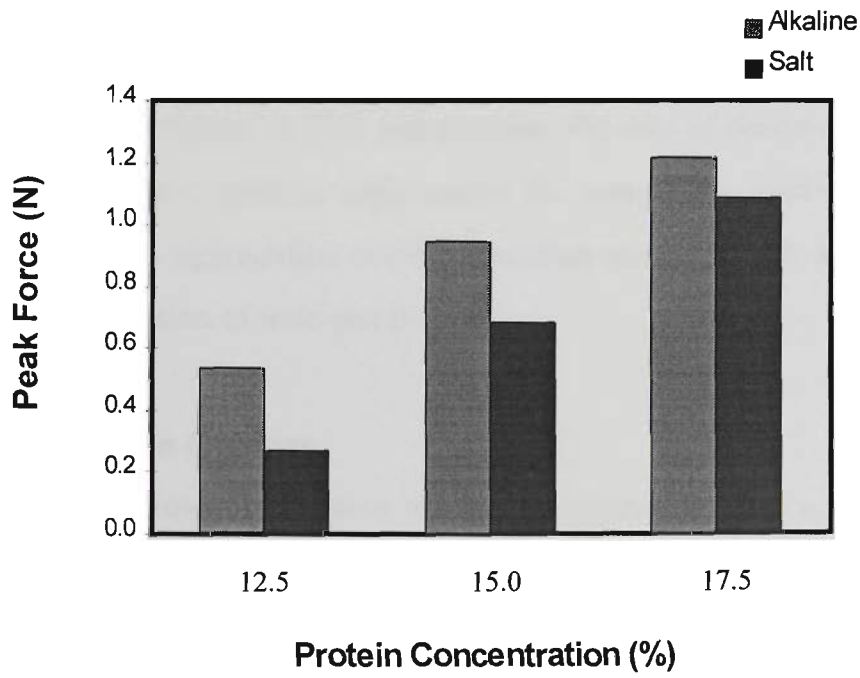


Figure 6.17 Effect of protein concentration on the gel peak force for field pea proteins. Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

were opaque, weak and not coherent. If the protein concentration was higher than 17.5%, it was difficult to obtain uniformity in the protein slurry prior to heating, since some portions of the material could not be totally wetted. Hsu et al. (1982) also reported that yellow pea protein isolates exhibited poor gelling properties since the heated slurries showed only a pastelike consistency after cooling. The protein concentration used in their studies was 10%.

Damodaran (1996) pointed out that the formation of a protein gel network is the result of the balance between protein-protein and protein-solvent interactions. In a thermodynamic sense, the formation of a self-supporting gel network is dependent on the number of cross-links, both covalent and noncovalent, resulting from these interactions. If the sum of the energies of these interactions is greater than the thermal energy, the gel network should be stable. Field pea proteins probably lack the ability to form a sufficient number of cross-links from the protein-protein interactions and protein-solvent interactions, and thus weak gel formation ability was observed. On the other hand, Hermansson (1979b) suggested that the relative rates of denaturation and aggregation processes during heating might play a role in determining the type of gel formation. If the rate of aggregation of protein is faster than the rate of denaturation, random aggregation of the denatured molecules could occur. This might result in the formation of an unordered gel network high in opacity and low in elasticity and water-holding capacity. With respect to field pea proteins, the rate of denaturation would be slow since the solubility remains high under the conditions applied during heat treatment. Thus random aggregation could be another reason which accounts for the weak gel network formation of field pea proteins.

6.1.6.3. Effect of pH on Gelation

The pH of the heated protein dispersion would be expected to have a profound effect on the gelation reactions (Schmidt, 1981). In order to study the effect of pH on gel formation and peak force, the pH values of field pea protein slurries were varied from 3 to 9, and the results are shown in Fig. 6.18. The maximum gel strength was observed at pH 6-7, whereas the gels formed at acidic pH values were very weak and nonelastic. If

the pH was increased above 8, a weak, sticky gel was observed and the colour was relatively dark. Bora *et al.* (1994) studied heat induced gelation of pea globulins, vicilin and legumin, and found that gel formation occurred at pH values above 6.4 with the greatest peak force at pH 7.1. At highly acidic and alkaline pH, proteins assume a net charge and the strong electrostatic repulsion inhibits gel network formation (Damodaran, 1996). On the other hand, at the pI, proteins have zero net charge and tend to aggregate via hydrophobic interactions. This leads to formation of a coagulum-type gel with a coarser network and lower gel strength. Thus only at the optimum pH, which permits an optimum balance of protein-protein and protein-solvent interactions, can a uniform gel matrix with high gel strength be formed. The optimum pH value is different for different types of proteins. However, it is typically in the range of 7-8 for many proteins (Damodaran, 1996).

6.1.6.4. Effect of Salt (NaCl) on Gelation

The presence of neutral salts affects gelation and gel properties via charge neutralisation of protein molecules and reflects the importance of electrostatic interactions (Phillips *et al.*, 1994c; Damodaran and Kinsella, 1982). As is demonstrated in Fig. 6.19, the gel strength of field pea proteins is decreased with the increase of the salt concentration. This is possibly due to the excessive repulsive forces which prevent the denatured protein molecules from associating to form a strong network and consequently there is no formation of a self-supporting gel upon cooling (Phillips *et al.*, 1994c). Bora *et al.* (1994) also reported that sodium chloride had an adverse effect on the gel peak force of mixed globulin from peas. Similar results have also been found with soy isolate and 11S protein (Utsumi and Kinsella, 1985; Wang and Damodaran, 1991). However, with these studies, the interior gelation properties were not observed with 7S soy globulin in the presence of NaCl. This suggested that in addition to the charge effect, other molecular properties of proteins including hydrophobicity and intermolecular disulphide or hydrogen bond formation may also play an important role in gel network formation (Damodaran, 1996). In the case of 7S globulin the contribution of ionic interactions were limited. On the other hand, the contribution of hydrogen

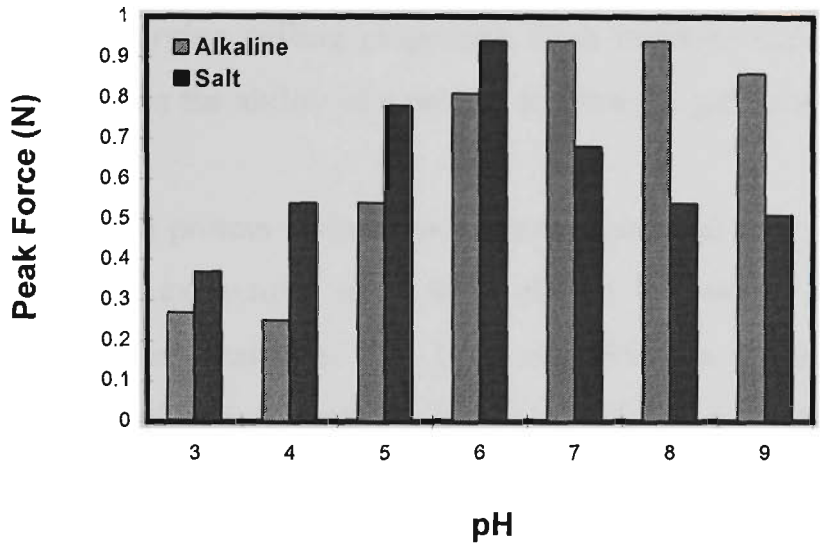


Figure 6.18 Effect of pH on the gel peak force of field pea proteins (at 15% protein concentration). Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

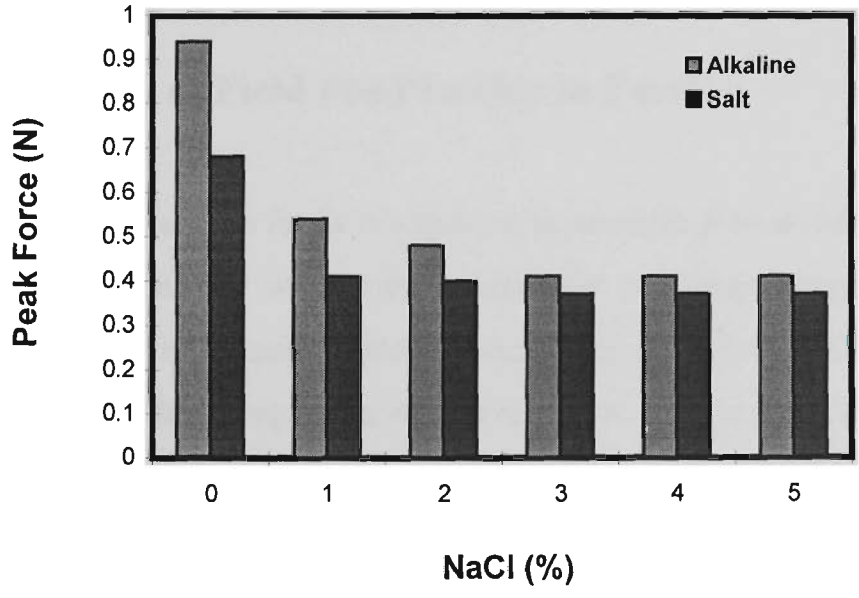


Figure 6.19 Effect of NaCl on the gel peak force of field pea proteins (at 15% protein concentration). Alkaline: pea proteins extracted with alkaline solution; Salt: pea proteins extracted with salt solution.

bonds was important and these were involved in the elasticity and the hardness of 7S gel (Utsumi and Kinsella, 1985). Hence proteins, because of their dynamic structures, possess varying gelling properties. Both intrinsic and extrinsic factors have great influence on the ability of a protein to form the gel network.

In summary, field pea protein isolates, both those extracted with salt solution and those from alkaline extraction, have been shown to posses many functional properties which are desirable for food applications. Field pea proteins demonstrated good solubility, water binding, emulsifying and foaming properties. In particular, salt extracted pea proteins exhibited very good water binding and foaming ability in comparison with the alkaline extracted proteins. Although the various molecular factors and physico-chemical principles that are involved in each of the functional properties are complex, the current study of the behaviour of field pea proteins in model systems provides a basis for assessing the potential of pea proteins as novel food ingredients. For example, the heat-stable foaming ability of pea proteins might be considered important in baked foods, whereas the good emulsifying property would be useful in salad dressing.

6.2. Applications of Field Pea Proteins in Foods

The use of plant proteins in foods is expected to increase substantially in the future as a means of meeting the worldwide demand for economical sources of protein. Among the reasons for interest in plant proteins are the ever increasing number of vegetarians and of the rising costs of conventional protein sources such as eggs (Sethi and Kulkarni, 1994). However, for a long time, soybean has been the principal plant protein resource for food applications including dairy products, meat or fish products, confectionery and bakery products. Undoubtedly, soy protein ingredients have made a significant impact in the food industry. Field pea proteins, which have now been found to exhibit comparable functional properties with soy proteins, provide significant potential in a variety of food applications. It has been previously reported that field pea flour and pin-milled protein concentrate

were used as protein supplements in bread, baking powder biscuits, ground beef patties and blended milk products (Sosulski and Mahmoud, 1979; McWatters, 1980; McWatters and Heaton, 1979; Sosulski *et al.*, 1978). The results indicated that the baking and organoleptic qualities of the products were not adversely influenced by the addition of pea flour as the replacement for milk protein. However, if the unheated flour was used at a higher concentration, undesirable effects of the protein supplements on dough or baking properties including crust and crumb colour and texture of the products were observed. Adverse flavours may also be a major limitation in the use of these flour and protein concentrates. Currently, little research has been reported on the evaluation of potential food uses of pea protein isolates extracted by wet methods.

Previous sections in this chapter have demonstrated that field pea proteins extracted with alkaline and salt solution on a pilot scale exhibited good functional properties including protein-water interaction, as well as emulsifying and foaming properties. Since the successful applications of plant-derived proteins will largely depend upon the physical and functional qualities they impart to foods and upon their acceptability to consumers (McWatters, 1980), the selection of suitable food systems for assessing the possibility of the new protein ingredients is important. Accordingly, for this study, sponge cake and mayonnaise have been chosen as model foods in order to study the potential of field pea proteins as a replacement for egg proteins. Whereas foaming and emulsifying properties of the proteins are desirable in sponge cakes, the good emulsifying capacity and stability are most important in mayonnaise.

6.2.1. Sponge Cake

6.2.1.1. Characteristics of Cakes Supplemented with Field Pea Proteins

Sponge cakes were prepared with varying levels of replacement of egg protein with field pea protein isolates. The replacement levels studied were 10, 25, 50, 75 and 100%. Cake quality was assessed in terms of total volume, crumb colour and firmness.

Table 6.7 Characteristics of Sponge Cakes Containing Field Pea Protein Isolates

Protein Source and Level	*Volume (mL)*	*Firmness (N)*	*Colour*		
			*L**	*a**	*b**
Control	940	5.30	77.23	-3.07	+21.84
SPI[a]					
10%	950	4.99	76.55	-2.79	+20.57
25%	945	5.46	73.93	-2.41	+21.15
50%	920	5.24	70.24	-1.72	+21.37
75%	795	4.73	67.40	-1.04	+20.18
100%	675	3.21	65.81	-0.22	+20.35
API[b]					
10%	945	5.75	76.20	-2.45	+21.39
25%	915	4.87	71.62	-1.73	+21.92
50%	870	4.05	67.06	-0.63	+20.43
75%	715	3.23	61.97	+0.53	+19.16
100%	685	3.06	60.17	+1.45	+19.54

a: Pilot scale salt (0.5M NaCl) extracted protein isolate
b: Pilot scale alkaline extracted protein isolate

The resulting characteristics of sponge cakes supplemented with pea proteins are shown in Table 6.7. For the protein isolate extracted with salt solution (SPI), at the level of 10 and 25% substitution of egg protein, the cake volumes were not reduced and were even slightly higher than that of the control (100% egg protein). Similarly, cake volume was not changed at up to 10% substitution with API. This is due to the good foaming properties of field pea proteins, particularly for SPI. Compared with breads fortified with non- wheat flours including legume proteins, a reduction in loaf volume is generally reported (Liu, 1996; Finney *et al.*, 1980; Sathe *et al.*, 1981). This reduction in loaf volume is normally attributed to the impact on wheat gluten, which is very important in dough formation, fermentation and the texture of the final product. In the case of cakes in the current study, the purpose was to use field pea protein to replace egg protein. If pea proteins were used to substitute egg protein at levels in excess of

50%, the volume of the cakes gradually decreased with increasing pea protein contents. Since the sponge cake contains a relatively large amount of oil, the adverse effect of field pea proteins on the total volume may result from their poor abilities to bind fat. From organoleptic assessment, at the high levels of fortification with pea proteins, the texture of the cakes became coarse, oily and lacking coherence with slices of the cake crumbling readily.

In further assessing the texture of the different cakes, firmness values were measured using the Instron Universal Testing Machine (Table 6.7). The apparent drop in firmness (over 50% substitution with pea proteins) did not represent a softening of the crumb which may have been desirable. The lower values reflect the more crumbly characteristics which resulted in a loss of coherence under the compressive force applied to the slice during testing.

The effects of substitution of egg proteins with pea protein on cake colour were measured instrumentally and the results are also shown in Table 6.7. The whiteness of the cakes remains similar to the control up to 25% replacement of egg protein with pea proteins. However, brown colour and yellowness are more desirable for the cakes. Although the darker colour was observed with the increase of the amount of pea proteins, the yellowness did not change significantly. The $-a^*$ value reflected the slight greenness of the product and this is perhaps because of the colour of vegetable oil contained in the cakes. Generally, only a minor impact on the colour was observed during the fortification with field pea proteins in cakes. The appearance and texture of the sponge cakes fortified with salt extracted pea proteins are shown in Fig. 6.20. It can be seen that up to 50% substitution of egg proteins with pea proteins, the cake quality is acceptable in terms of product colour and internal texture.

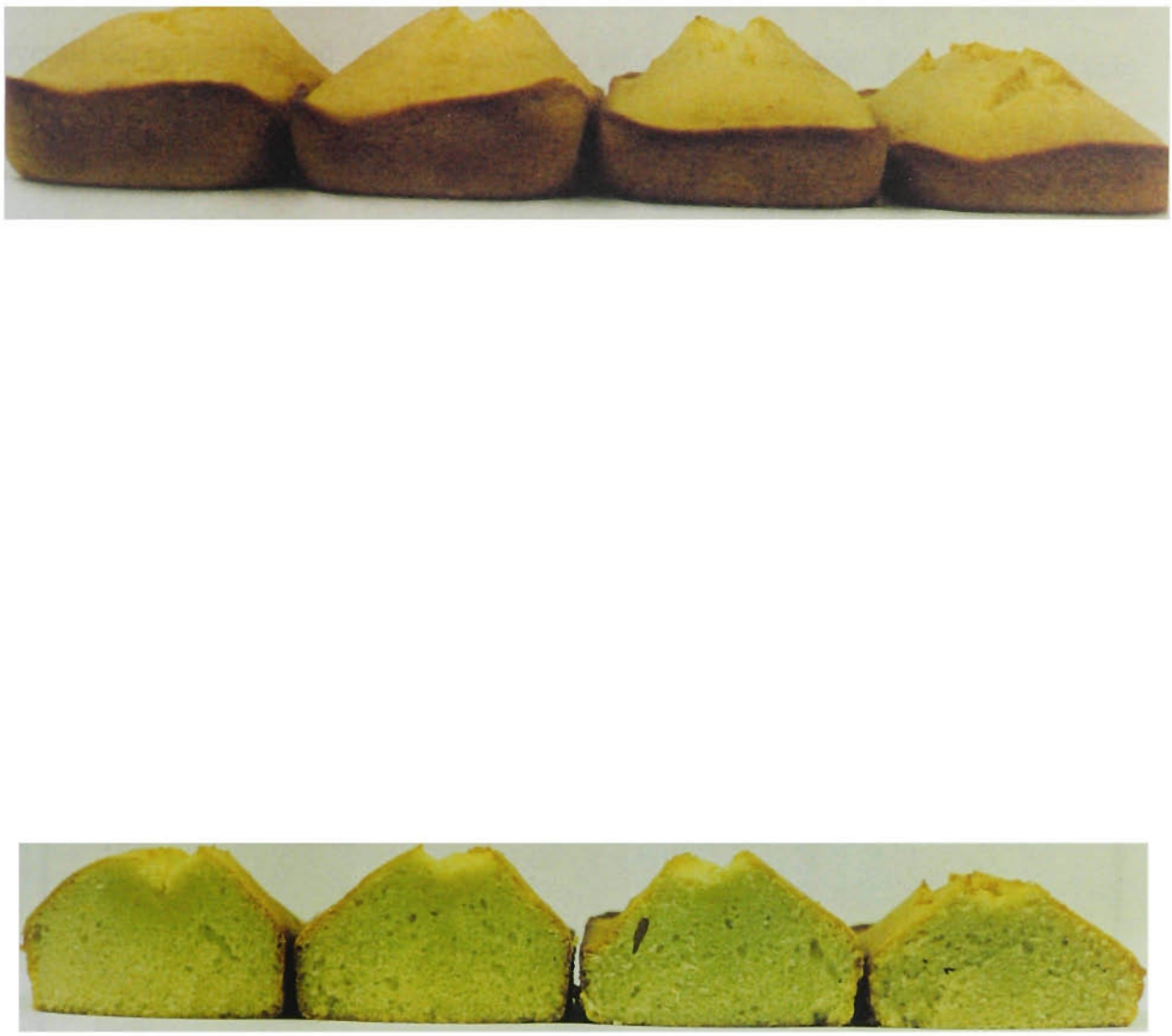


Figure 6.20 The appearance and texture of sponge cakes fortified with salt extracted pea proteins. From left to right: at 0%, 25%, 50%, 75% substitution of egg proteins with pea proteins, respectively.

6.2.1.2. Sensory Evaluation of Cakes Supplemented with Field Pea Proteins

In order to further assess the impact of replacement of egg by field pea proteins in sponge cakes, a sensory panel was established. This panel consisted of twelve members of varying backgrounds and they were asked to assess the products for overall acceptability and also the presence of a beany flavour. The results (Table 6.8) showed that no difference in the product was found at a level of 25% substitution. In addition, the panellists made the comment that up to 75% substitution with pea proteins, the quality of all of the cakes was acceptable. In relation to flavour, no panel members detected any beany flavour at 25% substitution levels. At higher substitutions, the flavour was detectable, but a number of panel members specifically noted that they preferred pea flavour in the

Table 6.8 Sensory Evaluation of Cakes and Mayonnaise Containing Pea Proteins

	Cakes		*Mayonnaise*	
	Overall acceptability	***Pea protein taste***	***Overall acceptability***	***Pea protein taste***
Control	9	-	7	-
SPI[b]				
10%	9	-	7	-
25%	9	-	7	-
50%	8	+	5	+
75%	6	++	4	++
100%	5	++	3	+++
API[c]				
10%	9	-	6	-
25%	9	-	6	-
50%	8	+	5	+
75%	5	++	4	++
100%	4	++	3	++

a: Score key: 8-9= very good; 6-7= good; 4-5= fair; 2-3= poor; 1= very poor
+: detectable; -: not detectable
b: Pilot scale salt (0.5M NaCl) extracted protein isolate
c: Pilot scale alkaline extracted protein isolate

products, reflecting wide cultural and dietary backgrounds of the panellists. Some also commented that the only adverse effect of the pea proteins was the coarse, crumbly mouthfeel of the cakes supplemented at higher concentrations of the pea proteins.

6.2.2. Mayonnaise Supplemented with Field Pea Proteins

The results of sensory evaluation of mayonnaise supplemented with field pea proteins are also included in Table 6.8. Similar to sponge cakes, the overall acceptability of the products was ranked the same as the control up to 25% substitution of egg yolk with field pea proteins. At this level, the beany flavour was not detectable. However, with substitution over the level of 50%, the overall quality of the mayonnaise was generally not acceptable. The major negative comment related to the watery texture and the coarse, oily mouthcoating. When the emulsifying properties of field pea proteins were studied, the proteins worked as an emulsifier in typical oil-in-water systems, where the hydrophilic properties of the proteins are more important. On the other hand, in the formulation of mayonnaise, the oil content is very high and the lipophilic properties of the emulsifier are particularly required. However, the results of the functional evaluations showed that field pea proteins exhibited good solubility properties but poor fat-binding abilities. This indicates that pea proteins might lack sufficient hydrophobic groups so that they could not successfully produce amphiphilic functions in highly fatty food systems such as mayonnaise. Flavour problems resulting from pea protein ingredients were more significant in mayonnaise than in sponge cakes since baking at high temperature for cake minimised the effect of volatile odour components present with the pea proteins. As a result, field pea proteins offer better potential as a new protein ingredient in cakes than in mayonnaise. Nevertheless, if applied at a lower concentration, pea proteins still appear to have potential significance in salad dressing including mayonnaise particularly because of their important functional behaviour and consequently, of their capacity to be used as the new plant protein source to replace conventional egg proteins.

6.3. Conclusions

In the current study, the functional properties of field pea proteins have been extensively evaluated. Generally, field pea proteins exhibit good solubility, emulsifying and foaming properties, whereas the oil absorption, viscosity and gelation properties showed lower potential. In addition, it was found that salt-extracted pea proteins and traditional alkaline extracted proteins have some differences in their functional properties. The former gives a better water adsorption capacity and foaming properties. Proteins extracted with alkaline solution produce higher viscosity in solutions compared with that of salt-extracted proteins. For the emulsification and gelation properties, the two preparations are quite similar. These results indicate that different protein products would be useful in particular food applications.

Different temperatures, salt (NaCl) concentration and pH have strong effects on the functional properties of field pea proteins. In particular, solubility seems to be positively related to the emulsifying and viscosity behaviours of the proteins. Foaming properties appear to be well correlated with the results obtained for protein hydrophobicity measurements. However, although protein solubility and surface properties including hydrophobicity and surface tension are very important, they cannot fully explain the changes of the emulsifying and foaming properties under different conditions. Molecular factors such as amino acid composition, secondary, tertiary and quaternary structures, net charge and distribution may all have a relationship to the changes in functional properties. Physical properties of the proteins including particle size and shape and processing-induced differences may also contribute to each particular functional characteristic of the proteins. In addition to emulsifying and foaming properties, viscosity and gelation properties of the pea proteins are also influenced by their intrinsic physico-chemical properties as well as various extrinsic factors. For a better understanding of the structure-functionality relationships of food proteins, further basic research is needed in this area.

With good foaming and emulsification ability, field pea proteins were found to be a good substitute for egg in cakes and mayonnaise. Instrumental assessment and sensory evaluation indicated that at levels of up to 50% replacement of egg proteins with pea proteins in cakes and 25% replacement in mayonnaise, the quality of the food products were similar to that produced with 100% egg proteins. In order to produce good functional applications in a wide variety of food systems, plant proteins including field pea proteins should possess multiple functionalities. In order to further evaluate the potential of the pilot scale protein isolates, the impact of chemical modification was also studied and the results will be discussed in the next chapter. Such treatments offer potential to tailor food proteins having very different functional properties for food processing.

CHAPTER 7

Chemical Modification of Field Pea Proteins

CHAPTER 7

Chemical Modification of Field Pea Proteins

During the recent decades, a number of non-conventional proteins have been identified as potential human food ingredients, for example, single-cell proteins, leaf, cereal and legume proteins. However, successful utilisation of these protein materials depends on their nutritive value as well as overall functional and organoleptic properties related to processed food formulations. Many of them, although to varying degrees, fail to meet one or more of these utilisation criteria (Shukla, 1982). In particular, it is recognised that no single protein is likely to meet all the functional properties required in different foods (Kinsella, 1982).

In order to make legume proteins including field peas more attractive as food ingredients, it is desirable to improve their functional characteristics. Modification of functional properties of vegetable proteins can be achieved by physical, chemical, and biological methods (Lee and Lopez, 1984). Physical modification of proteins generally makes use of heat (dry or moist) to bring about partial denaturation of proteins (Sathe *et al.*, 1984). For example, in the current study it has been found that heat treatment results in the improvement of foaming properties of field pea proteins (Chapter 6). Soluble proteolytic enzymes have also been used to modify food proteins. However, there are problems associated with enzymatic modification of proteins. In particular there is risk of excessive hydrolysis which deteriorates functional properties and results in bitter tastes of the hydrolysate (Lee and Lopez, 1984). In addition, the elimination or inactivation of enzymes used to treat proteins is another critical problem once the desired modification is achieved (Phillips and Beuchat, 1981).

Another alternative is the chemical modification of proteins which is more attractive since it is easy to carry out and is relatively inexpensive (Nakai, 1996). Among various chemical approaches used to improve protein functionality, acylation, most commonly

involving either acetylation or succinylation, is one of the most effective means. It has been applied to some plant proteins including wheat (Grant, 1973), oat (Ma, 1984), soybean (Franzen and Kinsella, 1976a), chickpea (Liu and Hung, 1998b), canola (Paulson and Tung, 1988a), cottonseed (Rahma and Rao, 1983), and peanut (Beuchat, 1977). In recent years, phosphorylation has also been found useful in several cases to improve the functional properties of food proteins such as soybean, yeast, casein and lysozyme (Sung *et al.*, 1983; Kim *et al.*, 1988; Huang and Kinsella, 1986a,b; Matheis, 1991). However, data on the phosphorylation of other plant proteins including field pea has not been found. In addition, literature on the functional properties of modified proteins in comparison to the native proteins, particularly under different conditions such as pH, temperature and salt addition, are lacking.

Field pea proteins have been demonstrated to possess many functions desirable for food ingredients in processed foods, including solubility, foaming and emulsification (Chapter 6). However, further enhancement of these functions would make pea proteins even more attractive as a food component. In addition, some other functional properties of the pea proteins are relatively poor and it is desirable that these be improved so the proteins may perform multiple functions in food products. In Chapter 6 it has been demonstrated that pea protein isolate extracted with alkaline solution is less desirable than that extracted with salt solution in terms of physico-chemical properties. However, alkaline extraction is still the most widely used method to isolate plant proteins because of the high recovery rate. Hence in the current study, the pea protein isolate which was extracted with alkaline solution on the pilot scale has been chosen for the evaluation of chemical modifications. This protein isolate has been subjected to acylation with acetic anhydride and succinic anhydride at different levels. Phosphorus oxychloride ($POCl_3$) has also been used to treat the proteins in order to assess the effect of phosphorylation on the functionality of field pea proteins. The purpose of the study was to characterise the modified proteins in terms of the extent of modification, amino acid analysis, SDS-electrophoresis patterns and *in vitro* digestibility. A further objective was to investigate the resultant changes in functionality of the proteins in comparison with the original isolate which had not been chemically modified.

7.1. Preparation and Characterisation of Modified Field Pea Proteins

7.1.1. Extent of Modification

The pilot scale isolate extracted with alkaline solution was modified using varying levels of three modifying reagents. The amount of free amino groups available to react with the reagent trinitrobenzenesulphonic acid (TNBS) in untreated and modified proteins was used to determine the extent of modification, and the results are shown in Fig. 7.1. The extent of modification of the free amino group increased as the ratio of acetic or succinic anhydride or of $POCl_3$ to the protein increased. However, after the ratio of 0.4g chemical/g protein had been exceeded, the degree of succinylation and phosphorylation did not increase significantly. In addition, the rate of modification with acetic anhydride was greater than with succinic anhydride or $POCl_3$. From Fig. 7.1, at the level of 0.2g acetic anhydride/g protein, the extent of acetylation was 88% and did not increase significantly with higher levels of treatment. For the proteins

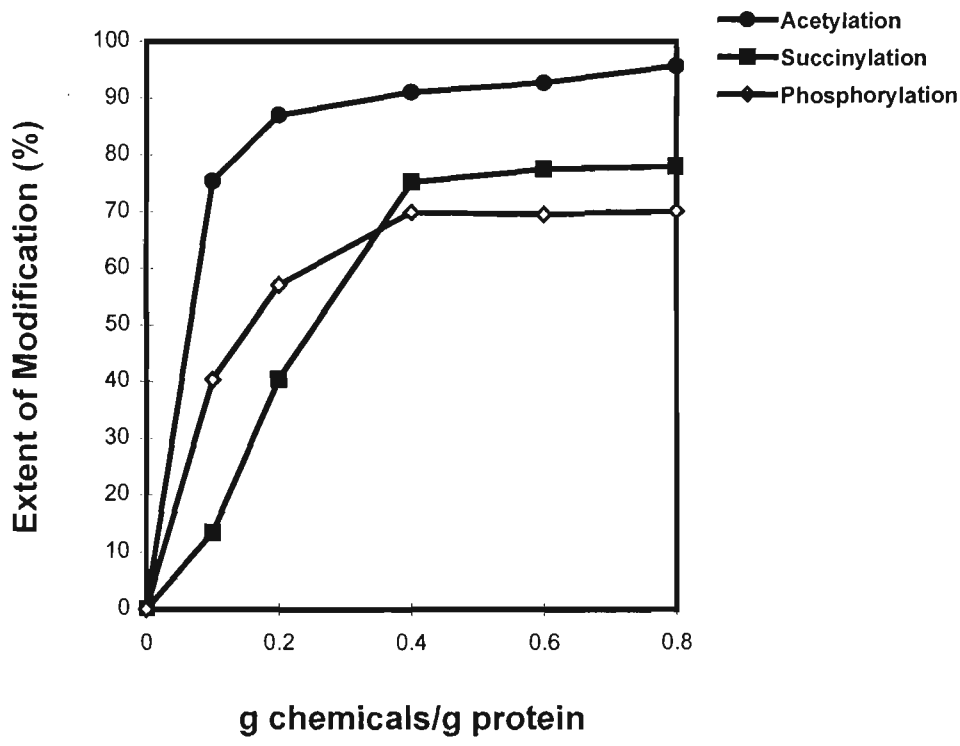


Figure 7.1 Extent of modification as a function of acetic anhydride, succinic anhydride and $POCl_3$ concentration.

modified by succinylation and phosphorylation, at the level of 0.4g chemicals/g protein, about 68% and 73% free amino groups were blocked respectively and the graphs reached a plateau. Acetic anhydride has also been found to be more reactive than succinic anhydride with other protein modifications such as oat (Ma, 1984) and cottonseed (Rahma and Rao, 1983). On the other hand, Shyamasundar and Rajagopal Rao (1982) studied the acylated arachins (peanut proteins) and reported that the rate of succinylation and acetylation are comparable at the highest level of reagent to protein ratio (0.2g/g) used in their study. In addition, with the lower ratios of reagent to protein, succinic anhydride resulted in far higher rates of modification than acetic anhydride. Thus the extent of modification is affected not only by the type of reagent but also by the amino groups of the protein which could be involved in the reactions.

7.1.2. Amino Acid Composition of Modified Field Pea Proteins

Since the extent of modification did not increase significantly after the ratio of chemicals to the proteins reached 0.2g/g for acetylation, and 0.4g/g for succinylation and phosphorylation, the modified protein samples selected for amino acid analysis were the acetylated protein at the level of 0.2g/g protein, and the succinylated and phosphorylated proteins at the level of 0.4g/g protein.

Amino acid profiles of native and modified proteins were analysed and the results are given in Table 7.1. These show that modification did not cause any significant changes in the amino acid patterns among the protein isolates. However, lysine content was slightly reduced due to the chemical modifications. The amino groups including lysine blocked during modification procedures are expected to be liberated during the hydrolysis step prior to amino acid analysis. Thus the amino acid profiles might not normally expected to exhibit significant differences between the modified and unmodified proteins. Kabirullah and Wills (1982) suggested that a decrease in lysine content might be due to degradation of lysine residues during modification and to their loss during the dialysis step following modification. Both acylation and phosphorylation reactions are facilitated in alkaline

Table 7.1 Amino Acid Composition of Native and Modified Field Pea Proteins (g/100g protein)[a]

Amino acid	NPI[b]	PRO-1[c]	PRO-2[d]	PRO-3[e]	FAO/WHO/UNU Reference protein		
Essential					**Infant**	**Child**	**Adult**
Lysine	6.68	6.02	6.13	5.97	6.6	5.8	1.6
Threonine	3.17	3.22	3.26	3.43	4.3	3.4	0.9
Valine	4.56	4.71	4.52	4.66	5.5	3.5	1.3
Methionine	0.93	0.87	0.96	0.91	4.2	2.5	1.7
Cysteine	0.92	0.96	0.89	0.94			
Isoleucine	4.73	4.68	4.86	4.82	4.6	2.8	1.3
Leucine	8.51	8.39	8.67	8.09	9.3	6.6	1.9
Phenylalanine	5.28	5.37	5.31	5.42	7.2	6.3	1.9
Tyrosine	2.32	2.29	2.46	2.38			
Histidine	2.65	2.73	2.66	2.79	2.6	1.9	1.6
Subtotal	39.8	39.2	39.7	39.4			
Nonessential							
Arginine	8.67	8.45	8.40	8.59			
Aspartic acid	10.78	10.81	10.92	10.84			
Serine	5.21	5.06	4.97	4.83			
Glutamic acid	17.07	17.32	17.51	17.41			
Proline	4.93	5.01	4.96	5.26			
Glycine	4.65	4.58	4.73	4.28			
Alanine	3.83	4.02	3.87	3.91			

a: Mean of duplicate determinations. Tryptophan not determined
b: Native protein isolate extracted with alkaline solution on a pilot scale
c: Protein isolate modified with acetic anhydride (0.2g/g protein)
d: Protein isolate modified with succinic anhydride (0.4g/g protein)
e: Protein isolate modified with $POCl_3$ (0.4g/g protein)

in alkaline conditions. During treatment, the pH of the aqueous protein solutions drops with the inclusion of acidic modification reagents and therefore the addition of sodium hydroxide is required to maintain the pH between 7.5- 8.5. The degradation of lysine residues could possibly occur as a result of the long exposure to alkali (2-3 hours). Nevertheless, with regard to essential amino acids, all of the native and modified proteins

exhibited adequate proportions of most amino acids for children and adults (FAO/WHO/UNU, 1985). Sulphur containing amino acids are still the limiting amino acids in all of the pea protein isolates and need to be supplemented from other protein sources when they are incorporated as ingredients in food formulations.

7.1.3. SDS Polyacrylamide Gel Electrophoresis of Modified Field Pea Proteins

A selection of the modified protein preparations at different levels of treatment was examined by polyacrylamide gel electrophoresis in SDS-containing buffers, and the resultant patterns are shown in Fig. 7.2. It can be seen that for the proteins modified with succinic anhydride, the major bands appear to be less mobile with mobility decreasing as the extent of succinylation increases. Furthermore, as the extent of modification increased, some of the bands were partially dissociated into a number of faint, low molecular weight bands following succinylation. Similar results have been observed by Beuchat (1977) with succinylated peanut flour proteins and by Sheen (1991) with

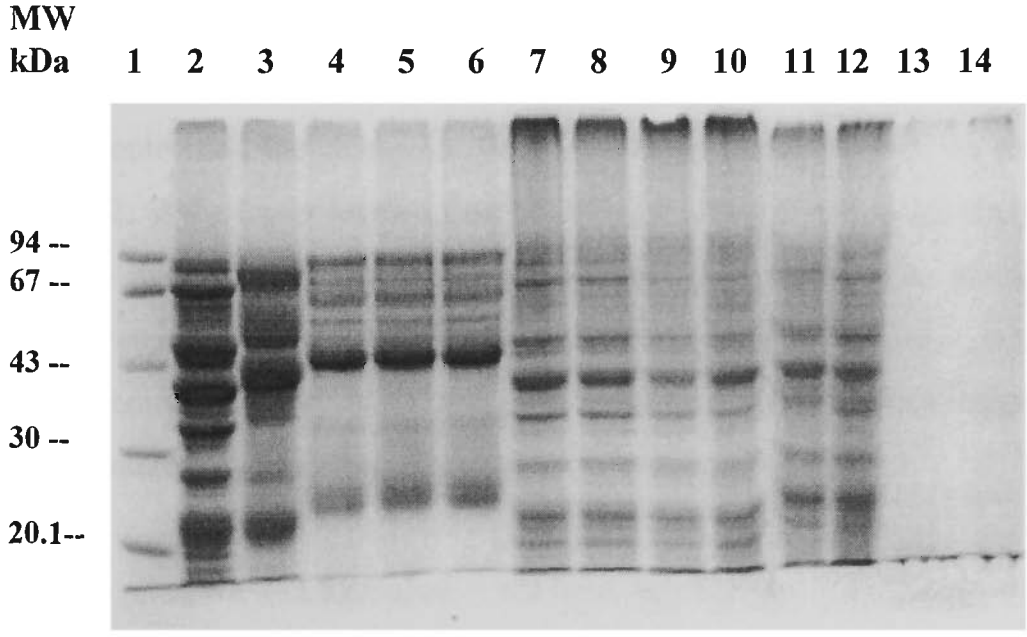


Figure 7.2 SDS-PAGE of native and modified field pea proteins. 1, standard proteins; 2, native protein; 3-6, proteins modified with 0.2g, 0.4g, 0.6g, 0.8g succinic anhydride/g protein, respectively; 7-10, proteins modified with 0.2g, 0.4g, 0.6g, 0.8g $POCl_3$/g protein, respectively; 11-14, proteins modified with 0.2g, 0.4g, 0.6g, 0.8g acetic anhydride/g proteins, respectively.

succinylated tobacco leaf proteins. SDS gel electrophoresis has not been widely applied for the characterisation of acetylated or succinylated plant proteins, since the acyl groups introduced are relatively small and would not be expected to change molecular weights significantly (Schwenke *et al.*, 1991b). However, the increase in overall negative charge by succinylation could cause molecular expansion and this had been confirmed by a decrease in the α-helix content of proteins measured by circular dichroism spectroscopy (Howell, 1996). Limited succinylation decreased the amount of α-helix but increased the amount of β-sheet conformation. Therefore the changes of the SDS-PAGE patterns of succinylated proteins found in the current study may be due to the unfolding of the proteins and the molecular expansion caused by the increase in overall negative charge. However, as is shown in Fig. 7.2, when the level of treatment with succinic anhydride exceeded 0.4g/g protein, there were only minor changes in the gel patterns. This is possibly because the extent of free amino group modification remains similar after this level (Fig. 7.1) and no more negative succinate residues could be introduced into the protein molecules.

In contrast to succinylation, acetylation involves covalent attachment of neutral acetyl groups. Accordingly, whilst modification by acetylation has made some difference to the protein molecules, the change in charge has only a minor impact. As can be seen from Fig. 7.2, at the lower levels of modification (< 0.2g acetic anhydride/g protein), there are no major differences in the SDS-PAGE patterns between the native and acetylated proteins. However, with the increase of the level of treatment, it was found that a large amount of acetylated protein did not enter the gel. This suggests that a network structure may have formed by the cross-linking of proteins.

In relation to phosphorylated proteins, it has been reported that modification with $POCl_3$ leads to protein cross-linking with bovine β-lactoglobulin, yeast, casein and lysozyme (Woo *et al.*, 1982; Huang and Kinsella, 1986a; Matheis *et al.*, 1983). This was indicated by the lower mobility of the protein bands in the polyacrylamide gel electrophoresis or by the absence of significant amounts of phosphorylated proteins in the gel. However, as can be seen from the gel patterns (Fig. 7.2) of the phosphorylated

field pea proteins, there is no evidence of cross-linking except that the bands are faint compared with the control. It was observed that proteins modified with $POCl_3$ had poor solubility in the loading buffer and thus only a small portion of the proteins could enter the gel, consequently the bands were weak even though the loading amount is similar to that for the control.

In some other studies, phosphorylation did not always appear to be accompanied by cross-linking. For example, when soybean isolate and lysozyme were treated with STMP for the phosphorylation of the proteins, there was no change in the electrophoretic behaviour of the proteins (Matheis and Whitaker, 1984). No data has been found on the phosphorylation of plant proteins other than soybean glycinin by $POCl_3$ (Shih, 1993). Although Woo (*et al.*, 1982) suggested that the possible cross-links formed by $POCl_3$ with β-lactoglobulin include phosphate bridges or isopeptide linkages, the nature of the cross-links is not clear (Matheis *et al.*, 1983). More research is needed to further clarify these issues. It seems that the changes in protein structure resulting from phosphorylation may depend upon the type of phosphorylating reagents as well as the origin, the amino acid composition and conformation of the proteins.

7.1.4. *In vitro* Digestibility of Modified Field Pea Proteins

In vitro digestibility of unmodified and modified field pea proteins was measured by the multi-enzyme hydrolysis procedure (Hsu *et al.*, 1977), and the results are presented in Table 7.2. The *in vitro* digestibility was determined from the extent of protein hydrolysis with the multi-enzyme solution, calculated from the pH after a period of 10 min. The graphs of the process of hydrolysis for acetylated, succinylated and phosphorylated pea proteins are shown in Fig. 7.3 (a), (b) and (c), respectively.

The results indicate that with the increase of the level of modification, there is a gradual decrease of multi-enzyme hydrolysis rates. However, this change is relatively small,

Table 7.2 Effect of Acetylation, Succinalytion and Phosphorylation on *in vitro* Digestibility of Field Pea Protein Isolates[a]

	g chemicals/g protein	*In vitro* protein digesbility (%)
Control	0.0	92.61
Acetylation[b]	0.1	93.52
	0.2	92.98
	0.4	92.43
	0.6	92.43
	0.8	91.53
Succinylation[c]	0.1	90.80
	0.2	89.54
	0.4	89.35
	0.6	86.82
	0.8	86.80
Phosphorylation[d]	0.1	92.98
	0.2	92.43
	0.4	90.26
	0.6	88.09
	0.8	87.91

a: Multienzyme system using trypsin, chymotrypsin, peptidase
b: Protein modified by acetic anhydride (0.2g/g protein)
c: Protein modified by succinic anhydride (0.4g/g protein)
d: Protein modified by $POCl_3$ (0.4g/g protein)

especially for the proteins modified with acetic anhydride. As can be seen from Table 7.2, the *in vitro* digestibility of the control is 92.61% and the acetylated proteins show a similar value of digestibility even at the high levels of treatment (up to 0.8g acetic anhydride/g protein). Succinylation and phosphorylation reduce the digestibility of the proteins with the increase of the extent of modification. However, in comparison to the control, the differences are only minor. Some other studies have indicated that acetylation and succinylation resulted in an improvement of the *in vitro* digestibility of the proteins (Johnson and Brekke, 1983; Ma, 1984). The results in the current study demonstrated

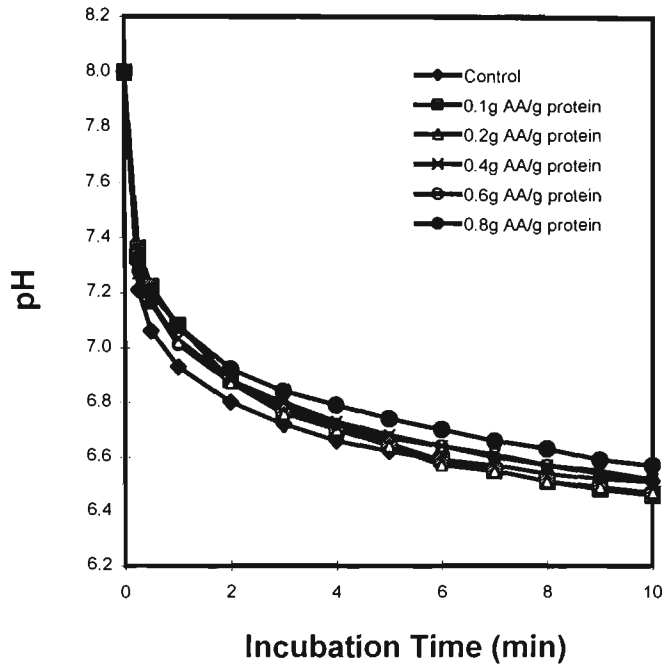


Figure 7.3a The hydrolysis of acetylated field pea proteins by a multi-enzyme system (AA: acetic anhydride).

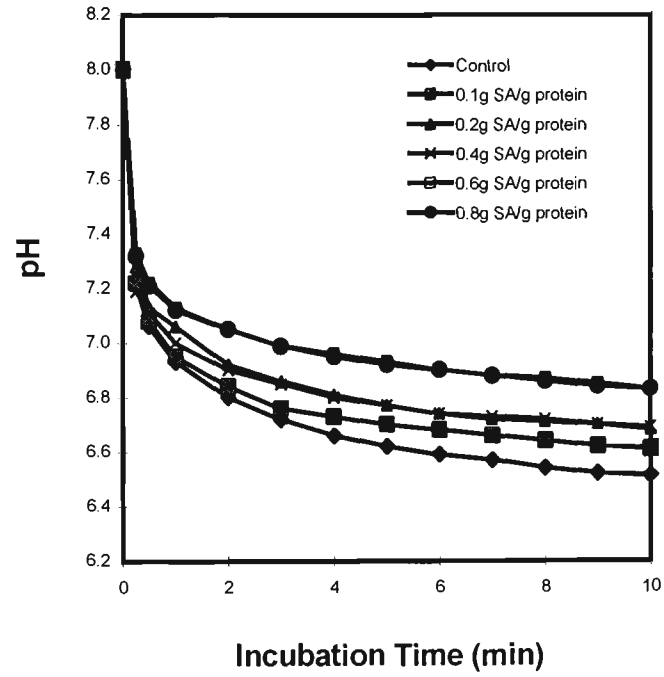


Figure 7.3b The hydrolysis of succinylated field pea proteins by a multi-enzyme system (SA: succinic anhydride).

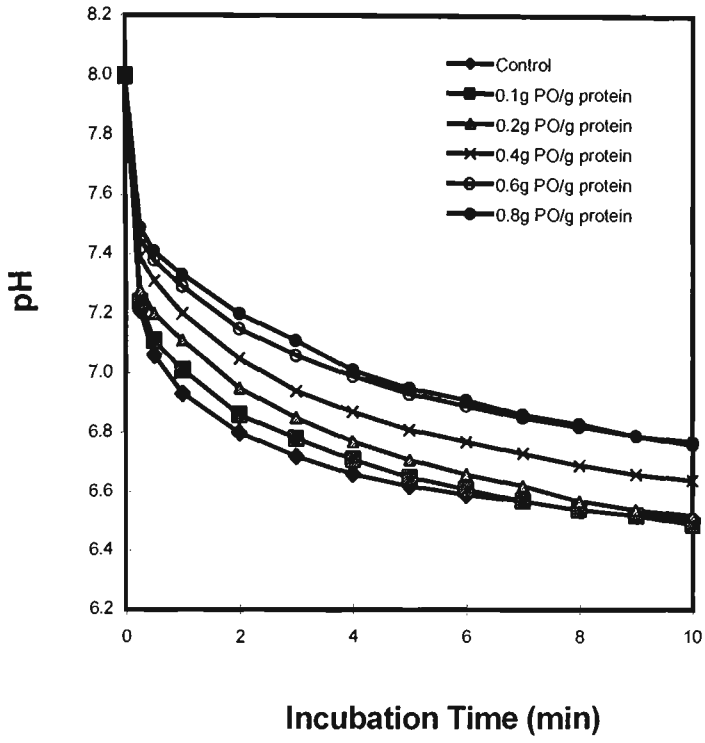


Figure 7.3c The hydrolysis of phosphorylated field pea proteins by a multi-enzyme system (PO: $POCl_3$).

that at low levels of modification (0.1 and 0.2g chemicals/g proteins), the digestibility of the proteins was slightly increased by acetylation and phosphorylation. The increase in digestibility may be due to dissociation and unfolding of the protein molecules, making them more susceptible to enzymic attack (Johnson and Brekke, 1983).

A decrease in *in vitro* digestibility has been reported for a number of succinylated proteins (Wanasundara and Shahidi, 1997; Matoba and Doi, 1979; Siu and Thompson, 1982) and this was confirmed by the current study at the higher levels of modification with field pea proteins. This may be attributed to the reduced availability of lysine which was susceptible to the chemical modification (Wanasundara and Shahidi, 1997). Nevertheless, the results in the current study indicated that overall there is not any significant adverse effect on *in vitro* digestibility of field pea proteins due to acetylation, succinylation and phosphorylation. More detailed information on the digestibility and nutritional quality of field pea proteins which have been subjected to chemical modification would require *in vivo* studies.

7.2. Functional Properties of Modified Field Pea Proteins

7.2.1. Solubility Characteristics of Modified Pea Proteins

Protein solubility curves for native (control) and modified field pea protein isolates are shown in Fig. 7.4a, b, c, d, for varying ratios of chemical to protein. There was a considerable decrease in the solubility of the phosphorylated proteins compared with the unmodified proteins and lower solubility was observed with increasing levels of modification. Similar effects have been reported for other proteins including casein, lysozyme and soybean glycinin which were phosphorylated using $POCl_3$ (Matheis *et al.*, 1983; Shih, 1993). Phosphorylation with $POCl_3$ possibly leads to protein crossing-linking (Matheis and Whitaker, 1984) and this may account for the decreased water solubility. However, the nature of the phosphate linkage in chemically phosphorylated proteins depends on the origin of the protein (Matheis and Whitaker, 1984) and the functional properties of the phosphorylated proteins can be improved or impaired depending on the particular characteristics of the proteins (Schwenke, 1997). As can be seen from the gel patterns (Fig. 7.2) of the phosphorylated field pea proteins, there is no evidence of cross-linking. This suggests that other factors, such as the charge density, surface properties of proteins and denaturation during processing may also affect the solubility of the modified proteins.

For the acetylated field pea protein isolates, there was a slight increase in nitrogen solubility compared with the control at the lower level of modification, but then showed poor solubility after the amount of acetic anhydride reached 0.4g/g protein. Rahma and Rao (1983) also reported that the nitrogen solubility of acetylated cottonseed protein showed a marginal increase up to 73% acetylation and then decreased. Acetylation involves covalent attachment of neutral acetyl groups which means it has slight effect on protein solubility (Howell, 1996). However, at high levels of acetylation, the excess hydrophobic groups introduced could reduce the solubilities (Liu and Hung, 1998b). On the other hand, from Fig. 7.2, it was found that a large amount of acetylated protein did not enter the gel. This indicates that a network

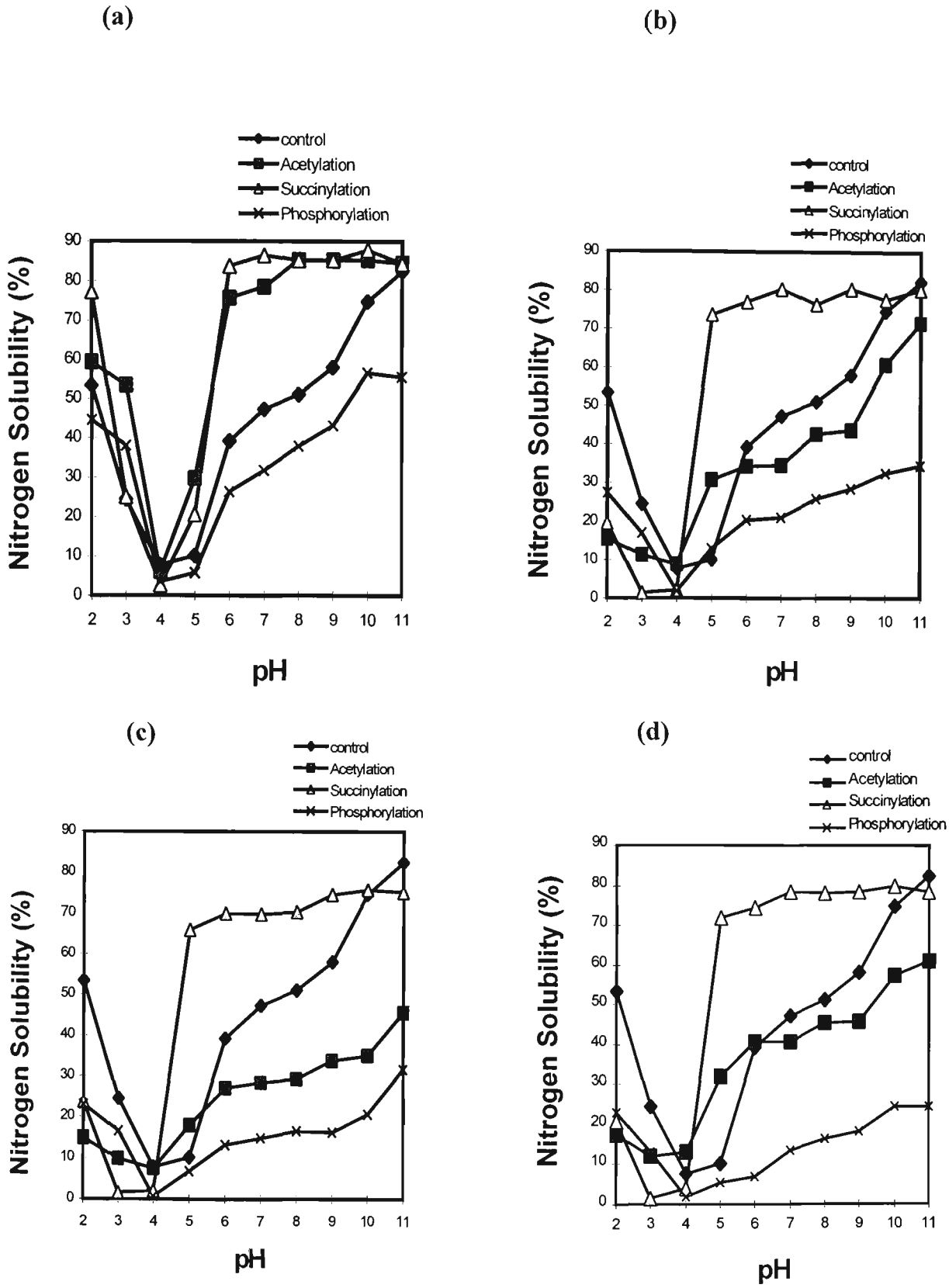


Figure 7.4 Solubility profiles of control and modified field pea proteins at different levels of treatments with acetic anhydride, succinic anhydride and $POCl_3$. (a) 0.2g chemicals/g protein; (b) 0.4g chemicals/g protein; (c) 0.6g chemicals/g protein; (d) 0.8g chemicals/g protein.

structure may have formed by cross-linking of protein. This may be another reason that the acetylated pea proteins displayed the decreased water solubility at higher extents of modification.

Compared with acetylation, succinylation has attracted more widespread interest because it affords more intensive changes in charge (from positive to negative) which is accompanied by major conformational changes and greater solubility (Nakai, 1996). The results in the current study confirmed this point. At pH values in the range of 5-7, solubility increased dramatically as a result of succinylation. However, if the ratio of chemical to protein was increased beyond 0.4g/g, there was little change in solubility patterns. This is possibly because the extent of modification did not change significantly after this level of treatment (Fig. 7.1).

As can be seen from Fig. 7.4, on the acid side of the isoelectric point, nitrogen solubility of the succinylated proteins decreased progressively and the isoelectric point shifted to a more acidic pH. The negatively charged residues introduced by N-acylation account for this shift (Beuchat, 1977). Similar solubility profiles have also been found with succinylated peanut, canola, flaxseed proteins (Beuchat, 1977; Paulson and Tung, 1987; Wanasundara and Shahidi, 1997). Succinylation has been found to increase protein solubility and alter protein conformation by promoting unfolding and increasing dissociation of subunits as well as shifting the isoelectric points to lower values (Paulson and Tung, 1987). The altered conformation of succinylated proteins results from the replacement of short-range attractive forces (ammonium, carboxyl) with short-range repulsive forces (succinate carboxyl, native carboxyl) (Habeeb *et al.*, 1958). The combination of intra- and intermolecular charge repulsion promotes protein unfolding and produces fewer protein-protein and more protein-water interactions, with the result that aqueous solubility is enhanced (Paulson and Tung, 1987). The enhancement of solubility of the succinylated proteins at neutral conditions may prove important for the successful employment of these proteins in food applications, particularly in low-acid food systems.

7.2.2. Water Adsorption Characteristics of Modified Field Pea Proteins

The abilities of the modified field pea proteins to adsorb water was determined using a relative humidity method (Chapter 3, 3.4.2.1). The water adsorption curves for the native and modified field pea protein isolates at different relative humidities are shown in Fig. 7.5. The protein samples used were those modified at levels of 0.2g, 0.4g, 0.4g chemicals/g protein for acetylated, succinylated and phosphorylated proteins, respectively. Both acetylation and succinylation result in increased water adsorption compared to the unmodified proteins. This is partly due to the general unfolding and expansion of protein molecules (Beuchat, 1977). In addition, the increased net negative charge of succinylated proteins would increase the number of potential water-binding sites which especially promote protein-water interaction (Johnson and Brekke, 1983). However, the phosphorylated proteins did not show discernible increases in water adsorption abilities.

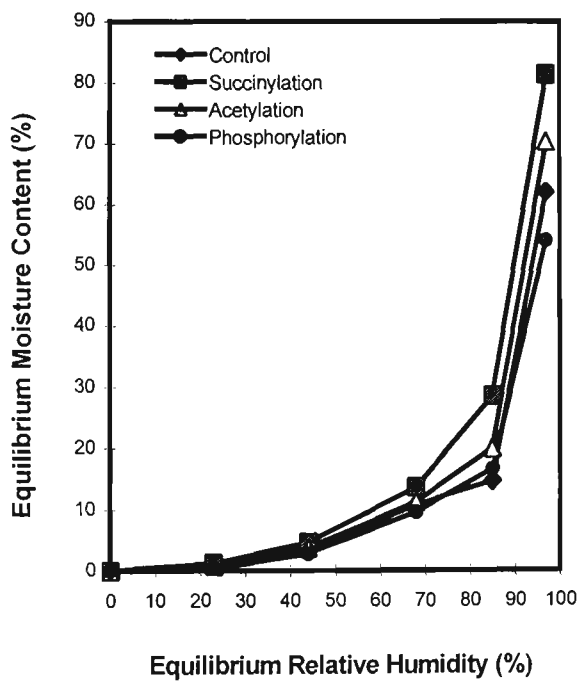


Figure 7.5 Water adsorption isotherms of control and modified field pea proteins. Succinylation, acetylation, phosphorylation: protein modified with succinic anhydride, acetic anhydride and $POCl_3$ at 0.4g, 0.2g, 0.4g/g protein, respectively.

With respect to the water-binding capacity of other acylated proteins, the results are contradictory (Schwenke, 1997). While an improvement in water binding was observed for succinylated or acetylated wheat gluten (Barber and Warthesen, 1982), cottenseed (Rahma and Rao, 1983), peanut (Beuchat, 1977) and chickpea (Liu and Hung, 1998b) proteins, among others, negative effects have been reported for sunflower and oat proteins (Canella *et al.*, 1979; Ma, 1984). Note that in most of these reports, the results were obtained by the excess water-centrifugation method. In the current study, the water absorption capacity of modified field pea proteins was also measured with the excess water-centrifugation method (Chapters, 3.4.2.2) for comparison purposes, and the results are shown in Table 7.3.

Table 7.3 Water Absorption Capacity of Modified Field Pea Protein Isolates[a] (g/g of sample)

Sample treatment level[b] (g/g)	Control	Acetylation	Succinylation	$POCl_3$ modified
0 (Control)	3.41			
0.1		*	5.39	2.63
0.2		*	5.35	3.15
0.4		5.37	*	2.87
0.6		5.81	*	3.03
0.8		6.46	*	2.58

a: Mean of duplicate analysis
b: Concentration values indicate ratios of acetic anhydride, succinic anhydride and $POCl_3$ to proteins (w/w), during modification procedure
*: Protein slurries could not be separated as supernatant and precipitate after centrifugation

For this method, it can be seen that at the higher levels of acetylation and at lower levels of succinylation, the water absorption of the proteins was increased. However, when protein was treated with acetic anhydride at 0.1 and 0.2g/g protein, and with succinic anhydride above 0.4g/g protein, the water absorption could not be satisfactorily measured because of the high solubility of the proteins. Most of the

proteins remained in the solution after centrifugation and could not be recovered as the wetted solids.

Phosphorylation resulted in the decreased water absorption of field pea proteins. It has previously been reported that highly soluble proteins exhibit poor water absorption (Hermansson, 1973). However, the current results show that phosphorylation causes the solubility to decrease with no any evidence of water binding abilities being enhanced. In addition, while succinylation increased the water solubility of the proteins, it also showed a positive effect on the water binding ability as well. Shih (1993) and Huang and Kinsella (1986a) reported that the increased water binding was observed for glycinin and yeast proteins treated with $POCl_3$. They suggested that the increases in water absorption could be partially due to the ionisation of the phosphoryl groups. However, in addition to the charge effect, many other factors such as structural differences between proteins, the isolation procedures used, and the technological treatments applied to proteins prior to modification seem to influence the functional properties including water binding (Schwenke, 1997). Protein-water interactions could also be related to the surface properties of the proteins, which can be changed considerably by chemical modifications. Therefore the decreased water binding ability of field pea proteins modified by phosphorylation may be attributed to a combination of effects and further study is required particularly with respect to the structural changes occurring during the modification process.

7.2.3. Oil Absorption of Modified Field Pea Proteins

The oil absorption capacity of modified field pea proteins is demonstrated in Fig. 7.6. Acetylation has been shown to have minor effects on the oil binding abilities of the proteins. Succinylation and phosphorylation showed an initial increase up to the level of modification of 0.4g chemical/g protein and then showed a decrease. The published effects of chemical modification on oil absorption capacity of food proteins are not uniform. Beuchat (1977) reported that the greatest increases in oil retention of peanut flour were noted for proteins treated with 10 and 40% succinic anhydride. The oil

absorption capacity of acetylated cotton seed protein was unaffected up to 73% lysine modification and then showed a decrease (Rahma and Rao, 1983). On the other hand, acetylation increased oil absorption capacity of chickpea and oat proteins (Liu and Hung, 1998b; Ma, 1984). Fat absorption capacity is partly related to the physical entrapment of oil by the protein matrix (Kinsella, 1976), therefore the origin of the protein may be important. Liu and Hung (1998b) hypothesised that the relatively high oil absorption capacity of chickpea proteins may be attributed to the degree of denaturation and thereby, the exposure of hydrophobic groups during chemical modifications. In general, field pea proteins demonstrated relatively low oil absorption capacity (Chapter 6) and chemical modification did not produce significant enhancement in oil absorption ability of the proteins.

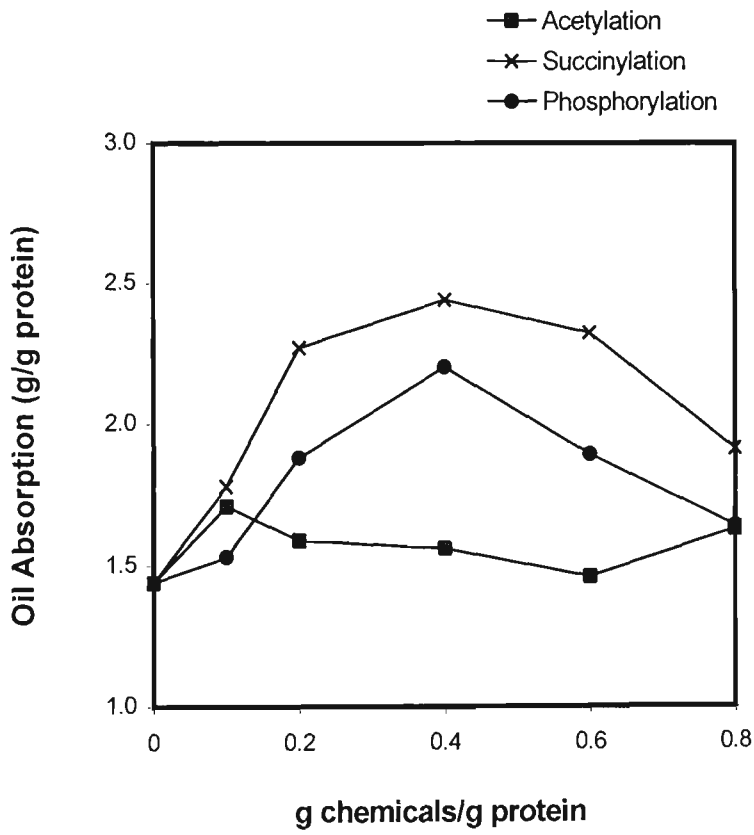


Figure 7.6 Oil absorption capacity of modified field pea proteins as a function of treatment levels of modifying agent (acetic anhydride, succinic anhydride and $POCl_3$).

7.2.4. Emulsifying Capacity and Stability of Modified Field Pea Proteins

7.2.4.1. Effect of the Extent of Chemical Modification on Emulsification

The effect of the ratio of chemicals to protein on the emulsifying capacity and stability of modified pea proteins is shown in Fig. 7.7. Succinylation resulted in the enhancement of emulsifying capacity and stability of the proteins. However, at levels of treatment over 0.4g succinic anhydride/g protein, only slight further increases were observed. Acetylation enhanced the emulsifying capacity and stability at the lower levels of modifications (0.1g and 0.2g acetic anhydride/g protein), and then decreased the emulsifying properties with the further increase of the level of treatment. Phosphorylation substantially reduced the emulsifying properties of field pea proteins. When the results for the solubility curves of the modified pea proteins are considered (Fig. 7.3), the emulsifying capacity and stability of the proteins clearly correspond to their solubility characteristics.

Succinylation has been reported to improve the emulsifying properties of plant proteins including wheat gluten (Barber and Warthesen, 1982), canola (Paulson and Tung, 1988a), soybean and leaf (Franzen and Kinsella, 1976a,b). As a reflection of increased solubility and looser structure of succinylated proteins, diffusion and migration of protein molecules to the oil/water interface and rearrangement within the interfacial film is facilitated (Wanasundara and Shahidi, 1997). As proteins become more soluble, they form layers around the fat globule and promote association with the aqueous phase which encloses the fat globule, thereby rendering the emulsion more stable and resistant to coalescence (Halling, 1981). However, emulsifying properties of proteins do not depend solely on solubility (Chapter 6). The unfolding of the protein structure due to succinylation may expose more hydrophobic groups normally buried within the molecule and could change hydrophobicity and hence the emulsifying properties (Wanasundara and Shahidi, 1997).

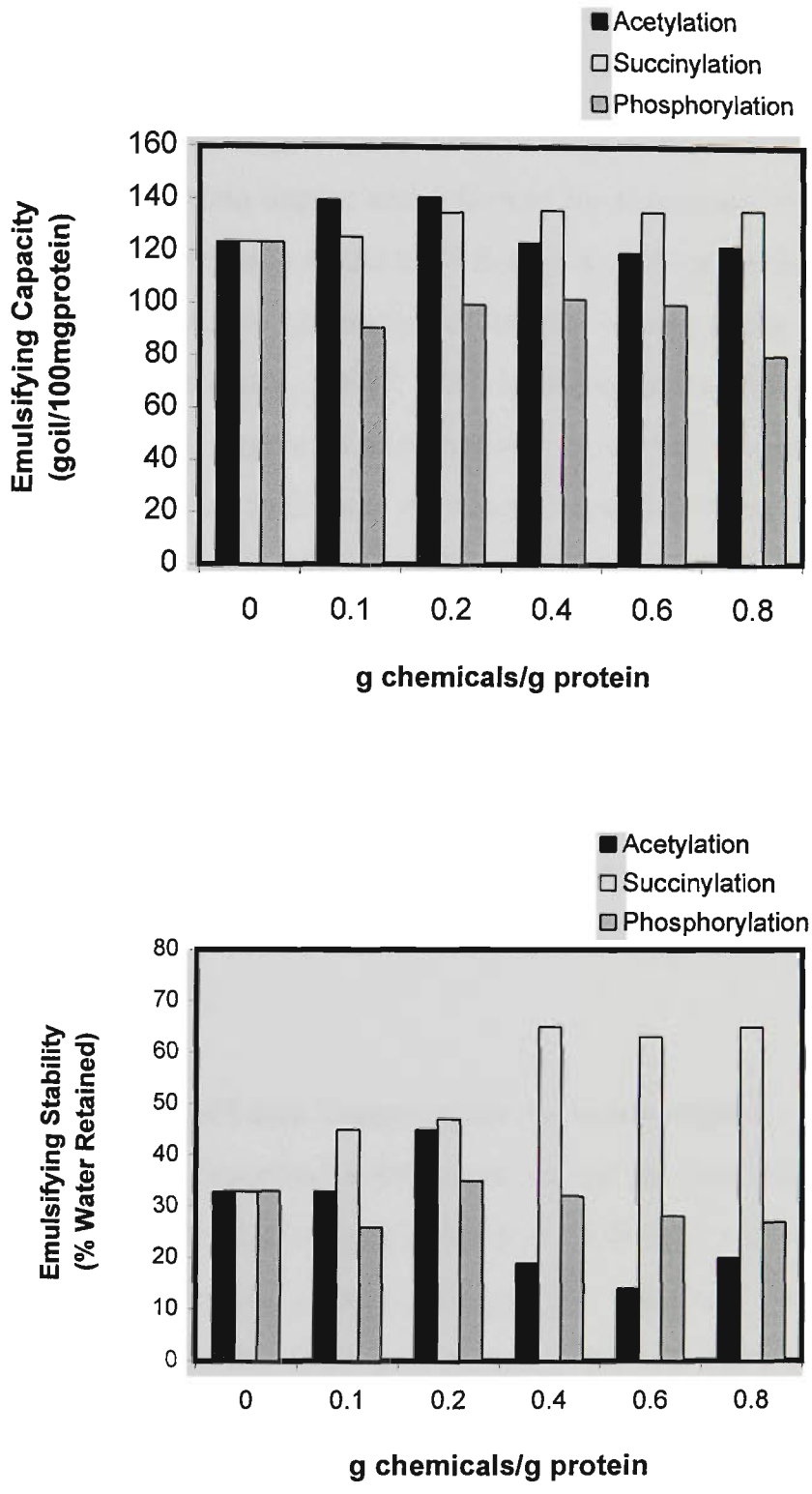


Figure 7.7 Emulsifying capacity and stability of modified field pea proteins as a function of treatment levels for acetic anhydride, succinic anhydride and $POCl_3$.

The published data on the effects of acetylation on the emulsifying properties vary widely for different proteins. An enhancement in the emulsifying properties after acetylation has been reported for several proteins including soybean, wheat gluten, oat and chickpea proteins (Franzen and Kinsella, 1976a; Barber and Warthesen, 1982; Ma, 1984; Liu and Hung, 1998b). On the other hand, acetylation increased emulsification up to a certain degree and followed by a decrease (Rahma and Rao, 1983). Similar results have been found with field pea proteins in the current study. The emulsifying properties are generally not linearly related to the number of acyl residues introduced (Schwenke, 1997). The decreased emulsifying properties of pea proteins at the higher degree of acetylation are possibly attributed to reduced solubility with the greater inclusion of neutral acetyl groups introduced. For proteins phosphorylated with $POCl_3$, both increased and decreased emulsifying properties have previously been reported (Matheis and Whitaker, 1984). The emulsifying activity was decreased in phosphorylated casein (Matheis *et al.*, 1983), but increased in phosphorylated soybean proteins (Hirotsuka *et al.*, 1984). Therefore the emulsifying properties of phosphorylated proteins depends, at least partially, on the source of the proteins. The poor emulsifying properties of the phosphorylated pea proteins was most probably due to their decreased water solubilities.

7.2.4.2. Effects of pH, NaCl and Temperature on Emulsification

The preliminary studies described above have shown that the modification with acetic anhydride at the level of 0.2g/g protein or with succinic anhydride at the level of 0.4g/g protein could greatly increase the solubility and enhance the emulsifying properties of the field pea proteins. Phosphorylation substantially reduced the solubility and emulsifying behaviour of the proteins. Since solubility is often considered to be a prerequisite for the performance of a protein in food applications (Kinsella, 1976), acetylation (0.2g/g protein) and succinylation (0.4g/g protein) have been chosen for studies of other functional properties of the proteins under varying conditions.

The results of the effects of pH on the emulsifying capacities and stabilities of native and modified field pea proteins are presented in Fig. 7.8. Generally, the emulsifying capacity and stability were increased with the increase of pH and both the native and modified proteins showed a similar trend. This pattern is again directly related to the solubility of the proteins. Similar observations were reported for acetylated chickpea proteins (Liu and Hung, 1998b). The addition of salt (NaCl) improved the emulsifying capacities of the control but greatly decreased the emulsifying capacities and stabilities of the modified proteins (Fig. 7.9). This is possibly due to the increased ionic and hydration repulsion forces which impair the mechanical stability of the emulsions. Salting out effects may also become significant for the acetylated and succinylated proteins with the increase of the ionic strength and consequently, impaired emulsifying properties were observed. The slight improvement of the emulsifying capacity of the native proteins with the addition of salt may be associated with the increased solubility and this has been discussed in Chapter 6.

The effects of temperature on the emulsion properties of native and modified proteins are shown in Fig. 7.10. With the increase of temperature, both the emulsifying capacity and stability of the control have dropped. On the other hand, the emulsifying properties of acetylated and succinylated proteins have been enhanced. Positive correlations between surface hydrophobicity and emulsifying properties of proteins have been observed in several cases (Kato and Nakai, 1980; Kato *et al.*, 1981). As can be seen from Table 7.4, the higher the hydrophobicity, and the lower the surface tension with the increase of temperature, the better are the emulsifying capacities for both the acetylated and succinylated protein isolates.

Partial denaturation of the proteins could occur during the process of chemical modification as well as under the heat treatment. This may result in the proteins unfolding and the subsequent exposure of the hydrophobic groups of the protein molecules. Therefore the protein molecules become more amphiphilic and consequently the emulsifying properties are improved. However, this is not true for the control. As discussed in Chapter 6, both surface hydrophobicity and solubility cannot

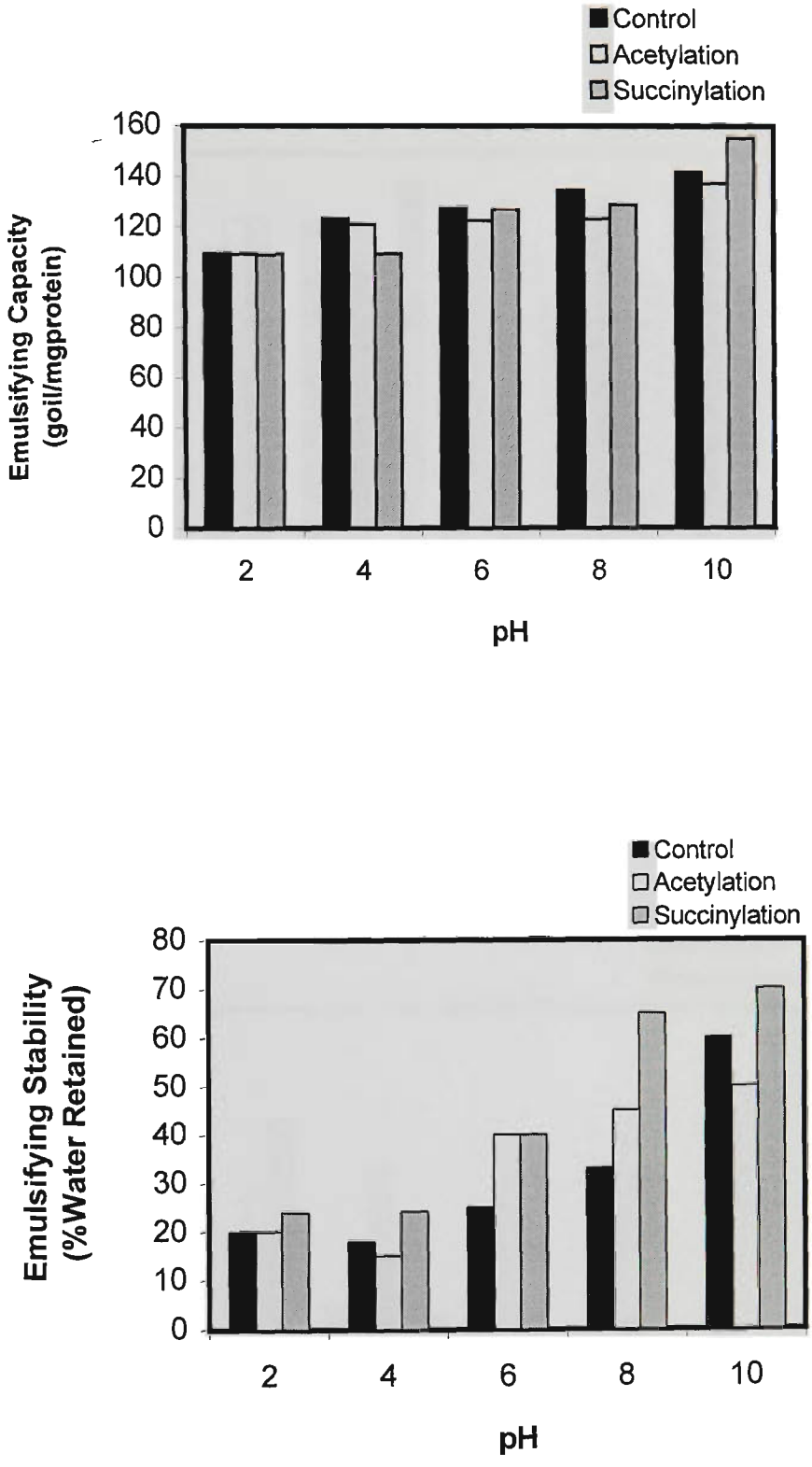


Figure 7.8 Effect of pH on the emulsifying capacity and stability of acetylated and succinylated field pea proteins.

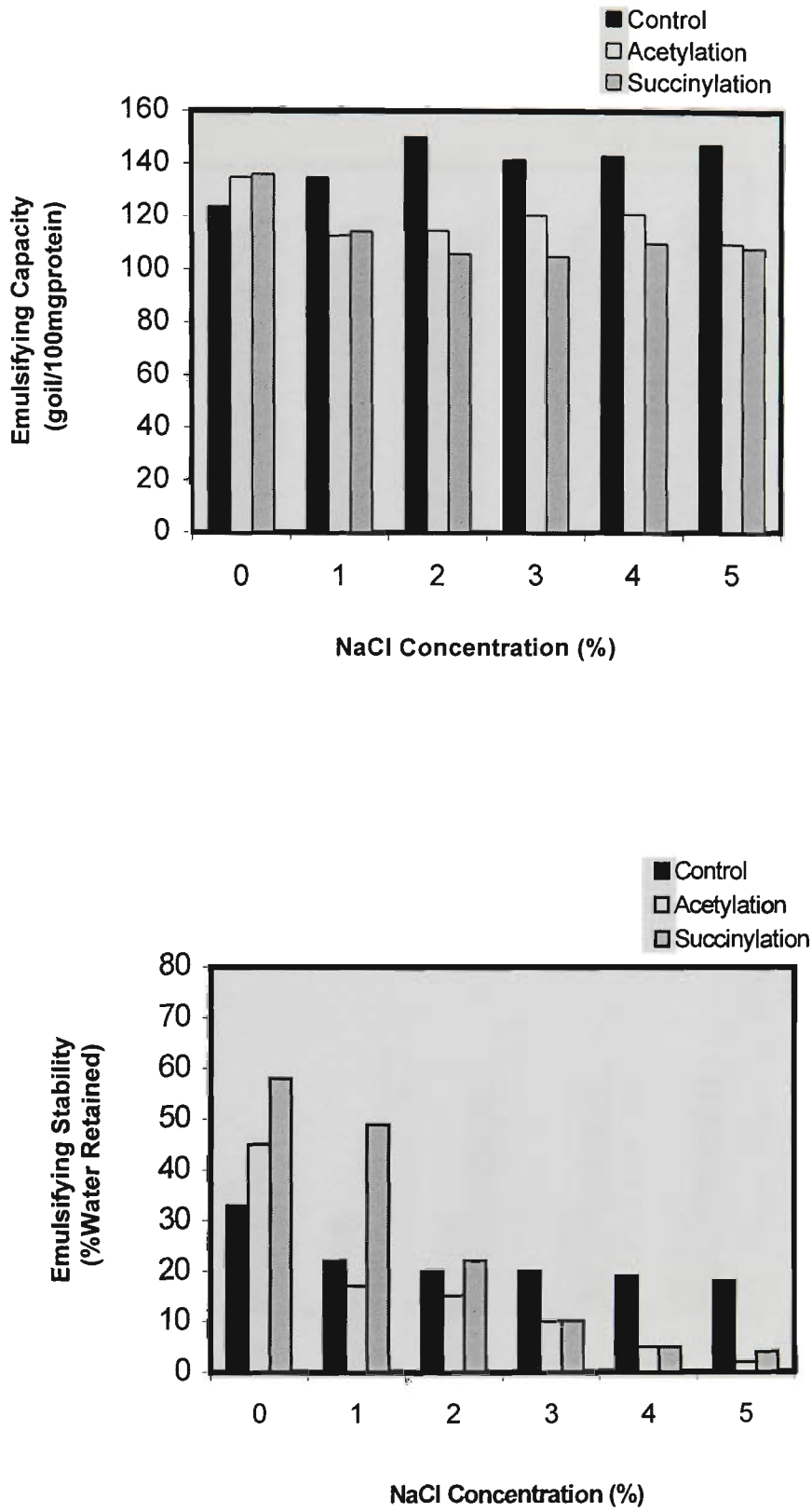


Figure 7.9 Effect of salt (NaCl) on the emulsifying capacity and stability of acetylated and succinylated field pea proteins.

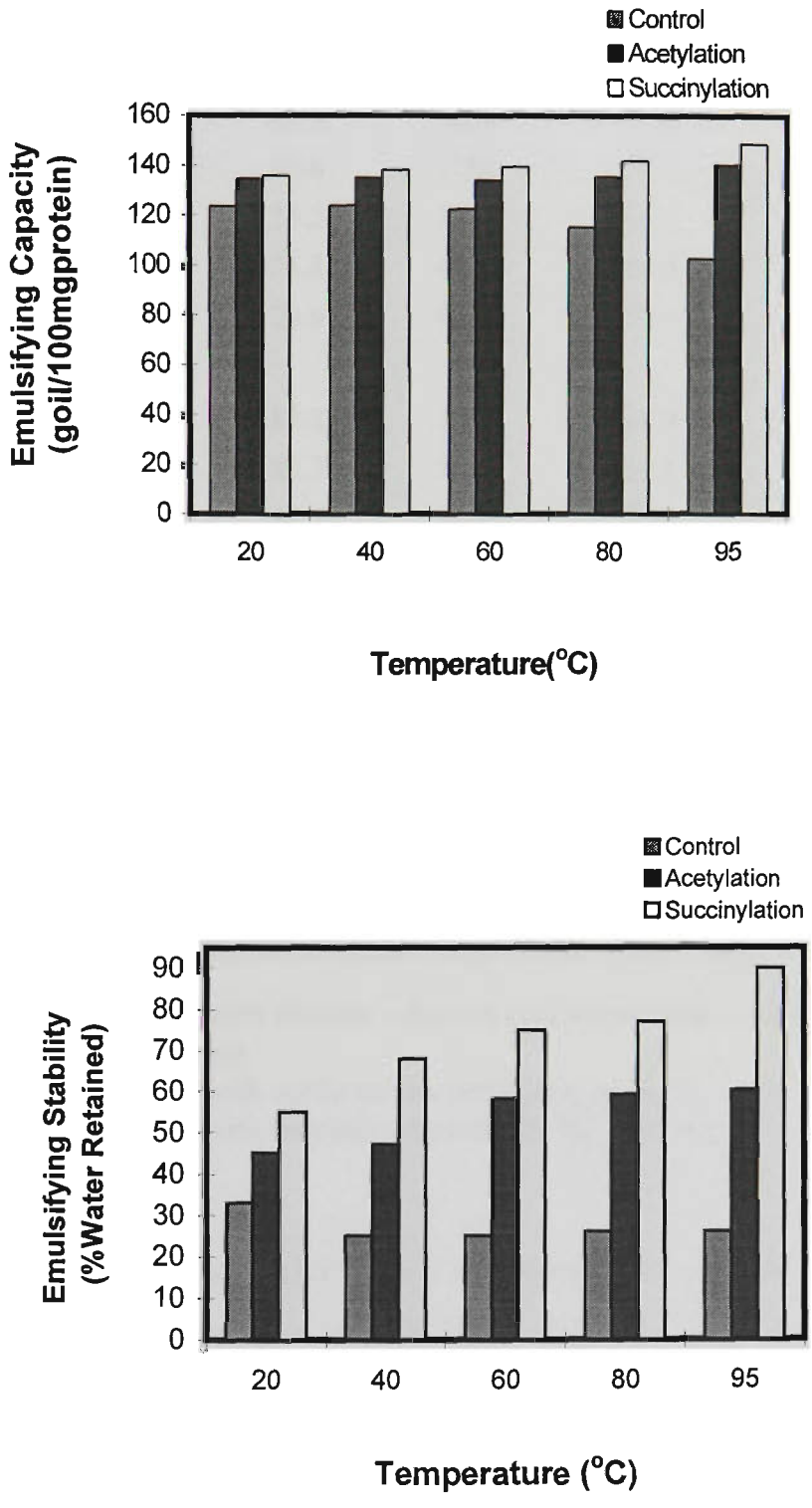


Figure 7.10 Effect of temperature on the emulsifying capacity and stability of acetylated and succinylated field pea proteins.

Table 7.4 Relationships between Protein Solubility, Hydrophobicity, Surface Tension, Emulsifying Capacity and Stability of Control and Modified Field Pea Protein Isolates

Protein	Temperature (°C)	Solubility (%)	Hydrophobicity (S_o)	Surface Tension (Dynes/cm)	Emulsifying capacity (g oil/100mg)	Emulsion stability (% water retained)
Control[a]	20	63.8	1024	57.0	123.7	33
	40	62.6	1160	59.3	123.9	25
	60	73.3	2420	56.1	122.3	25
	80	76.8	4820	54.8	115.3	26
	95	79.9	5062	51.7	102.6	26
Acetylated	20	83.2	1909	57.4	134.6	45
Isolate[b]	40	88.7	2472	59.8	134.9	47
	60	84.2	5400	58.7	134.0	60
	80	85.9	7882	56.4	135.5	60
	95	83.7	8824	50.8	140.4	61
Succinylated	20	81.5	2968	63.1	135.8	56
Isolate[c]	40	87.3	3920	65.1	138.2	68
	60	81.9	4010	64.6	139.5	74
	80	88.7	5948	60.5	141.9	77
	95	86.4	5893	49.2	148.8	90

a: Protein isolate extracted with alkaline solutions (pH 9) and recovered by isoelectric precipitation on the pilot scale
b: Protein isolate modified with acetic anhydride (0.2g/g protein)
c: Protein isolate modified with succinic anhydride (0.4g/g protein)

fully explain the emulsifying behaviour of the proteins. Molecular factors such as the conformational rearrangement at the interface may also have great effects on the emulsifying properties of proteins (Damodaran, 1996).

7.2.5. Foaming Capacity and Stability of Modified Field Pea Proteins

7.2.5.1. Effect of the Level of Chemical Modifications on Foaming Characteristics

The effect of the level of modification on foam capacity of field pea proteins is given in Fig. 7.11. The foam capacity increased slightly due to succinylation and this increase was not clearly related to the extent of modification. Acetylation enhanced the foam capacity of the proteins especially at the high levels of modification. Similar patterns have been found with the foam stability of the acetylated and succinylated proteins (Table 7.5). Thus the foaming properties of the proteins did not exhibit a close relationship with the solubility characteristics. Acetylation and succinylation have been shown to have a positive effect on foam capacity of some other proteins including those from soybean (Franzen and Kinsella, 1976a), cotton seed (Rahma and Rao, 1983) and oat (Ma, 1984). In several cases, foam stability has been found to decline with increasing degree of modification, especially for succinylation (Ma, 1984; Johnson and Brekke, 1983).

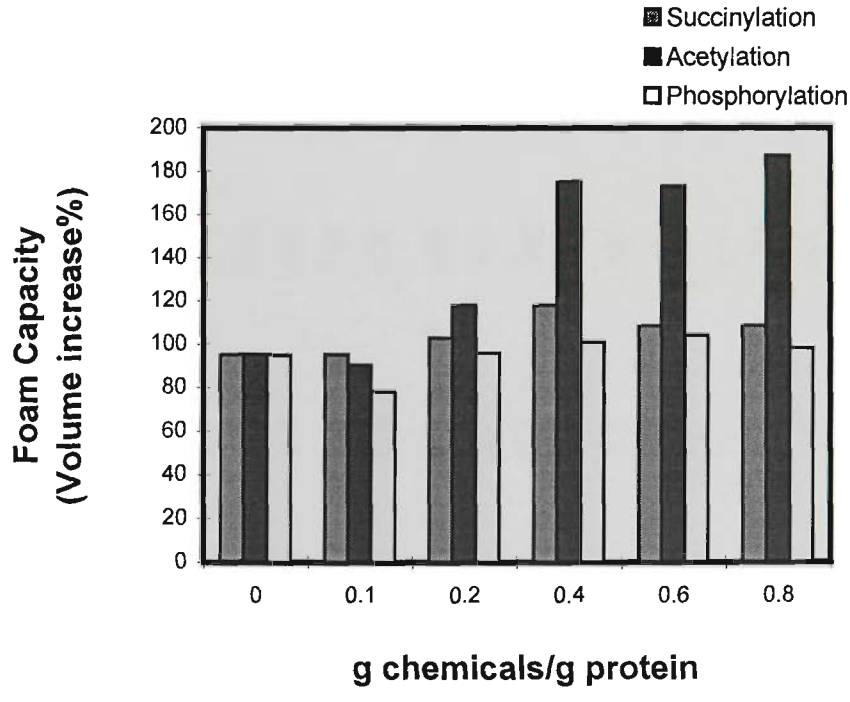


Figure 7.11 Foaming capacity of modified field pea proteins as a function of treatment levels for acetic anhydride, succinic anhydride and $POCl_3$.

Table 7.5 Foam Capacity and Stability of Modified and Unmodified Field Pea Protein Isolates

Sample	Level of treatment (g/g)	Volume before whipping (mL)	Volume after whipping (mL)	Volume increase (%)	Volume (mL) at room temperature (21°C) after time (hr)				
					0.25	0.5	1	2	3
Control		200	390	95	370	360	325	270	245
Succinylation[b]	0.1	200	390	95	380	370	360	315	280
	0.2	200	430	115	420	415	405	300	245
	0.4	200	435	118	432	430	420	320	260
	0.6	200	440	120	428	425	415	315	255
	0.8	200	435	118	425	420	415	320	265
Acetylation[c]	0.1	200	380	90	378	377	370	360	345
	0.2	200	435	118	430	425	420	410	395
	0.4	200	550	175	545	545	540	535	515
	0.6	200	545	173	540	540	535	533	520
	0.8	200	575	187	570	570	565	540	525
Phosphorylation[d]	0.1	200	365	78	350	310	270	250	260
	0.2	200	390	96	365	335	300	285	265
	0.4	200	400	102	370	335	295	285	270
	0.6	200	405	104	375	345	305	290	275
	0.8	200	395	98	360	315	280	260	255

a: The ratio of chemicals to protein isolate (g chemicals/g protein)
b: Protein isolate modified by succinic anhydride
c: Protein isolate modified by acetic anhydride
d: Protein isolate modified by $POCl_3$

The increase in net charge density as a result of acylation possibly prevents optimum protein-protein interactions, which are required for development of a continuous film around air bubbles (Schwenke, 1997). On the other hand, stable foams were obtained with some proteins although the extent of modification was high (Franzen and Kinsella, 1976a; Kabirullah and Wills, 1982; Canella *et al.*,1979). Clearly, other intrinsic (structural) and extrinsic factors may be important and override the charge repulsion in these cases (Schwenke, 1997). The enhanced foaming properties of the pea proteins at the higher levels of acetylation may be due to certain structural changes such as cross-linking which could promote the formation of a continuous viscoelastic film at the interface.

From Table 7.5 and Fig. 7.11, it can be seen that phosphorylation did not significantly alter the foaming properties of field pea proteins. Protein phosphorylated with $POCl_3$ has been reported to show increased foam capacity and stability for soybean glycinin (Shih, 1993). STMP-treated soy protein also showed increased foaming property (Sung *et al.*, 1983). It was suggested that the covalent attachment of anionic phosphate groups to polypeptide chains and the resultant increase in net electro-negativity altered the physicochemical character of the proteins. However, Matheis and Whitaker (1984) failed to detect any covalently bound phosphate by means of polyacrylamide gel electrophoresis when soybean and lysozyme were treated with STMP. In the current study, gel patterns of the phosphorylated field pea proteins also showed no evidence of cross-linking (Fig. 7.2). Therefore the foaming properties of phosphorylated proteins may be related to their solubility and charge effects as well as the specific type of protein rather than molecular changes.

7.2.5.2. Effects of pH, NaCl Concentration and Temperature on Foaming Characteristics

The effects of pH on the foaming capacity and stability of native and acylated pea proteins are shown in Fig. 7.12 and Table 7.6. In general, the foam capacity and stability was improved in the acid pH range for both native and acylated proteins.

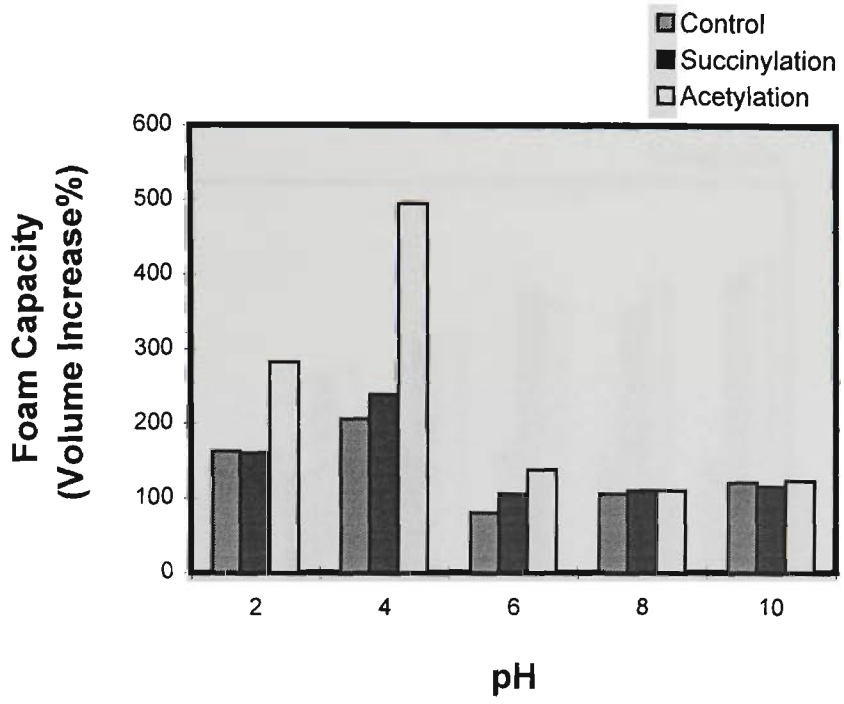


Figure 7.12 Effect of pH on the foaming capacity of acetylated and succinylated field pea proteins.

In particular, the foam capacity of acetylated protein was significantly increased at the isoelectric pH range. This is possibly due to the reduced electrostatic repulsion which allows greater protein adsorption at the interface (Mita *et al.*, 1977; Graham and Phillips, 1980). Since pH did not appear to influence foaming characteristics above the isoelectric points and similar effects were seen for the control and modified proteins, the increase in net charge density as a result of acylation did not affect the foaming properties of the proteins.

Addition of salt also enhanced the foaming capacity of native and modified pea proteins (Fig. 7.13), and the same is true for the foam stability of the proteins (Table 7.7). However, the maximum improvement was observed at a salt concentration of 0.5% (w/v) for the control, whereas the increases for acetylated and succinylated proteins occurred at higher salt concentrations. The excess electrostatic repulsion due to the increased ionic strength may inhibit the foam formation and stability of the native proteins. The modified proteins are not

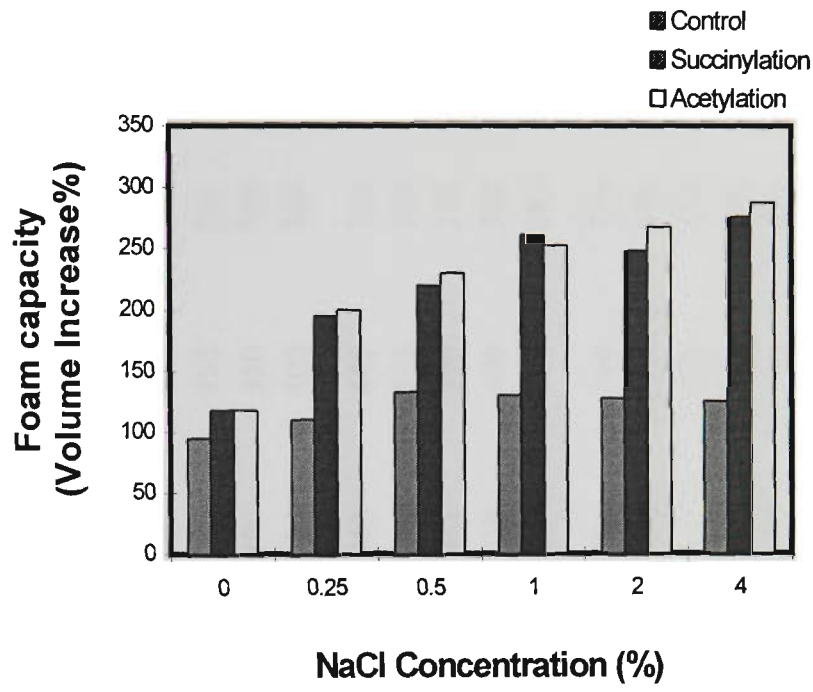


Figure 7.13 Effect of salt (NaCl) on the foaming capacity of acetylated and succinylated field pea proteins.

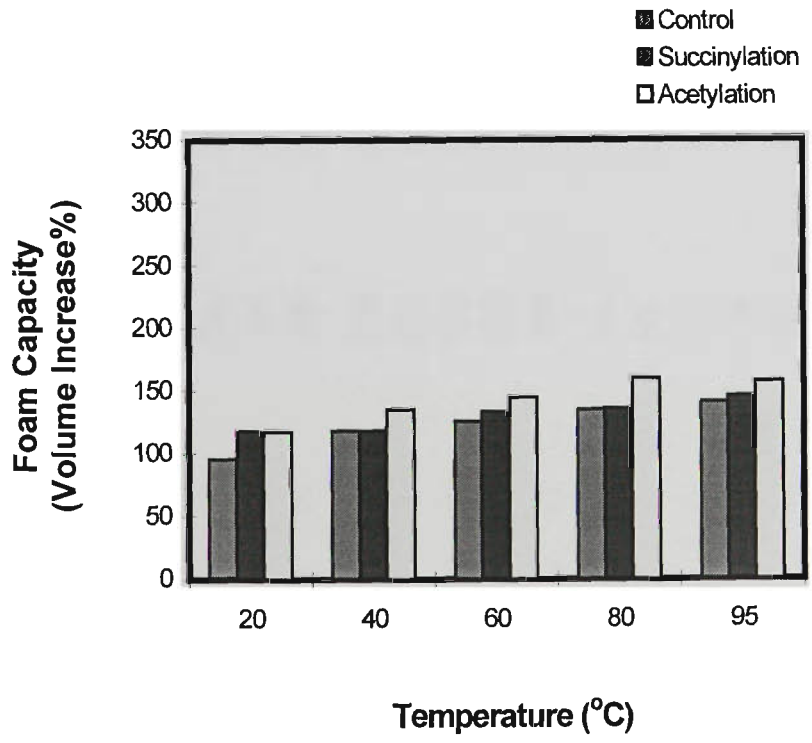


Figure 7.14 Effect of temperature on the foaming capacity of acetylated and succinylated field pea proteins.

Figure 7.6 The Effect of pH on the Foam Capacity and Stability of Modified and Unmodified Field Pea Protein Isolates

Sample	pH	Volume before whipping (mL)	Volume after whipping (mL)	Volume increase (%)	Volume (mL) at room temperature (21°C) after time (hr)				
					0.25	0.5	1	2	3
Control[a]	2	200	525	163	515	510	465	330	220
	4	200	610	205	610	605	605	595	580
	6	200	360	80	360	360	355	355	335
	8	200	410	105	405	400	350	300	260
	10	200	440	120	435	425	400	320	290
Succinylation[b]	2	200	520	160	520	460	445	370	320
	4	200	675	237	665	635	550	300	250
	6	200	410	105	405	405	390	320	280
	8	200	420	110	415	410	400	360	255
	10	200	430	115	430	415	355	265	230
Acetylation[c]	2	200	765	283	765	765	760	755	735
	4	200	1200	500	1200	1200	1100	830	290
	6	200	475	238	475	475	475	470	475
	8	200	420	110	415	410	405	400	390
	10	200	445	123	435	425	420	420	410

a: Protein extracted with alkaline solution (pH9) and then recovered by isoelectric precipitation on pilot scale
b: Control protein modified by succinic anhydride (0.4g/g protein)
c: Control protein modified by acetic anhydride (0.2g/g protein)

Table 7.7 The Effect of NaCl on the Foam Capacity and Stability of Modified and Unmodified Field Pea Protein Isolates

Sample	NaCl (%)	Volume before whipping (mL)	Volume after whipping (mL)	Volume increase (%)	Volume (mL) at room temperature (21°C) after time (hr)				
					0.25	0.5	1	2	3
Control[a]	0	200	390	95	370	360	325	270	245
	0.25	200	420	110	420	420	415	370	350
	0.5	200	465	133	460	460	445	420	405
	1	200	460	130	455	455	440	410	390
	2	200	455	128	445	435	415	375	305
	4	200	450	125	448	445	425	330	260
Succinylation[b]	0	200	435	118	432	430	420	330	270
	0.25	200	595	197	585	580	565	490	415
	0.5	200	640	220	635	635	610	550	410
	1	200	720	260	720	715	705	630	500
	2	200	695	248	690	685	670	600	270
	4	200	750	275	750	750	735	330	225
Acetylation[c]	0	200	435	118	430	425	420	410	395
	0.25	200	600	200	600	590	590	580	570
	0.5	200	660	230	660	655	655	650	645
	1	200	705	253	705	700	700	695	690
	2	200	735	268	730	730	725	710	695
	4	200	755	278	750	750	750	745	725

a: Protein extracted with alkaline solution (pH9) and then recovered by isoelectric precipitation on pilot scale
b: Control protein modified by succinic anhydride (0.4g/g protein)
c: Control protein modified by acetic anhydride (0.2g/g protein)

Table 7.8 The Effect of Temperature on the Foam Capacity and Stability of Modified and Unmodified Field Pea Protein Isolates

Sample	Temperature (°C)	Volume before whipping (mL)	Volume after whipping (mL)	Volume increase (%)	Volume (mL) at room temperature (21°C) after time (hr)				
					0.25	0.5	1	2	3
Control[a]	20	200	390	95	370	360	325	270	245
	40	200	435	118	435	410	335	270	235
	60	200	450	125	445	420	330	245	225
	80	200	468	134	455	410	315	225	210
	95	200	480	140	470	350	305	225	210
Succinylation[b]	20	200	435	118	432	430	420	330	270
	40	200	435	118	435	435	425	370	267
	60	200	465	133	462	462	445	340	285
	80	200	470	135	465	465	415	320	265
	95	200	490	145	490	480	455	380	290
Acetylation[c]	20	200	435	118	430	425	420	410	395
	40	200	470	135	465	460	455	445	435
	60	200	490	145	488	485	482	470	450
	80	200	520	160	515	512	485	475	445
	95	200	515	158	515	505	495	435	410

a: Protein extracted with alkaline solution (pH9) and then recovered by isoelectric precipitation on pilot scale
b: Control protein modified by succinic anhydride (0.4g/g protein)
c: Control protein modified by acetic anhydride (0.2g/g protein)

sensitive to this external charge effect due to the internal changes in charge produced by the attached acetyl or succinate groups. The differences in the molecular flexibility and molecular rigidity of the proteins resulting from the chemical modifications are probably important in explaining the foaming properties of the proteins. Meanwhile, as can be seen from Table 7.7, at the increased levels of salt addition (2-4%, w/v), succinylated proteins produced less stable foams compared with the acetylated ones. This may be partially due to succinylation affording more intensive changes in charge (from positive to negative). This effect becomes more significant when combined with the higher salt concentrations which prevent optimum protein-protein interactions required for formation of a continuous film around air bubbles.

The effects of temperature on the foaming capacity and stability are demonstrated in Fig. 7.14 and Table 7.8. Heat denaturation of native and acylated pea proteins causes the foam properties to improve. This property is related to the protein surface hydrophobicity (So), since the higher the temperature, the lower was the surface tension and the higher was the protein hydrophobicity observed (Table 7.4). It was also found that at the higher temperatures, the So value for acetylated proteins is increased much more than that for control and succinylated proteins. This may partially explain the significant improvement of the foaming properties due to acetylation rather than succinylation, especially with the increase of temperature.

In summary, as in the case of emulsifying properties, foaming properties of field pea proteins are greatly influenced by variations in environmental conditions including pH, temperature and salt addition. These factors, on the other hand, sometimes produced differing effects for native and modified proteins, consistent with the charge and structural changes induced by chemical modification. Furthermore, while foaming properties of the proteins are correlated with the surface hydrophobicity, many other intrinsic and extrinsic factors such as solubility

and molecular properties may also be important. Some of these factors have already been discussed in some detail in Chapter 6.

7.2.6. Viscosity Characteristics of Modified Field Pea Proteins

7.2.6.1. Effect of the Level of Chemical Modifications on Viscosity

The effect of the ratio of chemical to protein on apparent viscosity of acetylated and succinylated pea proteins is shown in Fig. 7.15 and Table 7.9.

For the phosphorylated proteins, viscosity could not be measured satisfactorily. Since phosphorylation resulted in the extremely low solubility of the products, the protein samples separated into two layers of water and precipitate in the ultra-low adaptors used with the Brookfield viscometer for this study. When the spindle of the viscometer was running, a portion of the solids gradually moved up along the wall of the adaptor and spindle and it was not possible to obtain the stable readings. The viscosities increased with time significantly and within 30 min, the readings were off-scale (> 2000 cP). As the characteristics of the fluid system were not uniform, the study of the flow properties via viscosity is not appropriate. Huang and Kinsella (1986a) reported that the viscosity of yeast proteins was greatly increased by phosphorylation and this improvement could be attributed to the presence of the high molecular weight protein aggregates and the highly hydrated protein network formed during phosphorylation. In their studies, the greater solubility of phosphorylated protein compared to the yeast nucleo-protein has also been found and this may be due to the added charged phosphoryl groups and the loosened structure of the derivatised proteins. However, to confirm this point, further study is needed to clearly elucidate the molecular and structural changes occurring as a result of chemical modification. The origin of the proteins may also be important in influencing the interaction of chemical modification reagent and protein molecules.

As can be seen from Fig. 7.15 and Table 7.9, acetylation and succinylation greatly improved the viscosities of field pea proteins, especially at a higher concentration of the proteins. However, the extent of succinylation showed only minor effects on the

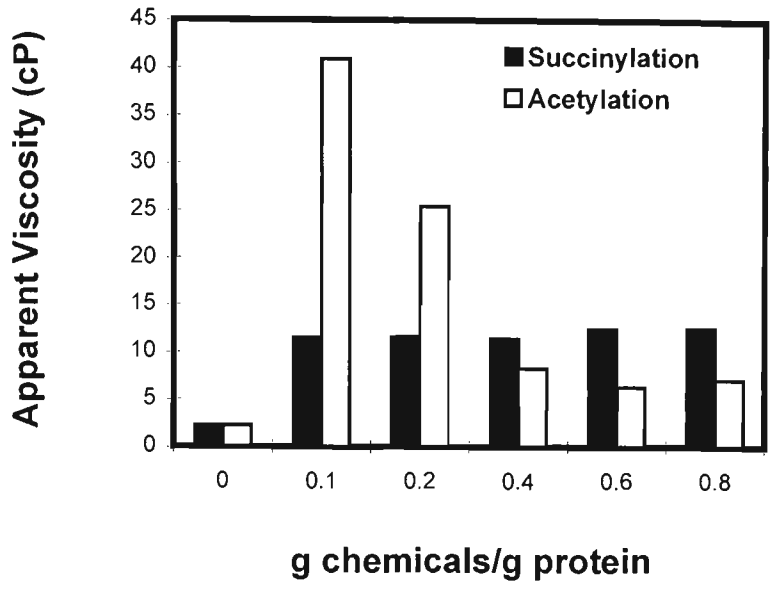


Figure 7.15 Apparent viscosity of modified field pea proteins as a function of acetic anhydride and succinic anhydride treatment level (at pH 7, 20°C, 4% dispersion).

Table 7.9 Effect of Succinylation and Acetylation on the Apparent Viscosity of Field Pea Protein Isolates[a]

	g Chemicals/g Protein	Apparent Viscosity (cP)	
		4 % dispersion	8 % dispersion
Control	0	2.25	7.06
Succinylation	0.1	11.5	57.6
	0.2	11.6	58.8
	0.4	11.4	57.7
	0.6	12.5	64.2
	0.8	12.6	64.6
Acetylation	0.1	40.9	863
	0.2	25.4	271
	0.4	8.28	187
	0.6	6.31	162
	0.8	7.06	178

a: Modification based on the protein isolate extracted by alkaline solution on pilot scale

viscosities of the proteins. The greatest improvement in viscosity of acetylated proteins were at the level of 0.1 and 0.2g acetic anhydride/g proteins and then the viscosities decreased gradually. This pattern appeared to be related to the solubilities of the acetylated pea proteins. Paulson and Tung (1988b) have also found that the solubility of succinylated canola proteins had a great influence on the viscosity of the solutions.

Acetylation and succinylation have been reported to increase the apparent viscosities of several other proteins including those from field bean (Schmidt and Schmandke, 1987), canola (Paulson and Tung, 1988b), peanut (Beuchat, 1977) and chickpea (Liu, 1996). In addition to solubility, Paulson and Tung (1988b) suggested that many other factors including protein hydrophobicity, the size, shape and number of aggregates, hydrodynamic volume, protein-solvent, and protein-protein interactions all contribute to the flow properties of protein dispersions. The hydrodynamic volume is dependent upon molecular size and degree of hydration of the molecule in solution (Frisch and Simha, 1956). The molecular expansion resulting from succinylation and the increased water hydration ability via the introduction of bulky succinyl and acetyl groups could increase the hydrodynamic volume, and consequently, contribute to the increase in viscosity. Liu (1996) hypothesised that acetylation may result in small protein aggregates and stronger protein-protein interactions by the acetyl residues bound to protein molecules, which in turn, produces a higher viscosity. In the current study, it has been found that acetylation resulted in a more profound rheopectic phenomenon than succinylation did. Rheopectic behaviour is one form of non-Newtonian viscosity characteristics in which viscosity increases with time. Small protein aggregates may have been formed by acetylation and therefore increased the apparent viscosity of the protein dispersions. This may partially explain the much higher viscosity of the acetylated proteins especially at the lower levels of modification compared with native and succinylated proteins. With the increase of the extent of acetylation, the solubility effect may have become more significant and consequently the viscosity was decreased.

7.2.4.2. Effects of NaCl Concentration and Temperature on Viscosity of Modified Field Pea Proteins

As in the case of native proteins, the viscosities of acetylated and succinylated pea proteins are also greatly influenced by the addition of salt (NaCl) and the changes of temperature. The results are shown in Fig. 7.16 and Fig. 7.17, respectively. For this study, viscosities were measured at a only one pH value of 7. The effect of varying pH was not included due to limitations of the instrument. In preliminary determinations it was found that generally the viscosity of the modified proteins was pH dependent. However, for the acetylated and succinylated proteins, there was so much variation in the viscosity values at different pH values that it was not possible to obtain stable readings for all samples at the same shear rate. Furthermore, for some samples, especially at higher pH, readings were beyond the range of measurement (2000 cP) at all eight shear rates defined by the instrument.

From Fig. 7.16, it can be seen that with the increase of salt concentration, the viscosities of succinylated and acetylated protein dispersions were significantly decreased. There are some differences between the viscosity patterns of native and modified proteins. In Chapter 6, it has been demonstrated that the viscosity of alkaline extracted protein (native protein) decreased at a low salt concentration and then gradually increased with further salt addition (Fig. 6.15). This reversal may have been due to a particular salt concentration being critical for protein solubilisation (Megen-van, 1974). For the acetylated and succinylated proteins, the salting-out effect may become significant with the combined effects of the increase in salt addition as well as the increase in net charge density as a result of the introduction of succinyl and acetyl groups. Consequently, the viscosity of the modified protein dispersions was apparently reduced. Liu (1996) also indicated that acetylation and succinylation resulted in the decrease in viscosity of chickpea proteins. The possible reason may be due to reduced water-protein interactions. Without salt addition, all of the water in the dispersion is available for protein association and a strong network can be formed, resulting in higher viscosity values. When salt is added, more water in the system tends to associate with sodium chloride rather than with protein molecules and therefore, the viscosity of the dispersions is reduced (Liu, 1996).

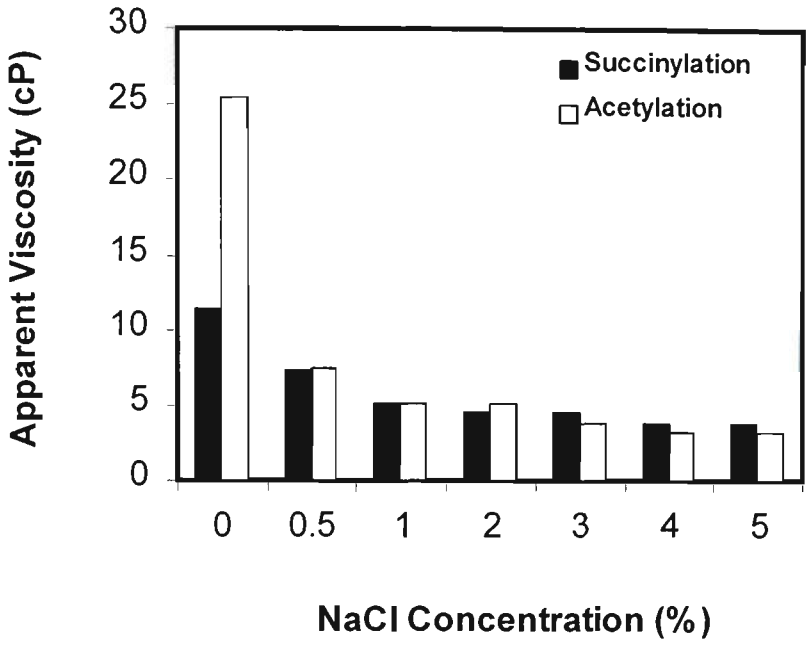


Figure 7.16 Effect of salt (NaCl) on the apparent viscosity of succinylated and acetylated field pea proteins (at pH7, 20°C, 4% dispersion).

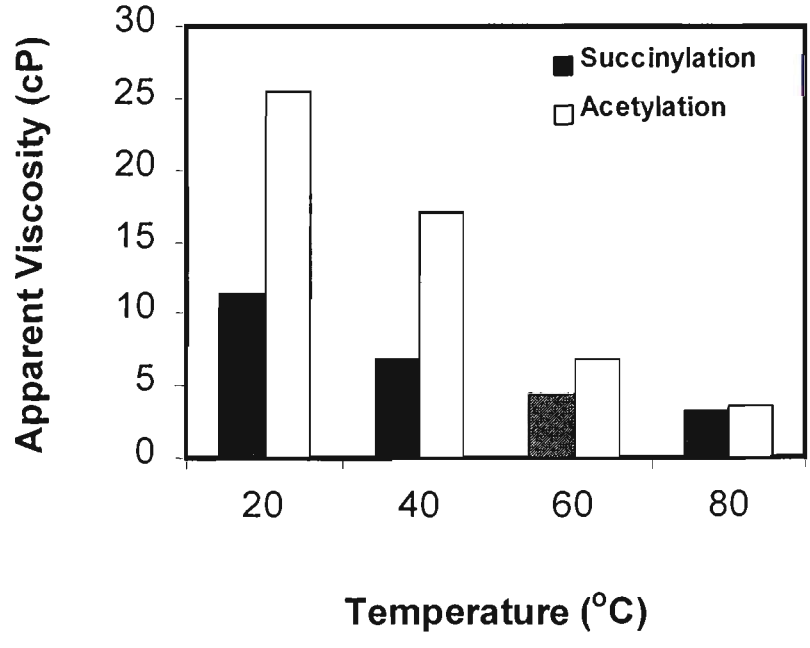


Figure 7.17 Effect of temperature on the apparent viscosity of succinylated and acetylated field pea proteins (at pH7, 4% dispersion).

With the increase of temperature, the apparent viscosities of the acetylated and succinylated proteins were also progressively reduced (Fig. 7.17). This pattern is generally consistent with the viscosity behaviour of the native proteins (Fig. 6.16). The higher temperatures probably reduced viscosity by destabilising both protein-protein and protein-water interactions (Huang and Kinsella, 1986a) and this is more significant for the proteins which had been chemically modified. For the native proteins, a slightly higher viscosity was observed at 80°C compared with those at 40°C and 60°C. This may be due to the unfolding accompanying the partial denaturation of the proteins at the higher temperatures. Catsimpoolas and Meyer (1970) concluded that with most proteins above a certain high temperature, thermal denaturation causes the viscosity to increase. However, for the succinylated and acetylated proteins, the strong electrostatic repulsions between the protein molecules might minimise noncovalent associations between the thermally altered proteins and progressively reduce the hydrodynamic volume of the protein in solution (Huang and Kinsella, 1986a). Therefore in the current study, with the increase of temperature, no improvement in viscosity of the modified proteins was observed. Similar results have been found with phosphorylated yeast proteins (Huang and Kinsella, 1986a).

7.2.7. Gelation Properties of Modified Field Pea Proteins

In an earlier phase of the current research it has been shown that field pea proteins do not readily form gel structures (Chapter 6). When the modified proteins were tested it was found that acetylation and succinylation did not produce any enhancement of the gelation properties of the proteins (Fig. 7.18). The results of the gel-forming ability of phosphorylated proteins have not been included. Since the solubility of the phosphorylated proteins was very low, no gels formed when the samples were heated up to 97°C for 30 min and then cooled. In the sample container, the protein slurry separated into two layers, the liquid layer at the top and the precipitate layer at the bottom. Therefore phosphorylation appeared to impair the functional properties of field pea proteins. It has been reported that gel-forming properties of casein and gluten were improved after $POCl_3$ treatment, possibly due to cross-linking of these proteins

(Matheis *et al.*, 1983; Matheis and Whitaker, 1984). However, for the pea proteins phosphorylated with $POCl_3$ in the current study, no evidence of cross-linking of the proteins has been found. Therefore, in different cases, phosphorylation can either improve or impair the functional properties including viscosity and gelation, largely depending on the nature of the protein used (Schwenke, 1997).

With the increase of the extent of acetylation and succinylation, the gel strength of the modified proteins gradually decreased (Fig. 7.18). This is possibly due to the increased charge repulsion between the protein molecules resulting in the inhibition of gelation (Howell, 1996). Succinylation of proteins including BSA and those from egg and fish gave modified products which did not gel upon heating (Murphy and Howell, 1990; Groninger and Miller, 1973; Ma and Holme, 1982). As discussed in Chapter 6 (6.1.6), the formation of a heat-induced gel is a complex process but usually involves two major steps. The first step includes dissociation and denaturation of the protein molecule by heating above its denaturation temperature. The next step is the formation of protein aggregates which set into a gel during cooling. The denaturation process is

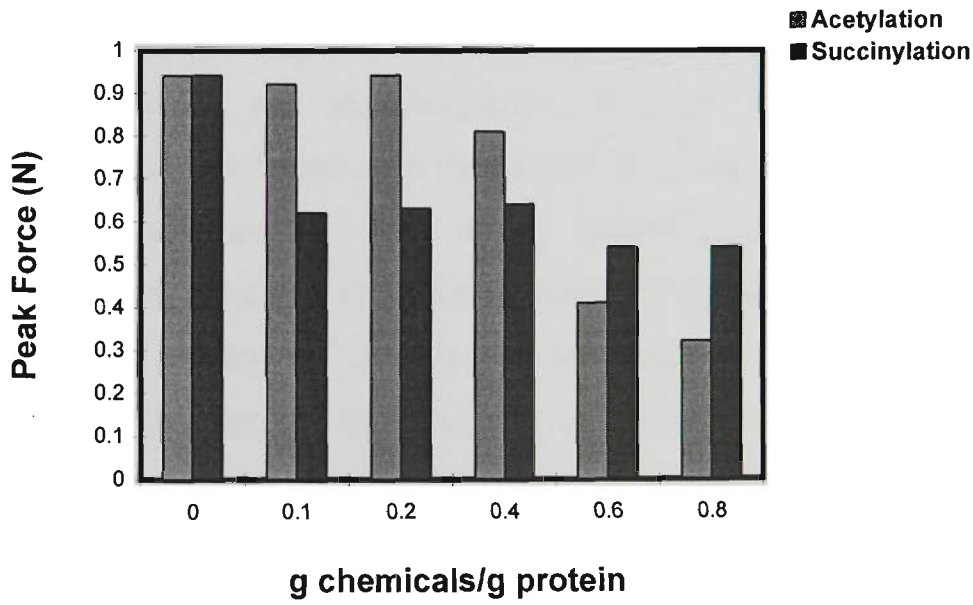


Figure 7.18 Gel peak force of modified field pea proteins as a function of treatment levels for acetic anhydride and succinic anhydride.

of primary importance since it exposes functional groups of the proteins which, under appropriate conditions, interact to form a three-dimensional gel network (Damodaran, 1996). However, examination of DSC thermograms demonstrated that no peaks were identified for the acetylated, succinylated and phosphorylated pea proteins (data not shown). This indicates that the proteins have may have already been denatured during the modification process.

It is known that extremely acidic or alkaline conditions reduce the thermal stability of proteins (Harwalkar and Ma, 1987). Chemicals such as acetic anhydride, succinic anhydride and $POCl_3$ are all acidic and when they are added to the protein solutions, a large amount of concentrated NaOH has to be used to maintain the reaction in the required range of pH 7.5-8.5. The pH and process-induced denaturation may result in the hydrophobic and conformational changes of the proteins and thus affecting the number of the functional groups available for gel network formation during subsequent heating. This could be a further explanation accounting for the weak gel formation of the acetylated, succinylated and phosphorylated proteins.

In summary, the functional properties of field pea proteins were greatly affected by acetylation, succinylation and phosphorylation. However, different modification processes caused significant variations in the functional behaviours of the proteins. The levels of chemicals used also had large effects. Phosphorylation did not appear to enhance the functional properties of field pea proteins, indicating that $POCl_3$ is not a suitable reagent to modify this protein for food applications. Acetylation and succinylation generally improved the functional characteristics of field pea proteins, including solubility, viscosity, emulsifying capacity and stability, as well as foaming properties. In particular, the solubility of succinylated proteins increased greatly at pH values of 6-7 and the viscosity behaviour of the proteins was significantly enhanced by acetylation. Therefore protein preparations from different chemical modification procedures at appropriate degrees of modification can present unique functional properties, which in turn, may find particular application in specific food systems.

7.3. Application of Modified Field Pea Proteins in Foods

Chemical modification is potentially an important tool for tailoring food proteins into products with very different functional properties. Currently few modified proteins have found application in food for human or animal consumption. One of the major obstacles in the commercial production of modified proteins is the expensive, time-consuming process of safety evaluation of these novel products (Howell, 1996). Concerns surrounding chemical modifications of food proteins include toxicity, deterioration of organoleptic properties, loss of nutritional value, interaction with other foods consumed, and the reversibility of modification. In addition, possible barriers to chemically modified proteins entail aesthetic, cultural, legal, and economic issues (Feeney and Whitaker, 1985; Feeney, 1977).

Although further work is needed for the assessment of the safety and nutritional value before modified proteins can be applied into human foods, it is necessary to evaluate the functional behaviour of the proteins in model food systems. In Chapter 6, it has been demonstrated that native field pea proteins are a good substitute for egg in cakes and mayonnaise. Since acetylation and succinylation improved the emulsifying, foaming and viscosity behaviours of the proteins, isolates modified with acetic anhydride and succinic anhydride at levels of 0.2g and 0.4 g/g protein, respectively, have been chosen for evaluation of food applications. These have been incorporated into sponge cakes at varying levels of replacement of egg protein. The quality of the cakes has been assessed in terms of volume, firmness and colour in comparison with those produced with native protein and 100% egg protein (control). The application of modified proteins in mayonnaise was not undertaken since this work would have required sensory evaluation. It is deemed inappropriate to ask panellists to ingest foods containing the chemically modified proteins until further research into the safety of the products has been completed.

The results for the characteristics of sponge cakes supplemented with modified field pea proteins are shown in Table 7.10. It can be seen that in terms of volume, firmness and colour of the cakes, with 25% replacement of succinylated proteins for egg proteins, the quality of the cake was similar to the control and better than that prepared with 25% substitution by native field pea proteins. The volume of the cake was slightly reduced at the level of 50% replacement of succinylated proteins for egg proteins and the colour was slightly darker than the control, but the quality was still better than that prepared with the same amount of native proteins for substitution of egg proteins. The cakes supplemented with succinylated proteins presented a softer texture compared with the control. The coherence of the cake was good and no oily-surface of the crumb was observed.

Table 7.10 Characteristics of Sponge Cakes Containing Modified Field Pea Proteins

Source and Level of Addition	*Volume (mL)*	*Firmness (N)*	*Colour*		
			*L**	*a**	*b**
Control[a]	940	5.30	77.23	-3.07	+21.84
NPI[b]					
25%	915	4.87	71.62	-1.73	+21.92
50%	870	4.05	67.06	-0.63	+20.43
Succinylation[c]					
25%	935	4.21	73.30	-1.93	+21.32
50%	890	4.89	69.24	-0.86	+21.97
Acetylation[d]					
25%	890	5.81	72.13	-1.96	+20.09
50%	835	5.68	67.06	-0.69	+20.02

a: Cake containing 100% egg proteins
b: Cakes containing native proteins (alkaline extracted proteins on the pilot scale)
c: Cakes containing protein modified with succinic anhydride at 0.4g/g protein
d: Cakes containing protein modified with acetic anhydride at 0.2g/g protein

For the sponge cakes supplemented with acetylated proteins, the cake quality did not show improvement compared with those produced with the same level of substitution of native proteins for egg proteins. This indicates that the acetylated pea proteins may show lower stabilities for both emulsifying and foaming properties compared with succinylated proteins upon heating. The poor gelation properties of the modified proteins may also partially account for the deterioration of the cake quality when the products were supplemented with acetylated pea proteins instead of egg proteins, especially at a higher level of substitution. It is also noted that the functional behaviour of proteins determined from simple model aqueous systems may fail to predict the functional properties of the proteins in real food systems (Damodaran, 1996). The extensive conformational changes that occur in proteins under industrial processing conditions, as well as multilateral interactions of the protein with other food constituents, make it difficult to translate the results of model system studies into predictions of behaviour in real food systems (Harper, 1984; deWit, 1989). However, at the current stage of food protein research, the study of functional behaviour of the proteins in model systems is critically important as it could provide a better understanding of the relationship between the structure and the functional properties of the proteins. Certainly more research is needed in this area.

7.4. Conclusions

In the present study, field pea protein isolate extracted with alkaline solution (pH 9) on the pilot scale, has been subjected to acetylation, succinylation and phosphorylation at different levels. The extents of free amino group modification increased as the ratio of reagents to the protein increased. However, the rates of modification varied with different reagents and beyond a certain level of treatment, the degree of modification did not increase significantly. The amino acid profiles of the modified proteins were not significantly changed by chemical modification and *in vitro* digestibility analysis shows that the nutritional value of modified field pea proteins was not impaired. Acetylation, succinylation and phosphorylation resulted in some differences in the SDS gel electrophoresis patterns of the proteins. A significant amount of acetylated proteins did

not enter the gel at the higher levels of modifications. The major bands of succinylated proteins were retarded and appeared to be less mobile. Phosphorylated proteins showed gel patterns similar to those of native proteins except that the bands were relatively weak.

The functional properties of field pea proteins were greatly affected by chemical modification. Nitrogen solubility of the proteins was increased by succinylation, as well as by acetylation at the lower levels of treatment. In particular, the isoelectric point of succinylated proteins was shifted to a more acid pH range and the solubility increased greatly at pH values of 6-7, which is of potential significance in a variety of food systems. There was a considerable decrease in the solubility of the phosphorylated proteins compared with the unmodified isolates. Succinylation and acetylation also improved the emulsifying, foaming and viscosity behaviour of the proteins. These functional properties, like those of the native proteins, were influenced by variations in pH, salt addition and temperature. However, for the modified proteins, the effects of the environmental changes were not always the same as those found for the native proteins. This indicated some alterations in structure as well as net charge density and distribution resulting from the chemical modifications. Acetylation and succinylation did not appear to enhance the oil absorption and gelation properties of the proteins. In addition to reducing solubility, phosphorylation resulted in the deterioration of the other functional properties of the proteins included in this study. This indicated that $POCl_3$ is not a suitable reagent for the modification of field pea proteins, and possibly, analogous plant proteins, for the improvement of functional properties for the food industry.

Succinylated and acetylated field pea proteins were found to be a good substitute for egg protein in sponge cakes. With the excellent solubility and viscosity behaviours, these modified proteins offer good potential in other food applications including protein beverages and in systems requiring food-thickening agents. For the realisation of this potential, further work must be directed to the careful evaluation of the modified proteins in terms of the safety and acceptability. In addition, further research effort clearly needs to be directed to the elucidation of the structure-functionality relationships of food proteins.

CHAPTER 8

General Conclusions

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8.1. Summary of the Results

Field pea (*Pisum sativum* L.) proteins, which offer potential as a novel protein source for food applications, have been extensively studied in the current research. Four major parts of the program were included and the results are reported in this thesis. The first phase involved the isolation and fractionation techniques of field pea proteins on a laboratory scale, which provided the useful information for the pilot scale production of the protein isolates. Different fractions of these proteins were further characterised in terms of gel electrophoresis, ion exchange and gel filtration chromatography, amino acid analysis and scanning electron microscopy. The second phase described the pilot scale isolation of field pea proteins via two different procedures, the extraction with salt solution and the extraction with alkaline solution. Based on the two different products obtained, the next part of the research covered the assessment of the functional properties of the proteins and the further applications in model food systems. Various factors which affect the functional behaviour of the proteins were also studied. Following this, the last part of the program evaluated the feasibility of chemical modification of the protein isolate in order to enhance the functional characteristics. The possible structure-functionality relationships between these proteins were also discussed based on the measurement of surface properties of the proteins and the resultant change in gel electrophoresis patterns.

8.1.1. Isolation, Fractionation and Characterisation of Field Pea Proteins

Field pea proteins have been extracted with distilled water at different pH values (pH 2, 7, and 9) and recovered by isoelectric precipitation. A neutral salt solution (0.5M NaCl) has also been used to extract the proteins and the isolate was recovered by the reduction of ionic strength. The four protein isolates exhibited similar gel electrophoresis patterns

but showed some difference in terms of the recovery rate and nitrogen solubility. The use of alkaline solution (pH 9) is an economic method for isolation of the proteins because it gave the highest recovery rate. The extraction with salt solution (0.5M NaCl) is also a feasible way to obtain the protein isolate on a pilot scale if an alternative procedure for removing salt could be used, for example, ultrafiltration and diafiltration, instead of dialysis with cold water.

Osborne protein fractions (albumins, globulins, prolamins and glutelins) were prepared with different buffer solutions and solvent. The quantities of globulin and albumin fractions showed considerable variation depending upon the extraction conditions used. The albumin fraction represents a larger proportion of the soluble proteins than previously reported. For example, when extracted with 0.2M phosphate buffer (pH 7), the recovery of albumin fractions was nearly the same amount as the globulin fractions. Like other grain legumes, prolamin content in field peas is very low. The recovery of glutelin was 9% and the isolate is most likely to have been contaminated with carbohydrates. Different pea protein isolates and fractions presented similar amino acid profiles except that a slightly higher amount of lysine, methionine and cysteine was found in albumins. Similar to other legume proteins, the amino acids containing sulphur are also the limiting amino acids in peas.

When albumin fractions were further purified using column chromatography and preparative electrophoresis, the major protein subunit had molecular weight of 27-28 kDa. Albumin fractions isolated using a variety of extracting buffers showed similar patterns on SDS-PAGE but these differed from those of protein isolates and globulin fractions. Nitrogen solubility analysis demonstrated that the albumin fraction had enhanced solubility characteristics compared with protein isolate and globulin fractions, particularly in the range of the isoelectric point. The scanning electron microscopic observations indicated that pea albumins possessed thin wafers and a large surface area whereas globulin and protein isolate showed a more tightly packed structure and denser mass. These results suggest that specific fractions of field pea

proteins may well find different applications in foods due to their structural differences and variations in the functional properties.

8.1.2. Pilot Scale Isolation of Field Pea Proteins

One of the procedures to produce pea protein isolate in the pilot scale involved alkaline extraction followed by decantation and recovery by isoelectric precipitation and neutralisation. The other included extraction with salt solution, followed by ultrafiltration and diafiltration to remove salt and obtain the concentrated proteins. Overall, the salt extracted proteins exhibited better physical properties than alkaline extracted proteins in terms of colour and particle size. The solubilities showed little variation and the electrophoretic patterns were similar. However, most of the major bands in albumin fractions were not included in the isolates. This indicated that a significant amount of the albumin proteins were lost during processing.

From the technological point of view, freeze drying is relatively simple to control, but is time-consuming and the resultant product is of a dark colour and non-uniform particle size. Freeze drying also resulted in isolates with different surface microstructure, which showed a denser mass, compared with the spherical shape of spray-dried proteins. From the results of differential scanning calorimetry, pilot-scale isolates by spray drying showed relatively low transition enthalpies, indicating the partial denaturation of the proteins. However, partial denaturation was not associated with a deterioration in functionality of proteins and on the contrary, this effect might be beneficial for special food applications.

8.1.3. Functional Properties of Field Pea Proteins and Applications in Foods

Functional properties of the two different pea protein isolates produced on the pilot scale have been extensively studied. Both of the products exhibited good solubility, emulsifying and foaming properties, in comparison to the commercial soy isolate as well as those of some other grain legumes from published results. Oil absorption,

viscosity and gelation properties of field pea proteins showed lower potential. In addition, salt extracted protein isolate demonstrated a stronger ability to bind water and showed enhanced foaming properties. The emulsifying capacity and stability of the two preparations are quite similar. Some relationships between particular functional properties were found. In particular, emulsifying and viscosity behaviours were both related closely to the solubility characteristics of the proteins.

Different environmental conditions including variations in temperature, salt (NaCl) concentration and pH have strong effects on the functional properties of field pea proteins. In the acid pH range, the viscosity, emulsifying capacity and stability were found to be decreased but the foaming properties were enhanced. Heat denaturation resulted in the reduction of emulsifying properties of the proteins but showed an improvement in foaming capacities. Foaming properties appear to be positively related to the protein hydrophobicity (So) values determined for each isolate. However, in addition to solubility and surface properties, many other factors may contribute to the change of functional properties under different conditions. Molecular factors such as amino acid composition, secondary, tertiary and quaternary structures, net charge and distribution are very important in the explanation of functional properties of the proteins. Processing-induced differences in physical properties, including particle size and shape, may also be related to the functional characteristics of the proteins. In summary, the functional properties of field pea proteins are affected by their intrinsic physico- chemical and structural properties, as well as being related to various extrinsic factors including the method of isolation and the conditions selected for the measurement of functionality.

Field pea proteins were found to be a good substitute for egg in cakes and mayonnaise due to their good emulsifying and foaming properties. The characteristics of the cakes were studied in terms of volume, colour and firmness. Sensory evaluation was also involved in the assessment of the quality of the cakes and mayonnaise in comparison to the product produced with 100% egg proteins. It is suggested that the replacement of egg proteins with pea proteins up to 50% is

acceptable. From the published literature, it is generally believed that the beany flavour is one of the major obstacles in food applications of grain legumes including soybeans. However, the results in the current study showed that the flavour problem was not significant in the application of field pea proteins in the model food systems.

8.1.4. Modification of Field Pea Proteins

Intentional chemical modification which leads to acetylation, succinylation and phosphorylation has been investigated with field pea proteins in the current study. The extents of free amino group modification increased as the ratio of reagents to the protein increased and acetic anhydride was more reactive than succinic anhydride and phosphorus oxychloride. The amino acid profiles of the modified proteins were not significantly changed by chemical modification and *in vitro* digestibility analysis showed that the nutritional value of modified field pea proteins was not impaired. The results of SDS gel electrophoresis show that the major bands of succinylated proteins were retarded and appeared to be less mobile. A significant amount of acetylated proteins did not enter the gel at the higher levels of modifications, indicating the possibility of crosslinking which might lead to the alteration of the functional behaviour. Phosphorylation did not result in any significant changes in gel patterns but greatly impaired the functional properties of the proteins. This suggests that phosphorus chloride ($POCl_3$) provides limited potential for modifying pea proteins for application in the food industry.

Generally, succinylation and acetylation enhanced the functional properties of field pea proteins including nitrogen solubility, viscosity, emulsifying and foaming properties. However, when modified with acetic anhydride at higher amounts (> 0.2 g/g protein), the proteins showed decreased functional behaviour, indicating some structural changes by extensive acetylation. The solubility of succinylated proteins increased greatly at pH values of 6-7 and the isoelectric point was shifted to a more acid pH range. This property offers great potential particularly in acid-based food systems. Like native proteins, functional properties of modified proteins were also influenced by the variations in pH,

salt addition and temperature. However, in several cases, the resultant changes of the functionality between the native and modified proteins were different. For example, with the increase of temperature, the emulsifying capacity and stability of native proteins decreased but showed an increase for acetylated and succinylated proteins. This indicated some alterations in structure as well as net charge density and distribution resulting from the chemical modifications. In addition to being a good substitute for egg protein in sponge cakes, acetylated and succinylated proteins may provide good potential in a variety of other food applications due to the enhancement of the functional properties.

8.2. Recommendations for Future Work

8.2.1. Recovery of Albumin Fractions

Up to this point, albumin fractions have been investigated far less extensively than the globulins with respect to the legume proteins. One of the major obstacles is the fractionation procedure. The method usually applied is the use of salt or buffer solution for the extraction of proteins from legume flours and then dialysis for a period of time. When the ionic strength is reduced the globulins are recovered as precipitate and the albumins are remained dissolved in the top layer. The albumin fractions are then concentrated and freeze-dried. Obviously this procedure is not practical using current technology in the large scale recovery of albumin fractions. In the production of protein concentrates and isolates on a pilot scale or even in the laboratory by the iso-electric precipitation procedures, a significant amount of the albumins have been lost, as indicated in the current study with field pea proteins.

The result in the current research shows that the albumin fraction in peas represents a larger proportion of the soluble proteins than previously reported and this fraction demonstrated unique solubility characteristics. The nutritional value of albumins is generally believed to be higher than that of globulins in terms of the contents of essential amino acids. Therefore it is recommended that further effort should be directed on the recovery of albumin fractions on a large scale. This could then lead to extensive research

on the functional properties of this fraction in food systems. It may well find particular technical and economic significance in food processing due to the different functional behaviours expected.

8.2.2. Standardisation of the Methodology for the Determination of Functional Properties

There are currently no standard methods available for the evaluation of the functional properties of food proteins including protein-water interactions, emulsifying and foaming properties, viscosity and gelation, as well as flow properties. The variations in sample size and preparation, equipment used from laboratory to laboratory make it very difficult to compare the published results. Hence it is important to develop methods. These would need to be standardised, subjected to collaborative evaluation and accepted internationally so that ultimately comparable results can be obtained by different laboratories

8.2.3. Relationships between Structure and Functionality

In the current research, some of the techniques including gel electrophoresis (SDS-PAGE, 2-D electrophoresis and preparative electrophoresis), scanning electron microscopy, differential scanning calorimetry, the determination of surface hydrophobicity and surface tension have been applied. The purpose was to evaluate the possible structure-functionality relationships of field pea proteins. Several extrinsic factors including the change of pH, temperature and salt concentration which affect the functional properties have also been studied. These studies have not previously been applied to field pea proteins. However, for a better understanding of the structure-functionality relationships of food proteins, further basic research is needed in this area. Knowledge in elucidating the change of functional behaviours on molecular basis remains elusive. More research is also needed to develop reliable methods to quantitate molecular factors such as charge distribution and molecular flexibility which could greatly affect the functional properties of the proteins. The information obtained will

help in the development of new protein ingredients with particular functional behaviours.

8.2.4. Nutritional and Safety Assessment of Modified Proteins

It has been recognised that chemical modification is potentially an efficient and economic tool for improving functional properties as well as for studying structure-function relationships of food proteins. However, in addition to functional properties, these protein products should meet the requirement of biological stability, nutritional value, safety, and acceptability for food applications. Therefore *in vivo* nutritional experiments and safety evaluation are needed although these processes are expected to be expensive and time-consuming. Research on the colour and flavour change due to the addition of the modifying chemical reagents is also needed. This information will help in the determination of whether or not a new protein ingredient is competitive in food market. Currently literature in this area is scarce and commercial application of modified proteins in food systems remains limited. However, with the increased interest in plant proteins and the protein shortage in relation to population growth, legume proteins including those of field peas should fulfil an important role in the global food industry in the near future.

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Appendix I Sensory Evaluation of Cakes

Product: Sponge Cake Date: Name:

I. **Colour**: **Please rate each sample for Overall acceptability (✓)**

	very poor								very good
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

II. **Texture: a. Overall acceptability:** Please (✓) (1-9 hedonic scale)

	very poor								very good
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

b. Comment: How do you describe the texture of each sample? e.g. softness or hardness, finesse or coarseness, adhesiveness, oily mouthcoating, crumbly, good or bad coherence (You may not need all of them)

Sample 1	
Sample 2	
Sample 3	
Sample 4	

III. **Flavour: a. Overall acceptability:** Please (✓) (1-9 hedonic scale)

	very poor								very good
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									
Sample 4									

b. Comment: How do you describe the flavour of the sample? e.g. strong or fair, poor flavour from egg, any bean flavour which are not acceptable, or other.

Sample 1	
Sample 2	
Sample 3	
Sample 4	

Appendix II Sensory Evaluation of Mayonnaise

Product: Mayonnaise Date: Name:

I. **Colour**: **Please rate each sample for Overall acceptability (✓)**

	very poor								very good
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									

II. **Texture:** **a. Overall acceptability:** Please (✓) (1-9 hedonic scale)

	very poor								very good
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									

b. Comment: How do you describe the texture of each sample? e.g. smoothness, finesse or coarseness, adhesiveness, oily mouthcoating, good or bad coherence (You may not need all of them)

Sample 1	
Sample 2	
Sample 3	

III. **Flavour:** **a. Overall acceptability:** Please (✓) (1-9 hedonic scale)

	very poor								very good
	1	2	3	4	5	6	7	8	9
Sample 1									
Sample 2									
Sample 3									

b. Comment: How do you describe the flavour of the sample? e.g. strong or fair, poor flavour from egg, any bean flavour which are not acceptable, or other.

Sample 1	
Sample 2	
Sample 3	