An Alternative Quantitative Method and Molecular Genetics of Amylose in Australian Wheats

Hemalatha Jegasothy

Doctor of Philosophy

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An Alternative Quantitative Method and Molecular Genetics of Amylose in Australian Wheats

A thesis submitted to the Centre for Bioprocessing and Food Technology in fulfillment of the requirements for the degree of Doctor of Philosophy

by

Hemalatha Jegasothy

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Centre for Bioprocessing and Food Technology
Victoria University of Technology, Werribee campus
Victoria
AUSTRALIA

This work is dedicated to my parents R. Jayaram and Jeyam Jayaram who inspired me.

Certificate of Originality

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to substantial extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgement is made in the text.

Hemalatha Jegasothy

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Abstract

In this study, a simple and rapid electrophoretic procedure was developed to determine the amylose content in wheat starch. The experimental approach was based on agarose gels using a borate buffer and entailed optimisation of gel concentration, staining method, electrophoretic conditions and starch solubilisation conditions. Laser densitometry was used to quantify the amylose component.

Agarose gel with a concentration of 0.5% was selected as a compromise between band mobility and gel handling characteristics. Iodine proved to be the most suitable stain to visualise the polysaccharide components in the gel. Amylopectin did not migrate from the origin and was detected from its reddish brown colour and amylose was detected from its blue colour. Cathodic movement of amylose indicated that the starch component was moving under the influence of electro-endosmotic flow of the borate buffer. The best resolution of the polysaccharide components was achieved using 30 volts for 16 hours. Optimum condition for starch solubilisation was achieved using 0.5 M KOH at 80°C for 15 minutes. A linear relationship existed between amount of amylose and its peak area determined by laser densitometry, thus allowing quantitation of amylose.

Small scale preparations of wheat amylose were carried out. The preparations involved a modified fractionation procedure using concanavalin A and the traditional butanol precipitation method with the former being more effective where wheat amylose free of amylopectin was obtained. Although laser densitometer showed a linear response to wheat amylose levels, HPLC analysis after hydrolysis to glucose, indicated the presence of other impurities in the samples obtained by concanavalin A fractionation.

The agarose gel electrophoresis method developed in this study was used to study starches isolated from twenty five Australian wheat cultivars and the results compared with existing colorimetric and concanavalin A precipitation methods. The amylose contents determined for the wheat starches by the agarose gel electrophoresis method

were within the range reported previously for Australian wheat cultivars. The method showed a positive correlation but a weak association with the colorimetric and concanavalin A methods. It was found that further refinement of the technique was required to improve reproducibility and reliability.

The advantages of agarose gel electrophoresis are as follows; It is a simple and rapid method for separation and detection of the principal starch components. Since amylose was quantitated after separation from amylopectin, interference from iodine complexes with linear portions of amylopectin was avoided. The method allows multiple simultaneous determinations of amylose to be carried out. The method can be used to monitor the purity of amylose and amylopectin preparations.

Molecular genetic studies were conducted on four normal and four null 4A cultivars to investigate the nature of mutations in the 4A *waxy* gene of null 4A Australian wheat cultivars, responsible for the absence of the Wx-B1 protein. Oligonucleotide primers were constructed for amplification of various sections of the *waxy* genes. Polymerase Chain Reaction (PCR) of genomic DNA indicated a deletion of the intron 1 in the 4A *waxy* gene of null 4A cultivars. Further, the intron 1 of the *waxy* genes (atleast the 7A and 7D) in the normal and null 4A cultivars was found to be longer in length than that of barley intron 1 by ~100 bp. Northern hybridisations were performed with total RNA, isolated from 20 dap seeds of the null 4A and normal cultivars and developing seeds of a null 4A (Rosella) and a normal (Chinese Spring) cultivar harvested at 5, 10, 15, 20 and 25 days after pollination, using *waxy* cDNA probes. *Waxy* transcripts of expected size, ~2.3 kb, were exhibited by all the cultivars. However, a significant reduction was observed in the *waxy* transcripts of null 4A ($P < 0.05$) compared to that of normal cultivars. This observation explains the possibility of complete lack of expression of the 4A *waxy* gene in the null 4A cultivars. Transcripts of altered sizes were not found in the null 4A cultivars, indicating that no large insertions or deletions or incorrect splicing events in the *waxy* transcripts were responsible for the lack of 4A *waxy* gene expression in these cultivars. However, the deletion of the intron 1 region observed in the null 4A cultivars, probably a part of a mutation in the 4A *waxy* gene, could be responsible for the absence of expression of this gene in these cultivars.

Preliminary work showed overall similar patterns in the *waxy* gene expression during development of seed in the normal (Chinese Spring) and null 4A (Rosella) cultivars. The *waxy* gene appeared to be activated during the early period (5-10 dap) of seed development and was shown to be strictly regulated to occur over a very short period, with a peak expression at 15-20 dap and a decrease in expression by 25 dap.

The relationship between waxy protein amount and amylose content during seed development was investigated in developing seeds of a normal (Chinese Spring) and a null 4A (Rosella) cultivars. Quantitative and qualitative analysis of waxy protein were carried out using SDS-PAGE (Laemmli, 1970) and modified SDS-PAGE (Kagawa *et al.* 1988) respectively, with the developing and mature seeds. Waxy protein levels were determined by scanning the bands corresponding to the waxy protein (~60 kD) with a laser densitometer (Nakamura *et al.* 1993). The amylose contents of developing and mature seeds were determined using the amylose/amylopectin assay kit (Megazyme Ltd.). It was found that during active transcription of the *waxy* gene, waxy protein and amylose accumulated in a linear fashion in both the cultivars. Amylose synthesis commenced soon after pollination (5 dap) and exhibited a high correlation with waxy protein production, up to 25 dap during seed development. However, the null 4A cultivars, lacked the Wx-B1 protein and exhibited lower amylose content (compared to the normal cultivars) at all stages of seed development. In mature seeds, the amylose content and relative waxy protein amount of null 4A cultivars were significantly lower ($P < 0.05$) than that of normal cultivars and a positive correlation existed between the two traits. This study thus established a direct relationship between production of waxy protein and amylose synthesis during seed development in wheat.

List of Abbreviations

au	Arbitrary units
bp	Base pair
cm	Centimetre
°C	Degree celcius
Con A	Concanavalin A
dap	Days after pollination
DEPC	Diethylpyrocarbonate
dH_2O	Deionised water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
g	Gram
GPC	Gel permeation chromatography
GPR	General Purpose Reagent
h	hour
HMW	High molecular weight
HPLC	High Performance Liquid Chromatography
I_2	Iodine
kd	Kilo Dalton
KI	Potassium iodide
LMW	Low molecular weight
M	Molar
mA	Milli amphere
mg	Milligram
mm	millimetre
µg	Microgram
min	Minute
mL	Millilitre
µL	Microlitre
mM	Millimolar

MOPS	3-(N-morpholino) propane sulfonic acid
ng	Nanogram
nm	Nanometre
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
s.d.	Standard Deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate poly acrylamide gel electrophoresis
sec	Second
SsDNA	Salmon sperm De-oxy ribonucleic acid
TAE	Tris-acetate
TCA	Trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
V	Volt
v/v	Volume by volume
w/v	Weight by volume
Wx	Waxy

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Jegasothy, H., Wootton, M. and Fairclough, R.J. 2000. Agarose gel electrophoresis of wheat starch. *Journal of Cereal Science* 31 (1): 75 – 78.

Jegasothy, H., Bhave, M.R. and Fairclough, R.J. 1997. Molecular studies on null 4A wheat cultivars. Proceedings of the 47th Australian Cereal Chemistry Conference. Eds. A.W. Tarr., A.S. Ross and C.W. Wrigley, Cereal Chemistry Division, RACI, 461-465.

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Davis, B.R., Jegasothy, H., Bhave, M.R. and Fairclough, R.J. 1997. Molecular Studies of null 4A waxy wheat cultivars. Presented at the 41st Annual Australian Society for Biochemistry and Molecular Biology, Melbourne, Australia.

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General Introduction

Starch is the major reserve carbohydrate of higher plants and it consists of essentially two types of polymers of glucose, linear amylose and branched amylopectin. Starch has a wide range of uses in the food and non-food industry depending on its structure and composition. In the non-food industry, starch has gained significant interest because of its abundance and low cost, and also because of growing concern for environmental pollution caused by non-biodegradable petroleum based polymers. In the food industry, the quality of end product of a starch-based food is determined by many of the functional properties of starch and this, in turn, is dependent on its amylose/amylopectin ratio. Hence, estimation of the ratio of these two components plays a major role in determining its end use. Breeding plant varieties for altered amylose content requires a simple, quick and reproducible method of amylose estimation from a small starch sample. As discussed in chapter 1 (Section 1.9), many methods based on different principles have been developed to measure the amylose content in starch. However, there are disadvantages for most of these methods.

Agarose gel electrophoresis has proven to be a very powerful technique for characterisation of high molecular weight bio-polymers, such as nucleic acids. However, the application of agarose gel electrophoresis for separation of starch components has not been reported. Thus, in the present study, a simple and rapid agarose gel electrophoresis method was developed, to separate the major starch components and to quantify the amylose content in wheat starch.

The factors that control the relative proportions of amylose and amylopectin in starch are primarily genetic. In cereals such as rice, maize and wheat, the starch granule bound waxy protein in the endosperm, which is encoded by the *waxy* gene, is known to be involved in amylose synthesis (Sano, 1984; Echt and Schwartz, 1981; Yamamori *et al.* 1992). In waxy mutants of rice and maize it has been shown that mutations in the *waxy* gene were associated with the absence or a drastic reduction in the waxy protein and amylose content of the endosperm starch (Sano, 1984; Echt and Schwartz, 1981). In hexaploid bread wheat (common wheat) there are three *waxy* loci (*Wx-A1*,

Wx-B1 and *Wx-D1*) (Chao *et al.* 1989) encoding three waxy proteins (Wx-A1, Wx-B1 and Wx-D1) (Nakamura *et al.* 1993b). Among the Japanese wheat cultivars, the waxy protein level of the starch granules was found to vary and it correlated to the amylose content of the starch (Yamamori *et al.* 1992). Yamamori *et al.* (1994) studied the deficiency of waxy protein in several hexaploid wheat cultivars and reported that wheats deficient for the Wx-B1 protein (null 4A cultivars) were found to be fairly common among wheats of Australian origin. This deficiency correlated with lower amylose content, higher starch pasting characteristics and superior quality of white salted Japanese noodles (Miura and Tanii, 1994).

The focus of wheat breeders and geneticists is currently on wheat starches having reduced amylose contents and altered starch properties, which would find many novel applications in food and non-food industries. Hence, studies on the genetic control of starch composition in wheat will not only be of theoretical interest but also of importance in agricultural and industrial applications. Little is known about the molecular nature of mutations in *waxy* genes of null 4A Australian cultivars. Thus an investigation was carried out with developing and mature wheat seeds of null 4A cultivars, to obtain a molecular explanation for the lack of expression of the Wx-B1 protein in these cultivars.

CHAPTER 1

Literature Review

1.1 Classification and evolution of wheat

1.1.1 Allopolyploid series

Wheat was first found as a wild grass native to the arid countries of Western Asia and it has been used as a food source since the stone-age era. Domestication of wheat from the wild grass, by early farmers, gave rise to cultivated wheats. Wheat belongs to the family *Gramineae* and is a member of the tribe *Triticeae,* a subdivision of the grass family. The two sub-tribes of *Triticeae* are, *Triticinae* (consisting of about 35 genera, such as *Triticum, Aegilops, Secale*), and *Hordeinae* (consisting of *Hordeum* and related genera) (Breiman and Graur, 1995). The genus *Triticum* contains wheat species with cultivated forms, such as the hexaploid common wheat, *T. aestivum*, while *Aegilops* (goat grass) includes the wild relatives of common wheat such as *Aegilops speltoides* (Breiman and Graur, 1995).

Linnaeus was the first to include all cultivated wheats in one genus *Triticum*, in the year 1753 (Feldman, 1977). The discovery of the wild prototypes of the one-grained and two-grained cultivated wheats enabled Schulz (1913) to assemble the first natural classification of wheats. He divided the genus *Triticum* into three major taxonomic groups, einkorn, emmer and dinkel. This classification was supported by Sakamura (1918) and Sax (1918, 1922) who showed that Schulz's three groups of wheats also differed in their chromosome number. The einkorns were characterised by 2n = 14, the emmers by 2n = 28 and the dinkels by 2n = 42. The analysis of hybrids between the groups indicated that the higher chromosome numbers were the result of allopolyploidy. This observation allowed the assignment of genome formulae (Kihara, 1919; Sax, 1922); Thus the einkorn group is considered to be diploid and genomically AA; emmer, tetraploid and AABB; and dinkel, hexaploid and AABBDD.

The einkorn is the cultivated diploid wheat, *T. monococcum*, which is separated into the two subspecies, *T. monococcum* ssp. *boeoticum* and *T. monococcum* ssp. *urarta* (Johnson *et al.* 1976). The wild equivalent of *T. monococcum* is *T. boeoticum* (Takumi *et al.* 1993; Heun *et al.* 1997). The emmer group of wheat is the cultivated tetraploid *T. turgidum* that includes three subspecies, *T. turgidum* ssp. *turgidum*, *T. turgidum* ssp. *dicoccum* and *T. turgidum* ssp. durum. Their wild relative is *T. turgidum* ssp. *dicoccoides* (Kerby and Kuspira, 1987). The dinkle or bread wheat, which is the common wheat, is hexaploid *T. aestivum*. In addition, the tetraploid *T. timopheevi* and the hexaploid *T. zhukovskyi* are also cultivated in the Caucasus region (Kimber and Sears, 1987).

In this polyploid series, 7 chromosome pairs of the diploid wheat (with genome A) plus 7 additional pairs (genome B) constitute the 14 pairs of the tetraploid wheat. These 14 pairs (genome AB), plus an additional 7 (genome D), make up the 21 pairs of the hexaploid wheat. These findings indicated that the tetraploid wheats were derived from crosses between the diploids, followed by chromosome doubling. Some of these allotetraploids then crossed with other diploids, and the hybrids, after chromosome doubling, evolved into the hexaploid species. Hence hexaploid wheats are allopolyploids which evolved as a result of hybridisation followed by chromosome doubling.

1.1.2 Genomes of polyploid wheat

It has been suggested that *T. monococcum* (AA), *T. urartu* (AA and/or BB), *T. tauschii* (DD) and the five diploid species belonging to section *Sitopsis* (genomes BB or SS), *T. bicornis*, *T. longissima*, *T. searsii*, *T. sharonensis* and *T. speltoides* are the candidates for being the diploid progenitors of the A, B, D or G genomes of the polyploid wheat (Kerby and Kuspira, 1987; Breimann and Graur, 1995).

Given the strong morphological similarities between diploid wheat *T. monococcum* and tetraploid wheat *T. turgidum*, and the chromosome pairing observed in hybrids between them, it was suggested that the A genome of the polyploid wheats had come from *T. monococcum* (Kimber and Sears, 1987). However, analysis of restriction

fragment length polymorphism (RFLP) of several repeated DNA families and unique sequences identified *T. urartu* as the donor species of A genome in *T. turgidum*, *T. timopheevi* and *T. aestivum* (Dvorak and Zhang, 1990; Dvorak *et al.* 1993). Further evidence for *T. urartu* being the donor of A genome has been provided by the comparison of isoenzymes (acid phosphatase, esterase and superoxide dismutase) between diploid and tetraploid wheat (Jaaska, 1997). The donor of the D genome of hexaploid wheat had been identified as *T. tauschii* (McFadden and Sears, 1944; Kihara, 1944) and this was confirmed in the recent investigations (Lagudah *et al.* 1991; Hohmann *et al.* 1993; Gill *et al.* 1991; Freibe and Gill, 1996).

Various theories up to date have been proposed for the origin of the B genome. The first proposal to gain wide acceptance was that of Sarkar and Stebbins (1956), who concluded that *T. speltoides* had contributed either the whole of the B genome or a substantial part of it. Recently Blake *et al.* (1998) have reported that, among the *Sitopsis* diploids, *T.speltoides* was the most closely related to the B genome sequences of Chinese Spring (hexaploid), but also had clearly diverged from the wheat genome B for all of the loci. Their data showed that the wheat B genome had undergone a severe bottleneck and diverged from the diploids since the amphiploidisation event. Some reports indicate that more than one species could be the likely donors, i.e., the origin of B genome of common wheat might be polyphyletic (Kerby *et al.* 1990; Takumi, 1994). Rhandhawa *et al.* (1997) reported that some species other than *T. speltoides* in the *Sitopsis* section contributed the B genome to polyploid wheat, based on the variations in the glutenin subunit composition in wheat. Three other suggestions are that the donor of B genome is now extinct, it is yet to be discovered and the B genome originates from an introgression of two or more parental species (Breiman and Graur, 1995; Dau and Gustafson, 1996). Dvorak and Zhang (1990) and Talbert *et al.* (1991) were unable to find any evidence of such introgression into *T.* aestivum, based on the studies of repeated DNA sequences in wheat. Although the majority of the reports support *T. speltoides* as the donor, the origin of B genome in polyploid wheat is yet to be resolved.

A and D genomes of polyploid wheats have changed relatively little at the polyploid level (Feldman, 1977). Approximately twice as much variation has been reported in the B genome than in A and D genomes by McIntosh (1988). Chao *et al.* (1989) have suggested the following two possible reasons for the difference between the genomes; (i) The variation in modern hexaploid subsequently arose by mutation due to the inherent mutable nature of the B genome chromosomes, which were characterised by far more heterochromatin and repeated sequences than those from the A and D genomes (Flavell *et al.* 1987; May and Appels, 1987). (ii) B genome progenitor was more variable (an outbreeding species or mixture of species) and this variability has been maintained through the allopolyploidization events which have led to the evolution of hexaploid wheat. Figures 1.1 and 1.2 illustrate the probable evolutionary pathway of the group *Triticinae*.

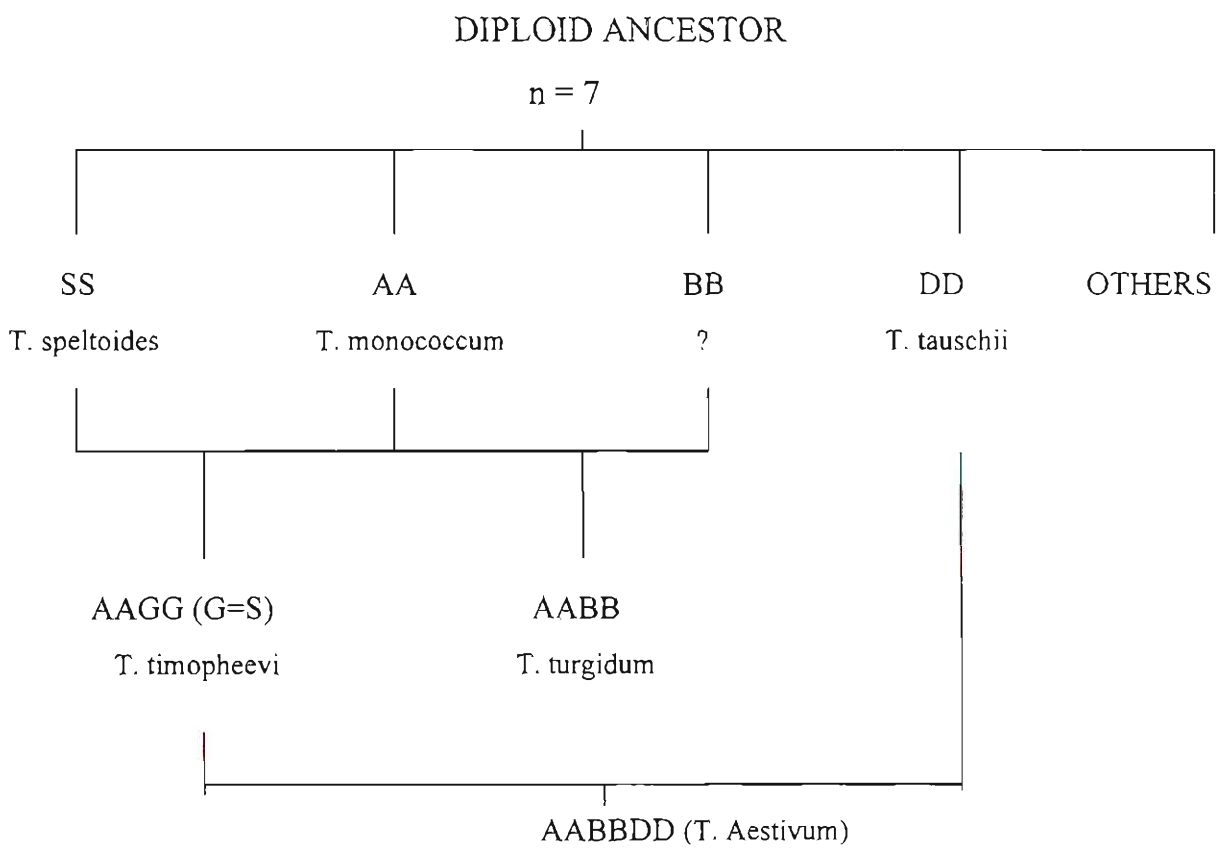


Figure 1.1 A diagramatic representation of part of the evolutionary pathway of the group *Triticinae*, as summarised by Kimber (1973).

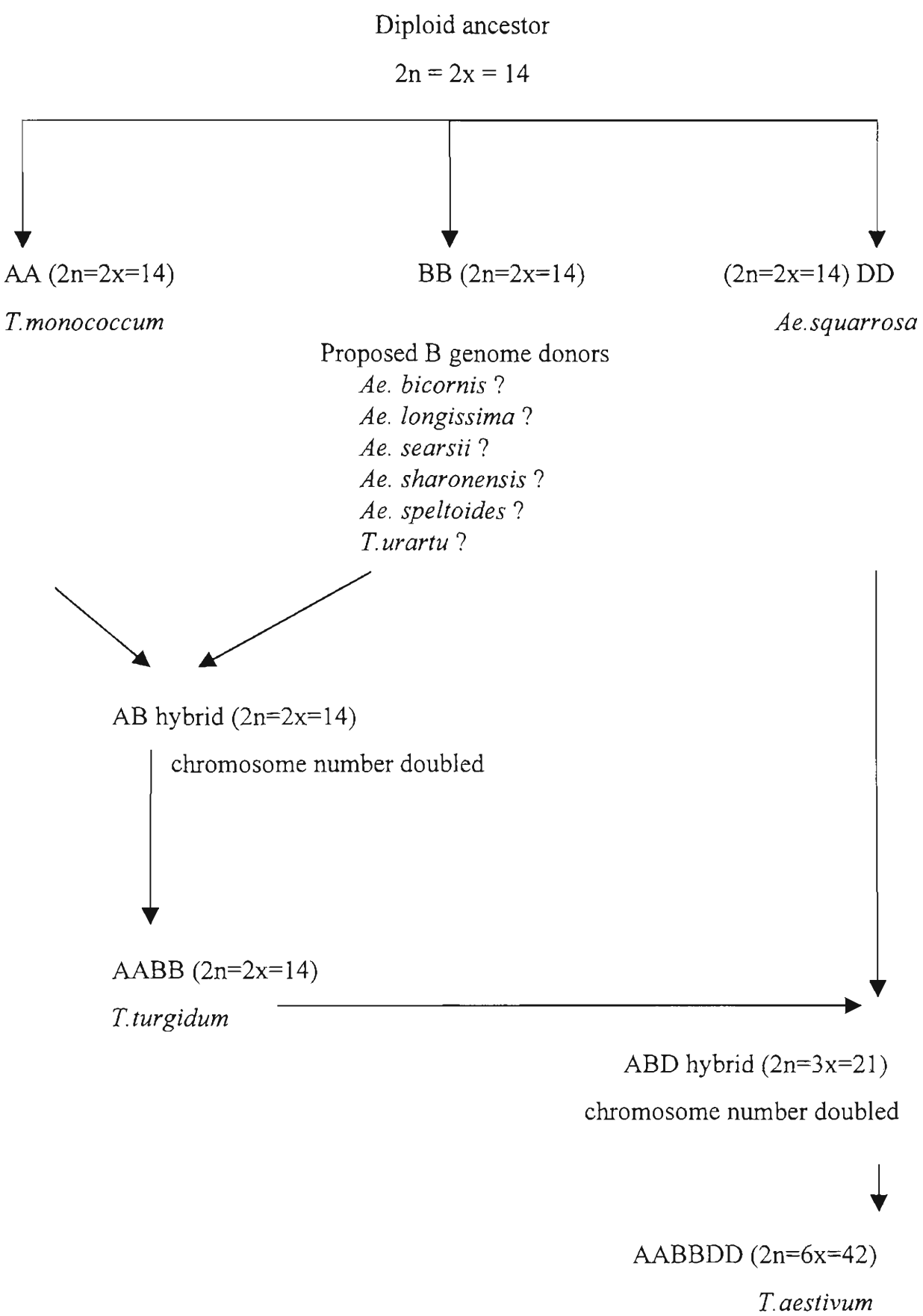


Figure 1.2 Probable origin of *T.turgidum* and *T.aestivum* as summarised by Kerby and Kuspira (1987).

1.2 Wheat production in Australia

Wheat is Australia's largest grain crop, producing an average of 14 million tonnes a year, with all other grains together amounting to a total of nearly 11 million tonnes. Approximately 80% of Australia's wheat is exported to more than 40 countries, largely concentrated in Asia and the middle-east. Australia's total share of the global wheat market is, on an average, approximately 11 percent. The wheat industry is one of the top export earners in the Australia economy, amounting to more than $A 3 billion each year.

The selection of suitable sites for growing wheat is an important consideration. Factors such as soil type, moisture levels, nutrient content and weather are of prime importance. In Australia, wheat is grown in all the states, with the major production area being in a zone called the `wheat belt'. This area stretches in a curve from North East Australia down the east coast, into the southern part of the continent and around into Western Australia. Rainfall, temperature, soil type, fertility and topography interact to provide favourable growing conditions. Much of the 'wheat belt' receives an average annual rainfall of between 230 to 380 mm during the growing period from May to October.

Wheat can be classified as spring or winter type. The former is planted in the spring and is harvested in the late summer and the latter is planted in the summer or autumn and harvested in the spring. Yields of spring wheats are usually lower than winter wheats because the latter are planted in late summer and are able to make more effective use of sunshine and moisture.

1.3 The wheat grain

The wheat grain or kernel (Figure 1.3; Cornell and Hoveling, 1998), known botanically as caryopsis, is the fruit of the plant and is normally about 4-8 mm long, depending on the variety and conditions of growth. The kernel contains only one seed, which is not shed at maturity. The outermost layer of the kernel is the pericarp, which consists of an epidermis, a hypodermis and a layer of thin walled cells. The pericarp, is about 50 μm thick and next to this is the thin seed coat, covering a nucellar

epidermis. The aleurone layer is found beneath these, covering the starchy endosperm. Bran is chiefly the outer layers of the kernel including the aleurone layer.

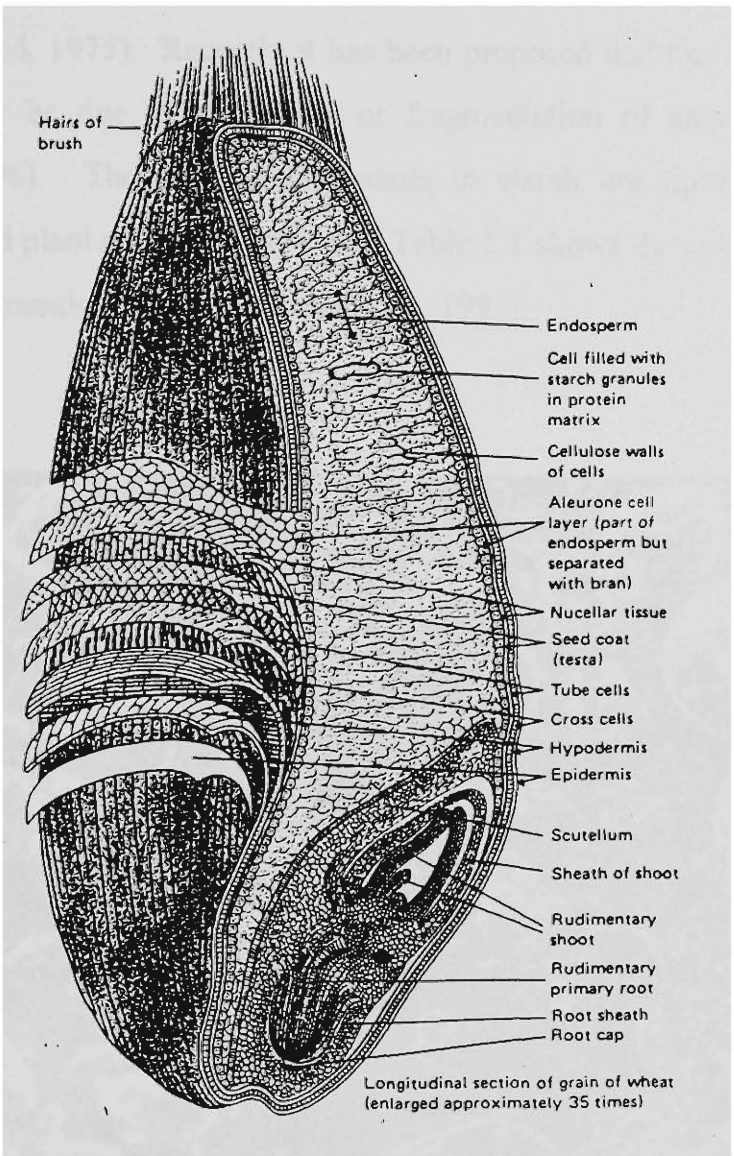


Figure 1.3 Longitudinal section of wheat grain (Cornell and Hoveling, 1998)

1.3.1 Wheat starch

Starch is the major energy reserve in many of the world's most important foods such as the cereal grains, the potato tubers and the root of yams. Starch is embedded within the protein matrix of the cereal endosperm and comprises approximately 65% of the dry weight of the mature wheat grain. Wheat starch consists of simple granules

(Figure 1.4), which are formed as single entities within the amyloplast of the developing wheat endosperm (Lineback and Rasper, 1988). It is comprised of predominantly two major polysaccharide components, amylose and amylopectin. It has been believed for a number of years that some starches contain a third type of polysaccharides, an intermediate material between amylose and amylopectin (Banks and Greenwood, 1975). Recently it has been proposed that this intermediate material may probably be due to hydrolysis or fragmentation of amylopectin (Tester and Karkalas, 1996). The minor components in starch are lipids, protein, inorganic substances and plant cell wall materials. Table 1.1 shows the composition of a typical wheat starch granule (Lillford and Morrison, 1997).

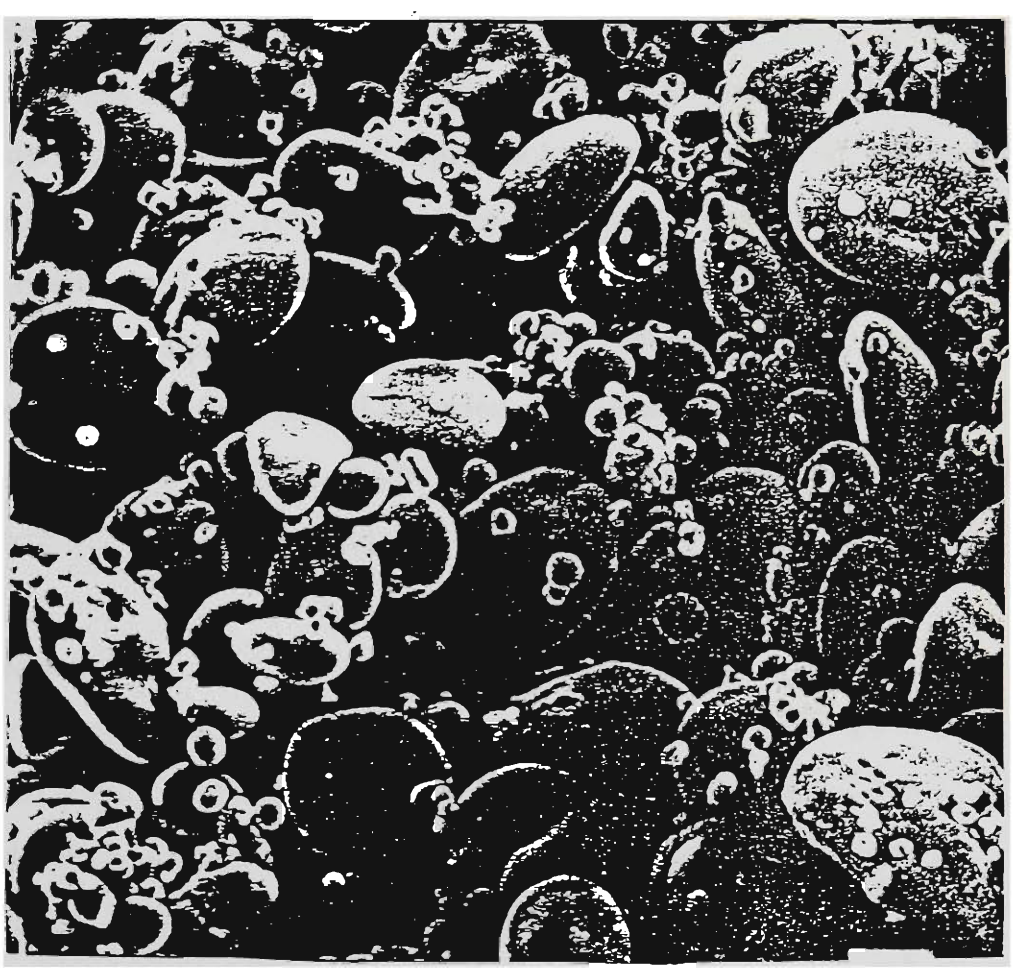


Figure 1.4 Scanning electron photomicrograph of isolated wheat starch granules (Lineback and Reasper, 1988).

Table 1.1 Composition of a typical wheat starch granule

Amylose		23 – 27%
Amylopectin		73 – 77%
Lipids (wheat)	- on surface	0.02 – 0.6%
	- in interior	0.1 – 0.2%
Proteins (wheat)	- on surface	0.006 – 0.5%
	- in interior	0.07%

Amylose

Amylose has traditionally been considered to be an essentially linear polymer of ∝-(1→4)- linked D-glucose units with a molecular weight 10^5-10^6 and an average chain length approximately 1000 glucose units (Figure 1.5; Fennema, 1976). However, it is well documented that the linear amylose molecule also contains a limited number of branch points (Banks and Greenwood, 1975; Manners, 1985). Amylose in solution has the ability to form helical-inclusion complexes with iodine, lipids, surfactants and primary aliphatic alcohols. It also has the ability to participate in strong intermolecular interactions leading to precipitation or gelation.

Amylopectin

Amylopectin is a ∝-(1→4)-D-glucan containing α-(1→6)-D-glucosidic linkages as branch points (Figure 1.5; Fennema, 1976). The average chain length of wheat amylopectin is reported in the range of 17-23 (Banks and Greenwood, 1967b; Lii and Lineback, 1977). This highly branched polymer has one of the highest molecular weights (>10^8) known among naturally occurring polymers. The basic organisation of the amylopectin chains is described in terms of the A,B and C chains as defined by Meyer and Bernfield (1940). The A chains are non-branched, the B-chains are branched at the C-6 positions and the C chains have a reducing end. Figure 1.6 illustrates the chain arrangement of amylopectin. Nowadays, the cluster structure

concept originally proposed by Robin *et al.* (1974) and French (1972) has found wide acceptance. An important feature in this model is the presence of three chain populations, with chain lengths of 15-20, 45 and about 60 (Figure 1.7; Lineback and Rasper, 1988). Similar chain lengths and distributions have been reported for a number of de-branched amylopectins from other sources. The ratio of A-chains to B-chains is about 1:1.

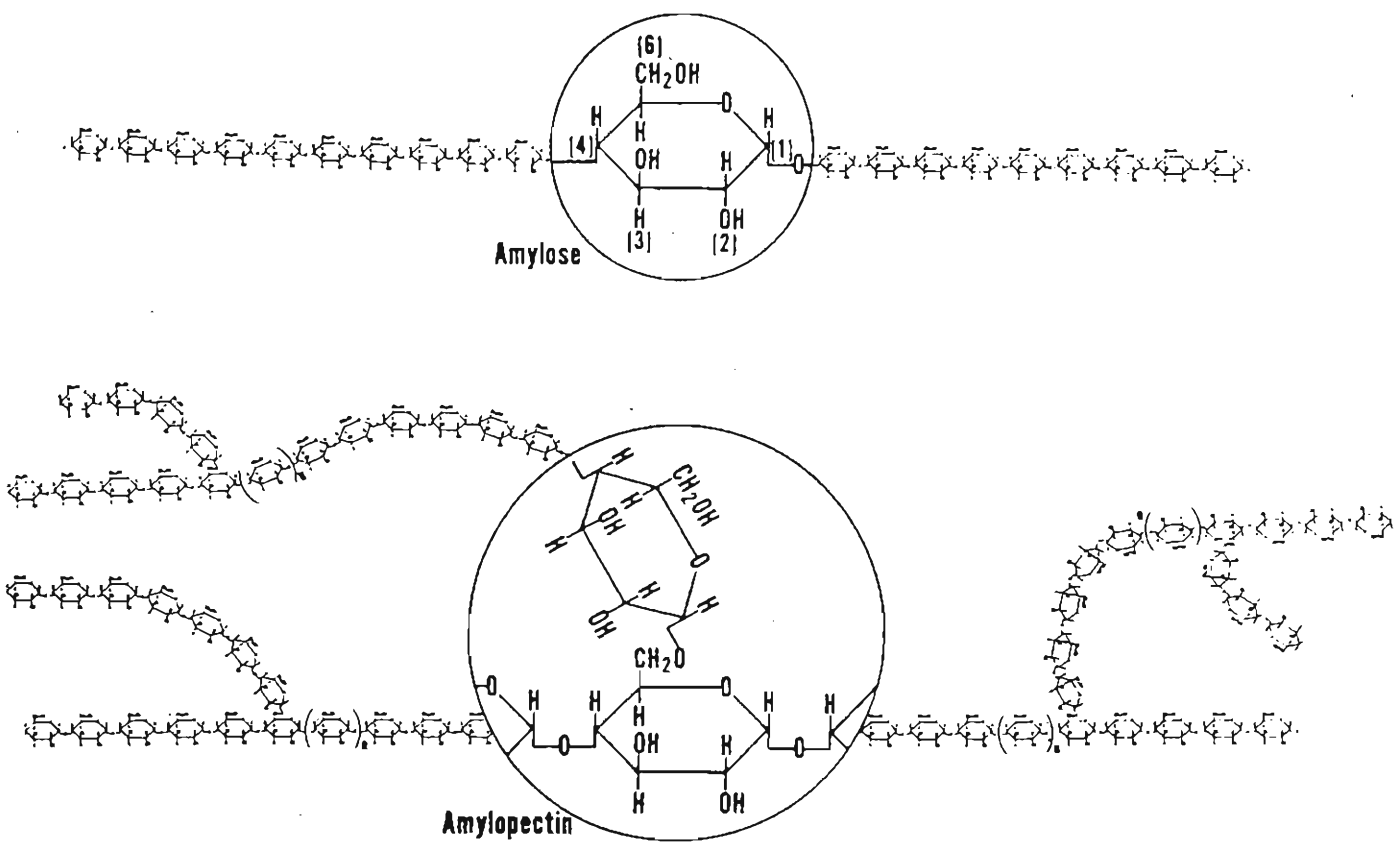


Figure 1.5 Linear and branched chains of starch fractions (Fennema, 1996).

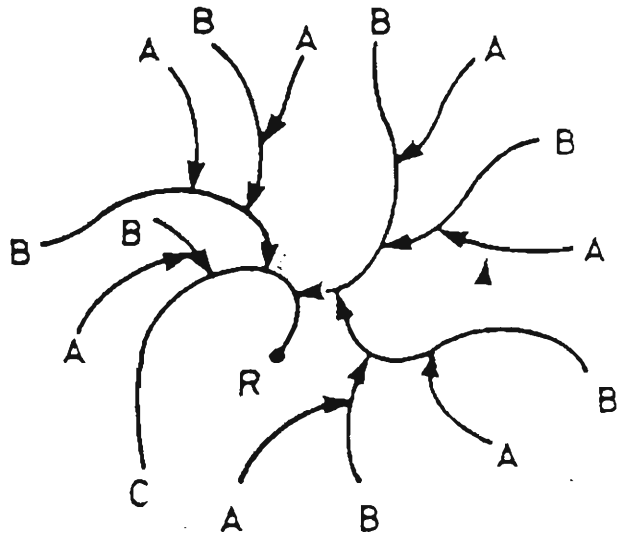


Figure 1.6 Molecular structure of amylopectin involving three types of chains (A, B and C) as described by Meyer and Bernfield (1940) (Lineback and Rasper, 1988).

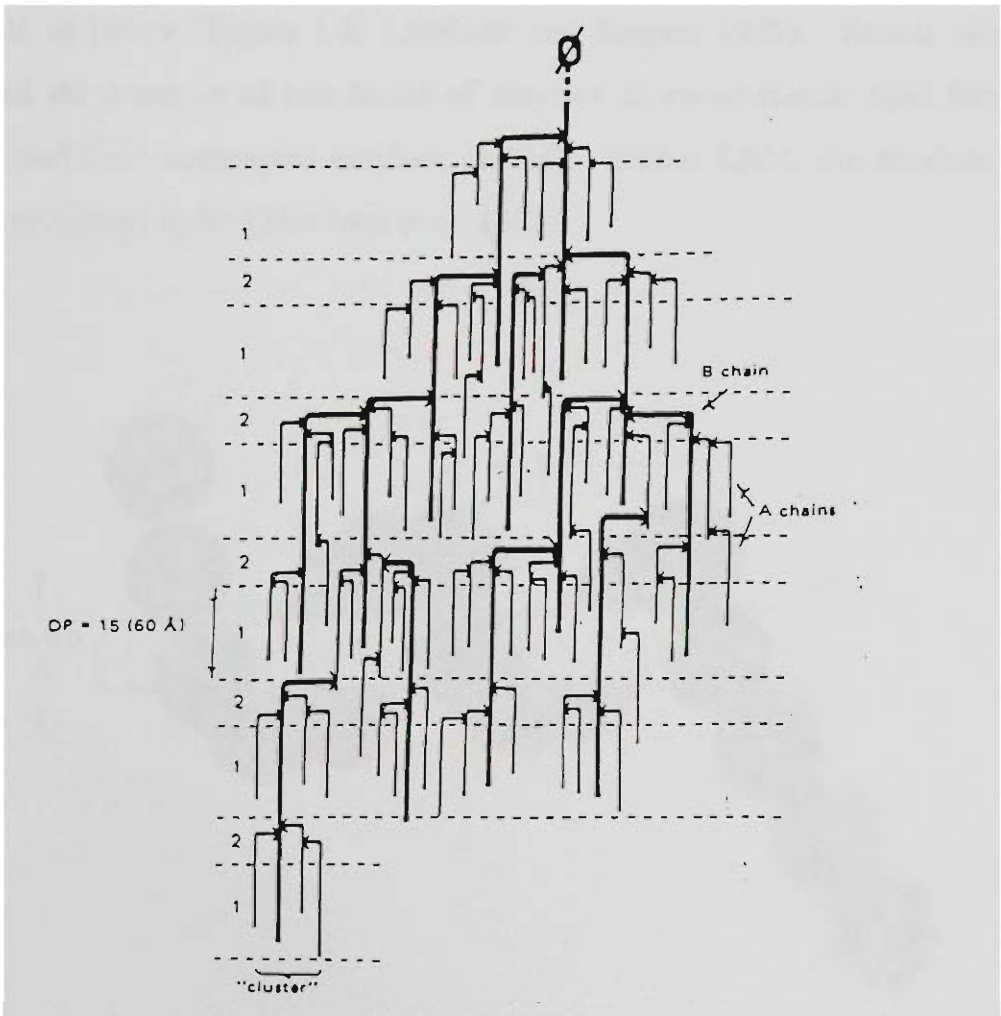


Figure 1.7 Molecular structure of amylopectin as proposed by Robin and co-workers. DP = degree of polymerisation (Lineback and Rasoer, 1988).

Starch lipids

Cereal grains are unusual in having lipids throughout the starch granules. There are three categories of lipids, based on the way they are associated with the starch granules. Lipids located inside the native starch granules are known as starch lipids (Morrison, 1981), and these are invariably monoacyl lipids. Non-starch lipids consist of membrane and spherosome lipids from the starchy endosperm, aleurone and germ (Morrison and Hargin, 1981). A third category known as starch surface lipids (Morrison, 1981) includes the monoacyl non-starch lipids, which could become firmly absorbed onto or into the starch granules, during the preparation of starch.

Starch lipids and starch surface lipids, which are monoacyl lipids, are capable of forming helical inclusion complexes with amylose. This can give rise to anomalous values for the amylose content of starch, if it is determined by the conventional iodine binding method, since the lipids occupy the same sites in the amylose helices as those available to iodine (Figure 1.8; Lineback and Rasper, 1988). Recent research has indicated the presence of two forms of amylose in cereal starch: lipid free amylose (FAM) and lipid complexed amylose (LAM). Within LAM, the amylose is coiled around monoacyl lipids (Morrison *et al.* 1993).

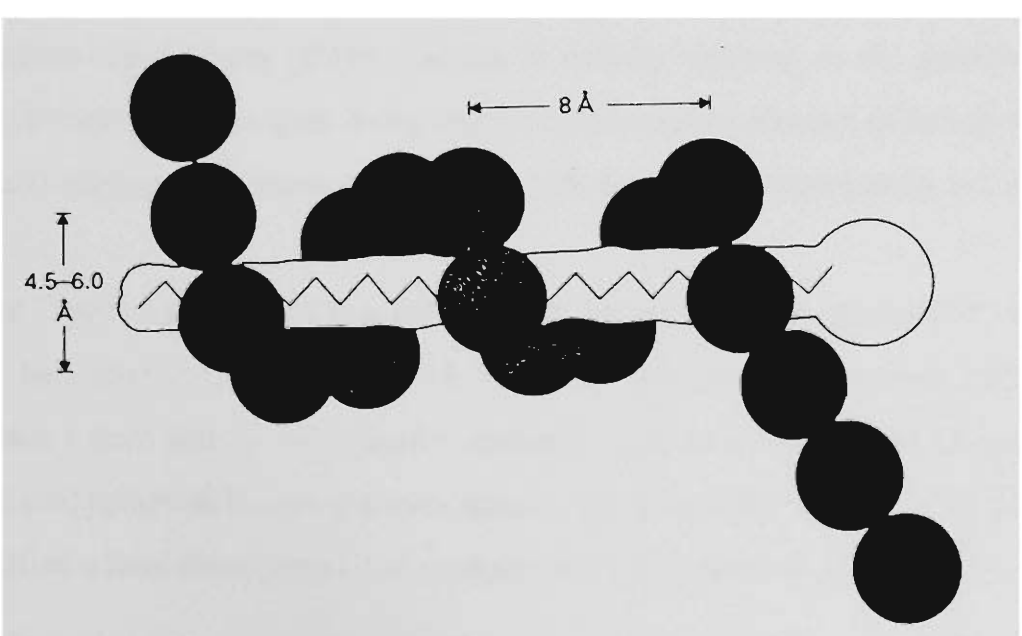


Figure 1.8 Representation of the amylose-lipid complex (Lineback and Rasper, 1988).

1.4 Starch granule initiation

The physical and molecular processes involved in the initiation of starch granules are still not well defined. According to Bechtel *et al.* (1982), early in endosperm development, rapid nuclear division occurs before cell walls are laid down and the endosperm resolves into a discrete organ. Initiation of starch granule begins during this phase and continues through the cell division and cell enlargement phases of endosperm growth (Parker, 1985; Baruch *et al.* 1979; Brocklehurst and Evers 1977). Lineback and Rasper (1988) have reported that during a relatively early stage in the biosynthesis of starch in the amyloplast, the amylose and amylopectin components being synthesised accumulate as a separate phase or coacervate droplet. At a critical point this droplet apparently undergoes spontaneous crystallisation to form the hilum of the developing grain; This is the point at which the granule begins its growth.

1.5 Starch granule organisation

The amylose and amylopectin molecules in the starch granules are radially oriented from the hilum (Lineback, 1984). A more recent model of cereal starch granule structure describes the existence of four polysaccharide fractions (Morrison and Tester, 1994). These fractions are the crystalline amylopectin, the amorphous amylopectin, the lipid-complexed amylose and the lipid-free amylose. A schematic diagram of the structure of a starch granule is presented in Figure 1.9 (Morell *et al.* 1995). The major crystalline amylopectin (CAP) fraction is radially oriented in the granule with the characteristic ring structures being formed by alternating clusters of helices formed by external amylopectin chains, interspersed with amorphous amylopectin zones (AAP).

Parker (1985) has reported two distinct populations of starch granules in wheat. The large lenticular A-type granules, 16-50 μm in diameter, are initiated in the period between 4 days and 12-14 days after anthesis. And, 14 -18 days after anthesis smaller (5-16 μm) spherical B-type granules appear. More recently, Bechtel *et al.* (1990) have identified a third class (type-C) of granules in the size range 0-5 μm.

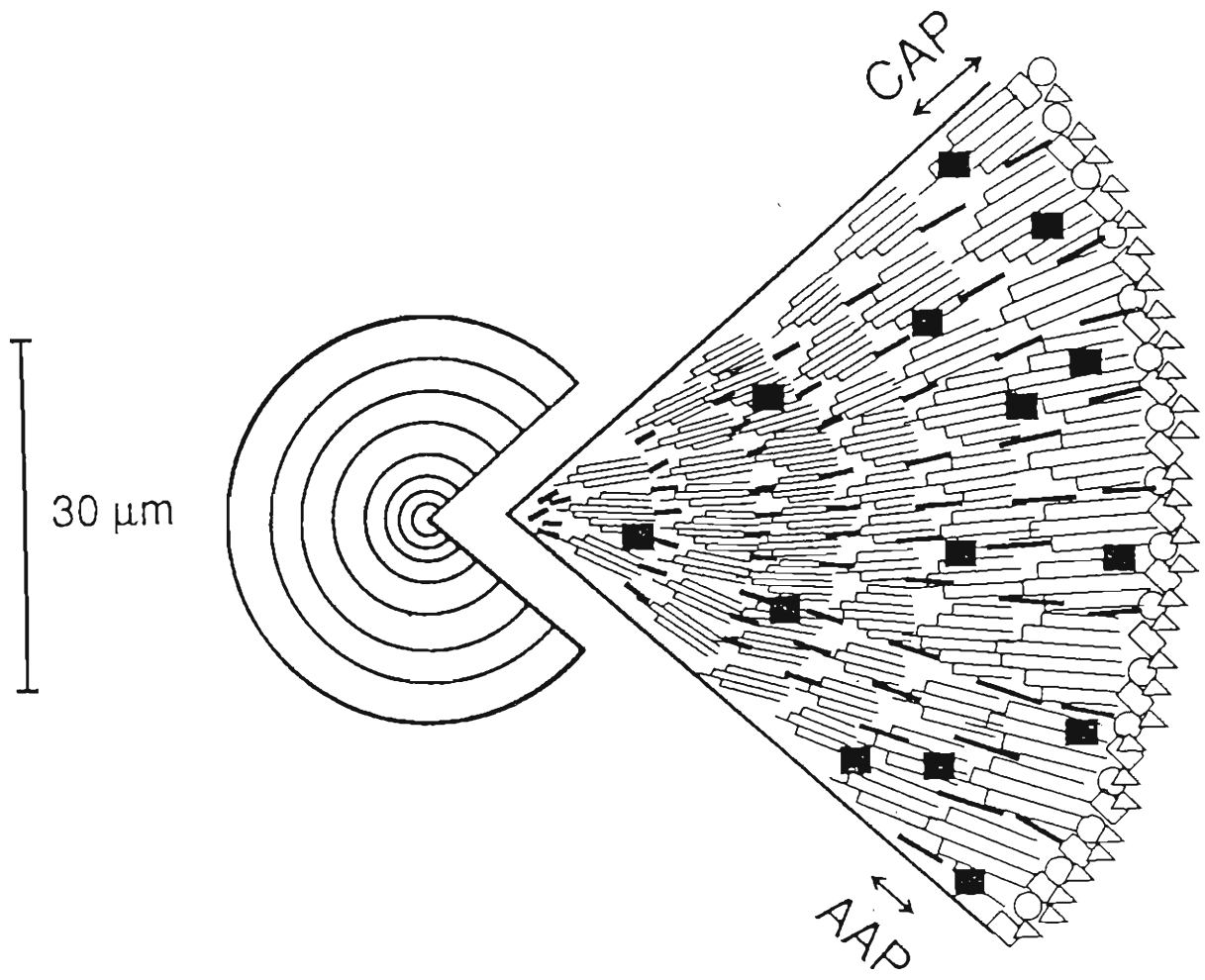


Figure 1.9 Schematic representation of starch granule structure. Amylopectin is represented by the branched structures, and amylose by the thick unbranched lines. Open symbols represent protein molecules associated with the granule surface and closed symbols represent proteins located within the granule matrix (Morell *et al.* 1995).

1.6 Uses of amylose and amylopectin in food and non-food industry

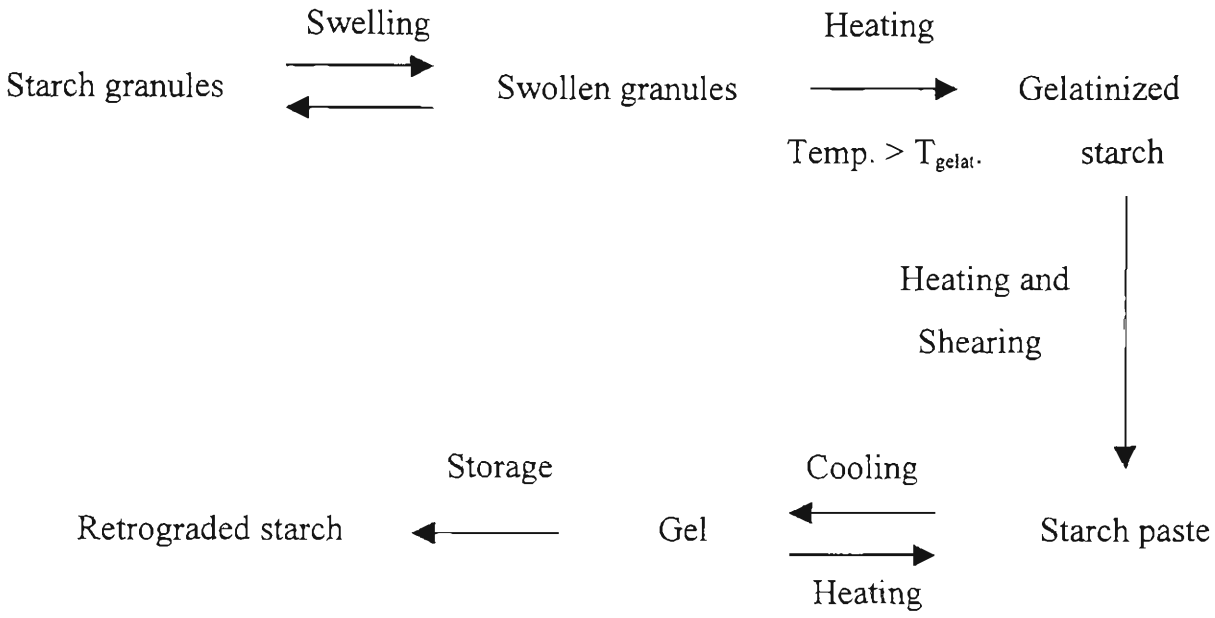
BeMiller (1973) and Powell (1973) have reviewed the industrial uses of polysaccharides. Amylose and amylopectin have found several applications in the non-food industry. In the paper industry, amylose xanthate is used for coating paper to increase its wet strength, as an adhesive for paper and as a carrier for ink. In the textile industry, highly oriented filaments are spun from amylose. Amylose fibres are also used for making medical sutures and bandages. In the food industry, amylose tubes and films are used for making edible sausage casings, and packaging food products such as instant coffee, soup and tea. Water dispersible amylose is useful in making instant pudding and gravies. High amylose cornstarch is useful as a thickener for cooked foods.

Amylopectin and unmodified waxy (amylose-free) starches when heated in water give pastes that are clear and high in viscosity. Although these starches produce a degree of stringiness, which is undesirable for food products, the starch pastes do not retrograde or gel on cooling and aging. Hence, they are of great value in foods and adhesives, where shelf-life is a problem. Unmodified waxy starches and modified waxy starches such as pre-gelatinised waxy starch, acid-thin boiled waxy starch and cross-linked waxy starch have many applications in textile, paper and food industries. In the non-food industry these starches are used in, paper manufacture, laundry starches, textile-printing pastes, photographic films and emulsions, adhesives and thermoplastics. In the food industry, the waxy starches are used in making salad dressings, white sauces, gravies, oriental soups, pie fillings, pudding mixtures, confectionery, infant foods, glazes and coatings for foods.

1.7 The importance of starch in food processing

Starch plays a structural role and contributes towards textural quality and acceptability, in starch based food products. The key features of the deposition of starch in the wheat endosperm that control functionality are starch content, grain hardness, granule size distribution and shape, the presence of endogenous lipids in the granule, amylopectin structure and the ratio of amylose to amylopectin (Rahman *et al.* 2000). The textural properties of a starch based food is, in turn, determined by the functional properties of starches which include, sheeting and gelling characteristics of starch pastes, solubility, the formation of resistant starch, and cooking and textural characteristics of whole grains.

The transformation of starch during cooking in the presence of water is shown below (Jane, 1997).



Eliasson and Larson (1993) reported on the importance of the amylose component of starch for the formation of bread crumb structure. Complete retrogradation of the amylose, has been reported by Schoch and French (1947) during baking and cooling of bread. Sundberg and Falk (1994) have observed the collapsed structure of bread-crumb made from waxy barley flour (containing only amylopectin fraction). The bread crumb structure of normal barley flour showed the normal structure, indicating the importance of amylose in the formation of breadcrumb.

In rice, the amylose content influences such characteristics as water absorption, volume expansion, dissolved solids in cooking and the gloss, stickiness and firmness of the cooked grain (Juliano, 1979). Reddy *et al.* (1993) reported amylopectin as the main determinant of rice quality. They have showed the presence of a large number of long, unbranched chains at the exterior region of the amylopectin molecule, apparently leading to the formation of strong and elastic starch granules and hence to a firm, dry, non-sticky cooked rice. Absence of such chains, on the other hand, is thought to make the granules weak and fragile and hence the cooked rice soft, moist and sticky.

Extrusion cooking process is one of the fastest growing technologies in the food industry. The percentage and the type of starch in the feed ingredients have significant effects on the extrusion process and product characteristics (Murray *et al.* 1968). Williams and Baer (1966) found that expansion of the extrudate was directly related to the starch content when comparing extrusion of corn grits. Gueriviere (1976) reported that amylose provided surface and textural regularity, elasticity and sticky characteristics to extrudates. Harper (1986) indicated that typically high amylose products, such as amylomaize, were dense and less radially expanded when extruded. Bhuiyan and Blanshard (1982) reported that corn flours and grits with a 35% amylose content expanded the best.

The effect of starch type on spaghetti cooking quality showed the importance of amylose in imparting firmness to cooked pasta (Dexter and Matsuo, 1979). Waxy maize and waxy barley starches were found to be unsuitable to spaghetti cooking quality (Dexter and Matsuo, 1979).

Glutinosity is one of the factors responsible for the palatability of Japanese noodles in which some degree of stickiness is desirable. Wheat starches that exhibit high peak viscosity (Crosbie 1991, Konik *et al.* 1992) have been associated with the desirable soft and elastic eating quality of Japanese white salted noodles (Miura and Tanii, 1994) and to high quality Korean white salted noodles (Lee *et al.* 1987). These starches have also been associated with a lower amylose content (Yamamori *et al.* 1992, Nakamura *et al.* 1993).

The examples provided above thus indicate that the amylose content of starch is an important quality parameter for starch processing.

1.8 Fractionation of amylose and amylopectin in starch

1.8.1 Aqueous leaching

Fractionation by leaching involves selectively removing the amylose from the starch granule by holding the granule in an aqueous suspension at or slightly above the gelatinisation temperature of the starch. After appropriate heating, the solubilised amylose is separated from the granule residue by centrifugation and is recovered from the supernatant solution by precipitation with butanol (Whistler, 1965). Major problems reported with this procedure are that the amylose isolated is often contaminated with small amounts of amylopectin and normally all the amylose is not leached from the granule.

1.8.2 Dispersion of the starch granule

Several methods have been used to destroy the internal organisation or structure of the starch granule and to disperse the amylose and amylopectin components. These include autoclaving in aqueous solution, treating with liquid ammonia, solubilising in aqueous alkali and solubilising in dimethyl sulphoxide (Lineback and Rasper, 1988). Banks and Greenwood (1967b) developed a general procedure for the fractionation of amylose and amylopectin from cereal starches, which involved dispersion of the starch in DMSO and precipitation of the amylose as the amylose-butanol complex. Amylopectin was obtained from the supernatant solution by precipitation with alcohol or by freeze-drying. Many other compounds such as thymol and nitro-methane form complexes that can be used in the separation of amylose from aqueous starch dispersions. Such complexation methods are unsuitable for a number of starches, such as those from immature cereal seeds (Matheson, 1971), leaf starches and high amylose starches (Banks and Greenwood, 1975). In each of these starches, the amylose fraction also contains amylopectin. In the leaf starches and high amylose starches, the amylopectin fraction includes amylose of low molecular size.

1.8.3 Lectins in starch fractionation

Carbohydrate-binding proteins from plant sources have gained prominence in recent years as analytical probes for the detection and preliminary characterisation of complex carbohydrates in solution. These proteins, also referred to as lectins and

phytohaemagglutinins, have been shown to interact with carbohydrates, with the formation of an insoluble precipitate. Many of these interactions are highly specific in nature. For example, the lectin concanavalin A (con A) isolated from *Canavalia ensiformis* (jack bean lectin) interacts with non-reducing terminal alpha-D-glucosyl group (Goldstein and Hayes, 1978). Two other lectins which show similar carbohydrate specificity as con A have been isolated from *Lens culinaris* (lentil lectin) (Howard *et al.* 1971) and *Pisum sativum* (pea lectin) (Trowbridge, 1974).

Matheson and Welsh (1988) have reported on the use of the lectin con A to separate the starch fractions. Elution profiles of the starch fractions using the Gel Permeation Chromatography (GPC) method showed that a more effective fractionation of starches could be obtained from potato tuber, rice grains, tobacco leaves and high-amylose pea seeds, by con A than by complexing with 1-butanol (Matheson and Welsh, 1988). The lectin has the ability to bind amylopectin due to its affinity to non-reducing end groups of polysaccharides, and separates starch into the highly branched (amylopectin) and unbranched or lightly branched (amylose) fractions.

1.9 Quantitative determination of amylose and amylopectin in starch

1.9.1 Iodine complex methods

Most commonly used methods, for determination of amylose, are based on the iodine binding capacity of amylose. These methods include, in general, amperometric and colorimetric assays (Williams *et al.* 1970; Banks and Greenwood, 1975; Juliano, 1971; Morrison and Laignelet, 1983) and are based on the formation of an intense blue iodine inclusion complex with amylose. However, these methods are subject to uncertainties because iodine may also form complexes with the long B chains of amylopectin, which absorb at similar wavelengths to the amylose-iodine complex (Takeda *et al.* 1987; Banks and Greenwood, 1975) and would over estimate the amylose content. On the other hand, the presence of short chain amylose (DP < 200) would underestimate the amylose content (Morrison and Laignelet, 1983). Hovenkamp-Hermelink *et al.* (1988) reported a two-wavelength spectrophotometric method for determining amylose/amylopectin ratios in potato leaf and tuber tissues.

This method was improved further by Jarvis and Walker (1993), by measuring the ratio at six wavelengths. The latter two methods were developed to reduce colour interferences with amylopectin.

1.9.2 Chromatographic methods

Many chromatographic methods have been employed to separate the polysaccharide components of starch. Smykova and Stepanenko (1969) have reported the separation of polysaccharide components of starch by traditional column chromatography. Adsorption chromatography on cellulose column has been used by Patil and Kale (1973) to fractionate the starch components in potato. Karve *et al.* (1981) reported on the separation of starch components by affinity chromatography on cross-linked gelatine granules in acetate buffer (pH 4.8, 0.1 M) containing urea, iodine, KI and SDS. These methods however are tedious and time consuming.

More recently, size-exclusion chromatography (SEC) has been employed to elucidate the profiles of starch components. Matheson (1971) and Yamada and Taki (1976), investigated the fractionation of wheat and maize starch respectively, on an agarose gel column. Praznik *et al.* (1986) used a system consisting of two columns, sephacryl gels S-500 and S-1000 to separate potato starch. Wang *et al.* (1993) reported the fractionation of maize starch on sepharose CL-2B gel. For rapid analysis, Kobayashi *et al.* (1985) developed a high performance size-exclusion chromatography (HPSEC) system using a two-column system (E-linear and E-1000 m-bonda gel) and dimethylsulphoxide as the mobile phase, to separate corn and wheat starches. Jane *et al.* (1992) found that the amylose content of taro starches measured by the SEC method were, in general, greater than those obtained from the potentiometric titration method. The difference have been attributed to the presence of the intermediate components, molecules with branched structures and sizes smaller than amylopectin, which were eluted at the same time as amylose. Thus, accurate measurement of the amylose content was difficult to achieve in these SEC methods due to the limited resolution of the polysaccharide components. Fishman and Hoagland (1994) have reported that, by using SEC in combination with on-line viscosity detection, refractive index detection and curve fitting of the chromatograms, amylopectin and amylose can

be determined quantitatively in each other's presence. Size-exclusion chromatography followed by enzymic de-branching of starch, has been performed by Sargeant (1982), Praznic *et al.* (1994) and Salomonsson (1994). These are useful for fine structure studies but are not adaptable to large sample numbers (Gibson *et al.* 1997). Batey and Curtin (1996) developed a size-exclusion high performance liquid chromatography method using hydrophilic columns, to quantitate amylose/amylopectin ratio. It has been reported that samples with amylose content higher than 40% always showed some precipitation. The use of isoamylase also can change the starch by de-branching and hence the actual molecular entities in native starches are not examined in these methods.

1.9.3 Electrophoretic methods

Electrophoresis has been widely used to separate macromolecules of various types, including polysaccharides. The technique offers several advantages in terms of ability to run multiple samples concurrently. Thus it has a great potential for determining the amylose/amylopectin ratio and providing further information on the molecular species present in starches. However, few reports have been published on the use of electrophoresis to separate and quantify starch components. Northcote (1954) has reported using a moving boundary electrophoretic method using the Tiselius apparatus for the analysis of neutral polysaccharides, including potato, rice, wheat and waxy maize starch, using borate buffer (0.05 M, pH 9.2). In this study, the polysaccharides moved towards the anode, and each starch sample showed two boundaries except that from waxy maize, which gave a single sharp peak. This method requires complex equipment and is very time consuming. Foster *et* al. (1956) described the zone electrophoretic behaviour of potato amylose and amylopectin on paper, using borate buffer at a pH of 10. Although both polysaccharides migrated toward the anode, the amylose remained at the origin and showed only slight migration. The polysaccharides were detected on the neutralised paper by spraying it with ethanolic iodine. A technique known as fluorophore-assisted carbohydrate electrophoresis, developed by Jackson (1991), separates fluorescently-labelled oligosaccharides on polyacrylamide gels. This technique is suitable for carbohydrate chain length analysis but involves lengthy derivatisation steps. Based on this technique Oshea and Morell

(1996) developed an electrophoretic method for the analysis of oligosaccharides using DNA sequencer technology. Malto-oligosaccharide distributions were obtained following isoamylase digestion of glycogen, wheat starch and potato starch. A capillary electrophoresis method was developed by Brewster and Fishman (1995) to resolve amylopectin and amylose from starches, using iodine containing buffers in unmodified capillaries. However, separation of the starch components was not optimised and further work is required to improve reproducibility. Oshea *et al.* (1998) developed a capillary gel electrophoretic method using laser-induced fluorescence (LIF) detection to fully resolve and quantify malto-oligosaccharides up to approximately dp 100. An agarose gel electrophoresis method was developed in the present work and described in chapter 3 (Jegasothy *et al.* 1995). This method allows separation of amylose and amylopectin in starch, using iodine staining and laser densitometry to visualise and quantify the components.

1.9.4 Other methods

A Differential Scanning Calorimetry (DSC) method for the determination of amylose as its amylose-L-alpha-lysophosphatidyl choline (LPC) complex has been described by Sievert and Holm (1993). They reported that the amylose content of various native starches, rice flours, wheat flours and lyophilised raw potato could be predicted on the basis of the melting enthalpy of their amylose-LPC complexes, using a standard linear calibration. Near-infra red reflectance (NIR) spectroscopy has been used for amylose determination of rice flours (Bean *et al.* 1990) and near-infra red transmittance (NIT) spectroscopy has been used for amylose determination of unground brown rice or milled rice (Villareal *et al.* 1994). These methods require a reference method for calibration.

The use of lectin concanavalin A for quantitation of amylose has been developed by Matheson and Welsh (1988). This method has been modified recently by Gibson *et al.* (1997). The method is based on the precipitation of amylopectin from a lipid free starch sample with the lectin, followed by the determination of amylose as glucose after hydrolysis (for details see Section 2.4.4.2). The lectin approach is potentially the

most accurate in that it specifically measures amylose after selective removal of amylopectin from lipid free starch. However, the method is complex and tedious.

1.10 Starch biosynthesis pathway in cereals

Starch biosynthetic enzymes in leaf tissue are localised in the chloroplast while in non-photosynthetic tissue they are localised in the amyloplast (Preiss, 1991). Amyloplasts, a specialised subcellular organelle that has a limiting double lipoprotein membrane (Badenhuizen, 1969), are present in the plant storage cells that accumulate starch. The enzymes found within the amyloplasts catalyse the biosynthesis of amylose and amylopectin from the starting material sucrose. The assembly of the starch polymer chains takes place on a lipoprotein matrix (Duffus, 1979).

Original mechanism for starch biosynthesis

Although the current prevailing view is that variable mechanisms operate in different plant tissues and organs, it is often argued that the enzymic steps described below are essential in the overall step of sucrose to starch conversion (Pozueta-Romera, 1999).

The original mechanism proposed for starch biosynthesis in cereal endosperm is summarised in Figure 1.10. This figure shows the pathway of carbon interconversion from sucrose entering the cytosol through to the biosynthesis of starch in cereal endosperm (Morell *et al.* 1995b). Sucrose derived either directly from photosynthesis or following the mobilisation of stem reserves is the predominant form of carbon used for the growth and development of the endosperm (Morell *et al.* 1995b). The initial hydrolysis of sucrose is catalysed primarily by sucrose synthase in the cereal endosperm (Chourey and Nelson, 1979). Subsequent synthesis of hexose phosphate, from the products of the sucrose synthase hydrolysis reaction, is carried out by a series of enzymes; UDPglucose pyrophosphorylase (Duffus, 1992; Turner, 1969) and a hexokinase with fructose phosphorylating activity (Higgins and Easterby, 1976). These enzymes have been shown to be very active in wheat endosperm. Carbon uptake and utilisation studies using both isolated amyloplasts and isolated endosperm tissue have been consistent with the view that hexose-phosphates are the predominant carbon compounds translocated into the amyloplasts to support starch biosynthesis

(Keeling *et al.* 1988; Neuhaus *et al.* 1993). Starch is predominantly synthesised within the amyloplast by a pathway involving the enzymes ADPglucose pyrophosphorylase (EC 2.7.7.27; reaction 1), starch synthase (EC 2.4.1.21; reaction 2) and branching enzyme (EC 2.4.1.18; reaction 3) (Preiss, 1991), as shown below,

1) α-glucosyl-1-P + ATP $\longleftrightarrow$ ADPGlc + PP_i

2) ADPGlc + $(Glucosyl)_n$ $\longrightarrow$ ADP + $(glucosyl)_{n+1}$

3) Linear glucose chain of α-glucan $\longrightarrow$ Branched chain of α-glucan with α-1$\rightarrow$ 6 linkage branch point

The starch granule continues to develop in the amyloplast until its internal volume is completely occupied by starch, as seen in the fully developed granule.

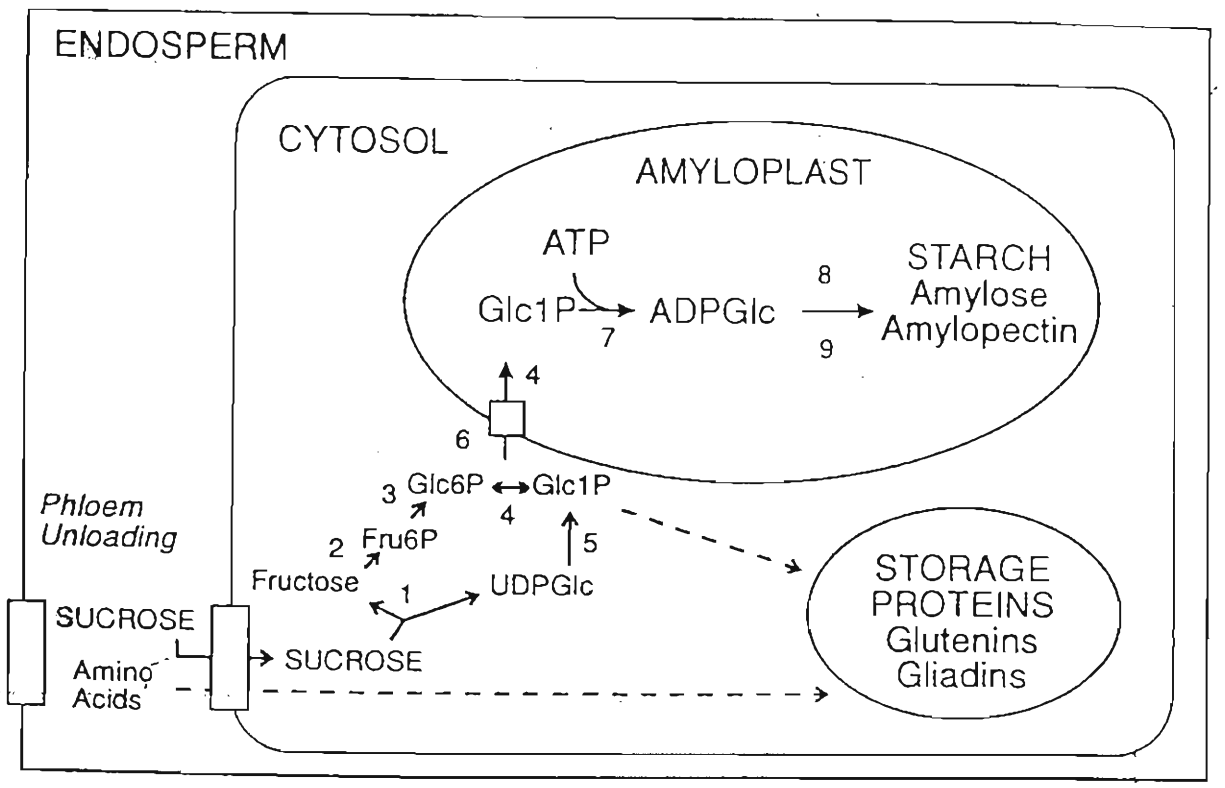


Figure 1.10 Proposed pathway of carbon incorporation into starch and protein in cereal endosperm. 1. Sucrose synthase; 2. Fructokinase; 3. Phosphoglucose isomerase; 4. Phosphoglucomutase; 5. UDPglucose pyrophosphorylase; 6. Hexose phosphate translocator 7. ADPglucose pyrophosphorylase; 8. Starch synthase; 9. Branching enzyme (Morell *et al.* 1995).

Newly proposed mechanism for starch synthesis

Although it was initially believed that ADPglucose pyrophosphorylase is present exclusively in the amyloplast compartment, the presence of a cytosolic enzyme has been shown in some cereals. The presence of an ADPGlc-specific translocator in the amyloplast has been demonstrated in a number of plant sources, which indicates the potential role of ADPGlc-synthesising machineries located in the cytosol of starch-storing cells (Pozueta-Romero *et al.* 1999). In maize and barley it has been clearly shown that cytosolic ADPglucose pyrophosphorylase is the major contributor to starch biosynthesis, however, the situation in wheat is yet to be resolved (Rahman *et al.* 2000). The newly proposed mechanism for starch synthesis (Figure 1.11) is based on studies in species such as maize, pea, potato and chlamidomonas and the principles of synthesis are expected to be similar in wheat (Rahman *et al.* 2000).

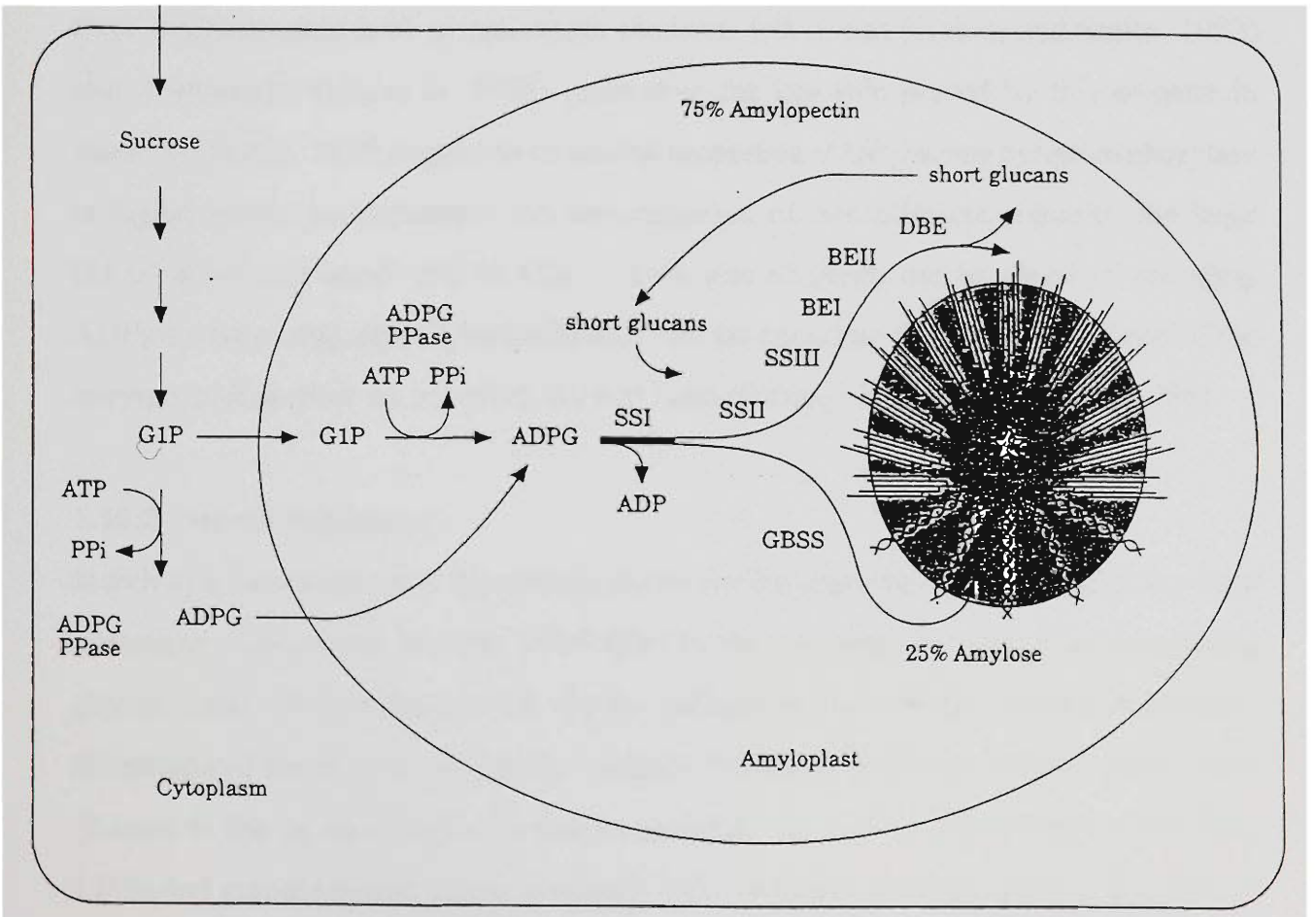


Figure 1.11 The newly proposed mechanism for starch biosynthesis in cereals (Rahman *et al.* 2000).

1.10.1 ADPglucose pyrophosphorylase

The formation of ADPglucose from glucose-1-phosphate and ATP, the first committed step in the starch biosynthetic pathway, is catalysed by the enzyme ADPglucose pyrophosphorylase. The enzyme was originally believed to be specifically localised within the amyloplast (Preiss, 1991). However, more recently barley and maize have been shown to possess a cytosolic ADPglucose pyrophosphorylase in addition to that in plastid (Pozueta-Romero *et al.* 1999).

The invivo evidence supporting the function of ADPglucose pyrophosphorylase in starch biosynthesis can be traced back to the pioneering work of Tsai and Nelson (1996) who found a selective defect in ADP-glucose pyrophosphorylase in the *sh2* (shrunken 2) mutants of maize that displayed a 70% reduction in starch content. Low starch mutants with reduced levels of ADPglucose pyrophosphorylase was isolated in three different species of plants, maize (Nelson, 1985), pea (Hylton and Smith, 1992) and *Arabidopsis* (Lin *et al.* 1988), indicating the key role played by this enzyme in starch synthesis. With respect to structural properties, ADPglucose pyrophosphorylase in higher plants are tetrameric and are composed of two different subunits, the large (55-60 kDa) and small (50-55 kDa). Two sets of genes are involved in encoding ADPglucose pyrophosphorylase subunits, one set encoding the endosperm form of the enzyme, and another set encoding the leaf form (Preiss, 1991; Krishnan *et al.* 1986).

1.10.2 Starch synthases

Starch synthase, a glucosyl transferase, catalyses the stepwise addition of glucose from adenosine diphosphate glucose (ADP-Glc) to the non-reducing end of an elongating glucan chain. Within the plastid, starch synthase exists both as a soluble enzyme in the stroma of the plastid and tightly bound to the starch granules (Preiss, 1988). ADP glucose is the *in vivo* sugar nucleotide substrate for both soluble (Preiss and Levi, 1980) and granule-bound starch synthases (MacDonald and Preiss, 1983). Recently it has been reported that in cereals there appear to be at least four classes of starch synthases important to starch synthesis in the endosperm: granule bound starch synthase (GBSS), starch synthase I (SSI), starch synthase II (SSII) and starch synthase III (SSIII) (Rahman *et al.* 2000). GBSS is essential for amylose synthesis. The

enzymes SSI, SSII and SSIII are predominantly in amylopectin synthesis (Rahman *et al.* 2000). In wheat, a 75 kDa starch synthase known as SSI, is partitioned between the soluble fraction and the granule. And, a group of three polypeptides of 100, 108 and 115 kDa apparent weight have been clearly shown to be starch synthase (Rahman *et al.* 2000).

Soluble starch synthase

Studies with different plant systems have indicated the presence of two major forms of soluble starch synthase designated as types I and II (Preiss, 1991). Soluble starch synthases have been purified from rice (55 and 57 kDa, Baba *et al.*1993), pea (77 and 60 kDa, Denyer and Smith 1992), maize (76 kDa, Mu *et al.* 1994) and potato (70 kDa, 85 kDa, 135 kDa, Koβmann, 1997).

The enzyme is highly sensitive to elevated temperature. Rijven (1986), Jenner and Hawker (1993) and Keeling *et al.* (1993) have shown that in wheat, high temperatures depress soluble starch synthase levels leading to a suppression of starch deposition and therefore grain filling. Jenner (1994) has reported that high temperature reduces the affinity of the enzyme for its substrates in Kalyanso wheat. The enzymatic activity of SSS can also be reduced by mutation in SSS genes as shown in barley or pea (Tynela and Schulman, 1993). Recent studies in transgenic potatoes (Koβmann, 1997) have shown that repression of the SSS-III gene encoding the 135 kDa SSS, reduced the amylose and phosphate contents and altered the gelation characteristics of the starch. However, specific inhibition of SSS-I gene encoding the 70 kDa SSS, did not change any significant structural properties and the repression of the SSS-II gene encoding the 85 kDa SSS led only to slight changes in the starch structure. The results of the study indicate that the different SSS enzymes might have different roles in starch synthesis.

Granule bound starch synthase

See Section 1.11.1.

1.10.3 Branching enzyme

Starch branching enzyme (SBE) introduces branches into the glucan polymer by cleaving the donor linear ∝-1,4 glucan chain (either amylose or a linear region of amylopectin) and attaching to a recipient chain via ∝-1,6 linkage. This is a very important reaction in determining starch quality parameters, because the rate and specificity of this reaction influences both the amylose/amylopectin ratio and the fine structure of amylopectin.

In transgenic potato plants created by introduction of the antisense RNA of the SBE gene, the repression of the native SBE gene altered the structure of some soluble glucans. However, it did not result in an increase in the amylose content of starch granules (Koβmann, 1997). These observations indicate the existence of additional SBE genes which could compensate for the repressed SBE gene. In most of the higher plants, two forms of branching enzymes are found, branching enzyme I and II. Studies on purified maize branching enzymes I and II demonstrated that these isoforms differed in their selectivity for the chain length transferred (Takeda *et al.* 1993). Maize branching enzyme I preferentially transfers longer chains than maize branching enzyme II. A decrease in the activity of branching enzyme II was observed in the high amylose mutants of maize (Hedman and Boyer, 1982), rice (Mizuno *et al.* 1993) and pea (Bhattacharyya *et al.* 1990). This suggests the importance of branching enzyme II in amylopectin biosynthesis.

Wheat endosperm also contains two isoforms of branching enzymes, WBE-I and WBE-II. Based on the analyses of homology of their deduced amino acid sequences these have been shown to be homologous to maize branching enzymes I and II respectively (Morell *et al.* 1995a). It has also been found that the homologues of maize SBE-I are pea SBE-II, rice SBE-I and potato SBE-I and that the homologues of maize SBE-II are pea SBE-I, rice SBE-III and potato SBE-I (Morell *et al.* 1995a; Smith *et al.* 1997)

1.10.4 Starch de-branching enzyme

Pan and Nelson (1984) have reported the involvment of starch de-branching enzyme in the synthesis of starch, in a maize mutant variety. These authors have suggested that the equilibrium between the activities of starch branching and de-branching enzymes controls the amylopectin component in starch. Ball *et al.* (1996) and Nakamura (1996) have suggested that they trim excess branches in the pre-amylopectin to yield amylopectin and short glucan chains. The released glucan chains may be converted into amylose molecules by the action of GBSSI. However, further research is required on starch de-branching enzyme.

1.11 Molecular genetics of starch synthesis

1.11.1 Granule-bound starch synthase (waxy protein)

The enzyme granule-bound starch synthase (GBSS), also known as the waxy protein, has a molecular weight of about 60 kDa and controls the synthesis of amylose in endosperm of cereals (Echt and Schwartz, 1981; Sano, 1984; Klosgen *et al.* 1986; Sivak *et al.* 1993). Nelson and Rhine (1962) have reported that the waxy protein or granule–bound starch synthase is responsible for amylose synthesis in storage tissue. In maize, a 65 kDa precursor polypeptide of waxy protein is processed to its mature size of 58 kDa during transport into amyloplasts (Shure *et al.* 1983). Similarly, the *in vitro* translation product of rice *waxy* transcript was identified as a 64 kDa precursor polypeptide, processed to its mature size of 60 kDa during transport into amyloplast (Sano, 1984). Waxy protein was first detected in wheat by SDS-PAGE as a 59 kDa protein (Schofield and Greenwell, 1988). Yamamori *et al.* (1992) detected the waxy protein of wheat, with the Laemmli's SDS-PAGE system, as a single 61 kDa band.

Starch granules isolated from the developing or mature endosperm of wheat contain two types of protein, those that are embedded within the starch granule and those that have come in contact with the starch granule and are exclusively associated with its surface (Schofield and Greenwell, 1987; Rahman *et al.* 1995). The enzymes embedded within the starch granules have been studied extensively and are believed to have important functions in starch synthesis. Electrophoretic analysis indicate that these enzymes fall into major groups based on their molecular weights. These include

the 60 kDa granule-bound starch synthase (GBSS I or the waxy protein), a 75 kDa starch synthase (SSS), a 85 kDa starch branching enzyme (SBE) and 100 - 115 kDa starch synthase (Baga, 1999). These polypeptides were demonstrated to be within the starch granule by their resistance to proteinase K digestion when granules were ungelatinised (Rahman *et al.* 1995). Among these the 60 kDa protein GBSS I, was found to be the most abundant protein embedded within starch granules and was exclusively granule-bound in endosperm of wheat. On the basis of the results described using Western blotting experiments (Taira *et al.* 1995; Rahman *et al.* 1995) and the sequences of amino acids at the N-terminus (Denyer *et al.* 1995; Taira *et al.* 1995; Rahman *et al.* 1995; Fujita *et al.* 1996), the 60 kDa protein has been confirmed to be starch synthase, in different types of wheat.

Since the *waxy* gene in common wheat is triplicated (see section 1.11.2), the single waxy protein band was assumed to comprise three products coded by the three *waxy* genes. A modified SDS polyacrylamide gel electrophoresis (SDS-PAGE) containing a lower concentration of bis acrylamide, separated the wheat waxy proteins into two bands, a high molecular weight (HMW) band and a low molecular weight (LMW) protein band (Kagawa *et al.*1988). Nakamura *et al.* (1992) analysed several Chinese Spring and Japanese wheat cultivars and suggested that the HMW protein was encoded by the 7A *waxy* gene and the more dense LMW protein to be a mixture of proteins produced by the 7B and 7D *waxy* genes. Subsequently, a modified two-dimensional gel electrophoresis (2D-PAGE) enabled to identify the three individual waxy proteins, which were found to have slightly different molecular weights and isoelectric points (Nakamura *et al.* 1993a). Hence, due to the allohexaploid nature, common wheat has three waxy proteins, which are encoded by the homoeologous *waxy* genes located on the group 7 chromosomes. The *Wx-A1* gene encodes for Wx-A1 protein, the *Wx-B1* gene (4A *waxy* gene) encodes for Wx-B1 protein and *Wx-D1* gene encodes for Wx-D1 protein.

Fujita *et al.* (1996) reported on the comparison of waxy protein sequences in polyploid wheats with those in related diploid species, which revealed that (i) waxy protein encoded by the A genome of polyploid wheats were identical to that of *T.*

monococcum (AA), (ii) the waxy protein encoded by the B genome of *T. turgidum* (AABB) was identical to that of *T. searsii* (S^sS^s), but differed from those of *T. speltoides* (SS) and *T. longissimum* (S^1S^1) by one amino acid substitution, (iii) the waxy protein encoded by the B genome of *T. aestivum* (AABBDD) differed from that encoded by the B genome of *T. turgidum* by one amino acid substitution; this point mutation is considered to have occurred after the establishment of hexaploid wheat, and (iv) the waxy protein encoded by the D genome of *T. aestivum* was identical to that of *T. tauschii* (DD).

1.11.2 *Waxy* gene

The *waxy* gene encodes for the waxy protein (GBSS) (Tsai, 1974) and is responsible for the synthesis of amylose in endosperm. In diploid plants such as maize (Klosgen *et al.* 1986), barley (Rhode *et al.* 1988) and rice (Wang *et al.* 1990; Hirano and Sano, 1991), the single *waxy* gene has been cloned and sequenced. Unlike other cereals, common wheat is allohexaploid and the *waxy* locus is triplicated. Chao *et al.* (1989) reported the presence of three homoeologous *waxy* genes in common wheat by Restriction Fragment Length Polymorphism (RFLP) mapping. These are the *Wx-A1*, *Wx-B1* and *Wx-D1* genes, located on the chromosomes 7A, 4A [to which part of the short arm of 7B is translocated (Naranjo *et al.* 1987)] and 7D. This makes the analysis of the products of the three individual *waxy* genes complicated. A cDNA clone of the *waxy* gene from common wheat has been isolated and sequenced (Clark *et al.* 1991; Ainsworth *et al.* 1993), but the correspondence between *waxy* genes and sequenced genes was not established. However, the sequence exhibited a high degree of homology with the *waxy* gene sequences of other cereals. A comparison of the primary structures of the *waxy* gene products (waxy proteins) among diploid cereals, including diploid wheat, rye, barley, rice and corn, indicated that the *waxy* locus had been highly conserved during the evolution of cereals (Taira *et al.* 1995).

More recently it has been reported that granule-bound starch synthase I (waxy protein) and II are encoded by separate genes that are expressed in different tissues (Vrinten and Nakamura, 2000). GBSS II is thought to be responsible for the elongation of amylose chains in non-storage tissues. The GBSS II genes were mapped to

chromosomes 2AL, 2B and 2D. Gel blot analysis indicated that genes related to GBSS II also occur in barley, rice and maize.

1.11.3 Expression of *waxy* genes

In wheat and rice the *waxy* gene is actively expressed throughout the grain filling period, where the *waxy* transcripts accumulate to higher levels and is temporarily regulated during seed development (Ainsworth *et al.* 1993; Hirano and Sano, 1991). Okagaki and Wessler (1988) have used northern blot analysis to detect the *waxy* transcripts in rice and other grasses, using maize *waxy* gene probes; the probes hybridised with a 2.4 kb transcript from maize, rice, millet and wheat, and a 2.6 kb transcript from sorghum. Wang *et al.* (1995) examined 31 rice cultivars, and reported the presence of three distinct patterns of *waxy* gene transcript accumulation. The high amylose cultivars (group I) contained a mature 2.3 kb *waxy* transcript. Intermediate level amylose cultivars (group II) indicated 10-fold lower levels of mature *waxy* transcript compared to the group I cultivars, and in addition a large 3.3 kb *waxy* transcript. Glutinous rice (group III), did not contain the 2.3 kb transcript, but exhibited the 3.3 kb transcript. Wang *et al.* (1995) suggested that, the presence of the 3.3 kb transcript was due to the failure to excise intron 1 from the *waxy* gene transcript. Hence it has been suggested, in rice, the excision of intron 1 from the 5' leader region of the *waxy* transcript plays an important role in regulating *waxy* gene expression.

1.11.4 Partial waxy mutants of wheat

A waxy (glutinous) mutant is characterised by the production of starch granules containing only amylopectin and no amylose. Such mutants have been identified in many cereals such as maize, rice, barley, sorghum and amaranth. Analysis of the proteins associated with the starch granule showed that waxy protein was absent in these waxy mutants (Echt and Schwartz, 1981; Sano, 1984; Rhode *et al.* 1988; Hseih, 1988; Okuno and Sakaguchi, 1982).

Waxy proteins were studied in wheat cultivars obtained from several countries and mutants lacking one or two waxy proteins have been identified (Nakamura *et al.* 1992,

1993; Yamamori, 1994). These mutants, which carry one or two null alleles at the *waxy* locus were termed "partial waxy mutants" The null allele for the Wx-A1 protein was found to occur frequently in Korean, Japanese and Turkish wheats; these did not produce the HMW band (Wx-A1 protein) in the gels. Wheat cultivars carrying the null allele for the Wx-B1 protein, were mostly of Australian and Indian origin, and showed a LMW and a HMW band of the same density. Since the LMW band comprises both the Wx-B1 and Wx-D1 proteins in normal, these cultivars might have lacked one of these proteins. 2D-PAGE revealed that almost all of them were lacking the Wx-B1 but none lacked the Wx-D1. Only one Chinese cultivar, Bai Huo, was identified as possessing a null allele for the Wx-D1 protein. Nine Japanese cultivars, which produced only a thin LMW band, were found to be deficient in both the Wx-A1 and Wx-B1 proteins. All of these "partial waxy mutants" often produced starches with reduced amylose (Nakamura *et al.* 1992, 1993; Yamamori *et al.* 1994).

More recently, the first demonstration of a genetic modification to wheat starch, which resulted in the production of waxy wheat (amylose-free) has been reported (Nakamura *et al.* 1995). Hybridisation of "partial waxy mutants" has resulted in the production of tetra- and hexaploid waxy mutants, with endosperms staining red-brown by iodine. Both mutants showed loss of waxy protein and amylose. Thus, based on the presence and absence of the three waxy proteins, wheat has been classified into eight types (Yamamori *et al.* 1994) (Table 1.2). The discovery of type 4 and type 7 wheats enabled plant breeders to produce the eight possible types by crossbreeding between the two wheats (Yamamoria and Quynh, 1997). Similarly, eight types of homozygous recombinant Chinese Spring lines carrying different null alleles at the *waxy* locus has been reported by Miura *et al.* (1998).

Table 1.2 Classification of common wheat based on presence and absence of three waxy proteins. + and – indicate presence and absence of waxy protein, respectively (Yamamori *et al.* 1997).

	Wx protein		
Type	**Wx-A1**	**Wx-B1**	**Wx-D1**
1	+	+	+
2	–	+	+
3	+	–	+
4	+	+	–
5	+	–	–
6	–	+	–
7	–	–	+
8	–	–	–

1.11.5 Waxy protein amount and amylose content

The apparent amylose content in some Japanese wheat cultivars declined in the following order, type 1 (28%) > type 2 (27.2%) > type 3 (25.3%) > type 7 (22.0%) (Kuroda *et al.* 1989). Recently, Yamamori and Quynh (1997) reported an increase in amylose content in Japanese wheat cultivars in the following order, type 8 (waxy, 0%) < 5 (21% - 23%) < 7 (23% - 25%) < 6 (25% - 26%) < 3 (26% - 27%) < 4 (27%) < 2 (27% - 28%) < 1 (~28%). Wheat cultivars lacking two waxy proteins (type 5, 6 and 7) produced less amylose than the types lacking one waxy protein (types 2, 3 and 4). An increase in amylose in the order of types 5, 7 and 6 indicated that among the three waxy proteins, the Wx-A1 protein produced the least amylose, then the Wx-D1, and the Wx-B1 protein produced the most amylose. 2D-PAGE analysis revealed that the amount of Wx-B1 protein was larger than that of the Wx-A1 protein (Nakamura *et al.* 1993a). An investigation on the amylose content of the eight types of homozygous recombinant Chinese Spring lines showed variation from 0% of the waxy CS to 25% of CS euploid, and the largest reduction in amylose was detected in the lines that produced only the Wx-A1 protein (Miura *et al.* 1998).

Miura *et al.* (1994) have interpreted the differential effects of the three *waxy* genes on amylose content in terms of the different potencies of these genes. It has been reported that, a sufficient reduction in the Wx-B1 protein to affect the level of amylose could be achieved by decreasing the dosage of the most potent *Wx-B1* gene and, a reduction in either of the Wx-A1 or Wx-D1 proteins could be compensated by the most abundant Wx-B1 protein (Miura *et al.* 1994). Comparison of the double deficient lines of waxy proteins with the single deficient or normal lines led to another interesting finding where a certain level of either the *waxy* gene activity or the waxy protein was shown to lead to a maximum amount of amylose (Miura *et al.* 1996). Further increases in the *waxy* gene products did not bring about any additional raise in amylose content. It has been suggested that this effect is due to the epistatic manner of the three *waxy* genes, inhibiting amylose synthesis when at least two genes produce the waxy proteins (Miura *et al.* 1996).

The waxy protein activity and amylose concentration have been reported to have an apparently positive relationship in different non-waxy mutant cultivars of cereals. In the endosperm of rice the waxy protein activity decreased with increase in the dosage of mutant *waxy* genes (Sano *et al.* 1986). Shimada *et al.* (1993) reported that the introduction and expression of the antisense RNA of the *waxy* gene into rice affected the level of expression of the target gene and reduced the amylose concentration in endosperm starch. In maize, the waxy protein activity as well as amylose concentration were found to decrease with the addition of inactive or mutant *waxy* genes in the series WX/WX/WX, wx/WX/WX, wx/wx/WX and wx/wx/wx in the triploid endosperm tissues (Tsai, 1974). In potato, Visser *et al.* (1991) and Koβmann (1997) reported that the expression of antisense RNA of the *waxy* gene inhibited the waxy protein activity in starch of potato tuber and gave rise to tubers containing amylose-free starch.

1.11.6 Mutations in *waxy* genes

Maize

In maize, many mutations affecting expression of the *waxy* gene have been identified, including unstable mutations caused by insertion of transposable elements (McClintock, 1961, 1965; Nelson, 1968). Among these are several alleles that arose by insertion of the autonomously transposing *Activator (Ac)* element, and/or by insertion of elements incapable of autonomous transposition which include the *Dissociation (Ds)* element and an element belonging to the *Suppressor-mutator (Spm)* controlling element family (McClintock, 1961, 1965; Nelson, 1968, 1976). Maize waxy mutants, having *Ds* elements inserted at the *waxy* locus, showed reduced or no waxy protein (Echt and Schwartz, 1981; Shure *et al.* 1983) due to defective transcription, translation or RNA processing (Echt and Schwartz, 1981). Some of the mutants, having *Ac* elements inserted at the *waxy* locus, did not produce amylose and showed the presence of inactive, structurally altered protein (Echt and Schwartz, 1981). These proteins were not detectably different in size from the active, normal waxy protein, but had different isoelectric points. Wessler and Varagona (1985) also have reported the presence of large insertions or deletions in several stable waxy mutants of maize. One of seven deletion mutants characterised by Wessler and Varagona (1985) involved the deletion of the entire *waxy* transcription unit. Wessler *et al.* (1990) reported the association of filler DNA with five spontaneous deletions at the *waxy* locus. Thus, from all the studies, it appears that transposable elements are frequently responsible for the origin of waxy mutants in maize.

Rice

In rice, Okagaki and Wessler (1988), could not detect large insertions or deletions among the waxy mutants. However, using RFLP it was suggested that the mutations were due to single base changes or small insertions or deletions that add or remove restriction sites rather than cause gross changes similar to those found in maize. Sano (1984) has reported the absence or drastic reduction of waxy protein in all of the examined waxy rice cultivars, instead of the formation of an inactive protein, suggesting the possibility of a defect in transcription or translation. The presence of Wx^a and Wx^b alleles has been reported in rice cultivars with high or intermediate level

of waxy protein respectively (Sano, 1984); This difference in waxy protein levels, was suggested to be controlled by *cis*-acting elements. Wang *et al.* (1995) have suggested the involvement of *cis*-acting elements in the excision of intron 1 from the 5' leader region of the *waxy* transcript, which plays an important role in regulating the *waxy* gene expression in rice (refer section 1.11.3).

Wheat

The discovery of two partial waxy mutants (one lacking Wx-A1 protein and the other lacking Wx-B1 protein) among Japanese wheat cultivars, was the first demonstration of mutations in the *waxy* gene of wheat (Nakamura *et al.* 1993). It has been suggested that these partial mutations may be due to very small insertions or deletions, since differences could not be detected between normal genotypes and these mutations, when restriction patterns were compared (Nakamura *et al.* 1993). Recently, Vrinten *et al.* (1999) characterised the mutations in the three *waxy* alleles in waxy hexaploid wheat (*Wx-A1, Wx-B1 and Wx-D1*). They have reported a deletion of the entire *Wx-B1* allele which appears to remove the entire GBSSI transcription unit and the presence of DNA homologues to *waxy* gene sequences in the *Wx-A1* and *W-D1* null alleles. A 23 bp deletion at an intron-exon junction in the *Wx-A1* null allele has been shown to affect the splicing of the transcript. And, a deletion at the 3' untranslated region and the polyadenylation signal in the *Wx-D1* null allele has been shown to cause a reduction in the level of transcript accumulation. Vrinten *et al.*(1999) have concluded that the absence of translation products may be either due to absence of translation process or due to the production of protein levels which is extremely low to be detected.

About 40% of the Australian wheat cultivars carry the null allele *Wx-B1* (Yamamori *et al.* 1994). These null 4A waxy mutants lack the Wx-B1 protein and consequently have a lower proportion of amylose in the starch, which is a desirable attribute for the production of Japanese Udon noodles (Yamamori *et al.* 1994). However, the reason for the lack of expression of the Wx-B1 protein in the endosperm of the null 4A cultivars is not yet fully understood.

At present, starches that possess novel and improved functionality are increasing in demand in the food industries, due to the desire to deliver new starch based products to the consumers. As discussed in this chapter, the functional properties of starch are determined by the fine structure of amylose and amylopectin. These polysaccharide starch components are synthesised by the enzymes responsible for starch biosynthesis, which in turn, is encoded by genes. It is therefore possible to alter the amount and/or type of starch produced in a plant by genetic manipulation. In order to develop valuable new genetically engineered starch, it is considered important to increase our understanding of the linkage between genetics, enzymes, starch structure and functionality.

The aims of this research investigation were as follows,

1. To develop an electrophoresis method to separate the polysaccharide components and to quantify the amylose component in wheat starch.
2. To develop a method for producing amylopectin free wheat amylose standard, using the lectin Concanavalin A.
3. To validate the new electrophoresis method for starches obtained from a range of Australian wheat cultivars, and, compare results with those from two other existing methods.
4. To investigate the nature of mutation responsible for the inactivation of the 4A *waxy* gene (*Wx-B1*), in the null 4A Australian wheat cultivars.
5. To determine the relationship between waxy protein amount and amylose content during seed development in null 4A and normal wheat cultivars.

CHAPTER 2

Materials and Methods

2.1 Chemicals and Reagents

The sources of all chemicals are shown in Appendix 3. All reagents were analytical grade and supplied by BDH (UK) or Sigma (USA), unless otherwise stated. Preparation of buffers and solutions are shown in Appendix 4.

2.2 Equipment and instrument

A Beckmann J2-HS centrifuge (Beckmann instruments Inc., Palo Alto, California, USA) was used for centrifuging 10-1000 mL quantities, while for volumes smaller than 2 mL a microcentrifuge (Beckmann) was used. For samples between 2-10 mL and requiring < 4000 g, a bench top centrifuge (Beckmann) was used. An Ultraspec model UV-Visible spectrophotometer (LKB) was used to measure absorbance of nucleic acids and a Cary 1E UV-Visible spectrophotometer (Varian) was used to measure absorbance of starch-iodine samples. Freeze-drying was performed in a Dynavac FD 300 freeze-dryer (Dynavac Engineering Pty. Ltd. Inc., Melbourne, Victoria, Australia). H1 8418 pH meter (Hanna instruments, New South Wales, Australia) was used to measure the pH of solutions and reagents. An Ultrascan™ XL laser densitometer (Pharmacia) with Gel Scan™ XL evaluation software was used for scanning the gels, negatives and autoradiographs. A vortex mixer MT19 (Chiltern) was used to vortex solutions and mixtures. Other general analytical instrumentation is described in the relevant method section.

2.3 Preparation of wheat amylose standards

2.3.1 Wheat starch

Wheat starch was kindly donated by Agrifood Technology, Werribee, Victoria.

2.3.2 Isolation of amylose fraction from wheat starch

2.3.2.1 *Fractionation of wheat starch with 1-butanol*

The procedure described by Matheson and Welsh (1988) was adapted for this investigation. Approximately 300 mg of wheat starch, which had been previously treated with DMSO and precipitated with ethanol, was dissolved by heating in a steam bath in 0.1 M sodium chloride (45 mL). The solution was cooled to 60°C and 1-butanol (2.5 mL) was added. After 48 hours, the mixture was centrifuged (JA-20, 14,000 rpm, 15 minutes, 20°C). The residue was re-dissolved in 0.1 M sodium chloride (30 mL) and 1-butanol (1.7 mL) was added at 60°C. After 48 hours the mixture was centrifuged (JA-20, 14,000 rpm, 15 minutes, 20°C) and the amylose precipitate was freeze-dried.

2.3.2.2 *Fractionation of wheat starch with concanavalin A (con A) followed by butanol precipitation.*

This method of fractionation using con A was based on the assay format described by Gibson *et al.*, (1996) and Matheson and Welsh (1988). Wheat starch (100 mg) was dissolved in 4 mL of DMSO in a boiling water bath for 15 minutes. Lipids were removed by pre-treating with 24 mL ethanol for 15 minutes. After centrifuging (JA-20, 14,000 rpm, 20°C), the supernatant was discarded and the residue was drained for 10 minutes and was re-dissolved in 4 mL of DMSO for 15 minutes in a boiling water bath. The starch solution was then diluted to 100 mL with 0.2 M sodium acetate buffer (pH 6.4) containing 1M Na^+, 1mM Mn^{2+}, Ca^{2+} and Mg^{2+} as chlorides. This was mixed with an equal volume of con A solution (which contained con A in 0.2 M sodium acetate buffer, pH 6.4) to precipitate the amylopectin. The concentration of con A solution and the precipitation time were optimised as described in Chapter 4, Section 4.2.1.

The amylopectin precipitate was then removed by centrifugation (JA-14, 14,000 rpm, 15 minutes, 20°C). The supernatant solution was mixed with EDTA (1 mL, saturated at 37°C) and heated in a steam bath to precipitate the protein (excess con A). After centrifugation (JA-14, 14,000 rpm, 15 minutes, 20°C), the supernatant was heated to 60°C in a steam bath and 11 mL of 1-butanol was added. After 24 hours, the amylose

precipitate was centrifuged (JA-14, 14,000 rpm, 15 minutes, 20°C), rinsed with distilled water and re-centrifuged. Rinsing and centrifuging was repeated 2-3 times and finally the amylose precipitate was freeze-dried.

2.3.2.3 *Fractionation of starch with Con A followed by ultra-filtration*

The procedure carried out was the same as in Section 2.3.2.2, except that the denatured protein (con A) was removed by centrifugation (JA-14, 14,000 rpm, 15 minutes, 20°C) and the supernatant was concentrated by membrane ultra-filtration. The ultra filtration unit used was a Sartorius Ultra-sart Cell 50 (Sartorius AG, Germany). Ultra-filtration was carried out under air pressure (300 kPa) using a membrane with a cut-off point of 30 kDa. The amylose residue was rinsed three times with 10 mL of distilled water and was freeze-dried.

2.3.3 Determining the purity of amylose standards

2.3.3.1 *Analysis by agarose gel electrophoresis*

The amylose samples (10 mg/mL) were solubilised in 0.5 M KOH at 80°C for 15 minutes and were subjected to agarose gel electrophoresis (Section 3.2.2.1). The electrophoresis was carried out at 30 V for 16 hours and the gel was stained in 0.2% I_2 in 2% KI solution for 1 minute and de-stained in distilled water by rinsing 3-4 times.

2.3.3.2 *Analysis by HPLC*

Hydrolysis of amylose samples was carried out by TFA hydrolysis (Cerning *et al.* 1986) with some minor modifications. Amylose (1 mg) standards were weighed in glass test tubes and 1 mL of 1 M TFA was added to each sample. The test tubes were closed with glass marbles and the samples were heated overnight at 100°C. The next morning the marbles were removed, and nitrogen was directed into each tube until the volume of the remaining liquid was reduced to approximately 0.25 mL. The temperature was reduced to 40°C and the samples were dried giving a transparent cover on the bottom of the glass tubes. The dried samples were re-dissolved in distilled water (1 mL) and 20 µL aliquots were applied on the HPLC column to determine the glucose concentrations. A calibration curve was plotted using the following standard glucose solutions, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL

and 1.0 mg/mL. HPLC was performed using a Varian 9012 solvent delivery system equipped with a Varian RI-4 refractive index detector and a Varian 9100 autosampler.

Column:	Aminex HPX-87H Ion exclusion column
Column temperature:	65°C
Mobile phase:	5 mM Sulphuric acid
Flow rate:	0.6 mL/minute

The amount of amylose was calculated based on glucose recovery, according to the following equation,

$$\text{Amount of amylose} = \frac{\text{Glucose recovered (mg/mL)} \times 100}{1.1}$$

The purity of amylose was based on amount of amylose determined by HPLC as a percentage of the 1 mg of amylose isolated by the various fractionation procedures.

2.4 Application

2.4.1 Wheat samples

Twenty five common wheat cultivars from the 1993/1994 Australian harvest were provided by Agrifood Technology, Werribee, Victoria.

2.4.2 Laboratory scale starch extraction

Starch was extracted from the wheat grains according to the method of Dexter and Matsuo (1979). This is a slightly modified method of Adkins and Greenwood (1966). Extraneous matter, damaged and diseased kernels were removed from each sample. Samples (approximately 500 g) were then washed thoroughly with distilled water and approximately twice the apparent volume of acetate buffer (0.02 M, pH 6.5) was added to the grains which were held at 4°C for 30 hours. During this period the pH of the system was maintained in the range 6-6.5. The softened kernels were drained, washed and reduced to a fine, porridge like grist in an electric blender (Waring commercial blender) at high speed. The cohesive grists were kneaded under water and screened

through a sieve (Mesh No. 40, Greer and Ashburner Pty. Ltd.). The steps of grinding, kneading and screening were repeated until the screen overs were dark in colour and free of visible fine endosperm particles. The slurry was centrifuged (JA-14 rotor, 5000 rpm, 10 minutes, 20°C) and the top starch and proteinaceous bands were removed. The white starch sediment was reslurried in water. Toluene-water (1:2 v/v) was then added to the slurry and stirred overnight. The toluene layer, bearing the denatured proteins was removed by suction. The process was repeated with fresh toluene-water, until the toluene layer was free of protein. The purified starch was filtered by suction, rinsed with ethanol and air-dried. The dried starch was sieved through a 600 μm sieve.

2.4.3 Extraction of free lipids and bound lipids from wheat starch

Free and bound lipids were removed according to the method of Greenblatt *et al.* (1995). Free lipids were removed from wheat starch with hexane (sample-to-hexane ratio of 1:10) for 30 minutes at room temperature, centrifuged (JA-20, 10,000 rpm, 15 minutes, 20°C) and the supernatant was discarded. The tubes were drained on tissue paper for 10 minutes. The starch was then air-dried. Bound lipids were removed from the air-dried starch by stirring with propan-2-ol and water (90:10) at a sample to solvent ratio 1:3, for 15 minutes at room temperature. The extract was then centrifuged (JA-20, 10,000 rpm, 15 minutes, 20°C), the supernatant discarded and the tubes were drained on tissue paper for 10 minutes. The starch was then air-dried.

2.4.4 Quantitation of amylose

2.4.4.1 *Measurement of amylose content of wheat starch by iodine colorimetry*

The amylose content of wheat starch was determined by the procedure of Williams *et al.* (1970). Starch (50 mg) was dispersed in KOH solution (0.5 M, 10 mL) and made up to 100 ml. An aliquot (10 mL) was placed in a 50 mL volumetric flask together with HCL (0.1 M, 5 mL) and iodine reagent (0.2% I_2 in 2.0% KI, 0.5 mL), and made up to volume. A blank (reagents only, no starch) was similarly prepared. Absorbance of the solution was measured at 625 nm after 5 minutes, using the blank as reference, in the UV-Visible spectrophotometer. A calibration curve was constructed by measuring the absorbances of potato amylose and potato amylopectin mixtures. The potato amylose and potato amylopectin were mixed in the following ratios

respectively, 0:50, 5:45, 10:40, 15:35, 20:30, 25:25. Amylose (%) of the starch sample was determined with reference to the calibration curve.

2.4.4.2 *Measurement of amylose content of wheat starch by con A method*

The Amylose/Amylopectin assay kit from Megazyme Int. Ireland Ltd. was used according to the company instructions, to determine the amylose content in wheat starch. The assay involved the removal of the amylopectin component of the lipid free starch by precipitation with con A, followed by the enzymatic hydrolysis of the resulting amylose to glucose which was measured colorimetrically (at 510 nm) using glucose oxidase/peroxidase (GOPOD) reagent. The total starch solution without con A treatment was also subjected to amylolytic hydrolysis to yield glucose, which was also measured colorimetrically (at 510 nm) using the glucose oxidase/peroxidase reagent. The amylose content was estimated as the ratio of GOPOD absorbance at 510 nm of the supernatant of the con A precipitated sample, to that of the total starch sample.

$$\text{Amylose \%} = \frac{\text{Absorbance of con A supernatant}}{\text{Absorbance of total starch aliquot}} \times 66.8^*$$

*Constant derived from dilution factors

2.5 Molecular genetic studies on Australian wheat cultivars

2.5.1 Solutions and Utensils

For DNA work, unless specified, all solutions and de-ionised water were sterilised by autoclaving for 20 minutes at 121°C. For RNA work, the following precautions were taken to prevent RNAse contamination: Where necessary, sterile, disposable plasticware were used. General laboratory glassware, mortars and pestles were treated by baking at 180°C overnight prior to use. Electrophoresis tanks, combs and gel plates were cleaned with RNAse away reagent (Gibco BRL, Life Technologies) and rinsed with DEPC treated water. The metal probe of the homogeniser was immersed in chloroform for 2 hours and rinsed with DEPC water prior to use. All solutions were prepared using the baked glassware, DEPC treated water, and chemicals reserved for RNA work that were handled with baked spatulas.

2.5.2 Wheat cultivars {*T.aestivum* (6n)}

Mature wheat grains were obtained from Department of Agriculture, Horsham, Victoria, Australia, and Australian Winter Cereals Collection, Tamworth, New South Wales, Australia. The wheat cultivars used for this study were as follows,

Normal cultivars	:	Chinese Spring, Goroke, Vectis and Wyuna.
Null 4A cultivars	:	Cadoux, Halberd, Machete and Rosella

2.5.3 Growing the wheat plants

Initially, the seeds of the eight wheat cultivars were allowed to germinate by partially immersing the seeds in water for approximately two days. The germinated seeds were then sown in vermiculite, in small plastic containers. The plants were grown in the dark for approximately 3 weeks (to ~20-25 cm in length). During this period the vermiculite was kept moist by sprinkling water every alternate day. The leaves were then harvested, snap-frozen in liquid nitrogen and stored at - 80°C. These were used for chromosomal DNA extraction, to study alterations in *waxy* gene structure.

For the *waxy* gene expression and GBSS protein study the eight wheat cultivars were grown in a glass house, owned by the State Chemistry Laboratory, Victoria. The main purpose of the glass house was to protect the plants from pests and extreme weather conditions. Since the temperature controlling facilities were not provided the conditions inside the glass house simulated field conditions. The germinated wheat seeds were sown (20 seeds per pot / diameter ~25 cm) in potting mixture (Debco brand), on the same day, and all of the plants were grown from May to October. Air circulation was maintained inside the glass house, by two large electric fans throughout the growth period. The plants were watered daily in order to keep the soil moist, until maturity. After four months (in September), a tablespoon full of fertiliser (Osmocote plus, Australia Pty Ltd.) was added to the potting mixtures, on the same day. At anthesis the heads were tagged for harvesting (Figure 2.1). 20 dap seeds of all cultivars were harvested, snap-frozen in liquid nitrogen and stored at -80°C. These were used for total RNA extraction to compare the extent of *waxy* gene expression between normal and null 4A cultivars. In addition, 5, 10, 15, 20 and 25 dap seeds of

Chinese Spring and Rosella were collected and stored as above to study the patterns of *waxy* gene expression and its correlation with GBSS protein and amylose content. The seeds of all eight cultivars were also grown to maturity for the subsequent analysis of waxy proteins and to determine the amylose content of mature seeds. It should be noted that sampling (harvesting) was carried out only once per wheat plant, because physiological changes are assumed to occur in the remaining seeds as a result of removal of nearby seeds.

2.5.4 Isolation of chromosomal DNA

DNA was extracted from the leaves of the eight wheat cultivars, by the method described by Sambrook *et al.* (1989). Frozen leaves (3 grams) were rapidly ground to a fine powder in a pre-cooled mortar and pestle, using liquid nitrogen. It was then suspended in 15 mL of extraction buffer and incubated for 10 minutes in a 65°C water bath. The mixture was centrifuged (JA-20 rotor, 6000 rpm, 15 minutes, 4°C). The supernatant was digested with 5 µL of 10 mg/ml RNAase A solution (DNAase-free) for 30 minutes at 37°C, to remove RNA. The DNA was then extracted with phenol-chloroform-isoamyl alcohol mixture (25 : 24 : 1) and precipitated with iso-propanol at -20°C. The DNA pellets were recovered by centrifugation (JA-20 rotor, 10,000 rpm, 10 minutes, 4°C), dried at room temperature and resuspended in approximately 200 µL TE buffer.

2.5.5 Estimation of DNA concentration

Concentrations of DNA and synthesised oligonucleotides (Section 2.5.6) were determined by the spectrophotometric method (Sambrook *et al.* 1989). Aliquots of nucleic acid samples and oligonucleotides were diluted in distilled water and absorbance readings taken at wavelengths of 260 nm and 280 nm. The A_{260}/A_{280} ratio was used to estimate the purity of the samples.

Nucleic acid concentrations of the undiluted samples were determined using the following formula (Sambrook *et al.* 1989),

A_{260} x dilution factor x 50* = μg/mL DNA

(*A_{260} = 1 for 50 μg/mL DNA)

A_{260} x dilution factor x 33** = μg/mL single stranded oligonucleotides

(**A_{260} = 1 for 33 μg/mL single stranded oligonucleotides)

2.5.6 Oligonucleotide (Primer) synthesis

Oligonucleotides were synthesised using the PCR-Mate EP™ DNA synthesiser (Model 391) from Applied Biosystems. The 40 nmole synthesis scale was used. Following synthesis, oligonucleotides were de-protected and cleaved from the solid support using concentrated ammonium hydroxide. Base protecting groups were removed by addition of fresh concentrated ammonium hydroxide and incubation overnight at 55°C. On evaporating the ammonia at 40°C overnight, the oligonucleotides were freeze dried and redissolved in sterile distilled water. Following the addition of sodium acetate (3 M, pH 5.2) to a concentration of 0.3 M, the oligonucleotides were precipitated using absolute ethanol. The oligonucleotide pellets were recovered by centrifuging in a microcentrifuge (10,000 rpm, 10 minutes), air-dried after an ethanol wash and were dissolved in sterile distilled water. The concentrations were determined spectrophotometrically as shown in Section 2.5.5 and the primers were stored as aliquots at -20°C.

The concentration unit was converted to pmoles of primers by the following equation,

μg of primer x 1,000,000/ (length of primer x 325*) = pmoles primer

(*Average molecular weight of dNMP is 325 Daltons)

The micromolar (μM) concentration of primer = pmoles/μL

2.5.7 PCR amplification

PCR was performed in a DNA Thermal Cycler 480 (Perkin Elmer) using the Gene Amp® PCR core reagent (Perkin-Elmer). Reaction conditions and PCR cycling parameters are shown in Tables 2.1 and 2.2 respectively.

Table 2.1 Reaction conditions for PCR amplification

Component	Volume (μL)	Final Concentration
DH_2O		
*10 X PCR buffer	5	1 X
*dNTP mix (2 mM each dNTP)	5	0.2 m M each dNTP
*25 mM $MgCl_2$	3	1.5 mM
30 μM upstream primer	1	0.6 μM
30 μM downstream primer	1	0.6 μM
Template DNA	0.5 μg	
*Amplitaq DNA polymerase (5U/μL)	0.5	0.05 U/μL

The reaction (50 μL) was incubated for 5 minutes at 94°C prior to addition of Taq DNA polymerase. (* Components from the kit)

Table 2.2 Cycling parameters for PCR amplification

Cycle	Denaturation	Annealing	Elongation
First cycle	5 minutes at 94°C		
Subsequent cycles (n = 35)	1 minute at 94°C	1 minute at T° C	2 minutes at 72°C
Last cycle			30 minutes at 68°C

The holding temperature at the end of the amplification was 4°C. The annealing temperature T°C, was based on the melting temperature (Tm) of the primers which was determined using the following formula (Sambrook *et al.*1989),

Tm = 4 (G+C) + (A+T)

A = No. of Adenine bases G = No. of Guanine bases

T = No. of Thymidine bases C = No. of Cytosine bases

2.5.8 Agarose gel electrophoresis

Electrophoresis of DNA was performed with 1% agarose slab gels in 1 X TAE buffer, (Gel Electrophoresis Apparatus GNA-100, Pharmacia, Sweden). Ethidium Bromide solution (10 mg/mL) was added to the agarose gels at a concentration of 0.5 µg/mL, during the preparation. Electrophoresis was carried out using 1 X TAE buffer, until the loading dye reached the bottom of the gel. The DNA bands were visualised over a UV transilluminator (λ = 302 nm) (LKB 2011 Macrovue transilluminator). Lambda DNA digested with *EcoR1* and *Hind III* (Promega) and 100 bp ladder (Promega) were used as markers for size determination. As per supplier's instructions, approximately 0.5 µg - 1 µg of size markers were used.

2.5.9 DNA elution from agarose gel

Purification of the PCR products of interest was performed, by excising the appropriate bands and eluting from the agarose gels using the Bandpure™ DNA Purification Kit (Progen), according to the manufacturer's instructions.

The purified PCR product was then subjected to agarose gel electrophoresis for quantification, with reference to the 100 base pair ladder from Promega. A 5 µL aliquot of this standard gives a distribution of bands each containing 50 ng DNA.

2.5.10 Sequencing

Fluorescence base cycle sequencing reactions were performed as per manufacturer's instructions, using the ABI Prism™ Dye termination cycle sequencing kit (Perkin-Elmer). Extension products were purified by ethanol precipitation after the addition of 3 μL 3 M sodium acetate (pH 4.6). The precipitate was recovered by centrifuging in a microcentrifuge (30 minutes, room temperature) and the pellets were rinsed briefly with 70% ethanol and air dried. Automated sequencing of the dried samples was performed at Monash University (Department of Microbiology), Victoria, using an ABI 373-Automated DNA sequencer (Perkin-Elmer). Sequence analysis of the data was done using the 'best fit' program of Webangis.

2.5.11 Total RNA extraction from wheat seeds

Total RNA was extracted from developing seeds of Chinese Spring and Rosella and 20 dap seeds of all eight wheat cultivars, with Trizol (Gibco, Australia). The protocol provided by Gibco was partially modified for this purpose. Wheat seeds (150-200 mg) were pulverised under liquid nitrogen with a mortar and pestle, without allowing the samples to thaw. The powder with the liquid nitrogen was transferred to a McCartyney bottle and the liquid nitrogen allowed to evaporate. Trizol reagent (4 mL) was added to the seed powder immediately after the evaporation of liquid nitrogen, and the tissue was homogenised using a Polytron (Model PT-2000, Kinematica AG) for 1 minute (at speed 3). Insoluble materials from the homogenate were removed by centrifugation (JA-20 rotor, 12,300 rpm, 10 minutes, 4°C) and the supernatant was transferred to a fresh tube. Chloroform (800 μL) was added to the supernatant, mixed vigorously for 15 seconds and allowed to stand at room temperature for 3 minutes. The sample was centrifuged (JA-20 rotor, 12,300 rpm, 15 minutes, 4°C) and the aqueous phase was transferred to a fresh tube. RNA was precipitated by adding 1 mL each of isopropanol and 4 M LiCl and allowed to stand for 10 minutes at room temperature. Following centrifugation (JA-20 rotor, 12,300 rpm, 10 minutes, 4°C) the precipitate was thoroughly resuspended in 3 mL of 2 M LiCl and centrifuged (JA-20 rotor, 12,300 rpm, 10 minutes, 4°C). The supernatant was discarded. This was repeated twice.

The RNA pellet was dissolved in approximately 100 µL RNAase free water by incubating for 10 minutes at 55-60°C. It was then centrifuged in a microcentrifuge (9000 rpm, 10 minutes, 4°C) to remove any insoluble materials. A 1/10 volume of 3 M sodium acetate (pH 5.2) was added to the supernatant followed by 2.5 volumes of absolute ethanol to precipitate the RNA. The mixture was stored at -80°C overnight and centrifuged in a microcentrifuge (9,000 rpm, 10 minutes, 4°C). The supernatant was discarded and the RNA pellet was washed once with 75% ethanol, adding at least 1 mL of 75% ethanol / mL of Trizol reagent used initially. The RNA pellet was air dried briefly (5-10 minutes) and resuspended in RNAase-free water. The samples were stored at -80°C.

2.5.12 Estimation of RNA concentration

An aliquot of the RNA sample was diluted to measure the absorbance at 260 nm and 280 nm. The concentration of the RNA sample was determined using the following formula (Sambrook *et al.*, 1989):

A_{260} x dilution factor x 40* = µg RNA/mL

(*A_{260} = 1 for 40 µg/mL RNA)

2.5.13 Formaldehyde gel electrophoresis

Denaturing formaldehyde gels were used to separate RNA species of different molecular weights for northern blot analysis. Preparation of the gels and electrophoresis was carried out according to the method of Sambrook *et al.* (1989). The gels contained 1% agarose and 6.6% formaldehyde in 1 X MOPS. Prior to loading, the RNA samples (10 µg) were mixed with freshly prepared sample buffer in the ratio 1 : 4 (v : v), then denatured by heating at 65°C for 10 minutes and placed on ice. Appropriate volume of 6 X loading dye and 1 µL (1 mg/mL) of ethidium bromide were added before sample application. RNA molecular weight marker (3 µL) from Promega, containing eight fragments (6583, 4981, 3638, 1908, 1383, 955, 623, 281), was also treated similarly before loading onto the gel. Electrophoresis was carried out in 1 X MOPS running buffer at 65 V until the blue dye reached 3/4 length of the length of the gel plate.

2.5.14 Northern blotting

The technique of transferring total RNAs from the gel to the nylon membrane (Boehringer mannheim) was carried out according to instructions (Sambrook *et al.* 1989). The flow of 10 X SSC due to capillary action aided in transferring the total RNAs to the membrane. The transfer continued overnight, after which the membrane was rinsed briefly in 4 X SSC, air-dried, wrapped in a plastic wrap and was exposed to UV for 5 minutes. It was then stored at 4°C until hybridisation.

2.5.15 Labelling of cDNA probes

A 840 bp cDNA representing the 5' sequences and a 1244 bp cDNA representing the 3' sequences were obtained by RT-PCR (Section 2.5.21), to be used as probes for northern hybridisation. According to the suppliers instructions the probes were radioactively labelled by nick translation, using a commercial kit from Promega. The radioactive label, α ^{32}P-dCTP (10 mCi/mL, specific activity, 3000 Ci/mmol), was purchased from Amersham Life Science.

The high molecular weight labelled DNA was separated from unincorporated excess nucleotides and short DNA fragments by elution on Micro-Spin Column (S-200 HR Columns, Pharmacia Biotech) according to the manufacturer's instructions.

2.5.16 Hybridisation

The blots were incubated in the pre-hybridisation buffer for 3-4 hours at 65°C with gentle shaking. The labelled cDNA probe was denatured for 10 minutes at 95°C, placed on ice for 5 minutes and then added to the bag containing the pre-hybridisation buffer and the blots. Hybridisation was carried out at 65°C overnight with gentle shaking.

2.5.17 Washes

After overnight hybridisation the solution was poured off and an equal volume of low stringency wash (1 X SSC / 0.1 % SDS) was added. The membranes were incubated at room temperature for 15 minutes. Further washes were carried out under more stringent conditions of lower salt and/or higher temperature (1 X SSC / 0.1% SDS at

65°C / 5 minutes; 0.2 X SSC / 0.1 SDS at 65°C / 10 minutes), which further reduced background adherence of the probe to the membrane and removed non-specific hybridisation products. The washed blots were air dried for 5 minutes and wrapped in a plastic wrap and exposed to a X-ray film (Hyperfilm™ MP from Amersham Life Science).

2.5.18 Autoradiography

Autoradiography was performed with a single intensifying screen at -80°C for 2-3 days, depending on the strength of the signal. After the required exposure time, the cassette was removed from the freezer and allowed to warm to room temperature prior to development of the film. The film was developed manually, allowing 5 minutes in the developer (AGFA-Gevaert, Australia), 1 minute in the stop bath (3% acetic acid v/v) and 3 minutes in the fixer (AGFA-Gevaert, Australia). Finally the film was washed extensively under running water and dried.

2.5.19 Quantifying the mRNA bands on X-ray films

mRNA expressions of *waxy* genes in the null 4A and normal wheat cultivars were evaluated by scanning the hybridisation signals on autoradiographs and the corresponding rRNA on the negative film, using the laser densitometer. The expression of the mRNA for each sample was determined by comparing the intensity ratio between the hybridisation signal obtained by *waxy* mRNA to that of rRNA, which verified a consistent loading of RNA.

2.5.20 Stripping the blot for reprobing

The blots were incubated 2 x 30 minutes at 68°C in stripping solution which contained 50% dimethyl formamide, 1% SDS, 50 mM Tris-HCl (pH 8). They were then rinsed first in water and then in 2 x SSC and stored wet in 2 x SSC in a sealed plastic bag at 4°C.

2.5.21 Reverse Transcriptase PCR (RT-PCR)

RT-PCR was performed using a RT-PCR kit (Promega), according to manufacturer's instruction. Table 2.3 and Table 2.4 show the reaction conditions and cycling parameters respectively.

Table 2.3 Reaction conditions for RT-PCR amplification

Component	**Volume (μ L)**	**Final concentration**
RNA-ase free water		
*AMV/*Tfl* 5 X Reaction buffer	10	1 X
*dNTP mix (10 mM each dNTP)	1	0.2 mM
50 μM upstream primer	1	1 μM
50 μM downstream primer	1	1 μM
*25 mM $MgSO_4$	2	1 mM
*AMV Reverse Transcriptase (5U/μL)	1	0.1U/μL
**Tfl* DNA Polymerase (5U/μL)	1	0.1U/μL
RNA template	1 μg	
Final volume	50 μL	

(* Components from the kit)

The RNA template was denatured at 65°C for 10 minutes and kept in ice for 5 minutes, prior to the addition of the remaining components. *Tfl* DNA Polymerase was added at the end of initial denaturation step (Table 2.4, Second cycle).

Table 2.4 Cycling parameters for RT-PCR amplification

Cycle	Reverse transcriptoion	Denaturation	Annealing	Elongation
First cycle	45 minutes at 48°C			
Second cycle		5 minutes at 94°C		
Subsequent cycles (n = 35)		1 minute at 94°C	1 minute at 58°C	2 minutes at 72 °C
Last cycle				30 minutes at 68°C

2.6 Analysis of waxy protein and amylose content

2.6.1 Starch extraction

Starch was extracted from the mature and developing seeds of wheat, by the method of Echt and Schwartz (1981). The pericarp and embryo were removed from all except the 5 dap seeds, which were extremely small in size and contained undeveloped embryo and pericarp. The endosperms were homogenised in buffer A (0.55 M Tris, pH 6.8, 2.3% SDS, 5% 2-mercaptoethanol, 10% Glycerol) and the suspensions were filtered through a layer of mira cloth and centrifuged (JA-20 rotor, 12,000 rpm, 1 minute). The pellets were washed by suspension and centrifugation, three times with buffer A, twice with distilled water and twice with acetone. The starch granules were then air-dried.

2.6.2 Extraction of waxy protein from starch

The method described by Nakamura *et al.* (1993b) was used to extract the waxy protein, from starch. Starch granules (5 mg) were heated for 5 minutes in 80 µL buffer A (refer Section 2.6.1) in a boiling water bath. The swollen solutions were cooled on ice, centrifuged in a micro-centrifuge (15,000 rpm, 10 minutes, 4°C) and the supernatant (10 µL) was subjected to SDS-Polyacrylamide gel electrophoresis.

2.6.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed using the BioRad Protein II unit. The waxy protein of the wheat cultivars were analysed by using a 10% separating gel with an acrylamide/bis acrylamide concentration of 30:0.8 (Laemmli, 1990) and 30:0.135 (Kagawa *et al.*1988) for quantitative and qualitative analysis respectively.

Preparation of 10% separating gel and 4% stacking gel using 30% acrylamide/bis stock solutions

10% Separating gel

The separating gel mix (10 mL) containing 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 10% Acrylamide/Bis, 0.05% fresh ammonium persulfate and 0.05% TEMED was prepared. Distilled water, Tris-HCl and Acrylamide/Bis were mixed and degassed under vacuum for 15 minutes at room temperature prior to the addition of SDS, TEMED and ammonium persulfate. A quantity of 3.5 mL of the solution was added per gel, overlayed with water/ butanol mixture (1:1) and allowed to set.

4% Stacking gel

The stacking gel mix (10 mL) containing 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 4% Acrylamide/Bis solution, 0.05% fresh ammonium persulfate and 0.1% TEMED was prepared as mentioned above, and was applied on to the top of the separating gel. The comb was inserted and was allowed to set.

Loading and Running the gel

To each 10 µL protein sample 5 µL sample buffer was added and the solution mixed. The unstained protein marker (Low Range Marker, BioRad) was diluted according to the manufacturer's instruction and sample buffer was mixed in the ratio 4:1 respectively. The mixture was heated in a boiling water bath for 5 minutes. The marker and the protein samples were then spun in a micro-centrifuge (14,000 rpm, 5 minutes, room temperature) and were loaded on to the gel carefully with the aid of a syringe. The gels were run at 30 mA constant current setting for approximately one hour and 10 minutes in 1 X electrode running buffer.

Silver staining

At the end of electrophoresis, the protein gels were fixed in a solution containing absolute ethanol, acetic acid and distilled water in the ratio 4 : 1 : 5 respectively, for 30 minutes. The gels were then transferred into a solution containing, 60 mL absolute ethanol, 10.25 g anhydrous sodium acetate, 1.04 mL glutaraldehyde (25% w/v), 0.4 g sodiumthiosulphate pentahydrate in 200 mL distilled water. After 30 minutes the gels were washed with distilled water three times and placed in silver staining solution (0.2 g silver nitrate and 40 μL formaldehyde in 200 mL distilled water) for 40 minutes. The gels were then placed in developing solution (5 g sodium carbonate and 20 μL formaldehyde in 200 mL distilled water) until the protein bands were visible and transferred to stop solution (2.92 g EDTA.Na_2 dihydrate in 200 mL distilled water) for 5 minutes. Then to preserving solution (10% glycerol) for 15 minutes and the gels were dried in a Gel Air Dryer (Bio-Rad) heater for approximately two hours.

Figure 2.1 Wheat plants with heads tagged at anthesis

CHAPTER 3

A gel electrophoretic approach to the quantitation of amylose in wheat starch

3.1 Introduction

As reviewed in Chapter 1, the ratio of amylose and amylopectin has a major impact on the functional properties of starch and its performance in end products and uses. As such, the amylose/amylopectin ratio is an important parameter for the food processing industry as well as for breeders and farmers. An overview of the current literature concerning quantitation of amylose in starch is given in Chapter 1 (Section 1.9). Many methods based on different principles have been developed to measure the amylose content in starch. However, most of these methods have disadvantages.

Electrophoresis is a valuable analytical technique for resolving individual components from complex mixtures of biological macromolecules. However, very few reports have been published on the use of electrophoresis to separate and quantify starch components. Northcote (1954) developed a moving boundary electrophoretic method to separate neutral polysaccharides such as amylose, amylopectin, waxy maize, potato starch, wheat starch and rice starch. This method requires complex equipment and is time consuming. Foster *et al.* (1956) reported on zone electrophoretic behaviour of amylose and amylopectin on paper. The more recent fluorophore-assisted carbohydrate electrophoresis (Hu, 1995, Oshea *et al.* 1998) is complex and involves lengthy derivatisation steps, although it is useful for structure analysis.

The aim of this research was to develop a simple and rapid electrophoretic procedure to separate amylose and amylopectin and to allow quantitation of amylose in wheat starch. The experimental approach was based on agarose gels. The objectives of this study were to (i) identify a suitable electrophoresis buffer and a staining method, (ii) determine the correlation between laser densitometer response (peak area of absorbance) and amylose concentration, (iii) select a suitable solvent and optimise the

conditions for starch solubilisation, (iv) optimise the electrophoresis conditions of gel strength, voltage and time.

3.2 Materials and Methods

3.2.1 Samples

The samples used for this study were as follows; potato amylose and potato amylopectin (purchased from Sigma Chemical Company), soluble starch (purchased from Hopkins and Williams Ltd.), wheat starch and waxy maize starch (donated by Agrifood Technology, Werribee, Victoria) and potato starch (donated by Australian Food Industry Science Centre now Food Science Australia, Werribee, Victoria).

3.2.2 Method development

3.2.2.1 *Agarose gel electrophoresis using Tris-borate buffer*

Preparing a 0.5% agarose gel

Agarose (0.25 g) was dissolved in 50 mL Tris-borate buffer (pH 8.3, 0.1 M) by heating in a microwave oven (Panasonic). It was then cooled to 50-55°C, poured into the gel tray slowly and allowed to set for 15-20 minutes. Electrophoresis of starch samples was performed with horizontal agarose slab gels (Gel Electrophoresis apparatus GNA-100, Pharmacia, Sweden).

Sample preparation

Solutions (10 mg/mL) of potato amylose and wheat starch were prepared in Tris-borate buffer (pH 8.3, 0.1 M) by heating in a boiling water bath for an hour in glass test tubes. The solutions were stirred gently for about 30 seconds in a vortex mixer. Prior to electrophoresis, 1 μL of gel loading buffer was added to each 5 μL of the potato amylose solution and 5 μL of the wheat starch solution. The contents were loaded into the wells.

Running the gel

Electrophoresis was carried out in a mini-gel tank (Gel electrophoresis apparatus, GNA-100, Pharmacia, Sweden). The gel was run at 100 V for about 3 hours. Electrophoresis buffer was Tris-borate (pH 8.3, 0.1 M).

3.2.2.2 *Evaluation of staining agents*

The following three stains, fuchsin red, alcian blue and iodine were evaluated.

Staining with Fuchsin reagent

The procedure described by Pechanek *et al.* (1982) was used to stain the gel. The gel was initially incubated in periodic acid solution and then was stained by immersing in fuchsin reagent until the gel turned intense red. Excess dye was removed with 15 mL portions of 90% methylated spirit until the gel cleared and the bands were visible as red zones.

Staining with Alcian Blue

The gel was stained using the procedure developed by Wardi and Michos (1972). The gel was initially treated with 12.5% TCA solution for 30 minutes, periodic acid (1% in 3% acetic acid) for 50 minutes and 0.5% potassium metabisulphite for 30 minutes. Finally it was placed in 0.5% Alcian blue (in 3% acetic acid) for 4 hours and de-stained in 7% acetic acid solution.

Staining with Iodine stain

The gel was immersed in I_2 solution (0.2% I_2 in 2% KI solution) for approximately 3 minutes and de-stained by rinsing in distilled water 3-4 times.

3.2.2.3 *Optimisation of the concentration of agarose gel*

Potato amylose and wheat starch solutions (10 mg/mL) were prepared in Tris-borate buffer (pH 8.3, 0.1 M) by heating in a boiling water bath for an hour in glass test tubes. The solutions were stirred gently for about 30 seconds in a vortex mixer. Electrophoresis of these samples was carried out on 0.3%, 0.5% and 0.8% agarose gels at 100 V for 3 hours and the gels were stained with iodine and de-stained as described above.

3.2.2.4 *Electrophoretic separation and identification of a range of starch samples*

Solutions (10 mg/mL) of soluble starch, potato amylose, potato amylopectin, wheat starch, potato starch and waxy maize were prepared in Tris-borate buffer (pH 8.3, 0.1 M) (Section 3.2.2.3). Electrophoresis of these solutions (Section 3.2.2.1) was on a 0.5% agarose gel, at 100 V for 3 hours. The gel was then stained with iodine stain and de-stained with distilled water (Section 3.2.2.2).

3.2.3 Development of the quantitative aspects of the method

Lipid extracted wheat starch was used for the following experiments. Lipids (non-starch lipids) were removed from wheat starch as mentioned in Section 2.4.3.

3.2.3.1 ***Quantitation using the laser densitometer*** The amylose bands (amylose-iodine complex) in the gels were scanned using an Ultra ScanXL laser densitometer, to quantify the amylose. The wavelength of the laser beam was 633nm and the parameter X-width was set at 5. The area of the peak corresponding to the amylose band was measured with the software supplied by the manufacturer.

3.2.3.2 *Evaluation of staining time with I_2/KI staining agent*

Wheat starch solution (10 mg/mL) was prepared in 0.5 M KOH by heating in a boiling water bath for 1 minute, then gently stirred in a vortex mixer for 30 seconds and kept in ice. The solution (5 μL) was then electrophoresed as described in Section 3.2.2.1 at 30 V for 16 hours. The gel was then immersed in the iodine stain, de-stained in distilled water by rinsing 3-4 times and scanned in the laser densitometer immediately to determine the peak areas corresponding to the blue amylose bands. The staining times used were 1, 3 and 5 minutes.

3.2.3.3 *Evaluation of time between de-staining and scanning*

Preparation and electrophoresis of wheat starch solution were performed as described in Section 3.2.3.2. The gel was stained for 1 minute in the iodine solution, de-stained with distilled water by rinsing 3-4 times and was scanned in the laser densitometer. The peak areas of absorbance of amylose were determined. The time evaluated between de-staining and scanning were 5, 20, 40 and 60 minutes.

3.2.3.4 *Establishment of solubilisation conditions for wheat starch*

The effects of temperature and time on starch solubilisation were studied with two solubilisation agents, KOH (0.125 M, 0.25 M and 0.5 M) and DMSO. Wheat starch solutions (10 mg/mL), in the various concentrations of KOH, were prepared by heating in a water bath at 60°C, 80°C and 100°C for times between 1 minute and 1 hour. Wheat starch solutions (10 mg/mL) were prepared in DMSO, by heating in a water bath at 80°C and 100°C for times between 1 minute and 1 hour. The solutions were gently stirred for approximately 30 seconds using a vortex mixer and placed in ice. Electrophoresis was carried out with the solutions as described in Section 3.2.2.1 on 0.5% gel at 30 V for 16 hours. The gels were stained in the iodine solution for 1 minute, de-stained in distilled water by rinsing 3-4 times and scanned immediately with the laser densitometer. The peak areas corresponding to the amylose bands were determined.

3.2.3.5 *Evaluation of electrophoresis voltage and time*

Wheat starch and potato amylose solutions (10 mg/mL) were prepared by solubilising in 0.5 M KOH at 80°C for 15 minutes. Electrophoresis of these solutions was carried out as mentioned in Section 3.2.2.1 on 0.5% gel. The following voltage and time combinations were applied: 50 V/3 hours, 75 V/3 hours, 100 V/3 hours and 30 V/16 hours. The gels were stained with iodine, de-stained with distilled water and scanned as described above.

3.2.3.6 *Calibration curve with potato amylose standard*

Potato amylose (10 mg/mL) in 0.5 M KOH was prepared by solubilising at 80°C for 15 minutes. This was diluted to produce 2, 4, 6 and 8 mg/mL solutions in 0.5 M KOH. The solutions were kept in ice. Electrophoresis was carried out as described in Section 3.2.2.1 on 0.5% gel, at 30 V/16 hours. The gels were then stained, de-stained and scanned as described above.

3.2.4 Electrophoresis of de-branched wheat starch

The de-branching procedure was based on the method developed by Matheson and Welsh (1988). Wheat and waxy maize starch solutions (10 mg/mL) in 0.5 M KOH were prepared by solubilising at 80°C for 15 minutes. The solutions were then neutralised with 0.5 M HCL (1 mL) and mixed with 2 M acetate buffer (pH 4.8, 0.20 mL) and isoamylase (Hayashibara) from *Pseudomonas amyloderamosa* (1200 units), and incubated overnight at 37°C. Potassium hydroxide (2.5 M, 0.20 mL) was added and an aliquot (10 µL) was electrophoresed (Section 3.2.2.1) on 0.5%, 0.8%, 1.5% and 2.0% agarose gels.

3.3 Results and Discussion

3.3.1 Borate buffers in electrophoresis of polysaccharides

The basic requirement for the application of electrophoretic techniques is that the components to be separated possess electrical charges. Borate esterification of carbohydrates has proved valuable in separating neutral polysaccharides by moving boundary electrophoresis (Northcote, 1954) and zone electrophoresis (Foster, 1956). Neutral sugars are converted into negatively charged complexes by reaction with borate ions at alkaline pH, which promotes anodic migration of the carbohydrate. The reaction between borate ions and carbohydrates is represented in Figure 3.1 (Weitzman *et al.* 1979) as shown below,

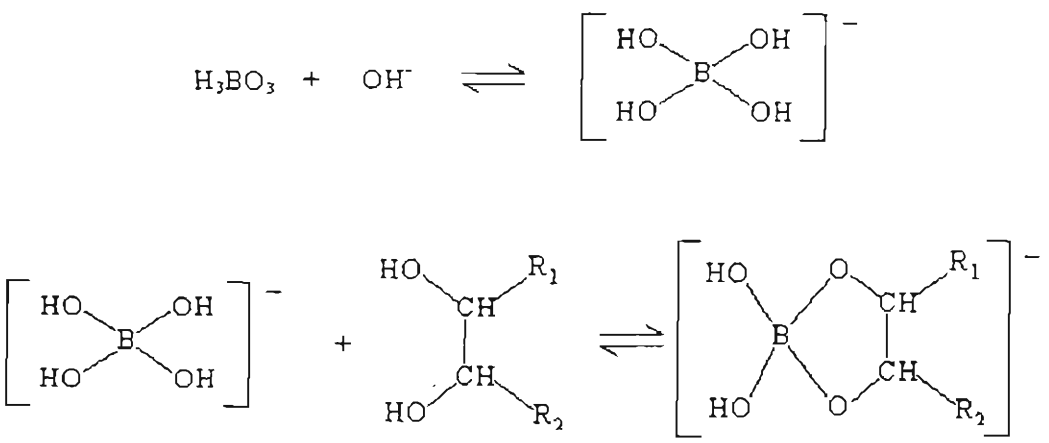


Figure 3.1 Borate complex formation with carbohydrates

A borate buffer system was therefore selected for this research. In the preliminary experiments, potato amylose and wheat starch were subjected to agarose gel electrophoresis in a borate buffer containing NaOH and boric acid at pH 10, as described by Padmoyo and Miseriz (1967). However, excess heat was generated during electrophoresis with this buffer. Ineffective or incomplete dissipation of this heat will lead to evaporation of the running buffer and melting of the gel. Hence an alternate running buffer, Tris-borate buffer (pH 8.3, 0.1 M) was used, which proved quite satisfactory. In the analysis of glycopeptides by polyacrylamide gel electrophoresis, the effectiveness of Tris-borate (pH 8.3, 0.1 M) has been attributed to the formation of a negatively charged complex with neutral polysaccharides (Weitzman *et al.* 1979).

Preliminary studies using potato amylose and wheat starch revealed that the polysaccharide components of starch could be separated by agarose gel electrophoresis with Tris-borate buffer (pH 8.3, 0.1 M) and visualised by iodine stain. Cathodic movement of amylose was observed in this buffer system. These findings indicate that the starch components are not forming a borate complex with the buffer system, which will lead to anodic migration. This view can be rationalised on the basis that, in starch, there are few 4-hydroxyl groups and these are trans with respect to the adjacent hydroxymethyl groups (ie 6-OH). The 2- and 3- OH groups are trans and steric hindrance of the 3-OH is indicated by low levels of substitution at this position in hydroxypropyl starch derivatives (Wootton and Haryadi, 1991). These factors precluded the formation of appreciable levels of borate complex with starch. Foster (1957) reported that in glycosidically linked sugars, adjacent hydroxyl groups in the pyranose ring react more readily in cis configuration than in trans configuration and borate buffers can form complex with C4 and C6 hydroxyl groups only if the stereo-chemistry was correct. According to Foster (1957) the electro-endosmotic flow of borate buffers is toward the cathode. Hence, the migration of the starch components toward the cathode in the present study is attributed to the influence of the flow of the buffer.

3.3.2 Evaluation of staining agents

Alcian blue and Fuchsin red proved to be unsuitable because de-staining of the agarose gel was extremely difficult. It was found that I_2/KI reagent was the most suitable stain. The colour development of the starch components in this reagent was rapid and the gel de-stained satisfactorily with distilled water allowing laser densitometer scanning to be carried out. The iodine stained amylose band was characterised by its blue colour and that of amylopectin by its reddish brown colour. This method of detection is specific for the polysaccharide components of starch. In the application of paper ionophoresis, potato amylose and amylopectin were detected on neutralised paper, by spraying with ethanolic iodine (Foster, 1956), as violet and reddish- brown zones respectively.

3.3.3 Electrophoresis of starch components

Figure 3.2 shows the electrophoresis profiles of soluble starch, potato amylose, potato amylopectin, potato starch, wheat starch and waxy maize. Electrophoresis was performed on 0.5% agarose gel at 100 V for 3 hours. Waxy maize starch remained at the origin and stained reddish brown, indicating the presence of only the amylopectin fraction. Wheat and potato starches showed two fractions, amylopectin, which stained reddish brown and did not migrate, and amylose, which stained blue and migrated towards the cathode. In contrast to these, commercial samples of potato amylopectin and soluble starch showed, in addition to amylopectin which remained at the origin and, in the case of soluble starch an amylose band, some broad bands which stained reddish brown and migrated towards the cathode at a slower rate than amylose. This implied the presence of some lower molecular weight branched chain components, which may have arisen during the commercial production of these two samples.

3.3.4 Concentration of agarose gel

A key aspect of this research was to select the optimum concentration of agarose gel for migration of the starch components. Migration of amylose was observed at all 3 concentrations of 0.3, 0.5 and 0.8% agarose, but migration of amylopectin did not occur even at 0.3% gel concentration. Since starch components are high molecular weight polymers, a gel of sufficiently low concentration to enable easy migration should be used. However, the 0.3% gel was too fragile for routine handling,

precluding the use of lower concentrations and therefore the 0.5% agarose gel was the concentration selected as a compromise between band mobility and gel handling characteristics.

3.3.5 Evaluation of staining time with I_2/KI staining agent

Table 3.1 shows the peak area corresponding to the amylose band of wheat starch, stained for 1, 3 and 5 minutes. As shown, there was no substantial change in the peak area by increasing the staining time to 5 minutes. This suggests that the staining of amylose is completed in 1 minute. Hence, the standard time chosen for staining the gel was 1 minute.

3.3.6 Evaluation of time between staining and scanning

Table 3.2 shows the peak area corresponding to the amylose band of wheat starch, when the band was scanned at different times after staining. Considerable change in the peak area was not observed up to an hour. However, the amylose band started fading after 2-3 hours. Hence, for this study, the standard time chosen between scanning the gel and staining was 5 minutes.

3.3.7 Relationship between potato amylose concentration and densitometer response

The electrophoresis of potato amylose standards is shown in Figure 3.3 and the laser densitometer profile of potato amylose standard (1%) is shown in Figure 3.4. Laser densitometer peak areas for the amylose bands had a strong linear correlation ($r = 0.999$, $p < 0.01$, $n = 4$) with amylose concentration (Figure 3.5), demonstrating that agarose gel electrophoresis could be used to quantify amylose.

3.3.8 Solubilisation of starch

Solubilisation of starch is crucial to the effectiveness of any method of amylose measurement since all amylose must be extracted without significant degradation. The effectiveness of solubilisation of wheat starch under the various conditions was assessed, by the densitometer peak area for amylose, obtained after electrophoresis of the solution.

Solubilisation of wheat starch in KOH

The results are summarised in Tables 3.3, 3.4 and 3.5. It can be seen that the maximum peak area for amylose was obtained when the starch was solubilised in 0.5 M KOH for 15 minutes at 80°C. Figure 3.6 shows the laser densitometer profile of this solution. The first big peak corresponded to amylose. However, there was a second small peak corresponding to a starch component, which migrated slower than amylose. Figure 3.7a shows the laser densitometer profile of wheat starch when the solubilisation conditions were less severe (0.125 M KOH, 60°C, 1 minute). There was no indication of the second peak at these conditions, however the peak area of the amylose band was much smaller (Table 3.3). With increased heating time, the second peak appeared (Figure 3.7b) and this was accompanied by an increase in the peak area of amylose (Table 3.3). The second peak corresponded to a starch fraction, most probably an "intermediate fraction" having a molecular size higher than amylose and lower than amylopectin, which appeared as a faint blue smear in the gel (Figure 3.8). These results indicate that solubilisation of starch increased with increase in heating time. It also appeared that, at 100°C in 0.25 M and 0.5 M KOH, peak areas of amylose decreased with solubilisation time and no amylose peak was detected after 60 and 45 minutes respectively (Tables 3.4 and 3.5). This indicates degradation of amylose with more rigorous alkali treatment. Figure 3.9 shows the effect of increasing the time of solubilisation at 100°C in 0.5 M KOH by the electrophoretic pattern. Extensive degradation, increasing with time at 100°C, is indicated. Thus, conditions chosen as optimal for wheat amylose extraction were heating in 0.5 M KOH at 80°C for 15 minutes.

It has been believed for a number of years that some starches contain a third type of polysaccharide, an intermediate material between amylose and amylopectin (Banks and Greenwood, 1975). Whistler and Doane (1961) have obtained intermediate materials ranging from 4.5% of the total starch for normal commercial corn starch to 6.6%-8.7% for high amylose corn starches. Yeh *et al.* (1981) and Boyer and Liu (1985) have reported the presence of intermediate material, consisting of a branched molecule with a molecular weight lower than that of amylopectin, which eluted between amylose and amylopectin fractions in GPC elution profiles of maize starch.

This suggests that, in the present study, the second peak observed in KOH solubilised wheat starch may be due to the "intermediate fraction" of the starch. However, the possibility of this being an artefact due to starch degradation should not be ignored. Recently, it has been proposed that, the intermediate material identified in some starches are probably derived from hydrolysis or fragmentation of amylopectin (Tester and Karkalas, 1996).

Solubilisation of wheat starch in DMSO

In DMSO, the recovery of amylose improved as extraction time and temperature increased, although it decreased slightly with heating at 100°C for greater than 30 minutes (Table 3.6 and Figure 3.10). The results are in agreement with those of Morrison and Laignelet (1983), who determined the optimum heating times for different starches in DMSO, at 100°C, from the blue values. They observed that maximum blue values were reached in 60-90 minutes (or earlier in few cases), then decreased linearly over the next 18 hours.

DMSO solubilisation for 30 minutes at 100°C gave highest densitometer readings, but this was lower than most of the alkali solubilisations. Based on the laser densitometer results, it appears that KOH gave better dispersion of starch than did DMSO. However, an alternative explanation for this may be that the iodine binding capacity of amylose is less in DMSO than in KOH. DMSO bonds temporarily with the starch hydroxyl groups and converts starch into a temporary organic derivative in which the hydroxyl groups have been replaced by hydrogen bonded DMSO (French, 1984). This may decrease the iodine affinity and hence lower iodine intensity colour.

The laser densitometer profile of wheat starch solubilised in DMSO at the optimum condition showed a single peak (Figure 3.11), which corresponded to amylose. The 'intermediate material' (second peak) was absent. Mua and Jackson (1995) have reported the presence of intermediate material in oat starch, solubilised by autoclaving in DMSO followed by sonication, due to depolymerisation of the starch.

Hence, in the present study, the intermediate material observed when KOH was used as the solvent may possibly be an artefact due to fragmentation of amylopectin by the alkali. However, solubilisation of wheat starch in KOH gave higher laser densitometer readings and hence KOH was chosen as the solvent in this study.

3.3.9 Electrophoresis voltage and time

The best separation of the starch components was achieved when electrophoresis of wheat starch was carried out at 30 V for 16 hours (Figure 3.12) and these conditions were selected for routine analysis. Although the electrophoresis conditions of 100 V for 3 hours gave satisfactory resolution and offered a substantially faster analysis, heating of the gel made it unsuitable as a routine method. It should be noted that no movement of amylopectin from the origin was observed for any of the voltage/time combinations evaluated.

3.3.10 De-branched wheat starch

Figure 3.13 presents the electrophoresis pattern of de-branched wheat starch and waxy maize starch. Cathodic movement of de-branched waxy maize starch was observed. Waxy maize starch contains only amylopectin, and it was seen that the native waxy maize starch remained at the origin during electrophoresis, due to its large molecular size (Figure 3.1). Isoamylase completely de-branches the amylopectin molecule to yield a population of linear chains (Gunja-Smith, 1970). Hence, in the present study, the de-branched amylopectin migrated towards the cathode due to its linear form and smaller molecular size.

In the native wheat starch it was seen that the amylopectin fraction remained at the origin and the amylose fraction migrated towards the cathode (Figure 3.1). However, in the de-branched wheat starch the amylopectin fraction migrated ahead of amylose towards the cathode (Figure 3.13), because of the lower molecular weight of the linear amylopectin chains compared to amylose. As discussed in the literature review, most of the starches contain amylose, with different proportions of linear and branched molecules, having a molecular weight less than that of amylopectin. On de-branching, they give linear chains much longer than that from amylopectin (South *et al.* 1991).

Hence, the de-branched amylose, which appeared blue, migrated slower than the de-branched amylopectin, which appeared reddish brown. However, the two fractions were not distinctly separated. This was reflected in the laser densitometer profiles. Figure 3.14 shows the laser densitometer profiles of de-branched wheat starch. Base-line separation between the amylose and de-branched amylopectin fraction could not be achieved even by increasing the agarose gel concentration from 0.5% to 2%.

3.3.11 Advantages of the method

Agarose gel electrophoresis of wheat starch provided a simple and a rapid method for separation and detection of the principal starch components. It allowed multiple simultaneous determinations of amylose to be carried out. As the starch components could be visualised, the method can be used to monitor the purity of amylose and amylopectin preparations. Lengthy derivatisation steps are not involved in this method.

3.3.12 Limitations of the method

This method requires the use of an amylose calibration curve, which, strictly speaking, should be based on amylose from the botanical source of the starch being analysed. Since the starch was solubilised by heating at 80°C for 15 minutes in 0.5 M KOH, it is likely that lipids would be saponified and values would be "true", rather than "apparent" amylose.

3.4 Conclusions

An agarose gel electrophoretic method has been developed to separate and detect amylose and amylopectin. Quantitation of amylose in wheat starch could be achieved by using laser densitometry after staining the gels with iodine. The starch solubilisation procedure was specifically developed for wheat starch and this aspect would to be re-evaluated prior to its application to other starch types.

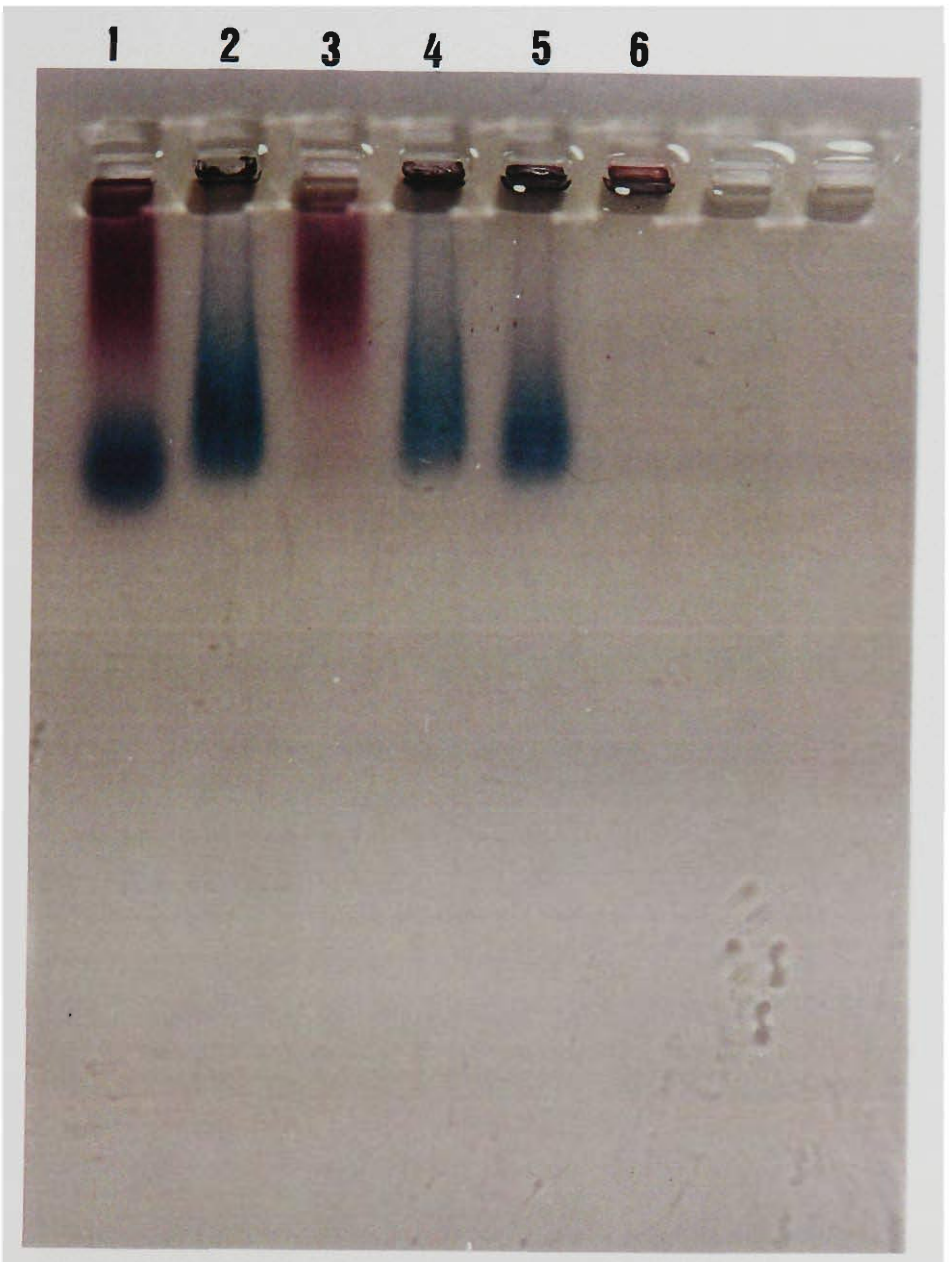


Figure 3.2 **Electrophoretic separation of polysaccharide components of starch solubilised in Tris-borate buffer. Electrophoresis was carried out at 100 V/3 hours on 0.5% agarose gel. Lanes, 1) Soluble starch 2) Potato amylose 3) Potato amylopectin 4) Potato starch 5) Wheat starch 6) Waxy maize.**

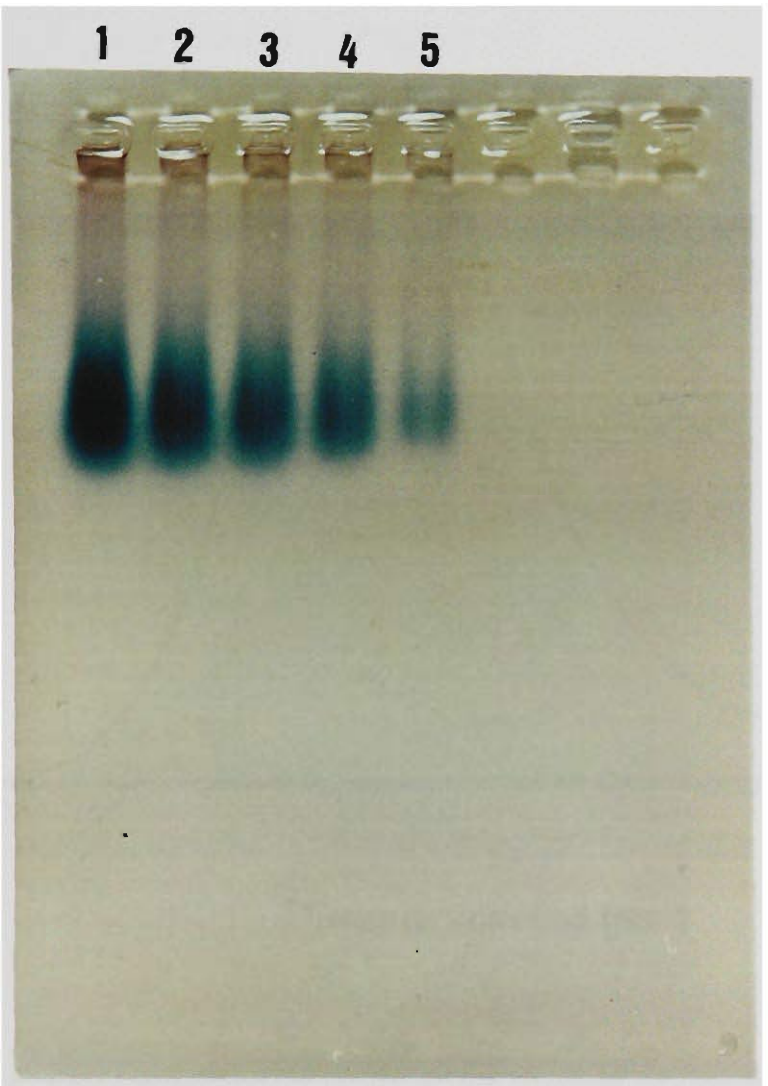


Figure 3.3 Electrophoretic separation of potato amylose standards solubilised in 0.5 M KOH. Electrophoresis was carried out at 30 V/16 hours on 0.5% agarose gel. Lanes, 1) 1.0% amylose 2) 0.8% amylose 3) 0.6% amylose 4) 0.4% amylose 5) 0.2% amylose.

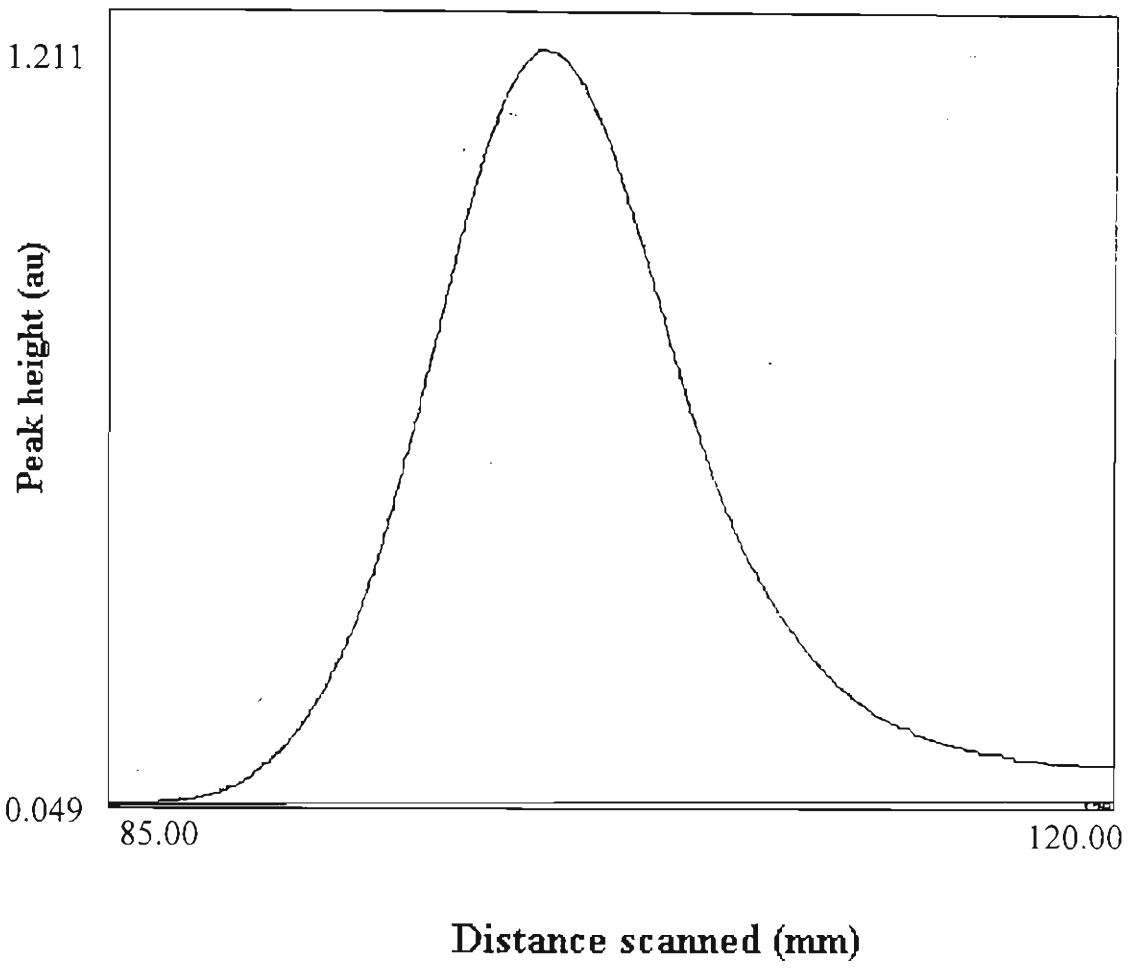


Figure 3.4 Laser densitometer profile of 1% amylose solubilised in 0.5 M KOH.

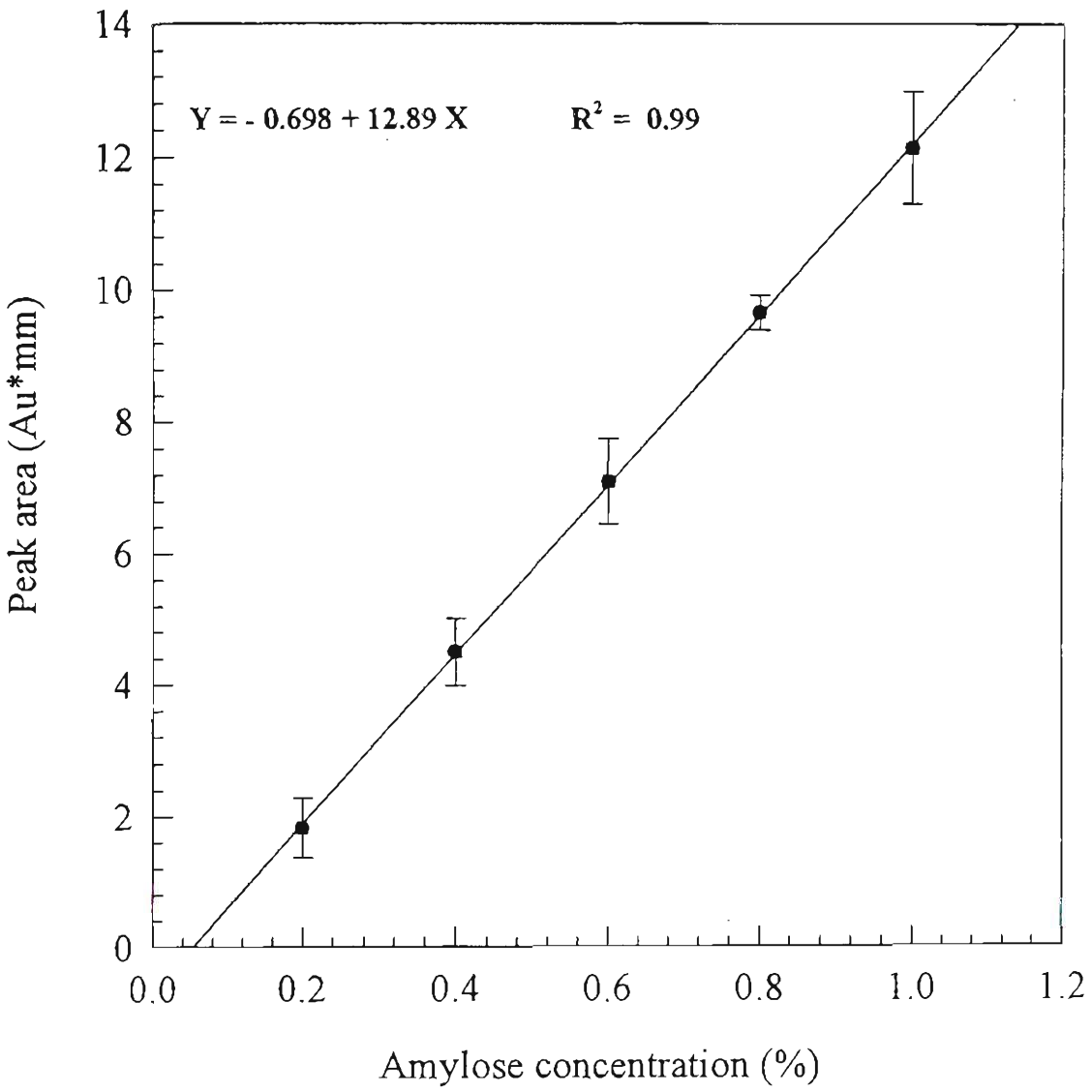


Figure 3.5 Calibration curve of potato amylose.

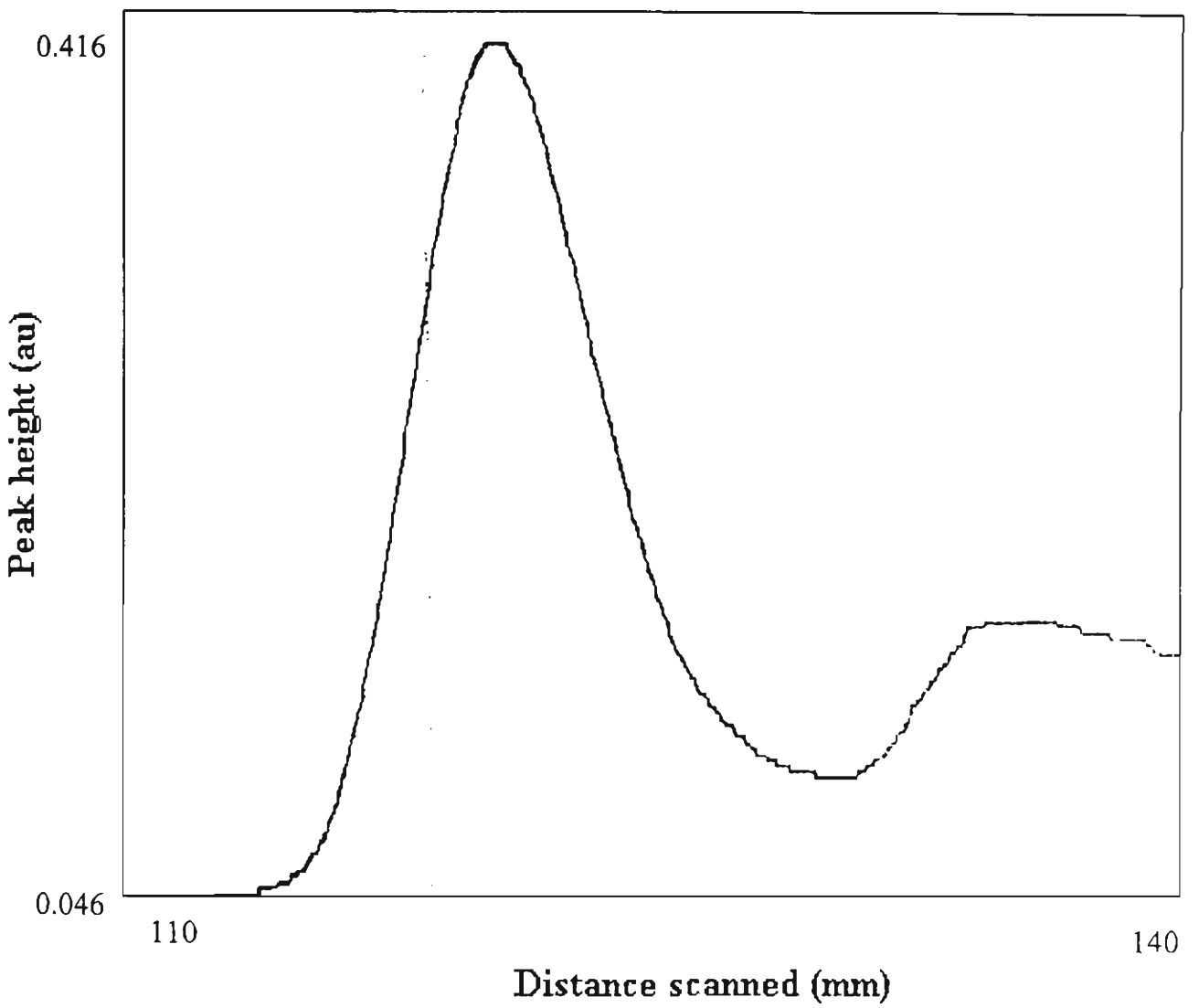


Figure 3.6 Laser densitometer profile of wheat starch solubilised in 0.5 M KOH at 80°C for 15 minutes.

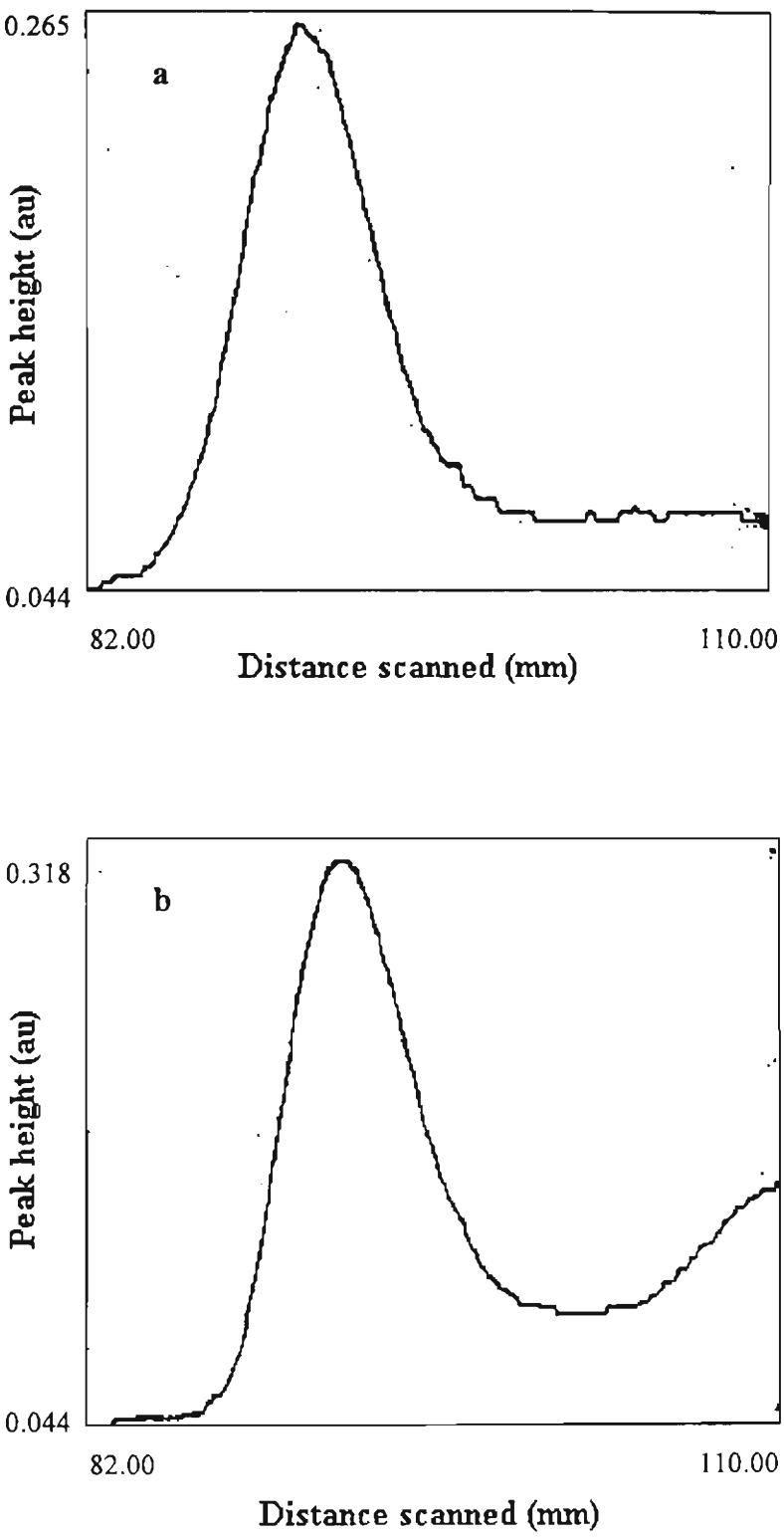


Figure 3.7 Laser densitometer profile of wheat starch solubilised in 0.125 M KOH at 60°C for, a) 1 minute b) 60 minutes.

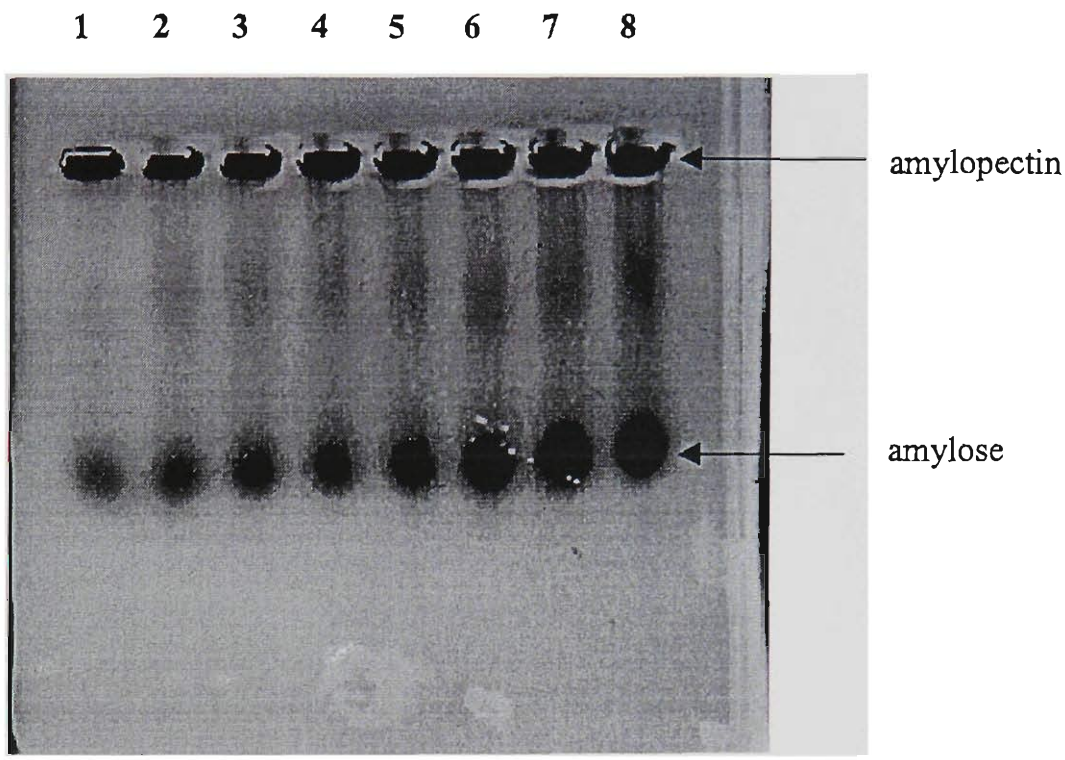


Figure 3.8 Electrophoresis of wheat starch solubilised in 0.125 M KOH at 60°C for 1, 3, 5, 10, 15, 30, 45 and 60 minutes, shown in lanes 1 to 8 respectively. Electrophoresis was carried out at 30 V/16 hours on 0.5% agarose gel.

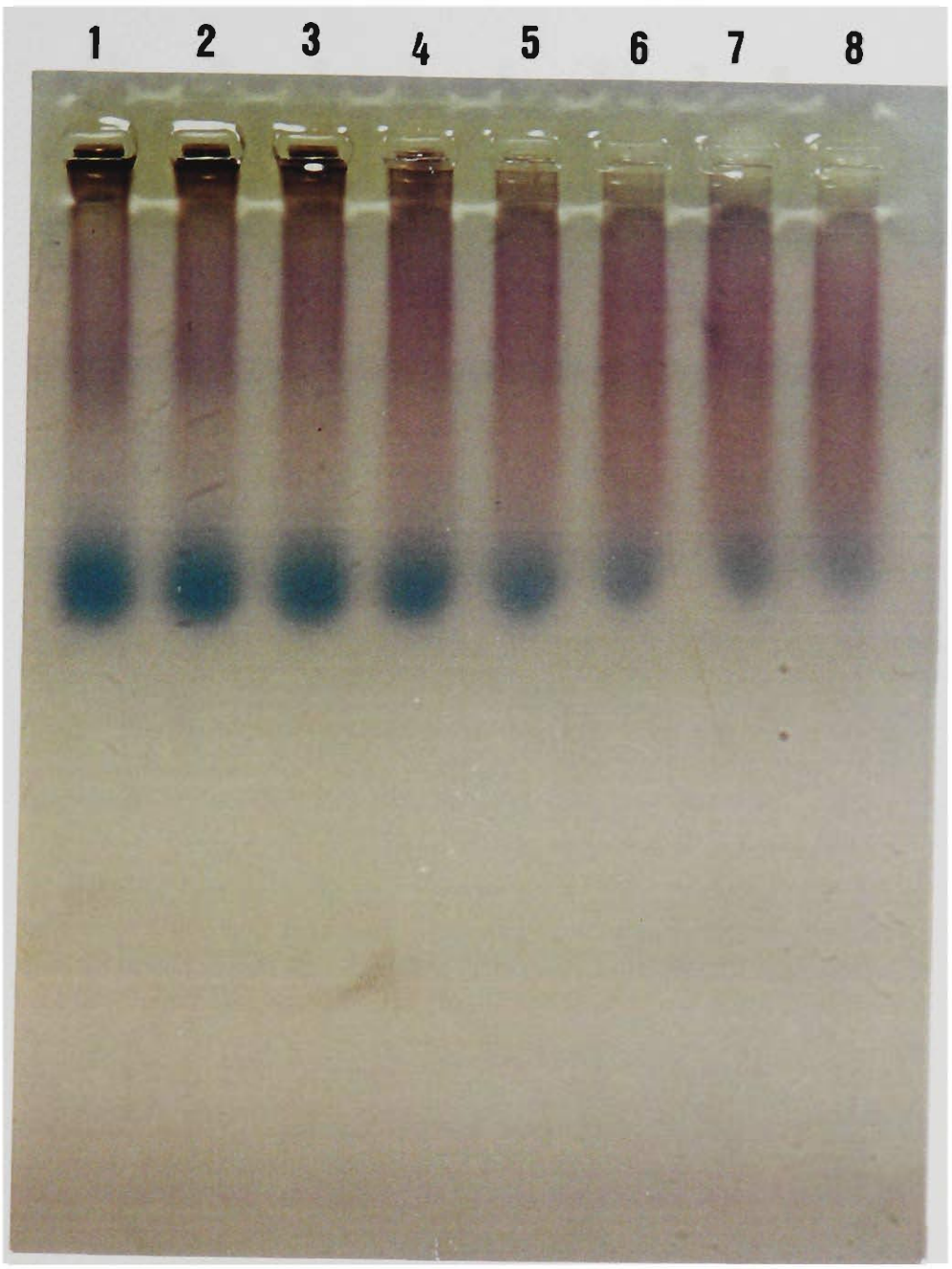


Figure 3.9 Electrophoresis of wheat starch solubilised in 0.5 M KOH at 100°C for 1, 3, 5, 10, 15, 30, 45 and 60 minutes, shown in lanes 1 to 8 respectively. Electrophoresis was carried out at 30 V/16 hours on 0.5% agarose gel.

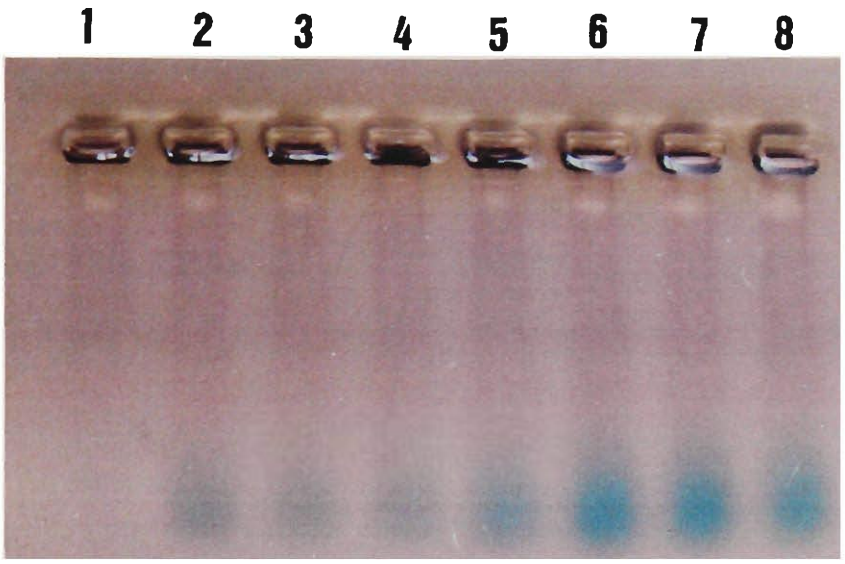


Figure 3.10 Electrophoresis of wheat starch solubilised in DMSO at 100°C for 1, 3, 5, 10, 15, 30, 45 and 60 minutes, shown in lanes 1 to 8 respectively. Electrophoresis was carried out at 30 V/16 hours on 0.5% agarose gels.

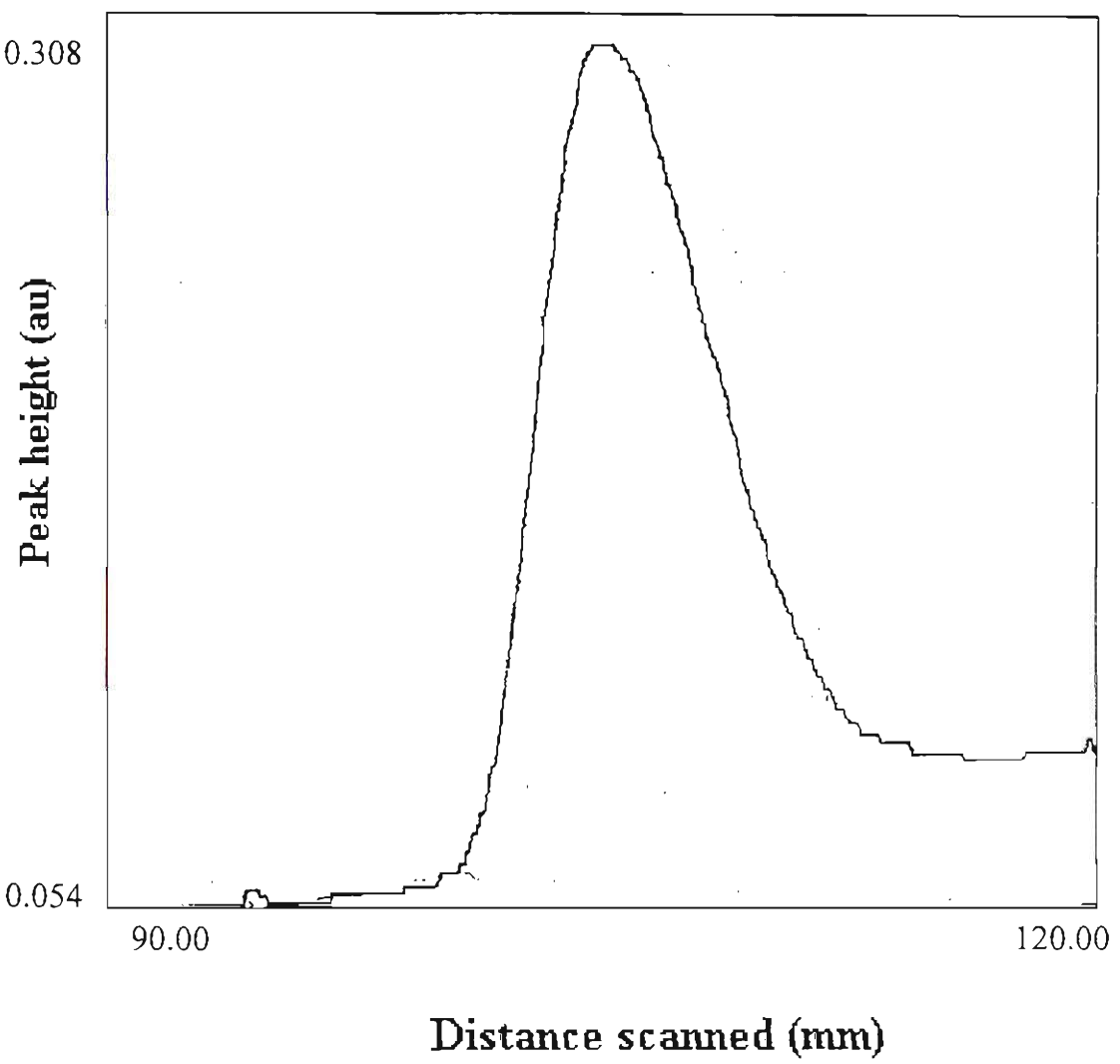


Figure 3.11 Laser densitometer profile of wheat starch solubilised in DMSO at 100°C for 30 minutes.

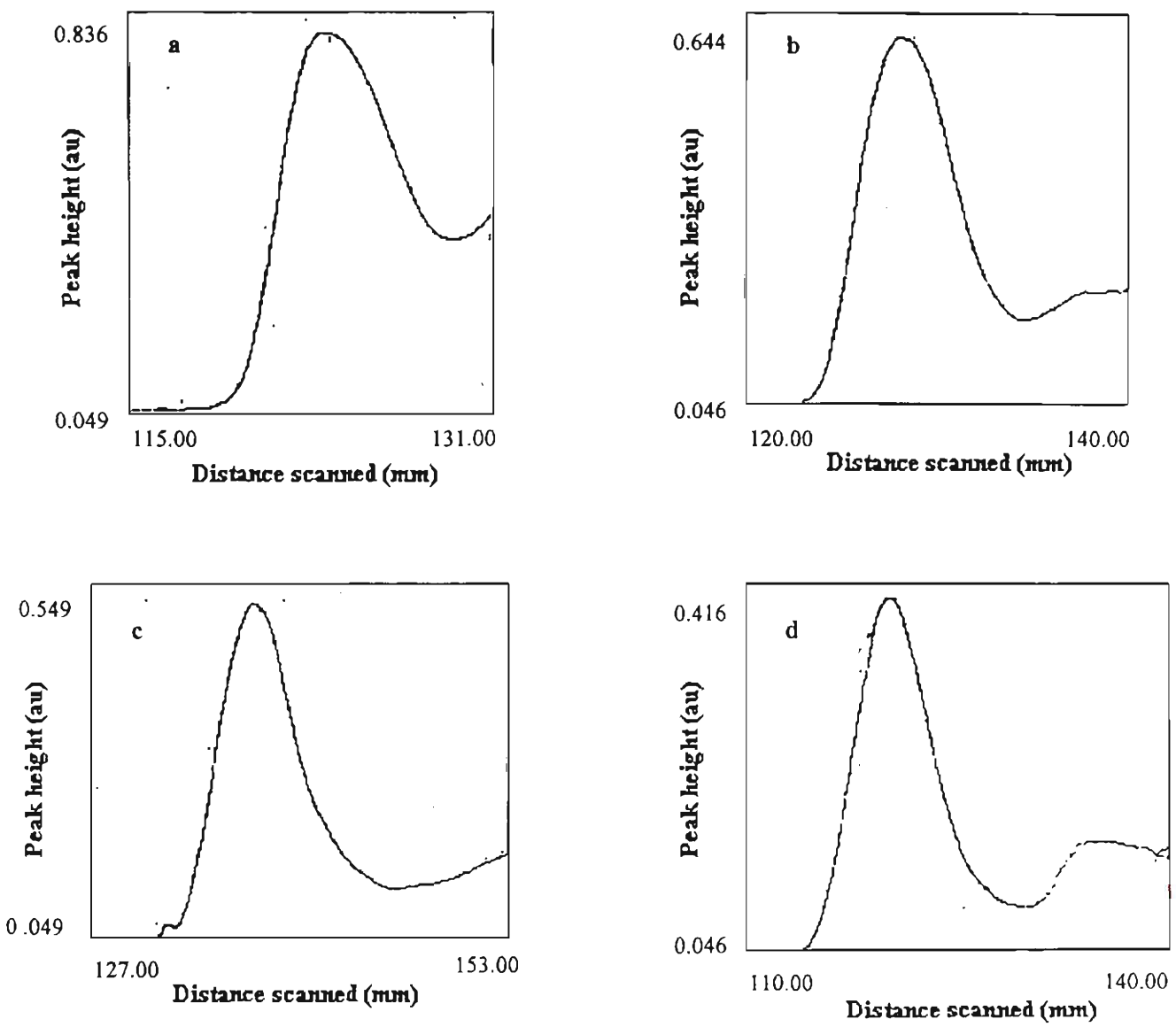


Figure 3.12 Laser densitometer profiles of wheat starch solubilised in 0.5 M KOH at 80°C for 15 minutes; electrophoresis was carried out at, a) 50 V/3 hours b) 75 V/3 hours c) 100 V/3 hours d) 30 V/16 hours on 0.5% agarose gel.

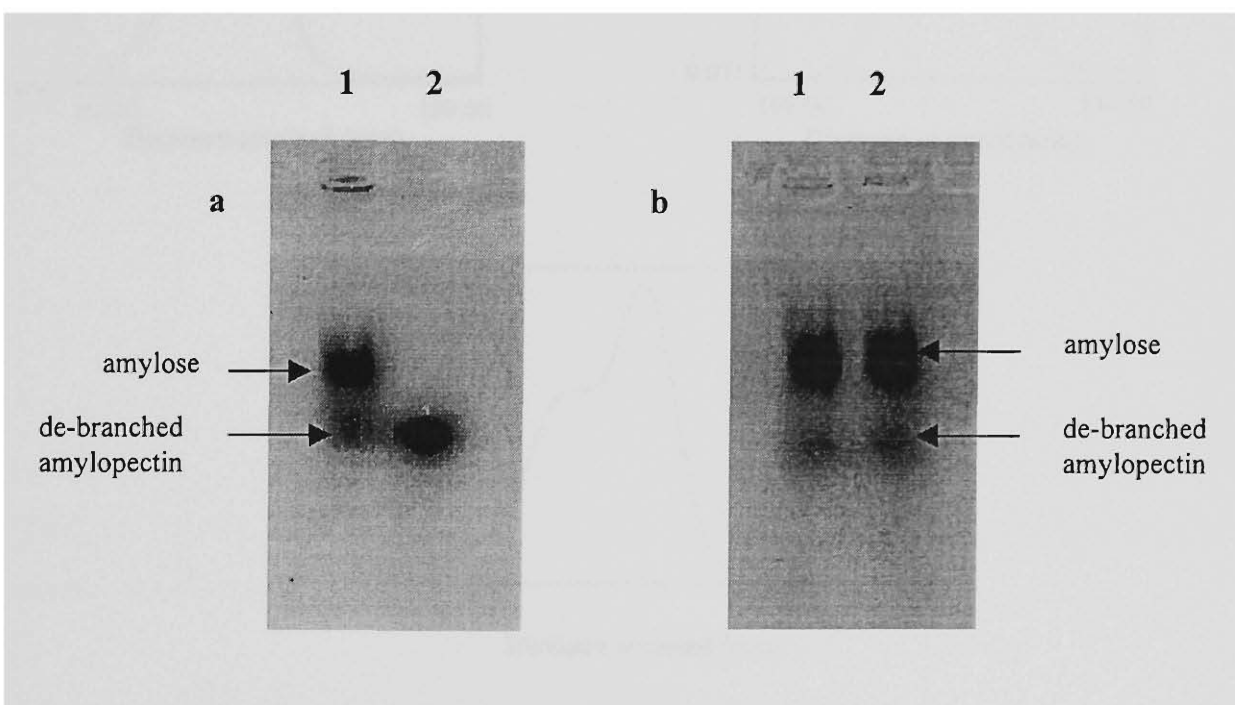


Figure 3.13 a) Electrophoresis of de-branched wheat starch (lane 1) and de-branched waxy maize starch (lane 2), at 100 V/3 hours on 1.5% agarose gel. b) Electrophoresis of de-branched wheat starch (lanes 1&2), at 100 V/4 hours on 2% agarose gel.

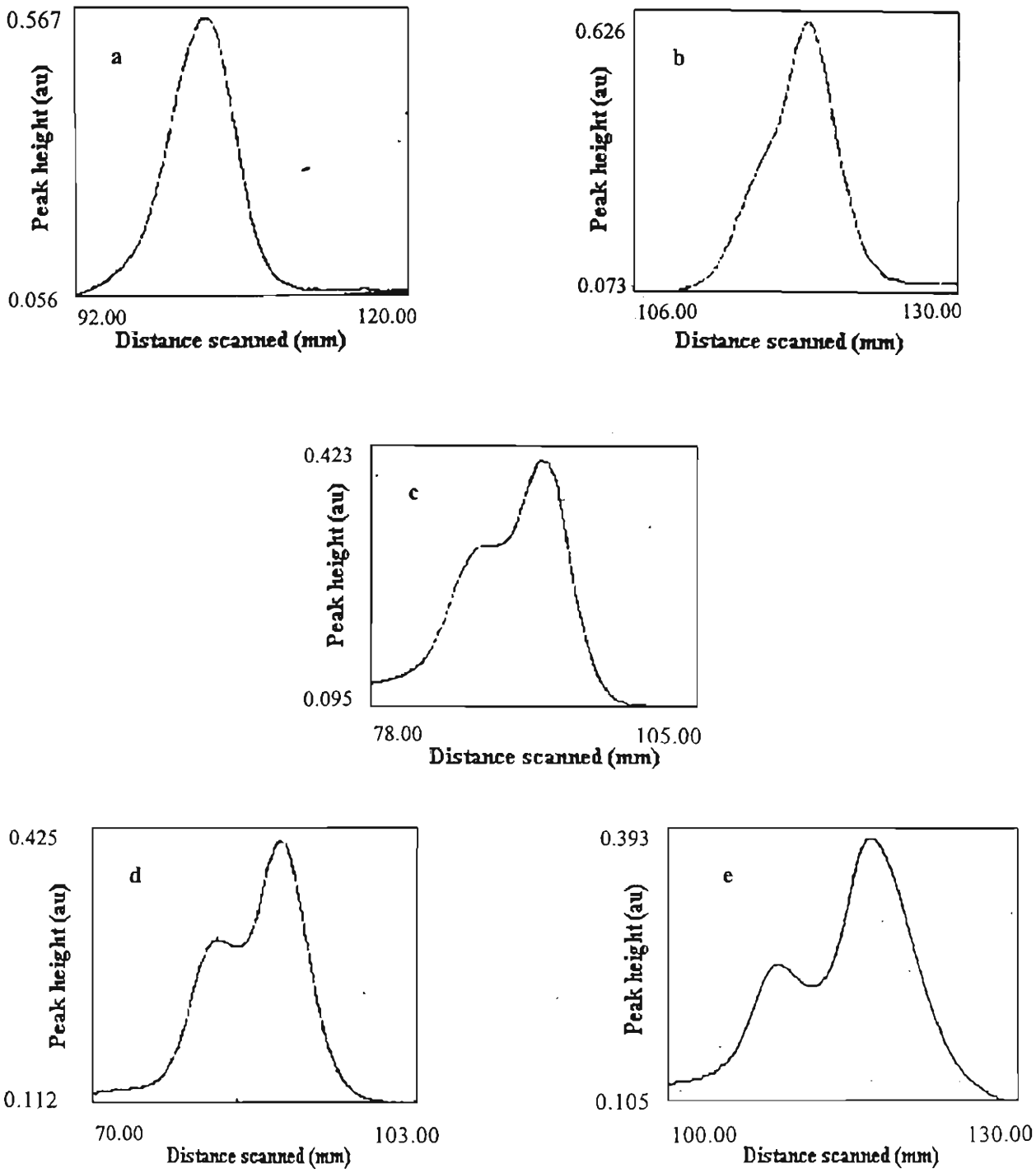


Figure 3.14 Laser densitometer profiles of de-branched wheat starch. Electrophoresis was carried out at 100 V/3 hours on, a) 0.5 % b) 0.8 % c) 1.25 % d) 1.5 % agarose gel e) Electrophoresis was carried out at 100 V/4 hours on 2% agarose gel.

Table 3.1 Effect of time of iodine staining on peak area of absorbance of amylose in wheat starch.

Time (Minutes)	Mean peak area (au*mm) ± s.d.
1	2.1 ± 0.2
3	2.2 ± 0.3
5	1.9 ± 0.2

Table 3.2 Effect of time between staining and scanning on peak area of absorbance of amylose in wheat starch.

Time (minutes)	Mean peak area (au*mm) ± s.d.
5	2.1 ± 0.2
20	2.4 ± 0.1
40	2.2 ± 0.2
60	2.4 ± 0.1

Table 3.3 Peak areas of wheat amylose determined by laser densitometer when wheat starch was solubilised in 0.125 M KOH at different time and temperature. Each value is the average of two determinations.

Time (min)	60°C Peak area (au*mm)	80°C Peak area (au*mm)	100°C Peak area (au*mm)
1	1.5	1.5	1.2
3	1.7	1.8	1.2
5	1.7	1.8	1.2
10	1.7	2.0	1.6
15	1.8	2.1	1.6
30	2.0	2.1	1.5
45	2.1	2.2	1.4
60	2.2	2.3	1.3

Table 3.4 Peak areas of wheat amylose determined by laser densitometer when wheat starch was solubilised in 0.25 M KOH at different time and temperature. Each value is the average of two determinations.

Time (min)	60°C Peak area (au*mm)	80°C Peak area (au*mm)	100°C Peak area (au*mm)
1	2.0	1.9	2.0
3	2.0	2.1	1.8
5	2.0	2.1	1.8
10	2.1	2.2	1.6
15	2.1	2.2	1.5
30	2.3	2.4	1.1
45	2.3	2.4	0.7
60	2.4	2.2	0.0

Table 3.5 Peak areas of wheat amylose determined by laser densitometer when wheat starch was solubilised in 0.5 M KOH at different time and temperature. Each value is the average of two determinations.

Time (min)	60°C Peak area (au*mm)	80°C Peak area (au*mm)	100°C Peak area (au*mm)
1	2.1	2.2	2.1
3	2.1	2.3	1.9
5	2.1	2.4	1.9
10	2.2	2.4	1.7
15	2.0	2.4	1.4
30	1.9	2.3	1.1
45	1.9	2.1	0.0
60	1.8	1.9	0.0

Table 3.6 Peak areas of wheat amylose determined by laser densitometer when wheat starch was solubilised in DMSO at 80°C and 100°C at different times. Each value is the average of two determinations.

Time (min)	80°C Peak area (au*mm)	100°C Peak area (au*mm)
1	0.0	0.0
3	0.0	0.7
5	0.0	0.6
10	0.0	0.7
15	0.0	1.2
30	1.0	1.8
45	1.1	1.8
60	1.2	1.7

CHAPTER 4

Preparation of wheat amylose standard

4.1 Introduction

Most methods for determining amylose content and amylose/amylopectin ratios in starches rely on the use of standards for either or both of these starch components. The standards are most commonly from potato or maize, which are used in the determinations involving starches from other plants, especially using iodine complexation for quantification. However, molecular weights and degree of branching differ between starch types and hence the nature of iodine binding will vary between starches resulting in differing characteristics of the starch/iodine complexes. It is therefore necessary to use standards from the starch being studied if precise values for amylose content are to be obtained.

Cereal starches are difficult to fractionate by butanol precipitation into two distinct, easily separated fractions (Welsh and Blakeney, 1992). The lectin concanavalin A (con A) has been used to fractionate starches on the basis of its ability to bind amylopectin due to its affinity with non-reducing end groups of polysaccharides. However, the ratio of con A to carbohydrate and the time allowed for precipitation of the amylopectin/con A complex are normally regarded as being crucial to ensure complete complex formation (So and Goldstein, 1967).

The objectives of this study were to (i) prepare amylose in small scale from wheat starch, by the butanol precipitation and con A precipitation methods, (ii) determine the purity of the wheat amylose samples, (iii) determine the correlation between concentration of amylose and laser densitometer response, with the wheat amylose samples.

4.2 Materials and Methods

Wheat starch was kindly donated by Agrifood Technology, Werribee, Victoria.

4.2.1 Preparations of amylose from wheat starch

Butanol precipitation method

Fractionation of wheat starch by butanol precipitation was carried out as described in Section 2.3.2.1.

Con A precipitation method

Recovery of amylose by butanol precipitation

Fractionation of wheat starch by con A precipitation was carried out as described in Section 2.3.2.2. The following conditions were examined to optimise the concentration of con A solution (containing con A in 0.2 M sodium acetate buffer, pH 6.4) and the time to precipitate amylopectin.

Con A solution	Precipitation time
(a) 150 mg con A/100 mL buffer	2 hours
(b) 200 mg con A/100 mL buffer	1 hour
(c) 200 mg con A/100 mL buffer	2 hours.

The amylose was recovered from the supernatant by butanol precipitation as described in Section 2.3.2.2.

Recovery of amylose by ultra-filtration

Precipitation of amylopectin from wheat starch and recovery of amylose from the supernatant were performed as mentioned in Section 2.3.2.3. The optimum conditions, con A solution (200 mg con A/100 mL buffer) and a precipitation time of 2 hours, were used to precipitate the amylopectin from wheat starch.

4.2.2 Determining the purity of wheat amylose

Analysis by agarose gel electrophorsis

The wheat amylose samples prepared in Section 4.2.1 were subjected to agarose gel electrophoresis (as described in Section 2.3.3.1) to detect amylopectin impurities.

Analysis by HPLC

The amylose samples were hydrolysed and glucose concentrations in the hydrolysates were determined by HPLC as mentioned in Section 2.3.3.2.

4.2.3 Calibration curves with wheat amylose standards

The correlations between amylose concentration and the corresponding peak area were examined for both the wheat and potato amylose standards. Solutions of potato amylose (Sigma) and the wheat amylose standards were prepared as mentioned in Section 3.2.3.6, with different concentrations covering the range of 0.2%-1.0%. Electrophoresis was carried out with the standard solutions at 30 V for 16 hours on 0.5% agarose gel. The gels were then stained in 0.2% I_2 in 2% KI solution for 1 minute, de-stained by rinsing in distilled water 3-4 times and scanned in the laser densitometer to determine the peak areas of the amylose bands.

4.3 Results and Discussion

4.3.1 Optimisation of concentration of con A solution and time to precipitate amylopectin

Examination of different concentrations of con A solution to precipitate amylopectin from wheat starch showed that the optimum wheat starch/con A ratio was 1:2 (w/w) and the optimum precipitation time was 2 hours at room temperature. The gel profiles and the corresponding laser densitometer patterns of wheat starch are shown in Figures 4.1 and 4.2. The second peak which appeared after the amylose peak (Figures 4.2a and 4.2b) corresponded to the "intermediate material" which was visible in the gels (Figures 4.1a and 4.1b). As mentioned in the previous chapter, this intermediate fraction may possibly be a breakdown product of amylopectin, which had not precipitated with con A. This fraction was not present when wheat starch/con A ratio was increased to 1:2 and the precipitation time was increased to 2 hours (Figures 4.1c and 4.2c), indicating complete precipitation of the branched fraction. These results suggest that the concentration of con A solution and the precipitation time are critical parameters in fractionating starch by this method.

Although starch is generally described as a mixture of two components, there appears to be a range of structures with overlapping molecular sizes and also possibly some that are intermediate between the unbranched chains and branched chains with a particular average chain-length and degree of branching (Matheson and Welsh, 1988). Con A precipitates essentially all, or most of, the material with (1→6) branches, including any intermediate fraction (Matheson and Welsh, 1988). As illustrated in this study, the concentration of con A is a critical parameter. It has been reported that amylopectin interacts with con A only in the presence of higher concentrations of lectin (Smith *et al.* 1968). Under the conditions of the assay proposed by Gibson *et al.* (1997), the determined amylose contents of waxy, normal and high amylose starch samples were affected only when the ratios of concentration (on weight basis) of con A to solubilised starch were well below 2. The results of the present study are in agreement with these previous reports.

It has been suggested previously that precipitation reaction times for con A and amylopectin should range from 10 minutes to 120 minutes at room temperature (Colona *et al.* 1985; Matheson *et al.* 1988). In the amylose assay procedure, Gibson *et al.* (1997) reported that although the amylopectin-con A precipitation reaction was complete within 30 minutes with little change in 120 minutes, the time required for effective precipitation of the amylopectin complex was 60 minutes. Since amylose tends to retrograde in solution it was suggested that the precipitation time should not exceed 2 hours (Gibson *et al.* 1997).

4.3.2 Determining the purity of amylose samples by agarose gel electrophoresis

The purity of the wheat amylose samples obtained by the butanol precipitation and con A precipitation methods, is shown in the electrophoresis profiles (Figure 4.3). The presence of amylopectin, which was indicated by a reddish brown smear moving at a slower rate than amylose, was observed in the wheat amylose sample prepared by butanol fractionation and in commercial potato amylose. However, wheat amylose free of amylopectin was obtained by the con A precipitation method, confirmed by the absence of the reddish brown smear (Figure 4.3). These results are in agreement with that of Matheson and Welsh (1988) who demonstrated a more effective fractionation

of starch from tobacco leaves and high amylose pea starch by the con A method than by complexing with 1-butanol. Elution profiles obtained by gel permeation chromatography on Sepharose CL-2B indicated the presence of amylopectin in the fraction that was precipitated by the butanol. Furthermore, butanol precipitation was found to be unsuitable for a number of other starches, such as those from immature cereal seeds (Matheson 1971), leaf starches and high amylose starches (Whistler *et al.* 1984; Banks and Greenwood 1975). In each of these starches, the amylose fraction was contaminated with amylopectin, and, in the leaf and high amylose starches, the amylopectin fraction included amylose of low molecular size.

4.3.3 Determining the purity of amylose samples by HPLC

Commercial potato amylose (ICN), stated as 95% pure was used for comparison. Table 4.1 shows the concentration of glucose determined by HPLC, after hydrolysing 1 mg of the amylose samples. Approximately 1 mg/mL of glucose was derived by hydrolysis, from potato amylose (ICN), potato amylose (Sigma) and wheat amylose obtained by butanol precipitation (wheat amylose 1). The purity of amylose was 92%, 99% and 89% respectively based on glucose recovery. In comparison, the amount of glucose derived from wheat amylose standards obtained by con A precipitation followed by butanol precipitation (wheat amylose 2) and by ultra-filtration (wheat amylose 3) were 0.37 mg/mL and 0.50 mg/mL respectively, implying purities of 33.6% and 45.5% respectively. These results indicate that although the potato amylose (Sigma) and the wheat amylose (obtained by butanol precipitation method) were contaminated with amylopectin, they were pure polyanhydroglucoses with minimal impurities. On the other hand, the wheat amylose standards derived by the con A precipitation methods, although free of amylopectin, contained impurities such as denatured con A and salts. The excess con A in the amylose supernatant was removed by denaturing it by heating in a steam bath and centrifuging, however if the excess con A was not completely removed from the supernatant, it would remain as a contaminant in the amylose.

4.3.4 Analysis of amylose standards by agarose gel method

Electrophoresis profiles of potato amylose, wheat amylose 1 (butanol precipitated), wheat amylose 2 (con A precipitated followed by butanol precipitation) and wheat amylose 3 (con A precipitated followed by ultra-filtration) samples are shown in Figure 4.4. Calibration curves of peak area vs amylose concentration, for the different samples, are shown in Figure 4.5.

Excellent linear correlations were obtained with all the amylose standards, potato amylose ($r = 0.999$, $p < 0.01$, $n = 4$), wheat amylose 1 ($r = 0.995$, $p < 0.01$, $n = 2$), wheat amylose 2 ($r = 0.996$, $p < 0.01$, $n = 2$), wheat amylose 3 ($r = 0.991$, $p < 0.01$, $n = 2$). However, differences were observed between the different amylose samples, and it was reflected in the peak areas (Table 4.2). The electrophoresis band of potato amylose standards (Figure 4.4a) appeared broader than wheat amylose 1 (Figure 4.4b) and gave a greater response with iodine (Table 4.2), which indicates that potato amylose is a bigger molecule than wheat amylose. Matheson and Welsh (1988) have reported a wide range of molecular sizes of the amylose fraction, with differences between starch types, in the gel elution profiles on Sepharose CL-2B; Potato amylose had more material of larger molecular size than rice grain amylose. Sargeant and Wycombe (1982) reported the presence of wide range of chain lengths in the gel elution profile of wheat amylose on Sepharose CL-6B. In the present study, the broad amylose bands exhibited by the potato amylose and wheat amylose 1 indicated the presence of a wide range of molecular sizes. The broader appearance of potato amylose compared to wheat amylose 1 also indicated that the former contained material of a wider range of molecular size than wheat amylose 1.

When comparing the peak areas of wheat amylose 1, 2 and 3 (Figure 4.4 and Table 4.2), wheat amylose 1 gave a better response with iodine. The decreased response to iodine by wheat amylose 2 and 3 is attributed to their lower purity as indicated by glucose recoveries (Section 4.3.3). The laser densitometer peak area for wheat amylose 3 with iodine was higher than that of wheat amylose 2 (Figure 4.5 and Table 4.2), consistent with its higher purity. Since wheat amylose 3 was recovered by ultra-filtration, after con A precipitation, this would have enabled the removal of

contaminating denatured protein and salts compared to wheat amylose 2. This was reflected in the HPLC analysis (Section 4.3.3), which indicated that wheat amylose 3 was purer than wheat amylose 2.

4.4 Conclusions

A small scale preparation of wheat amylose by butanol precipitation and con A precipitation was achieved. A more efficient fractionation of wheat starch was achieved by the con A method which produced wheat amylose free of amylopectin. However, glucose recoveries from the hydrolysed amylose samples reflected low purity of the wheat amyloses 2 and 3, and wheat amylose 1 contained some amylopectin.

Linear correlations were observed between laser densitometer response (peak area of absorbance) and concentrations of wheat and potato amylose samples. Although the correlations were high, the impurities in the amylose samples would preclude their use as standards for amylose determination.

Based on these results, potato amylose was used as the standard for applications in this research. The time and expense of preparing wheat amylose standards and their low purity outweighed shortcomings associated with the use of potato amylose for this purpose.

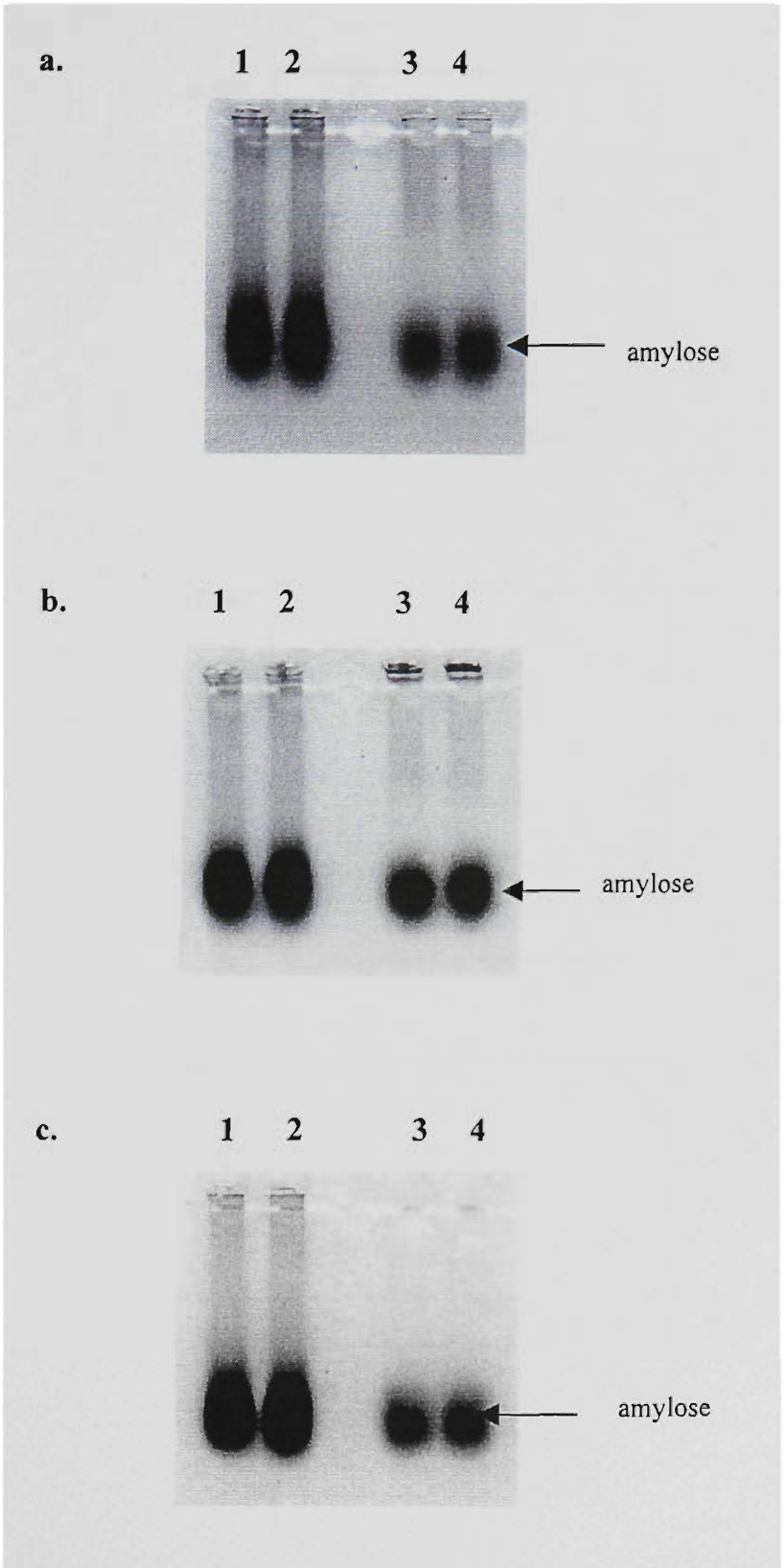


Figure 4.1 Electrophoresis of potato amylose (lanes 1 & 2) and wheat amylose isolated by the con A method (lanes 3 & 4). Wheat starch/con A ratio and the precipitation times are as follows, a) 1:1..5 and 2 hours b) 1:2 and 1 hour c) 1:2 and 2 hours.

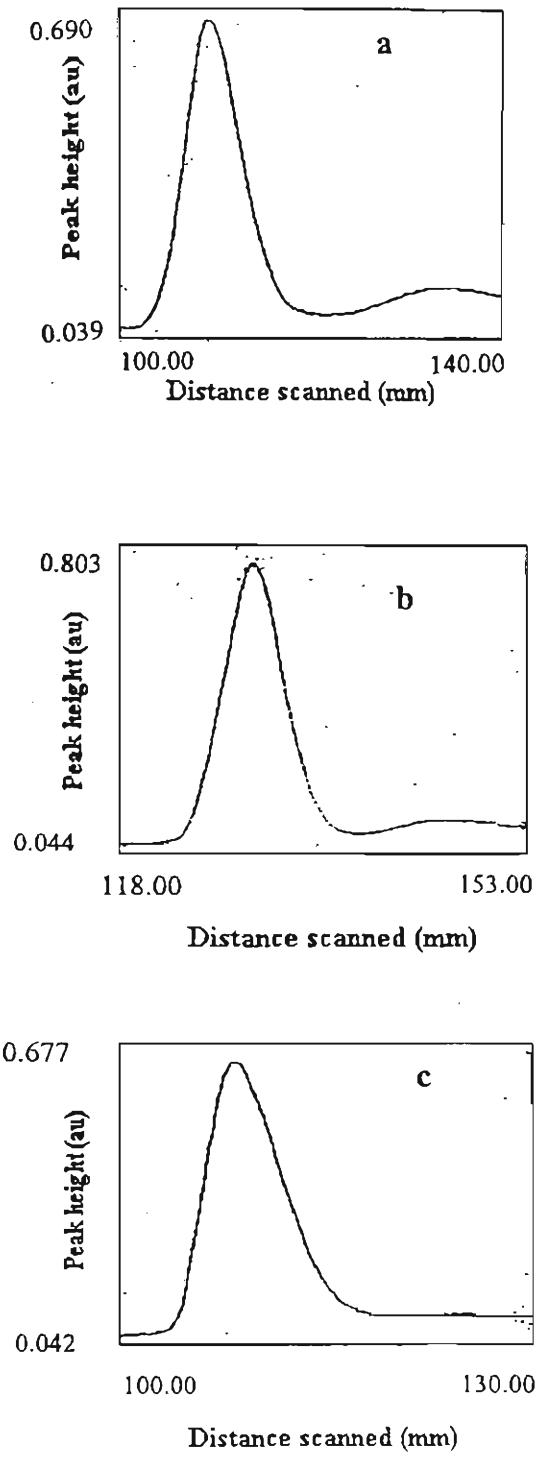


Figure 4.2 Laser densitometer profiles of wheat amylose isolated by the con A method. Starch/con A ratio and the precipitation times are as follows, a) 1:1.5 and 2 hours b) 1:2 and 1 hour c) 1: 2 and 2 hours.

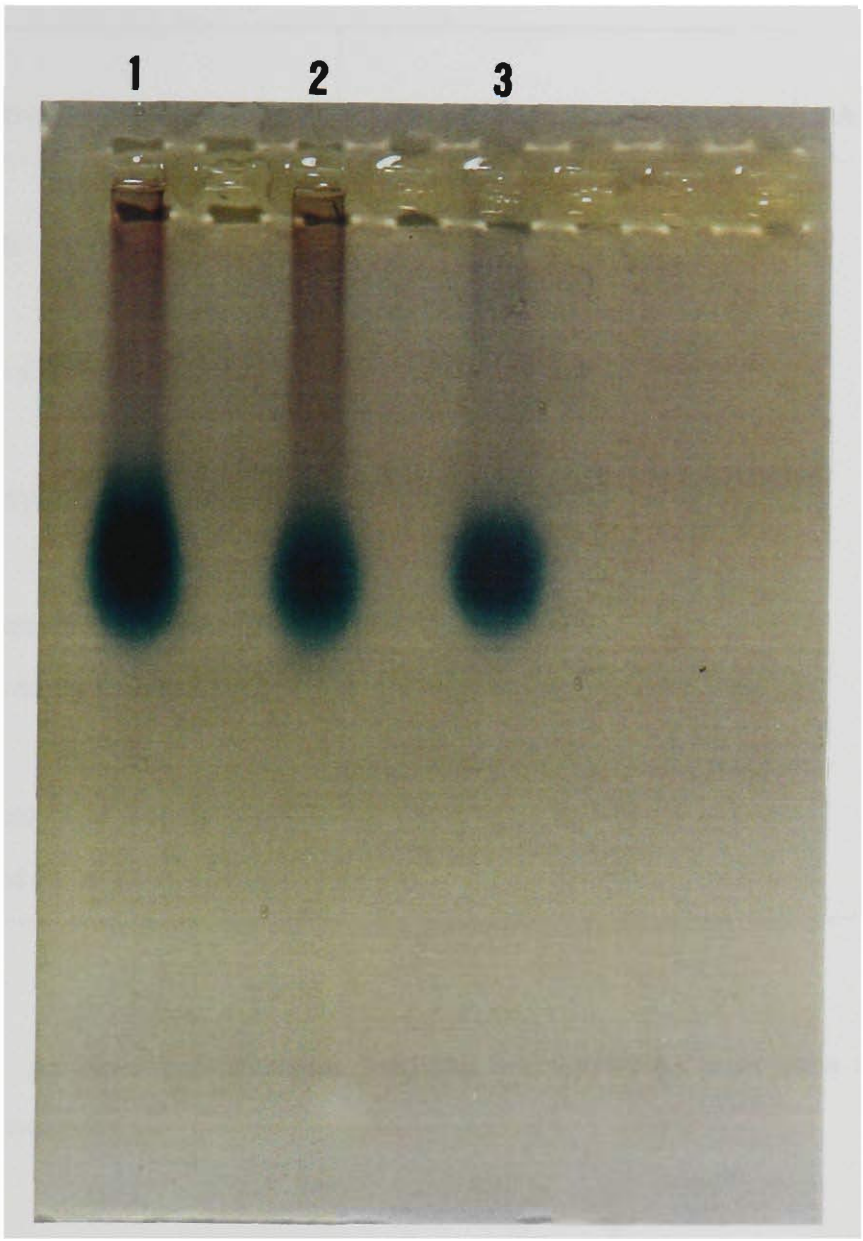


Figure 4.3 Electrophoresis of potato amylose (lane 1), wheat amylose isolated by the traditional butanol fractionation method (lane 2) and wheat amylose isolated by the con A method (lane 3).

Table 4.1 Glucose concentrations and purity of the amylose samples.

Amylose Sample	Glucose recovery (mg/mL)	Amylose purity (%)
Potato amylose (ICN)	1.0	92
Potato amylose (Sigma)	1.1	99
Wheat amylose 1 (butanol ppt)	0.9	89
Wheat amylose 2 (con A ppt followed by butanol ppt)	0.4	33.6
Wheat amylose 3 (con A ppt followed by ultra-filtration)	0.5	45.5

Table 4.2 Amylose concentration and the corresponding peak area for the different amylose standards.

	1% amylose	0.8% amylose	0.6% amylose	0.4% amylose	0.2% amylose
Potato amylose Peak Area (au*mm)	12.1	9.7	7.1	4.5	1.8
Wheat amylose 1 Peak area (au*mm)	5.6	4.9	3.3	1.9	0.6
Wheat amylose 2 Peak area (au*mm)	2.0	1.7	1.1	0.6	0.2
Wheat amylose 3 Peak area (au*mm)	3.1	2.3	1.7	0.5	0.00

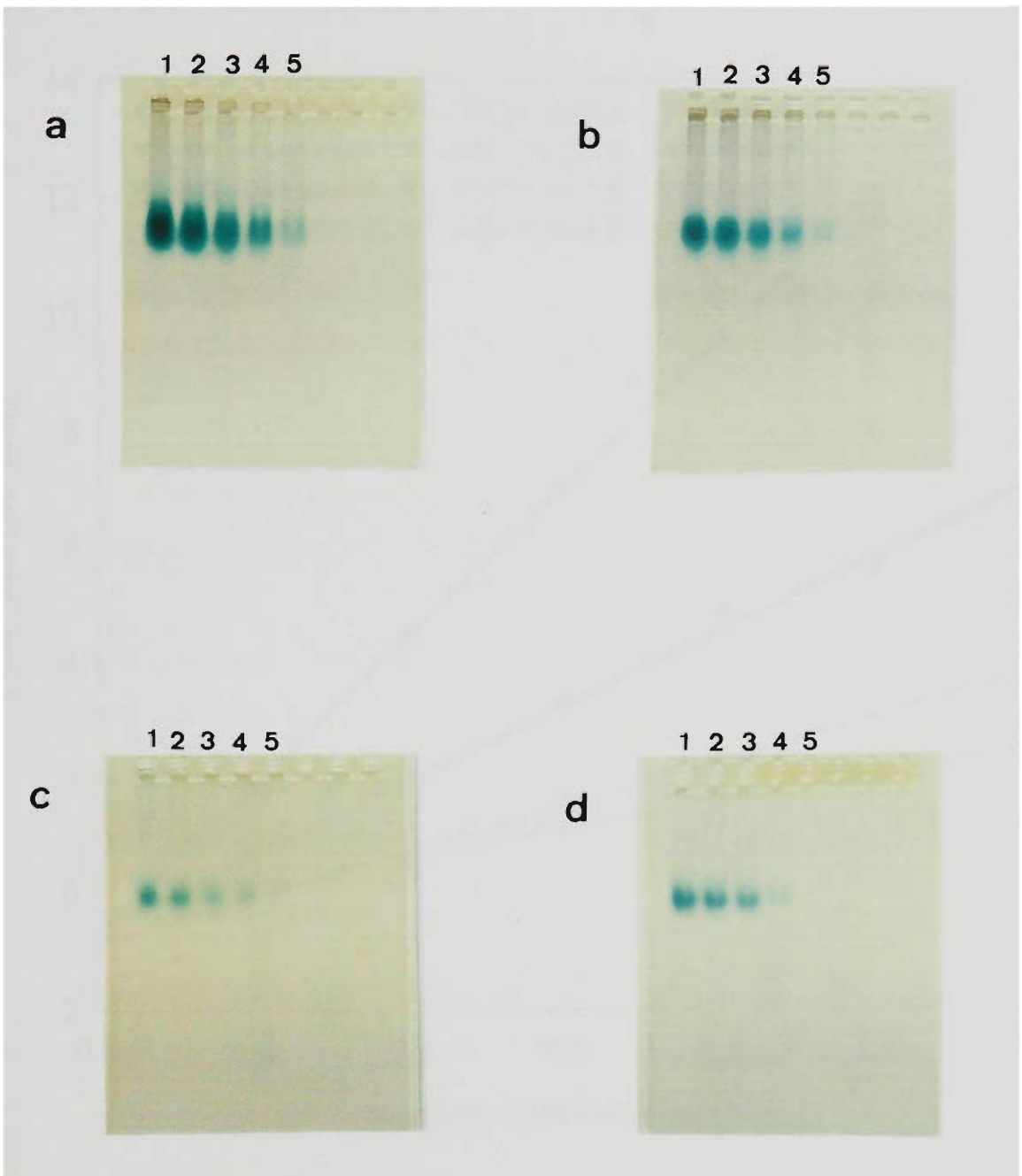


Figure 4.4 Electrophoresis of amylose standards. a) potato amylose b) wheat amylose 1 (butanol ppt) c) wheat amylose 2 (con A ppt followed by butanol ppt) d) wheat amylose 3 (con A ppt followed by ultra-filtration). Lanes, 1) 1% 2) 0.8% 3) 0.6% 4) 0.4% 5) 0.2% solutions.

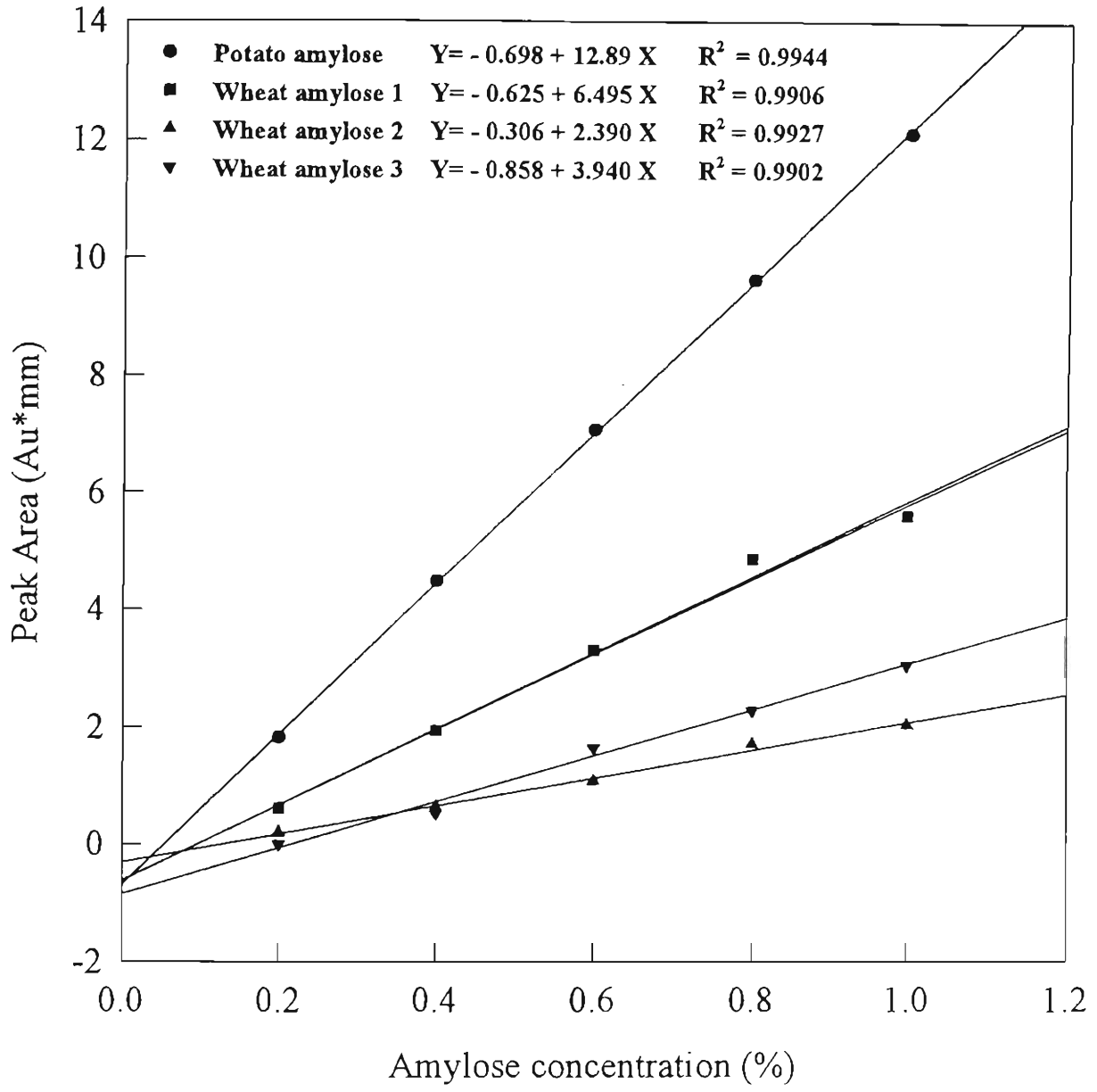


Figure 4.5 Calibration curves of amylose standards.

CHAPTER 5

Application of the new method

5.1 Introduction

A simple and rapid method of amylose estimation on a small starch sample is essential to assist in wheat breeding programs related to starch properties. In Chapter 3, a simple and rapid agarose gel electrophoresis method was described, to measure amylose content of starch from mature wheat. This method permits separation of amylose and amylopectin and uses iodine staining and laser densitometry for visualising and quantifying the amylose.

The following study aims to apply the newly developed agarose gel electrophoresis method to a range of wheat starch types and compare results with those from the iodine colorimetric and con A precipitation methods.

5.2 Materials and Methods

5.2.1 Wheat samples

Wheat samples (25) from the 1993/1994 Australian harvest were provided by Agrifood Technology, Werribee, Victoria, Australia. The varieties and their receival sites and their states of origin are shown in Table 5.1.

5.2.2. Laboratory scale starch extraction

Starch was extracted from the 25 varieties of wheat grains, as described in Section 2.4.2.

5.2.3 Determining the amylose contents of the wheat starches by colorimetry method

Starch surface lipids (Section 1.3.1) were removed from a sample of starch from the wheat cultivar Machete as described in Section 2.4.3, in three replicates. Amylose was determined in the three lipid extracted starch samples and in the unextracted starch sample of Machete wheat cultivar, by the colorimetric method (Section 2.4.4.1). The mean amylose contents of unextracted starch and each of the 3 lipid-extracted

starches were statistically compared using the independent sample t-test. And, the mean amylose contents of the three lipid-extracted starches were statistically compared using the one way ANOVA test.

Amylose determination was also carried out on all of the 25 wheat starch samples, before and after lipid (starch surface lipid) extraction (Section 2.4.3), by the colorimetric method (Section 2.4.4.1). The assay was carried out in duplicate.

5.2.4 Application of agarose gel electrophoresis to determine amylose content in wheat starch

5.2.4.1 *Measurement of amylose content of wheat starch by agarose gel electrophoresis*

Sample preparation

Potato amylose standard solutions (0.8%, 0.6%, 0.4% and 0.2%) were prepared and kept in ice (Section 3.2.3.6). Solutions of wheat starches (10 mg/mL) were also prepared by solubilising the wheat starch in 0.5 M KOH at 80°C for 15 minutes and kept in ice. Loading buffer (1 μL) was added to 5 μL of the standard solutions and the wheat starch solutions, which were then loaded onto the gel.

Electrophoresis

Electrophoresis of the potato amylose standards and wheat starch samples was carried out as described in Section 3.2.2.1, at 30 V for 16 hours, on 0.5% agarose gels. Potato amylose standards were run along with the wheat starch samples on all occasions.

Staining

The gel was immersed in 0.2% I_2 in 2% KI for 1 minute and de-stained in distilled water by rinsing 3-4 times. It was then scanned immediately in the laser densitometer.

Calculation of amylose content (%) in a wheat starch sample is as follows,

1. Initially, a calibration curve of absorbance (peak area) versus concentration of potato amylose was constructed.
2. The peak area corresponding to the amylose band in a wheat starch sample, determined from the laser densitometer scan, was referred to the calibration curve, to obtain the amylose (%) in the 10 mg/mL starch solution.

3. Amylose content (%) in starch = Amylose (%) in solution (from step 2) X 100*

*Constant derived from dilution factors.

5.2.4.2 *Validation of the method for a range of wheat starch*

(i) The amylose content was determined for lipid (starch surface lipid) extracted starch of Dollarbird wheat cultivar, by the agarose gel electrophoresis method described in Section 5.2.4.1. Lipid extraction was carried out as described in Section 2.4.3. Ten replicates were analysed with this sample.

(ii) The amylose content was determined in 25 lipid (starch surface lipid) extracted starch samples, by the agarose gel electrophoresis method described in Section 5.2.4.1. Lipid extraction was performed as described in Section 2.4.3. The assay was repeated four times for each sample.

5.2.4.3 *Validation of the method against colorimetric and con A methods*

The amylose content of the 25 lipid unextracted wheat starches were determined by the con A method described in Section 2.4.4.2. The assay was carried out in duplicate. The amylose contents determined by the agarose gel, colorimetric and con A methods were compared. Lipid (starch surface lipid) extracted starches were used in the agarose and colorimetric methods, and unextracted starches were used in the con A method.

5.3 Results and Discussion

5.3.1 Amylose content of wheat starch determined by the colorimetric method

It should be noted that, in this study, a hot extraction method was not employed to remove starch (internal) lipids. This is because, a non-invasive lipid extraction technique, which would keep the starch granules intact, was necessary for the development of the agarose gel electrophoresis method.

Table 5.2 presents the mean amylose contents of the lipid unextracted starch and the 3 samples of lipid extracted starches of Machete wheat cultivar. The independent sample t-test showed that the difference between the mean amylose content of each of the lipid-extracted starch (n = 6) and unextracted starch (n = 6) were statistically

significant at 95% level of confidence. This indicates that the mean amylose content measured in the unextracted starch was different from that of the extracted starches. The results of the one way ANOVA test, which compared the means of amylose contents of the three lipid extracted starch samples gave a F value (0.727) that was insignificant at the 95% level of confidence at degrees of freedom 2 and 15. This indicates that the mean amylose contents of the three lipid extracted starches are not different. Table 5.3 presents the mean amylose contents of 25 wheat starch samples before and after lipid (starch surface lipid) extraction. The amylose content measured in the lipid extracted starches were higher than that in the unextracted starches. The differences (ΔAM) reflect the impact of starch surface lipids on the iodine complexing ability of amylose and indicate the effect of lipids on measured amylose levels.

As reviewed in Section 1.3.1, starch surface lipids are predominantly monoacyl glycerols which are capable of forming helical inclusion complexes with amylose in solution, thereby interfering with iodine binding when measuring amylose content iodometrically. During the preparation of starch, these lipids have a tendency to become firmly absorbed onto or into the starch granules (Morrison, 1981). Morrison and Laignelet (1983) have reported that starch surface lipids in bean and potato starches can complex with amylose in solution to give a measurable ΔAM effect. According to Morrison (1988), non-starch lipids (which include starch surface lipids) can be extracted with polar solvents at ambient temperature. The results in the present study indicate that removal of starch surface lipids, with polar solvent at room temperature, has a measurable effect on observed amylose levels.

The results reported by Morrison and Laignelet (1983) and Bolling and El Baya (1975) show much greater ΔAM values ranging from 3.7%-6.8% upon removal of total lipids (including the starch internal lipids) in wheat. In comparison, the results obtained in the present investigation showed ΔAM values ranging from 1.2%- 3.4%. The internal starch lipids would not have been extractable at ambient temperature employed during lipid extraction (Morrison, 1988). Hence, in this study, the removal of only the starch surface lipids was responsible for the lower ΔAM values. These results reinforced the impact of starch surface lipids on amylose determination based on iodine complex formation.

5.3.2 Amylose content of wheat starch determined by agarose gel electrophoresis method

Validation of the method for a range of wheat starch types

Figure 5.1 presents the electrophoretic profile of 10 replicates of a lipid (starch surface lipids) extracted Dollarbird wheat starch, and the potato amylose standards. Table 5.4 illustrates the results obtained from laser densitometer scanning of the gel. The mean amylose content of the 10 replicates was 21.7 ± 1.4%. Table 5.5 presents the amylose contents of the 25 wheat starch samples measured by the agarose gel electrophoresis procedure. The data are the means of 4 replicates and standard deviations are also included. Relative standard deviation {(standard deviation/mean) X 100} ranged from 3.9% to 27.9%. Sunstar and Sunelg showed very high relative standard deviations, 21.6% and 27.9% respectively, and those for Bencubbin, Eradu, Janz 5 and Kulin 2 starches were between 10% and 16%. Although most of the varieties had standard deviation below 10%, the high standard deviation exhibited by some implies the necessity for improvement of this technique. In the colorimetric and con A methods, the relative standard deviations for all the cultivars were below 10% ranging from 0.6% to 6.9% and 0.5% to 8.5% respectively.

Validation of the method against existing methods

Table 5.6 presents the percentage amylose determined by the three different methods. The results obtained by the colorimetric method were lower than those obtained by the agarose gel electrophoresis and con A methods, by over 3% in some cases (eg. Halberd). This may be due to the difference in the conditions employed to extract the amylose from starch. Amylose was extracted at room temperature with 0.5 M KOH in the colorimetric method, and, in the gel electrophoresis and con A methods amylose extractions were carried out at 80°C for 15 minutes in 0.5 M KOH and at 100°C in DMSO respectively. The use of heat would have optimised amylose solubilisation, leading to an increase in the amylose content measured by the agarose gel and con A methods. In addition, saponification of lipids by the alkali at 80°C may have significantly altered their impact in the electrophoresis method. In the con A method, the procedure to remove lipids is incorporated into the assay format.

Comparisons of different methods for estimation of the amylose contents of different cultivars are shown in figures 5.2 – 5.4. The results show that none of the correlations coincide with the expected line. This indicates that no two methods provide an accurate measure of amylose content. However, amylose contents obtained by the colorimetric and con A methods show a high correlation coefficient of 0.839 ($p < 0.01$) and Figure 5.2 shows that the two methods are positively correlated. The con A vs. colorimetry line is approximately parallel to the expected line showing that the amylose content is consistently underestimated by the colorimetric method compared to the con A method. The correlations involving the agarose gel electrophoresis method with the colorimetric method ($r = 0.641$ at $p < 0.01$) and con A method ($r = 0.679$ at $p < 0.01$) (Figures 5.3 and 5.4 respectively) are both weak. The correlation line is far removed from the expected line and far from parallel to it, in both cases (Figures 5.3 and 5.4 respectively). Thus, the gel electrophoretic method may not be giving a reliable quantitative estimate of the amylose content. Further refinement of the technique will be necessary before the gel electrophoretic method can be used to reliably estimate amylose content.

It should be noted that the analytical procedures in these methods are based on different principles. The colorimetric and the agarose gel electrophoresis methods are based on quantitation of amylose/iodine complexes. The con A method is based on the precipitation of amylopectin by con A, and the amylose in the supernatant is determined spectrophotometrically as glucose equivalents, after hydrolysis by *alpha*-amylase and amyloglucosidase. As reviewed in Section 1.9.1, in the colorimetric method, the amylopectin-iodine complex also absorbs at similar wave-lengths to the amylose-iodine complex, which can affect the result. The con A and the agarose gel electrophoresis methods should be independent of the amylopectin fraction of starch, as the components are separated before the estimation of amylose.

Even though both con A and agarose gel electrophoresis methods measure amylose after separation from amylopectin, they are based on different separation principles. This may explain why the two methods may not correlate strongly. Although Gibson *et al.* (1997) showed a high correlation ($r = 0.99$) of the con A method with various colorimetric methods, Regina *et al.* (1997) reported a weaker correlation ($r = 0.626$)

between such methods. A need for more standardisation for the con A method has been suggested by Regina *et al.* (1997). Although the con A method is more complex than the colorimetry and agarose gel electrophoresis methods, it has the advantage over the latter two methods of not requiring the use of amylose and/or amylopectin standards.

In this context it should be noted that starch probably consists of a range of structures, varying in molecular weight, average-chain length, and degree of branching which occurs with differing frequency. Thus, it can be expected that fractions produced from the same starch may well vary with separation methods (Yun and Matheson, 1992).

Table 5.1 shows the receival sites and the states of origin of the 25 wheat cultivars. It is notable that significant differences in amylose contents were found between the starches (Table 5.5), including those isolated from wheats of the same varieties (Janz) grown in different locations. Graybosch *et al.* (1998) reported on the significant effects of environment, line, and line x environment interaction on amylose contents of North American hexaploid wheats. As such, the variation in amylose content observed in this study is probably due to the influence of environment and cultivar type.

5.4 Conclusions

The newly developed simple and rapid agarose gel electrophoresis method described in Chapter 3 has provided an alternative approach for the rapid separation and detection of the principal starch components in the Australian wheat cultivars. The amylose contents determined for the Australian wheat starches by this method were within the range reported previously for starches purified from a range of Australian wheat cultivars. However, comparison of this method with existing con A and colorimetric methods for the measurement of amylose contents in Australian wheat starches showed a weak correlation ($r = 0.641$ at $P < 0.01$ and $r = 0.679$ at $P < 0.01$, respectively). Thus it could be concluded that although the gel method is a useful technique for qualitative separation of amylose and amylopectin it does not at the present time, give a reliable quantitative estimate of amylose content in starch. Hence,

further refinement of this technique is required to improve reproducibility and reliability.

Table 5.1 Wheat varieties and origins

Wheat Variety	Receival Site	Australian Site
Aroona	Dandaragan	Western Australia
Bencubbin	Gutha	Western Australia
Cadoux	Goodilands	Western Australia
Canna	Milawing	Western Australia
Dagger	Dangara	Western Australia
Dollarbird	Yeovale	New South Wales
Eradu	Kalamanie	Western Australia
Halberd	Pingaring	Western Australia
Janz 1	Farrell Flat	South Australia
Janz 2	Matong	New South Wales
Janz 3	Dalby	Queensland
Janz 4	Wyonga	New South Wales
Janz 5	Kukarin	Western Australia
Janz 6	Yeoval	New South Wales
Kulin 1	Geraldton	Western Australia
Kulin 2	Bulyee	Western Australia
Machete	Greenough	Western Australia
Meering	Tandara	Victoria
Osprey	Jerdacuttap	Western Australia
Oxley	Goroke	Victoria
Rosella	St. Arnaud	Victoria
Spear	Dandaragan	Western Australia
Sunelg	Grasspatch	Western Australia
Sunstar	Edgeroi	New South Wales
Wilgoyne	Wonganhills	Western Australia

Table 5.2 Mean amylose content of starch from Machete cultivar, determined by the colorimetric method, before and after lipid (starch surface lipid) extraction.

Starch samples	Mean amylose content (%) ± s.d.	t-test value
Lipid unextracted starch	16.6 ± 1.2	—
Lipid extracted starch 1	18.6 ± 0.6	-3.7*
Lipid extracted starch 2	18.8 ± 0.5	-4.2*
Lipid extracted starch 3	18.5 ± 0.2	-3.9*

*P < 0.05

Table 5.3 Mean amylose contents of wheat starches determined by the colorimetric method, before and after lipid (starch surface lipid) extraction.

Wheat cultivars	Mean amylose content (%) Before lipid removal	Mean amylose content (%) After lipid removal	ΔAM
Aroona	18.6	20.3	1.7
Bencubbin	17.2	18.8	1.6
Cadoux	17.1	19.2	2.1
Canna	21.2	22.8	1.6
Dagger	18.2	21.1	2.9
Dollar Bird	18.1	21.5	3.4
Eradu	19.0	20.4	1.4
Halberd	15.5	17.3	1.8
Janz 1	21.7	23.2	1.5
Janz 2	21.8	24.3	2.5
Janz 3	20.1	22.6	2.5
Janz 4	21.6	23.6	2.0
Janz 5	20.4	22.4	2.0
Janz 6	21.3	22.8	1.5
Kulin 1	19.2	22.1	2.9
Kulin 2	20.7	23.4	2.7
Machete	16.9	18.3	1.4
Meering	19.2	21.7	2.5
Osprey	19.2	21.6	2.4
Oxley	21.8	24.2	2.4
Rosella	19.7	20.9	1.2
Spear	15.4	18.3	2.9
Sunelg	17.3	19.1	1.8
Sunstar	21.9	23.5	1.6
Wilgoyn	19.2	20.8	1.6

Table 5.4 Results of scanning of the gel from figure 5.1. A) Concentrations of potato amylose standards and the corresponding peak areas B) Peak areas and amylose contents of the 10 wheat starch replicates of Dollarbird cultivar.

A

Concentration of potato amylose standard	0.8%	0.6%	0.4%	0.2%
Peak area (au*mm)	13.2	9.9	8.2	2.5

B

Replicate	**Peak area (au*mm)**	**Amylose content (%)**
1	2.8	20.6
2	2.9	21.0
3	2.9	21.0
4	2.9	21.0
5	2.8	20.4
6	2.9	21.0
7	3.3	23.2
8	3.6	24.7
9	3.2	22.5
10	2.9	21.0
Mean ± s.d.	3.0 ± 0.2	21.7 ± 1.4

Table 5.5 Mean amylose contents of lipid (starch surface lipid) extracted wheat starches determined by the agarose gel electrophoresis method

Wheat variety	Agarose gel method Mean amylose (%) ± s.d.	% Standard deviation
Aroona	21.7 ± 2.2	10.1
Bencubbin	21.3 ± 3.5	16.4
Cadoux	22.3 ± 1.7	7.6
Canna	25.9 ± 2.5	9.7
Dagger	24.5 ± 2.2	8.9
Dollarbird	21.6 ± 1.0	4.6
Eradu	21.7 ± 2.9	13.4
Halberd	20.5 ± 1.2	5.9
Janz 1	24.6 ± 1.1	4.5
Janz 2	27.7 ± 1.4	5.1
Janz 3	25.6 ± 1.9	7.4
Janz 4	26.4 ± 2.5	9.5
Janz 5	25.9 ± 2.8	10.8
Janz 6	28.0 ± 2.8	10.0
Kulin 1	24.9 ± 2.0	8.0
Kulin 2	21.8 ± 3.3	15.1
Machete	22.6 ± 1.1	4.9
Meering	26.1 ± 2.5	9.6
Osprey	25.9 ± 2.0	7.7
Oxley	27.8 ± 1.1	3.9
Rosella	22.9 ± 1.3	5.7
Spear	22.1 ± 1.9	8.6
Sunelg	21.1 ± 5.9	27.9
Sunstar	19.9 ± 4.3	21.6
Wilgoyn	23.7 ± 2.4	10.1

Table 5.6 Comparison of amylose contents of wheat starches determined by the colorimetric, con A and agarose gel electrophoresis methods. Lipids (starch surface lipid) were extracted from wheat starches used in the colorimetric and agarose gel methods.

Wheat cultivars	Colorimetric Mean amylose (%) ± s.d.	Con A Mean amylose (%) ± s.d.	Agarose gel Mean amylose (%) ± s.d.
Aroona	20.3 ± 1.2	21.4 ± 1.4	21.7 ± 2.2
Bencubbin	18.8 ± 0.5	22.2 ± 0.4	21.3 ± 3.5
Cadoux	19.2 ± 1.3	21.7 ± 1.5	22.3 ± 1.7
Canna	22.8 ± 0.5	24.5 ± 0.5	25.9 ± 2.5
Dagger	21.1 ± 0.5	24.1 ± 0.1	24.5 ± 2.2
Dollar Bird	21.5 ± 0.4	22.5 ± 0.3	21.6 ± 1.0
Eradu	20.4 ± 0.3	22.7 ± 0.9	21.7 ± 2.9
Halberd	17.3 ± 0.9	21.9 ± 0.2	20.5 ± 1.2
Janz 1	23.2 ± 0.5	25.9 ± 1.1	24.6 ± 1.1
Janz 2	24.3 ± 0.9	25.8 ± 0.7	27.7 ± 1.4
Janz 3	22.6 ± 0.6	23.7 ± 0.5	25.6 ± 1.9
Janz 4	23.6 ± 1.1	25.1 ± 0.5	26.4 ± 2.5
Janz 5	22.4 ± 0.7	25.1 ± 0.2	25.9 ± 2.8
Janz 6	22.8 ± 1.1	25.3 ± 1.2	28.0 ± 2.8
Kulin 1	22.0 ± 0.8	25.4 ± 0.2	24.9 ± 2.0
Kulin 2	23.4 ± 0.1	24.5 ± 1.1	21.8 ± 3.3
Machete	18.3 ± 0.4	20.3 ± 0.5	22.6 ± 1.1
Meering	21.7 ± 0.3	23.9 ± 0.7	26.1 ± 2.5
Osprey J	21.6 ± 0.3	23.0 ± 0.6	25.9 ± 2.0
Oxley	24.2 ± 0.3	25.6 ± 0.7	27.8 ± 1.1
Rosella	20.9 ± 1.4	21.4 ± 1.8	22.9 ± 1.3
Spear	18.3 ± 0.2	22.9 ± 1.1	22.1 ± 1.9
Sunelg	19.1 ± 0.5	21.2 ± 0.3	21.1 ± 5.9
Sunstar	23.5 ± 0.7	24.3 ± 1.5	19.8 ± 4.3
Wilgoyn	20.8 ± 0.6	22.5 ± 0.8	23.7 ± 2.4

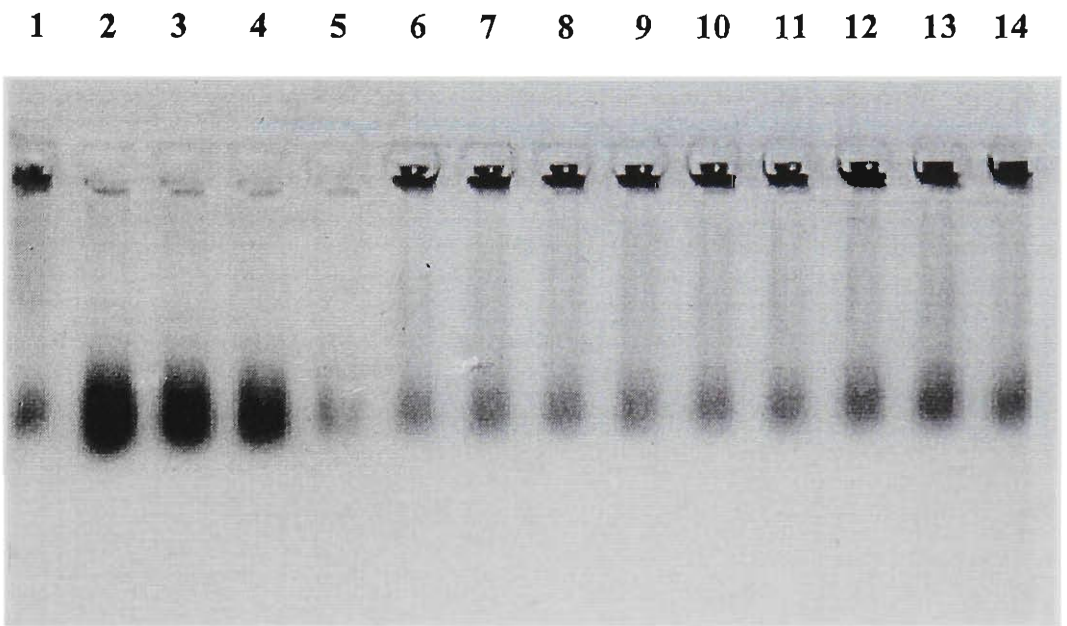


Figure 5.1 Electrophoresis profile of potato amylose standards and the ten replicates of wheat starch, solubilised in 0.5 M KOH at 80°C for 15 minutes. Lanes 2, 3, 4 and 5 : 0.8%, 0.6%, 0.4% and 0.2% potato amylose respectively. Lanes 6, 7, 8, 9, 10, 11, 12, 13, 14 and 1: replicates of wheat starch solution.

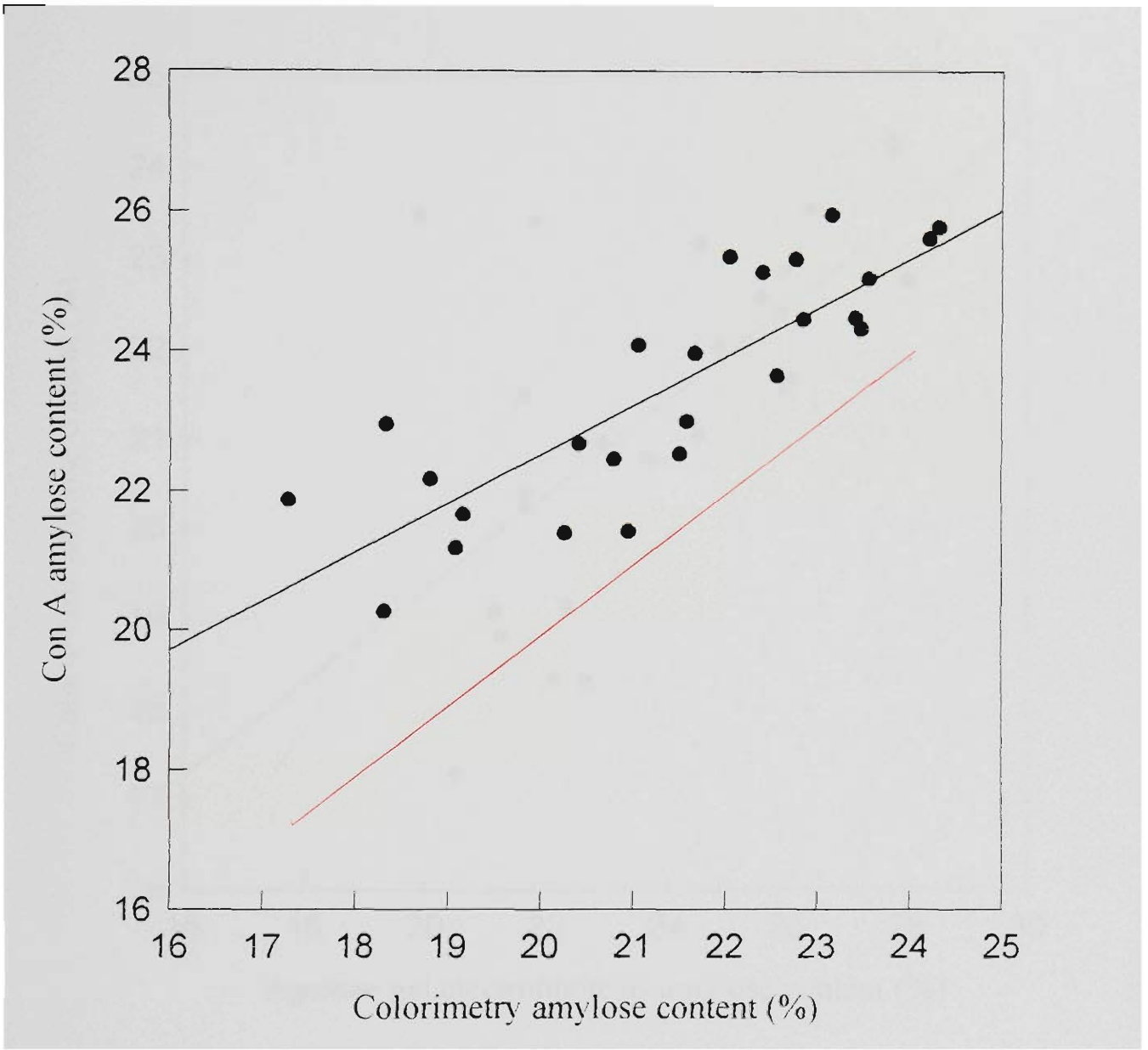


Figure 5.2 Relationship between amylose contents in various wheat starches, determined by the iodine colorimetric and con A methods.
($r = 0.839$ at $p < 0.01$).
——————— : Expected line

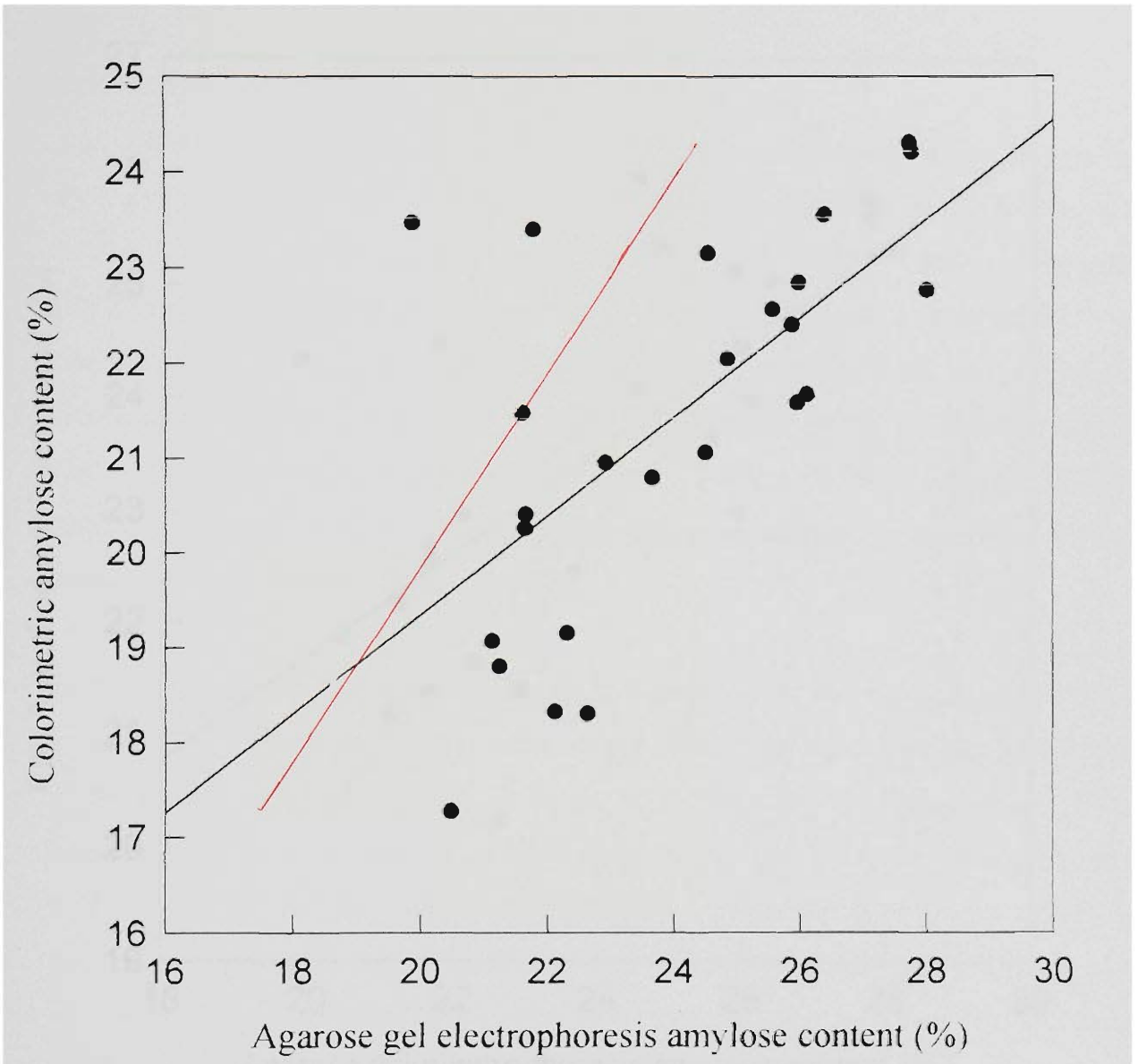


Figure 5.3 Relationship between amylose contents in various wheat starches, determined by the iodine colorimetric and agarose gel methods.
($r = 0.641$ at $p < 0.01$).
————: Expected line

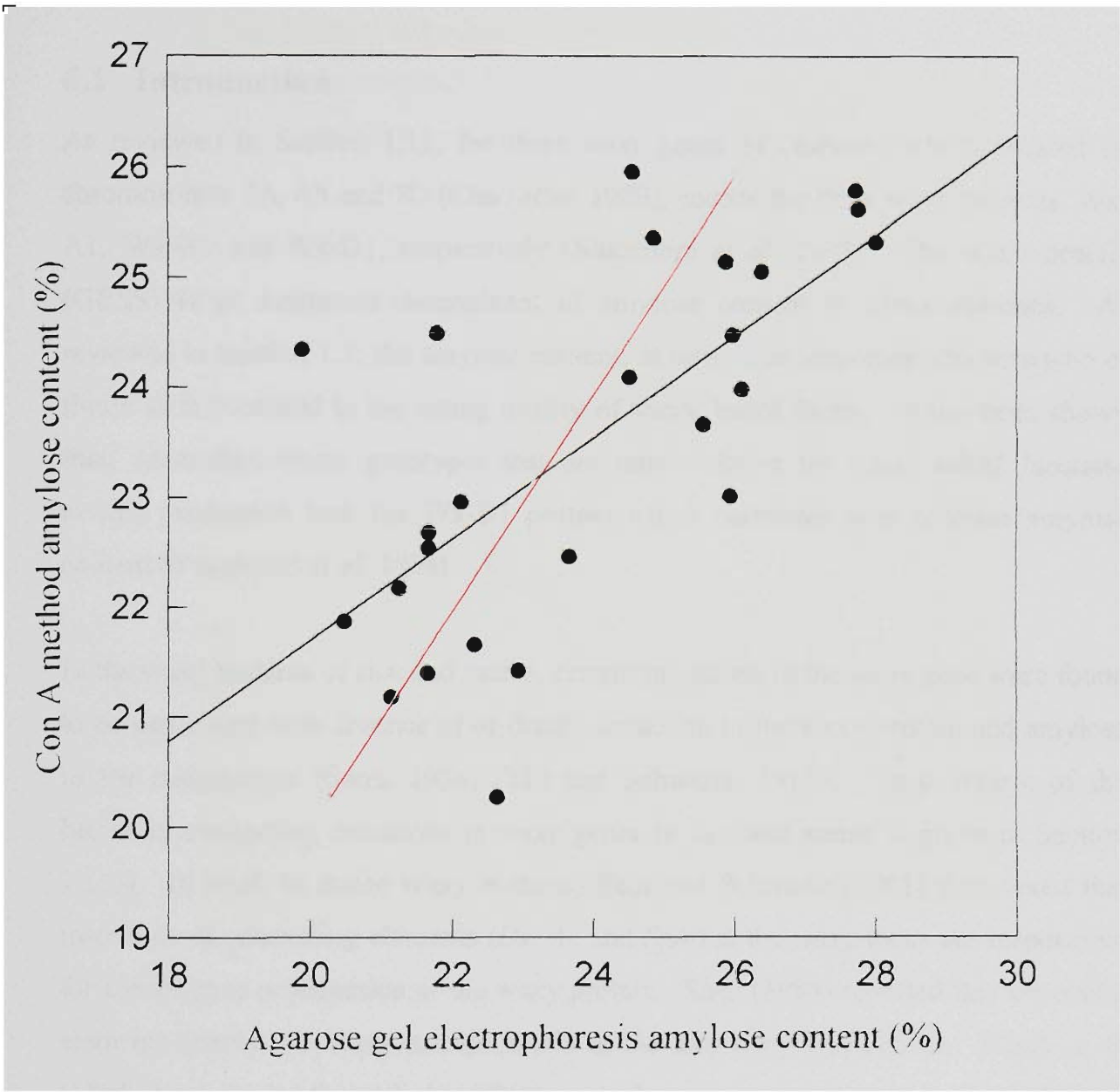


Figure 5.4 Relationship between amylose contents in various wheat starches, determined by the agarose gel and con A methods. ($r = 0.679$ at $p < 0.01$).
———— : Expected line

CHAPTER 6

Molecular genetic studies on Australian wheat varieties

6.1 Introduction

As reviewed in Section 1.11, the three *waxy* genes of common wheat, located on chromosomes 7A, 4A and 7D (Chao *et al.* 1989), encode the three waxy proteins, Wx-A1, Wx-B1 and Wx-D1, respectively (Nakamura *et al.* 1993). The waxy protein (GBSS) is an important determinant of amylose content in cereal starches. As reviewed in Section 1.7, the amylose content, in turn, is an important characteristic of flours as it is related to the eating quality of starch based foods. It has been shown that, Australian wheat genotypes that are most suitable for white salted Japanese noodle production lack the Wx-B1 protein which correlates with a lower amylose content (Yamamori *et al.* 1994).

In the waxy mutants of rice and maize, certain mutations in the *waxy* gene were found to be associated with absence of or drastic reduction in the waxy protein and amylose in the endosperms (Sano, 1984; Echt and Schwartz, 1981). An overview of the literature concerning mutations in *waxy* genes in rice and maize is given in Section 1.11.6. In brief, in maize waxy mutants, Echt and Schwartz (1981) discovered that insertions of controlling elements (*Ds*, *Ac* and *Spm*) at the *waxy* locus are responsible for the absence or reduction of the waxy protein. Sano (1984) reported that *cis*-acting elements control the waxy protein levels in the non-waxy rice strains. Wang *et al.* (1995) have shown that splicing of intron 1 plays an important role in regulating the *waxy* gene expression, which in turn, affects the waxy protein level and amylose content in rice cultivars. They have suggested that *cis*-acting elements may be involved in the splicing of intron 1 from the 5' leader region of the *waxy* transcript.

Amylose synthesis is regulated by one *waxy* gene in diploid plants such as maize and rice and by three homeologous *waxy* genes in hexaploid common wheat. Hence, the structural analysis of the individual *waxy* gene in common wheat is complicated. The Australian partial waxy mutants of common wheat (null 4A cultivars) have not been

investigated and the molecular basis for the absence of Wx-B1 protein is not clearly understood.

The aim of this study was to investigate the nature of the mutations that may be responsible for the inactivation of the 4A *waxy* gene in the Australian null 4A wheat cultivars. The specific objectives of this study were to (i) identify any major deletions or alterations in the organisation of *waxy* genes in null 4A cultivars, (ii) test the possibility of incomplete splicing of intron 1 playing a role in null 4A waxy mutations, (iii) compare the size of *waxy* gene transcripts and the level of *waxy* gene expression in seeds of null 4A and normal cultivars harvested at 20 days after pollination, (iv) determine the level of expression of the *waxy* gene during different stages of seed development in a normal and a null 4A cultivar.

6.2 Materials and Methods

6.2.1 Wheat cultivars

As mentioned in Section 2.5.2, four normal wheat cultivars (Chinese Spring, Goroke, Vectis and Wyuna) and four null 4A wheat cultivars (Cadoux, Machete, Halberd and Rosella) were used in this study. Plants were grown and developing seeds were harvested as mentioned in Section 2.5.3, for investigations of *waxy* gene structure and expression.

6.2.2 Analysis of intron/exon structure of *waxy* genes

DNA was extracted from the leaves of the eight wheat cultivars (Section 2.5.4) and the concentrations were estimated (Section 2.5.5). Five pairs of primers were designed based on wheat *waxy* gene cDNA sequence (Clark *et al.* 1991) and synthesised and purified as mentioned in Section 2.5.6. The positions of the primers in the wheat *waxy* gene sequence are indicated in Figure 6.1 and the primer sequences are given in Table 6.1.

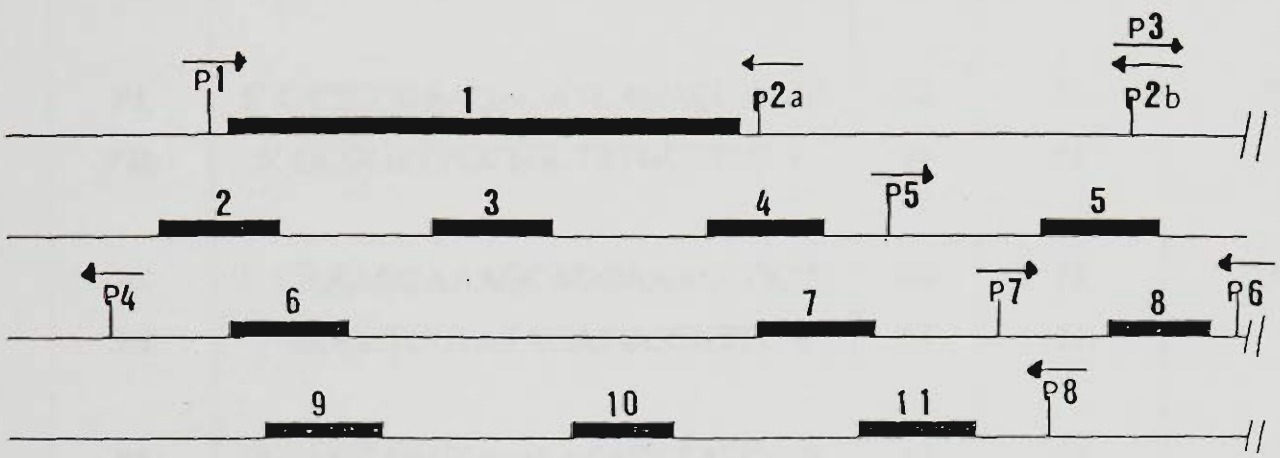


Figure 6.1 **Line diagram indicating the locations of the primers used for PCR, in wheat *waxy* gene.**

= Intron **= Exon** **P = Primer**

Table 6.1 Primers used for PCR amplification of wheat *waxy* gene. * The size of PCR amplification products were determined based on barley *waxy* gene sequence (Rhode *et al.* 1988) and wheat *waxy* gene sequence (Clark *et al.* 1991).

Primers	Sequence	Tm (°C)	Length of primers (bases)	*Size of PCR amplification product (bp)
P1	5' GTTCCGAAGAGATCAGACCAG 3'	64	21	599
P2a	5' GCTACCTGCAGCAAGAGACG 3'	64	20	
P1	5' GTTCCGAAGAGATCAGACCAG 3'	64	21	790
P2b	5' GCGGTTTCCTGCTTTGCTTTG 3'	64	21	
P3	5' CAAAGCAAAGCAGGAAACCGC 3'	64	21	1086
P4	5' GCCGTCCTATAGATGCCATTG 3'	64	21	
P5	5' GACCAAGGAGAAGATCTATGG 3'	62	21	1274
P6	5' CCGCTCAAACTTCTTCTTCCC 3'	64	21	
P7	5' GAGGAGGACGTCCAGATCG 3'	62	19	809
P8	5' CGTCCTCCCAGTTCTTGGC 3'	62	19	

PCR was performed as mentioned in Section 2.5.7, with the eight DNA samples and the five pairs of primers. The sizes of the PCR products were determined by agarose gel electrophoresis (Section 2.5.8). The PCR products of primers 1 and 2a, from a normal and a null 4A variety, were eluted from the agarose gel, purified and sequenced as described in Sections 2.5.9 and 2.5.10.

6.2.3 RT-PCR analysis to investigate the splicing of intron 1

RT-PCR was performed as mentioned in Section 2.5.21, with total RNAs isolated from 20 dap seeds of all eight cultivars (Section 2.5.11) and primers 1 and 2b.

6.2.4 Analysis of *waxy* gene expression

Preparation of Northern blots

Total RNAs were isolated (Section 2.5.11) from 20 dap seeds of the null 4A and normal wheat cultivars and from developing seeds (5, 10, 15, 20 and 25 dap) of the cultivars Chinese Spring and Rosella. The concentrations of the total RNAs were determined (Section 2.5.12) and 10 μg each of the above RNA samples was transferred to nylon membranes (Sections 2.5.13 and 2.5.14) for hybridisation.

Development of waxy cDNA probes

Two *waxy* cDNA probes for the wheat *waxy* gene were developed for northern hybridisation by the RT-PCR technique (Section 2.5.21). Total RNA from 20 dap seeds of a normal wheat cultivar (Chinese Spring) was used as the template. Primers P1 and P4 (Figure 6.1) were used to develop the 840 bp cDNA probe, and the 1244 bp cDNA probe was developed using the primers P5 and P8 (Figure 6.1). The concentrations of the probes were determined as mentioned in Section 2.5.9. Purification and sequencing of the probes were performed as mentioned in Sections 2.5.9 and 2.5.10.

Northern hybridisation

The *waxy* cDNA probes were labelled (Section 2.5.15) and Northern hybridisations of the blots performed as explained in Section 2.5.16 to 2.5.18. The blots with the total RNA of developing seeds were hybridised with the 1244 bp probe. The blots with the total RNA of 20 dap seeds were initially hybridised with one probe (1244 bp), and re-probing with the second probe (840 bp) was carried out following stripping of the blots (Section 2.5.20). The hybridisation signals of *waxy* gene expressions were quantified according to Section 2.5.19.

6.3 Results and Discussion

6.3.1 Analysis of the size and organisation of the *waxy* gene

The results of the Polymerase Chain Reactions of genomic DNA from the eight wheat cultivars, with the five sets of primers, are shown in Table 6.2. The expected sizes of the PCR products were calculated based on barley *waxy* gene sequence (Rhode *et al.* 1988) and wheat *waxy* cDNA sequence (Clark *et al.* 1991).

PCR using primers P1 and P2b

All of the wheat cultivars exhibited a single fragment of size ~900 bp when PCR amplification was carried out with primers P1 and P2b, at an annealing temperature of 62°C (Figure 6.2, Table 6.2). However, the expected size of the PCR product, was ~790 bp. These results indicate that the amplified regions of wheat *waxy* genes are ~100 bp longer than the expected length. Since primers P1 and P2b encompass intron 1 and sections of exons 1and 2, the results did not reveal whether the coding or the non-coding region had been altered in length.

PCR using primers P1 and P2a

When PCR amplification was carried out using primers P1 and P2a, at an annealing temperature 64°C, the null 4A and normal cultivars exhibited a ~700 bp fragment (Table 6.2 and Figure 6.3). However, the expected size of the PCR product with these primers was ~600 bp. Since the primers P1 and P2a are located at the intron 1 junctions, the result suggests that the intron 1 of wheat *waxy* genes is ~100 bp longer than that of barley *waxy* gene. Sequence alignments of the 700 bp fragments from a null (Halberd) and normal (Chinese Spring) cultivar are shown in Figures 6.4 and 6.5 respectively. The results of both cultivars showed 70% similarity with barley intron 1 sequence. The 700 bp fragment from a normal cultivar (Chinese Spring) showed approximately 94% similarity with the 700 bp fragment of null 4A cultivar (Halberd) (Figure 6.6).

Normal cultivars consistently exhibited an additional fragment of size ~600 bp during PCR amplification with P1 & P2a primers, which was lighter in intensity compared to the 700 bp fragment (Figure 6.3). Partial DNA sequence analysis of the ~600 bp fragment from a normal cultivar (Chinese Spring) indicated 75% similarity with the

700 bp fragment from the same cultivar (Figure 6.8) and 70% similarity with the sequence of intron 1 of barley *waxy* gene (Figure 6.7). However, this fragment was absent in the null 4A cultivars. This fragment may thus represent the amplification product of the intron 1 of the 4A *waxy* gene in the normal cultivar, which could have been deleted in the null 4A cultivars. The results strongly suggest that the intron 1 region of the 4A *waxy* gene in the null 4A cultivars is deleted. The results also indicate the following: the length of intron 1 of the 4A *waxy* genes of the normal wheat cultivars is the same as that of barley intron 1 and intron 1 of the 7A and 7D *waxy* genes of the normal and null 4A wheat cultivars are longer than the intron 1 of barley *waxy* gene by ~100 bp.

In this context it should be noted that the amplification product of primers P1 and P2b also encompasses the intron 1 region. Hence, the normal cultivars should have exhibited an ~800 bp fragment (4A *waxy* gene product) along with a ~900 bp fragment (7A and 7D *waxy* gene products), when PCR amplification was performed with primers P1 and P2b. However, the ~800 bp fragment was absent in these cultivars. This may be due to a mutation in the annealing region of primer P2b, in the 4A *waxy* gene of normal cultivars. Recently, the results of sequencing of the *waxy* genes in the diploids, *T.monococcum* (AA) and *T.speltoides* (BB) (L. Yan and M. Bhave, personal communication and unpublished data) revealed the existence of polymorphism in the annealing region of primer P2b, in *Wx-A* and *Wx-B* genes. Hence, it is possible that in the normal cultivars, there are no amplification products from 7A and 4A *waxy* genes with primers P1 and P2b and the ~900 bp fragment most probably would be the amplification product of 7D *waxy* gene.

The results of PCR amplification with primers P1 and P2b, in the null 4A cultivars, showed the presence of a ~900 bp fragment, and the ~800 bp fragment was absent. The ~900 bp fragment would most probably represent the amplification product of 7D *waxy* gene as explained above. The absence of the ~800 bp fragment in the null 4A cultivars would be due to the observed deletion in the intron 1 region of 4A *waxy* gene, which has prevented the annealing of primer P1.

Konik *et al.* (1996) has reported a deletion on the 4A *waxy* gene in the null 4A wheat cultivars. They observed the presence of a 2 kb band which appeared strong in the normal and very faint in the null 4A, when *Bam H1* digests were probed with a 5' end barley GBSS probe of size 400 bp. However, the extent of the deletion was not reported in the study of Konik *et al.* (1996).

PCR using primers P3 and P4, P5 and P6, P7 and P8

When PCR amplification was carried out with the three primer pairs P3 and P4, P5 and P6, and, P7 and P8, at an annealing temperature 58°C, all eight wheat cultivars exhibited single fragments of expected size (Table 6.2). The sizes of the PCR products were approximately 1086 bp, 1274 bp and 809 bp respectively. The absence of altered size products suggests that there are no alterations in intron/exon lengths in these sections of the *waxy* genes, in normal and null 4A cultivars (Figures 6.9, 6.10 and 6.11). _

6.3.2 Role of intron 1 in waxy phenotype

Wang *et al.* (1995) reported that in waxy rice cultivars, a major factor in the control of amylose content of rice endosperm was the efficiency of excision of intron 1 from the *waxy* transcripts. Failure to excise intron 1 resulted in the absence of mature *waxy* transcript, waxy protein and amylose in the waxy rice. In the present study, in order to investigate the splicing of intron 1, RT-PCR was performed with the total RNA of 20 dap seeds of null 4A and normal cultivars and primers 1 and 2b. Ainsworth *et al.* (1993) reported the presence of abundant *waxy* transcript in wheat grains harvested at 20 dap. On the basis of this information, wheat grains harvested at 20 dap were used in this study and the *waxy* gene expression study (Section 6.33). The RT-PCR results showed the presence of a single fragment of size ~231 bp in all eight cultivars (Table 6.3 and Figure 6.12), indicating that intron 1 is completely spliced from the *waxy* transcripts during transcription. The expected size of RT-PCR product based on barley *waxy* gene and wheat cDNA sequences would be ~231 bp if intron 1 was completely spliced and ~790 bp if intron 1 was not spliced. The results strongly suggest that defect in splicing of intron 1 from 4A *waxy* gene is not a factor responsible for the absence of the Wx-B1 protein in the null 4A cultivars. However, deletion of the 4A *waxy* genes in this region in the null 4A cultivars cannot be ruled out from these observations.

In Section 6.3.1, when PCR amplification was performed with primer pairs P1, P2a and P1, P2b, all eight wheat cultivars showed PCR products ~100 bp longer than the expected size. The above results from RT-PCR study using primers P1 and P2b showed that the coding region (Sections of exons 1 and 2) was unaltered in length, in the null 4A and normal cultivars. This study thus strongly supports the results reported in Section 6.3.1 indicating that intron 1 of common wheat is longer than that of barley intron 1 by ~100 bp.

6.3.3 Northern analysis on *waxy* gene expression

Waxy cDNA probes

The size of the *waxy* cDNA probes obtained by RT-PCR with primer pairs P1 and P4 and P5 and P8 were 840 bp and 1244 bp respectively, as expected (Figures 6.13 and 6.14). The partial sequencing data indicated 93% and 92% similarity respectively with the published sequence of wheat cDNA (appendix 1 and 2), confirming that the RT-PCR products did indeed represent *waxy* cDNA.

The RT-PCR product represents all three *waxy* genes in common wheat. Hence both the probes would allow the detection of any major alterations in size or quantity of RNA transcripts of all three *waxy* genes together.

Expression of waxy gene in 20 dap seeds

The total RNA profile of 20 dap seeds of the wheat cultivars, in a formaldehyde gel, is shown in Figure 6.15. The results of hybridisation of the 840 bp and 1244 bp *waxy* cDNA probes, to northern blots of RNA from 20 dap wheat cultivars, are illustrated in Figures 6.16a and 6.16b respectively.

RNA samples from the null 4A and normal cultivars contained a single RNA band of approximately 2.3 kb that hybridised to the *waxy* probes. The size of the *waxy* transcript was consistent with the observations of Okagaki and Wessler (1988) who reported the *waxy* transcripts of maize, rice, millet and wheat to be 2.4 kb in size, and the results of Ainsworth *et al.* (1993) who reported the size of the *waxy* transcript of wheat to be 2.3 kb. In the null 4A and normal wheat cultivars, the absence of *waxy* transcripts of altered size with both the probes indicates that (i) there was no defect in splicing of introns during the transcription process throughout the *waxy* transcript; (ii)

there was neither large partial deletions nor large insertions in the coding regions of *waxy* genes, in null 4A and normal wheat cultivars.

Analysis of the *waxy* transcript levels, in the 20 dap seeds of null 4A and normal cultivars are shown in Table 6.4. The independent sample t-test results showed that the difference between the mean *waxy* transcript level of null 4A (n = 6) and normal (n = 4) wheat cultivars was statistically significant at 95% level of confidence, when the ~840 bp and the ~1244 bp probe were used for hybridisation (Table 6.4). These results indicate that the *waxy* transcript level in the null 4A cultivars is significantly lower ($p < 0.05$) than in the normal cultivars. The ~840 bp probe represents a sequence at the 5' end and the ~1244 bp represents a sequence at the 3' end in the wheat *waxy* gene (Figure 6.1). Hence the reduction in the *waxy* transcript level in the null 4A cultivars using these probes, which together encompass the entire *waxy* gene, may be due to absence of expression of the 4A *waxy* gene, with the 2.3 kb transcripts representing the 7A and 7D *waxy* genes.

The deletion of intron 1 in the 4A *waxy* gene of null 4A cultivars, as discussed previously in Section 6.3.1, may be responsible for the complete lack of expression of this gene in these cultivars. This deletion could be probably extending further to the 5' end and could include the untranslated exon 1 and the promoter of the gene, thus preventing transcription. The extent of this deletion was not investigated further in this study.

Expression of waxy gene during seed development

The total RNA profiles of developing seeds of Chinese Spring and Rosella cultivars, in formaldehyde gels, are shown in Figures 6.17a and 6.18a respectively. The results of hybridisation of 1244 bp *waxy* cDNA probe to northern blots of RNA from the developing seeds are shown in Figures 6.17b and 6.18b, for Chinese Spring and Rosella respectively. Results of the level of expression of *waxy* gene during seed development in Chinese Spring and Rosella are shown in Figures 6.19a and 6.19b. These preliminary data showed a general trend in *waxy* gene expression for both the cultivars, where the waxy transcripts accumulated to higher levels from early (5-10 dap) to middle stages of seed development (15-20 dap) and decreased after the period of peak expression. These preliminary observations support similar results for wheat

(Ainsworth *et al.* 1993) and rice cultivars (Wang *et al.* 1995). These results suggest that there were no major differences in the regulation of the *waxy* gene during seed developmental period in the null 4A and normal cultivars.

6.4 Conclusions

This study led to two important findings. Firstly, the intron 1 of *waxy* genes (atleast the 7A and 7D *waxy* genes) in the normal and null 4A wheat cultivars was found to be longer than that of barley intron 1 by ~100 bp. Secondly, it was found that the intron 1 of 4A *waxy* gene is deleted in the null 4A cultivars. The significant decrease observed in the *waxy* transcript levels in 20 dap seeds of null 4A cultivars could be the result of absence of expression of the 4A *waxy* gene in these cultivars. It was also suggested that the deletion of the intron 1 region could probably be a part of the mutation in the 4A *waxy* gene, which could be responsible for the absence of expression of this gene in the null 4A cultivars. The extent of this deletion should be investigated in future studies. This study strongly suggests that unlike in rice (Wang *et al.* 1995), the incomplete excision of intron 1 from the 4A *waxy* transcript is not responsible for the lack of expression of Wx-B1 protein in the null 4A cultivars.

Table 6.2 Results of the Polymerase Chain Reaction of genomic DNA from null 4A and normal wheat cultivars. * The calculation was based on barley *waxy* gene sequence (Rhode *et al.* 1988) and wheat *waxy* gene cDNA sequence (Clark *et al.* 1991).

Primer pairs	Annealing temperature (°C)	*Expected size of product (bp)	Observed size of products in null 4As (bp)	Observed size of products in normals (bp)
P1 and P2a	64°C	599	~700	~600 and ~700
P1 and P2b	62°C	790	~900	~900
P3 and P4	58°C	1086	~1086	~1086
P5 and P6	58°C	1274	~1274	~1274
P7 and P8	58°C	809	~809	~809

Table 6.3 Results of Reverse Transcriptase-PCR (RT-PCR) performed with the total RNA from 20 dap seeds of the eight wheat cultivars and primers P1 and P2b.

Expected size of RT-PCR product if intron 1 was spliced (bp)	Expected size of RT-PCR product if intron 1 was not spliced (bp)	Observed size of products in null 4As (bp)	Observed size of products in normals (bp)
231	790	~231	~231

Table 6.4 *Waxy* transcript levels, determined with two *waxy* cDNA probes, in null 4A and normal wheat endosperms harvested at 20 dap.

Waxy cDNA probes	Wheat cultivars	*Waxy* transcript levels Mean ± s.d.	*Probability Value (p-value)
840 bp	Null 4A	3.9 ± 0.6	0.02
	Normal	9.8 ± 4.4	
1244 bp	Null 4A	3.4 ± 0.8	0.02
	Normal	8.3 ± 3.3	

* $p < 0.05$

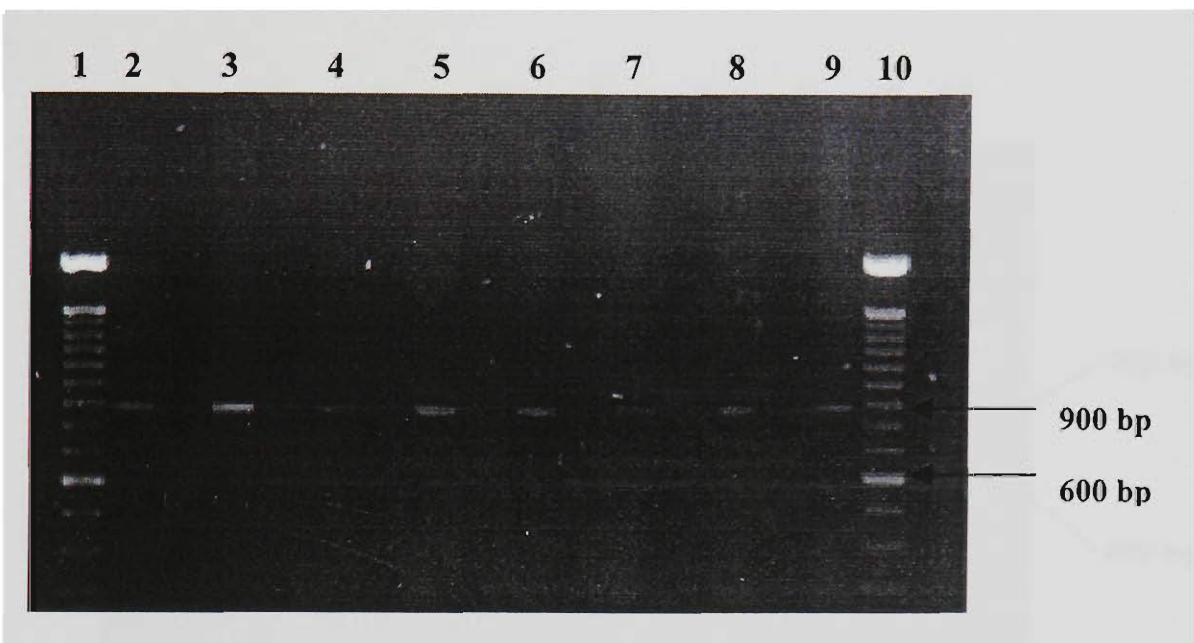


Figure 6.2 PCR amplification products of genomic DNA from null 4A and normal wheat cultivars, with primers P1 and P2b. Lanes 1 and 10: Marker, 100 bp ladder, Lanes 2-5: Cadoux, Halberd, Machete and Rosella respectively (null 4A cultivars) and Lanes 6-9: Chinese Spring, Goroke , Vectis and Wyuna (normal cultivars).

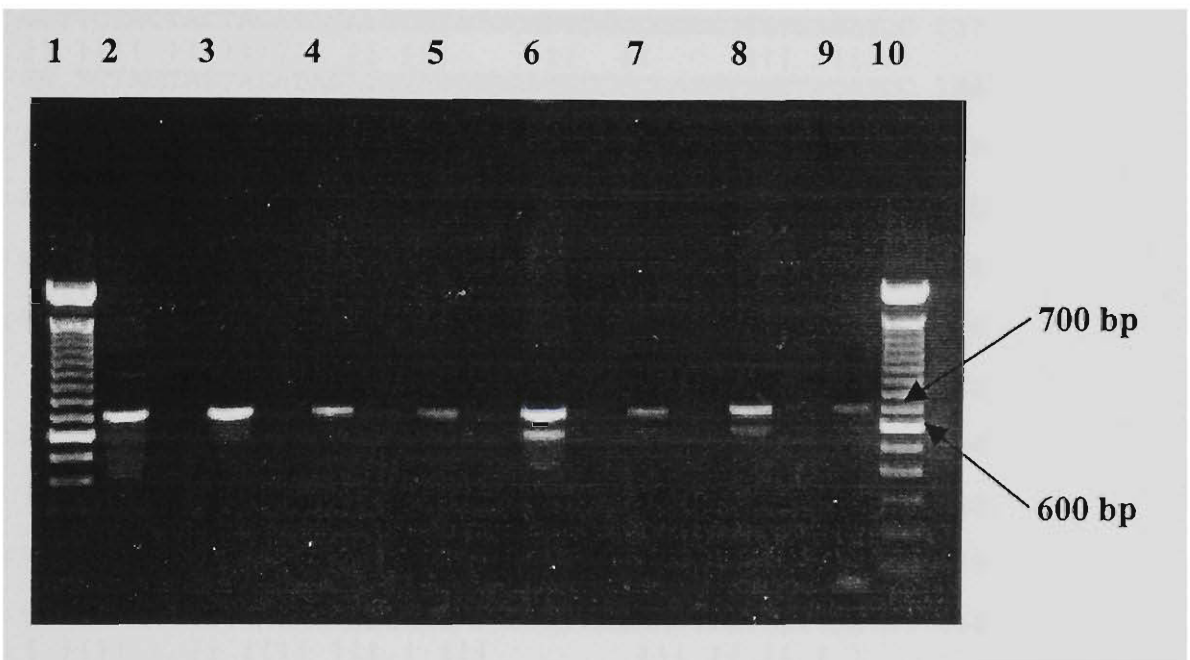


Figure 6.3 PCR amplification products of genomic DNA from null 4A and normal wheat cultivars, with primers P1 and P2a. Lanes 1 and 10: Marker 100 bp ladder, Lanes 2-5: Cadoux, Halberd, Machete and Rosella respectively (null 4A cultivars) and Lanes 6-9: Chinese Spring, Goroke , Vectis and Wyuna respectively (normal cultivars).

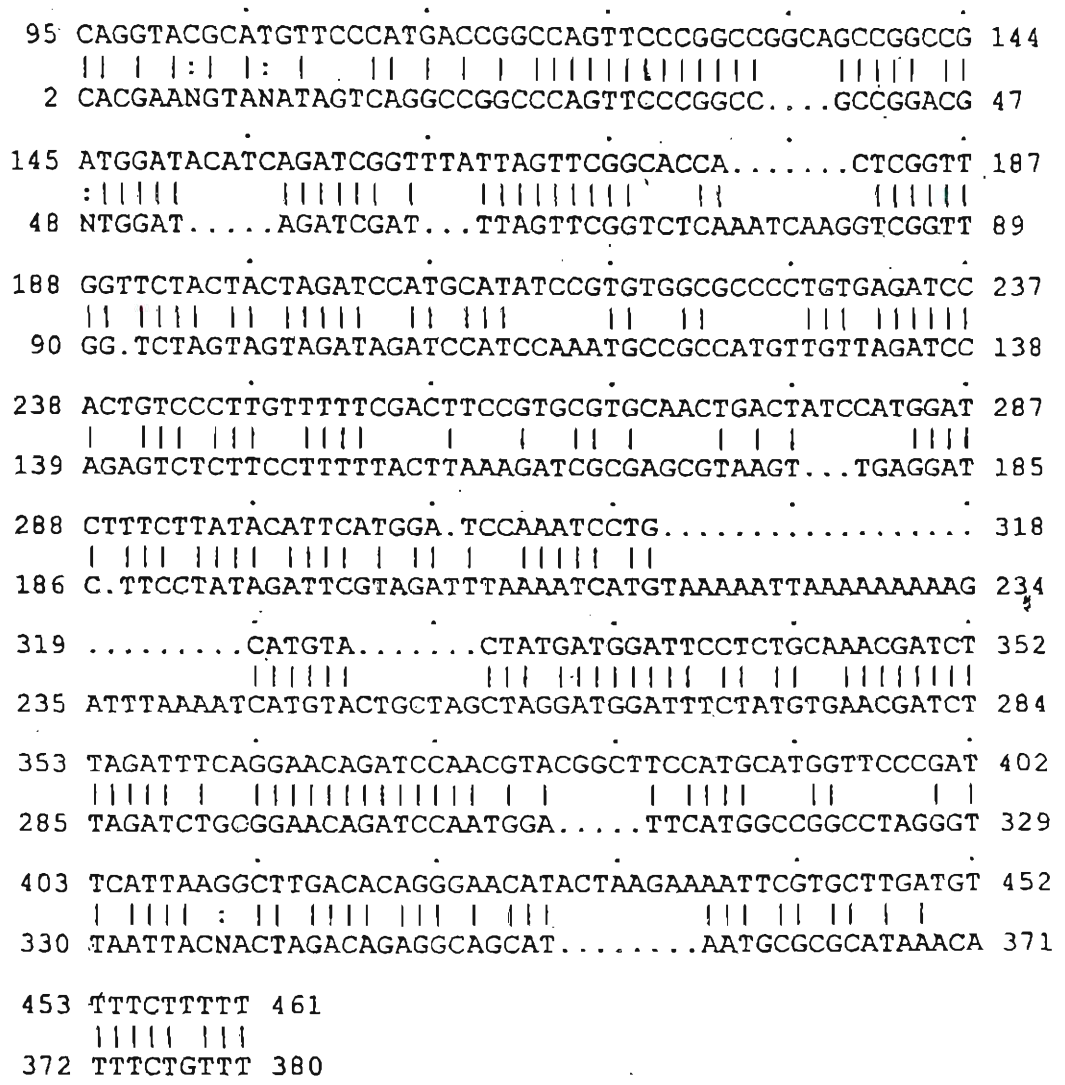


Figure 6.4 Partial sequence alignment of 700 bp PCR fragment of a null 4A cultivar (lower sequence) with the published intron 1 sequence of barley *waxy* gene (upper sequence).

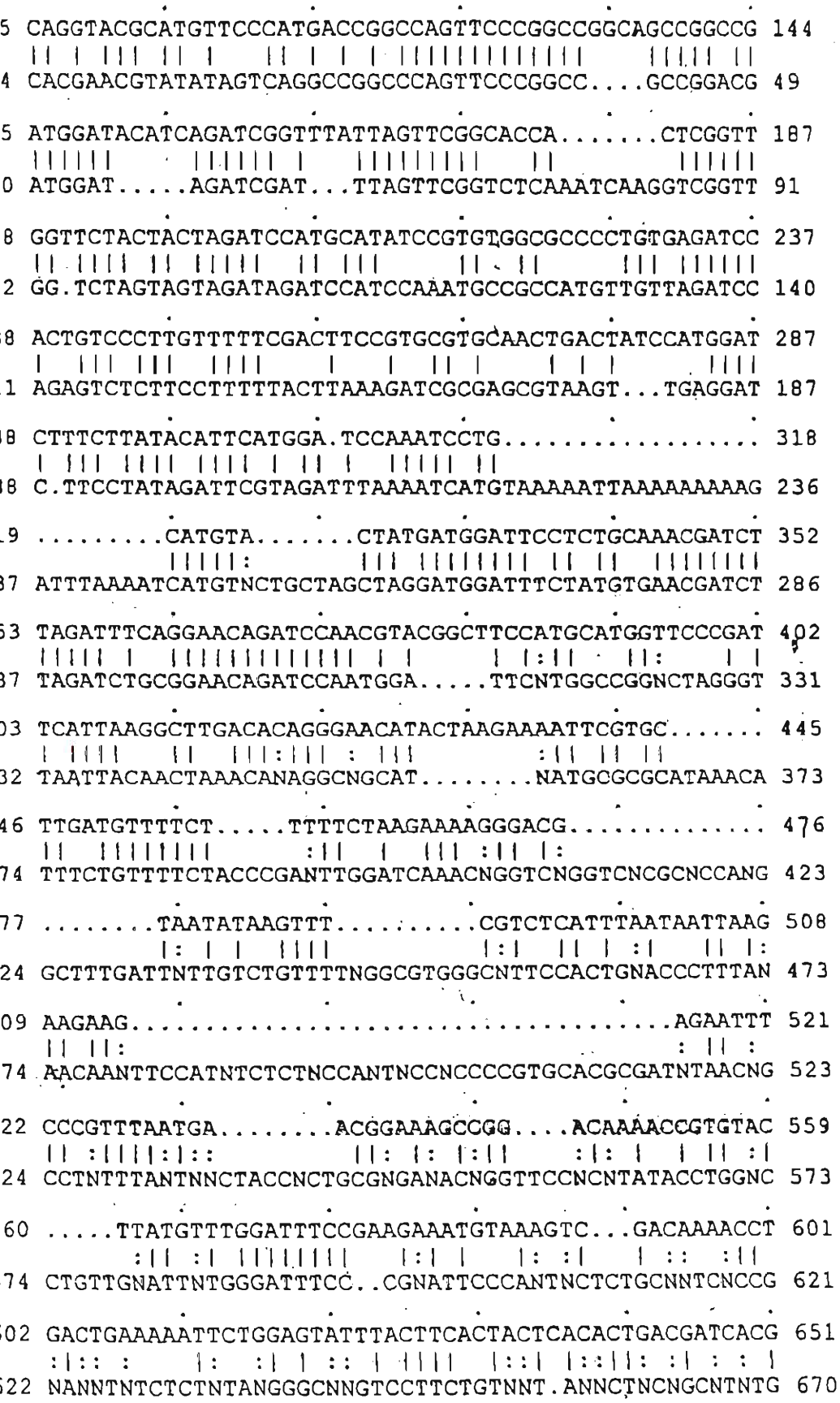


Figure 6.5 Partial sequence alignment of 700 bp PCR fragment of a normal cultivar (lower sequence) with the published intron 1 sequence of barley *waxy* gene (upper sequence).

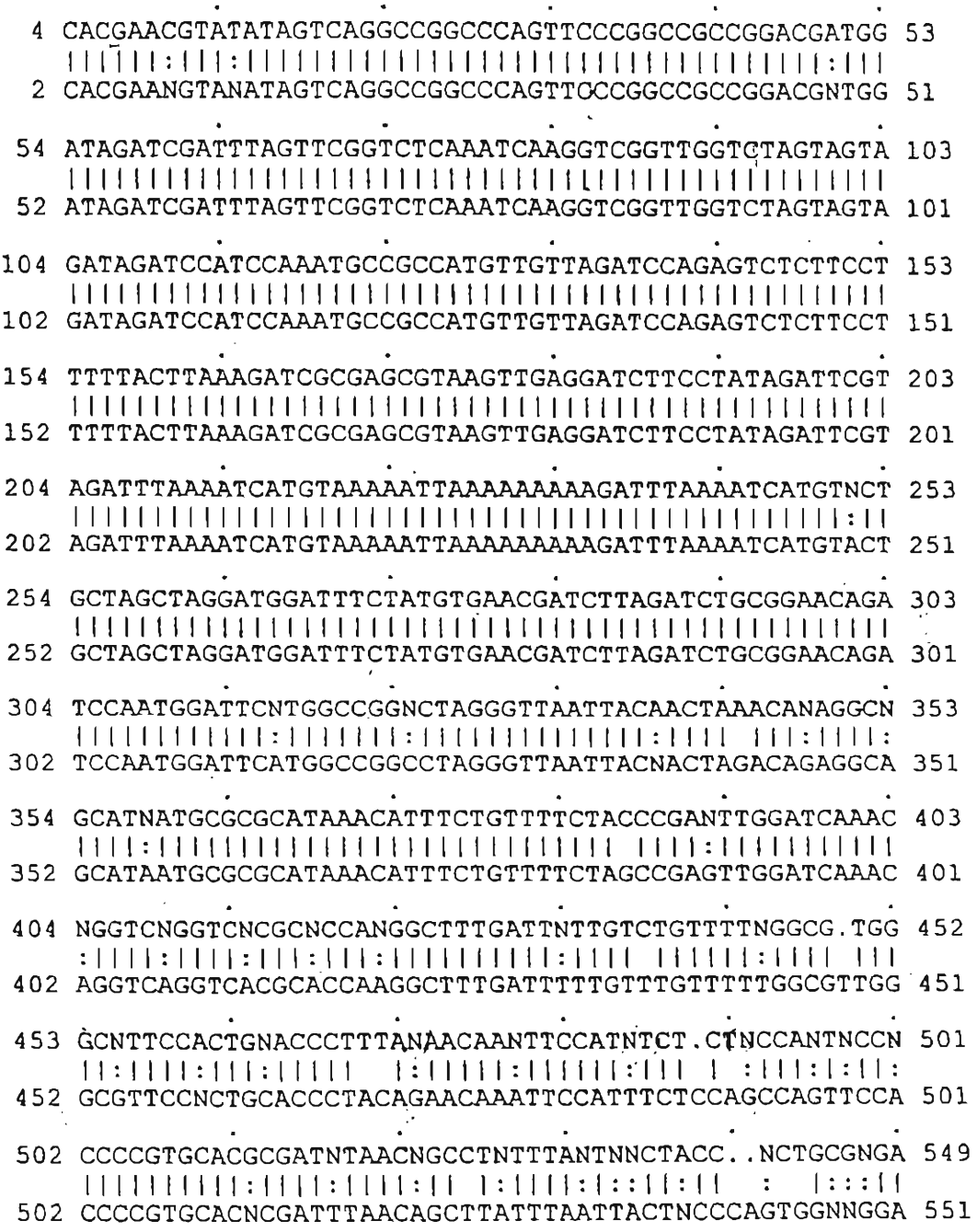


Figure 6.6 Partial sequence alignment of 700 bp PCR fragment of a normal cultivar (upper sequence) with the 700 bp fragment of a null 4A cultivar (lower sequence).

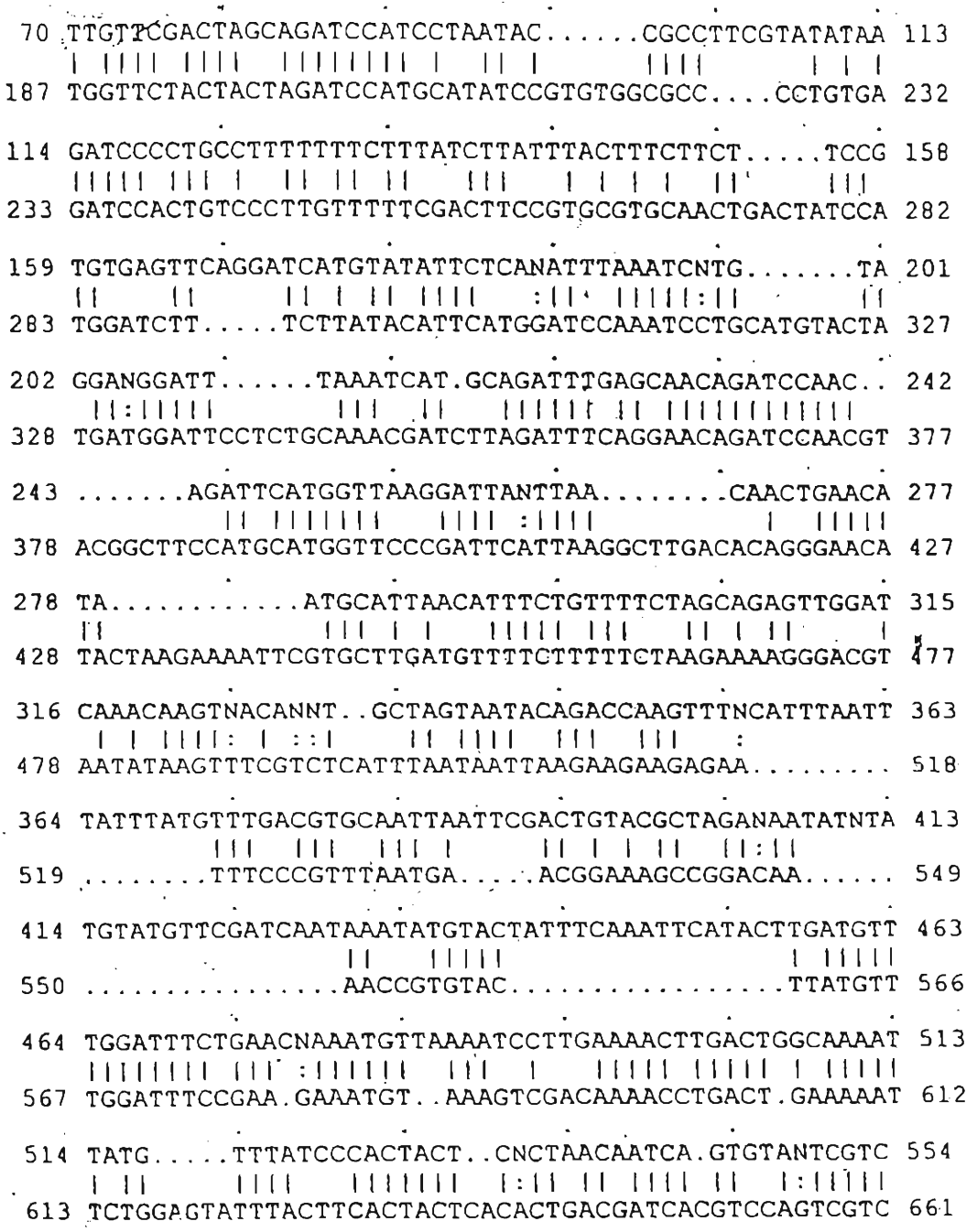


Figure 6.7 Partial sequence alignment of 600 bp PCR fragment of a normal cultivar (upper sequence) with the published intron 1 sequence of barley *waxy* gene (lower sequence).

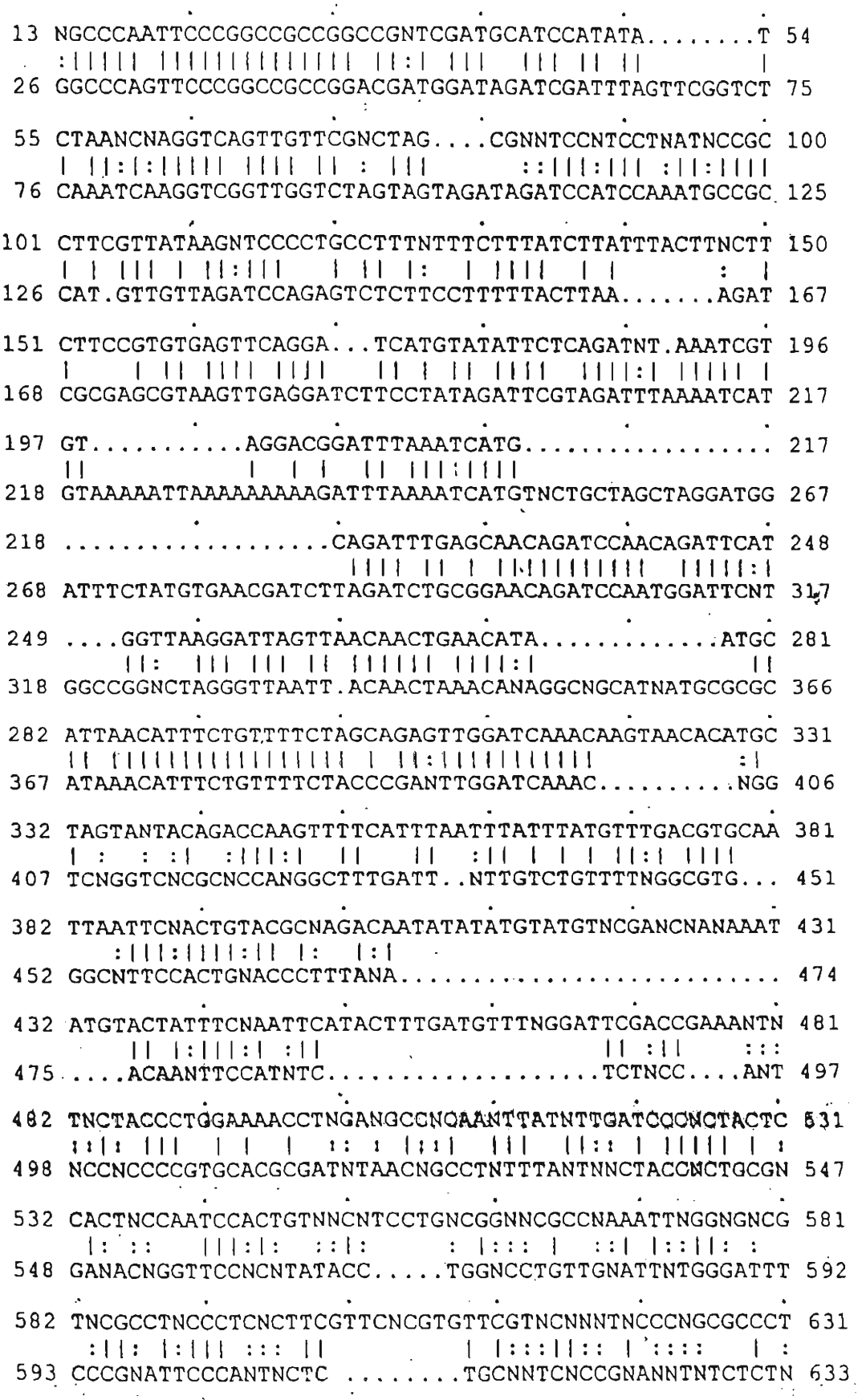


Figure 6.8 Partial sequence alignment of 600 bp (upper sequence) and 700 bp (lower sequence) PCR fragments of a normal cultivar.

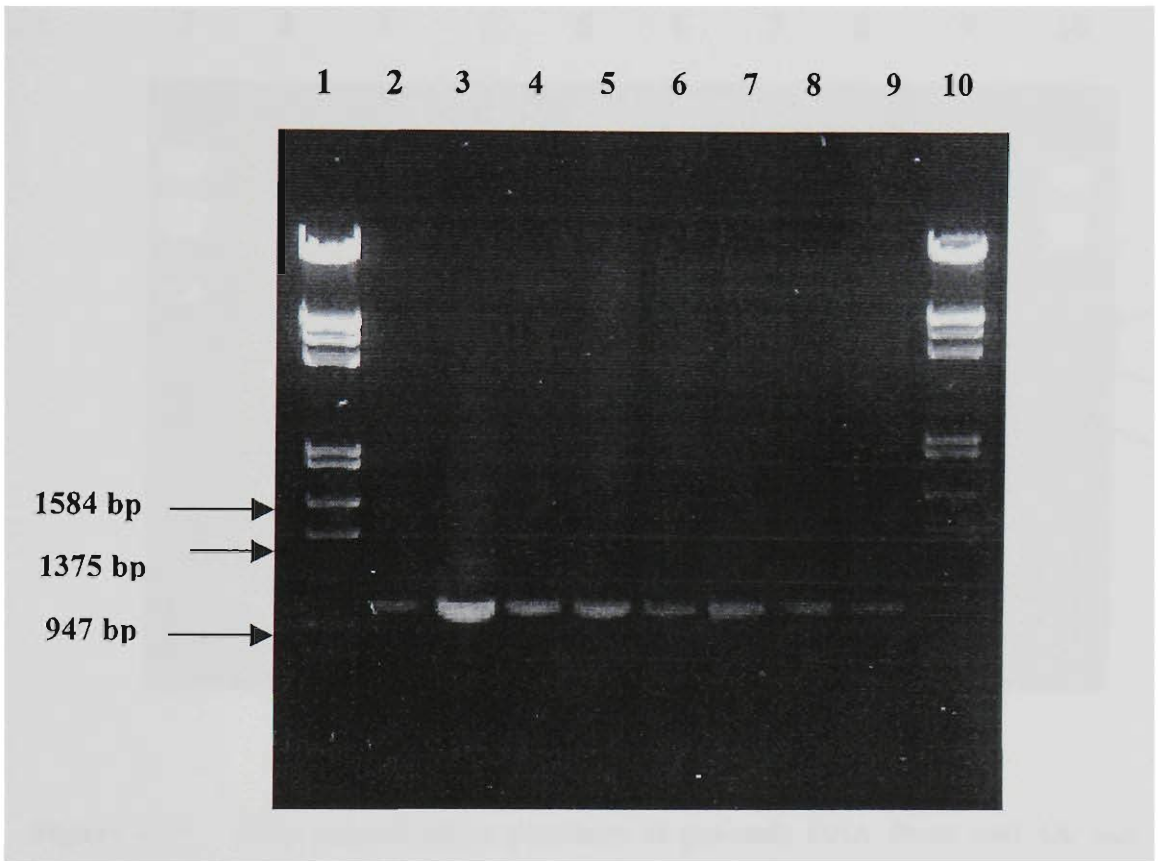


Figure 6.9 PCR amplification products of genomic DNA from null 4A and normal wheat cultivars, with primers P3 and P4. Lanes 1 and 10: Marker, Lambda DNA digested with *EcoR1* and *Hind111*, Lanes 2-5: Cadoux, Halberd, Machete and Rosella respectively (null 4A cultivars), Lanes 6-9: Chinese Spring, Goroke , Vectis and Wyuna respectively (normal cultivars).

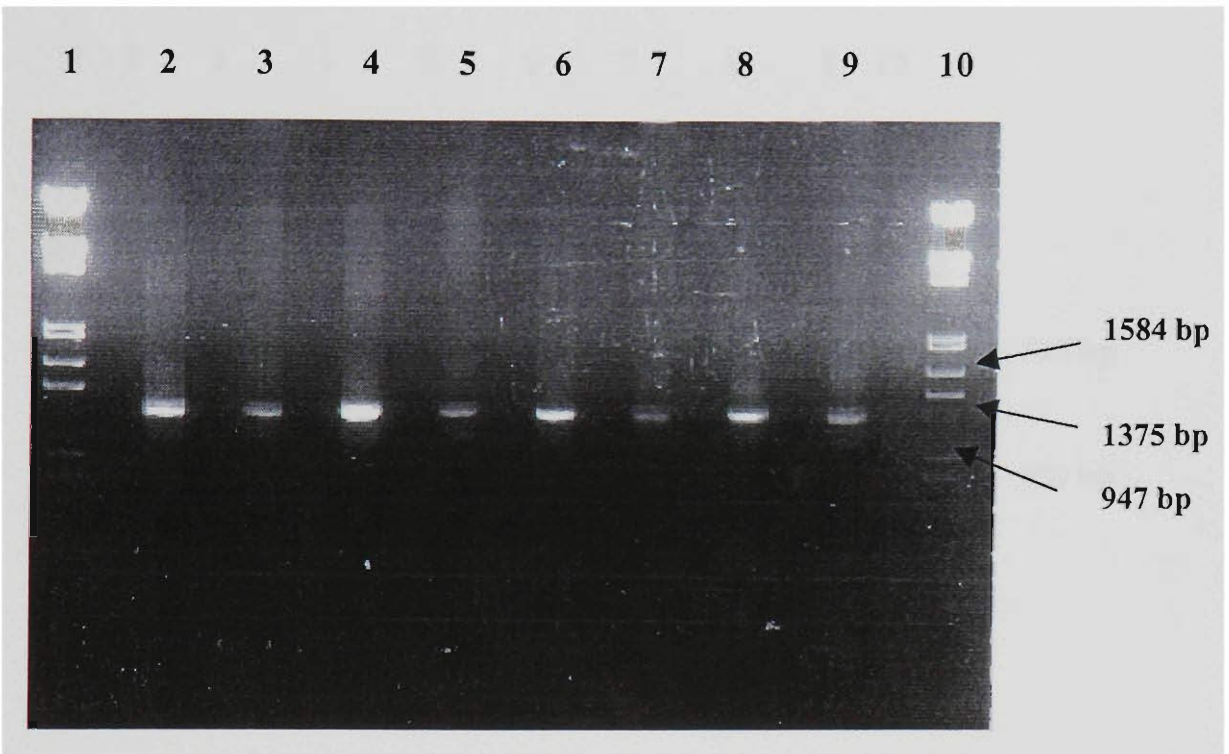


Figure 6.10 PCR amplification products of genomic DNA from null 4A and normal wheat cultivars, with primers P5 and P6. Lanes 1 and 10: Marker, Lambda DNA digested with *EcoR1* and *Hind111*, Lanes 2-5: Cadoux, Halberd, Machete and Rosella respectively (null 4A cultivars), Lanes 6-9: Chinese Spring, Goroke , Vectis and Wyuna respectively (normal cultivars).

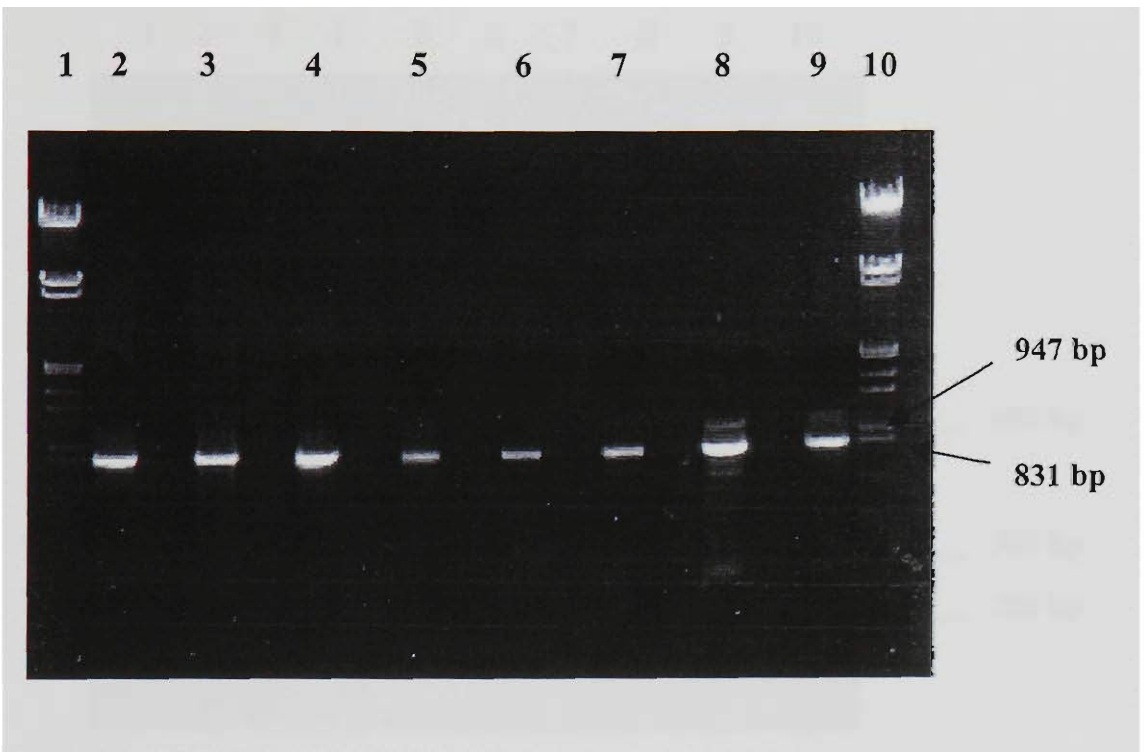


Figure 6.11 PCR amplification products of genomic DNA from null 4A and normal wheat cultivars, with primers P7 and P8. Lanes 1 and 10: Marker, Lambda DNA digested with *EcoR1* and *Hindlll*, Lanes 2-5: Cadoux, Halberd, Machete and Rosella respectively (null 4A cultivars) , Lanes 6-9: Chinese Spring, Goroke , Vectis and Wyuna respectively (normal cultivars).

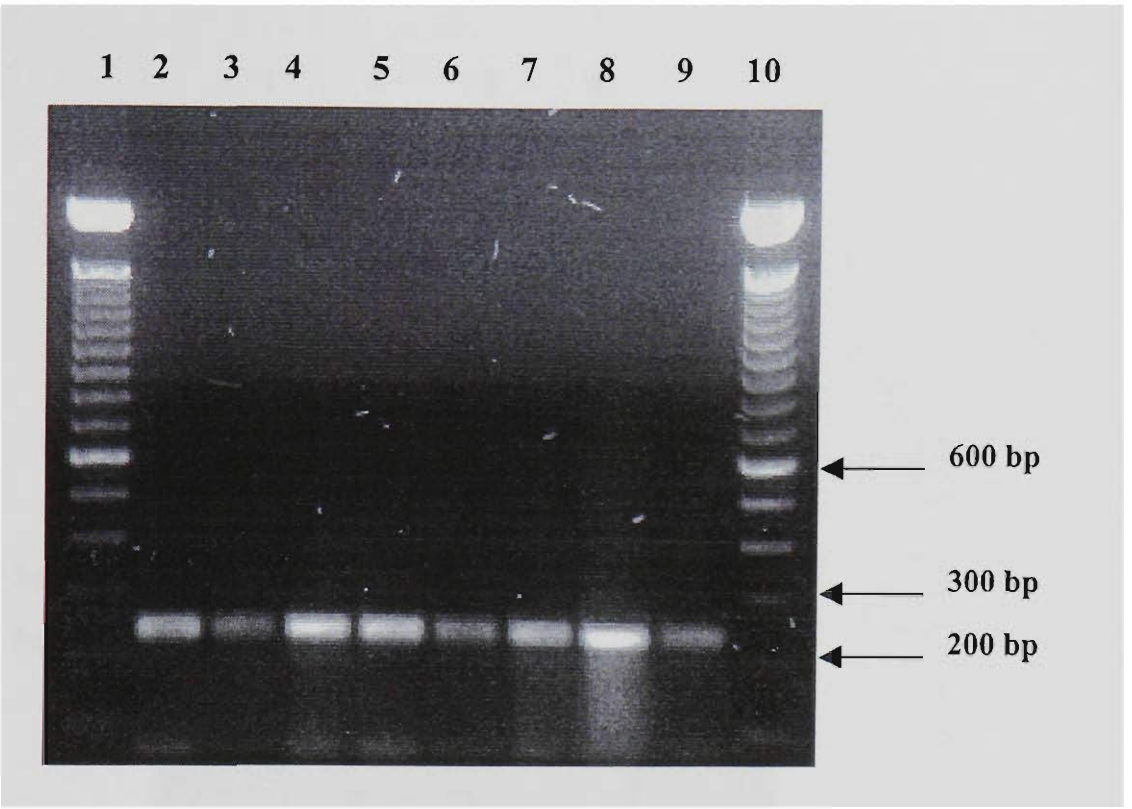


Figure 6.12 Reverse Transcriptase-PCR amplification products of total RNA from 20 dap seeds of null 4A and normal wheat cultivars, with primers P1 and P2b. Lanes 1 and 10: Marker, 100 bp ladder, Lanes 2-5: Cadoux, Halberd, Machete and Rosella respectively (null 4A cultivars) , Lanes 6-9: Chinese Spring, Goroke , Vectis and Wyuna respectively (normal cultivars).

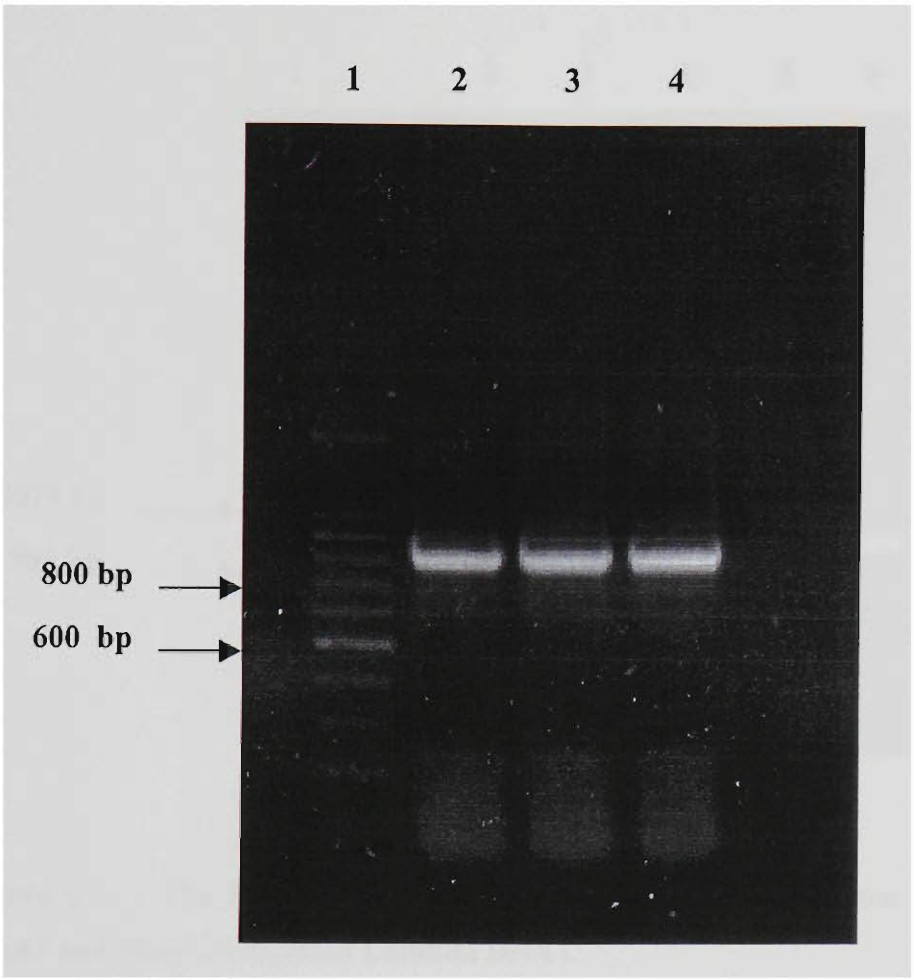


Figure 6.13 The 840 bp *waxy* cDNA probe, before purification. Lane 1: Marker, 100 bp ladder.

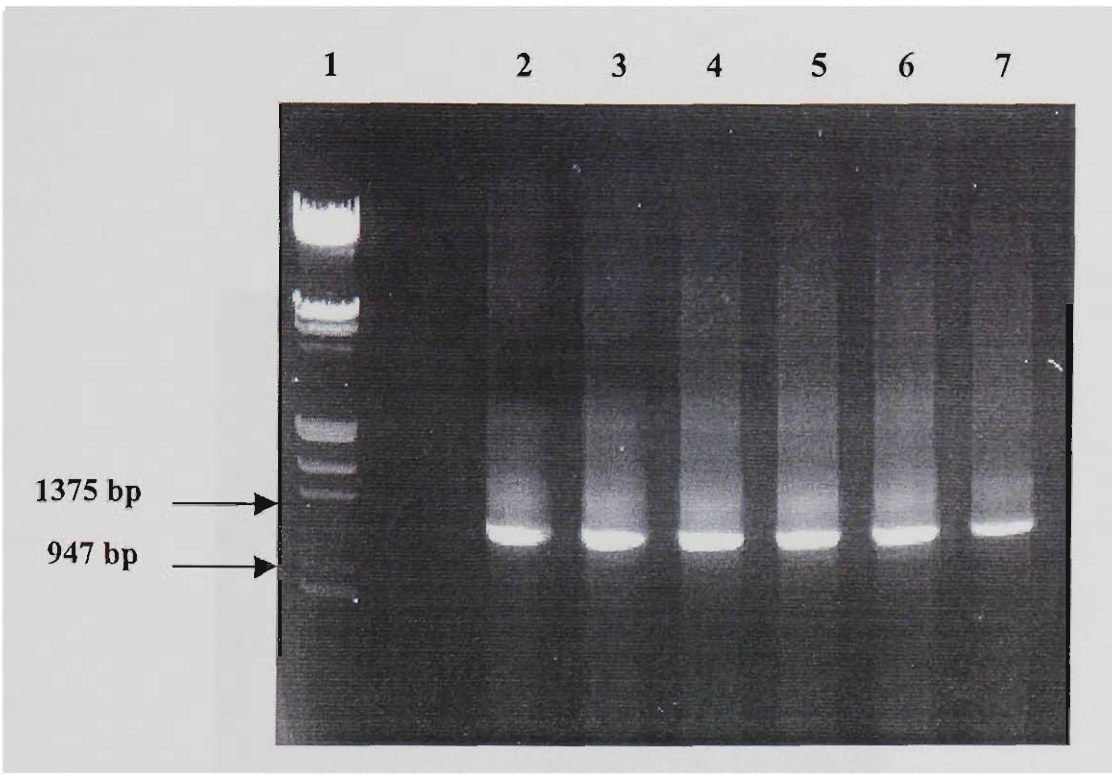


Figure 6.14 The 1244 bp *waxy* cDNA probe, before purification. Lane 1: Marker, *EcoR1* and *Hind111* digested Lambda DNA .

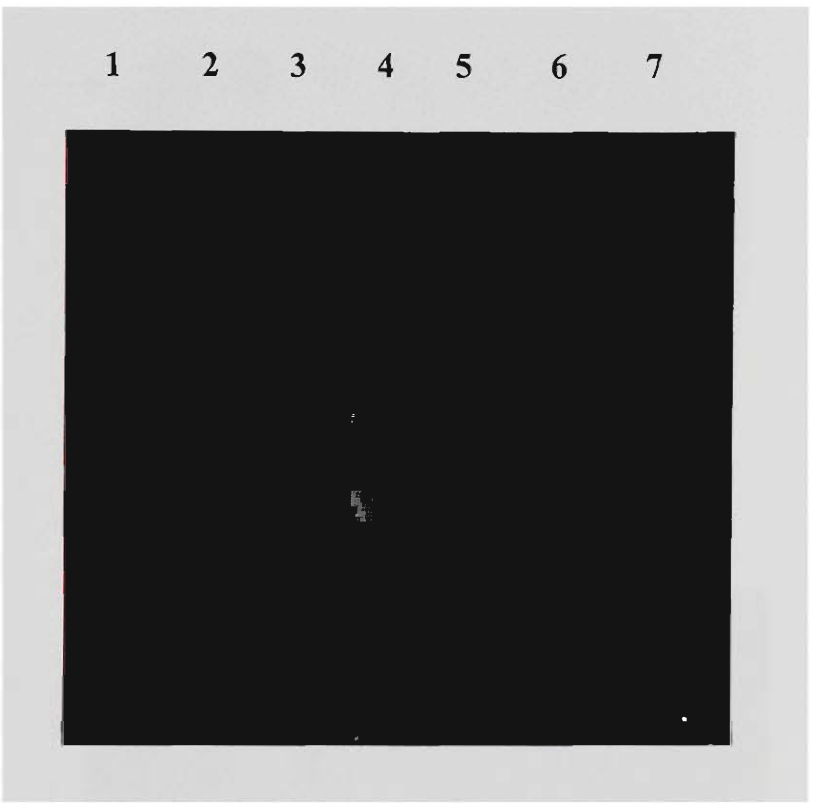


Figure 6.15 Formaldehyde gel electrophoresis of total RNA from 20 dap seeds of null 4A and normal wheat cultivars. Lanes 1 to 4: Cadoux, Halberd, Machete and Rosella respectively (null 4A cultivars), Lanes 5 to 7: Chinese Spring, Goroke and Vectis respectively (normal cultivars).

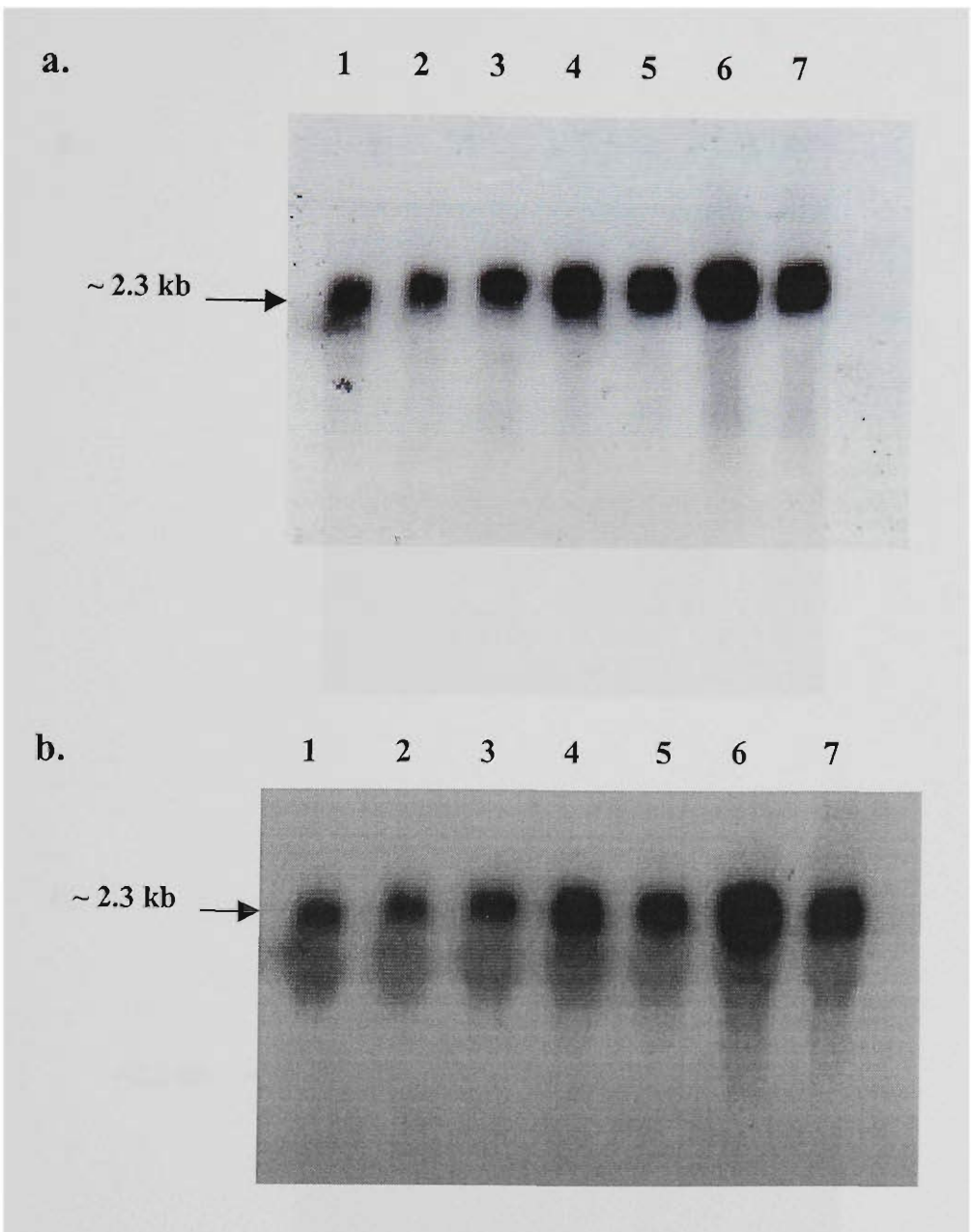


Figure 6.16 Northern blot analysis of total RNA isolated from 20 dap seeds of null 4A and normal cultivars, using a) the 840 bp *waxy* cDNA probe, b) the 1244 bp *waxy* cDNA probe. Lanes 1 to 4: Cadoux, Machete, Halberd and Rosella respectively (null 4A cultivars), Lanes 5 to 7: Chinese Spring, Goroke and Vectis respectively (normal cultivars).

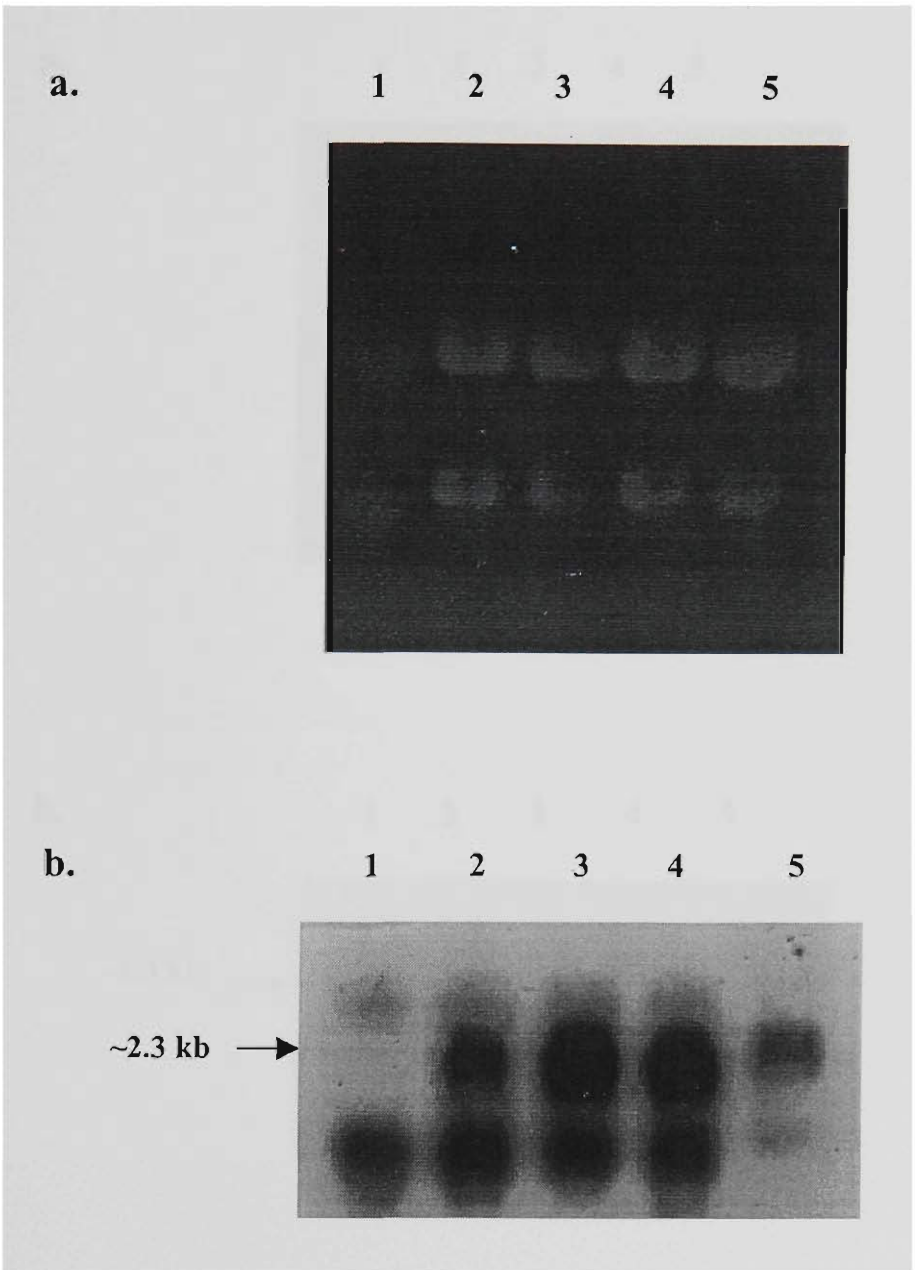


Figure 6.17 a) Formaldehyde gel electrophoresis b) Northern blot analysis, of total RNA isolated from developing seeds of cultivar Chinese Spring (normal). Lanes 1 to 5: 5, 10, 15, 20 and 25 dap seeds respectively.

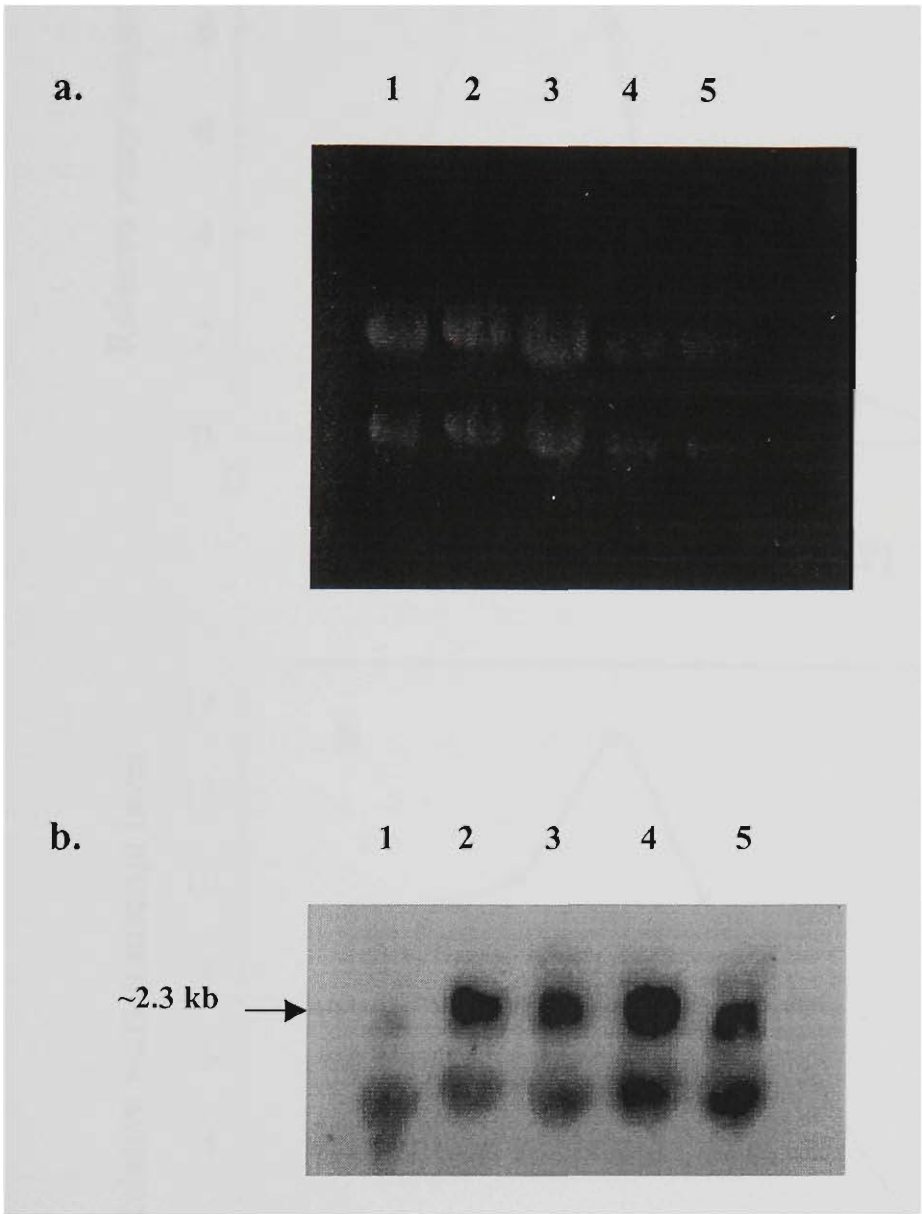


Figure 6.18 a) Formaldehyde gel electrophoresis b) Northern blot analysis, of total RNA isolated from developing seeds of cultivar Rosella (null 4A). Lanes 1 to 5: 5, 10, 15, 20 and 25 dap seeds respectively.

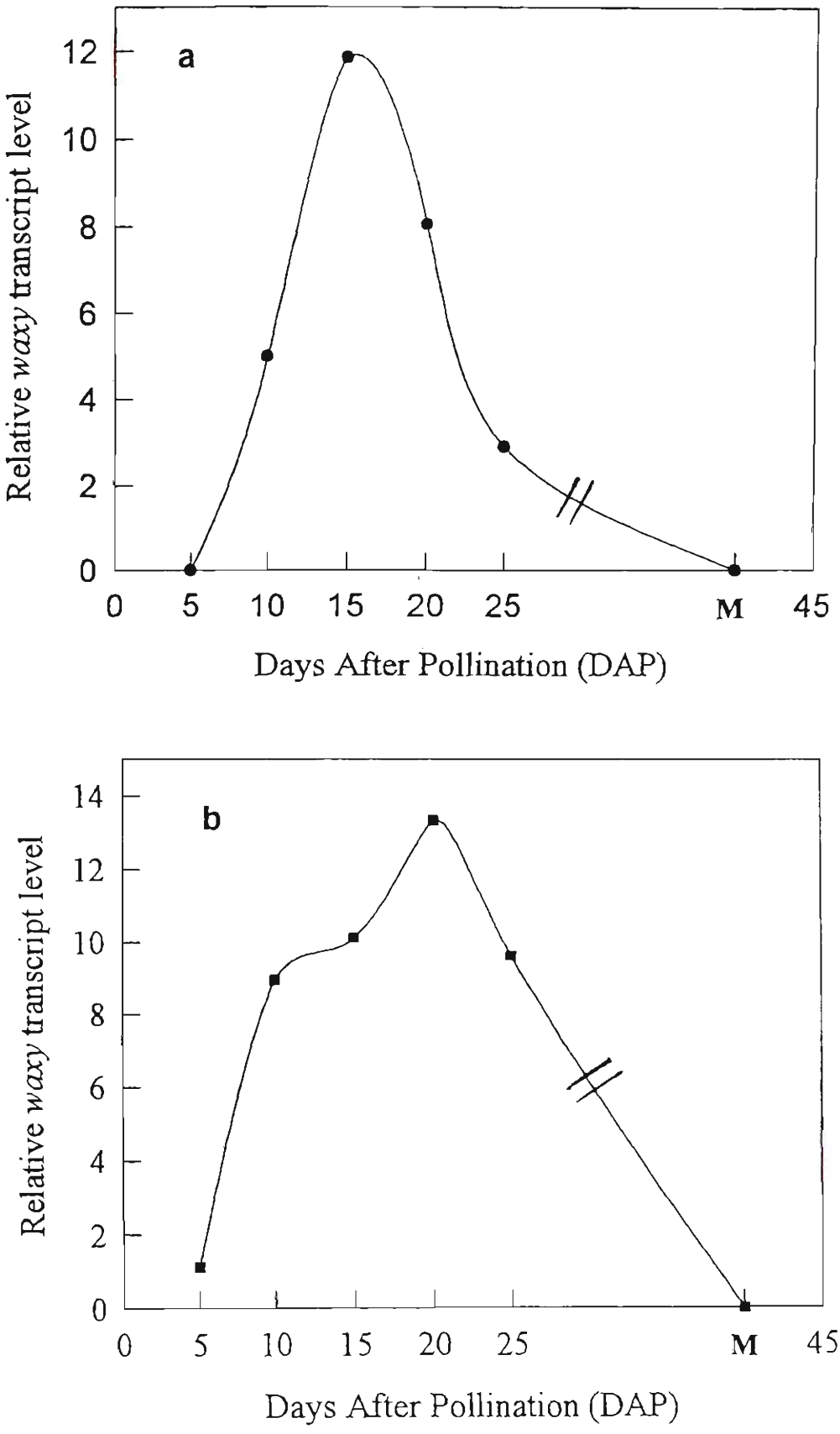


Figure 6.19 Relative *waxy* transcript levels during seed development in, a) Chinese Spring b) Rosella. M = mature seeds

CHAPTER 7

Analysis of waxy protein and amylose content

7.1 Introduction

Th electrophoretic profile of waxy proteins of wheat cultivars has been discussed at length in Chapter 1. Briefly, the three ~60 kDa waxy proteins of common wheat, Wx-A1, Wx-B1 and Wx-D1, can be separated into high molecular weight (HMW) and low molecular weight (LMW) proteins by a modified SDS-PAGE (Kagawa *et al.* 1988). The HMW waxy protein is encoded by the 7A *waxy* gene, and the LMW waxy protein is a mixture of proteins produced by the 4A and 7D *waxy* genes (Nakamura *et al.* 1992).

Yamamori *et al.* (1992) investigated the relationship between waxy protein and amylose content in Japanese common wheat cultivars and reported that the amount of waxy protein was associated with the amylose content in wheat, as in the case of rice (Sano *et al.* 1985) and maize (Imam, 1989). However, this relationship between waxy protein amount and amylose content during seed development has not been investigated in wheat. Further, it is of interest to determine whether the reduced amylose content, in the mature endosperm of null 4A mutants, is due to rapid degradation of the waxy protein B or lack of its expression at all stages of seed development.

The aim of this study was to establish the relationship between waxy protein amount and amylose content during seed development in a null 4A and a normal wheat cultivar. The correlation of *waxy* gene expression with waxy protein amount and amylose content in developing seeds of a null 4A and a normal wheat cultivar, was also investigated.

7.2 Materials and methods

7.2.1 Wheat cultivars

Developing seeds (5, 10, 15, 20 and 25 dap) of Chinese Spring (6n, normal) and Rosella (6n, null 4A) wheat cultivars and mature seeds of the eight wheat cultivars (Section 2.5.2) were harvested as described in Section 2.5.3 to determine the waxy protein amounts and amylose contents.

7.2.2 Extraction of wheat starch

Starch was extracted, from the above mentioned developing and mature wheat seeds, as described in Section 2.6.1. The pericarp and embryo were removed from all the seeds except the 5 dap seeds of both Chinese Spring and Rosella cultivars, where such separation was impossible.

7.2.3 Analysis of waxy proteins

Electrophoretic separation and quantitation of waxy proteins

Protein extracts were prepared from the starches (Section 7.22) as mentioned in Section 2.6.2. SDS-PAGE was carried out as mentioned in Section 2.6.3 (Laemmli, 1990), with acrylamide / bis acrylamide (30 : 0.8 respectively) in a 10% separating gel. The gels were stained as described in Section 2.6.3, dried in a Gel Air Dryer (Bio-Rad) for 2 hours and the bands corresponding to waxy protein (~60 kda) were scanned with a laser densitometer. The area under each peak was determined to estimate the relative amount of the waxy protein (Nakamura *et al.* 1993). In mature seeds, the relative waxy protein amount for each cultivar was calculated based on the amount in the cultivar Goroke as 100%, because this cultivar showed the maximum amount of waxy protein (n=4). In the developing seeds, the relative waxy protein amount for each developmental stage was calculated based on 25 dap as 100%, because this developmental stage showed the maximum waxy protein amount (n=4).

Qualitative analysis of waxy protein

Protein extracts were prepared as mentioned in section 2.6.2 from the wheat starches (Section 7.2.2). SDS-PAGE was performed as mentioned in section 2.6.3 (Kagawa *et al.* 1988) with 30 : 0.135 acrylamide / bis acrylamide in a 10% separating gel.

7.2.4 Determination of amylose content

Amylose contents were determined in the starches extracted in Section 7.2.2 using the amylose/amylopectin assay kit (Megazyme), estimations being carried out in duplicate.

7.2.5 Determination of *waxy* gene expression during seed development

Refer Chapter 6: section 6.2.4

7.3 Results and Discussion

7.3.1 Waxy protein amount in mature seeds

The ~60 kDa waxy protein was seen as a single band in all eight mature wheat cultivars, by Laemmli's SDS-PAGE method (Figure 7.1). The bands appeared thinner in null 4A cultivars compared to the bands of normal cultivars. Figure 7.2 shows the relative waxy protein amounts of the wheat cultivars. It can be seen that the amount of waxy protein in the null 4A cultivars was reduced to an amount that was approximately 2/3 of the level in normal cultivars. Statistical analysis of the results (Table 7.2) shows that the amount of waxy protein in the null 4A cultivars was significantly lower than that in normal cultivars ($P < 0.01$). Figure 7.3 shows the waxy protein profiles of the eight wheat cultivars in the modified SDS-PAGE system. The waxy proteins of all cultivars were distinctly separated into two main bands, the high molecular weight (HMW) and low molecular weight (LMW) proteins. In the normal cultivars the LMW bands were thicker than the HMW. In comparison, the null 4As displayed thinner LMW bands, their density being almost the same as that of HMW. These results indicate a clear decrease in one of the proteins that contributes to the LMW band. The reduction is most likely caused by the lack of Wx-B1 protein, as it has been reported by Yamamori *et al.* (1994). According to the classification of Yamamori *et al.* (1994) based on presence and absence of the three waxy proteins, Chinese Spring, Goroke, Vectis and Wyuna belong to Type 1 (Wx-A1/+ , Wx-B1/+ and Wx-D1/+) and Cadoux, Halberd, Machete and Rosella belong to Type 3 (Wx-A1/+ , Wx-B1/- and Wx-D1/+).

7.3.2 Amylose content in mature seeds

Table 7.1 shows the amylose contents of mature seeds of the eight wheat cultivars. The t-test analysis shows (Table 7.2) that the mean amylose content of null 4A cultivars were significantly lower than that of normal cultivars ($P < 0.01$). Approximately 3% reduction in the amylose content was observed in the null 4A compared to that of normal cultivars. This result is comparable to that obtained by Miura *et al.* (1998), who investigated the effects of null alleles upon amylose synthesis, using Chinese Spring and its aneuploid lines. They observed about a 3% reduction in amylose content in the nullisomic 4A compared to the CS euploid. They have reported that the action of the *Wx-B1* gene was most important in amylose synthesis and absence of this gene has the most striking effect in reducing amylose content.

7.3.3 Correlation between waxy protein amount and amylose content in mature seeds

Comparison of amylose content and the corresponding waxy protein level among the eight mature wheat cultivars (Figure 7.4 and Table 7.2) showed a positive correlation between the two traits. This indicates that the amount of waxy protein is highly associated with the amylose content in Australian wheat cultivars, as previously reported in Japanese wheat cultivars by Yamamori *et al.* (1992). This association between waxy protein and amylose has been reported in rice (Sano *et al.* 1985) and in maize (Imam, 1989).

7.3.4 Correlation between *waxy* gene expression and waxy protein amount during seed development

Figures 7.5 and 7.6 show the waxy protein profiles during the development of Chinese Spring and Rosella wheat cultivars, respectively, examined by Laemmli's SDS-PAGE system. In chapter 6, the preliminary study showed that the *waxy* gene was active from early (5-10 dap) to middle stage (15-20 dap) of seed development in Chinese Spring and Rosella (Figures 7.7a and 7.8a respectively). During this period the waxy protein appeared to accumulate linearly in both the cultivars (Figures 7.7b and 7.8b respectively). The *waxy* transcript level started decreasing beyond the peak expression

period, however, the waxy protein continued to increase until 25 dap and appeared to remain constant until seed maturity, in both of the cultivars. This indicates that the abundant transcripts, which accumulate at the time of maximum expression, may be responsible for the continued synthesis of waxy protein. Similar pattern was observed in the developing seeds of rice (Hirano *et al.* (1991), where the *waxy* transcripts were most abundant in the period from the 13 to 18 dap and waxy protein accumulated linearly from 4 to 23 dap and the amount remained almost constant thereafter. According to Hirano *et al.* (1991), the abundant transcripts formed during active transcription period may be required for the large-scale waxy protein synthesis.

Figures 7.9 and 7.10 show the waxy protein profiles during the development of the cultivars Chinese Spring and Rosella respectively, examined by the modified SDS-PAGE system (Kagawa *et al.* 1988). Throughout the developmental period the normal cultivar (Chinese Spring) exhibited two bands with the LMW band being thicker than the HMW band, while the null 4A (Rosella) exhibited the two bands with equal intensity. As discussed in Section 7.3.1, in the developing seeds of normal cultivar, the HMW band is comprised of Wx-A1 protein and the LMW band of Wx-B1 and Wx-D1 proteins. And, in the developing seeds of null 4A cultivar, the clear decrease in one of the proteins that comprises the LMW band could be attributed to lack of Wx-B1 protein, also discussed in Section 7.3.1. These results strongly suggest that the null 4A mutants lack the Wx-B1 protein throughout the development of the seeds until maturity.

The 5 dap seeds of both normal and null 4A cultivars exhibited an additional band smaller in size than the waxy protein band (Figures 7.9 and 7.10). This band cannot represent the precursor polypeptide due to its smaller size. During extraction of starch from the 5 dap seeds of both the cultivars, the pericarp and embryo could not be removed, due to the exceedingly small size of the seeds and lack of firmness and distinct morphology of most seed components. Hence, the low molecular weight protein is most likely to be a protein from the pericarp or embryo.

7.3.5 Correlation between waxy protein amount and amylose content during seed development

Tables 7.3 and 7.4 show that the amount of waxy protein amount is correlated with the amylose content during seed development up to 25 dap, in Chinese Spring and Rosella respectively. These results suggest that during seed development the increase in the amount of the waxy protein may contribute to the increase in the amount of amylose content. However, this is difficult to assess since the enzyme activity of waxy protein at any given time is not known. The presence of waxy protein and amylose at 5 dap in both the cultivars indicates that the onset of amylose synthesis occurs soon after pollination. Mature seeds of Chinese Spring and Rosella showed approximately the same amount of waxy protein as that of 25 dap seeds, however, their amylose content increased dramatically, as shown in Figures 7.7b and 7.7c for Chinese Spring, and in Figures 7.8b and 7.8c for Rosella. These results suggest that the abundant waxy protein produced at and up to 25 dap, may be responsible for the continued synthesis of amylose until seed maturation in these cultivars, thus indicating that the waxy protein is enzymatically active for long periods of time. However, for an accurate assessment a detailed study on the enzyme activity of waxy protein during seed development is recommended for future study as this will provide information on how much of the granule bound waxy protein is active at any one time.

The observed increase in the amylose content in developing seeds supports the results of Matheson (1971), who compared wheat grains at 14 and 42 dap and concluded that the increase in iodine binding of starch as the wheat grain matures is due to an increase in the amount of amylose. It was notable that Rosella (null 4A) exhibited lower amylose content compared to the cultivar Chinese Spring (normal) (Figure 7.11) which coincided with the observed lack of expression of Wx-B1 protein, throughout the developmental period.

7.4 Conclusions

This study has demonstrated a positive correlation between production of waxy protein and amylose synthesis in the endosperms of a normal (Chinese Spring) and a null 4A (Rosella) cultivar during seed development, until 25 dap. It was found that the waxy protein amount of mature seeds did not vary much from that of the 25 dap seeds, however, the amylose content showed a dramatic increase in the mature seeds of both the cultivars. It was suggested that the abundant waxy protein synthesised at 25 dap could probably be responsible for the continued synthesis of amylose until seed maturity. However, future studies involving the correlation of enzymatic activity of waxy protein with amylose content during seed development would aid in confirming the present results.

The preliminary study of *waxy* gene expression in the developing seeds of Chinese Spring and Rosella suggested the following; near linear accumulation of waxy protein and amylose in the endosperm occurred during active transcription of the *waxy* gene, and, the abundant transcripts formed at the period of peak expression may be responsible for the continued synthesis of waxy protein up to 25 dap.

In the null 4A mutant (Rosella), lack of expression of Wx-B1 protein was associated with a lower amylose content (compared to the normal), throughout seed development. This strongly suggest that in the mature seeds of null 4A cultivars, the reduced amylose content is due to lack of expression of Wx-B1 protein, and not due to degradation of this protein during seed development.

The mature seeds of Australian wheat cultivars also exhibited a high association between waxy protein and amylose content, as previously reported for Japanese wheat cultivars (Yamamori, *et al.* 1992).

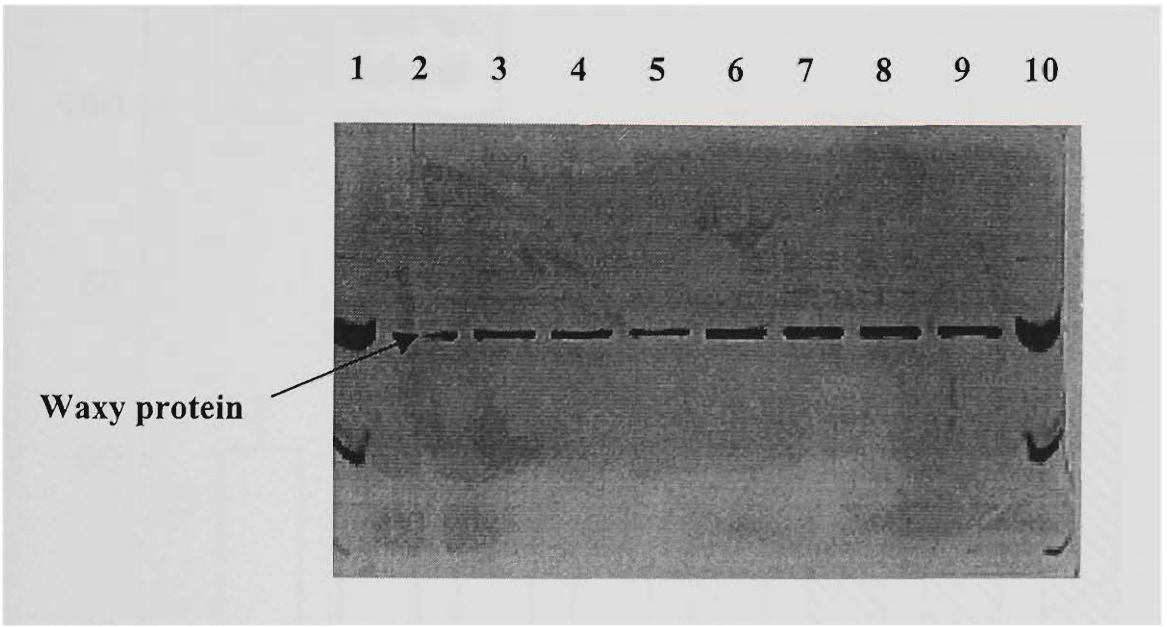


Figure 7.1 SDS-Polyacrylamide gel electrophoresis (30:0.8 Acrylamide / Bis) of protein extracts from mature endosperms of null 4A and normal wheat cultivars. Lanes 1 and 10: Low range protein marker, Lanes 2 to 5: Cadoux , Halberd, Machete, Rosella respectively (null 4A), Lanes 6 to 9: Chinese Spring, Goroke, Vectis and Wyuna respectively (normal).

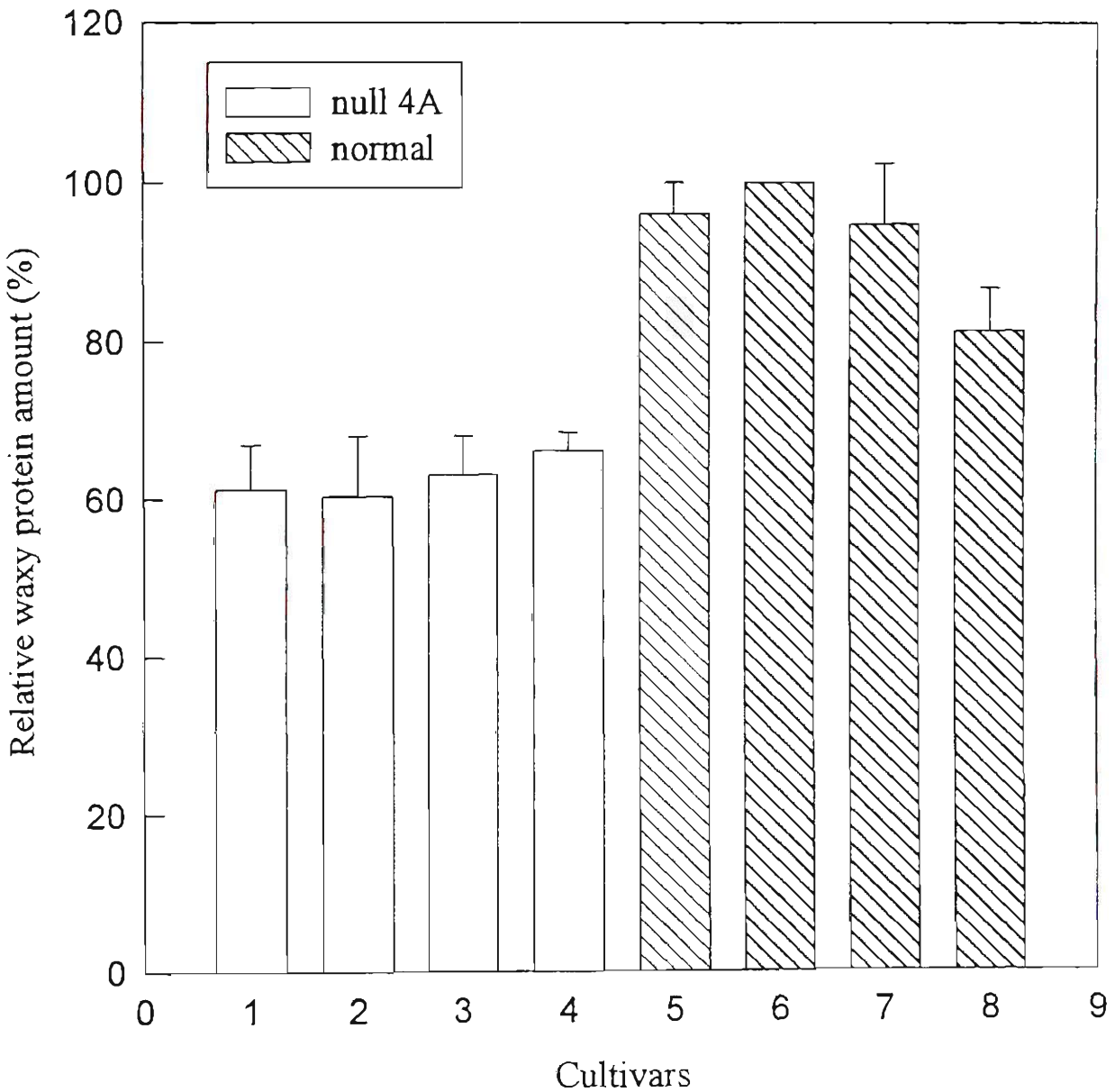


Figure 7.2 Relative waxy protein amount in the endosperms of mature wheat cultivars, 1. Cadoux 2. Halberd 3. Machete 4. Rosella 5. Chinese Spring 6. Goroke . Vectis 8. Wyuna, 1 to 5: null 4A cultivars, 5 to 8: normal cultivars.

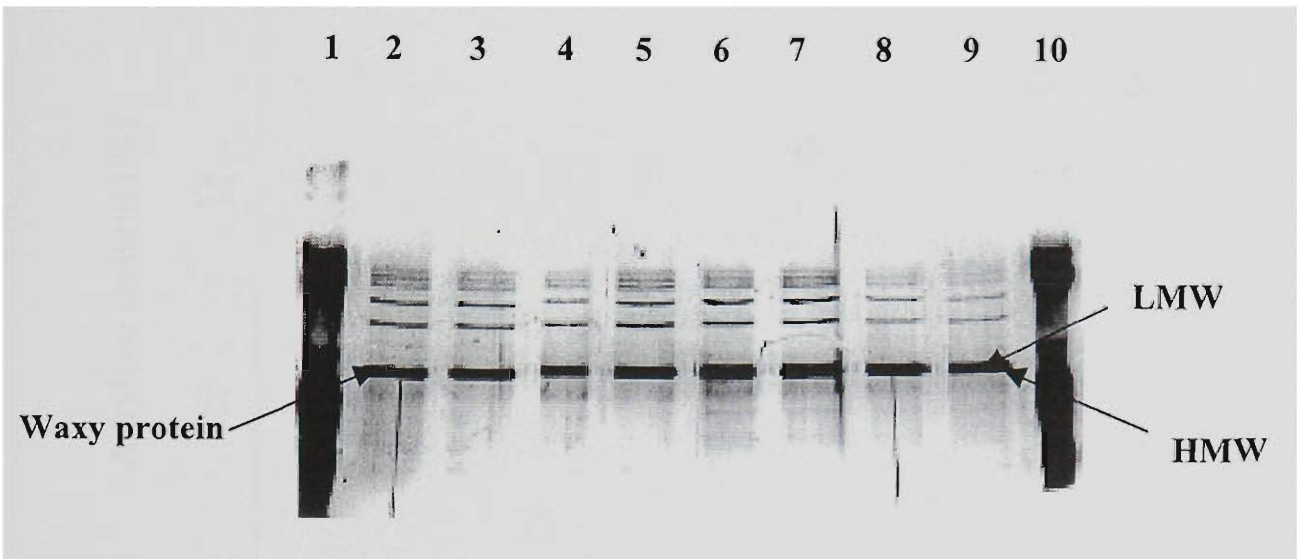


Figure 7.3 Modified SDS-Polyacrylamide gel electrophoresis (30:0.135 Acrylamide / Bis) of protein extracts from mature endosperms of null 4A and normal wheat cultivars. Lanes 1 and 10: Low range protein marker, Lanes 2 to 5: Cadoux , Halberd, Machete, Rosella respectively (null 4A), Lanes 6 to 9: Chinese Spring, Goroke, Vectis and Wyuna respectively (normal).

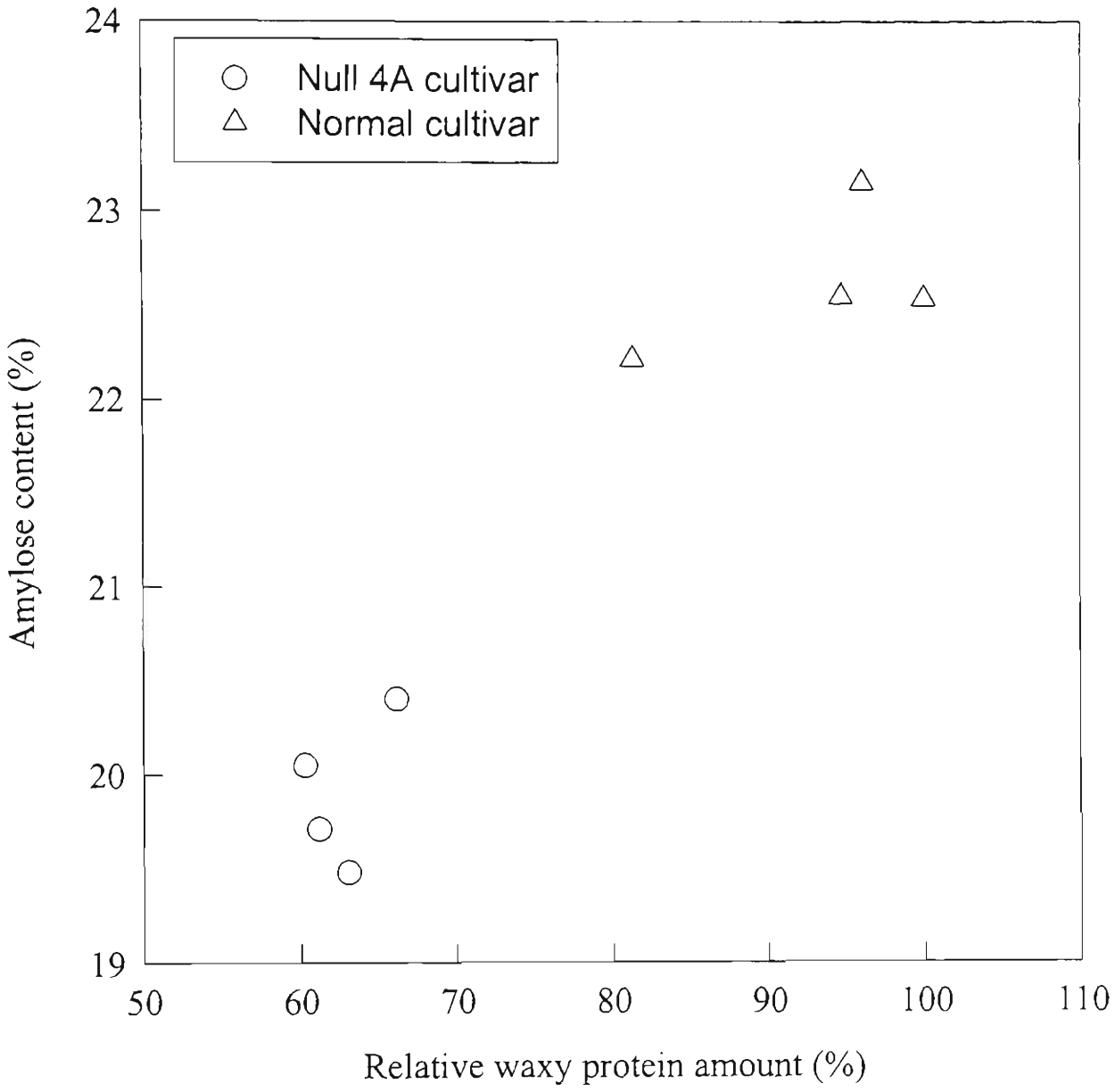


Figure 7.4 Relationship between amylose content and waxy protein amount in mature seeds of null 4A and normal wheat cultivars (r = 0.956).

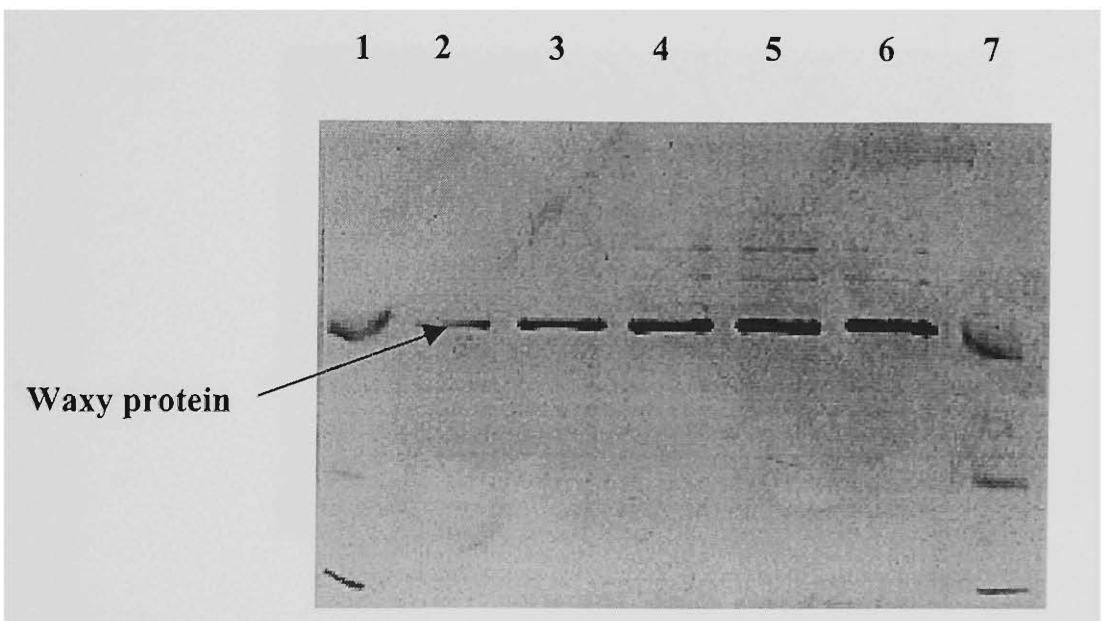


Figure 7.5 SDS-Polyacrylamide gel electrophoresis (30:0.8 Acrylamide / Bis) of protein extracts from endosperms of developing seeds of Chinese Spring (normal). Lanes 1 and 7: Low range protein marker, Lanes 2 to 6: 10, 15, 20, 25 dap and mature seeds.

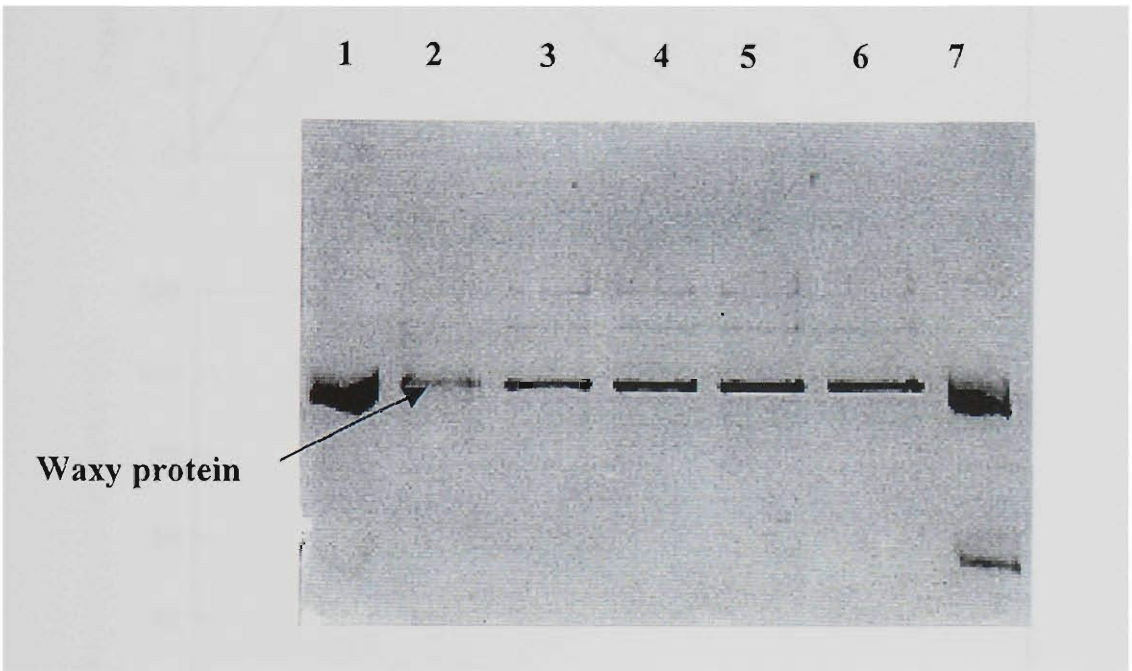


Figure 7.6 SDS-Polyacrylamide gel electrophoresis (30:0.8 Acrylamide / Bis) of protein extracts from endosperms of developing seeds of Rosella. Lanes 1 and 7: Low range protein marker, Lanes 2 to 6: 10, 15, 20, 25 dap and mature seeds.

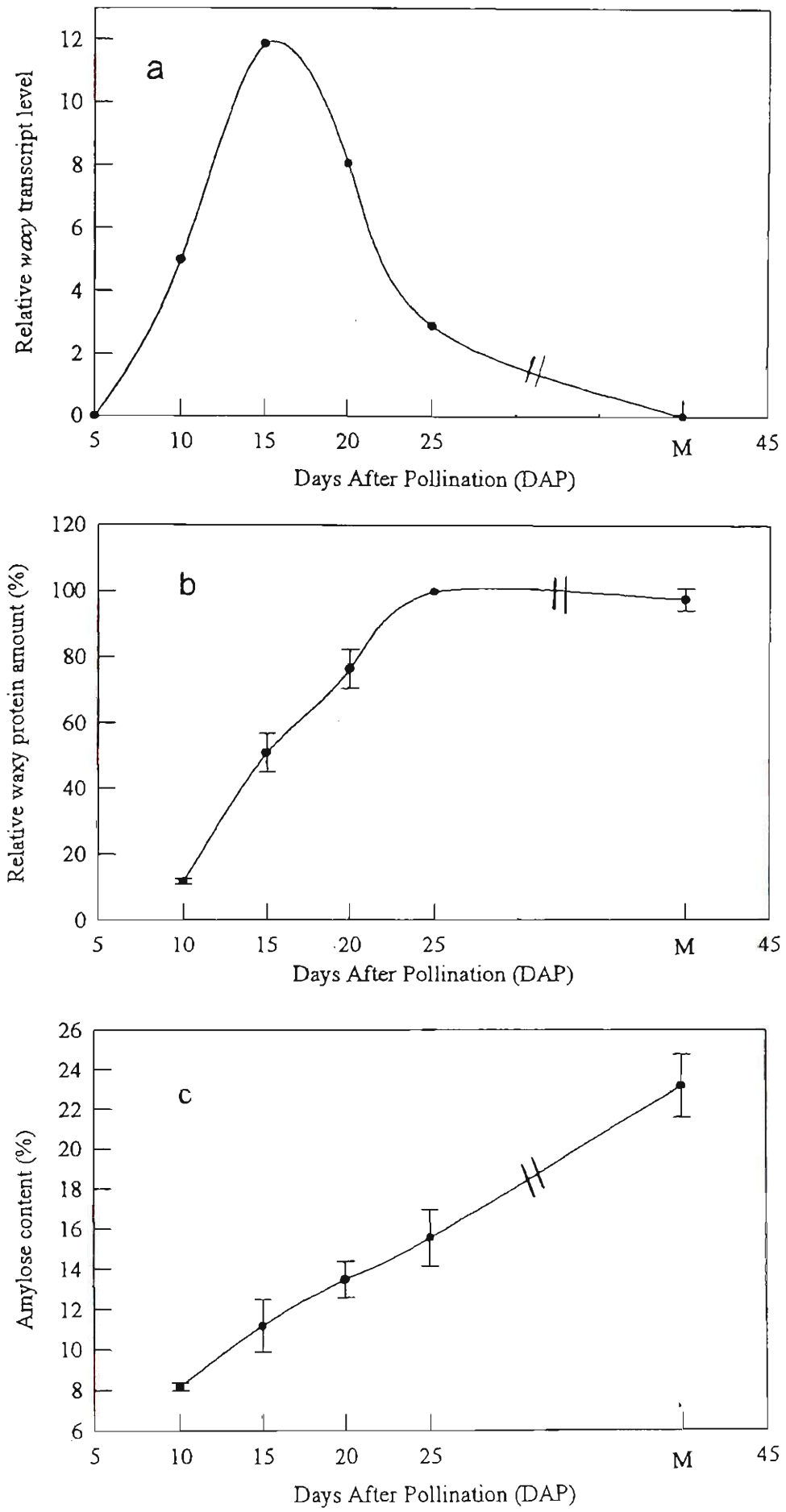


Figure 7.7 a) Relative waxy transcript level b) Relative waxy protein amount and c) Amylose content, during seed development in Chinese Spring. M = mature seeds

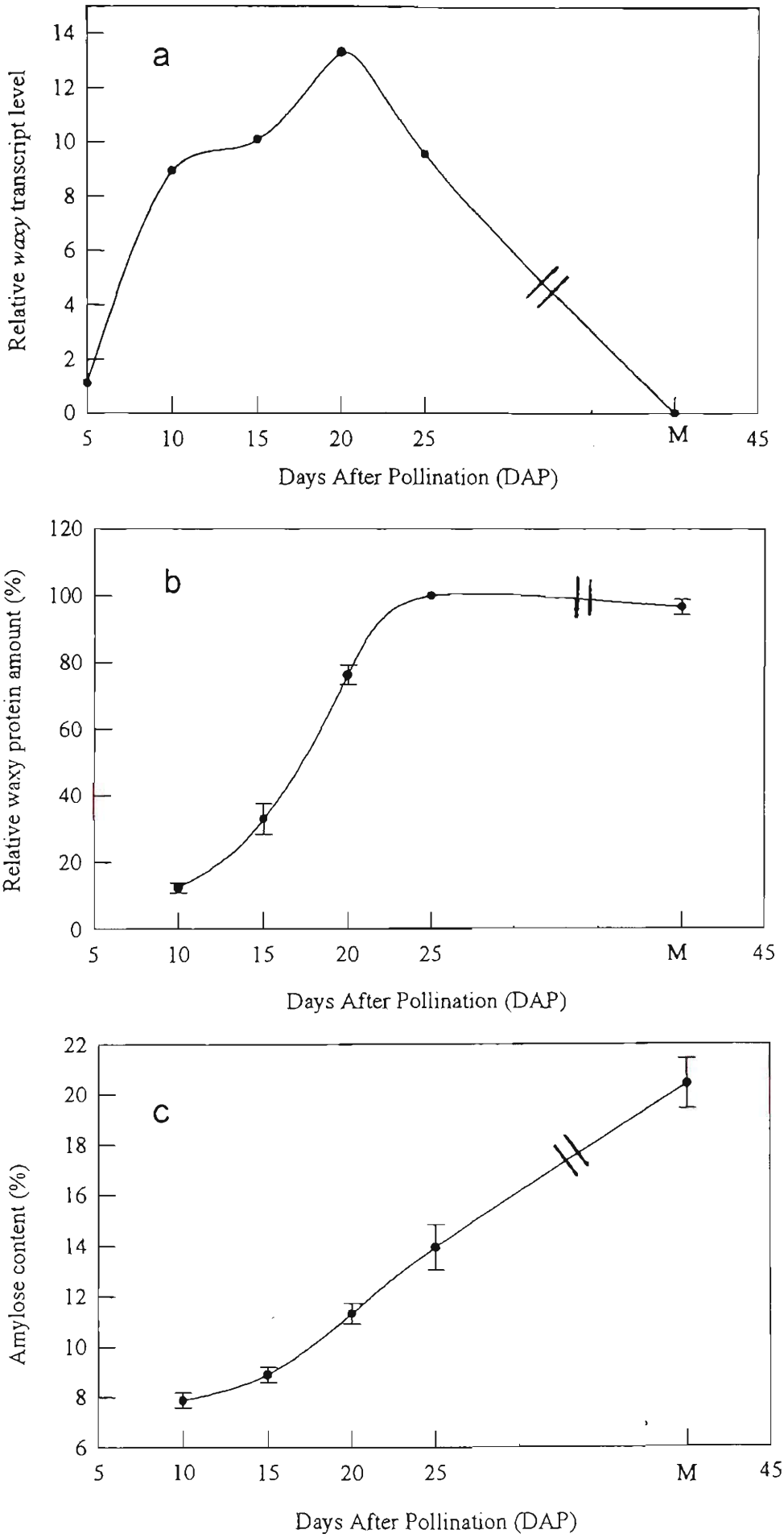


Figure 7.8 a) Relative waxy transcript level b) Relative waxy protein amount and c) Amylose content, during seed development in Rosella. M = mature seeds

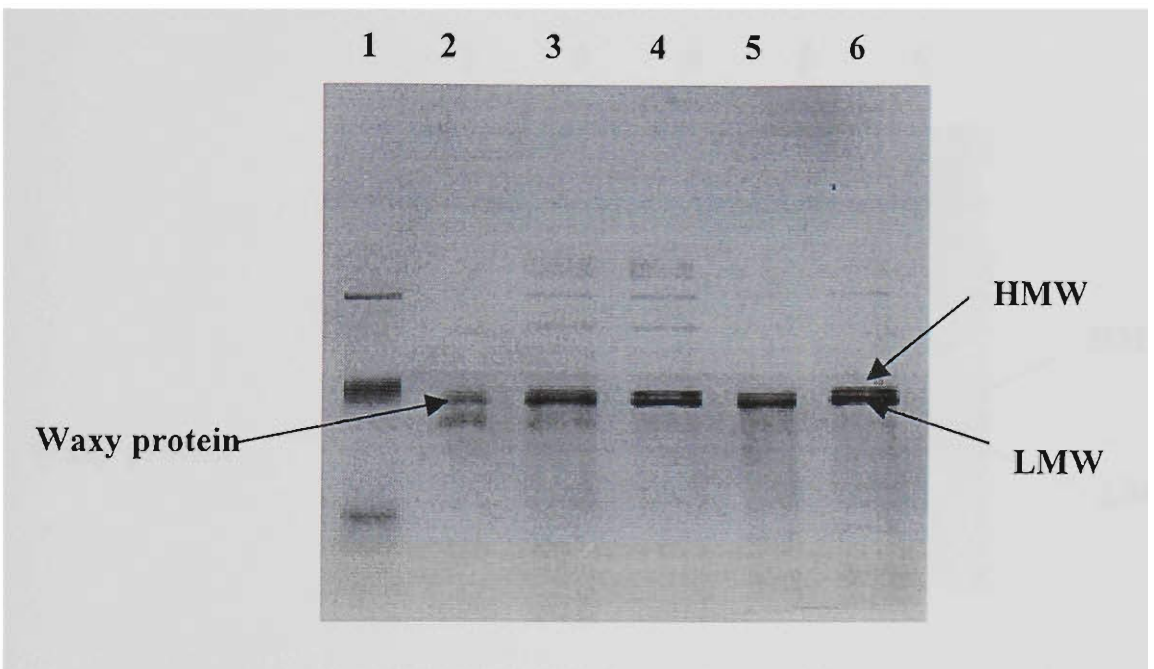


Figure 7.9 Modified SDS-Polyacrylamide gel electrophoresis (30:0.135 Acrylamide / Bis) of protein extracts from endosperms of developing seeds of Chinese Spring. Lane 1: Low range protein marker, Lanes 2 to 6: 5, 10, 15, 20 and 25 dap seeds.

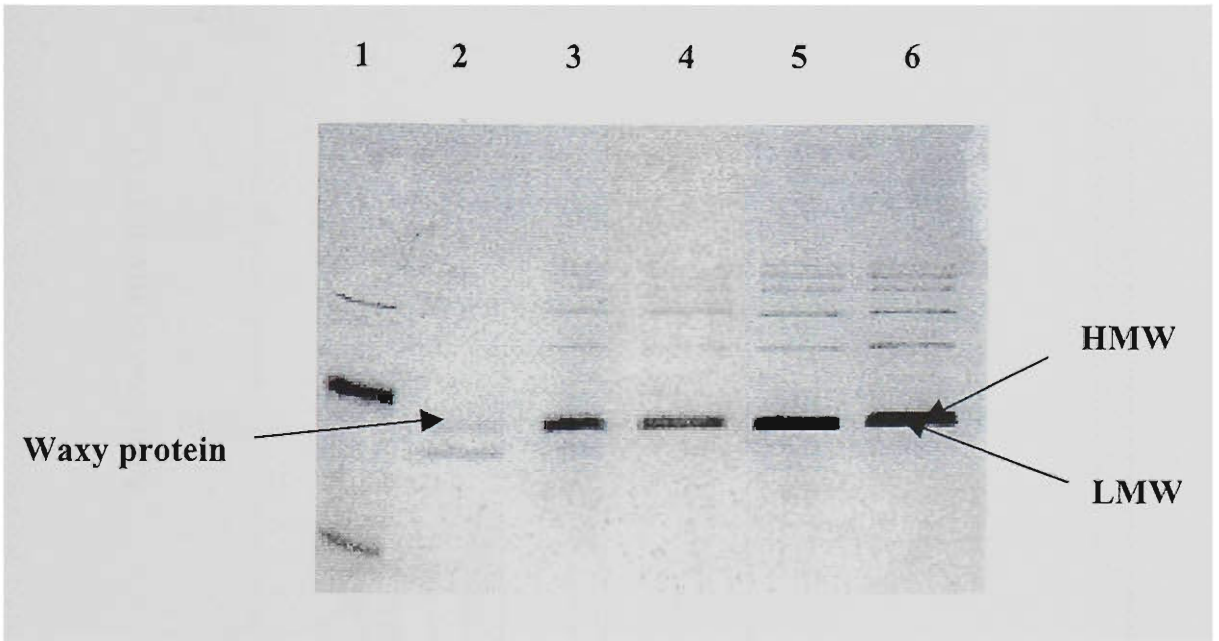


Figure 7.10 Modified SDS-Polyacrylamide gel electrophoresis (30:0.135 Acrylamide / Bis) of protein extracts from endosperms of developing seeds of Rosella. Lane 1: Low range protein marker, Lanes 2 to 6: 5, 10, 15, 20 and 25 dap mature seeds.

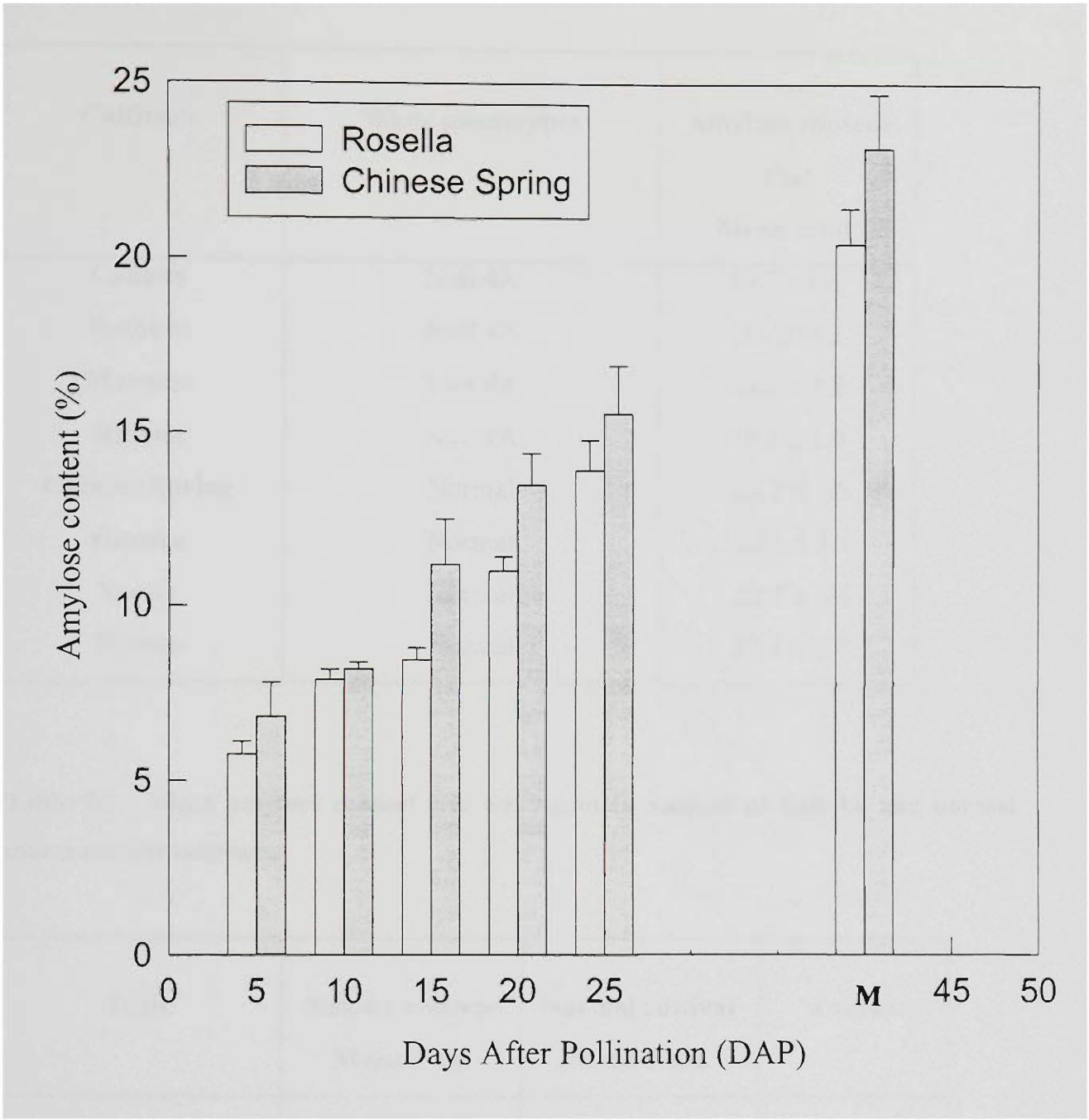


Figure 7.11 Amylose contents in the endosperms of Chinese Spring (normal) and Rosella (null 4A) during seed development. M = mature seeds

Table 7.1 Amylose contents of null 4A and normal mature wheat cultivars.

Cultivars	Waxy phenotypes	Amylose content (%) Mean ± s.d.
Cadoux	Null 4A	19.7 ± 0.9
Halberd	Null 4A	20.1± 0.3
Machete	Null 4A	19.5 ± 1.2
Rosella	Null 4A	20.4 ± 1.0
Chinese Spring	Normal	23.2 ± 1.6
Goroke	Normal	22.5 ± 1.1
Vectis	Normal	22.5 ± 0.5
Wyuna	Normal	22.2 ± 0.7

Table 7.2 Mean amylose content and waxy protein amount of null 4A and normal mature wheat cultivars.

Trait	Null 4A cultivar Mean ± s.d	Normal cultivar Mean ± s.d.	t value
Amylose content (%)	19.9 ± 0.4	22.6 ± 0.4	-9.6*
Relative amount of waxy protein (%)	62.7 ± 2.6	93.0 ± 8.1	-7.1*
No. of cultivars	4	4	

- $P \leq 0.01$

Table 7.3 Amylose content and relative waxy protein amount during seed development in Chinese Spring.

Age of seeds (DAP)	Amylose content (%) Mean ± s.d.	Relative amount of waxy protein (%) Mean ± s.d.
10	8.2 ± 0.2	11.8 ± 0.8
15	11.2 ± 1.3	51.1 ± 4.1
20	13.5 ± 0.9	76.8 ± 5.4
25	15.5 ± 1.4	100

Table 7.4 Amylose content and relative waxy protein amount during seed development in Rosella.

Age of seeds (DAP)	Amylose content (%) Mean ± s.d.	Relative amount of waxy protein (%) Mean ± s.d.
10	7.9 ± 0.3	12.5 ± 1.5
15	8.9 ± 0.3	32.9 ± 4.6
20	11.3 ± 0.4	76.2 ± 2.5
25	13.9 ± 0.9	100

CHAPTER 8

Conclusions and Recommendations

Agarose gel electrophoresis method development

No previous attempts to separate the polysaccharide components of starch by agarose gel electrophoresis have been reported in the literature. The experiments described in Chapter 3 have investigated the development of an agarose gel electrophoresis method for qualitative and quantitative determination of the major polysaccharide components of wheat starch. At the commencement of this study, the conditions of electrophoresis such as the buffer system, loading and running the gel, and method of staining were unknown.

The experimental approach was based on agarose gels because this eliminates the toxic hazards associated with the use of acrylamide. Preliminary experiments revealed that the amylose and amylopectin components of wheat starch could be separated by electrophoresis on agarose gel using a Tris-borate buffer system, and visualised by 0.2% I_2 in 2% KI solution. The blue colour of the amylose-polyiodide complex developed rapidly in the agarose gel after the addition of the iodine stain. Staining was completed in 1 minute. Since agarose is also a form of carbohydrate, it also stained in iodine and developed a dark brown background obscuring the blue amylose band. However, the background cleared rapidly upon de-staining the gel by rinsing with distilled water. It was found that the iodine stain was not permanent since the blue amylose and reddish brown amylopectin zones faded after 2-3 hours. Hence in the quantitative determination of amylose, laser densitometer scanning of the amylose band was performed immediately after de-staining the gel. This newly developed iodine staining is a simple and rapid method to detect amylose and amylopectin in agarose gels compared to the other staining methods of carbohydrate, Fuchsin red and Alcian blue, which were not useful because of difficulties in de-staining the gel.

The basic requirement for the application of electrophoretic techniques is that the components to be separated possess electrical charges. The results of this study indicate that the movement of the polysaccharide components toward the cathode is not due to acquisition of a charge but due to the flow of the borate buffer. Since the

larger size native amylopectin component remained at the origin and the smaller size amylose component migrated towards the cathode, the separation of the starch components was a result of filtration of the molecules based on differences in their molecular weights. Low voltage and longer time were preferred to high voltage and shorter time for electrophoresis, because under the latter conditions the buffer and gel were heated and could affect the results. Future work should investigate the use of iodine containing electrophoresis buffers, which could impart charge and favour anodic migration of the polysaccharide components of starch.

In quantitative analysis, the high linear correlation obtained between % amylose (from potato) and its peak area determined by laser densitometer indicated that this method could be used to quantitate amylose. Since all of the amylose should be extracted without significant degradation, two commonly used solvents, KOH and DMSO, were evaluated to solubilise wheat starch for amylose determination. Although DMSO is widely considered to be a better solvent than KOH, in the present study, wheat starch solubilised in KOH gave higher densitometer readings. Further, the probable saponification of lipids by KOH would significantly alter the impact of lipids on amylose determination. Hence, KOH was selected as the solvent and the optimum solubilisation condition of wheat starch was found to be 0.5 M KOH at 80°C for 15 minutes. Severe alkali treatment of wheat starch resulted in degradation of amylose and amylopectin, which was visible in the gel and was revealed in the laser densitometer response.

The presence of "intermediate material" in starch remains controversial. Several researchers have reported the "intermediate material" as a distinct component of starch having a molecular weight between amylose and amylopectin. However, others have reported the presence of "intermediate material" as a result of hydrolysis of amylopectin due to harsh treatment of starch to effect dissolution. The results of the present study support the explanation of the latter group of researchers since during alkali solubilisation of the wheat starch the "intermediate material" was absent when less severe heat treatment was employed, however, it appeared when the temperature and time of solubilisation was increased. DMSO solubilised wheat starch did not show the presence of "intermediate materials". It has been reported in the past that adding 5-15% water to the DMSO greatly enhances the rate of dissolution of starch.

Hence, the effect of adding water to DMSO on starch solubilisation could be further investigated for agarose gel electrophoresis method. Also an investigation on the "intermediate material" should be carried out with DMSO based solvent.

Wheat amylose standard

The accuracy of the newly developed agarose gel electrophoresis method relies on the use of an amylose standard. For amylose determination methods based on iodine complexation, it is appropriate to have a standard from the starch being studied. Chapter 4 describes the smallscale preparations of wheat amylose standards. It was interesting to find the reddish brown amylopectin contamination in the agarose gel when butanol precipitated wheat amylose was subjected to electrophoresis. This result is in agreement with previous reports where amylopectin contamination was observed in butanol precipitated starches. The con A procedure developed by previous researchers to estimate amylose content in starches was employed in this study with slight modification, to prepare amylopectin-free wheat amylose. Earlier studies on the interaction between con A and carbohydrates emphasised the effects upon the extent of precipitant formation on the relative concentrations of con A and polysaccharide and the time allowed for the precipitation to occur. In the present study, it was found that the optimum ratio of concentration (on a weight basis) of con A to solubilised starch for the precipitation reaction was 2 and the optimum amylopectin - con A precipitation time was 2 hours. These results are in agreement with previous reports. In this study, the amylose from the supernatant was recovered by two methods, butanol precipitation and ultra-filtration. Although these amylose samples were free of amylopectin, they were of low purity. The samples apparently contained non-starch impurities and were less than 50% amylose, based on glucose recovery from acid hydrolysed samples. The time and expense of isolating wheat amylose standards and their low purity outweighed shortcomings associated with the use of potato amylose. Therefore in this study, potato amylose was used as the standard for application of the agarose gel electrophoresis method.

Application of the new method

Agarose gel electrophoresis allowed the determination of amylose content in a range of wheat starches isolated from Australian wheat cultivars. The results are shown in Chapter 5. The amylose contents of the wheat starches were within the range reported previously for starches from Australian wheats and demonstrated a positive but a weak correlation with the con A and iodine colorimetry data on these starches. There are a number of factors that can influence the accuracy of this method. These include the concentration of the iodine solution and variations in loading of the sample on the gel. These variables emphasise the need for replication if reliable data are to be obtained using this method. For future work it is suggested that this newly developed method should be further refined to improve reproducibility and reliability and then be extended to other cereal starches.

Molecular studies

Molecular studies of *waxy* genes in hexaploid Australian wheat cultivars have provided information, relating to the nature of mutation in 4A *waxy* gene of null 4A cultivars. The investigation led to two important observations. Firstly, it was discovered that the intron 1 of *waxy* genes (atleast the 7A and 7D *waxy* genes) in the null 4A and normal cultivars is longer than that of barley intron 1 by 100 bp. Secondly, it was found that the region of 4A *waxy* gene that includes (atleast) the intron 1 is deleted in null 4A cultivars. It is possible that this deletion is responsible for the complete lack of transcription of this gene, indicated by the northern blots. This may be explained by the hypothesis that the mutation extends further to the 5' region of the gene and includes the untranslated exon 1 and perhaps the promoter of the gene. Similarly it could also extend further to the 3' region of the gene and include other introns and exons. However, this hypothesis could not be investigated in the present study due to the following reasons: 1) absence of gene-specific promoter sequences for the 7A, 4A and 7D genes, which could be used as probes for Southern hybridisation, 2) lack of size variation in the section 3' to exon 2, between the three *waxy* genes, made it difficult to analyse whether or not the PCR products came from one, two or three genes. However, new data has been obtained in our lab (L.Yan and M.Bhave, personal communication) that shows some polymorphic restriction sites in *waxy* gene. Further molecular work must, therefore, be conducted to determine the extent of deletion of the null 4A *waxy* gene in these waxy wheat varieties. The

question remains whether all null 4A variation have similar mutation and this needs to be investigated further.

It is known that development of a DNA based assay would allow a rapid identification of the waxy genotypes and aid in wheat breeding programs and for seed suppliers. The intron 1 deletion discussed above, in the null 4A wheat cultivars, could thus be used as a molecular marker linked to GBSS 4A *waxy* gene and to identify potentially "good" noodle wheats, early in the breeding cycle.

Waxy protein and amylose content

No previous studies have reported a relationship between waxy protein amount and amylose content, during seed development in wheat. In Chapter 7, a direct relationship was established between production of waxy protein and amylose synthesis in the endosperms of null 4A and normal wheat cultivars during seed development, until 25 dap. However, from these results it was difficult to assess whether the increase in the amount of waxy protein during seed development contributed to the increase in the amylose content, because the enzymatic activity of waxy protein at a given time was not known. Hence further research should be conducted to determine the correlation of enzyme activity of the granule bound waxy protein with amylose content during seed development. This would provide a more accurate assessment of the association between waxy protein and amylose content in seeds during development. It was found that the relative waxy protein amount of mature seeds were approximately the same as that of seeds harvested at 25 dap, in both the cultivars. Further, it was shown that the amylose content of mature seeds was much higher compared to that of 25 dap seeds. Thus, it appears from these results that the waxy protein produced at 25 dap may be responsible for the continued synthesis of amylose until seed maturity, in both the cultivars. However, as mentioned before, a future study on the enzymatic activity of the waxy protein would provide detailed and accurate information on the relationship between waxy protein and amylose throughout seed development.

Preliminary studies of *waxy* gene expression during seed development indicated a positive correlation between *waxy* transcript level and waxy protein amount during the period the *waxy* gene was actively expressed, in both the cultivars. Beyond the period

of maximum expression the *waxy* transcript level started to decline, however, the waxy protein amount increased up to 25 dap and appeared to remain constant thereafter until seed maturity. These data suggest that the abundant transcripts produced at the period of peak expression, are required for the continued synthesis of waxy protein until atleast 25 dap..

In the null 4A cultivars, a clear decrease in one of the proteins that contributes to the LMW band was observed at all stages of seed development. This reduction is most likely caused by the lack of Wx-B1 protein, as it has been reported for mature seeds by Yamamori *et al.* (1994), and this correlated with a decrease in the amylose content throughout the development of seeds in the null 4A cultivars. These observations considered together suggest strongly that the reduced amylose content in the mature seeds of null 4A cultivars is due to lack of expression of Wx-B1 protein and not due to degradation of this protein, during seed development.

In the first part of this study, an agarose gel electrophoresis method was developed to quantitate amylose in Australian wheat cultivars. The method provided a useful approach for rapid separation and detection of the principal starch components. Although further refinement of this technique is required to improve reproducibility and reliability, this seems to be a promising new technique for determination of amylose in wheat starch. In the second part of this study, the nature of mutation in the 4A *waxy* gene of null 4A Australian wheat cultivars was partially identified as a deletion of intron 1, which may be responsible for the inactivation of the gene in these cultivars. The hypothesis that, the mutation extends further and includes the untranslated exon 1 and the promoter region of the gene, should be further investigated in future studies.

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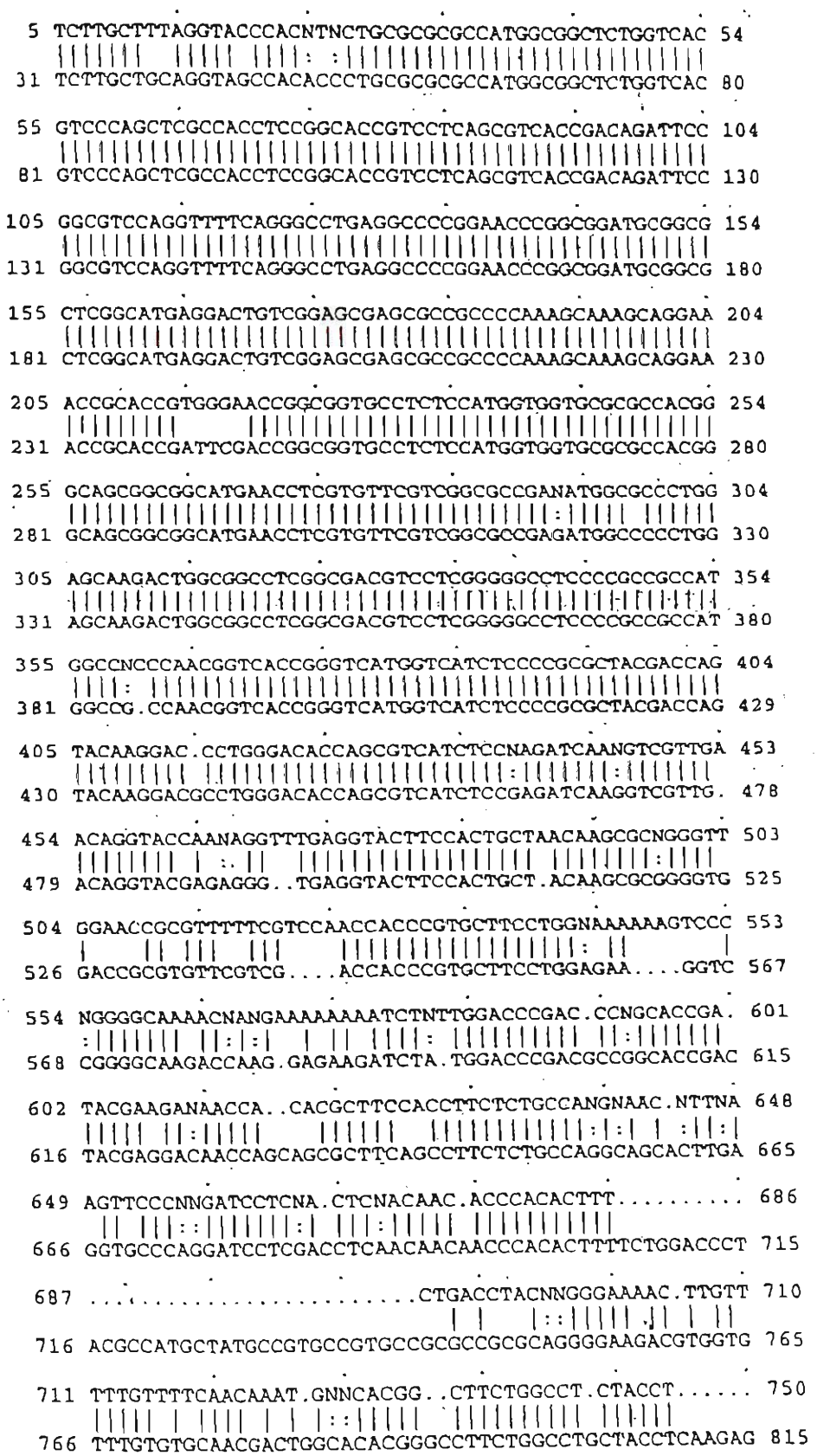
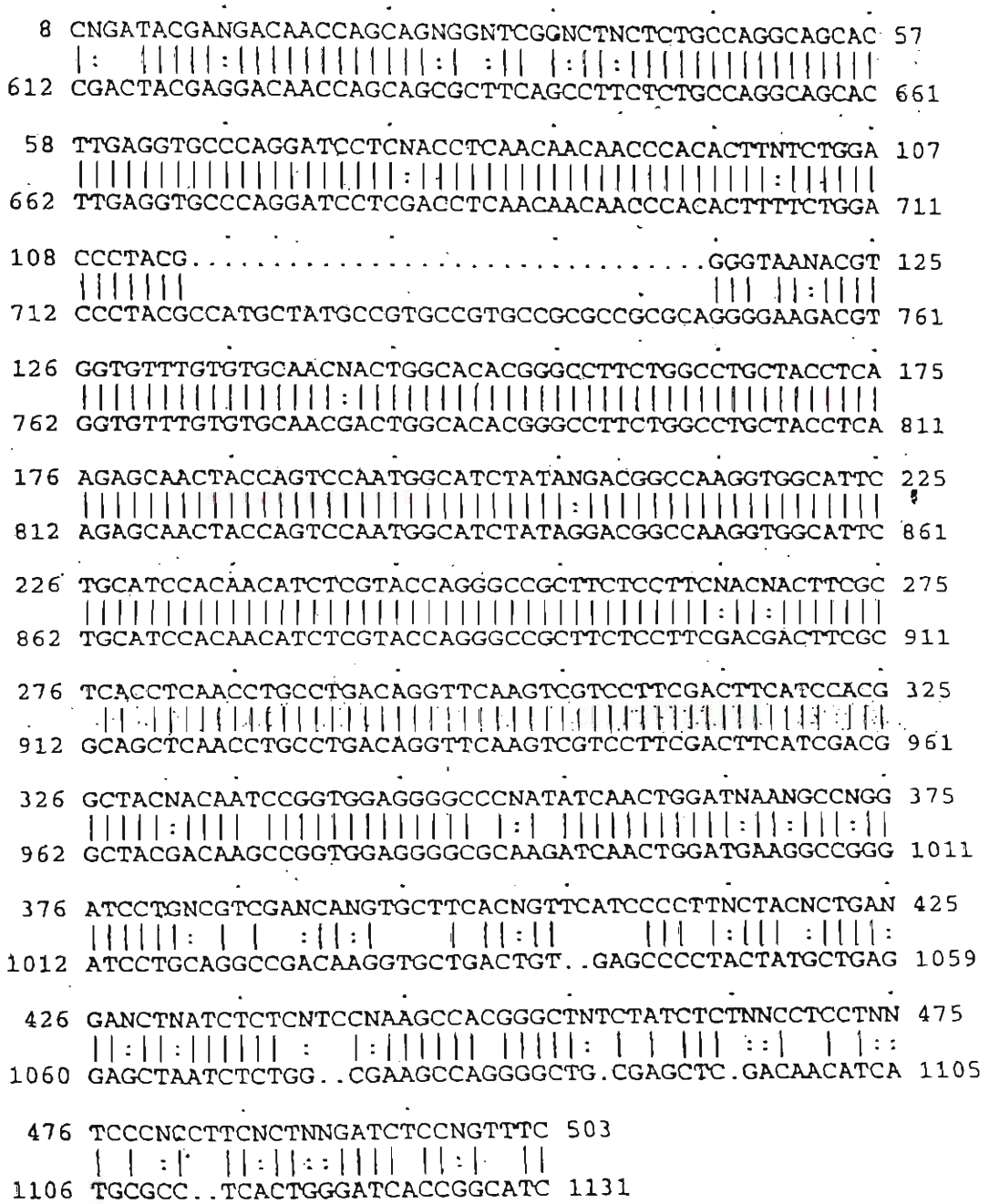
APPENDIX 1



Partial sequence alignment of the 840 bp *waxy* cDNA probe (upper sequence) with the published sequence of wheat cDNA (lower sequence).

APPENDIX 2



Partial sequence alignment of the 1244 bp *waxy* cDNA probe (upper sequence) with the published sequence of wheat cDNA (lower sequence).

APPENDIX 3

Sources of chemicals and reagents

Chemical / Reagent	Source
Acetic acid	BDH
Acrylamide	ICN
Alcian blue	Sigma
Ammonium persulfate	Biorad
Bis, N', N Methylene bis acrylamide	Biorad
Boric acid	Sigma
Bovine serum albumin	Sigma
Bromophenol blue	BDH
Chloroform	BDH
Concanavalin A	Sigma
Diethyl Pyrocarbonate	Sigma
Dimethyl formamide	BDH
Disodium hydrogen orthophosphate	BDH
DNA grade agarose	Progen
Ethidium bromide	Sigma
EDTA (di-sodium salt)	Sigma
Fuchsin red	Sigma
Glucose	BDH
Glycerol	BDH (GPR)
Hexane	BDH
Hydrochloric acid	BDH
Iodine (Re-sublimed)	Mallinckodt
Isopropanol	Sigma
Isoamyl alcohol	BDH
2-Mercaptoethanol	Sigma
Methylated spirit	BDH
Periodic acid	Sigma

Phenol	Sigma
Polyvinyl pyrrolidone (Molecular Biology Reagent)	Sigma
Potassium acetate	Sigma
Potassium hydroxide	BDH
Potassium iodide	BDH
Potassium meta-bisulphide	BDH
RNA-ase (DNA-ase free)	Sigma
SDS	Pierce
Sodium chloride	BDH
Sodium dihydrogen orthophosphate	BDH
Sodium thiosulphate pentahydrate	BDH
Sucrose	BDH
TEMED	Biorad
Trichloro acetic acid	Sigma
Tris base	Sigma
Xylene Cyanol FF	Kodak
Trizol	Gibco-BRL

APPENDIX 4

Solutions and Buffers

Acrylamide/bis acrylamide solution (30% T, 2.67% C)

Acrylamide (29.2 g) and N'N'-bis-methylene-acrylamide (0.8 g) were made up to 100 mL with dH_2O. The solution was stored at 4°C in an amber bottle.

Acrylamide/bis solution (30% T, 0.45% C)

Acrylamide (29.865 g) and N'N'-bis-methylene-acrylamide (0.135 g) were made up to 100 mL with dH_2O. The solution was stored at 4°C in an amber bottle.

Denhardt's reagent X 50

An amount of 5 g of Ficoll (Type 400, Pharmacia), 5 g of polyvinylpyrrolidone and 5 g of bovine serum albumin was dissolved and made up to 500 mL with DEPC water, filter sterilised (0.2 μm filter) and stored at -20°C.

DEPC water

Deionised water containing 0.1% of DEPC was shaken vigorously, incubated overnight at 37°C and autoclaved for 30 minutes.

DNA extraction buffer

The buffer constituted of Tris (pH 8.0) 100 mM, EDTA (pH 8.0) 50 mM, NaCl 100 mM, SDS 1% and 10 mM –mercaptoethanol added fresh. The solution was autoclaved.

EDTA, 0.5 M (pH 8.0)

Disodium ethylenediaminetetra-acetate.$2H_20$ (186.1 g) was dissolved in 800 mL dH_2O by continuous stirring and the pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 1 litre with dH_2O and autoclaved.

Electrode buffer (Running buffer) X 5, (pH 8.3)

Tris base (9 g), glycine (43.2 g) and SDS (3 g) to 600 mL with dH_2O. The solution was stored at 4°C. The working solution is 1 X buffer.

Ethidium Bromide (10 mg/mL)

Ethidium bromide (10 mg/mL) in autoclaved dH_2O was stirred for several hours to ensure that the dye has dissolved. The solution was stored in an amber bottle at 4°C.

Formaldehyde gel-loading buffer

The buffer constituted of 50% autoclaved glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophenol blue and 0.25% xylene cyanol FF.

Gel loading buffer (for starch electrophorsis)

Bromophenol blue (0.25 %) was prepared in 40% (w/v) sucrose in water and stored at 4°C.

MOPS X 10

The buffer constituted of 200 mM of 3-[N-Mopholino] propane sulfonic acid, 80 mM sodium acetate and 10 mM EDTA (The pH was adjusted to 7 with 2N NaOH). Following 0.2 µm filter sterilisation, the stock buffer was stored in an amber bottle at room temperature.

Phenol-chloroform-isoamyl alcohol (25:24:1; v / v)

Liquified phenol (melted at 65°C), to which 8-hydroxyquinoline was added at a concentration of 0.1% (w/v), was equilibrated by extracting several times with 50 mM Tris base and 50 mM Tris-Cl (pH 8) until the pH was 8. One part of this phenol was mixed with one part of chloroform-isoamyl alcohol (24:1) and stored under 50 mM Tris-Cl (pH 8) buffer in an amber glass bottle at 4°C.

Prehybridisation solution

The solution constituted of 2 parts of Denhardt's reagent, 5 mM EDTA, 10 mM Tris-Cl, 0.5 M Na_3PO_4 (pH = 7), 5% SDS and treated salmon sperm DNA solution (100 ng/mL) added fresh.

RNAase (DNAase-free)

Ribonuclease A (from bovine pancreas) was dissolved at a concentration of 10 mg/mL in 0.01 M sodium acetate (pH 5.2) and heated at 100°C for 15 minutes. It was then cooled slowly to room temperature, dispensed into aliquots and stored at -20°C.

RNA marker stain

Methylene blue (0.04% w/v) dissolved in 0.5 M sodium acetate (pH 5.2).

Salmon sperm DNA

Salmon sperm DNA (ssDNA) solution (from Gibco-BRL) was prepared at a concentration of 10 mg/mL by dissolving in autoclaved dH_2O. The required amount for prehybridisation was denatured in a boiling water bath for 10 minutes and chilled for 2-3 minutes.

Sample buffer (for RNA)

The buffer was made up by mixing 250 µL formamide, 83 µL of 37% formaldehyde (pH > 3.5), 50 µL of 10 X MOPS buffer and the volume was adjusted to 400 µL with DEPC water and stored -20°C.

SDS 10%

Sodium dodecyl sulphate (100 g) was dissolved in 900 mL dH_2O by heating it to 68°C and the pH was adjusted to 7.2 with concentrated HCL. The volume was made up to 1 litre.

SDS gel-loading buffer (for protein)

The buffer constituted of 50 mM Tris.Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol. The buffer was stored at –20°C.

Sodium acetate 3 M (pH 5.2)

Sodium acetate.$3H_2O$ (408.1 g) was dissolved in 800 mL dH_2O, the pH was adjusted to 5.2 with glacial acetic acid and the volume made to 1 litre with dH_2O. The solution was autoclaved.

Sodium phosphate 1 M (pH 7)

To prepare 100 mL of the solution, 68.4 mL of 1 M Na_2HPO_4 was added to 31.6 mL of 1 M NaH_2PO_4.

SSC X 20

Sodium chloride (175.3 g) and sodium citrate (88.2 g) were dissolved in 800 mL of dH_2O. The pH was adjusted to 7 with 10 N NaOH and the volume was made up to 1 litre with dH_2O and autoclaved.

Stripping Solution

The solution constituted of dimethylformamide 50%, SDS 1% and Tris-HCL (pH 8.0) 50 mM.

Tris-acetate buffer (TAE) X 10

An amount of 400 mM Tris (pH 7.4), 200 mM sodium acetate and 20 mM EDTA (pH 8.0) were mixed and the pH was adjusted to 7.4 with glacial acetic acid. The volume was made up to 1 litre with dH_2O and the solution was autoclaved. The working solution is 1 X TAE.

Tris-borate buffer 0.1 M (pH 8.3) X 5

Tris base (54 g) and boric acid (27.5 g) were made up to 1 litre. The working solution is 1 X Tris-borate.

Tris-HCl 1 M

Tris base (121.1 g) was dissolved in 800 mL of dH_2O. The pH was adjusted with concentrated HCl as follows when solution was at room temperature:

pH	HCl
7.4	70 mL
7.6	60 mL
8.0	42 mL

The volume was made up to 1 litre and the solution was autoclaved.

Tris-HCl 1.5 M (pH 8.8)

Tris-base (18.15 g) was dissolved in 60 mL dH_2O, the pH was adjusted to 8.8 with 1N HCl and the volume was made to 100 mL with dH_2O. The solution was stored at 4°C.

Tris-HCl 0.5 M (pH 6.8)

Tris base (6 g) was dissolved in ~60 mL dH_2O, the pH was adjusted to 6.8 with 1 N HCl and the volume was made up to 1 litre.

TE Buffer (pH 7.4)

The solution constituted of 10 mM Tris-Cl (pH 7.4) and 1 mM EDTA (pH 8.0). The solution was autoclaved.

X-ray developer

400 mL developer concentrate (AGVA GEVART)

2 litres water

APPENDIX 5

Amylose content of mature wheat cultivars

Cultivar	Amylose content 1 (%)	Amylose content 2 (%)	Amylose content 3 (%)	Amylose content 4 (%)	Mean (%)	Standard Deviation
Cadoux	18.5	20.7	19.8	19.9	19.7	0.9
Halberd	20.3	19.7	20.3	19.9	20.1	0.3
Machete	18.1	19.5	20.1	20.3	19.5	1.2
Rosella	20.7	20.4	21.3	19.3	20.4	1.0
Chinese Spring	21.9	21.8	24.1	24.9	23.2	1.6
Goroke	23.2	23.7	21.9	21.3	22.5	1.1
Vectis	22.8	22.4	23.0	22.0	22.5	0.5
Wyuna	22.1	22.5	21.4	22.8	22.2	0.7

Amylose content in developing seeds of Chinese spring wheat cultivar

Age of seeds (DAP)	Amylose content 1 (%)	Amylose content 2 (%)	Amylose Content 3 (%)	Amylose content 4 (%)	Mean (%)	Standard Deviation
5	8.1	6.9	5.9	6.2	6.8	0.9
10	8.4	7.9	8.1	8.3	8.2	0.2
15	11.1	10.0	10.6	13.1	11.2	1.3
20	13.3	12.4	13.8	14.5	13.5	0.9
25	14.4	14.4	17.0	16.4	15.5	1.4

Amylose content in developing seeds of Rosella wheat cultivar

Age of seeds (DAP)	Amylose content 1 (%)	Amylose content 2 (%)	Amylose Content 3 (%)	Amylose content 4 (%)	Mean (%)	Standard Deviation
5	5.3	6.2	5.7	5.8	5.8	0.4
10	7.9	7.8	7.5	8.2	7.9	0.3
15	8.9	9.1	9.1	8.4	8.9	0.4
20	11.7	10.8	11.1	11.6	11.3	0.4
25	12.9	13.4	14.7	14.6	13.9	0.9

APPENDIX 6

Relative waxy protein amount in mature seeds of wheat cultivars

Cultivar	Waxy protein 1	Waxy protein 2	Waxy protein 3	Mean	Standard Deviation
Cadoux	67.8	57.6	58.2	61.2	5.7
Halberd	66.1	51.5	63.3	60.3	7.7
Machete	67.9	63.4	57.9	63.1	5.0
Rosella	66.5	63.9	68.4	66.3	2.3
Chinese Spring	92.1	95.4	100.7	95.8	3.9
Goroke	100	100	100	100	0.0
Vectis	84.6	94.2	97.1	94.7	7.6
Wyuna	82.7	80.0	81.3	81.3	5.4

Relative waxy protein amounts in developing seeds of Chinese spring cultivar

Age of seeds (DAP)	Waxy protein 1	Waxy protein 2	Waxy protein 3	Waxy protein 4	Mean (%)	Standard Deviation
10	11.9	12.5	10.7	11.9	11.7	0.8
15	51.2	50.3	46.4	56.4	51.1	4.1
20	76.2	71.3	75.3	84.2	76.7	5.4
25	100	100	100	100	100	0.0
Mature	101.7	93.6	98.5	96.9	97.7	3.4

Relative waxy protein amounts in developing seeds of Rosella cultivar

Age of seeds (DAP)	Waxy protein 1	Waxy protein 2	Waxy protein 3	Waxy protein 4	Mean (%)	Standard Deviation
10	10.5	12.4	13.8	13.4	12.5	1.5
15	26.9	38.0	32.9	34.1	32.9	4.6
20	76.2	72.7	78.2	77.7	76.2	2.5
25	100	100	100	100	100	0.0
Mature	95.9	97.2	98.8	95.4	96.8	1.5

APPENDIX 7

Amylose content of wheat starch determined by the colorimetric method before lipid removal

Variety	Amylose 1	Amylose 2	Amylose 3	Amylose 4	Mean
Aroona	18.2	18.6	18.5	19.0	18.6
Bencubbin	16.9	17.4	16.4	18.0	17.2
Cadoux	17.1	17.2	17.0	17.2	17.1
Canna	21.0	21.3	20.5	21.9	21.2
Dagger	18.3	18.1	18.2	18.2	18.2
Dollarbird	17.6	17.9	17.6	19.4	18.1
Eradu	18.9	18.9	18.1	20.2	19.0
Halberd	16.3	15.9	15.5	14.5	15.5
Janz 1	21.9	21.3	21.1	22.3	21.7
Janz 2	22.3	21.9	21.8	20.9	21.8
Janz 3	20.3	19.9	19.8	20.4	20.1
Janz 4	21.5	21.2	21.7	22.0	21.6
Janz 5	20.4	20.5	20.9	19.8	20.4
Janz 6	20.9	21.5	21.1	21.8	21.3
Kulin 1	19.1	19.3	18.8	19.4	19.2
Kulin 2	21.5	20.1	20.4	20.8	20.7
Machete	17.4	16.5	17.7	16.3	16.9
Meering	19.2	19.7	18.8	18.9	19.2
Osprey	19.5	18.7	19.2	19.5	19.2
Oxley	22.3	22.2	21.8	21.1	21.8
Rosella	19.6	20.0	19.6	19.6	19.7
Spear	15.9	15.2	15.4	15.2	15.4
Sunelg	17.9	17.6	17.1	16.3	17.26
Sunstar	21.99	22.25	22.03	21.43	21.3
Wilgoyn	19.4	20.0	19.3	18.1	19.2

APPENDIX 8

Amylose content of wheat starch determined by the colorimetric method after lipid removal

Variety	Amylose 1	Amylose 2	Amylose 3	Amylose 4	Mean
Aroona	18.9	21.6	19.8	20.8	20.3
Bencubbin	18.8	18.8	19.4	18.2	18.8
Cadoux	17.7	20.7	19.8	18.6	19.2
Canna	22.3	22.6	23.2	23.3	22.8
Dagger	21.2	20.9	21.6	20.5	21.1
Dollarbird	21.8	21.2	21.9	21.1	21.5
Eradu	20.6	20.2	20.1	20.8	20.4
Halberd	17.4	17.7	15.9	18.1	17.3
Janz 1	23.8	23.3	22.9	22.6	23.2
Janz 2	25.4	24.3	24.3	23.2	24.3
Janz 3	22.5	22.3	22.0	23.4	22.6
Janz 4	24.8	22.3	23.3	23.8	23.6
Janz 5	23.2	21.6	22.2	22.6	22.4
Janz 6	22.0	22.7	22.0	24.3	22.8
Kulin 1	21.2	22.9	21.7	22.4	22.1
Kulin 2	23.3	23.4	23.4	23.6	23.4
Machete	17.9	18.3	18.2	18.8	18.3
Meering	21.3	22.1	21.7	21.6	21.7
Osprey	21.3	21.8	21.9	21.3	21.6
Oxley	24.6	23.9	24.1	24.3	24.2
Rosella	20.9	19.5	20.5	22.8	20.9
Spear	18.4	18.0	18.4	18.5	18.3
Sunelg	19.3	18.4	19.1	19.6	19.1
Sunstar	24.2	22.7	23.2	23.8	23.5
Wilgoyn	21.2	21.1	20.9	19.9	20.8

APPENDIX 9

Amylose content of wheat starch determined by the con A method

Variety	Amylose 1	Amylose 2	Mean
Aroona	20.4	22.4	21.4
Bencubbin	21.9	22.5	22.2
Cadoux	22.7	20.6	21.7
Canna	24.2	24.8	24.5
Dagger	24.2	24.0	24.1
Dollarbird	22.7	22.3	22.5
Eradu	21.9	23.4	22.7
Halberd	21.7	22.0	21.9
Janz 1	26.7	25.2	25.9
Janz 2	25.3	26.3	25.8
Janz 3	23.3	24.0	23.7
Janz 4	24.7	25.4	25.1
Janz 5	24.9	25.3	25.1
Janz 6	26.2	24.5	25.3
Kulin 1	25.5	25.3	25.4
Kulin 2	23.7	25.3	24.5
Machete	19.9	20.6	20.3
Meering	24.5	23.5	23.9
Osprey	22.6	23.4	23.0
Oxley	26.1	25.1	25.6
Rosella	20.1	22.7	21.4
Spear	22.2	23.8	22.9
Sunelg	20.9	21.4	21.2
Sunstar	23.3	25.4	24.3
Wilgoyn	21.9	23.0	22.5

APPENDIX 10

Amylose content of wheat starch determined by the agarose gel electrophoresis method

Variety	Amylose 1	Amylose 2	Amylose 3	Amylose 4	Mean
Aroona	18.5	22.5	22.0	23.5	21.7
Bencubbin	24.0	17.3	24.5	19.3	21.3
Cadoux	21.1	22.5	24.7	20.9	22.3
Canna	27.3	28.8	23.9	23.9	25.9
Dagger	23.10	26.9	25.5	22.5	24.5
Dollarbird	22.5	20.7	22.6	20.7	21.6
Eradu	19.0	21.4	25.6	20.7	21.7
Halberd	22.0	19.4	19.5	20.9	20.5
Janz 1	23.8	25.5	25.4	23.5	24.6
Janz 2	28.8	26.8	29.0	26.2	27.7
Janz 3	25.2	23.6	28.3	25.2	25.6
Janz 4	26.0	23.6	26.0	29.8	26.4
Janz 5	24.8	23.9	27.5	23.2/29.9	25.9
Janz 6	25.2	26.2	29.1	31.4	28.0
Kulin 1	27.5	23.0	25.1	23.9	24.9
Kulin 2	23.5	23.5	16.7	23.4	21.8
Machete	24.3	22.2	22.3	21.7	22.6
Meering	25.0	23.1	28.5	27.9	26.1
Osprey	23.7	25.1	28.5	26.4	25.9
Oxley	27.9	26.2	28.5	28.4	27.8
Rosella	23.1	21.6	22.4	24.7	22.9
Spear	20.3	20.6	23.3	24.2	22.1
Sunelg	15.5	16.7	26.6	25.7	21.1
Sunstar	25.9	16.8	16.9	19.9	19.9
Wilgoyn	25.7	25.6	20.9	22.3	23.7