PROCESSING INDUCED CONFORMATIONAL CHANGES OF FOOD PROTEINS IN RELATION TO ANTIGENICITY

A thesis submitted for the degree of

Doctor of Philosophy

by

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Dedicated to my beloved parents whose enormous contribution enabled me to pass long study journey in my life

Abstract

Food allergy is one of the major public health concerns worldwide and its prevalence has been increasing at an alarming rate over the time especially in the industrialised countries. Most of the foods are processed into a variety of products before consumption and undergo different physico-chemical alterations, which could affect their antigenic potential. β-lactoglobulin, the major cow milk allergen, and gluten, one of the main plant allergens, are also exposed to various processing steps prior to packaging, distribution and consumption. Thus, the present research has focused on effects of different applied processing conditions (pH, temperature and shear) on the conformational changes of β-lg and gluten in relation to their antigenicity.

β-Lg was treated at different pH (3, 5 and 7), temperature (80, 100 and 120ºC) and shear rate (100, 500 and 1000 s^{-1}) and its physico-chemical structure including surface hydrophobicity (Ho), thiol (SH) content, secondary structure and gel electrophoresis pattern were studied. Heating the solution of β-lg above 80ºC at pH 5 and 7 influenced participation of exposed reactive thiol groups in thiol-thiol oxidation and/or thiol-disulfide exchange reaction contributing to heat induced protein aggregation with concomitant reduced hydrophobicity, which resulted in masking and/or destroying of conformational epitopes leading to reduced antigenicity. However, the rate of reduction of antigenicity was greater was higher at pH 5 than pH 7 at a given temperature. In contrast, heating at low pH (pH 3) caused unfolding of protein molecule with incresead hydrophobicity, fragmentation of molecule by partial acid hydrolysis and thereby exposure of β-strands that might have contributed to appearance of some hidden epitopes resulting in higher antigenicity. Shear alone did not exert any effect on the antigenicity of β-lg but could have an influence in combination with pH and

temperature. Overall, heating β-lg solutions to 120°C at pH 5 with high shearing (1000 s^{-1}) resulted in a significantly lower antigenicity compared to low shear rate (100 s^{-1}) .

Thereafter, treatments showing higher (pH 3, room temperature and 0 s^{-1}; pH 3, 120°C and 1000 s^{-1}) and lower (pH 5, room temperature and 0 s^{-1}; pH 5, 120°C and 1000 s^{-1}; pH 7, room temperature and 0 s^{-1} and pH 7, 120°C and 1000 s^{-1}) antigenicity were selected for a further study to investigate the consequence in digestion and post digestion antigenicity. Irrespective of pH, unheated β-lg showed fairly high resistance to peptic digestion presenting high antigenic value while it was fairly susceptible to pancreatin with moderate reduction of antigenic potential. Treatment at high temperature (120°C) significantly improved both peptic and pancreatic digestion of β-lg, which was attributed to structural alterations and exposure of cleavage sites resulting in much lower antigenicity compared to the unheated samples. However, digestive patterns and levels of antigenic reduction of heated β-lg were greatly influenced by pH. At pH 7 and 3, β-lg was completely hydrolysed into smaller fractions some of which might still possess sequential epitopes showing residual antigenicity. In contrast, at pH 5 and 120°C, the antigenicity was lowest although the digestibility was not as high as it was at pH 3 or 7. Application of a high shear rate (1000 s^{-1}) slightly reduced susceptibility of β-lg to digestion and thereby enhanced antigenicity at pH 5 and 7 in the absence of heat treatment, whereas at 120°C, the effect was oposite for all pH.

Processing induced structural changes and related antigenicity of gluten was also investigated. Effects of pH (3, 5, 7) temperature (80, 90,100°C) and shear (500, 1000, 1500 s^{-1}) on the conformational changes (surface hydrophobicity, FTIR, SDS-PAGE and thiol content) of gluten and consequent change in antigenicity were studied. Overall, at pH 3, up to 90°C, acidic deamidation and possible burial of some antigenic hydrophobic residues resulted in minimum antigenicity. However, heating to 100° C

caused slight increase in antigenicity due to exposure of some hidden epitopes. However, heating gluten solution to 100°C at pH 5 or 7 prompted disulfide bonded cross-linking between gliadin and glutenin causing destruction and/or masking of some epitopes, which resulted in reduced antigenicity. Shear alone had no effect on antigenic response regardless of pH. However, at pH 7, combination of high rate shearing (1000 and 1500 s^{-1}) and 100°C temperature triggered slight increase of antigenicity ($P<0.05$) in comparison to low shear (500 s^{-1}) or without shear possibly due to exposure of hidden epitopes resulting in increased antigenicity($P<0.05$).

Furthermore, treatments (pH 3, room temperature, 0 s^{-1}; pH 3, 100°C 0 s^{-1}; pH 7, room temperature, 0 s^{-1}; pH 7, 100°C, 0 s^{-1}; and pH 7, 100°C, 1500 s^{-1}) having significant influence on antigenicity were selected for further study to investigate their digestibility and consequent impact on antigenicity. Lower antigenic reaction expressed by hydrolysate of gluten treated at pH 3 and room temperature was ascribed to its acidic deamidation and improved digestibility while heating to 100°C resulting in slightly increased values. In contrast, hydrolysate of gluten at pH 7 and room temperature exhibited highest antigenicity, attributed to partial resistance of α/β-gliadin to digestion and appearance of some new potential antigenic polypeptides. However, heating at 100°C caused heat induced protein aggregation, consequently lowered digestibility and less availability of antigenic components resulting in minimum (60% reduction) antigenicity. Overall, shear had no effect on digestibility and antigenicity irrespective of pH and temperature.

However, effects of processing on food protein antigenicity cannot be easily generalized, explained or predicted; expressed antigenicity appeared to depend on inherent molecular characteristics of a protein and also a type and extent of a treatment. In case of β-lg, combination of high temperature (120°C), isoelectric pH (pH 5) and

high rate shear (1000 s^{-1}) induced covalent protein aggregation resulting in modification of epitopes, enhanced digestibility and lowest post digestion antigenicity. For gluten, heating at 100°C and neutral pH prompted structural alterations related to lower digestibility and less availability of antigenic components leading to lowest post digestion antigenicity.

Certificate

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This is to certify that the thesis entitled "**PROCESSING INDUCED CONFORMATIONAL CHANGES OF FOOD PROTEINS IN REALATION TO ANTIGENIICTY**" submitted by Md. Toheder Rahaman in partial fulfilment of the requirement for the award of the Doctor of Philosophy with specialisation in Food Sciences and Technology at Victoria University is a record of the bonafide research work carried out by him under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title

Prof. Todor Vasiljevic

Date: 06.09.2016

II. Declaration

"I, Md. Toheder Rahaman, hereby declare that this thesis entitled "**PROCESSING INDUCED CONFORMATIONAL CHANGES OF FOOD PROTEINS IN REALATION TO ANTIGENIICTY**" is no more than100, 000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. The contents of this thesis in part or whole never been submitted previously to acquire any other academic degree or diploma. This thesis is my own work except where otherwise indicated.

Toheder Rahaman Date: 06.09.2016

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List of publications during candidature

Peer reviewed publications:

1. Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2015). Conformational changes of β-lactoglobulin induced by shear, heat, and pH—Effects on antigenicity. *Journal of dairy science,* 98, 4255–4265.

2. Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2016). Shear, heat and pH induced conformational changes of wheat gluten–Impact on antigenicity. *Food chemistry,* 196, 180-188

3. Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2016). Effect of processing on conformational changes of food proteins related to allergenicity. *Trends in Food Science & Technology*, 49, 24-34. http://dx.doi.org/10.1016/j.tifs.2016.01.001

4. Rahaman, T., Vasiljevic, T., Ramchandran, L. (2016). Effect of heat, pH and shear on digestibility and antigenic characteristics of wheat gluten. *European Food Research and Technology*. In press, DOI: 10.1007/s00217-016-2682-4

5. Rahaman, T., Vasiljevic, T., Ramchandran, L. (2017). Digestibility and antigenicity of ß-lactoglobulin as affected by heat, pH and applied shear. *Food Chemistry*. 217, 517–523 DOI: 10.1016/j.foodchem.2016.08.129

Oral presentation

1. Rahaman, T; Vasiljevic, T & Ramchandran, L; 2015, Effect of processing (thermal, pH and shear stress) on physico-chemical properties of β-lg and consequences in antigenic reactivity, In Proceedings of Postgraduate Research Conference, held on 2nd October 2014, St Albans Campus, Victoria University, Melbourne, Australia.
2. Rahaman, T; Vasiljevic, T &Ramchandran, L; 2015, Shear, heat and pH induced conformational change of βlg-Impact on antigenicity, In Proceedings of Australian Institute of Food Science and Technology (AIFST) Food Science Summer School , held on 28 January, 2015 at RMIT, Melbourne, Australia.

Table of Content

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List of abbreviations

α=alpha

β=beta

ϒ=gamma

κ= kappa

ω=omega

μ = micro

μg= microgram

μL= microliter

μm = micrometre

α-la= alpha-lactalbumin

AA=amino acids

ANS=1-anilinonaphthalene-8-sulfonic acid

Asn=asparagine

Asp=aspartic acid

β-lg= beta-lactoglobulin

BSA = bovine serum albumin

CD=celiac disease

CDR=complementary determining region

CMA=cow milk allergy

Cys=cysteine

DHPM=dynamic high pressure microfluidization

EDTA=ethylenediaminetetraaceticacid

ELISA=enzyme linked immunosorbent assay

f=fragment

Fab=facilitated allergen binding

FTIR=fourier transform infrared spectroscopy

g = gram

Glu= glutamic acid

GLM =general linear model

GMP=glutenin macropolymer

Gn=glutamine

Gy-gray

h = hour

H_2SO4 = sulphuric acid

HCl= hydrochloric acid

HMW-GS=high molecular weight gluten subunit

Ho=hydrophobicity

HP=high pressure

Hz=hertz

Ig = immunoglobulin

IL=interleukin

kDa=kilo dalton

kg=kilogram

kGy=kilogray

kHz=kilohertz

L = litre

LMW-GS=low molecular weight gluten subunit

mA=milliampere

MHC= major histocompatibility complex

min= minute

mL= millilitre

mm= millimetre

mM= millimolar

MPa=mega pascal

MW = molecular weight

ng= nanogram

OM=ovomucoid

OVA=ovalbumin

˚C = degree celsius

PAGE=polyacrylamide gel electrophoresis

pH = hydrogen ion concentration

pI=isoelectric point

rpm= revolution per minute

RFI=relative fluorescence intensity

RT=room temperature

s = second

SAS=statistical analysis software

SDS=sodium dodecyl sulphate

SEM=standard error of the mean

SH=thiol group

SS=disulfide

TCA = trichloroacetic acid

TLPs=thaumatin-like proteins

TMB=3,3,5,5-tetramethylbenzidine

tTG=tissue transglutaminase

UV = ultra violet

V = volts

W=watt

w/w= weight per weight

WGA=wheat germ agglutinin

v/v= volume per volume

Chapter 1

Introduction to the thesis

1.1 Introduction

Food allergy (food hypersensitivity) is the adverse health effect resulting from abnormal immune response to an antigen contained in food or a food additive (Rodgers, 2011). It can be IgE mediated (also called immediate type or type I) where food antigens bind with allergen specific IgE resulting in release of allergic mediators and subsequent allergic symptoms, or non IgE mediated which is a delayed type, less common and governed by Eosinophil and T cell (Sicherer, 2013). Food allergy is an evolving public health concern worldwide affecting 1-3% of adults and 4-6% of children and appears to have risen in the industrial countries (Sansonetti and Medzhitov, 2009). It is one of the major causes of fatal anaphylaxis, responsible for approximately 30,000 anaphylactic cases in a year with an estimated 200 deaths in USA (James et al., 2012). It can seriously affect daily social activities, mental growth and education of the sensitive individuals and overall the quality of their family life, even more than chronic childhood diseases (Skolnick, 2007). Although many foods are reported for their allergic reactions, more than 90% of food allergies are caused by cow's milk, egg, fish, crustaceans, peanuts, tree nuts, wheat and soybeans which are referred to as "The Big Eight" (Török et al., 2014). However, in general, cow milk allergy (CMA) is the most common among IgE mediated food allergies (Bossios et al., 2011) whereas among non IgE mediated food allergies, gluten allergy (celiac disease) is the most prevalent.

Since food allergy is one of the epidemic forms of health issues, its managements and control has drawn special attention from researches and health practitioners. The most common approach in managing food allergy is complete avoidance of allergenic food by sensitive individuals which may otherwise lead to serious nutritional deficiency health syndromes. Another strategy in controlling food allergy is oral and subcutaneous desensitisation treatment by epinephrine and corticosteroids that always does not give

satisfactory results (Patriarca et al., 2007) due to unpredictability of the incidence and to the variability of symptoms, pattern and duration of the episodes. Immunotherapy is also an option in the management food allergy where sensitive individuals are subjected to gradually increased doses of allergens over a period of time in order to help body's antibody to be familiar with allergen to weaken adverse immune reactions. However, effectiveness of such immunotherapy scheme is also not equally effective for all individuals (Nelson et al., 1997). Therefore, it is an urgent demand in food industries to seek alternative approaches such as selective processing to reduce allergenicity of foods and assist in managing severity of food allergy.

Foods are processed in diverse ways before consumption in order to improve functional, nutritional and sensory attributes, preservation and detoxification. Applied techniques in food manufacturing include thermal, high pressure, radiation, high intensity ultrasound, and bio-chemical processing. Different processing methods alter the structure of food proteins, which are the main food allergens, in various ways. Possible structural modifications include unfolding, aggregation, cross-linking between the ingredients and chemical modifications such as oxidation and glycosylation (Lepski and Brockmeyer, 2013). Such processing induced conformational changes can directly influence the allergenicity by disrupting conformational or linear epitopes. Conformational epitopes can be exposed or hidden by unfolding or aggregation of proteins (Rahaman et al., 2015) whereas sequential epitopes can be affected by acidic or enzymatic hydrolysis (Kasera et al., 2015) and extreme Maillard reaction (Toda et al., 2014). Processing induced physico-chemical changes of food proteins may further affect gastrointestinal digestibility, absorbance kinetics through mucosa as well as their presentation to the immune system and thereby influence their allergenicity. However, the degree of structural alteration and allergenicity depends on the processing method

used, extent and exposure time, and presence of other ingredients for example salt, sugar etc. (Verma et al., 2012).

Among different processing techniques heating is the most commonly applied method for most of the food and food products and therefore its effect on food protein allergenicity has been studied extensively. For example, heating of β-lactoglobulin (β-lg), the most abundant whey protein, up to 90°C caused unfolding of the protein molecule with exposure of conformational epitopes which bound with antibody resulting in increased allergenicity. Further temperature rise has led to irreversible aggregation with covalent and hydrophobic interaction, consequently masking and/or destroying of conformational epitopes and reduction of allergenicity (Kleber et al., 2004a, Bu et al., 2009). Through the heat induced aggregation, β-lg acquired such a compact structure that could also explain inaccessibility of the antibodies to inner linear epitopes and reduction of allergenicity (Kleber et al., 2004b). Heat induced conformational change can also influence digestibility of β-lg and thereby modulate its allergenic potential (Peyron et al., 2006). Heat induced unfolding of protein molecule results in exposure of some hydrophobic residues thereby increase susceptibility to hydrolysis (Peram et al., 2013). Heat treated β-lg aggregates also undergo rapid digestion by pepsin apart from some dimers. Similarly, heat application during baking can alter conformation, digestibility as well as allergenicity of potential wheat gluten allergens (Petitot et al., 2009a). Immunoblotting of *in vitro* digested unheated bread dough, bread crumb and crust with sera from wheat allergic patients resulted in almost complete disappearance of IgE binding protein in dough whereas such proteins persisted in bread crumb and crust (Simonato et al., 2001b, Pasini et al., 2001). Heat mediated protein aggregation both in bread crumb and crust leads to hindered susceptibility to

proteolysis of gluten allergens allowing them to cross gastrointestinal barrier to elicit adverse immune reaction.

Another important food processing parameter is pH. Variation of pH can substantially affect physico-chemical and functional properties of food proteins (Dufour et al., 1994) and thereby could influence their digestive susceptibility and allergenicity. For example, heating (>80°C) of β-lg at neutral pH (pH 7) caused dissociation of dimeric β-lg to monomers and partial unfolding which facilitated exposure of free thiol group and enabled their participation in thiol-thiol oxidation or thiol-disulfide exchange reactions contributing to heat-induced aggregation (de la Fuente et al., 2002, de Wit, 2009, Wada et al., 2006). Heating at low pH did not initiate such a thiol/disulfide exchange mediated covalent aggregation even at higher temperatures due to lower reactivity of free thiol groups (Wada et al., 2006, de la Fuente et al., 2002). Likewise, structural and functional properties of gluten are influenced by pH; lowering the pH by mild acid treatment has been reported to improve the solubility, foaming and emulsifying properties of gluten proteins through the mechanism of deamidation (Berti et al., 2007, Liao et al., 2010a, Liao et al., 2010b). Such deamidation could substantially affect allergenic potential of gluten proteins (Berti et al., 2007, Abe et al., 2014a)

Moreover, food proteins are also subjected to shear stress during many common manufacturing processes such as centrifugation, mixing, homogenization, ultrafiltration and microfiltration (Yim and Shamlou, 2000). Shear stress is also known to alter the structure of native protein molecules (Bekard et al., 2011) that in turn, could affect functional properties of proteins and their susceptibility to enzymatic digestion. For example, preheated β-lg solution (0.5 wt %) at low pH under moderate shear rate of 200 s^{-1} undergoes aggregation and fibril formation (Akkermans et al., 2006). Shear originating during high mixing rate (100 rpm) of mixing induces scission of disulfide

bonds in gluten resulting in an increase of transient reactive thiol groups that participate in SH/SS interchange reactions and lead to formation of new inter-chain SS bonds (Morel et al., 2002).

Although several studies as mentioned above, have focused on effects of pH and temperature separately on structural changes of β-lg and gluten and subsequent antigenicity; combination of different pH, temperature and shear mimicking common conditions during processing in food industries and their effect on conformational changes related to antigenicity has not been established and needs to be well studied. Even though baking temperature for most of the bakery products is quite high (around 177-205°C), due to low heat conductibility of outer crust, internal loaf temperature never reach above 100°C (Vanin et al., 2009). Hence, 100°C has been selected as highest temperature for gluten study. In contrast, milk undergoes both high (ultra-high temperature processing, around 135°C) and low temperature (pasteurization, around 72°C) during processing and therefore wide range of temperature (80, 100 and 120°C has been considered for β-lg study. Moreover, a characteristic feature of most food allergens is their resistance to gastrointestinal digestibility and therefore, understanding digestibility of processed food allergens and their post digestion antigenic potential is the fundamental to managing food allergenicity which is also ambiguous and need to be addressed.

1.2 Research aims

The overall aim of the proposed research was to establish the impact of combined effects of shear, pH and heat on the structure of β-lg and gluten in relation to their antigenic responses. The specific objectives were:

a) To investigate the effects of varying pH, temperature and shear all in combination on the conformational changes of β-lg and gluten and relate these changes to their perceived antigenicity;

b) To relate processing induced conformational changes of examined proteins to their digestibility and post digestion antigenic potential;

c) To identify potentially antigenic fractions of the examined proteins post digestion;

1.3 Thesis outline

This thesis has been organized into 7 chapters. Chapter 1 provides background, research objectives and outline of the Thesis. Chapter 2 critically reviews the literature relevant to the thesis and covers the fundamental concepts and recent advances in different processing techniques, their effects on the conformation of food allergens related to digestibility and antigenicity. Chapter 3 and 5 details the effects of pH, temperature and shear on the secondary and tertiary structure of gluten and β-lg, respectively, and how these structural changes affect their antigenicity. Chapter 4 and 6 encompass how the processing induced conformational changes affect digestibility of gluten and β-lg, respectively, and also the antigenic characteristics of the digests. The overall conclusions drawn from this study and the scope for the future work are delivered in Chapter 7. Chapter 8 contains the list of references.

Chapter 2

Review of literature

Review of literature is presented here under section 2A and the part of review that has been published is presented under section 2B

2A Review of literature

2A.1 Food allergy and its mechanism

2A.1.1 Introduction

Food is an essential part of everyone's lives providing energy for growth and development, to be healthy and active and to perform all the activities of life. Varieties of foods are consumed daily which contain five different nutrients such as protein, carbohydrate, fat, vitamins and minerals, each of which has a different physiological role in maintaining sound physical and mental health. Although most people enjoy a wide variety of foods without any problem, for a small percentage of people, however, specific food nutrients may cause adverse reactions which can be either a food intolerance or food allergy. It is reported that about a quarter of the world's population experiences adverse food reactions at least once in their life time; mostly in their infancy and childhood to any of the foods (James et al., 2012). Food intolerance occurs when the body cannot digest a food or food component properly whereas food allergy results from adverse immune response to certain food proteins. Food allergy may result in mild and less harmful to severe life threatening symptoms and it is now a worldwide public health issue, particularly among children. Its occurrence seems to be increasing (Burks et al., 2012a) and therefore research on its mitigation is of outmost importance.

2A.1.2 Underlining mechanism of food allergy

Food allergy, also known as food hypersensitivity, is an adverse health effect resulting from abnormal immune response to the food protein antigen. On the basis of mechanism of immune response food allergy is classified into two types - immunoglobulin E (IgE) mediated and non IgE mediated food allergy (Fig. 2A.1). Most

of the food allergies are IgE mediated and also known as immediate type/type I as it occurs within minutes to an hour after exposure to foods. In this type, allergen specific IgE binds to FC3RI receptor located on the surface of effector cells (Mast cell and Basophil) followed by subsequent degranulation of effector cells and release of allergic mediators such as histamine (Ortolani and Pastorello, 2006). Its symptoms usually involve skin (acute urticarial and angioedema), gastrointestinal tract and respiratory tract (bronchospasm). In contrast, non IgE mediated (T cell mediated) food allergy is delayed type and primarily affects gut mucosa. The example of cell mediated allergy is celiac disease (CD), a food protein-induced enteropathy. In certain cases both cell-mediated and IgE facilitated mechanism could play a combined role (Sampson, 2004) . In both type of allergies, the series of reactions are regulated by cytokines (Burks et al., 2012b) which is illustrated in Fig. 2A.1.

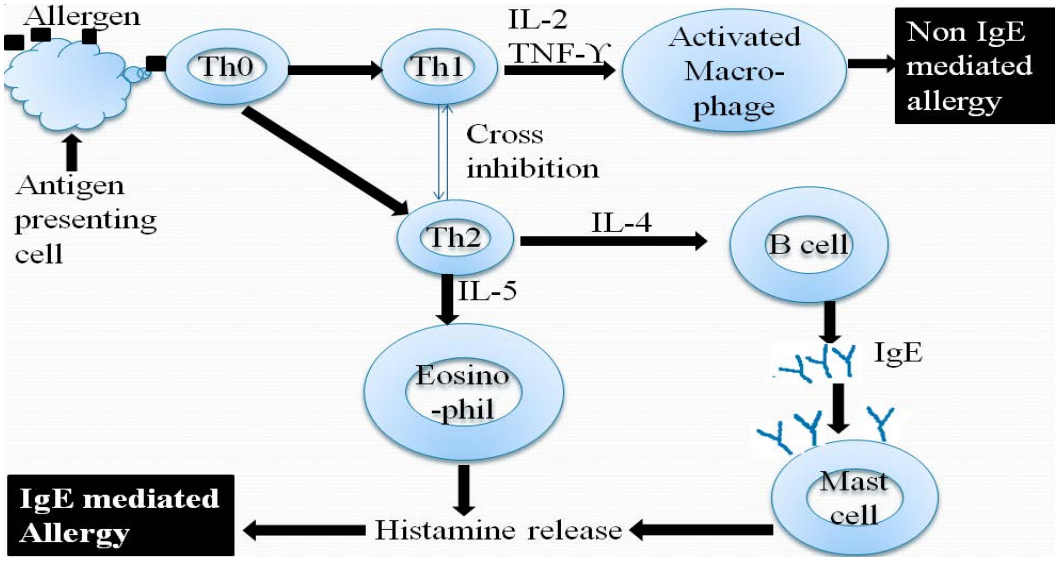

Fig. 2A.1 Schematic presentation of the pathway from exposure to allergen to allergic symptoms

The mechanism of food allergy involves immune sensitization, which is triggered by small structural parts rather than the whole food protein which are called allergenic epitopes. These are usually presented as two types: conformational and linear (sequential) (Fig. 2A.2) epitopes. Linear epitopes are composed of a series of few adjacent amino acids which are not affected by the change of secondary and tertiary structure whereas conformational epitopes are formed by special three dimensional arrangement of protein molecules and their stability can be altered or destroyed by changing tertiary structure of protein when subjected to different physical treatments such as heat, pressure, ultrasound and radiation (Restani et al., 2004). A food allergen may have single type of epitopes which is repeating or several different epitopes (Sathe et al., 2005).

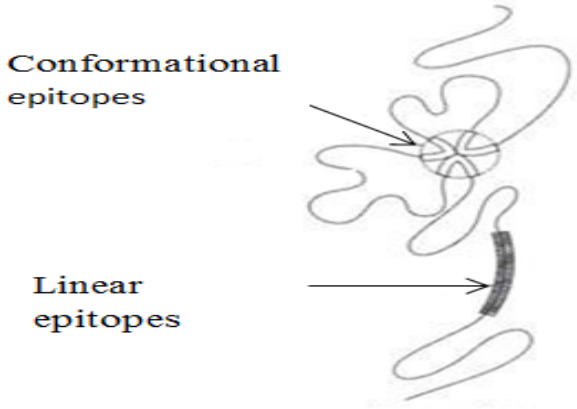

Fig. **2A.2** Two types of epitopes in a food allergen

2A.2 What makes a food allergic?

Why some food proteins, and not all, possess antigenic epitopes and trigger allergenic reactions to some individuals? What makes a food protein allergen? These questions are still unclear. Nevertheless, there are some common biochemical characteristics that enable food protein to be allergic (Bannon, 2004):

1. Abundance in the food: Most of the commonly allergenic food proteins comprise significant portion of total food proteins. For example, major allergen in shrimp, Pen a 1 makes up about 20% of total shrimp-tail protein. β-lactoglobulin, a major allergen of milk, comprises 10% of total milk protein.

2. Digestive stability: One of the important parameter of food protein to be allergenic is its stability to gastrointestinal degradation to preserve a minimum number of epitopes to bind with IgE on the surface of mast cell. Such resistance to digestion is often associated with their structural stability via intra and/or inter molecular disulfide bonds. For example, β-lg, major milk allergen, possesses two intra-molecular disulfide bonds Cys^{66}-Cys^{160} and Cys^{106}-Cys^{119} which contribute to its digestive resistance and reduction of these disulfide bonds by thioredoxin leads to susceptibility to digestion and disruption of IgE and IgG binding epitopes (Bannon, 2004). Likewise, wheat gluten contains intra and inter-molecular disulfide bonds and reduction of these bonds increase its susceptibility to digestion with mitigation of allergenicity. Similarly eight cysteine residues form four disulfide bonds in peanut allergen, Ara h 2 and reduction of those significantly enhance gastrointestinal digestibility with reduction of allergenicity (Sen et al., 2002)

3. Multiple linear epitopes: An allergen should have at least two epitopes to bind with IgE which can be different types or single repetitive (Lepski and Brockmeyer, 2013)

2A.3 Prevalence of food allergy

Food allergy is one of the major health concerns worldwide affecting 1-3% of adults and 4-6% of children and in the last two decades the rate has been increased at such a rate that it is now “second wave” of allergic diseases in the westernized countries

like Australia, UK & US (Lack and Du Toit, 2014) and one of the leading cause of anaphylaxis (Prescott and Allen, 2011a, Wu et al., 2012). About 18% increase of self-reported food allergy in USA has been reported during the period 1997 to 2007 (Branum and Lukacs, 2009). A recent study (Nwaru et al., 2014) has revealed increase of food allergy prevalence from 5.9 to 17.3% over the time from 2000-2012 in Europe through population based meta-analysis. Australia is one of the countries with high prevalence rate of food allergy in the world (Prescott and Allen, 2011b). During the period of 1995 to 2006, 10-fold increase of specialist referral for food allergy and 5-fold increase of hospital admission for food anaphylaxis have been reported in Australia (Mullins, 2007). Most of the studies on prevalence of food allergy are based on self-report and many cases remain ignored and unreported (Glick Robison, 2014). Thus, actual prevalence rate of food allergy could be more. It can seriously affect the physical and mental health and thereby the quality of life of both the patient and their family, even more than chronic childhood diseases (Arasi et al., 2014).

Although many foods are reported for their allergic reactions, more than 90% of food allergies are caused by cow's milk, egg, fish, crustaceans, peanuts, tree nuts, wheat and soybeans which are referred as "The Big Eight" (Török et al., 2014) (Fig. 2A.3). Sensitivity of different major food allergens is highly related to age group. For example, cow's milk, egg and soy allergies are reported to be mostly prevalent in childhood and outgrown by late childhood (Skripak et al., 2007, Savage et al., 2007) whereas peanut, nuts and fish allergies usually persist up to adulthood (Zeiger, 2003).Variation of allergen prevalence could also be due to differences in allergen exposure, traditional processing methods and also genetic factors (Sicherer, 2011). A survey on food allergy in Taiwan showed highest prevalent allergen for children less than 3 years of age was milk while with the increase of age, the major allergen shifted to

sea food such as shrimp and crab. Although cow milk and egg are the most common childhood allergen in most of the countries, wheat and buckwheat are the most widespread causes for food-induced anaphylaxis in Korea (Robison, 2014). However, in general, cow milk allergy (CMA) is the most common among IgE mediated food allergies whereas among non IgE mediated food allergies gluten allergy (celiac disease) is the most prevalent and are the focus of this review.

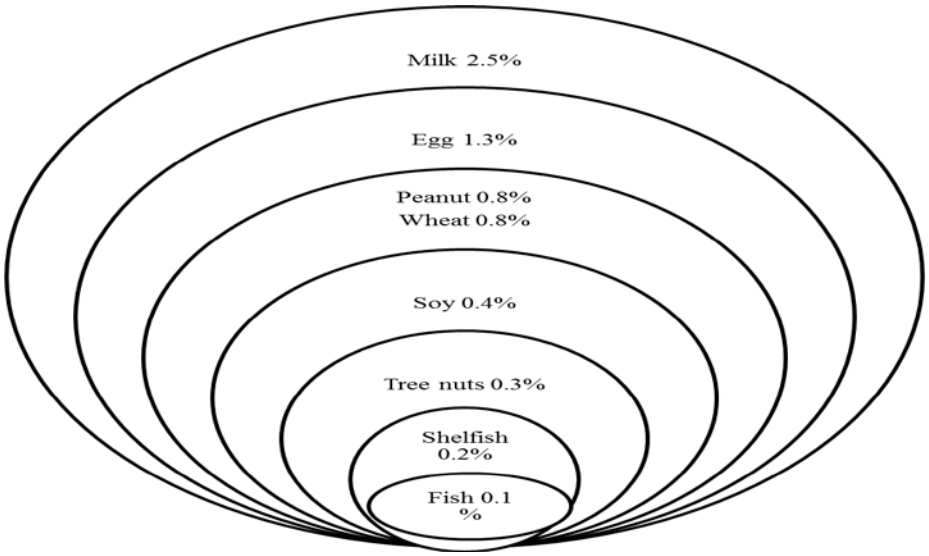

Fig. 2A.3 Prevalence of most common eight food allergies on total population based (not based on age group) (Sicherer, 2011).

2A.4 Beta lactoglobulin (β-lg) and its allergenicity

2A.4.1 Structure

Beta lactoglobulin (β-lg) is the major whey protein of bovine milk comprising about 50% of total whey protein and 10% of total milk protein (Hasni et al., 2011). It is a globular protein of 120 amino acids having molecular weight of 18 kDa (Brownlow et al., 1997). The amino acid composition is as follows: Asp (10), Asn (5), Thr (8), Ser (7), Glu (16), Gln (9), Pro (8), Gly (4), Ala (15), Cys (5), Val (9), Met (4), Ile (10), Leu (22), Tyr (4), Phe (4), Lys (15), His (2), Trp (2) and Arg (3) (Morr and Ha, 1993). Out

of its 5 cysteine residues four, Cys^{106}-Cys^{119} and Cys^{66}-Cys^{160} form 2 disulfide (SS) bonds, whereas Cys^{121} remains as a free thiol (SH) group (Sava et al., 2005)(Fig. 2A.4). Although more than two genetic variants of β-lg have been reported, the two most common variants are A and B which differ at position 64 and 118, aspartic acid (Asp) and valine (Val) in β-lg A, and glycine (Gly) and alanine (Ala) in β-lg B (de Wit, 2009) and both are known to possess allergenic potential (Maier et al., 2006). X-ray crystallography (Croguennec et al., 2004) revealed that that secondary structure of both variants consists of two anti-parallel β-sheets formed by 9 strands labelled A to I, and eight of them form somewhat flattened β-barrel with one α- helix on outer surface of barrel which folded into a calyx and act as a carrier (lipocalins) for some hydrophobic ligands such as retinoids, fatty acids, vitamin D, and lipids (Qin et al., 1999). One side of both the β-sheets are hydrophobic which face each other forming a hydrophobic core (Considine et al., 2007). In native state, the free thiol group (Cys^{121}) of β-lg is disguised in the hydrophobic core and does not participate in formation of disulfide interactions and its reactivity can be initiated through unfolding of molecule by physical treatment such as heat (Sava et al., 2005). At neutral pH and room temperature β-Lg remain as non-covalently linked dimer stabilized by hydrogen bonds (De Wit, 1998).

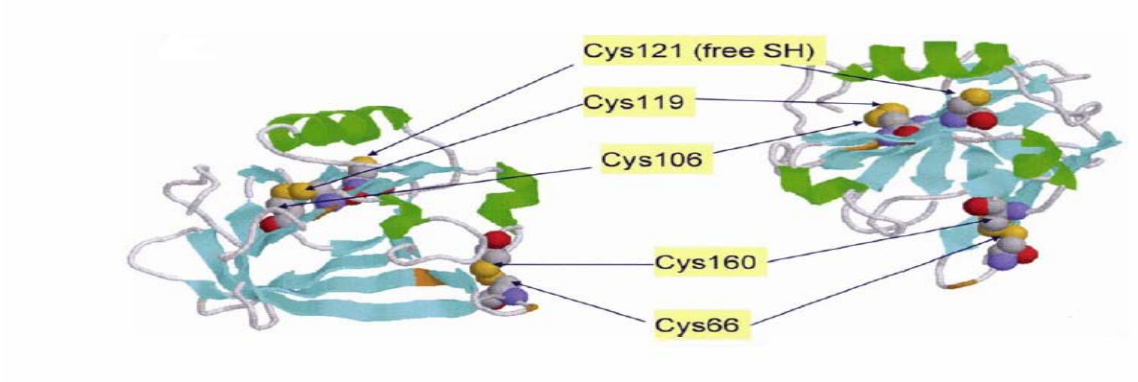

Fig. 2A.4 Three dimensional structure of β-lg showing relative position of cysteine residues. Adapted from (Patel, 2007).

2A.4.2 Functional properties of β-lactoglobulin

Beta lactoglobulin has many beneficial nutritional and functional characteristics that make it an ingredient of choice in the formulation of many modern foods and beverages. Its uses in food industries has been increasing due to its excellent gelling, emulsifying and foaming properties (Chevalier et al., 2001). Structural arrangement of β-lg makes it a member of lipocalin protein family and act as transport protein. Because of its stability at low pH, β-lg is able to bind and protect retinol or some other small hydrophobic molecule during passage through the stomach, in order to deliver to a specific receptor located in the intestine (Cho et al., 1994). Bioactive peptides released from digested β-lg is reported to have opioid (Teschemacher, 2003), angiotensin I-converting enzyme (ACE) inhibitory (Pihlanto-Leppälä, 2000), antimicrobial (Pellegrini et al., 2001), anticarcinogenic (McIntosh et al., 1995), hypocholesterolemic (Nagaoka et al., 2001) and immune modulatory (Gauthier et al., 2006) activity. In spite of its diversified functional properties and health benefits, its application in food manufacturing is limited due to its allergenic potential (Hattori et al., 2004)

2A.4.3 Allergenicity

Beta lactoglobulin is one of the major bovine milk allergens, known as bos d5 (allergenic nomenclature), being responsible to about 80% cow milk allergic patients (Bu et al., 2009). Relative resistance of β-lg to peptic digestion due its native conformation and its absence in human milk may explain for why it is a potent allergen for cow milk allergic patients (Bossios et al., 2011). Both the conformational and sequential epitopes are known to show allergenic reaction of β-lg (Wal, 1998). According to Sélo et al. (1998) linear epitopes are widely spread all along the β-lg molecule and some of them are short sequences while other are quite large fragments

that might encompass conformational epitopes. The major linear epitopes of β-lg has been demonstrated in Fig. 2A.5.

Modifying the epitopes in β-lg can modulate its antigenic responses. Various methods have been adopted to destroy or modify such epitopes in β-lg. Conformational epitopes can be destroyed or hidden through various physical treatments such as heat (Fritsche, 2003, Shandilya et al., 2013), high pressure (Zhong et al., 2012) and combination of heat and pressure treatments (Kleber et al., 2007, Zhong et al., 2011) whereas sequential epitopes can be affected by acidic or enzymatic hydrolysis (Wroblewska et al., 2004, Kim et al., 2007).

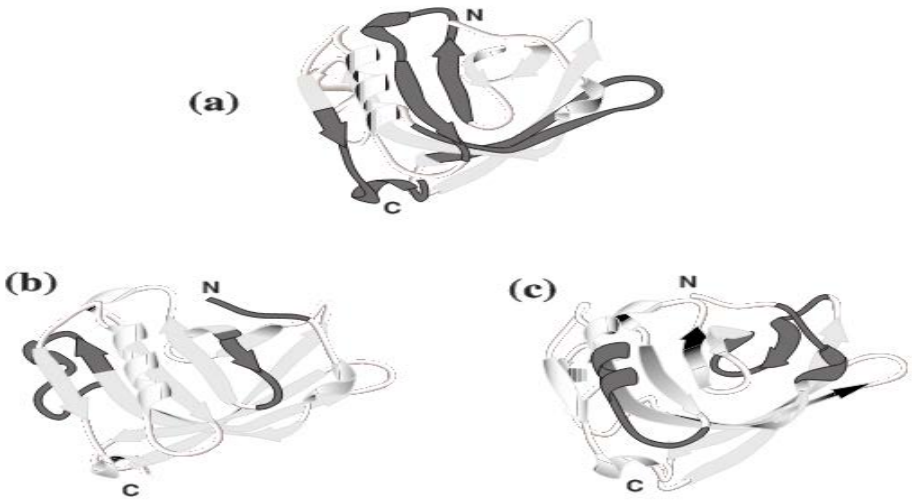

Fig. 2A.5 Position of immunoreactive fractions in three-dimentional structure of β-lg: (a) Major allergenic epitopes f(41-60), f(102-124) and(149-162) recognized by serum anti β-lg IgE in 90-100% allergic patients (b) Second major epitopes f(1-8), f(25-40) and f(92-100) recognized by serum IgE in 50-65% allergic patients (c) Minor epitopes f(9-14), f(78-83), f(84-91) and f(125-135) recognized by serum IgE in <40% allergic patients. (Selo et al., 1999)

2A.4.4 Modifications of β-lg allergenicity

2A.4.4.1 Thermal impact

Thermal denaturation of β-lg involves some consecutive steps involving both covalent and noncovalent interactions (Harwalkar and Ma, 1989, Sava et al., 2005). At the initial step of heat treatment at neutral pH, β-lg tends to dissociate into monomers at the critical temperature (≈60°C). Then the molecule undergoes partial unfolding with exposure of hydrophobic residues as well as a free thiol group which is usually buried at the interface between the monomers in their native state (Zuniga et al., 2010). The exposed thiol group become reactive and participate into intra-molecular thiol-disulfide interchange reaction with the formation of non-native reactive monomers with exposed Cys^{121} and Cys^{119} residues (Croguennec et al., 2003). Further heating (above 90°C) leads to formation of intermolecular disulphide cross liking via thiol/disulphide interchange reaction resulting in non-native dimers and polymers. Some non-covalent interactions such as hydrophobic interaction also become involved in the aggregation mechanisms leading to larger aggregates. However, this heat induced denaturation depends on different factors such as heating time and temperature, environmental pH and ionic strength (Stănciuc et al., 2012).

Several studies have reported that such heat induced structural alterations of β-lg could noticeably affect its immunoreactivity. Heating up to 90°C caused unfolding of protein molecule with exposure of conformational epitopes to bind with antibody resulting in increased allergenicity whereas further raising the temperature led to irreversible aggregation with covalent and hydrophobic interaction, consequently masking and/or destroying of conformational epitopes and reduction of allergenicity (Kleber et al., 2004a, Bu et al., 2009) . Similar trend was also observed by Ehn et al.

(2004), Taheri-Kafrani et al. (2009) although they reported significant reduced allergenicity at 74°C and increasing the temperature to 90°C further abrogated allergenicity. Through the heat induced aggregation, β-lg achieve such a compact structure that reasons inaccessibility of the antibodies to inner linear epitope which in turn results in reduction of allergenicity (Kleber et al., 2004b).

In addition to conformational changes, heating can also alter the susceptibility of β-lg to proteolysis and thereby modulate allergenic potential (Peyron et al., 2006). Native β-lg is resistant to peptic hydrolysis due to its unique structural feature (Corzo-Martínez et al., 2010). Hydrophobic amino acids are the cleavage site for pepsin which is buried inside the hydrophobic core of β-lg molecule. Heat induced unfolding of protein molecule results in exposure of some hydrophobic residues thereby increase susceptibility to hydrolysis (Peram et al., 2013). In the same study it has been shown that heat induced aggregates and intermediates except some dimers also underwent rapid digestion by pepsin.` Morisawa et al. (2009a) reported increased proteolysis of β-lg due to heating at 80 and 100°C facilitated cleavage of protein sequences with disruption of B cell linear epitopes. Therefore, basophil sensitized with heat treated and digested β-lg released less histamine compared to unheated and digested β-lg.

2A.4.4.2 Effect of pH

Physico-chemical properties of β-lg is largely influenced by pH (Dufour et al., 1994); at neutral pH, β-lg remains as dimer in solution whereas below pH 3 and above 7 it dissociates into monomers. Above the isoelectric point (pH 5.2), β-lg assumes net negative charge whereas below the isoelectric point the net charge becomes positive (Kella and Kinsella, 1988, Gotham et al., 1992). Several studies have reported increased stability of β-lg with decreasing the pH in the range 3.0-1.5 attributed to the additional

hydrogen bonding in the protein due to neutralization of carboxyl groups (Kella and Kinsella, 1988). Either two titrated carboxyl groups or one amide group and one titrated carboxyl group might form a pair of hydrogen bonds giving such structural stability at low pH. Reactivity of free thiol group in β-lg is also pH dependent and thus, its heat mediated denaturation is also influenced by environment pH.

At low pH, lower reactivity of free thiol groups (de la Fuente et al., 2002; Wada et al., 2006) hinders thiol/disulfide exchange mediated covalent aggregation even at high temperature (120°C). Akkermans et al. (2008) have reported that heating β-lg at lower pH (<3) led to formation of small peptides, which are the building block of a fibril formation. In contrast, heating (>80°C) at around neutral pH (7) caused dissociation of dimeric β-lg to monomer and partial unfolding which facilitates exposure of free thiol group to participate in thiol-thiol oxidation or thiol-disulfide exchange reactions contributing to heat-induced aggregation resulting in masking of conformational epitopes and reduced allergenicity (Davis and Williams, 1998). Zhang and Vardhanabhuti (2014) reported that morphological characteristics as well as digestion behaviour of heat induced β-lg aggregates greatly varied with heating pH; aggregates formed at pH 6.0 were more susceptible to pepsin digestion than at pH 7.0. Another study (Zheng et al., 2008) showed that heating β-lg at 45°C and subsequent hydrolysis caused increase of antigenicity between pH 8-9 and above that range it decreased.

2A.4.4.3 Effect of shear

2A.4.4.3.1 What is shear force?

Shear is a stress that is produced when any fluid moves along the solid boundary (Fig. 2A.6). Exposure of proteins to shear stress is a common phenomenon during

bioprocessing through different operations like centrifugation, mixing, homogenization, ultrafiltration and microfiltration in food and pharmaceutical industries (Yim and Shamlou, 2000). Likewise, various proteaceous therapeutic and biochemical reagents such as immunoglobulins, hormones and enzymes may also experience shear stress during shipping and handling (Bekard et al., 2011).

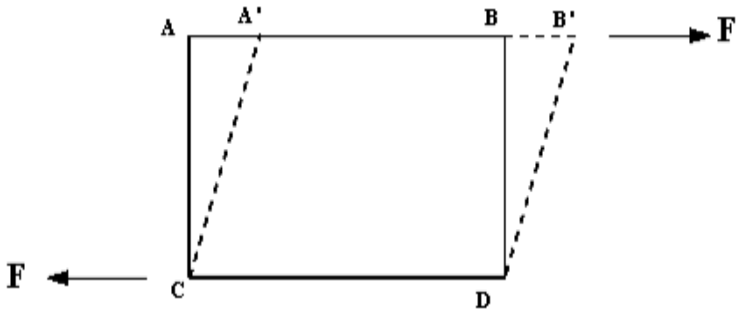

Fig. 2A.6 Shearing force, F acting tangentially on a rectangular (solid lined) element ABDC and produces the (dashed lined) rhombus element A'B'DC.

2A.4.4.3.2 Shear induced changes in β-lg structure

Shear stress can result in perturbation of native protein molecule with the exposure of hydrophobic groups leading to hydrophobic interactions followed by protein aggregation and subsequently fibril formation (Bekard et al., 2011). Hill et al. (2006) have shown that exposure of β-lg to controlled shear flow *in vitro* promotes amyloid like fibrillogenesis by generating precursors that act as seed-like initiators; the rate and extent of precursor formation increases with shear rate. Dunstan et al. (2009) also reported shear induced amyloid fibril formation of β-lg and the rate of fibril formation, morphology and mechanical response of the resulting fibril depended on type of shear flow such as controlled (coquette) or uncontrolled (stirring) as well as on the rate of applied shear. The influences of temperature (70-90°C) and shear rate (111-625 s^{-1}) upon the rate of growth and size of aggregates in whey protein concentrate

solutions (WPC) (1.5%w/w) has been investigated (Simmons et al., 2007) where it has been concluded that above 70°C, WPC formed aggregates and the particle growth rate were enhanced by increasing shear rate due to an increase in the number of particle collisions. Preheated (90°C) β-lg solution (0.5 wt %) at low pH and moderate shear rate of 200 s^{-1} underwent aggregation and fibril formation (Akkermans et al., 2006); the length of the fibril was more with pulsed and continuous shear treatment as compared to the fibrillar growth under quiescent conditions. The aggregation behaviour of whey proteins under shear at different pH was studied by Spiegel and Huss (2002); where they found that β-lg at pH 4-5.5 regardless of heating temperature formed small particle size aggregates (<5 μm) at a shear rate of 1200 s^{-1}(Spiegel and Huss, 2002). However, although several studies have focused on the effect of shear flow on β-lg morphology separately or in combination with pH and temperature, their effect on its antigenic consequences has never been addressed.

2A.5 Wheat Gluten and its allergenicity

2A.5.1 Gluten structure

Gluten is a cohesive and viscoelastic protein complex comprising about 80-85% of total protein in wheat grain. It appear as a rubbery mass after washing out soluble protein, fat and starch from dough. The structure of gluten is characterized by (a) high content of glutamine (Gln), particularly in gliadin, contains about 35% molar glutamine (b) rich in proline (Pro) (about 20% molar) which leads to sharp bending in the structure (c) much intra and intermolecular disulfide bonds contributed by cysteine residues and (d) significant amount of amino acids with hydrophobic side chains for example, Valine (Val), Isoleucine (Ile), Phynylalanine (Phe) and Proline (Pro) leading to its very poor solubility in water (Cornell and Hoveling, 1997).

On the basis of solubility, gluten is divided into two major fractions gliadin, soluble in aqueous alcohol and glutenin, soluble in dilute acid and alkali which are further divided into many sub fractions.

2A.5.1.1 Gliadin

Gliadin appears as a heterogeneous mixture of monomeric proteins having molecular weight 30-50 kDa. It is rich in Gln and Pro accounting near about 35% and 20% of total amino acid respectively (Cornell and Hoveling, 1997). High content of proline is supposed to be responsible for its much β turns. It is well solubilised in 50-70% ethanol or propanol but insoluble in water due to its less content of amino acids with charged side chains. On the basis of mobility in acid polyacrylamide gel electrophoresis (PAGE), gliadin is classified into four groups such as α-, β-,ϒ- and ω-gliadins in the order highest to lowest mobility (Wieser, 2007). Although a high performance liquid chromatography (HPLC) technique allows separation of hundred components of gliadin, on the basis of amino acid composition and sequence and molecular weight, these components can be grouped into main four types: ω5-, ω1,2-, and α/β-, and ϒ- gliadins (Wieser, 1996). ω-gliadins are rich in Pro, Gln and Phe which together account for 80% of total composition and they lack cysteine residues resulting in absence of disulfide bonds. Molecular weight of ω5- gliadin (≈ 50 kDa) is bit higher than ω1, 2- gliadins (40 kDa) (Hamer et al., 2000). The repetitive unit of ω1,2- gliadins is PQQPFPQQ which is rich in Gln and Pro. On the other hand, α/β-, and ϒ- gliadins have molecular weight (28-35 kDa) below ω- gliadins and comparatively less percentage of Pro and Gln. The repetitive unit of α/β-gliadins are dodecapeptides such as QPQPFPQQPYP whereas for ϒ- gliadins it is QPQQPFP (Wieser, 2007). α/β-, and ϒ- gliadins contain six and eight cysteine residues in the C-terminal domains

respectively and all them are involved in the formation of intramolecular disulfide linkage (Grosch and Wieser, 1999) which contribute to their globular structure.

2A.5.1.2 Glutenin

Glutenin proteins occur as un-associated (15-150 kDa) and associated (150-3,000 kDa) fractions. In the associated form, the fractions are linked by inter-chain disulfide bonds and considered as the largest protein in the nature (Cornell and Hoveling, 1997). The molecular weight of glutenin polymer is one of the important determinants of dough properties and baking performance. The largest polymer of glutenin is called glutenin macropolymer (GMP), which contributes most significantly to the dough quality. Once the disulphide bridges of glutenin protein are reduced, it yields glutenin subunits which have solubility in aqueous alcohol like gliadins. On the basis of molecular weight, these subunits are classified into two types: low molecular weight glutenin subunits (LMW-GS) and high molecular weight glutenin subunit (HMW-GS) at the proportion of 1:2 (Wieser, 2007). LMW-GS has molecular weight 28-35 kDa, similar in amino acid composition to α/β-, and ϒ- gliadins and their repetitive unit (QQQPPFS) is rich in Gln and Pro which also resembles to α/β-, and ϒ-gliadins. Out of eight cysteine residues in LMW-GS, six are homologous in position to α/β-, and ϒ- gliadins and participate in intra-chain disulfide linkage whereas other two form inter-chain disulfide linkage (Grosch and Wieser, 1999). They contain two different domains; N-terminal domain consists of Pro and Gln rich repetitive units such as QQQPPFS whereas C terminal domains have similar composition as α/β-, and ϒ-gliadins. HMW-GS less abundant than LMW-GS, accounts for 10% of total gluten proteins. It has three structural subunits (Fig. 2A.7): (A) non repetitive N terminal domain containing about 80-85 residues (B) a repetitive central domain having 480-700 residues and (C) a C terminal domain of 42 residues (Shewry et al., 1992). The most of

the residues in A and C are cysteine and charged. Domain B has main repetitive unit of QQPGQG upon which some other hexapeptides (YYPTSP) and tripeptides (QQP or QPG) units lie. HMW-GS is divided into two types such as x and y having molecular weights 83-88 kDa and 67-74 kDa respectively (Wieser, 2007). The difference between x and y types is based on domains A and B. For example, in domain B of y type, the hexapeptide constituent unit is less frequently repeated than that of x type. The x type contains four cysteine residues in domain A, two of which form intra-chain and other two form inter-chain disulfide bonds and domain C has only one cysteine residue. In contrast, for y type, there are five cysteine residues in domain A and one in each of domain B and C. Cysteine residues of domain A form inter-chain disulfide links with adjacent residues of another y types whereas cysteine from domain B form cross linking with that of LMW-GS. Secondary structure of HMW-GS reveals that the repetitive domain B has overlapping reverse β turns forming spiral loops that contribute to elastic behaviour of gluten. On the other hand domain A and C has globular structure containing α helix.

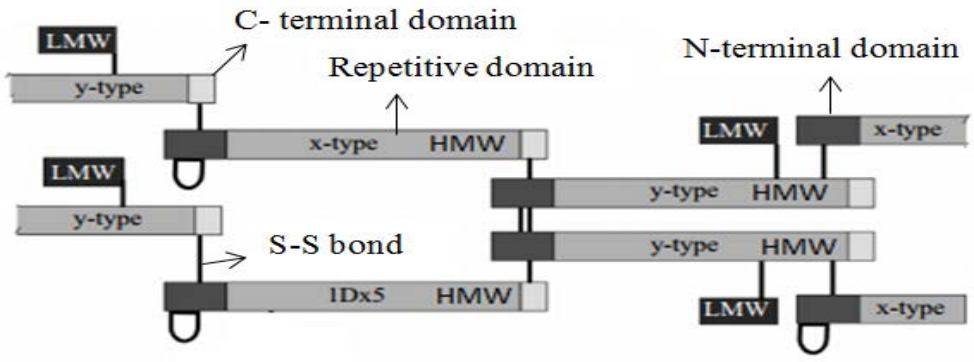

Fig. 2A.7 Schematic model for arrangement of high molecular weight (HMW) and low molecular weight (LMW) glutenin subunits forming glutenin polymers. Adapted from (Shewry et al., 2002).

2A.5.2 Functional properties of gluten

Exclusive functional properties such as viscoelastic, film-foaming, thermosetting and water holding ability of gluten proteins make wheat flour suitable for wide range of bakery products such as breads, cakes, noodles, pasta and pastries. Due to unique physico-chemical properties, availability and low cost; gluten is not only used in cereal products but also in other foods such as meat and fish products, ice-cream and drugs. By restructuring, gluten contributes to conversion of less desirable fresh meat cuts into more palatable steak type products (Day et al., 2006b). It is also used as a meat replacement in vegetarian foods and for the preparation of synthetic cheese in combination with soy. Preparation of edible and biodegradable coatings and films from wheat gluten and study of their properties has drawn special attention (Kayserilioğlu et al., 2003). Considering the environmental hazards of normal packaging materials, researchers are now focusing on bio-based and biodegradable packaging materials (Petersen et al., 1999). Unique network forming characteristics, low water solubility and biodegradability of gluten make it a suitable candidate for such type of alternate packaging (Jansens et al., 2011). Use of gluten is not only limited to food products but also in non-food products such as natural adhesives and cosmetics due its unique cohesive and elastic properties (Day et al., 2006a). Peptides from controlled hydrolysis of gluten are being used in cosmetics, lotions, hair preparations and other personal care products.

2A.5.3 Gluten Allergy

Although gluten is used extensively in food products and draws consumer's attention, it creates adverse immune reaction to some individuals which is called celiac disease (CD). It is an autoimmune disorder of small intestine upon ingestion of gliadin,

a prolamin of gluten, in genetically predisposed individuals. Triggering genetic factor for CD is human leukocyte antigen (HLA) class II alleles especially in HLA-DQ region. It is characterised by mucosal inflammation, villous atrophy and consequent malabsorption of various nutrientsThe mechanism of celiac disease has been illustrated in figure 2A.8. About 1% of most of the population is affected with CD while in some countries the rate is higher (Rubio-Tapia and Murray, 2010) and over the time the rate is considerably increasing worldwide (Rubio–Tapia et al., 2009). Although many epitopes have been identified related to CD, three common epitopes that are recognized by T cells of most of the patients are QPFPQPELPY, PQPELPYPQP and QPQQSFPEQER (Sollid, 2002). A common feature for all these epitopes are multiple Pro and Gln residues which gives rise to unique structural and functional properties such as resistance to gastrointestinal stability, preferred conformation (left-handed polyproline II helix) to bind with MHC (major histocompatibility complex) class II ligands and easily deamidation tendency by tissue transglutaminase (tTG) (Kim et al., 2004).

Moreover, gluten proteins are involved in some IgE mediated food allergies (Simonato et al., 2001a) such as baker's asthma, exercise-induced anaphylaxis and atopic dermatitis in some sensitive individuals. Baker's asthma is one of the most prevalent occupational respiratory allergies reported to affect 0.03-0.24% bakery workers (Baatjies et al., 2009). (Baatjies et al., 2009). Although salt soluble proteins (albumins and globulins) of wheat flour are mostly responsible for baker's asthma, gluten proteins are also reported to cause such sensitivity (Malo and Chan-Yeung, 2009). Wheat-dependent exercise-induced anaphylaxis (WDEIA) is another form of type I food allergy which is induced by ingestion of wheat products before physical exercise. ω-5 Gliadin is considered as the major allergen for WDEIA and the specific serum IgE from WDEIA patients against ω-5 gliadin can show cross reactivity with γ-

gliadin (Morita et al., 2003). QQIPQQQ, QQFPQQQ, QQSPEQQ, and QQSPQQQ are found to be the dominant linear epitopes to bind with IgE of WDEIA (Matsuo et al., 2004) patients.

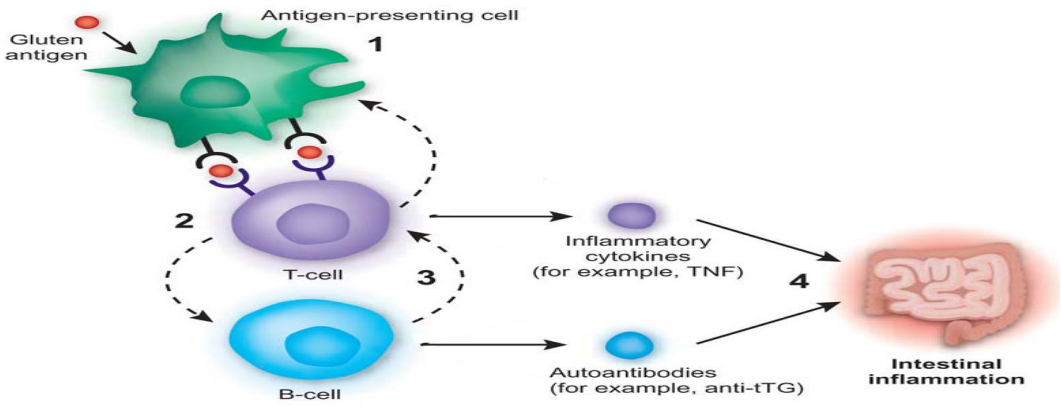

Fig. 2A.8 Schematic representation of a mechanism underlining initiation of celiac disease

However, structural stability and reactivity of different epitopes in gluten proteins are subjects to modification upon various physical such as heat (Ouahidi et al., 2011, Simonato et al., 2001a), chemical such as acid hydrolysis (Kroghsbo et al., 2014) and enzymatic (Berti et al., 2007) treatment which could alter their allergenic potential.

2A.5.4 Modification of gluten allergy

2A.5.4.1 Heat induced changes

Heat application during baking can alter conformation, digestibility as well as allergenicity of potential wheat flour allergens (Petitot et al., 2009a). Immunoblotting of *in vitro* digested unheated bread dough, bread crumb and crust with sera from wheat allergic patients resulted in almost complete disappearance of IgE binding protein in

dough whereas persistence of such proteins in bread crumb and crust (Simonato et al., 2001b, Pasini et al., 2001). Heat induced covalent protein aggregation in bread crumb (<100° C) and crust (>180°C) make the proteins less susceptible to proteolysis thereby allowing passage of large IgE-reactive fragments through gastrointestinal tract where they could elicit more allergic reaction. Reduction in bioavailability of lysine residues at high temperature treated wheat products ascertain the Maillard reaction in addition, as lysine undergoes reaction with reducing sugars during the process. Interestingly, α-amylase inhibitor protein (responsible for wheat flour inhalation allergy called “baker's asthma”) disappeared in digested bread crumb and crust although this protein remained in bread dough even after pancreatic digestion. This could explain why patients suffering from baker's asthma do not show allergic reaction upon ingestion of baked products like bread. In contrast to bread, in vitro digestion of pasta dried at up to 110°C resulted in complete breakdown of most of the potential wheat allergens (>1 kDa) and became unavailable to bind with IgE (De Zorzi et al., 2007). Thus, the patients with wheat allergy may tolerate pasta better than the bread. However, drying pasta at extreme temperature (180°C) caused formation large aggregate of proteins as a result of Maillard reaction resulted in reduction of protein digestibility consequently persistence of IgE binding prolamins. Heat mediated changes in allergenicity of gluten proteins also depend on environmental factor such as moisture content. Petitot et al. (2009b) reported that drying (90°C) pasta at low moisture (12%) content favoured formation of large protein aggregates via SS and iso-peptide bonds resulting in decreased digestibility and production of more IgE reactive peptides compared to high moisture content (20%).

2A.5.4.2 pH induced changes

pH plays a significant role on the structural and functional properties of gluten proteins and thereby on their antigenic potential as well. Lowering the pH by mild acid

treatment has proved to improve the solubility, foaming and emulsifying properties of gluten proteins through the mechanism of deamidation (Berti et al., 2007, Liao et al., 2010a, Liao et al., 2010b). Deamidation is a post-translational modification of protein in which glutamine (Gln) and asparagine (Asn) are converted to glutamic acid (Glu) andaspartic acid (Asp), respectively, due to removal of their amide groups. Changes of such physico-chemical properties through deamidation could also affect the immune reactivity of proteins. The gluten prolamins (gliadins) responsible for celiac disease (CD) are rich in Gln and Pro residues and their CD triggering epitopes are characterized by multiple Pro and Gln residues (Berti et al., 2007). As deamidation process converts glutamine to glutamic acid, such changes in amino acid sequence lead to modification of epitopes with lower allergenicity (Abe et al., 2014b). Acid hydrolysed deamidated gliadins showed significantly lowered immune reactivity with IgA anti-gliadin antibodies (AGAs) from celiac patients (Berti et al., 2007). Similar finding was also reported by Kroghsbo et al. (2014), acid deamidated gluten sensitized Norway rats produced lower biological activity of the IgE assessed by RBL (Rat Basophilic Leukemia) assay degranulation. Moreover, deamidation process can affect the digestibility of gluten, its absorption kinetics as well as immune response. *In vitro* digestion of gluten proteins showed its partial resistance to digestion which led to absorption of intact protein with allergenic reaction (Mittag et al., 2004) whereas acid induced deamidation at low pH could improve the digestibility of wheat gluten and thereby reduced its allergenicity (Kumagai et al., 2007).

2A.5.4.3 Effect of shear

Effect of shear on the physico-chemical properties of gluten protein in relation to dough qualities has been studied previously (Lindborg et al., 1997; Peressini et al., 2008). Shear stress originated during mixing of dough caused dispersion of gluten

throughout the dough followed by formation of large gluten network which is important for satisfactory bread-making performance. Graveland et al. (1985) demonstrated that high molecular weight glutenin polymers were almost completely broken down due to shear stress imparted via mixing and upon releasing the stress the fractions were reassociated. Peressini et al. (2008) showed that originated shear stress did not lead to considerable changes in glutenin macro polymer (GMP) content below the mixing rate speed 50 rpm whereas above that rate, a slight decrease in GMP content was observed attributed to cleavage of disulphide bonds which rearranged and reassociated upon resting. Detailed mechanism of shear mediated wheat gluten aggregation upon mixing was explained by Morel et al. (2002). At a low speed of mixing, SS bonds in gluten were reduced with increase of solubility whereas during high speed (100 rpm) of mixing, shear induced scission of disulfide bonds in gluten resulted in increase of transient reactive thiol groups that participated in SH/SS interchange reaction and led to formation of new inter-chain SS bonds.. Nevertheless, though alteration of structural and functional properties of gluten proteins with applied shear has been focused in few studies, relation of such alterations with their antigenic behaviour has not been outlined.

2B Published review article

Effect of commonly applied processing techniques in food industries on structural changes and allergenic consequences of the most prevalent food allergens has been summarized and published as the title "**Effect of processing on conformational changes of food proteins related to allergenicity**" by Rahaman, T., Vasiljevic, T., & Ramchandran, L. in the peer review journal, Trends in Food Science & Technology, 49, 24-34, 2016.doi: http://dx.doi.org/10.1016/j.tifs.2016.01.001

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]:	**Signature:**	**Date:**
Md. Toheder Rahaman		30/03/2016

Paper Title:

Effect of processing on conformational changes of food proteins related to allergenicity

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Md. Toheder Rahaman	80	Concept development and writing manuscript
Todor Vasiljevic	10	Concept development and manuscript editing
Lata Ramchandran	10	Manuscript editing and submission for publication

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The undersigned certify that:

1. They meet criteria for authorship in that they have participated in the conception, execution or interpretation of at least that part of the publication in their field of expertise;
2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. There are no other authors of the publication according to these criteria;
4. Potential conflicts of interest have been disclosed to **a)** granting bodies, **b)** the editor or publisher of journals or other publications, and **c)** the head of the responsible academic unit; and
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Review

Effect of processing on conformational changes of food proteins related to allergenicity

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ABSTRACT

Background: Food allergy is one of the major health concerns worldwide that has been increasing at an alarming rate in recent times. Foods undergo various processing steps before consumption that could affect conformation of food proteins, their digestion and thereby allergenicity.
Scope and approach: This review summarizes the effect of various processing methods on structural changes of major food allergens and how these changes affect their digestibility as well as allergenicity. This information could be a base line for selecting suitable food processing parameters for management of food allergies.
Key findings and conclusions: Most physical processes (heat, pressure, radiation, and ultrasound) affect conformational epitopes (destroy, mask or expose) of food proteins by altering their secondary and tertiary structures whereas the linear/sequential epitopes are affected mainly through bio-chemical (fermentation and enzymatic hydrolysis) treatments. Processing may also influence the interaction of food proteins with other ingredients via Maillard reaction and give rise to formation of new allergenic compound (neo-allergens). Processing induced changes to food proteins can largely affect their susceptibility to gastrointestinal digestion, absorption kinetics and consequently their allergenic response to immune system. Therefore, allergenic potential of food proteins may be minimized by selecting appropriate parameters during processing. Allergenicity of certain food proteins can also be modulated through optimized formulation with other food matrices. However, depending on the method of processing, intensity of treatment and molecular characteristics of allergen food proteins, allergenicity can be increased, decreased or remain unaltered.

1. Introduction

Food allergy is one of the major health concerns worldwide affecting 1–3% of adults and 4–6% of children, and in the last two decades the rate has increased considerably (Lack & Du Toit, 2014). It can seriously affect the quality of life of both, patients and their family, even more than chronic childhood diseases (Arasi et al., 2014). Food allergies are the adverse immune reactions to specific foods that result in either instant severe life threatening symptoms such as acute urticaria, angioedema, bronchospasm and anaphylaxis or delayed symptoms including atopic dermatitis and allergic gastrointestinal disorders. Although many foods are reported for their allergic reactions, more than 90% of food allergies are caused by cow's milk, egg, fish, crustaceans, peanuts, tree nuts, wheat and soybeans, which are referred as "The Big Eight" (Török et al., 2014).

In the early history of food allergy, a report of sensitivity to cooked but not raw fish (Mills & Mackie, 2008) created an interest to know whether processing affected food allergy or not. It is now understood that processing may either reduce or enhance the allergic potential of food proteins or sometimes have no effect at all. For example, Chinese traditional water boiling and frying of egg showed higher allergenic potential than steamed egg and tea-boiled egg (Liu et al., 2013). In addition, some treatments can induce formation of new allergenic compounds (neo-allergens) by prompting interactions between different ingredients (Verma, Kumar, Das, & Dwivedi, 2012).

Foods are processed in diverse ways before consumption in order to improve functional, nutritional and sensory attributes, as well as for preservation and detoxification. Commonly applied processing techniques include thermal, high pressure, radiation, high intensity ultrasound, and bio-chemical approaches. Different processing methods alter the structure of food proteins in different

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ways and possible structural modifications include unfolding, aggregation, cross-linking between the ingredients and chemical modifications such as oxidation and glycosylation (Lepski & Brockmeyer, 2013). Such processing induced conformational changes can directly influence the allergenicity by disrupting conformational or linear epitopes. Conformational epitopes can be exposed or hidden by unfolding or aggregation of proteins (Rahaman, Vasiljevic, & Ramchandran, 2015), respectively, whereas sequential epitopes can be affected by acidic or enzymatic hydrolysis (Kasera, Singh, Lavasa, Prasad, & Arora, 2015) and extreme Maillard reactions (Toda, Heilmann, Ilchmann, & Vieths, 2014). Processing induced physico-chemical changes of food proteins may further affect gastrointestinal digestibility, absorbance kinetics through mucosa as well as their presentation to the immune system and thereby influence their allergenicity (Table 1). However, the degree of structural alteration and allergenicity depends on the processing method used, extent and exposure time, and presence of other ingredients for example salt, sugar etc. (Verma et al., 2012).

Avoidance of allergic foods is the most common management strategy for sensitive individuals which may consequently lead to a number of nutritional deficiency syndromes. Although oral and subcutaneous desensitization therapies have also been practiced for a long time, their efficacies are not always satisfactory (Patriarca et al., 2007). Thus, there is a need to seek alternate strategies such as selective processing for minimizing the allergenic severity of foods. To select appropriate processing methods, it is very important to understand how these procedures alter the structure of food proteins both at a microscopic and macroscopic level and their subsequent gastrointestinal digestibility, all of which can influence their allergenicity. Several reviews (Lepski & Brockmeyer, 2013; Mills, Sancho, Rigby, Jenkins, & Mackie, 2009; Paschke, 2009; Shriver & Yang, 2011; Verhoeckx et al., 2015) have compiled the effect of processing on allergenicity of various foods but it is not well explained how the processing induced conformational changes affect digestibility. Resistance to gastrointestinal digestion is one of the main characteristics that allow food proteins retaining intact epitopes to invoke allergic reaction. Therefore the present review focuses on processing induced conformational changes of major food proteins and its relation to their digestibility and allergenicity. Such information is critical in the selection of appropriate parameters during food processing as an effective alternate in the management strategies of food allergies.

2. Various processing and their effect on food protein structure, digestibility and allergenicity

2.1. Effect of thermal treatments

Thermal treatment is the conventional and most commonly used processing technique for many foods in order to reduce their

Table 1
Summary of effect of different processing methods on conformation, digestibility and allergenicity of food allergens.

Allergen	Processing methods	Conformational change	Digestibility and allergenic consequence	References
Ara h1 and Ara h2 from peanut	Roasting	Compact globular covalent aggregates and Maillard products (neo-allergen)	Less susceptible to protease and enhanced allergenicity	(Blanc et al., 2011; Maleki & Hurlburt, 2004)
	Boiling	Loss of β barrel with adopting random coil and formation of branched rod-shaped aggregates	More susceptible to hydrolysis and decreased allergenicity	
Wheat protein allergen	Baking	Formation of aggregates through Maillard reaction and inter-peptide linkage	Decreased digestibility and enhanced allergenicity	(Pasini et al., 2001)
β-lg in cow milk	Sterilization	Unfolding followed by covalent aggregation and Maillard reaction	Increased susceptibility to peptic hydrolysis and reduced allergenicity	(Bu et al., 2009; Peram et al., 2013)
	Pasteurization	Exposure of conformational epitopes	Enhanced uptake through epithelium with increased allergenicity	(Bu et al., 2009)
	Heating with wheat matrix	Complex structure formation between wheat and β-lg	Reduced digestibility and bio-availability to immune system	(Bloom et al., 2014)
	High pressure	Unfolding of protein molecule with exposure of cleavage site	Enhanced digestibility and reduced allergenicity	(López-Expósito et al., 2012)
	Radiation	Protein agglomeration	Unaltered	(Lee et al., 2001)
	Ultrasound	Formation of oligomers and β sheet to α helix transition	Increased digestibility but allergenicity is unaltered	(Stanic-Vucinic et al., 2012)
Casein in cow milk	Pasteurization, Sterilization	Rheomorphic, no conformational change	Unaffected	(Morisawa et al., 2009)
Egg ovalbumin	Moist heat	Denaturation and aggregation	Lower permeability through enterocyte resulting in reduced allergenic potential	(Shin et al., 2013; Watanabe et al., 2014)
	High pressure	Loss of conformational and sequential epitopes	Enhanced digestibility and reduced allergenicity	López-Expósito et al., 2008
Egg ovomucoid	Moist heat	Heat stable	Unaltered	(Julià et al., 2007; Shin et al., 2013)
	Heating with wheat flour in pasta	Formation of insoluble aggregates	Reduced allergenicity	(Kato et al., 2001)
Tropomyosin from shrimp	Moist heat	Formation of new allergic compound through Maillard reaction	Digestibility remain unaltered and allergenicity increased	(Kamath et al., 2013)
	High Pressure	Unfolding of protein with loss of α helix	Improved digestibility and reduced allergenicity	(Jin et al., 2015)
	Ultrasound	Denaturation and fragmentation	Increased digestibility but allergenicity remain unaltered	(Li, Lin, Cao, & Jameel, 2006)
Walnut	Moist heat	Fragmentation of protein molecules	Enhanced susceptibility to digestion and reduced allergenicity	(Cabanillas et al., 2014)
Soy allergen (glycicnin)	Moist heat	Formation of soluble aggregates	Slight decrease of peptic digestibility but no change of allergenicity	(van Boxtel, van den Broek, Koppelman, & Gruppen, 2008)
	High pressure	Increased hydrophobicity, SH and α helix content	Increased digestibility and reduced allergenicity	(Penas et al., 2006)

pathogen load, increase shelf life and improve quality. It includes boiling, cooking, baking, roasting, frying, grilling, pasteurization and sterilization. Heat induced structural changes of food proteins have been extensively investigated (Blanc et al., 2011; Rahaman et al., 2015; Verma et al., 2012) and the suggested mechanisms include initial unfolding of a protein molecule, loss of secondary and tertiary structure, formation of intra and/or inter-molecular covalent and non-covalent interactions. Such structural alterations can express, mask or destroy conformational epitopes in food proteins and thereby influence allergenicity, while the sequential epitopes remain unaffected (Fig. 1). Thermal treatment not only affects the allergenicity of food proteins through conformational changes, but also by influencing their interactions with other food ingredients. One such interaction is Maillard reaction. It is a non-enzymatic condensation of Nε-group of amino acid residues (lysine) in protein with the carbonyl group of reducing sugars to form glycosamine (Renzone, Arena, & Scaloni, 2015). These glycosamines undergo further rearrangement to form advanced glycation end products (AGEs). In addition, heating can also alter the susceptibility of proteins to gastrointestinal digestion and their absorption through mucosa and thereby modulate their allergenic potential (Fig. 2).

2.1.1. Heat labile food allergens and their allergenic consequence

Heat induced antigenic changes of beta lactoglobulin (β-lg), a major cow milk allergen, has been summarized in Fig. 3. Heating up to 90 °C causes unfolding of the β-lg molecule, exposure of conformational epitopes and enhanced susceptibility to proteolysis resulting in increased allergenicity (Bu, Luo, Zheng, & Zheng, 2009; Kleber, Krause, Illgner, & Hinrichs, 2004) as determined by *in vitro* ELISA inhibition test with sera from allergic patients. An *in vivo* study (Roth-Walter et al., 2008) detailed the mechanism of higher IgE reactivity as a consequence of pasteurization. Aggregated β-lg could be taken up more extensively by Peyer's patches in the intestinal mucosa, which leads to greater production of IgE although anaphylactic symptoms were elicited only by absorbed soluble β-lg. Thus measuring IgE levels by ELISA may not always reflect accurately the anaphylactic score. This makes it necessary to compare serum level of IgE with anaphylactic degree after oral challenging with processed milk allergens. Raising the temperature further to 100 and 120 °C can lead to irreversible aggregation with covalent and hydrophobic interactions, consequently masking and/or destroying conformational epitopes and reduction of allergenicity. Such heat induced changes showed higher susceptibility to peptic hydrolysis (Peram, Loveday, Ye, & Singh, 2013), which resulted in cleavage of protein sequences with disruption of linear epitopes (Morisawa et al., 2009) consequently weakening their allergenicity. Moreover, heat induced glycation also influenced the allergenicity of β-lg. Most of the β-lg epitopes contain one or more lysine residues and condensation of these lysine residues with reducing sugars (Maillard reaction) during heating results in modification of linear epitopes (Taheri-Kafrani et al., 2009) with significant reduction of IgE reactivity. However, high level of glycation reduced the susceptibility of β-lg to proteolysis resulting in increased IgE reactivity of hydrolysate (Corzo-Martínez, Soria, Belloque, Villamiel, & Moreno, 2010) regardless of the type of reducing sugar. Similar reduction in allergenicity of egg white proteins subjected to heating has also been reported. Heating of egg white at 100 and 120 °C resulted in denaturation and aggregation of ovalbumin (OVA). Although the aggregation showed higher stability to

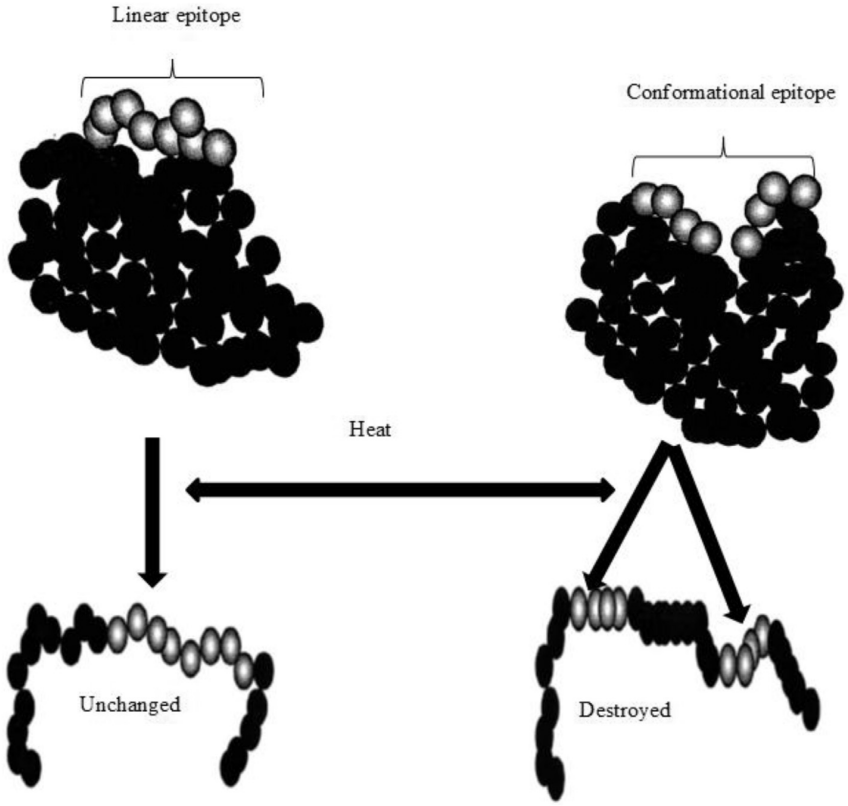

Fig. 1. Schematic diagram of heat induced changes of antigenic epitopes.

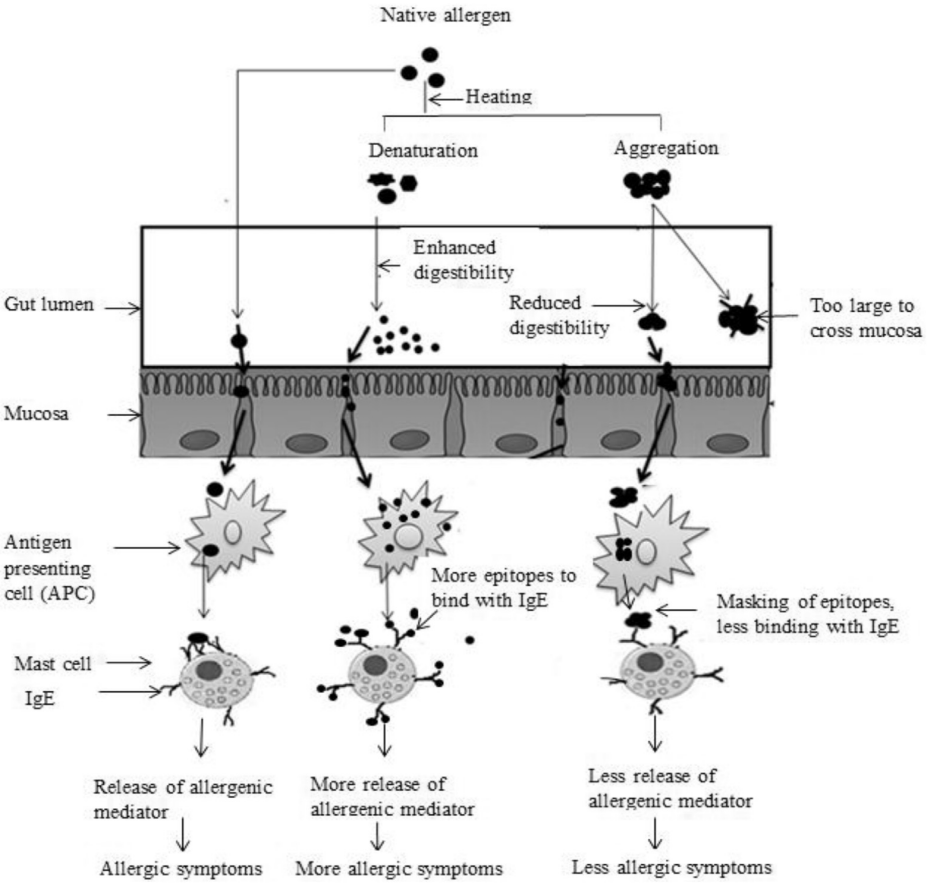

Fig. 2. In general heat induced conformational changes and their consequences in digestion, absorption and immune reactivity of food allergens.

digestion, its lower absorption and poor/delayed sensitivity to immune system resulted in reduced IgE production in mice model (Watanabe et al., 2014).

Conditions such as pH and ionic strength are important factors for heat mediated denaturation of some proteins such as β-lg (Schmitt et al., 2009) and thus can influence related changes in allergenicity. For example, compared to neutral pH, heating of β-lg at acidic pH (pH 3) leads to unfolding of protein molecule, exposure of β strands and partial acid hydrolysis, which could contribute to the appearance of some new epitopes resulting in enhanced antigenicity (Rahaman et al., 2015). Decreased immune reactivity of egg ovomucoid (OM) has been reported to be higher at an alkaline pH (9.5) than at pH 7.5 (Lee et al., 2002) which was attributed to irreversible denaturation of OM with alteration of allergenic epitopes at basic pH.

2.1.2. Heat stable food allergens

Although many food allergens are sensitive to heat and their allergenicity is changed accordingly, some are stable to heat denaturation and digestion (Mills et al., 2009), consequently their allergenicity remains unaltered or can be affected only when exposed to extreme temperatures. A characteristic feature of such proteins is presence of intra or inter molecular cystine that determines their structural integrity as well as antigenic stability (Maeno, Matsuo, & Akasaka, 2013). One such major heat stable allergen is egg OM that is stable to digestion even after boiling for 1 h. Immune reactivity of OM did not decrease after heating regardless of temperature and heating time (Shin, Lee, Ahn, Lee, & Han, 2013). This is attributed to its clearly defined three tandem domains interlinked by strong disulphide bonds (Julià et al., 2007) which allows for maintained solubility after heating rather than aggregating thereby retaining its reactivity to patients' IgE (Kato, Oozawa, & Matsuda, 2001). Tropomyosin, a major allergen in crustacean food (Lopata, O'hehir, & Lehrer, 2010) also exhibits heat resistance, which is attributed to its α-helical coiled-coil dimeric form (Ozawa, Watabe, & Ochiai, 2011). Although α-helical secondary structure of tropomyosin collapsed upon heating above 80 °C, the native structure could be regained upon cooling and consequently its antigenicity measured as binding to specific IgG by ELISA remains unaltered (Usui et al., 2013). Contrary to this, increased binding affinity of tropomyosin to patients' sera IgE after boiling has also been observed (Kamath, Rahman, Komoda, & Lopata, 2013) that has been ascribed to its high content of lysine residue which readily reacts with reducing sugars to form some non-native structures (neo-allergens). Such discrepancies between the studies could be due to use of different antibody, IgG vs IgE. Variation of primary structure, binding pattern and heat response of the epitopes for two different antibodies could also have resulted in these contrary findings.

IgE reactivity of the milk protein casein is also unaffected when milk is heated at 90 °C (Bloom et al., 2014). The four components of casein α_{s1}-, α_{s2}-, β- and κ-casein possess ill defined, disordered mobile structure (rheomorphic) and they lack a co-operative transition of unfolding or partial folding during heating. Also due

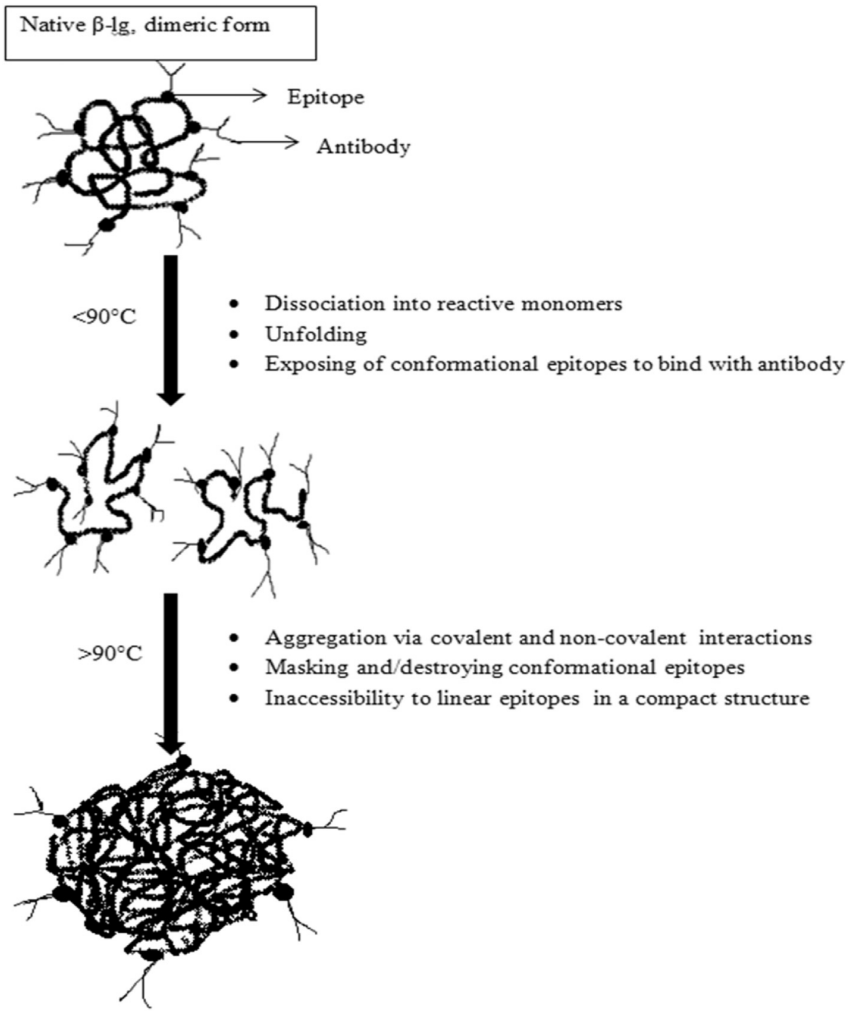

Fig. 3. Schematic model for alteration of epitopes in β-lg to bind with antibody at different level of heat treatment.

to their dynamic structure, casein components contain many linear epitopes rather than conformational that could explain why their immune reactivity remains unaltered upon heating (Mills et al., 2009).

2.1.3. Dry vs moist heat

Stability and allergenic potential of allergens can also be affected by type of heat i.e. dry or moist heat. For example, allergenicity of seed storage globular protein Ara h1 and Ara h2 found in peanuts is enhanced by dry roasting but diminished after boiling in water (Blanc et al., 2011). Upon roasting, Ara h1 undergoes covalent cross linking and hydrophobic interactions and forms compact polymers. Such aggregation makes the protein inaccessible to gastrointestinal digestion to some extent, allowing absorbance of large fragments through intestinal mucosa containing more IgE binding sites resulting in enhanced allergenicity (Maleki & Hurlburt, 2004). In contrast, boiling of Ara h1 formed branched rod-shaped aggregates with loss of some secondary structure and consequently lowers IgE binding ability (Blanc et al., 2011). Likewise significantly higher IgE reactivity of OVA in fried egg compared to boiled egg was also reported (Shin, Han, & Ahn, 2013). However, none of these studies explained the cause of such structural and allergenic variation between roasted and boiled peanut allergens. Variation of temperature arising from the two different methods of heating, mode of heat treatment, as well as variations in moisture content in dry vs moist heat could govern differently the rate of certain interactions (hydrophobic, covalent etc.) in protein molecules that could have influenced their allergenicity. Samadi and Yu (2011) have also reported that dry and moist heating (autoclaving) of soybean to the same temperature (120 °C) resulted in significantly different variations in protein structure, digestibility and consequently allergenicity. Further, it has been shown that (Kroghsbo et al., 2014) roasted peanuts had lower rat basophilic leukemia (RBL) degranulation capacity than blanched peanut whereas extracted Ara h1 from roasted peanut had higher RBL elicitation. Thus, although many studies referred to the allergenic potential of purified allergen rather than that of whole food, the values always do not represent each other possibly due to the effect of other ingredients in the allergic foods. Therefore, it is essential to determine effect of processing on allergenicity of both purified allergens and whole food to obtain a more holistic evaluation for its application in food manufacturing.

Baking, another form of dry heat application can alter conformation, digestibility as well as allergenicity of potential wheat flour

allergens (Petitot, Abecassis, & Micard, 2009). Immunoblotting of *in vitro* digested unheated bread dough, bread crumb and crust with sera from wheat allergic patients resulted in almost complete disappearance of IgE binding protein in dough but showed persistence of such proteins in bread crumb and crust (Pasini, Simonato, Giannattasio, Peruffo, & Curioni, 2001; Simonato et al., 2001). Heat induced covalent protein aggregation of bread crumb (<100 °C) and Maillard reaction, in addition, for bread crust (>180 °C), make the proteins less susceptible to proteolysis thereby allowing passage of large IgE-reactive fragments through gastrointestinal tract where they could elicit more allergic reaction. Interestingly, α-amylase inhibitor protein (responsible for wheat flour inhalation allergy called "baker's asthma") disappeared in digested bread crumb and crust although this protein remained in bread dough even after pancreatic digestion. This could explain why patients suffering from baker's asthma do not show allergic reaction upon ingestion of baked products like bread.

2.1.4. Heat mediated interaction of food allergens with other food proteins and allergenicity

Several studies have established that allergenicity of certain food proteins could be modulated through optimized formulation. Cooking of food proteins with other food matrix can affect gastrointestinal susceptibility and thereby potentially influence their allergenicity (Nowak-Wegrzyn & Fiocchi, 2009). About 50–70% of cow milk allergic children tolerated baked milk products (muffin and waffle) (Nowak-Wegrzyn et al., 2008) and adding baked milk products into the daily diet accelerated development of their tolerance to unheated milk compared to strict avoidance diet (Kim et al., 2011). Whey proteins (β-lg and α-la) of baked milk in muffin and waffle showed significantly lower immune activity with patients' sera by Western blotting than milk heated alone but reactivity of casein remained unaltered (Bloom et al., 2014). Formation of disulphide bonded protein complexes through the interaction of wheat and milk proteins could possibly result in decreased bioavailability of allergic proteins to immune system consequently reducing their allergenicity. Similarly, more than 50% of egg allergic patients tolerated baked egg products with wheat flour in the form of muffins (baked at 176 °C for 30 min) and waffles (baked at 260 °C for 30 min) whereas 19% patients tolerated only heated egg (Lieberman, Huang, Sampson, & Nowak-Węgrzyn, 2012). Antigenicity of egg OM reduced significantly when these were baked with wheat flour at 180 °C for 10 and 30 min and rate of reduction was positively correlated with increase of heating time (Shin, Lee, et al., 2013). Irreversible denaturation of egg proteins due to disulphide exchange reaction with gluten proteins during baking could have resulted in reduced immune reactivity. Thermal treatment of hazelnut proteins alone did not affect the stimulatory activity of basophil of patients with systemic allergy while presence of wheat proteins decreased such activities. Presence of protein rich food matrix such as hazelnut and peanut extract with cow milk and apple allergens can also significantly reduce gastrointestinal digestibility, epithelial transport and thereby reduce their allergenicity (Schulten, Lauer, Scheurer, Thalhammer, & Bohle, 2011). Incorporation of almond flour in chocolate mousse and Victorian sponge cake decreased the enzymatic degradation of almond proteins and their allergenicity (Mandalari et al., 2014).

Thus, it is apparent that matrix proteins create a competitive environment with allergen for enzymatic cleavage as well as active epithelial transport which resulted in insufficient/delayed presentation of allergic protein to immune system that could help in formulating another effective means of managing allergies.

2.2. Non thermal applications

Although thermal application can be used to alter allergenic potential of many foods, sometimes it can have a negative impact on product qualities by changing organoleptic properties, colour and nutrient content (Shriver & Yang, 2011). With non-thermal procedures, food preserves its original characteristics, appears fresher and sometimes is more nutritious than heat treated foods. Thus, alternative non thermal approaches have been experimented and their effects on food allergenicity have been investigated. These procedures include high pressure, ultrasound, gamma radiation, microbial fermentation and enzymatic hydrolysis.

2.2.1. High pressure treatment

High pressure (HP) is an emerging non-thermal technique in food industries to inhibit the growth of microorganism as well as increase the shelf life of food without affecting its organoleptic properties. High pressure techniques mostly affect the non-covalent interactions (hydrogen, ionic and hydrophobic bonds) in protein molecules thereby affecting secondary and tertiary structures and consequently modulating their digestibility and allergenicity. The possible mechanisms for modulation of allergenicity of food proteins through high pressure treatment are summarized in Fig. 4.

2.2.1.1. Susceptible allergens and their response to HP. Combined high hydrostatic pressure (600 MPa) and enzymatic hydrolysis of β-lg significantly reduced its *in vitro* reactivity with IgE in allergic individuals when tested by indirect ELISA (Bonomi et al., 2003). *In vivo* studies (López-Expósito, Chicón, Belloque, López-Fandiño, & Berin, 2012) in sensitized mice also proved that hydrolysate under high hydrostatic pressure (400 MPa) are immunologically inert. HP induces unfolding of β-lg and loss of β sheet and α helix at the expense of unordered structure. Such high pressure induced unfolding facilitated enzymatic digestion resulting in shorter peptide fragments (7–10 residues long, MW < 1.5 KDa) which might not have enough epitopes to react with antibodies leading to reduced allergenicity (Zeece, Huppertz, & Kelly, 2008). In contrast, Chicón, López-Fandiño, Alonso, and Belloque (2008) reported that although HP treatment (up to 400 MPa) enhanced susceptibility to proteolysis, its *in vitro* IgE reactivity did not decrease. Such contradictory findings could be the result of differences in methods of combined HP and enzymatic treatment vis-a-vis enzymatic hydrolysis followed by HP. Moreover, pressure level, holding time and temperature could also be influencing factors that modulate allergenicity of HP treated β-lg.

Similarly, egg OVA treated at 400 MPa (López-Expósito et al., 2008) and Gly 1 allergen in soybean whey at 200–300 MPa (Penas, Préstamo, Polo, & Gomez, 2006) showed much more enhanced peptic digestion and significantly lower *in vitro* reactivity of resulting hydrolysate with specific antibody compared to that treated at atmospheric pressure. However, allergenicity was not totally abolished due to residual antigenic effect of some peptide fragments. Application of high pressure (300 MPa, 15 min) to soy seed during sprouting could be a novel approach to produce hypoallergenic soybean sprouts (Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011). Pressure treatment of squid tropomyosin (Tod p1 allergen) up to 400 MPa (Jin et al., 2015) caused unfolding of protein molecule with conversion of α helix to β sheet, increased free SH and surface hydrophobicity. Such unfolding resulted in exposure of target residues for enzymatic hydrolysis consequently increasing proteolysis and reducing allergenicity. However, further increase of pressure up to 600 MPa caused slight decrease of surface hydrophobicity without remarkable change in proteolysis and hence in allergenicity.

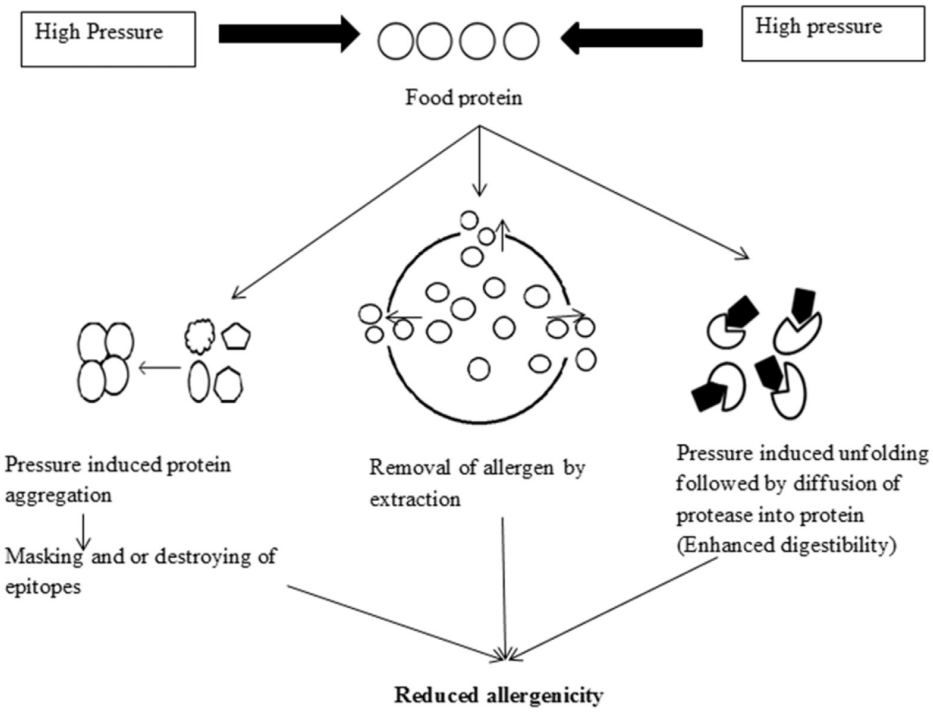

Fig. 4. Mechanisms for reducing food protein allergenicity using high pressure processing.

High pressure treatment up to 200 MPa did not affect the immune reactivity of crushed peanut whereas at 400, 600 and 800 MPa immune reactivity was reduced significantly (Huang, Yang, & Wang, 2014). Dhakal et al. (2014) investigated the effect of high pressure treatment (450–600 MPa, for up to 600 s) on immune reactivity of both conformational and linear epitopes of almond milk using monoclonal antibody against both types of epitopes. High pressure treated almond milk showed total inhibition in binding of conformational epitopes with specific antibody and 50% inhibition for linear epitopes. Pressure induced denaturation, formation of aggregation and loss of solubility caused inaccessibility of antibody to the epitopes.

Dynamic high pressure microfluidization (DHPM) is a new high pressure technique that differs from hydrostatic pressure and involves integrated action of strong shear, high speed-bumping, prompt pressure release and cavitation in the foods being processed. Treatment of β-lg with DHPM below 80 MPa caused increase of antigenicity (Zhong et al., 2011) whereas above 80 MPa allergenicity reduced significantly. DMPH up to 180 MPa caused unfolding of purified Ara h2 from peanut that exposed hydrophobic residues, decreased α helix at the expense of β sheet and caused reduction of S–S bonds resulting in reduced immune reactivity (Hu et al., 2011). However, both these studies used polyclonal antibody against specific proteins to assess the allergenicity of DHPM treated proteins which does not always mimic the original allergenic response. Therefore, there is a need to further investigate the efficacy of DHPM on allergenicity of food allergens using patients' sera *in vitro* as well as by challenging the treated allergens *in vivo*.

2.2.1.2. Some allergens resistant to HP. Allergenicity of apple allergens Mald 1 and Mald 3 under the superfamily of thaumatin-like proteins (TLPs) are least affected by HP (Husband et al., 2011). Eight intra-molecular disulphide bonds contribute to the folding of protein that could also offer resistance to pressure and proteolysis (Marzban et al., 2009). Similarly, cod allergen (Gad m1) (Somkuti, Bublin, Breiteneder, & Smeller, 2012) and largemouth bass allergen (Liu, Tao, Liu, Chen, & Xue, 2012) did not show any change in immunogenic potential after treatment with high pressure. Thus, pressure induced changes in allergenicity largely depend on variation of microstructure in different food proteins.

2.2.2. Radiation

Radiation has been a successful technique for food preservation with minimal alteration in nutritive and sensory characteristics of the food. Radiation brings about conformational changes such as fragmentation, aggregation, cross linking and amino acid modification in food proteins that can modulate their immune reactivity (Luo et al., 2013). Such changes are triggered by free oxygen radicals generated through radiolysis of water during radiation of proteins in solution.

Irradiation (10 and 50 kGy, applied at 10 °C at the rate of 10 kG/h) brought about modification in conformational epitopes found in peanuts that resulted in significantly lowered allergenic cytokine production (IL-4) by splenocytes of sensitized mice (Oh et al., 2009). In another study (Luo et al., 2013), irradiation (1, 3, 5 and 10 kGy at 10 °C) of isolated peanut allergen Ara h 6 and whole peanut extract also caused remarkable decrease in IgG binding activity in ELISA with the increase of irradiation dose. However, up to 5 kGy, IgG reactivity of whole peanut extract was higher than Ara h6 that could be attributed to the effect of other components which could help in protecting the epitopes on Ara h6. A similar trend was also observed (Zhenxing, Hong, Limin, & Jamil, 2007) for irradiated (3–15 kGy, at 10 °C at 1 kGy/h) shrimp. Shrimp protein extract showed decrease of IgE reactivity with increasing dose whereas whole shrimp muscle presented a higher reactivity only up to 5 kGy after which it decreased.

Irradiation of wheat germ agglutinin (WGA) caused initial fragmentation of polypeptide chain followed by formation of large

insoluble amorphous aggregates resulting in lowered allergenicity (Vaz et al., 2012). Dose dependent effect of irradiation on cow milk allergy was investigated by Lee et al. (2001) where they found that up to 5 kGy, the IgE reactivity of isolated β-lg increased and above this dose, the protein underwent agglomeration with masking of some epitopes resulting in reduced allergenicity. In contrast, irradiation (3–10 kGy applied at 13 Gy/min) of liquid and lyophilized cow milk and whey showed increased recognition of anti β-lg IgG (Kaddouri et al., 2008). Such differences could be due to the forms of sample viz. isolated β-lg and β-lg in milk matrix and also type of antibody used. Thus it is apparent that allergenic response of whole food extract and purified allergen after radiation are different which can be of significance to the food industries. Secondly, different studies used different dose rates and hence the effect of various dose rates for a given dosage of radiation on the allergenicity food proteins is not yet clear.

However, irradiation treatment of whole almonds, cashew nuts and walnuts did not alter conformation of allergens as well as their allergenicity (Su, Venkatachalam, Sathe, Teuber, & Roux, 2004). In contrast, negative impact of radiation on gliadin and wheat flour with increased immune reactivity has been reported (Leszczynska, Łącka, Szemraj, Lukamowicz, & Zegota, 2003). Such opposing observation could be due to the variation of transmission media i.e. solid state vs solution which can impact the effectivity of the radiation treatment. In general, radiation of allergens in liquid medium could be the most effective mode of treatment to reduce allergenicity.

2.2.3. Ultrasound

High intensity ultrasound is an emerging technique in food industries, frequently used for homogenization (mayonnaise), filtration (dairy whey solutions and fruit juice), tenderization (meat), and dehydration (fruits and vegetables) processes. High intensity ultrasound uses high energy mechanical waves (20–100 kHz) which induces cyclic generation and collapse of cavities (sonication bubbles) followed by formation of localized region of high pressure and temperature surrounding these collapsed cavities which can bring about conformational changes to food proteins and thereby influence their allergic reactivity (Shriver & Yang, 2011).

There was no effect of high intensity ultrasound (30 Hz, 800 W) at 0 °C on *in vitro* (ELISA) IgE reactivity of a shrimp protein extract whereas at 50 °C reactivity reduced by 2.5 fold (Zhenxing, Caolimin, & Jamil, 2006). Such differences was possibly due to the heat induced decrease in viscosity of sample matrix which allowed better penetration of ultrasound into the food system resulting in conformational changes as well as reduction of allergenicity compared to ultrasound treatment alone. However, so far there are no reports on the digestibility of ultrasound treated shrimp allergens and allergenic consequence of resulting hydrolysates. Also, all the studies are limited to *in vitro,* and hence, *in vivo* studies with oral food challenge are required for proper assessment. Exposure of β-lg to high intensity ultrasound can lead to formation of non-native oligomers and dimers, β-sheet to α helix conversion, and exposure of tryptophan (Trp) residues providing a less compact and more dynamic structure compared to untreated β-lg. Although these conformational changes enhanced the susceptibility of β-lg to peptic hydrolysis, no remarkable change was observed in patients' sera IgE reactivity, basophil activation test and skin prick test (Stanic-Vucinic et al., 2012). Ultrasound treatment alone reduced IgE-binding affinity of major peanut allergens Ara h1 and Ara h2 moderately (by about 10%) whereas ultra-sonication followed by protease digestion (trypsin–alpha chymotrypsin) significantly increased the solubility of proteins and decreased IgE reactivity (Li, Yu, Ahmedna, & Goktepe, 2013).

2.2.4. Fermentation

Fermentation is one of the traditional techniques for processing and preservation of food. Microbial enzymatic hydrolysis of food proteins during fermentation produces some bioactive peptides with potential health benefits such as anticancer and antihypertensive properties (Sah, Vasiljevic, McKechnie, & Donkor, 2014) while destroying some antigenic epitopes resulting in decreased allergenicity (Fotschki, Szyc, & Wróblewska, 2015). Moreover, consumption of probiotics in fermented foods can influence secretion of certain type of cytokines that could also affect allergenicity through immune-modulatory mechanism (Bu, Luo, Zhang, & Chen, 2010).

Lactic acid bacteria (LAB) (*Lactobacillus acidophilus* 5622, 20243, 20242 and *Lactobacillus kefiri* 20587) can potentially reduce (measured by indirect ELISA using polyclonal antibody) the antigenic response of β-lg by 70% in sweet whey and more than 90% in skim milk (Kleber, Weyrich, & Hinrichs, 2006) compared to unfermented milk. Usage of co-culture (*Streptococcus thermophilus* subsp. *salivarius*) synergistically accelerated the hydrolysis of β-lg resulting in further splitting the linear epitopes and reducing their antigenic potential. *In vitro* IgE-binding affinity of α-la, β-lg, α- and β-casein decreased by 55.12%, 49.66%, 2.62% and 2.02% respectively, when reconstituted skim milk was fermented with *Lactobacillus casei* (Shi et al., 2014). Combining strains of *Lactobacillus bulgaricus* with *Lactobacillus helveticus* caused much higher reduction in allergenicity of α-la and β-lg in skim milk (Bu et al., 2010) compared to the individual strains alone. The extent of changes in antigenicity also depends on the fermentation time. Antigenicity gradually decreased from 0 to 12 h of fermentation and slightly increased thereafter (Bu et al., 2010) which was attributed to breaking down of oligopeptides by bacterial peptidase into smaller fragments with exposure of some hidden epitopes. Fermentation with *L. helveticus* (Ehn, Allmere, Telemo, Bengtsson, & Ekstrand, 2005) did not affect the reactivity of β-lg with IgE although it caused 80% hydrolysis, indicating strain specificity in the allergenic potential of LAB. Thus, pre-treatment such as heating, selection of bacterial strain and fermentation time are the important factors affecting changes of milk protein allergenicity by fermentation. All these studies assessed changes in antigenicity of milk allergens as a result of their partial hydrolysis during fermentation, however, further changes during gastrointestinal digestion, absorption through mucosa and final allergenic consequences have not been studied. Thus, oral challenges with fermented products and resulting anaphylaxis needs to be further investigated.

Lactobacillus fermentation of sourdough caused acidification and reduction of disulphide bonds of gluten resulting in increased activity of cereal proteases, which improved the digestibility of gluten by the consumers (Gänzle, Loponen, & Gobbetti, 2008). *In vitro* digestion of fermented sourdough bread showed almost complete disappearance of potential peptides to induce gut associated T cell mediated immune response (celiac disease) due to pre-proteolytic activity by selected LAB which resulted in tolerance of gluten by 80% patients (Di Cagno et al., 2004).

2.2.5. Enzymatic hydrolysis

One of the common characteristics of food allergen is their resistance to gastrointestinal digestion; therefore pre-hydrolysis with enzymes is one of the most effective methods of modifying immune reactivity of food proteins. As a result of hydrolysis conformational epitopes rapidly collapsed whereas linear epitopes are cleaved and their further existence depends on the degree of hydrolysis and type of enzyme used (Sabadin, Villas-Boas, de Lima Zollner, & Netto, 2012).

Many studies have been performed to assess the allergenicity of hydrolysed milk or whey protein concentrate and all showed

significantly lower reactivity of resulting hydrolysate with serum from allergic patients (Duan, Yang, Li, Zhao, & Huo, 2014; Sabadin et al., 2012). Although extensively hydrolysed whey and casein formulae and amino acid preparations were tolerated by most of the allergic patients without any reactions, allergic reactions such as atopic dermatitis have been reported for several individuals (Meulenbroek et al., 2014). Such reactions of cow milk allergy patient to hydrolysed formula are attributed to the residual antigenicity of the small peptides (Bu, Luo, Chen, Liu, & Zhu, 2013). There is no straight relationship between molecular mass of peptides in hydrolysate and their residual allergenicity. Peptides with molecular weight of 3000 Da (Bu et al., 2013) or even smaller peptides (Puerta, Diez-Masa, & de Frutos, 2006) can provoke allergic reaction. Such discrepancies could be ascribed to the type of enzyme and hydrolysis model used degree of hydrolysis and sensitivity of the patients. Whey protein hydrolysate prepared with alcalase enzyme significantly lowered immune reactivity of α-la and β-lg (Wróblewska et al., 2004) and the effectivity can be maximized by controlling pH, temperature and enzyme-substrate ratio (Zheng, Shen, Bu, & Luo, 2008). Peptic and tryptic hydrolysis of heated whey protein had significantly lower allergenicity than the unheated hydrolysate (Kim et al., 2007). Heat induced unfolding of protein molecule with exposure of cleavage sites for enzymes resulting in enhanced proteolysis, destruction of epitopes and thereby reduction of allergenicity could be the most plausible explanation.

Peptic hydrolysis of soybean 2S protein only slightly reduced the allergenicity while chymotrypsin hydrolysis following peptic hydrolysis increased the allergenicity (Sung, Ahn, Lim, & Oh, 2014). Thus, allergenic epitopes of soybean 2S protein may not be fully destroyed after peptic digestion and chymotrypsin treatment possibly due to exposure of some hidden epitopes.

3. Conclusion

Different processing approaches affect physico-chemical properties of food proteins in different ways which in turn influence their gastrointestinal digestion, bioavailability and allergenicity. Inherent molecular characteristics of allergen, type of method used in processing, intensity of treatment, environment condition (pH, ionic strength etc.) and food matrix structure largely influence the conformational changes related to digestibility and allergenicity of food proteins. In thermal processing, moist heat usually reduces the allergen reactivity of food proteins by changing protein structure, alteration of IgE binding conformational epitopes and increased digestibility. In contrast, dry heat such as baking of wheat flour and roasting of nuts most often leads to formation of new epitopes (neo-allergens) via Maillard reaction and reduction of digestibility resulting in increased allergenicity. Reduction of allergenicity by non-thermal techniques is attributed to conformational change of protein with enhanced susceptibility to proteolysis. High pressure followed by enzymatic hydrolysis appears to be one of the effective approaches in minimizing allergenicity of many foods. Microbial fermentation and selective enzymatic hydrolysis can break the sequence of linear epitopes and extensively reduce the allergenic potential of dairy proteins.

Although total elimination of allergenic potential by processing is unlikely, minimization of elicitation threshold could be achieved through selection of proper conditions. Moreover, development of tolerance to allergic individuals by challenging hypoallergenic processed foods may also be an alternative therapeutic strategy. Although many studies have linked processing induced change of IgE reactivity of food proteins to their allergenic potentials, this change always does not translate to clinical symptoms. Thus, other assessments such as release of allergic mediator (histamine and cytokines), oral challenge and skin prick test also need to be performed before concluding the effect of any processing method on allergenic potential of any food protein. Increased understanding of the impact of various processings on structure, digestibility and allergenic consequence of food allergens could be applied at industrial level to develop novel processing strategies aimed at reducing the prevalence of food allergies.

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Chapter 3

Conformational changes of β-lactoglobulin induced by shear, heat, and pH—Effects on antigenicity

This chapter has been published as the title "**Conformational changes of β-lactoglobulin induced by shear, heat, and pH—Effects on antigenicity**" by Rahaman, T., Vasiljevic, T., & Ramchandran, L in the peer review journal, Journal of Dairy Science, 98, 4255–4265, 2015

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: Md. Toheder Rahaman

Signature: [redacted]

Date: 30/03/2016

Paper Title:

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In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Md. Toheder Rahaman	80	Designing and conducting the experiment, data collection and manuscript writing
Todor Vasiljevic	10	Concept development, experiment designing, data analysis and manuscript editing
Lata Ramchandran	10	Concept development, experiment designing, manuscript editing and submission for publication

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

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Conformational changes of β-lactoglobulin induced by shear, heat, and pH—Effects on antigenicity

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ABSTRACT

Structural modifications influence the immune-reactivity of food proteins. We investigated effects of pH (3, 5, 7), temperature (80, 100, 120°C), and shear (100, 500, and 1,000 s^{-1}) on conformational changes (monitored by surface hydrophobicity, total thiol content, Fourier transform infrared spectroscopy, and gel electrophoresis) and their relation to antigenicity (determined by indirect ELISA) of β-lactoglobulin (β-LG). Overall, heating at low pH (3) caused unfolding of proteins and fragmentation due to partial acid hydrolysis and thereby exposed β-strands that contributed to appearance of some hidden epitopes, resulting in higher antigenicity. Heating at pH 5 and 7 decreased the allergenic response due to covalently bonded molecular polymerization and aggregation, which destroyed or masked some epitopes. Shear alone had no effect on the antigenic response of β-LG but may have an effect in combination with pH or temperature. Overall, heating β-LG solutions to 120°C at pH 5 with shearing (100–1,000 s^{-1}) resulted in minimal antigenicity. Structural modifications of β-LG via denaturation or disulfide- or thiol-mediated interactions can either enhance or decrease its antigenicity.
Key words: antigenicity, shear, heat, pH, conformation

INTRODUCTION

Cow milk allergy (**CMA**) is one of the most common childhood food allergies, affecting about 2% of newborns under 2 yr of age (Bossios et al., 2011). Several proteins in cow milk can stimulate allergic reactions, such as caseins, α-LA, β-LG, and BSA. Of these, β-LG is considered the main allergen for children sensitive to milk protein (Bu et al., 2009), being responsible for allergic reactions in 60 to 80% of CMA patients. The resistance of β-LG to proteolysis by gastric enzymes means that it reaches the intestine intact, and thus has the potential to cause allergic reactions (Zhong et al., 2011). Such allergenicity limits the consumption of milk by certain groups of consumers and is a disadvantage in the manufacturing of infant milk products. Measuring the antigenic response of a protein by ELISA is a common method to measure the allergenicity of a protein ingredient.

Allergic reactions are triggered by parts of protein called epitopes that bind with specific antibodies and provoke adverse immune reactions (Lehrer et al., 1996). In β-LG, both conformational epitopes (discontinuous AA sequences whose function is based on the tertiary or quaternary structure of protein) and linear or sequential epitopes (continuous sequences of AA that determine the primary structure of protein) contribute to the antigenic response (Kleber et al., 2004). Therefore, modifying these epitopes in proteins may modulate the antigenic responses of milk proteins.

Various methods have been adopted to destroy or modify such epitopes in β-LG. These include heat treatment to temperatures above 90°C (Davis and Williams, 1998; Fritsche, 2003), combination of heat and pressure treatments (Zhong et al., 2011), enzymatic hydrolysis (Ena et al., 1995; Heyman, 1999; Wróblewska and Troszyńska, 2005), microwave treatment (Grar et al., 2009), gamma radiation (Lee et al., 2001), and exposure to high-intensity ultrasound (Stanic-Vucinic et al., 2012). All of these studies have attributed changes in antigenicity to alterations in the secondary and tertiary structure of the protein caused by processing. Specifically, the degree of unfolding, content of β-strands, and formation of intermolecular disulfide bonds are linked to increases and decreases of protein antigenicity (Zhong et al., 2012).

Reports to date have focused on some physical as well as enzymatic treatments of β-LG and its subsequent structural deformation associated with antigenic potentiality; however, no reports have outlined the effects of combinations of different conditions, such as pH and temperature, on conformational changes of β-LG and their relation to antigenicity. Further, food proteins are exposed to shear stress during many common manufacturing processes such as centrifugation, mixing, ho-

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mogenization, ultrafiltration, and microfiltration (Yim and Shamlou, 2000). Shear stress is also known to alter the structure of native protein molecules (Bekard et al., 2011), which, in turn, could affect functional properties of proteins and their susceptibility to enzymatic digestion. However, there are no reports on shear-induced conformational changes of β-LG and their effects on antigenicity. Thus, a gap exists in our understanding of the combined effects of various processing conditions, including temperature, pH, and shear, on the structure of β-LG and consequences of changes in processing conditions on its antigenicity. Therefore, the objective of this study was to establish effects of different shear, pH, and temperature conditions on conformational changes of β-LG in relation to perceived antigenicity.

MATERIALS AND METHODS

Preparation of Protein Samples

Samples of β-LG (85% purity) containing 96% protein (as analyzed in the laboratory) were provided by Davisco Co. Ltd. (Le Sueur, MN). A 0.3% (wt/vol) solution of β-LG was prepared in citric acid-phosphate buffer at different pH values (3, 5, or 7). Citric acid-phosphate buffers at pH 3, 5, and 7 were prepared by mixing appropriate volumes of 0.1 *M* citric acid and 0.2 *M* Na_2HPO_4 and contained 0.02% sodium azide to prevent microbial growth. A solution of β-LG prepared in milliQ water (Millipore Inc., Billerica, MA) served as the control (pH 7.6). The concentration chosen was similar to the reported concentration of β-LG in milk (Dupont et al., 2013).

Treatment of Samples

Each prepared sample of β-LG was subjected to different shear rates (100, 500, or 1,000 s^{-1}) and temperatures (80, 100, or 120°C) in a pressure cell (CC25/PR-150) of a rheometer (Physica MCR 301 series; Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with constant pressure of 250 kPa following the method of Liyanaarachchi et al. (2015). Samples at control pH (7.6) not subjected to shear were the controls under each condition of sample preparation. An aliquot of 15.14 mL of each sample at 20°C was poured into the measuring cup of the pressure cell. The samples were then heated at 2°C/min to the required temperature, held for 1 min at that temperature, and then cooled at 2°C/min to 20°C. Fixed shear rate was applied throughout the period. Samples were then taken immediately for Fourier transform infrared (**FTIR**) analysis. The remaining portion was divided and stored at −80°C for subsequent analysis. The aliquot for SDS-PAGE analysis was stored in SDS sample buffer.

Determination of Total Thiol Content

Total thiol (**SH**) content was determined according to the method of Sava et al. (2005). The control and treated samples of β-LG solutions were diluted 10 times with standard buffer (0.086 *M* Tris, 0.09 *M* glycine, 4 m*M* Na_2-EDTA, and 8 *M* urea; pH 8). Ten microliters of Ellman's reagent [5,5′-dithiobis-2-nitrobenzoic acid (DTNB); 4 mg/mL of standard buffer] was added to 1 mL of diluted sample, vortexed, and incubated for 2 min. The absorbance was measured at 412 nm at room temperature with a UV spectrometer (Libra S11, Biochrom, Cambridge, UK), using standard buffer as a blank. The absorbance value was divided by molar extinction coefficient of 13,600 to obtain the thiol content, expressed as micromoles per gram of protein.

Determination of Surface Hydrophobicity

Surface hydrophobicity was determined fluorometrically using 8-anilino-1-naphthalenesulfonic acid (**ANS**; Sigma Aldrich, St Louis, MO) as a fluorescence probe, according to the method of Alizadeh-Pasdar and Li-Chan (2000) with some modifications. A stock solution of ANS was prepared in 0.1 *M* phosphate buffer (pH 7.6) and stored in a screw-capped bottle, wrapped with aluminum foil to prevent exposure to light. The treated and untreated samples of β-LG were diluted in respective pH buffer to the concentration range of 0.0002 to 0.0012%. To 4 mL of diluted β-LG solution was added 20 μL of ANS stock solution, and the mixture was vortexed and incubated for 15 min in dark. Relative fluorescence intensity (**RFI**) was measured at excitation and emission wavelengths of 390 and 470 nm, respectively, using a Shimadzu fluorescence spectrophotometer (model-RF5301PC, Shimadzu Corp., Kyoto, Japan). The RFI of protein dilution blank (without ANS) was also measured and subtracted from that of corresponding protein dilutions with ANS to obtain net RFI. The initial slope of net RFI versus protein concentration was calculated by linear regression analysis and used as an indicator for surface hydrophobicity.

PAGE

Both reducing and nonreducing SDS-PAGE were performed to study the status of protein in control and treated samples. For nonreducing SDS-PAGE, 400 μL of sample was mixed with 600 μL of SDS sample buffer [500 mL of MilliQ water, 125 mL of 0.5 *M* Tris-HCL

buffer, pH 6.8; 100 mL of glycerol, 200 mL of 10% (wt/vol) SDS solution, and 25 mL of 0.4% (wt/vol) bromophenol blue solution]. For reducing SDS-PAGE, in addition, 50 μL of β-mercaptoethanol was added to the SDS sample buffer and the solution heated for 5 min at 90°C. A 1% solution of β-LG and BSA in distilled water, suitably diluted with SDS sample buffer, was used as protein standard. Broad-range unstained standard from Bio-Rad Laboratories (Gladsville, NSW, Australia) was used as a molecular marker. Molecular weight markers (15 μL), β-LG and BSA standards (8 μL each), and samples (10 μL) were loaded onto a Mini-Protean TGX Any KD (Bio-Rad Laboratories) precast gel. The gels were placed in a Bio-Rad Protean II xi cell filled with Tris-glycine-SDS buffer. Electrophoresis was performed at 50 mA, 180 V, and 6.5 W. The gels were stained by immersing the gels in staining solution (0.025% Coomassie Brilliant Blue, 40% methanol, 7% acetic acid, and 50% distilled water) overnight. Then, the gels were transferred into destaining solution (40% methanol, 7% acetic acid, and 53% distilled water) for 2 h followed by immersion in distilled water until the background became clear. Gel images were taken using a ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories)

FTIR

About 15 μL of each sample was placed between a pair of CaF_2 discs. Sample-containing discs were set on a sample holder assembly that was then put into the sample compartment of FTIR instrument (IRAffinity-1, Shimadzu Corp.). The FTIR spectrum was obtained at room temperature from 20 scans at a resolution of 4 cm^{-1} using Happ Genzel apodization. The spectrum thus obtained was smoothed by 5 points to reduce the noise and deconvoluted using IRSolution software (Shimadzu Corp.). To observe the effect of treatments, the final spectra were obtained after subtracting the spectra obtained for each of the treated samples from the spectra for corresponding sample buffers.

Determination of Antigenicity

Antigenicity of all samples (control and treated) was determined by sandwich ELISA using the bovine β-lactoglobulin ELISA quantitation kit (Bethyl Laboratories Inc., Webster, TX). Individual wells of a 96-well microtiter plate (Nunc Maxisorp C bottom well) were coated with capture antibody diluted in 0.05 *M* carbonate-bicarbonate coating buffer. After a 1-h incubation at room temperature, the plate was washed 5 times with ELISA wash solution (50 m*M* Tris, 0.14 *M* NaCl, 0.05% Tween 20, pH 8). Then, the wells were filled with 200 μL of blocking solution to block the residual free binding sites in the wells. After a 30-min incubation at room temperature, the blocking solution was drained and the plate was washed 5 times to remove unbound antibody. Thereafter, 100 μL of standards or samples diluted in sample diluent was added to each well, incubated for 1 h followed by washing the plate. Then, 100 μL of horseradish peroxidase-conjugated β-LG-detecting antibody was added to each well. After a 1-h incubation and subsequent washing, the wells were filled with 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and color was allowed to develop for 15 min in the dark. The reaction was stopped by adding 0.18 *M* H_2SO_4 and absorbance measured on a microplate reader (iMark, Bio-Rad Laboratories). The antigenicity was calculated from the standard curve as equivalent nanograms of protein per milliliter of sample.

Statistical Analysis

The experiment was designed as a randomized, blocked split-plot design with pH as the main plot and shear and temperature as the subplots. This block was replicated with 3 subsamplings. The results of the various determinations were analyzed using the GLM procedure of SAS (SAS Institute, 1996), establishing effects of main factors (pH, temperature, shear) and their interactions. The level of significance was set at $P = 0.05$.

RESULTS AND DISCUSSION

Total Thiol Content

Among the whey proteins of milk, β-LG contains 5 cysteine residues. Four of these, Cys^{106}-Cys^{119} and Cys^{66}-Cys^{160} form 2 disulfide (SS) bonds, whereas Cys^{121} remains as a free thiol (SH) group (Sava et al., 2005). The amount of free SH groups present can be influenced by modifying the conditions that influence the structural arrangement of β-LG. Our results indicated that different treatment conditions had varying effects on the total thiol content of the samples (Figure 1). Overall, thiol content was affected not only by pH, temperature, or shear alone ($P < 0.0001$), but also by the interaction of pH with shear and with temperature ($P < 0.0001$) and by the interaction of all 3 parameter ($P = 0.0159$). On average, the total thiol content of freshly prepared solution of β-LG (control, pH 7.6) was 46.5 μmol/g of protein, which is close to the reported values for native β-LG (50.9 μmol/g of protein; Sava et al., 2005). Adjusting the solution pH to 7 did not result in any major change in thiol content (46.4 μmol/g of protein), whereas lowering the pH to 5 or 3 decreased

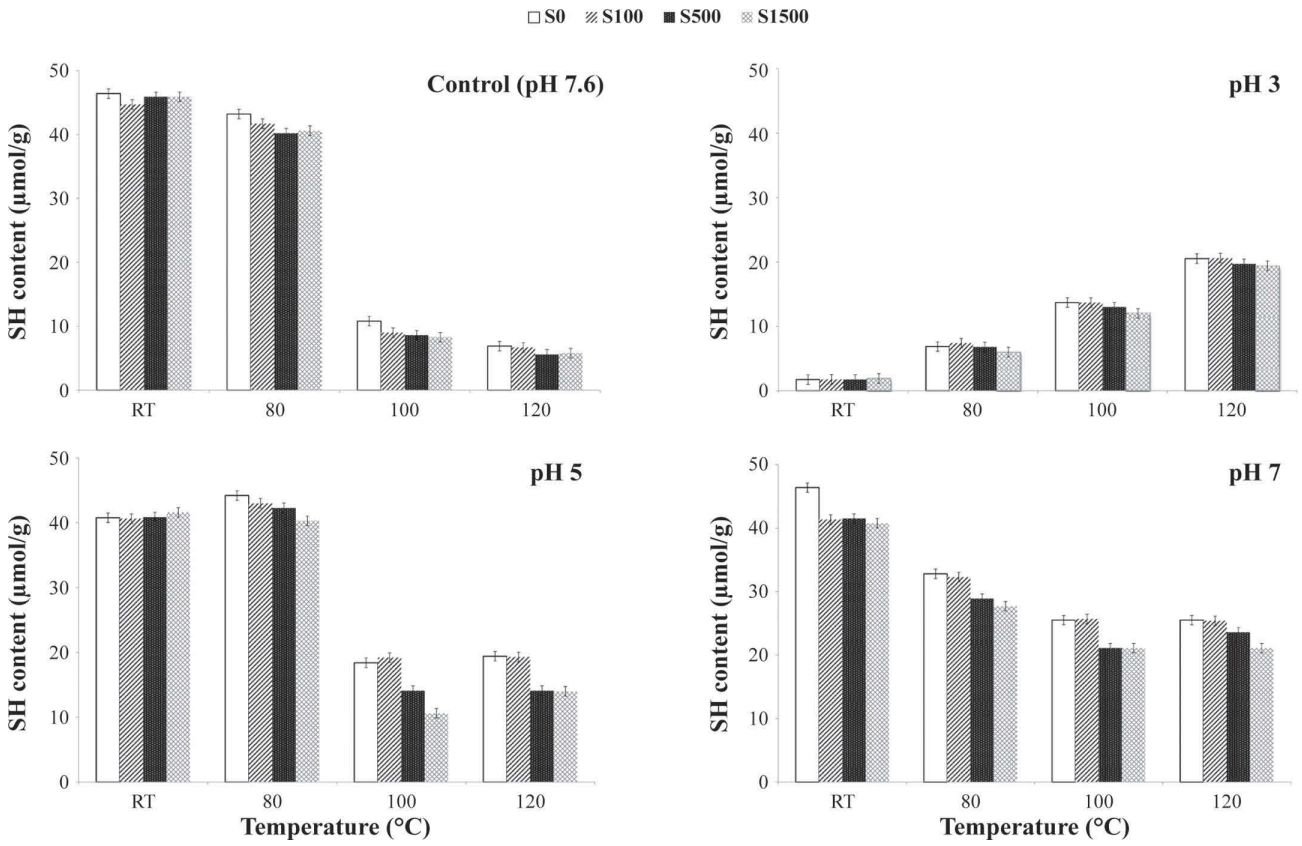

Figure 1. Thiol (SH) content of β-LG solutions at different pH [pH 7.6 (control), 3, 5, or 7], temperature [room temperature, RT (~22°C; control), 80, 100, or 120°C], and shear [S; 0 (control), 100, 500, or 1,000 s^{-1}]. Error bars indicate standard error of the mean (SEM = 0.741).

($P < 0.05$) thiol content to 40.7 and 1.7 μmol/g of protein, respectively (Figure 1). The structural changes that could have occurred due to the low pH may have reverted upon changing the pH during analysis of thiol groups. Therefore, the minimum thiol content observed at pH 3 could be due to lower exposure, slow reactivity, and lesser accessibility of thiols to DTNB at acidic pH (Wada et al., 2006).

Heating β-LG caused variable changes in thiol content depending on the pH (Figure 1). At control pH, the total thiol content decreased ($P < 0.05$) as the temperature increased, with a substantial change occurring between 80 and 100°C. At pH 7, the thiol content declined following the temperature increase to 100°C, after which it remained steady. The trend was similar at pH 5, although no change in thiol content was observed beyond 100°C. Interestingly, the trend was reversed at pH 3: thiol content increased with the increase in temperature. Such an increase in thiol content could be attributed to unfolding as well as fragmentation of β-LG by acid hydrolysis at pH 3 and higher temperature. Dissociation of dimers and partial unfolding of β-LG could have exposed reactive SH groups at pH 7 but not at pH 5 or 3, which could explain, in part, the lower thiol content at a lower pH. Participation of reactive SH groups in thiol-thiol oxidation or thiol-disulfide exchange reactions contributing to the heat-induced aggregation could account for the decrease ($P < 0.05$) in thiol content when the temperature was increased above 80°C at control pH and pH 5 and 7. The reversed trend of changes in thiol group content with temperature observed at pH 3 could be due to exposure of certain thiol groups but lack of participation in thiol-disulfide–mediated polymerization at elevated temperature due to their lowered reactivity in an acidic environment (de la Fuente et al., 2002; Wada et al., 2006). In addition, β-LG has a positive charge at pH 3, which would further hinder interactions between individual molecules due to electrostatic repulsion (de la Fuente et al., 2002). These observations are similar to those of Monahan et al. (1995), Visschers and de Jongh (2005), and Mudgal et al. (2011).

Surface Hydrophobicity

The hydrophobicity of β-LG was markedly affected by different pH values and temperatures (Figure 2), with shear ($P < 0.05$) becoming an important factor at >80°C and at low (100 s^{-1}) or high (1,000 s^{-1}) shear rates. Interestingly, a decline of average hydrophobicity values coincided with an increase in shear rate under different temperatures and pH (Figure 2). The opposite observation was made at pH 3. Heat-induced initial aggregation through nucleation-mediated coagulation pathway (Dunstan et al., 2009) is modulated by shear. Such a shear-modulated aggregation may lead to a structural rearrangement that would render some hydrophobic binding sites unavailable, resulting in decreased hydrophobicity because of greater shear at pH 5 and 7.

Overall, the highest hydrophobicity was observed in samples adjusted to pH 3, whereas those adjusted to pH 5 exhibited the lowest values, irrespective of temperature. These observations are in line with those of Shimizu et al. (1985) and Alizadeh-Pasdar and Li-Chan (2000). Denaturation of β-LG molecules leading to exposure of hydrophobic AA residues could explain the greater hydrophobicity of samples adjusted to pH 3. The prevalence of positive charges on β-LG at pH 3 could prevent aggregation due to electrostatic repulsion, which could further account for increased hydrophobicity when the shear rate was increased. In addition, partial acid hydrolysis of β-LG at pH 3 and higher temperature, as shown by SDS-PAGE, might expose new hydrophobic residues, resulting in increased hydrophobicity. In contrast, because pH 5 is near the isoelectric point of β-LG, the tendency of proteins to interact and aggregate could explain the very low values of hydrophobicity observed at this pH.

At a given pH, hydrophobicity generally increased with increasing temperature. However, this observation could not be applied to the samples adjusted to pH 5. Instead, at pH 5, hydrophobicity declined ($P < 0.05$) as the treatment temperature increased—an observation that coincides with the observations of Monahan et al.

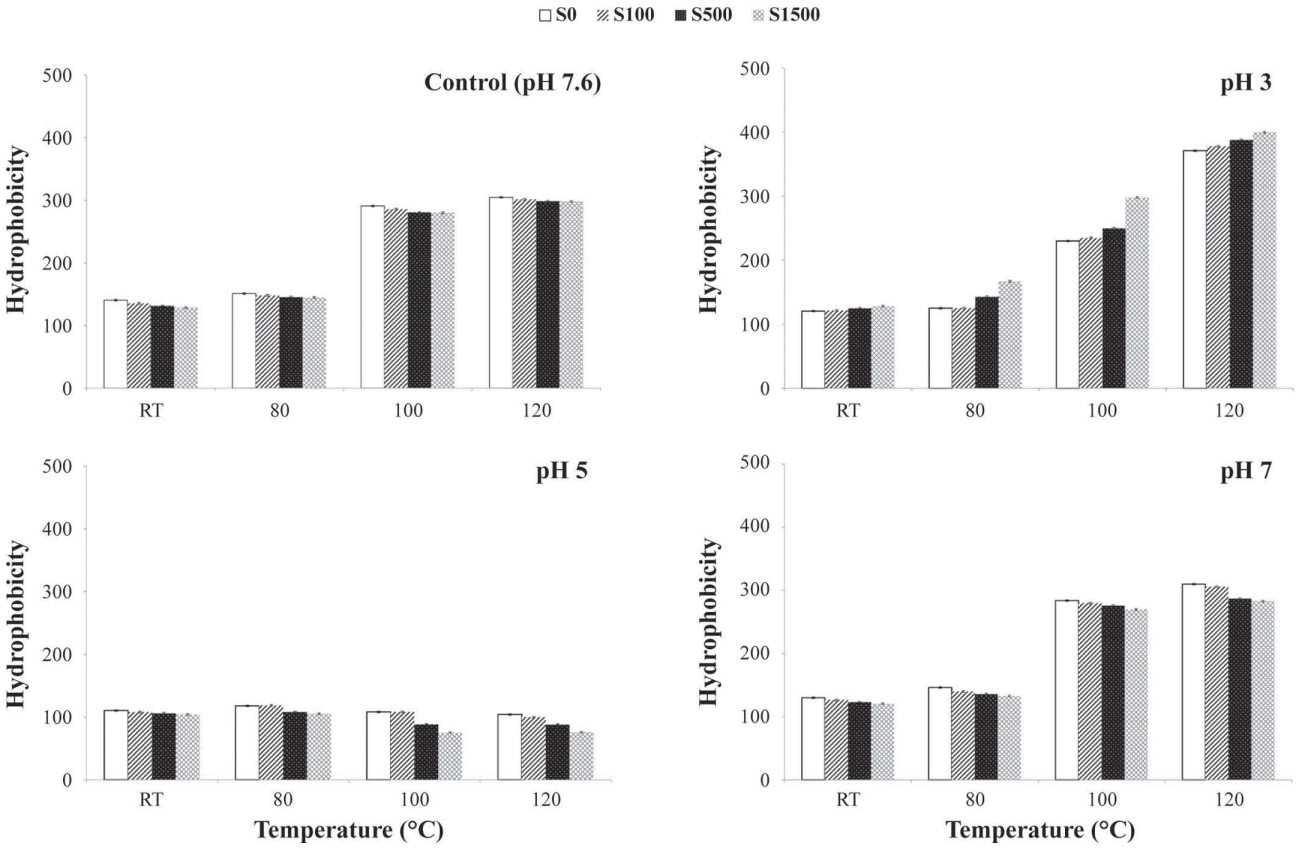

Figure 2. Effect of treatment pH [pH 7.6 (control), 3, 5, or 7], temperature [room temperature, RT (~22°C; control), 80, 100, or 120°C], and shear [S; 0 (control), 100, 500, or 1,000 s^{-1}] on surface hydrophobicity of β-LG. Error bars indicate standard error of the mean (SEM = 1.286).

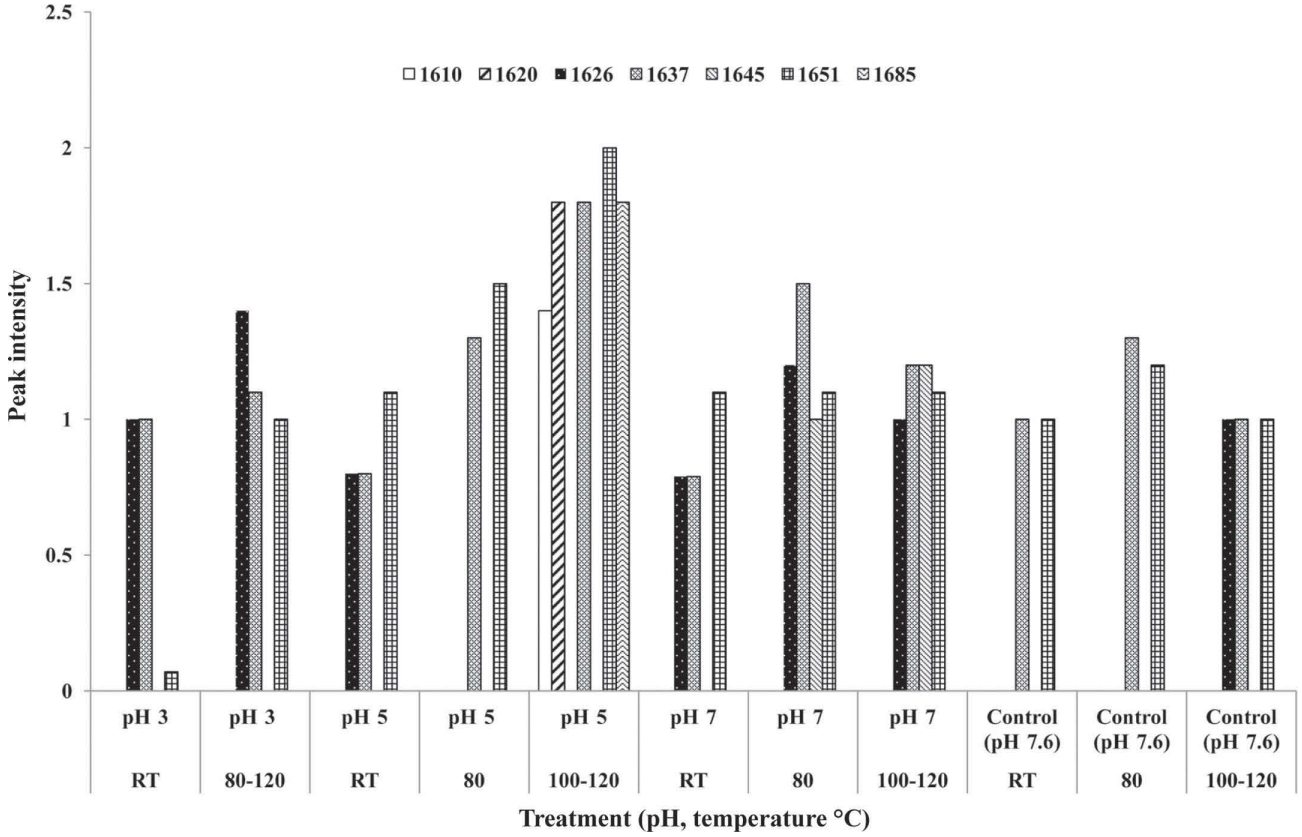

Figure 3. Average peak intensity at specific wave numbers (1,610 to 1,685 cm^{-1}) obtained from interferograms for β-LG treated at different pH and temperatures and their corresponding controls. RT = room temperature (~22°C).

(1995), who also observed decrease in hydrophobicity of whey protein isolate heated at pH 5. Extensive intermolecular disulfide bonding of β-LG at higher temperatures and pH 5 (as observed in SDS-PAGE) could have resulted in burial of hydrophobic AA residues, thereby resulting in decreased hydrophobicity values. Although disulfide-mediated polymerization can occur at higher temperatures at both pH 5 and pH 7 (de la Fuente et al., 2002), isoelectric precipitation could have preceded this polymerization and aggregation at pH 5, which explains the lower hydrophobicity values at pH 5 than at pH 7.

FTIR

The effect of pH and temperature on the secondary structures of β-LG was ascertained by deconvoluted FTIR interferograms (Figure 3). Overall, we did not observe any major variations in peak intensities in the amide I range (1,600–1,700 cm^{-1}) that would indicate structural changes as a function of shear. However, marked variations in amide I bands were exhibited by β-LG samples subjected to different pH and temperatures. Deconvoluted amide I interferograms showed peaks between 1,608 and 1,610 cm^{-1} (corresponding to side-chain residues of AA), 1,626 to 1,627 cm^{-1} (exposed β-sheet), 1,645 to 1,647 cm^{-1} (related to unordered structures), 1,649 to 1,654 cm^{-1} (defining α-helix), 1,681 to 1,685 cm^{-1} (intermolecular β-sheet aggregation), and 1,693 cm^{-1} (representing β-turns and β-bends; Casal et al., 1988; Lefèvre and Subirade, 2000; Bhattacharjee et al., 2005).

Untreated β-LG in milliQ water (control pH) showed 2 major peaks, representing intramolecular β-sheet (1,637 cm^{-1}) and α-helix (1,651 cm^{-1}) structures. Heating at control pH did not show any change in the profile up to 80°C, with only an increase in peak intensity at 80°C indicating minor structural modifications. However, at 100 to 120°C, another peak appeared at 1,626 cm^{-1} related to additional exposure of β-sheets that is indicative of partial unfolding of β-LG. This extra peak at 1,626 cm^{-1} also appeared when solutions of β-LG adjusted to pH 3 were exposed to higher temperatures (80 to 120°C). This finding suggested minimal confor-

mational modifications during heating at pH 3, which further explains the observations of changes in hydrophobicity values (Figure 2).

However, at pH 5, increasing the temperature to 80°C resulted in a similar FTIR spectrum as in control β-LG, only with greater intensity. At 100 and 120°C, however, some additional peaks appeared at 1,608 to 1,610 cm^{-1} (corresponding to side-chain residues of AA), 1,618 to 1,620 cm^{-1} (related to intermolecular cross linking of β-sheet), 1,654 cm^{-1} (indicating α-helix), 1,684 to 1,685 cm^{-1} (related to β turns, as well as antiparallel β-sheet associated with aggregation), and 1,693 cm^{-1} (representing β-turns and β-bends). The appearance of such a wide array of structural features indicated that β-LG passed through more structural changes, denaturation, and aggregation when heated near the isoelectric pH than at any other pH. Similar findings have been reported by Casal et al. (1988). This further supports the observations in our study related to decline of hydrophobicity values during heating at pH 5 (Figure 2). On the other hand, heat treatment at pH 7 gave a spectrum with peaks at 1,626, 1,637, 1,645–1,647, and 1,651–1,654 cm^{-1} when heated to 80°C. When heated to 100 to 120°C, the intensity for β-sheet bands (1,626 and 1,637 cm^{-1}) increased, whereas that for unordered structures (1,645–1,647 cm^{-1}) decreased, indicating extensive denaturation and aggregation. Overall, we observed complex structural modifications, particularly during heating of β-LG solutions at pH 5 compared with that at pH 7; minimal changes were observed at pH 3.

SDS-PAGE

Reducing and nonreducing SDS-PAGE was performed to investigate the extent of covalent aggregation under experimental conditions (Figure 4 A to F). Gel patterns of untreated β-LG samples (lane 10) exhibited monomeric bands under both reducing and nonreducing conditions, along with a faint band corresponding to BSA, indicating the presence of some BSA in the analyzed samples.

The gel patterns obtained by heating β-LG at 80, 100, and 120°C at pH 3 (Figure 4 A and B) were similar under nonreducing conditions, indicating the absence of aggregates formed through thiol interchange and formation of disulfide bonds, which supports our FTIR observation (Figure 3) of relatively high structural stability. This observation is in agreement with that of Wada et al. (2006), who reported the absence of covalently bonded aggregation at low pH, even after heating up to 95°C. The enhanced stability of β-LG to heat denaturation at low pH (Kella and Kinsella, 1988; Wada et al., 2006; Dissanayake et al., 2013) resulted in reduced exposure and minimal reactivity of thiol groups and thereby decreased thiol or disulfide exchange-mediated polymerization. Appearance of some low-molecular-weight bands (Figure 4 A and B) suggest the formation of some peptide fragments as a result of partial acid hydrolysis of β-LG at pH 3 and higher temperature (120°C).

The patterns for samples adjusted to pH 5 and heated to 80°C (Figure 4 C and D) also indicated the absence of thiol- or disulfide-mediated polymerization. However, upon increasing the temperature to 120°C, the typical bands for β-LG did not appear under nonreducing conditions compared with those obtained under reducing conditions, indicating that at high temperatures β-LG created polymer aggregates, which were cleaved into the monomeric form upon dissociation by 2-mercaptoethanol under the reducing conditions of electrophoresis. This observation is supported by the spectral features related to intermolecular cross-linking of β-sheets and related aggregates (Figure 3). The absence of repulsive forces at the isoelectric point could further explain the formation of aggregates at pH 5. This is in line with the observations of Monahan et al. (1995). This further explains why we observed the lowest hydrophobicity at pH 5 (Figure 2).

Antigenicity Related to Conformational Changes

The 3-dimensional structure of β-LG is an important feature that dictates its antigenicity. Previous studies have shown that people exhibit allergic responses to both linear and conformational epitopes of β-LG (Sélo et al., 1998, 2002; Clement et al., 2002). Allergenic epitopes are spread throughout the structure of β-LG. Major potential allergenic epitopes of β-LG identified are located on f(41–60), f(102–124), and f(149–162) (Sélo et al., 1999; Wal, 2001). The peptide f(41–60) forms a loop between β-strands C and D and also f(102–124), which is stabilized by hydrogen and disulfide bonds. Another potential antigenic fragment, f(149–162), is located at the C terminal and forms a flexible turn structure that, in the native state, is buried in the molecule and therefore inaccessible to IgE and IgG antibodies unless denaturation occurs (Kleber et al., 2004). More recently, Niemi et al. (2007) indicated a flat epitope area on the β-strands of β-LG that consisted of 6 different short fragments that bind to all 6 CDR (complementary-determining region) loops of IgE Fab (facilitated allergen binding). Thus, the availability of epitopes in β-LG for antibody binding is linked to its secondary or tertiary structure and can be influenced by conformational changes in response to various treatments.

The antigenicity of β-LG subjected to various pH, temperatures, and shear (Table 1) related well to the

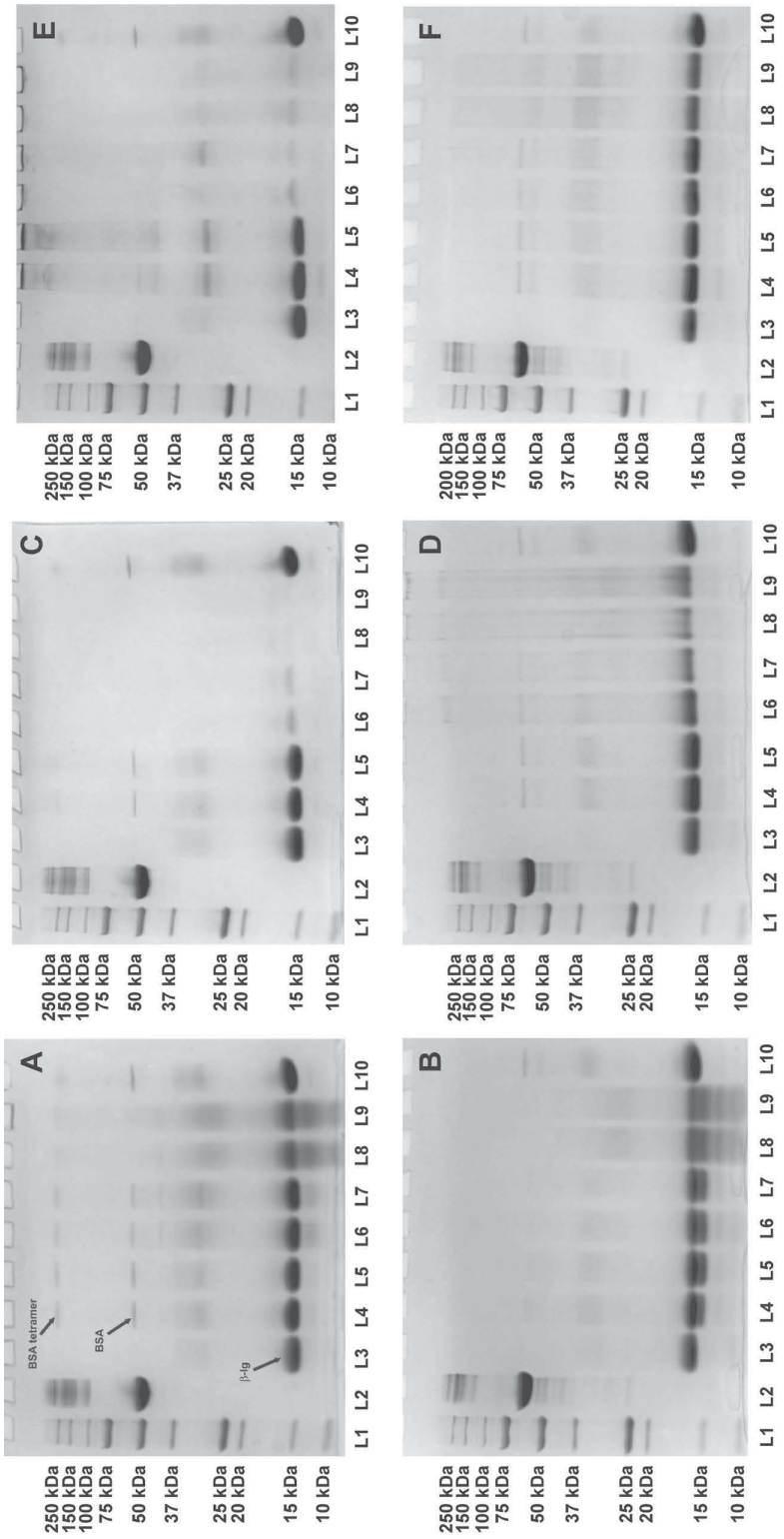

Figure 4. Sodium dodecyl sulfate-PAGE analysis showing the effect of heat and shear on polymerization of β-LG at pH 3 (A = nonreducing SDS-PAGE, B = reducing SDS-PAGE), pH 5 (C = nonreducing, D = reducing), pH 7 (E = nonreducing, F = reducing). For all gels, lane (L) 1 = molecular weight marker, L2 = BSA standard; L3 = β-LG standard; L4 = 80°C, shear 100 s^{-1}; L5 = 80°C, shear 1,000 s^{-1}; L6 = 100°C, shear 100 s^{-1}; L7 = 100°C, shear 1,000 s^{-1}; L8 = 120°C, shear 100 s^{-1}; L9 = 120°C, shear 1,000 s^{-1}; L10 = untreated β-LG (control).

structural modifications observed. In general, the antigenic potential of β-LG was strongly influenced by variations in pH, temperature, and shear. The antigenicity of control samples (pH 7.6) was unaffected ($P > 0.05$) by shear and temperatures up to 80°C. It increased ($P < 0.05$), however, at 100°C and then remained stable with a further increase up to 120°C. Lowering the pH to 3 resulted in increases ($P < 0.05$) in antigenicity as the temperature was increased to 120°C. Interestingly, although shear had a minor effect on the extent of antigenicity, application of shear at pH 3 resulted in reduced ($P < 0.05$) antigenicity when the temperature was increased above 80°C. Exposure of the proteins to increasing temperature at pH 3 caused unfolding of the protein molecules: we observed enhanced hydrophobicity (Figure 2) as well as exposure of β-sheet structures (Figure 3). These structural changes, although limited, suggested the possible exposure of conformational or linear epitopes, which were otherwise buried inside the structure of β-LG molecules, resulting in increased antigenicity (Table 1). Increased antigenicity at pH 3 and elevated temperature could also be attributed to the formation of some peptide fragments (Figure 4A and B) containing exposed linear epitopes. On the other hand, the decrease in antigenicity due to increased shear rate at temperatures above 80°C could be attributed to alterations in the integrity of conformational epitopes brought about by intramolecular SH or SS exchange reactions in a manner that decreased their antibody-binding capability.

We found that the influence of increasing temperature at pH 5 on antigenicity of β-LG was opposite to that observed at pH 3. At pH 5, antigenicity was similar to that of untreated β-LG heated to 80°C. Similar structural features (Figure 3), thiol contents (Figure 1), and hydrophobicity (Figure 2) suggested limited structural rearrangement and denaturation, which resulted in similar antigenicity values. However, when β-LG was heated to 100 or 120°C, heat- and shear-induced aggregation resulted in a reduction ($P < 0.05$) in the antigenic response. This result concurs with the observation of Kleber et al. (2004) and Bu et al. (2009), who reported heat-induced aggregation above 90°C that decreased the antigenicity of β-LG. Such a decrease in antigenicity could be attributed to the destruction of some conformational epitopes due to intermolecular disulfide-mediated aggregation as well as a resultant compacting of structure of β-LG that resulted in some inner epitopes becoming inaccessible. Increasing the temperature from 100 to 120°C further enhanced the aggregation process, as was evident from the electrophoretic patterns (Figure 4 C and D), leading to a further reduction in antigenicity. Additionally, increasing the shear rate that caused a reduction in thiol content (Figure 1) and surface hydrophobicity (Figure 2), indicating greater polymerization and more compactness in the structure, could have contributed to additional masking of some surface epitopes, thereby further lowering the antigenicity.

Table 1. Antigenicity of β-LG (ng/mL) as affected by treatment temperature [room temperature (RT; ~22°C; control), 80, 100, or 120°C], pH [pH 7.6 (control), 3, 5, or 7], and applied shear [0 (control), 100, 500, or 1,000 s^{-1}]

		Shear, s^{-1}			
pH	Temperature	0	100	500	1,000
Control (7.6)	RT	$50.9^{a,A}$	$49.7^{a,A}$	$48.6^{a,A}$	$47.8^{a,A}$
	80°C	$52.9^{a,A}$	$50.6^{a,A}$	$50.4^{a,A}$	$49.3^{a,A}$
	100°C	$62.3^{a,B}$	$60.6^{a,B}$	$59.1^{a,B}$	$59.1^{a,B}$
	120°C	$63.46^{a,B}$	$61.5^{a,B}$	$60.3^{a,B}$	$60.2^{a,B}$
3	RT	$67.2^{a,A}$	$63.7^{a,A}$	$61.4^{a,A}$	$61.0^{a,A}$
	80°C	$70.8^{a,A}$	$69.8^{ab,B}$	$66.1^{ab,A}$	$65.7^{b,AB}$
	100°C	$81.1^{a,BC}$	$78.7^{ab,C}$	$75.9^{b,B}$	$69.1^{c,B}$
	120°C	$86.0^{a,C}$	$81.3^{ab,C}$	$78.0^{b,B}$	$77.5^{b,C}$
5	RT	$55.9^{a,A}$	$53.2^{ab,A}$	$51.9^{ab,A}$	$49.4^{b,A}$
	80°C	$50.7^{a,B}$	$45.36^{b,B}$	$40.6^{bc,B}$	$39.7^{c,B}$
	100°C	$18.8^{a,C}$	$18.6^{a,C}$	$10.7^{b,C}$	$9.8^{b,C}$
	120°C	$8.8^{a,D}$	$6.6^{a,D}$	$4.5^{ab,D}$	$1.0^{b,D}$
7	RT	$47.5^{a,A}$	$46.0^{a,A}$	$45.7^{a,A}$	$45.8^{a,A}$
	80°C	$60.4^{a,B}$	$57.0^{a,B}$	$55.7^{ab,B}$	$51.8^{b,B}$
	100°C	$19.7^{a,C}$	$20.2^{a,C}$	$15.7^{a,C}$	$15.4^{a,C}$
	120°C	$18.1^{a,C}$	$19.3^{a,C}$	$19.0^{a,C}$	$19.0^{a,C}$
Pooled SEM			1.79		

[a–c]Means in the same row at a given pH are different ($P < 0.05$).

[A–D]Means in the same column at a given shear and pH are different ($P < 0.05$).

[1]All values presented are the mean of at least 6 independent observations (n = 6).

Increasing the pH of β-LG solutions to 7 resulted in an antigenicity trend opposite to that observed at pH 3. At 80°C, the increased hydrophobicity (Figure 2), indicative of β-LG unfolding, could have exposed hidden epitopes, resulting in increased antigenic potential of protein molecules. Increasing the temperature to 100 or 120°C followed by a decline in total thiol content (Figure 1) due to SH or SS exchange-mediated polymerization (Figure 4 E and F) could have masked some conformational epitopes, resulting in decreased antigenicity. However, because some β-LG molecules persisted as monomers (Figure 4 E and F) at 100 and 120°C, relatively more epitopes could have remained available for binding with antibodies, which might explain the relatively higher antigenicity at pH 7 compared with pH 5. Shear alone did not affect the antigenic potential at pH 7. Although the mechanism remains to be explained, high shear rate might have contributed to stability of the epitopes.

Overall, under defined processing conditions such as heating at 120°C, pH 5, and high shear rate (1,000 s^{-1}), β-LG can undergo substantial structural modifications, resulting in maximum reduction in its antigenicity. However, the stability or further modification of these structures during digestion needs to be investigated to confirm the carryover effect from processing to the physiological level. It remains to be seen whether such changes in antigenicity translate to modified allergenic potential of β-LG.

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Chapter 4

Digestibility and antigenicity of β-lactoglobulin as affected by heat, pH and applied shear

This chapter has been submitted as the title "**Digestibility and antigenicity of β-lactoglobulin as affected by heat, pH and applied shear**" by Rahaman, T., Vasiljevic, T., & Ramchandran, L in the peer review journal, Food Chemistry. 217, 517–523, doi: 10.1016/j.foodchem.2016.08.129

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: Md. Toheder Rahaman

Signature: [redacted]

Date: 30/03/2016

Paper Title:

Digestibility and antigenicity of β-lactoglobulin as affected by heat, pH and applied shear

In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Md. Toheder Rahaman	80	Designing and conducting the experiment, data collection and manuscript writing
Todor Vasiljevic	10	Concept development, experiment designing, data analysis and manuscript editing
Lata Ramchandran	10	Experiment designing, manuscript editing and submission for publication

Page - **1** - of **2**

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DECLARATION BY CO-AUTHORS

The undersigned certify that:

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Digestibility and antigenicity of β-lactoglobulin as affected by heat, pH and applied shear

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ABSTRACT

Processing induced conformational changes can modulate digestibility of food allergens and thereby their antigenicity. Effect of different pH (3, 5, 7), temperature (room temperature, 120 °C) and shear (0 s^{-1}, 1000 s^{-1}) on simulated gastrointestinal digestibility of β-lg and post digestion antigenic characteristics have been studied. At all pH levels unheated β-lg showed resistance to peptic digestion with high antigenic value while it was fairly susceptible to pancreatin with moderate reduction in antigenicity. Heating at 120 °C significantly improved both peptic and pancreatic digestion attributed to structural alterations that resulted in much lower antigenicity; the level of reduction being pH dependant. The lowest antigenicity was recorded at pH 5. Shearing (1000 s^{-1}) had a minor impact reducing digestibility and thereby enhancing antigenicity of unheated β-lg at pH 5 and 7 slightly; however in conjunction with heating (120 °C) it reduced antigenicity further irrespective of the pH. Overall, treatment at pH 5, 120 °C and 1000 s^{-1} could potentially reduce post digestion antigenicity of β-lg.

1. Introduction

Beta-lactoglobulin (β-lg) is the major whey protein of bovine milk comprising about 50% of total whey proteins and 10% of total milk proteins (Lozano, Giraldo, & Romero, 2008). It is a globular protein composed of 120 amino acids with two disulfide bridges and one free cysteine residue with a molecular weight of 18 kDa (Brownlow et al., 1997). It has nine antiparallel β-sheets (β barrel) and one α-helix on outer surface of barrel which folds into a calyx and acts as a carrier for some hydrophobic ligands such as retinoids, fatty acids, vitamin D, and lipids (Ron, Zimet, Bargarum, & Livney, 2010). β-Lg is a good source of many essential amino acids (Smithers, 2008) and also has some highly regarded physical functional properties such as gelling, emulsifying and foaming (Chevalier, Chobert, Popineau, Nicolas, & Haertlé, 2001). In spite of its diversified functional properties and nutritional benefits, its application in food manufacturing is limited due to its allergenic potential (Hattori et al., 2004). It is one of the major bovine milk allergens, responsible for almost 80% of cow milk allergies (Bu, Luo, Zheng, & Zheng, 2009). The relative resistance of β-lg to peptic digestion appears to be due its native conformation and its absence from human milk could be the underlying reasons for observed allergenicity in individuals allergic to cow's milk (Villas-Boas, Vieira, Trevizan, de Lima Zollner, & Netto, 2010).

Digestive stability is one of the common features for most food allergens, which allows them to pass intact through the gastrointestinal mucosa that consequently lead to retention of a sufficient number of epitopes to bind with present antibodies thereby triggering allergenic reactions (Astwood, Leach, & Fuchs, 1996; Moreno, 2007). For this reason, it is necessary to gain a thorough understanding how known allergens present in food behave during gastrointestinal passage and what impact this passage may have on their allergenic potential in order to properly manage food allergenicity. Most food products are subjected to various processing operations in order to produce diversified end products. Processing conditions affect conformation of food proteins, recognized as the most important allergen property, which in turn impacts on accessibility of antigenic epitopes to digestive enzymes (Rahaman, Vasiljevic, & Ramchandran, 2016a, 2016b) and thereby increase (Maleki et al., 2003), decrease (López-Expósito, Chicón, Belloque, López-Fandiño, & Berin, 2012) or maintain (Bu, Luo, Chen, Liu, & Zhu, 2013) their allergenic potential. Milk, in particular, undergoes various processes such as heating (pasteurization, sterilization), high pressure treatment, fermentation, homogenization, centrifugation and filtration prior to distribution and consumption. Among these, effect of heat treatment on the conformation of milk allergens and subsequent impact on digestibility and allergenicity has been well documented (Morisawa et al., 2009; O'Loughlin, Murray, Kelly, FitzGerald, & Brodkorb, 2012; Rudloff & Lönnerdal, 1992). Heating above 90 °C induced conformational changes in β-lg resulting in exposure of susceptible peptic bonds and disruption

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of conformational and sequential epitopes that consequently reduced the extent of antigenic reaction (Rahaman et al., 2016b). Another important environmental factor is pH, which modulates heat induced changes in the conformation of β-lg and its digestive behaviour (Zhang & Vardhanabhuti, 2014). Since diverse milk products are prepared at different pH levels and treated at various temperatures accompanied with flow induced shear, it is necessary to establish the impact of pH in combination with temperature on digestibility of β-lg and its post digestion antigenic potential. Shear is an another important processing factor that milk proteins are subjected to during various processing operations such as mixing, centrifugation, homogenization, general flow and membrane processing (ultrafiltration, microfiltration) (Yim & Shamlou, 2000). Shear stress can affect the native physico-chemical and functional properties of protein molecules (Bekard, Asimakis, Bertolini, & Dunstan, 2011). Our previous work (Rahaman, Vasiljevic, & Ramchandran, 2015) has shown that shear in combination with pH and temperature exerts substantial conformational changes on β-lg and its antigenicity. However, stability of such changes during digestion and post digestion antigenic potential has not been well studied and requires to be investigated in order to establish if any carryover effect exists from processing to a physiological level. Therefore, the present work was aimed at establishing effects of selected pH, temperatures and applied shear on the digestion of β-lg and antigenicity of the resulting digests.

2. Materials and methods

2.1. Treatment of sample

Based on the result of our earlier work (Rahaman et al., 2015), treatments that significantly influenced the antigenicity of β-lg were selected for further investigation. These included prepared samples of β-lg with high antigenicity observed at pH 3, room temperature, 0 s^{-1}; and pH 3, 120 °C, 1000 s^{-1} and samples with low antigenicity at pH 5, room temperature, 0 s^{-1}; pH 5, 120 °C, 1000 s^{-1}; pH 7, room temperature, 0 s^{-1}; and pH 7, 120 °C, 1000 s^{-1}. As reported previously (Rahaman et al., 2015), a dispersion (0.3%, w/v) of β-lg powder (Davisco Co. Ltd., Le Sueur, MN, USA) was prepared in citric acid-phosphate buffer at pH 3, 5 or 7 with 0.02% sodium azide to prevent microbial growth. The concentration was selected on the basis of reported concentration of β-lg in bovine milk (Dupont, Croguennec, Brodkorb, & Kouaouci, 2013). Samples at each pH were treated to different temperatures (room temperature or 120 °C) and shear (0 or 1000 s^{-1}) using a pressure cell (CC25/PR-150) of an Anton Paar rheometer (Physica MCR 301 series, Graz, Austria) as described previously (Rahaman et al., 2015). Thereafter the samples were subjected to digestion and then assessed for post digestion antigenicity. The control (0.3% β-lg solution in MilliQ water, pH 7.6, without any shear and heating) was also included in the assessment. It was assessed intact (undigested) and subjected to digestion protocol and antigenicity determination to establish effects of different treatments on digestibility and antigenicity.

2.2. Sample digestion

All the samples and controls were digested following the method described by Corzo-Martínez, Soria, Belloque, Villamiel, and Moreno (2010) with some modifications. The samples were subjected to the conditions of the stomach by lowering the pH to 2.0 with 2 M HCl, followed by addition of 0.3% (w/v) solution of porcine pepsin (EC 3.4.23.1, Sigma-Aldrich, 2500 units mg^{-1} protein activity) in 2 M HCl to each sample to obtain enzyme-substrate ratio 1:20. The gastric digestion was performed at 37 °C for two hours in a shaking water bath to ensure continuous mixing. The pH of the mixture was then increased to 7.5 by addition of 1 M NaOH to inactivate the pepsin. A 0.1% solution of pancreatic protease (United States Pharmacopeia (USP) in Milli Q water) was then added to obtain enzyme-substrate ratio of 1:100. The simulated intestinal digestion was performed for 2 h at 37 °C. At the end of digestion, the pancreatin was inactivated by placing the samples in an oil bath at 100 °C for 10 min. The resulting digests were frozen and stored at −80 °C until required.

2.3. Determination of degree of hydrolysis (DH)

The DH of different treated samples was assessed by measuring the ratio of nitrogen soluble in 10% trichloroacetic acid (TCA-SN) to the total nitrogen following a method described previously (Drago & González, 2000). Briefly, at the end of digestion, 2 ml of the digested samples was mixed with the equal volume of 20% TCA. The mixture was allowed to stand for 1 h and then centrifuged at 3000×*g* (RT7, Sorvall, Newtown, CT) at room temperature (20 °C). The nitrogen content of the supernatant was determined by Kjeldahl method (Helrich, 1990). The DH% was calculated as follows:

$$\mathrm{DH}(\%) = \frac{(\text{Soluble nitrogen in 10\% TCA in hydrolysate} - \text{soluble nitrogen in 10\% TCA in sample without hydrolysate}) \times 100\%}{\text{Total nitrogen in dispersion}}$$

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Non-reducing SDS-PAGE of the digests was used to establish the fate of proteins in all β-lg samples, including the control before and after digestion and treated samples after digestion. The protocol has been described previously (Rahaman et al., 2015). Briefly, aliquots of exactly 20 μl of each sample were mixed with equal volume of Tris-Tricine sample buffer and loaded onto a 16.5% Mini-PROTEAN® Tris-Tricine Gel (Bio-Rad Laboratories, NSW, and Australia). Electrophoresis was carried out at a constant voltage of 100 V for 100 min. The gels were then stained with a staining solution followed by destaining for 2 h and immersion in distilled water until the background became clear (Rahaman et al., 2015). Gels were visualized using an imager (Chemidoc MP, Bio-Rad Laboratories, NSW, Australia).

2.5. Antigenicity of digests

The digested samples (Section 2.2) were assessed for antigenicity following a protocol reported previously (Rahaman et al., 2015) using sandwich enzyme-linked immunosorbent assay (ELISA) with

a bovine β-lg quantitation kit (Bethyl Laboratories Inc., TX, USA). Microtiter plate wells were coated with anti-bovine β-lg coating antibody diluted in 0.05 M carbonate-bicarbonate buffer (1:1000). After incubation at 20 °C for an hour, the plate was washed once with an ELISA wash solution (Rahaman et al., 2015). Residual free binding sites in each well were blocked with 200 µl blocking solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8). After 30 min incubation at room temperature the plate was washed five times. Thereafter, 100 µl of standards or samples diluted in sample diluent (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8) were added in duplicate wells and incubated for 1 h and washed five times followed by adding 100 µl of diluted horseradish peroxidase (HRP, as suggested in the kit) conjugated β-lg detecting antibody to each well and incubation for 1 h at room temperature. After washing, the wells were filled with 100 µl of TMB (3,3′,5,5′-tetramethylbenzidine) substrate and allowed to develop colour in the dark for 15 min. The reaction was terminated with 0.18 M H_2SO_4. The absorbance of the mixtures was measured at 450 nm with a iMarkTM microplate reader (Bio-Rad Laboratories, NSW, Australia). Antigenicity, expressed as an equivalent nanogram of protein/ml of sample, was derived from a standard curve.

2.6. Immunoblot analysis

Immunoblotting of samples (as indicated in Fig. 4) was carried out according to our recent report (Rahaman, Vasiljevic, & Ramchandran, 2016c). Briefly, the proteins from electrophoretic gels were transferred to a nitrocellulose membrane of a Mini trans-Blot® cell (Bio-Rad Laboratories, NSW, Australia) using Tris-glycine transfer buffer system (Rahaman et al., 2016c). The membrane was first washed with a Tris-buffered saline solution and then incubated in a blocking solution for 1 h at 20 °C. The membrane was then transferred to a diluted solution of a rabbit anti-bovine β-lg antibody (Bethyl Laboratories Inc., Webster, TX, USA) and incubated for 1 h at the same temperature. The blot was washed five times with Tris-buffered saline containing 0.1% Tween 20 (TTBS) and then incubated with secondary antibody diluted in TTBS for another hour. The membrane was then washed five times with TTBS and the immobilized protein-antibody complex in the membrane was detected (Clarity™ Western ECL Blotting Substrate, Bio-Rad Laboratories, NSW, Australia) and recorded using a ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories, NSW, Australia).

2.7. Statistical analysis

All experiments were conducted in a randomised blocked split-plot arrangement with pH as the main plot. The subplots included effects of shear and temperature. The whole block was at least replicated with 3 subsamplings ($n \geqslant 6$). The data were analysed using the General Linear Model (GLM) procedure of SAS (SAS, 1996) and the means were assessed using a pdiff statement. Differences among the values were considered significant when $P < 0.05$.

3. Result and discussion

3.1. Degree of hydrolysis (DH)

Degree of hydrolysis (DH), expressed as trichloroacetic acid-soluble nitrogen (TCA-SN), was measured after both peptic and pancreatic digestion of the control and treated samples, which reflected effects of various treatment conditions on the digestive susceptibility of β-lg. All three tested factors (pH, temperature, shear) substantially influenced the DH of all samples (Fig. 1). At all pH levels and irrespective of shear, unheated β-lg had lower TCA-SN indicating greater resistance to peptic digestion due to

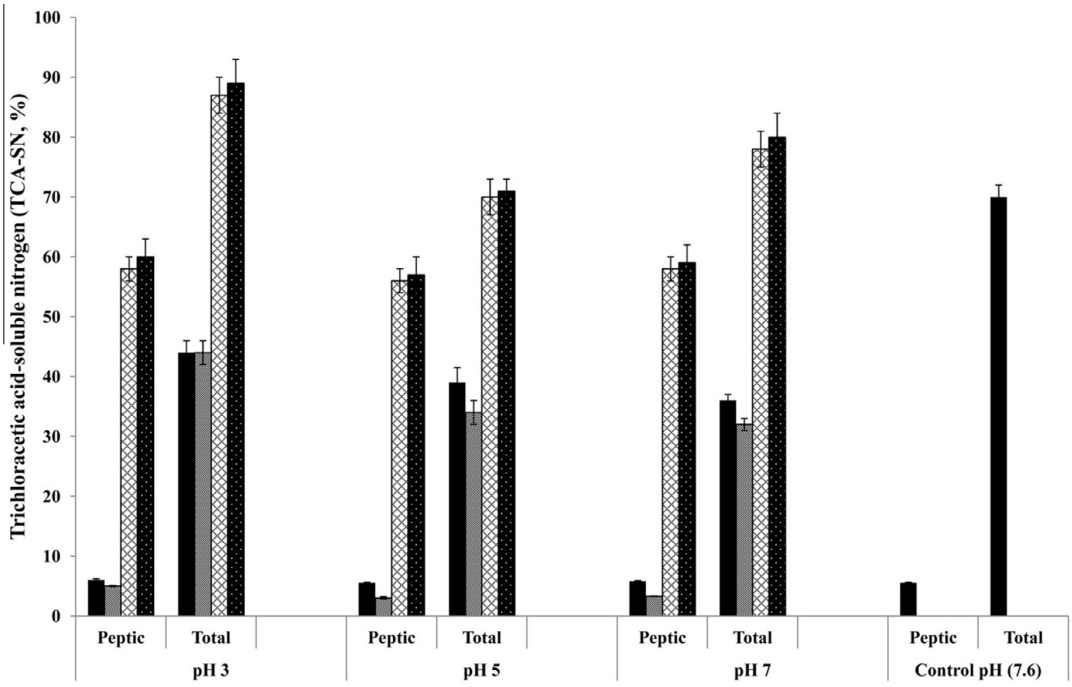

Fig. 1. Trichloroacetic acid-soluble nitrogen (TCA-SN) content (%) after peptic and total (both peptic and pancreatic) digestion of control and treated β-lg at different pH, temperature and shear. The values in this figure are the means of three replications, with ■ representing TCA-SN at RT and 0 s^{-1}, ▨ TCA-SN at RT and 1000 s^{-1}, ▧ TCA-SN at 120 °C and 0 s^{-1}, and ▩ TCA-SN at 120 °C and 1000 s^{-1}. Control sample was 0.3% β-lg solution in MilliQ water at pH 7.6 without any shear and heating.

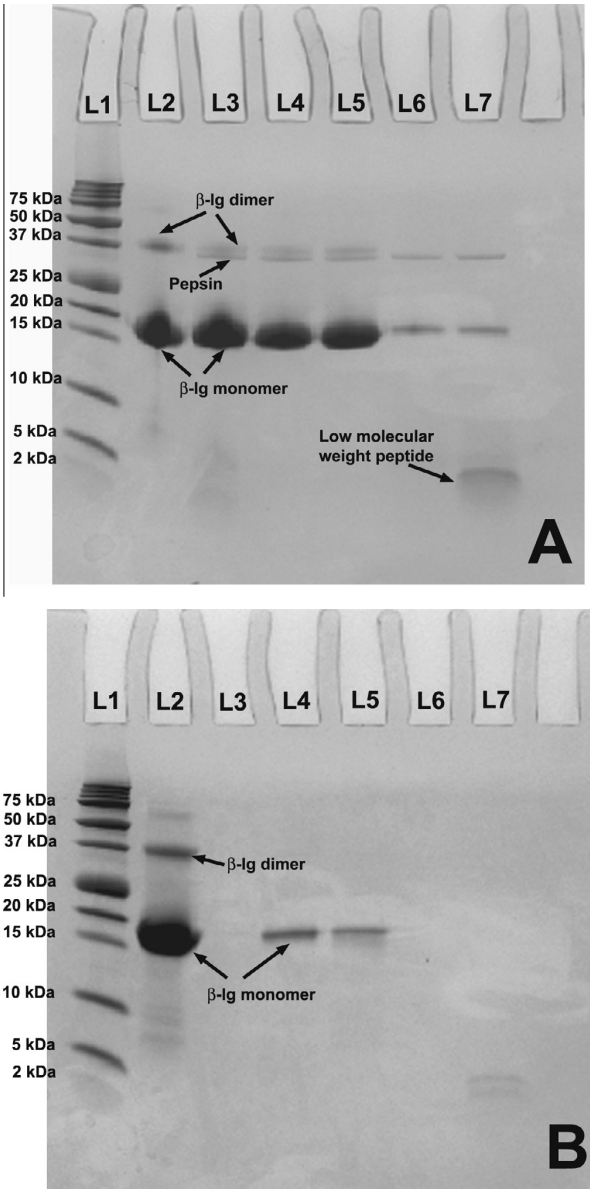

Fig. 2. Non-reducing SDS-PAGE profiles of *in vitro* (A) peptic and (B) total (both peptic and pancreatic) digestion of β-lg at pH 3. L1 = Molecular marker, L2 = Undigested control, L3 = Digested control, L4 = Room Temperature, 0 s^{-1}; L5 = Room Temperature, 1000 s^{-1}; L6 = 120 °C 0 s^{-1}; L7 = 120 °C 1000 s^{-1}. Control sample was 0.3% β-lg solution in MilliQ water at pH 7.6 without any shear and heating.

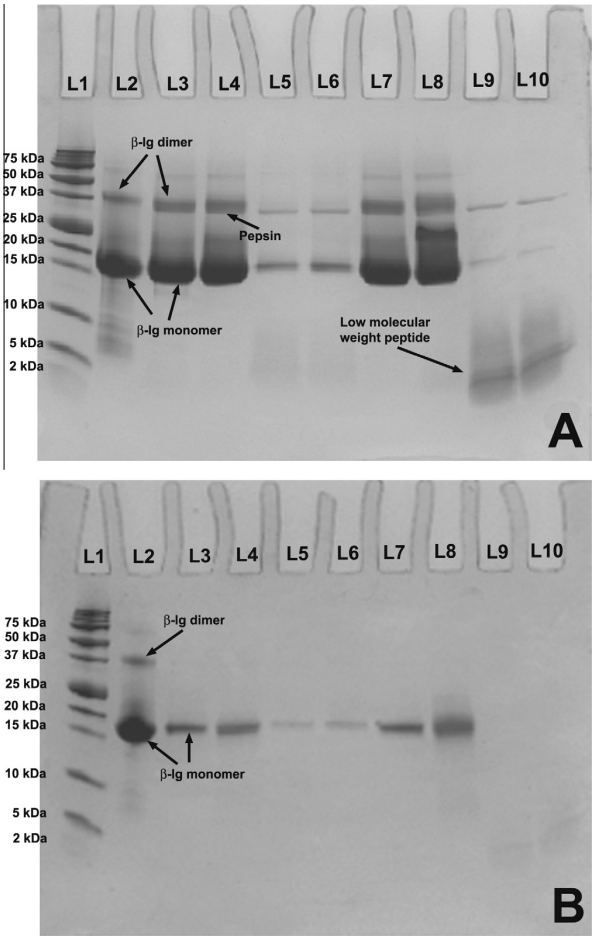

Fig. 3. Non-reducing SDS-PAGE profiles of *in vitro* (A) peptic digestion and (B) total (both peptic and pancreatic) digestion of β-lg at pH 5 and 7. L1 = Molecular marker, L2 = Undigested control, L3 = pH 5, Room Temperature, 0 s^{-1}; L4 = pH 5, Room Temperature, 1000 s^{-1}; L5 = pH 5 120 °C 0 s^{-1}; L6 = pH 5 120 °C 1000 s^{-1}; L7 = pH 7, Room Temperature, 0 s^{-1}; L8 = pH 7, Room Temperature, 1000 s^{-1}; L9 = pH 7 120 °C 0 s^{-1}; L10 = pH 7 120 °C 1000 s^{-1}. Control sample was 0.3% β-lg solution in MilliQ water at pH 7.6 without any shear and heating.

its unique structural stability (Macierzanka et al., 2012; Moreno, 2007; Reddy, Kella, & Kinsella, 1988; Sarkar, Goh, Singh, & Singh, 2009). β-Lg adopts an eight-stranded antiparallel β barrel structure with a central lipid binding calyx stabilized by two intramolecular S–S bonds (Breiteneder & Mills, 2005). At low pH, many hydrophobic amino acids residues, which are potential cleavage sites for pepsin, are buried inside the core of the β barrel (Peram, Loveday, Ye, & Singh, 2013), even during gastric digestion resulting in lower digestibility. Following pancreatic digestion, TCA-SN of unheated samples increased remarkably ($P < 0.05$) (Fig. 1) that could be ascribed to multiple accessible sites of β-lg to two pancreatic enzymes (trypsin and chymotrypsin). Heating at 120 °C further increased ($P < 0.05$) TCA-SN levels ($P < 0.05$) at all pH. Heat induced conformational changes with exposure of cleavage sites have been reported to increase the susceptibility of β-lg to pepsin and pancreatin (Iametti et al., 2002; Peyron, Mouécoucou, Frémont, Sanchez, & Gontard, 2006). However, our present study has shown that the extent of such heat induced rise in digestibility of β-lg greatly depended on pH (Fig. 1). After completion of digestion, highest TCA-SN was recorded at pH 3 and 120 °C followed by pH 7 and pH 5 at the same temperature, which was in concurrence with the observations of Kitabatake and Kinekawa (1998) and Reddy et al. (1988). Our previous work (Rahaman et al., 2015) has indicated that β-lg heated to 120 °C at pH 3 had highest surface hydrophobicity, which could have contributed to exposure of more hydrophobic residues accessible to enzymes and lead to a greater DH ($P < 0.05$) under these conditions than that at pH 7 or pH 5. In addition, effect of acid hydrolysis at low pH and high temperature, as evident in our earlier work (Rahaman et al., 2015), could also contribute to such a high TCA-SN content at pH 3 and 120 °C.

Shear noticeably affected both peptic and pancreatic digestion of β-lg (Fig. 1), especially in interaction with pH. For unheated

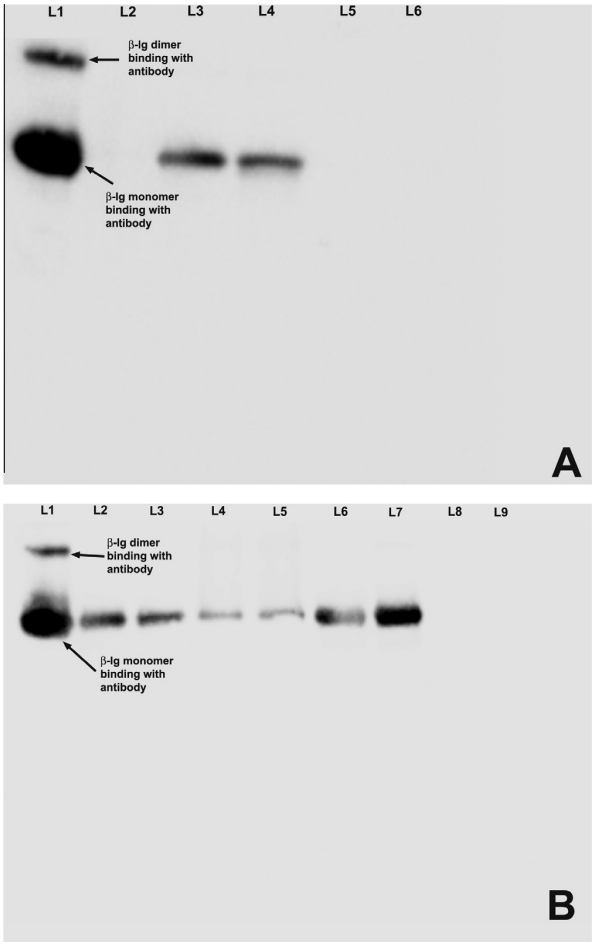

Fig. 4. Western blots depicting binding of anti-β-lg antibody to the reactive sites of digested β-lg treated at selected temperature and shear at (A) pH 3 and B) pH 5 and 7. For (A), L1 = Undigested control, L2 = Digested control, L3 = pH 3, Room Temperature, 0 s^{-1}; L4 = pH 3, Room Temperature, 1000 s^{-1}; L5 = pH 3, 120 °C 0 s^{-1}; L6 = pH 3, 120 °C, 1000 s^{-1}. For (B), L1 = Undigested control, L2 = pH 5, Room Temperature, 0 s^{-1}; L3 = pH 5, Room Temperature, 1000 s^{-1}; L4 = pH 5, 120 °C, 0 s^{-1}; L5 = pH 5, 120 °C, 1000 s^{-1}; L6 = pH 7, Room Temperature, 0 s^{-1}; L7 = pH 7, Room Temperature, 1000 s^{-1}; L8 = pH 7, 120 °C, 0 s^{-1}; L9 = pH 7, 120 °C, 1000 s^{-1}. Control sample was 0.3% β-lg solution in MilliQ water at pH 7.6 without any shear and heating.

samples, high shear (1000 s^{-1}) lowered ($P < 0.05$) the TCA-SN content at pH 5 and 7. Shear induced conformational rearrangement of β-lg leads to reduced availability of some hydrophobic residues (Rahaman et al., 2015) that could minimize susceptibility of the protein to digestion when exposed to high shear in comparison to that without shear at pH 5 and 7. In contrast, at pH 3, such an effect was not observed probably due to electrostatic repulsions of positively charged molecules at low pH (Castelletto & Hamley, 2007; Rahaman et al., 2015). However at 120 °C, irrespective of the pH, only a slight increase ($P > 0.05$) of the TCA-SN content was observed as a function of shear. Unheated β-lg was hardly susceptible to gastric digestion but fairly susceptible to pancreatic digestion. Heating significantly enhanced the susceptibility depending on pH, being at maximum at low (pH 3, 89%). It was notably lower at neutral (pH 7, 80%) and intermediate pH (pH 5, 71%). High shear reduced the degree of hydrolysis to a lesser extent at pH 5 and 7 when unheated and had only a slight impact on heated samples.

3.2. Electrophoretic patterns of β-lg

Electrophoresis of both peptic and pancreatic hydrolysates of β-lg provided additional insights into the effects of applied pH, temperature and shear on the digestive patterns (Figs. 2 and 3). Bands corresponding to intact β-lg monomer and dimer in the peptic digest of the unheated β-lg (Fig. 2A, Lane 4) showed slight degradation compared to undigested β-lg irrespective of pH and shear. Lower digestibility of intact β-lg, as reflected by diminished TCA-SN value of unheated samples (Fig. 1), has been ascribed to the burial of some hydrophobic residues such as tyrosine (Tyr), phenylalanine (Phe), leucine (Leu) and isoleucine (Ile), as potential active sites for pepsin cleavage (Kim et al., 2007). Upon pancreatic digestion of unheated samples (Fig. 2B, Lane 4), β-lg dimers were completely degraded and monomeric β-lg was also extensively digested compared to peptic digestion, which was also evident by higher value of TCA-SN after pancreatic digestion than that after peptic digestion alone (Fig. 1). Thus, although β-lg showed reasonable resistance to pepsin, it was quite susceptible to pancreatic enzymes when unheated.

Heating at 120 °C enhanced ($P < 0.05$) both peptic and pancreatic digestion of β-lg. The electrophoretic patterns of hydrolysates varied with pH (Figs. 2 and 3). In contrast to pH 3 and 5, β-lg monomers were almost completely hydrolysed at pH 7 (Fig. 3A, Lane 9 and 10) during peptic digestion, which gave rise to formation of some small polypeptides of molecular weight <10 kDa. Heating of β-lg at neutral pH (7) causes substantial conformational changes including dissociation of dimer to non-native monomers and extensive unfolding (Chicón, Belloque, Alonso, & López-Fandiño, 2008; Kim et al., 2007), which resulted in higher peptic degradation of intact β-lg monomer compared to that at pH 3 and 5. After subsequent pancreatic digestion of the heated samples, monomeric β-lg was completely hydrolysed as observed by the disappearance of corresponding band at pH 3 (Fig. 2B, Lane 6 and 7) and pH 7 (Fig. 3B, Lane 9 and 10). Higher TCA-SN content of heated β-lg observed at pH 3 and 7 (Fig. 1) could explain for disappearance of β-lg monomer. However, at pH 5, β-lg monomer was only partially hydrolysed (Fig. 3B, Lane 5 and 6). Synergistic effect of heat induced protein aggregation and isoelectric precipitation of β-lg at pH 5 could have resulted in the burial of some hydrophobic amino acid residues (Rahaman et al., 2015) that could have provided some resistance to pancreatic digestion.

Shear had a significant ($P < 0.05$) effect on electrophoretic patterns of both peptic and pancreatic hydrolysates of unheated β-lg. At pH 5 and 7, and at room temperature, β-lg sheared at high rate (1000 s^{-1}) underwent peptic and pancreatic digestion to a lesser extent, as evident by higher band density for intact β-lg, compared to sample without shear (Fig. 3A and B). Interestingly, at pH 7 and high shear a protein band appeared between monomeric and dimeric forms of β-lg, of approximately 25 kDa, which could indicate changed pattern of pepsin digestion (Fig. 3A. Lane 8), consequently resulting in slightly diminished pancreatic digestion (Fig. 3B, Lane 8). High shear rates could provide sufficient force for nucleation mediated condensation (Bromley, Krebs, & Donald, 2005), which could lead to burial of some peptic and pancreatic cleavage sites resulting in reduced digestion and lower TCA-SN value (Fig. 1). At low pH (3) and room temperature, due to a net positive charge, electrostatic repulsions of molecules could prevent such condensation reactions and therefore hinder digestion (Fig. 2A and B). Furthermore, α-helical structure was the most affected upon shearing under these conditions (Rahaman et al., 2015), which might have prevented access of pepsin to for example leucine as one of amino acid residues with a high α-helical propensity and one of the preferential cleavage sites. Although shear did not exert any apparent effect on digestive patterns of heated β-lg at pH 5 and 7, digestion of β-lg at pH 3 and 120 °C resulted in

Table 1
Antigenicity after peptic and total (both peptic and pancreatic) digestion of control and treated β-lg at different pH, temperature and shear.

pH	Digestion	Antigenicity (ng/ml)			
		RT		120 °C	
		0 s^{-1}	1000 s^{-1}	0 s^{-1}	1000 s^{-1}
3	Peptic	110.0[a]	109.0[a]	59.0[b]	51.0[c]
	Total	60.0[a]	59.0[a]	43.2[b]	24.9[c]
5	Peptic	112.0[a]	126.0[b]	32.2[c]	19.2[d]
	Total	60.0[a]	58.0[a]	10.0[b]	6.0[c]
7	Peptic	114.0[a]	130.0[b]	52.3[c]	45.0[d]
	Total	53.0[a]	63.0[b]	35.8[c]	28.1[d]
Control pH (7.6)	Peptic	119.3			
	Total	46.0			
Undigested β-lg		150.0			

[a–d] Means in the same row with different small letter superscripts are significantly ($P < 0.05$) different.

formation of some low molecular peptides of <3 kDa at 1000 s^{-1}, which were absent in unsheared samples (Fig. 2A, Lane 6 and 7). Some studies (Akkermans et al., 2008; Loveday, Wang, Rao, Anema, & Singh, 2011) have reported that at high temperature and pH 3, β-lg is hydrolysed into peptides between 2 and 8 kDa that could be the building blocks of amyloid like β-lg fibrils.

Hence, electrophoretic patterns of β-lg hydrolysates in non-reducing SDS-PAGE were greatly influenced by treatment conditions. The gel patterns of peptic digests of unheated samples was almost similar to the control (undigested β-lg) whereas pancreatic digests showed reasonably reduced intensity of intact β-lg in all treatments. Digestion of heated β-lg significantly degraded β-lg yielding some low molecular weight fractions (<10 kDa) at pH 3 and 7.

3.3. Antigenicity

Antigenicity of the peptic and pancreatic digests from treated and control samples was studied by indirect ELISA (Table 1) and western blotting (Fig. 4). In general, antigenicity of peptic digests was reduced after subsequent pancreatic digestion and the extent of such reduction was significantly affected by all three parameters – pH, temperature and shear. High antigenic reaction was recorded for peptic digests from unheated β-lg regardless of pH and the values were similar to that for control. Lower digestibility (Fig. 1) and consequent persistence of intact β-lg (Figs. 2 and 3) with sufficient conformational and sequential epitopes likely resulted in such a higher antigenicity. Subsequent hydrolysis of the unheated samples at all pH with pancreatin significantly degraded the remaining intact molecules of β-lg, as observed by electrophoresis, that resulted in decreased ($P < 0.05$) antigenicity. Thus, although β-lg was scarcely digested with pepsin, multiple proteolytic actions of pancreatic enzymes (trypsin and chymotrypsin) were fairly efficient in hydrolysing it to an extent that lowered its antigenicity significantly (Chicón et al., 2008). Heating significantly affected both peptic and pancreatic digestion and thereby their antigenicity. As reported in many previous studies (Peyron et al., 2006; Rahaman et al., 2016b; Wada & Lönnerdal, 2014), heat induced conformational changes lead to exposure of peptic and pancreatic cleavage sites resulting in enhanced digestibility and consequently reduced allergenicity. Our present result showed that such heat induced reduction of antigenicity was also noticeably influenced by pH and shear. Highest reduction was recorded for digests from heated samples at pH 5, which was 78% in peptic digest and 93% in total digest compared to undigested β-lg. In contrast, at pH 3 and 7, antigenicity was reduced by 60% and 65% for peptic digest and 71% and 76% for the total digest, respectively. SDS-gel electrophoresis patterns showed that heated β-lg at 120 °C at pH 3 (Fig. 2B, Lane 6 and 7) and pH 7 (Fig. 3B, Lane 9 and 10) was completely hydrolysed upon pancreatic digestion having higher TCA-SN content (Fig. 1) compared to that at pH 5, but interestingly, the antigenic reduction was highest at pH 5. Our earlier studies showed that at pH 5 and 120 °C, β-lg underwent covalent aggregation and most of such aggregates could have been completely hydrolysed during digestion with cleavage of potential peptide bonds maintaining epitopic stability while some aggregates were partially digested leaving a small portion of intact β-lg (Fig. 3B, Lane 5 and 6) accounting for minimum antigenicity. Zhang and Vardhanabhuti (2014) also found that the morphological characteristics as well as digestion behaviour of heat induced β-lg aggregates greatly varied with pH during heating. In contrast, at 120 °C, at pH 3 and 7, although β-lg monomer was totally hydrolysed showing no binding reaction with antibody in the Western Blot (Fig. 4A and B), the smaller yielding peptides with molecular weight, which were not retained in the gel due to their size, might still have possessed sequential epitopes expressing a residual antigenicity. From such an observation it is apparent that although reduction of antigenicity is positively correlated with digestibility, such a decline not only depends on overall digestion but also on relative susceptibility of peptide bonds involved in integrity of antigenic epitopes to digestive enzymes, which may vary according to treatment conditions.

Shear stress substantially influenced the antigenic potential of pepsin and pancreatin digests of both heated and unheated samples (Fig. 4 and Table 1, respectively) depending on pH. As discussed in Section 3.1, in the unheated samples, at pH 5 and 7, shear induced structural denaturation could mask some of hydrophobic residues, making them less accessible to proteolytic enzymes resulting in lower TCA-SN content (Fig. 1) and diminished degradation of β-lg (Fig. 3 A) with the consequence of higher ($P < 0.05$) antigenicity compared to unsheared sample. In contrast, at high temperature (120 °C) regardless of pH, shear exerted an opposite effect; digests from samples subjected to high shear (1000 s^{-1}) showed slightly lower antigenic value compared to zero shear although no apparent differences in their electrophoretic patterns were observed. Synergistic effect of heat and shear could have altered the conformation of β-lg in such a way that could facilitate the hydrolysis of peptide bonds associated with integrity of sequential epitopes resulting in lower antigenicity.

4. Conclusion

Heat, pH, and shear combined induced conformational changes substantially affecting digestibility of β-lg and its post digestion antigenicity. Unheated β-lg, at all pH, had lower peptic digestibility attributed to its unique structural stability and consequently

higher antigenicity. However, subsequent pancreatic digestion noticeably increased the digestibility with simultaneous decline in antigenic value. Heating at 120 °C considerably enhanced both peptic and pancreatic digestion due to structural alterations with exposure of cleavage sites that in turn resulted in greater decrease in antigenicity compared to unheated samples. Nevertheless, extent of such heat attributed changes was pH dependant. At pH 3 and 7, although β-lg was completely hydrolysed, some of smaller fragments could still preserve sequential epitopes with residual antigenicity. In contrast, at pH 5 β-lg had minimum antigenicity compared to pH 3 and 7 at 120 °C even though it was hydrolysed to a lesser extent, indicating diminished presence of peptides with residual antigenicity. High shear ($1000\,s^{-1}$) slightly reduced digestibility of β-lg and thus enhanced antigenic potential in unheated samples at pH 5 and 7 but had a reverse effect in combination with heat at all pH examined. Further research is required to confirm the observed effect of selected conditions by studying *in vivo* allergenic response in sensitive individuals through oral challenges, skin prick tests and release of allergic mediators.

Conflict of interest

The authors declare that there is no conflict of interest in regards to publication of this research work.

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Chapter 5

Shear, heat and pH induced conformational changes of wheat gluten – Impact on antigenicity

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

Declaration by [candidate name]: Md. Toheder Rahaman

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In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Md. Toheder Rahaman	80	Designing and conducting the experiment, data collection and manuscript writing
Todor Vasiljevic	10	Concept development, experiment designing, data analysis and manuscript editing
Lata Ramchandran	10	Experiment designing, manuscript editing and submission for publication

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Shear, heat and pH induced conformational changes of wheat gluten – Impact on antigenicity

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ABSTRACT

Processing can induce conformational changes of food proteins depending on the conditions used that may affect their antigenicity. This study investigated the effect of pH (3,5,7) temperature (80,90,100 °C) and shear (500,1000,1500 s^{-1}) on the conformational changes (surface hydrophobicity, FTIR, SDS–PAGE and thiol content) of gluten in relation to its antigenicity (determined by Enzyme-linked Immunosorbent Assay). Overall, at pH 3, up to 90 °C, conformational changes and possible burial of some antigenic hydrophobic residues resulted in reduction of antigenicity to one-third that of control. Further heating to 100 °C caused increase in antigenicity due to exposure of some hidden epitopes. However, at pH 5 and 7, the antigenicity declined only at 100 °C due to modification in thiol content and related structural changes causing destruction and/or masking of some epitopes. Shear alone had no effect on antigenicity of gluten but could have a synergistic influence at pH 7 and 100 °C.

1. Introduction

Wheat is a staple food in many parts of the world and consumed in varieties of baked products. Widespread use of wheat flour in food is attributed to the visco-elastic properties of its protein gluten. Gluten, a complex protein, consists of aqueous alcohol soluble gliadins and insoluble glutenins. Gliadin is a mixture of heterogeneous monomeric proteins, divided into α/β, ϒ and ω-gliadins. α/β and ϒ-gliadins are low molecular weight proteins (MW 28–35 kDa) with six and eight cysteine residues respectively whereas ω-gliadins (MW 40–75 kDa) lack cysteine. Glutenin is a polymeric protein composed of high molecular weight (HMW-GS, MW 65–90 kDa) and low molecular weight glutenin subunits (LMW-GS, MW 30–45 kDa) which are linked by inter-chain disulphide bonds (Wieser, 2007). Due to their unique physico-chemical properties, availability and low cost, gluten is used not only in cereal products but also in other foods such as sausages, ice-cream and drugs. Preparation of edible and biodegradable coatings and films from wheat gluten and study of their properties has also drawn special attention (Kayserilioğlu, Bakir, Yilmaz, & Akkaş, 2003).

Although gluten is used extensively in food products, it has been associated with the Celiac disease (CD) in some individuals. Celiac disease is an autoimmune disorder of small intestine upon ingestion of gliadin, a prolamine of gluten, characterised by mucosal inflammation, villous atrophy and consequent malabsorption of various nutrients (Gujral, Freeman, & Thomson, 2012). Almost 1% of the population worldwide is affected by CD, being more prominent in some countries such as USA and Sweden (Rubio-Tapia & Murray, 2010), and over the years, the rate has been increasing considerably. Moreover, gluten proteins also cause IgE mediated food allergy such as baker's asthma, exercise-induced anaphylaxis and atopic dermatitis (Simonato et al., 2001) in some sensitive individuals.

Different food processing can alter the antigenicity of food proteins in different ways, either by causing conformational changes in the structure of the epitopes (Rahaman, Vasiljevic, & Ramchandran, 2015) and/or by changing their digestibility (Soler-Rivas & Wichers, 2001). Commonly wheat is subjected to various processes e.g. baking, extrusion, cooking etc. before consumption. The processing affects the physico-chemical structure of wheat proteins and consequently their role in human physiology. For example, baking affected the digestibility of wheat proteins and the immunological characteristics of resulting hydrolysate were different from that of unprocessed flour proteins (Pasini, Simonato, Giannattasio, Peruffo, & Curioni, 2001). Immunoreactivity of gliadin initially increased while heating at 90 and 100 °C but decreased with extended heating times (Rumbo, Chirdo, Fossati, & Añon, 1996). In contrast, while drying of pasta at 20, 60, 100 or 180 °C, temperature did not affect the allergenic potentiality of wheat proteins although at 180 °C the

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digestibility was reduced significantly (De Zorzi, Curioni, Simonato, Giannattasio, & Pasini, 2007). Radiation (Leszczynska, Łącka, Szemraj, Lukamowicz, & Zegota, 2003) may also affect gluten antigenicity. Although several researches have focused on some physical as well as enzymatic treatments of gluten and its subsequent structural deformation associated with antigenic potentiality, there are no reports that outline effects of combinations of different conditions such as pH and temperature on conformational changes of gluten and their relation to antigenicity. For example pH is an important factor which influences the conformation of food proteins during processing. Several kinds of wheat products are prepared at different pH; however, so far no study has been conducted on the effects of pH in combination with temperature on antigenicity of gluten. Another phenomenon which appears during various food processing (mixing, homogenisation etc.) is shear. Shear stress can alter the structure of native protein molecules that could affect not only the functional properties but also the susceptibility of proteins to enzymatic digestion (Bekard, Asimakis, Bertolini, & Dunstan, 2011). To the best of our knowledge, there are no studies on shear induced conformational change of gluten in relation to its antigenicity. This study thus aimed to investigate the combined effect of pH, heat and shear on the structural changes of gluten proteins and evaluate the consequences of such changes on their antigenicity.

2. Materials and methods

2.1. Sample preparation

Plain wheat flour (Manildra Flour Mills Pty. Ltd, NSW, Australia) was mixed with distilled water and gently kneaded to form a dough ball. The dough was soaked in water for 6 hours and then washed under running tap water with kneading to remove starch until clear water was observed leaving gluten as a rubbery mass. Then the gluten mass was washed in NaCl solution (0.4 M) to remove salt soluble proteins (globulin). The wet gluten mass was then freeze dried (Alpha 1-4 LSC freeze dryer, John Morris Scientific Pty Ltd, Melbourne, Australia). Dried gluten was ground to fine powder by mortar and pestle and sieved (No. 50) to obtain fine powder (<300 μm). Exactly 8% (w/w) gluten dispersion was prepared in citric acid-phosphate buffer at different pH (3, 5 and 7). The gluten dispersion in MilliQ water at room temperature and zero shear was the control (pH 6.6). The gluten concentration chosen in the dispersion was similar to the reported concentration in wheat flour (Van Der Borght, Goesaert, Veraverbeke, & Delcour, 2005).

2.2. Treatment of sample

Gluten dispersion prepared in buffers at various pH was subjected to shear and heat in a pressure cell (CC25/PR-150) of a rheometer (Physica MCR 301 series, Anton Paar, GmbH, Germany) following the method of Rahaman et al. (2015). An aliquot of 15.14 mL of each sample was poured into the measuring cup of the pressure cell at 20 °C. The samples were first sheared at a different rate (500, 1000 or 1500 s^{-1}) for 5 min and then subjected to heating to different temperatures (80, 90 or 100 °C). The samples were held at these temperatures for 1 min and then cooled down to 20 °C. Both heating and cooling rate was set at 5 °C/min. The treated gluten was then freeze dried, ground with mortar and pestle, sieved (<300 μm) and stored at room temperature (20 °C) until analysis.

2.3. Determination of surface hydrophobicity

Surface hydrophobicity (Ho) of all samples and their controls was determined fluorometrically using 8-anilino-1-naphthalenesulfonic acid (ANS; Sigma Aldrich, St Louis, MO, USA) as a fluorescence probe according to a previously reported method (Stathopoulos, Tsiami, David Schofield, & Dobraszczyk, 2008) with some modifications. A dispersion of 4 mg of freeze-dried gluten was prepared in 10 ml of 50 mM acetic acid solution and mixed intermittently every 15 min for 45 min. Then the dispersion was centrifuged at 3000×*g* for 10 min using a bench top centrifuge (RT7, Sorvall, Newtown, CT) at room temperature. A series of dilutions of each supernatant were made with 50 mM acetic acid solution. Then 28 μl of ANS solution (8 mM in 0.1 M phosphate buffer, pH 7.4) was added to 4 ml of these serially diluted supernatants and incubated for 15 min in dark. Relative fluorescence intensity (RFI) was measured at excitation and emission wave length 405 and 480 nm respectively using a Shimadzu fluorescence spectrophotometer (model-RF5301PC, Shimadzu Corp., Kyoto, Japan). The initial slope of net RFI versus protein concentration was calculated by linear regression analysis and used as an indicator for surface hydrophobicity.

2.4. Determination of free thiol (SH) content

Free thiol content was measured colorimetrically using DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) according to the reported method (Jansens et al., 2011) with some modifications. A dispersion of the samples (2 mg/ml) was prepared in sample buffer [0.05 M phosphate buffer (pH 6.5) containing 2% (w/v) SDS, 3.0 M urea and 1.0 mM EDTA (Ethylenediaminetetraacetic acid)]. The dispersion was left for 1 hour with intermittent vortexing every 15 min. Then 400 μl of DTNB reagent [0.1% (w/v) in respective sample buffer] was added, incubated for 45 min with intermittent vortexing and centrifuged at 3000×*g* at room temperature for 10 min using a bench top centrifuge (RT7, Sorvall, Newtown, CT). The supernatant was collected, filtered (0.45 μm RC, Millipore) and the absorbance of the filtrate was taken at 412 nm using UV spectrometer (Libra S11, Biochrom). The absorbance value was divided by molar extinction coefficient of 13,600 to obtain the thiol content, expressed as μmol/g of protein.

2.5. Gel electrophoresis

Both reducing and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) were performed to study the status of protein in both the control and treated samples. For non-reducing SDS–PAGE, 10 mg of gluten powder was suspended in 1 ml of SDS sample buffer containing 62.5 mM Tris-HCL (pH 6.8), 2% (w/v) SDS, 40% (v/v) glycerol and 0.4% (w/v) bromophenol blue. For reducing SDS–PAGE, in addition, 50 μL of β-mercaptoethanol was added and the solution was heated for 5 minutes at 90 °C. 150 μl of gliadin extracted in 70% ethanol mixed with 1 ml of sample buffer was used as standard. Ten microlitres of gluten sample, gliadin standard and molecular weight marker was loaded onto a 10% Mini-Protean TGX precast gel (Bio-Rad Laboratories, NSW, Australia). Electrophoresis was performed at 50 mA and 180 volt. The gel was stained overnight in the solution composed of 0.025% Coomassie brilliant blue, 40% methanol, 7% acetic acid and 50% distilled water. Then it was placed in destaining solution containing 40% methanol, 7% acetic acid and 53% distilled water for 2 hours followed by immersing in distilled water until the background became clear. Gel images were taken using a Chemi-Doc imager (Chemidoc MP, Bio-Rad Laboratories, NSW, Australia). Quantification of the bands was carried out using image analysis software (Image LabTM, Bio-Rad Laboratories, NSW, Australia).

2.6. Fourier transform infrared spectroscopy (FTIR)

FTIR spectra of treated and untreated gluten were recorded using FTIR spectrometer (IRAffinity-1, Shimadzu Corp., Kyoto,

Japan) following the method of Cui et al. (2013) with some modifications. Briefly, 4 mg of gluten powder was mixed with 60 mg of spectroscopy-grade KBr, after homogenising by mortar and pestle, the mixture was pressed into pellets (1–2 mm thick films) with a 10-ton hydraulic press. The FTIR spectra of the pellets were obtained between 400–4000 cm^{-1} from 20 scans at a resolution of 4 cm^{-1} using Happ Genzel apodization. The spectra were smoothed by 7 points to reduce noise and a secondary derivative of the spectrum was obtained using PerkinElmer Spectrum Version 10.3.6 software (PerkinElmer Corp., USA).

2.7. Antigenicity determination

Antigenicity of all samples (control and treated) was determined by sandwich Enzyme-linked Immunosorbent Assay (ELISA) using Ridascreen®Gliadin ELISA kit (R-Biopharm AG Darmstadt, Germany). For gliadin extraction, 50 mg of gluten powder was dispersed in 4 ml of 70% ethanol, mixed for 1 hour at room temperature and centrifuged at 3000 g for 10 min at room temperature. The supernatant was diluted with sample diluent and 100 µl of diluted samples or standards were added to each separate duplicate well pre-coated with gliadin antibody. After 30 min incubation at room temperature the liquid was poured out and the plate was tapped upside down against absorbent paper. Then the plate was washed three times with washing buffer (R-Biopharm AG Darmstadt, Germany) to remove unbound antigen. Thereafter, 100 µl of diluted horse radish peroxidase (HRP) conjugated detecting antibody was added to each well, incubated at room temperature for 30 min followed by three times washing. Then the wells were filled with 50 µl of substrate and 50 µl chromogen [3,3′,5,5′-tetramethylbenzidine, (TMB)], incubated for 30 min at room temperature in dark to develop the colour. The reaction was stopped by adding 100 µl 1 N H_2SO_4 to each well and absorbance was measured with a microplate reader (iMarkTM, Bio-Rad Laboratories, NSW, Australia). The antigenicity was calculated from the standard curve as equivalent mg of gluten/kg of sample.

2.8. Statistical analysis

The experiments were designed as a randomised block, full factorial design with pH as the main plot and shear and temperature as the subplots. This block was replicated with 3 subsampling ($n = 6$). The results of the various determinations were analysed using the General Linear Model (GLM) procedure of SAS (SAS, 1996). The level of significance was set at $P = 0.05$.

3. Results and discussion

3.1. Surface hydrophobicity (Ho)

Surface hydrophobicity determines the extent of available hydrophobic groups on the protein surface in contact with polar environment and is an indication of changing tertiary structure of proteins. Gluten has very low solubility in water due to its low content of amino acids with charged side chains and high content of hydrophobic amino acid proline (Jansens et al., 2011). Consequentially, the highest Ho was recorded for samples sheared or unsheared at room temperature at control pH (6.6) (Table 1). Isoelectric pH (pI) of gluten protein is reported around 6.4 (Majzoobi & Abedi, 2014) which is near to control pH of the sample. Minimal solubility of gluten around pI along with maximum exposure of hydrophobic groups on protein surface could explain the highest Ho observed for the control sample. On the either side of pI (pH 5 and 7), Ho decreased ($P < 0.05$) and was at minimum at pH 3. Increasing net charge of ionised protein and burial of some hydrophobic residues might contribute to such a lowering of Ho when pH is adjusted to values higher or lower than pI (Mejri, Rogé, BenSouissi, Michels, & Mathlouthi, 2005). However, this net surface charge inclined to positive or negative values depending on whether pH value was below (pH 3) or above (pH 7) the pI (Bengoechea, Romero, Aguilar, Cordobés, & Guerrero, 2010).

During heating, hydrophobicity changed differently depending on the pH (Table 1). In general, Ho decreased with rise of temperature at control pH (6.6), pH 5 and 7 but not at pH 3. For control pH, initial reduction of Ho was at 80 °C followed by further decline at 100 °C. A similar pattern for reduction of Ho was also observed at pH 7 whereas at pH 5, Ho decreased consistently at 90 and 100 °C. Initial unfolding of the protein molecules, causing exposure of hydrophobic groups followed by re-formation of irreversible hydrophobic interactions and disulphide bonded inter-molecular aggregation (evident in SH measurement and SDS–PAGE) leading to folding of proteins with subsequent hiding of hydrophobic groups, could have caused the decline in Ho observed during heating at pH 5–7. Similar findings have been reported previously (Stathopoulos et al., 2008). It also appeared that gluten became structurally stable at pH 5 and required higher temperatures to initiate denaturation than at control pH or pH 7. However, at pH 3, Ho did not exhibit any change up to 90 °C and thereafter the values tended to rise ($P > 0.05$). Surplus of positive charge at such a low pH and high temperature (>90 °C) could lead to electrostatic repulsions within the molecules leading to unfolding of protein that in turn could prevent intermolecular hydrophobic association. Apparently at pH values much below the isoelectric point, protonation of the carboxyl group and ionisation of glutamine amide resulted in greater net positive-charge and induced side chain repulsion, which lead to a partial protein unfolding, rupture of hydrogen bonds and dissociation of hydrophobic interactions (Sun, Song, & Zheng, 2008).

Shear alone did not affect Ho regardless of pH. However, at 100 °C, both at pH 7 and control pH, Ho tended to increase ($P > 0.05$) when shearing rate was raised to 1000 and 1500 s^{-1} in comparison to low shearing rates (500 s^{-1}), irrespective of the pH (Table 1). This could be due to conformational changes of proteins since shear stress can cause mechanical dissociation and unravelling of protein molecules (Morel, Redl, & Guilbert, 2002) which can lead to scission of hydrophobic interactions followed by exposure of some hydrophobic residues.

3.2. Thiol content

Among the gluten proteins, α/β, and ϒ- gliadins contain six and eight cysteine residues, respectively, in their C terminal domains, all of which form homologous intra-chain disulphide bonds, whereas in glutenin fractions cysteine forms both intra and inter-chain disulphide bonds and a low level of cysteine remains free (Jansens et al., 2011). The amount of free thiol can be influenced by various conditions such as pH, temperature and hydrostatic pressure (Kieffer, Schurer, Köhler, & Wieser, 2007; Singh & MacRitchie, 2004). In our study, thiol content was affected ($P < 0.05$) by pH and temperature whereas shear alone had no effect (Table 2). Highest amount of free thiol (5.4 µmol/g) was measured in control sample (pH 6.6) and the value reduced ($P < 0.05$) to around 4 µmol/g when pH was adjusted to both 5 and 7. Such findings revealed the fact that near isoelectric pH (6.4), the exposure of free thiol group in gluten was at maximum and deviation to either side of pI resulted in reduced exposure of thiol groups as a consequence of their participation in SS/SH interactions.

Heating affected the free thiol content of gluten depending on the pH. At control pH and pH 7, heating to 80 °C caused remarkable decrease ($P < 0.05$) in thiol content due to disulphide bonding

Table 1
Surface hydrophobicity of wheat gluten at different pH as a function of temperature and shear rate.

pH	Temperature, °C	Surface hydrophobicity			
		Shear, s^{-1}			
		0	500	1000	1500
Control (6.6)	RT	339^{aA}	338^{aA}	336^{aA}	340^{aA}
	80	267^{aB}	266^{aB}	267^{aB}	263^{aB}
	90	263^{aB}	262^{aB}	262^{aB}	267^{aB}
	100	189^{aC}	190^{aC}	199^{aC}	200^{aC}
3	RT	260^{aA}	261^{aA}	263^{aA}	262^{aA}
	80	262^{aA}	259^{aA}	259^{aA}	262^{aA}
	90	258^{aA}	257^{aA}	261^{aA}	259^{aA}
	100	279^{aA}	276^{aA}	280^{aA}	277^{aA}
5	RT	319^{aA}	315^{aA}	317^{aA}	320^{aA}
	80	315^{aA}	318^{aA}	320^{aA}	317^{aA}
	90	271^{aB}	269^{aB}	272^{aB}	273^{aB}
	100	240^{aC}	241^{aC}	244^{aC}	243^{aC}
7	RT	313^{aA}	314^{aA}	312^{aA}	310^{aA}
	80	262^{aB}	263^{aB}	262^{aB}	258^{aB}
	90	258^{aB}	260^{aB}	258^{aB}	263^{aB}
	100	166^{aC}	162^{aC}	182^{aC}	185^{aC}
SEM	9.0				

All values presented are the means of six independent observations ($n = 6$).
a–c – Means in the same row are significantly different ($P < 0.05$).
A–D – Means in the same column are significantly different ($P < 0.05$).
SEM – pooled standard error of the mean; RT – room temperature (~22 °C).

Table 2
Thiol content of wheat gluten treated at different pH as a function of temperature and shear rate.

pH	Temperature, °C	Thiol content ($\mu mol.g^{-1}$)			
		Shear, s^{-1}			
		0	500	1000	1500
Control (6.6)	RT	5.4^{aA}	5.2^{aA}	5.3^{aA}	5.2^{aA}
	80	3.6^{aB}	3.5^{aB}	3.8^{aB}	3.8^{aB}
	90	3.5^{aB}	3.7^{aB}	3.6^{aB}	3.7^{aB}
	100	2.2^{aC}	2.4^{aC}	2.3^{aC}	2.4^{aC}
3	RT	1.5^{aA}	1.4^{aA}	1.3^{aA}	1.4^{aA}
	80	1.4^{aA}	1.3^{aA}	1.0^{aA}	1.5^{aA}
	90	2.8^{aB}	2.6^{aB}	2.7^{aB}	2.6^{aB}
	100	2.7^{aB}	2.8^{aB}	2.6^{aB}	2.8^{aB}
5	RT	4.2^{aA}	4.1^{aA}	4.3^{aA}	4.2^{aA}
	80	4.1^{aA}	4.0^{aA}	4.2^{aA}	4.3^{aA}
	90	3.1^{aB}	3.0^{aB}	3.1^{aB}	3.0^{aB}
	100	2.1^{aC}	2.0^{aC}	2.1^{aC}	2.0^{aC}
7	RT	4.0^{aA}	4.0^{aA}	3.9^{aA}	4.1^{aA}
	80	2.4^{aB}	2.2^{aB}	2.3^{aB}	2.4^{aB}
	90	2.3^{aB}	2.2^{aB}	2.3^{aB}	2.2^{aB}
	100	1.2^{aC}	1.3^{aC}	1.1^{aC}	1.3^{aC}
SEM	0.23				

All values presented are the means of six independent observations ($n = 6$).
a–c – Means in the same row are significantly different ($P < 0.05$).
A–D – Means in the same column are significantly different ($P < 0.05$).
SEM – pooled standard error of the mean; RT – room temperature (~22 °C).

driven cross linking of glutenin molecules mainly via thiol oxidation and to a lesser extent SH-SS interchange reactions (Lagrain, Brijs, Veraverbeke, & Delcour, 2005). Up to 90 °C the content remained stable, and further temperature rise to 100 °C caused additional decline ($P < 0.05$). At 100 °C, in addition to glutenin polymerization, covalent cross linking of some gliadins with glutenin molecules initiated by free thiol groups in glutenin likely resulted in further reduction of thiol content. Similar heat mediated changes of thiol content of gluten proteins in neutral pH has been reported by other researchers (Lagrain, Thewissen, Brijs, & Delcour, 2008; Lagrain et al., 2005; Singh & MacRitchie, 2004; Stathopoulos et al., 2008). Heat induced changes to free thiol content was also observed at pH 5 except that unlike the samples at pH 6.6 and 7, the first reduction ($P < 0.05$) was observed only at 90 °C followed by another reduction ($P < 0.05$) at 100 °C. Such a finding also corresponded with the changes in surface hydrophobicity (Table 1) confirming that gluten was more stable to thermal denaturation at pH 5 than at pH 6.6 (control) and 7 which are closer to its pI. Overall, heating of gluten up to 100 °C at pH 5, control pH or pH 7 caused about 50%, 60% or 70% reduction in free thiol content, respectively. This degree of free thiol content reduction reflected as an extent of SH/SS mediated cross linking of gluten

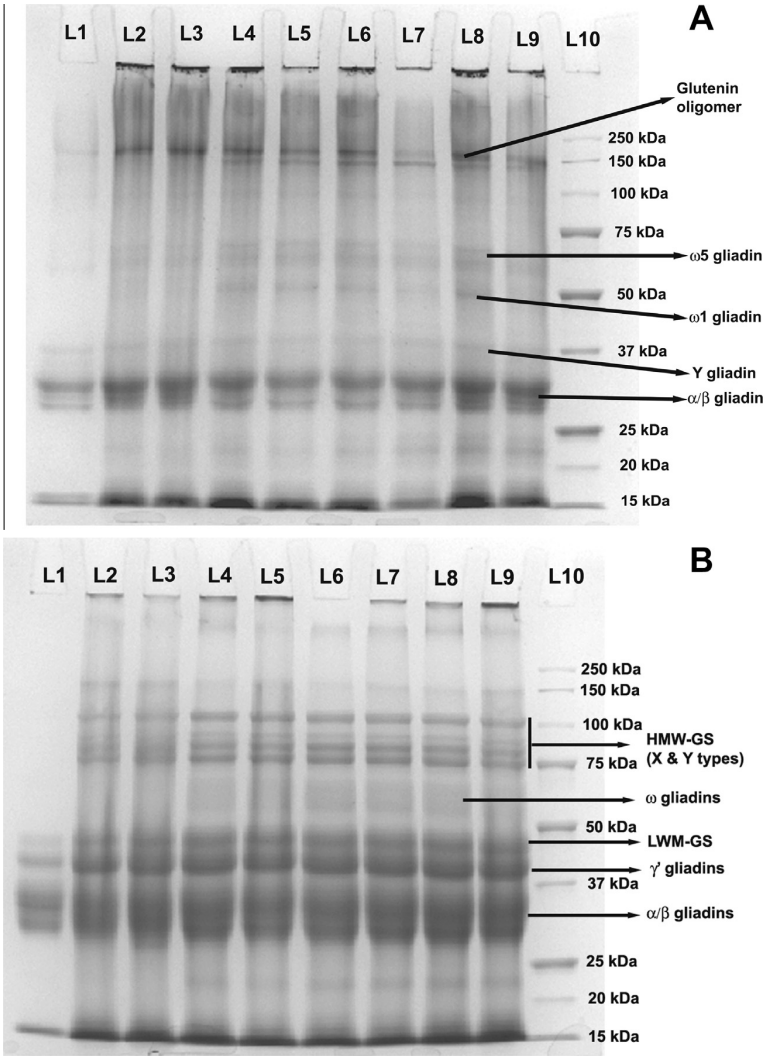

Fig. 1. Non-Reducing (A) and Reducing (B) SDS–PAGE pattern of gluten proteins sheared at 1000 s^{-1} and subjected to different pH and temperatures. L1 = gliadins standard, L2 = pH 3 Room Temp., L3 = pH 3 Temp. 100 °C, L4 = pH 5 Room Temp, L5 = pH 5 Temp. 100 °C, L6 = pH 7 Room Temp., L7 = pH 7 Temp. 100 °C, L8 = Control pH Room Temp., L9 = Control pH Temp. 100 °C, L10 = molecular marker.

proteins was in order of pH 7 > control pH (6.6) > pH 5. Such differences in thiol reduction could be a function of pH as thiol availability and reactivity of protein vary according to pH (Rahaman et al., 2015). This observation indicated that thiol mediated polymerisation of gluten proteins was mostly favoured at pH close to pI and below pI, the tendency decreased.

In contrast, free thiol content was minimal at pH 3. This lower content of SH was more likely due to the lower exposure and reactivity of cysteine residues at acidic pH (Wada, Fujita, & Kitabatake, 2006). Heating the samples at pH 3 up to 80 °C did not result in any change in thiol content and when temperature was raised to 90 °C, the thiol content increased ($P < 0.05$) and then remained steady until 100 °C. At higher temperature and low pH, partial unfolding of proteins along with exposure of some cysteine residues might be responsible for increased value of free SH content.

3.3. SDS–PAGE

Variations in shear rate at particular pH and temperature had no obvious effect on changes to protein conformation or their aggregation. Preliminary runs indicated that the electrophoretic patterns obtained for samples heated to 80, 90 and 100 °C were similar, but remarkably different to that at room temperature. Hence, only the samples sheared at 1000 s^{-1} at various pH, at room temperature and 100 °C were selected for assessment by both reducing and non-reducing SDS–PAGE to identify any changes in the proteins due to breakdown or aggregation. Under non-reducing condition the SDS–PAGE patterns of unheated gluten at pH 5, 7 and control pH (6.6) (Fig. 1A) showed specific bands corresponding to α/β-gliadins (MW 28–35 kDa), ϒ-gliadins (MW 35 kDa) ω1,2-gliadins(45 kDa), ω-5gliadins (MW 55–60 kDa) and glutenin oligomers (MW 150 kDa). Shear alone had no apparent influence on the electrophoretic pattern. Heating the samples at any pH up to 100 °C caused fading of all of gliadins and glutenin bands indicating heat induced glutenin-gliadin covalent aggregation (Lagrain et al., 2005; Lagrain et al., 2008). However, this degree of polymerisation was more pronounced at control pH and pH 7 than at pH 5. Since both control pH and pH 7 are closer to pI (6.4), it is reasonable to say that heating at such pH was more favourable to covalent aggregation than lower pH (5). Such

Table 3
Band intensities of ω-gliadins in non-reducing SDS–PAGE of gluten proteins sheared at 1000 s^{-1} at different pH and temperatures representing gel image in Fig. 1A. Lane numbers represent the same samples mentioned in Fig. 1A.

Band	Band volume (Intensity)							
	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9
$\omega 5$	284,100	0	486,476	618,048	498,464	501,881	582,528	383,653
$\omega 1$	0	0	934,731	915121	583786	444,932	887,112	715,876

observation was also supported by the earlier observation that extent of free thiol reduction due to SS link formation was more prominent at pH 7 and control pH (6.6) than at pH 5 (Table 2). Since glutenin components naturally remain as inter-molecular disulphide bonded polymers, large glutenin polymers were stacked on wells and no bands corresponding to HMW-GS (MW 65–90 kDa) and LMW-GS (MW 30–45 kDa) appeared in non-reducing condition. However, bands corresponding to some oligomers of HMW-GS and/or LMW-GS appeared.

Interestingly electrophoretic patterns of gluten at pH 3 were different. Unlike other pH, bands corresponding to ω-1, 2 and 5 gliadins (MW 45–60 kDa) disappeared or became faint both in unheated and heated samples (Fig. 1A) which could be attributed to deamidation of ω-gliadins with concomitant hydrolysis of some peptide bonds leading to degradation of ω-gliadins into very low molecular fractions. Such degradation of ω-gliadins was confirmed by quantification of band intensities (Table 3) obtained via image analysis. Deamidation is a post-translational modification of protein in which glutamine (Gln) and asparagine (Asn) are converted to glutamic acid (Glu) and aspartic acid (Asp)+ respectively due to removal of their amide groups (Abe et al., 2014). Deamidation of gluten proteins in acidic pH could lead to partial cleavage of peptide bonds (Zhao, Tian, & Chen, 2011). Although these studies mentioned hydrolysis of some gluten proteins due to acidic deamidation, it was not clear which particular proteins were subjected to these changes. However, our study clearly indicated that only ω-gliadins were affected (Table 3). Among the gluten proteins, ω-gliadins possess highest amount of glutamine residues, which account for more than 50% of total amino acids (Lasztity, 1995). Such high glutamine content as well as more accessible glutamine residues in repetitive N-terminal domain of ω-gliadin made it the most suitable candidate for deamidation in acidic pH in comparison to the other forms of gliadin. However, heating up to 100 °C at pH 3 did not result in any remarkable change in electrophoretic pattern which could be due to high electrostatic repulsion at low pH that prevented protein–protein association even at higher temperature.

However, in reducing SDS–PAGE (Fig. 1B), the band corresponding to monomeric HMW-GS and LMW-GS appeared in all the samples prepared at different temperatures and pH, which were absent in non-reducing PAGE as the glutenin proteins naturally remain as disulphide bonded polymer. Moreover, under reducing SDS–PAGE, the gliadin bands became more prominent in the samples treated at 100 °C and pH 7 or control pH, which were faint in non-reducing PAGE likely due to their involvement in covalent aggregation with glutenin.

3.4. FTIR

Effect of different pH, temperature and shear on the secondary structure of gluten was also evaluated by Fourier transform infrared spectroscopy (FTIR). Variation in shear at particular pH and temperature did not show any remarkable change in FTIR spectrum, therefore, only the sample sheared at 1000 s^{-1} was studied as the function of different pH and temperature (Fig. 2). However, at pH 7 and 100 °C, variations in shear rate affected the secondary

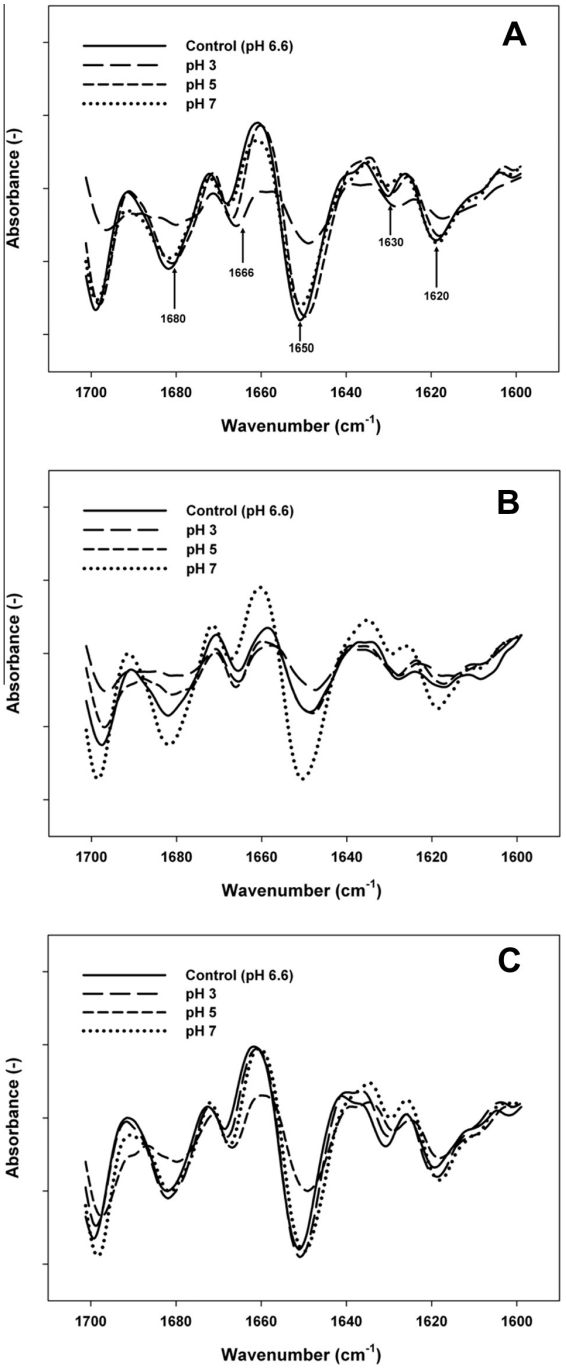

Fig. 2. Secondary derivatives of FTIR spectra of amide I region of gluten as a function of pH at room temperature (A), 80 °C (B) and 100 °C (C) and sheared at 1000 s^{-1}.

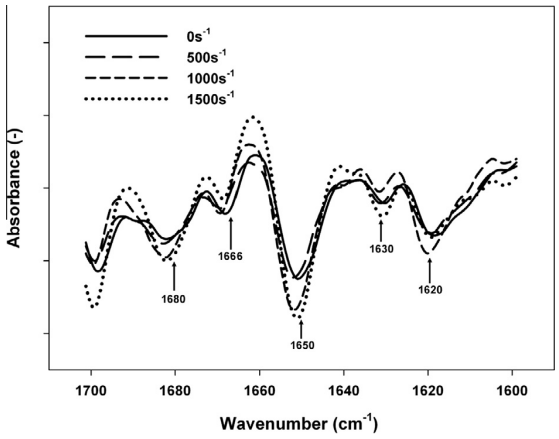

Fig. 3. Secondary derivatives of FTIR spectra of amide I region of gluten as a function a different shear rate (0, 500, 1000 and 1500 s^{-1}) at pH 7 and 100 °C.

structure (Fig. 3). Second derivative of amide I region (1600–1700 cm^{-1}) of the spectrum was investigated and the peaks at 1620 and 1630 cm-1 (β sheet), 1650 cm-1 (α helix), 1666 cm-1 (β turn structure) and 1680 cm-1 (intramolecular β sheet) (Yong, Yamaguchi, & Matsumura, 2006) were evident (Fig. 2).

Highest β turn (1680 cm^{-1}) and lowest α helix (1650 cm^{-1}) at pH 3 (Fig. 2A) indicated helix to β turn transition whereas for pH 5, 7 and control the trend was opposite. As glutamic acid (Glu) and aspartic acid (Asp) contribute to the formation of β turn (Cui et al., 2013), high exposure of these amino acids at lower pH, as a result of deamidation, might have contributed to such prominence of the turn structure at pH 3. Such increased β turn and decreased α helix conformation has been reported previously (Cui et al., 2013; Yong et al., 2006). Further, proline-rich regions in protein are known to be responsible for formation of polyproline helical conformation (Williamson, 1994). High exposure of hydrophobic proline residues as evident by greater Ho value (Table 1) could explain the higher prominence of helical structure observed at pH 5, 7 and 6.6 (control) than at pH 3. Upon heating to 100 °C, hiding of some hydrophobic proline residues might have caused disappearance of helical structure more noticeably at pH 5 than at pH 7 or control pH. In contrast, greater helical arrangements at pH 3 and 100 °C could be associated with exposed proline residues that could also explain the increased Ho (Table 1) observed for these samples. The presence of intramolecular β-sheet (1980 cm^{-1}) was recorded with maximum intensity at pH 5, 7 and control pH and minimum at pH 3 at room temperature (Fig. 2A) and heating to 100 °C caused slight increase of this structure in all samples. Another two peaks representing the β sheet region viz. 1630 cm^{-1} and 1620 cm^{-1} were present in all pH samples (Fig. 2A). However, at 80 °C and pH 7 (Fig. 2B), the peak at 1620 cm^{-1} grew at the expense of 1630 cm^{-1} indicating intermolecular β sheet aggregation while for other pH, there was no change. Nevertheless, at 100 °C increasing peak intensities at around 1620 cm^{-1} in all samples (Fig. 2C) could have resulted from some intermolecular β sheet association.

Shear alone had no effect on secondary structure of gluten regardless of pH. However, at pH 7, heating to 100 °C with high shearing (1000 or 1500 s^{-1}) caused relatively higher intensity of peaks representative of α helix and random coil (1653 cm^{-1}) and also intramolecular β sheet structures (1680 cm^{-1}) in comparison to other applied shear rates (Fig. 3). Hydrodynamic shear can trigger disruption of secondary and tertiary structure of protein, which includes unfolding of protein molecule and exposure of hydrophobic residues (Singh, Themistou, Porcar, & Neelamegham, 2009). However, shear driven structural changes are dependent on nature and molecular weight of proteins, shear rate, duration and also pH and temperature of the environment (Maa & Hsu, 1996; Walkenström, Panighetti, Windhab, & Hermansson, 1998). In our study, high rate shearing at pH 7 followed by heating at 100 °C led to exposure of hydrophobic proline residue which might have contributed to formation of α helix. Thus, near pI, high shearing rate and heating synergistically affect the quaternary and tertiary structure of gluten, leading to a shift from stretched and flexible form to helical and random coiled structure.

Table 4
Wheat gluten antigenicity as a function of temperature, pH and shear rate.

pH	Temperature, °C	Gluten antigenicity equivalent (mg kg^{-1})			
		Shear, s^{-1}			
		0	500	1000	1500
Control (6.6)	RT	63.5^{aA}	62.0^{aA}	61.0^{aA}	62.0^{aA}
	80	62.0^{aA}	61.0^{aA}	61.0^{aA}	64.5^{aA}
	90	62.6^{aA}	61.1^{aA}	63.3^{aA}	62.5^{aA}
	100	46.0^{aB}	46.5^{aB}	53.1^{bB}	54.5^{bB}
3	RT	40.8^{aA}	41.8^{aA}	41.5^{aA}	42.0^{aA}
	80	39.6^{aA}	39.8^{aA}	40.0^{aA}	39.8^{aA}
	90	38.6^{aA}	40.6^{aA}	41.1^{aA}	38.3^{aA}
	100	52.1^{aB}	50.1^{aB}	49.6^{aB}	49.1^{aB}
5	RT	60.3^{aA}	60^{aA}	61.3^{aA}	62.8^{aA}
	80	62.1^{aA}	63.0^{aA}	60.5^{aA}	59.1^{aA}
	90	61.5^{aA}	61.3^{aA}	58.3^{aA}	58.1^{aA}
	100	53.6^{aB}	53.0^{aB}	53.5^{aB}	54.3^{aB}
7	RT	63.5^{aA}	62.0^{aA}	62.3^{aA}	62.8^{aA}
	80	63.3^{aA}	65.3^{aA}	64.0^{aA}	64.1^{aA}
	90	63.1^{aA}	63.3^{aA}	62.0^{aA}	63.5^{aA}
	100	47.5^{aB}	48.8^{aB}	55.1^{bB}	55.3^{bB}
SEM	1.57				

All values presented are the means of six independent observations ($n = 6$).
a–c – Means in the same row are significantly different ($P < 0.05$).
A–D – Means in the same column are significantly different ($P < 0.05$).
SEM – pooled standard error of the mean; RT – room temperature (~22 °C).

3.5. Antigenicity related to conformational change

The short sequence of amino acids that binds with antibody to elicit immune reaction is called antigenic epitope. The most common antigenic epitopes of gluten protein are HLA (Human Leukocyte Antigen) DQ2 genetic factor associated and these are characterised by multiple proline (Pro) and Gln residues (Kim, Quarsten, Bergseng, Khosla, & Sollid, 2004). Tanabe (2008) also reported Gln-Gln-Gln-Pro-Pro as the major motif of wheat gluten protein to act as antigenic epitope. Availability of these antigenic fragments in gluten depend on secondary and tertiary structure of protein as well as the SS bonds that stabilize particular conformation of epitopes to bind with antibody (Waga, 2004).

Control gluten sample (pH 6.6) had highest antigenicity and changing the pH to 5 and 7 did not affect the antigenic potentiality remarkably (Table 4). Maximum exposure of antigenic hydrophobic residues with the evidence of highest Ho (Table 1), prominent helical conformation and intramolecular β sheet structure (peaks at 1650 and 1680 cm^{-1}, respectively; Fig. 2A) could facilitate exposure of epitopes to bind with antibody. In contrast, adjusting the pH to 3 reduced ($P < 0.05$) antigenicity by one-third compared to that of the control sample. As described above, at pH 3, acidic deamidation resulted in conversion of glutamine (Gln) to glutamic acid (Glu) with partial hydrolysis of some peptide bonds. Such type of changes could lead to modifications of some epitopes resulting in lower antigenic value in pH 3 than other pH values tested. Such finding agreed with Berti et al. (2007) who reported modification of immunogenic epitopes in gluten proteins due to conversion of Gln to Glu as a result of acidic deamidation resulting in significantly lowered immunoreactivity. Masking of some antigenic hydrophobic residues as evident by lower Ho (Table 1) might also have contributed to reduced antigenicity at pH 3. In addition, least helical arrangement (1650 cm^{-1}) and intramolecular β sheet structure provided the protein molecule with such a conformation that exposed least number of antigenic epitopes to bind with antibody. Similar observations were obtained by Yong et al. (2006) who reported decreased allergenicity of deaminated gluten associated with reduced intensity of the peak at 1680 cm^{-1}. However, no further changes in antigenicity were observed up to 90 °C and further heating to 100 °C caused substantial increase ($P < 0.05$) of immunoreactivity. At this temperature, exposure of new antigenic hydrophobic Pro residues together with progression of helical structure (Fig. 2C) contributed by exposed Pro (Rucker & Creamer, 2002b) could have resulted in appearance of some new epitopes resulting in higher antigenicity.

In contrast, the trend of changes in antigenicity of gluten when heated at pH 5 and 7 and control pH (6.6) was opposite to that observed at pH 3. At these pH values, antigenic potentiality remained unaffected up to 90 °C. However, at 100 °C both glutenin and gliadin are inter-linked through disulphide bonds and due to this aggregation, destruction and/or inaccessibility of some epitopes could have occurred that brought about reduction ($P < 0.05$) in antigenicity. Changes in secondary structural features as observed by reduced helical conformation as well as anti-parallel β sheet (Fig. 2C) could be related to such a decline. Shear alone had no effect on antigenic response regardless of pH. However, at pH 7, combination of high rate shearing (1000 and 1500 s^{-1}) and temperature (100 °C) triggered slight increase of Ho (Table 1) as well as shifting its secondary structure towards more helical and random coiled arrangement and formation anti-parallel β-sheet (Fig. 3) in comparison to low shear (500 s^{-1}) or without shear which could possibly have led to exposure of more epitopes resulting in increased antigenicity ($P < 0.05$).

4. Conclusion

Overall, gluten underwent considerable conformational changes in response to changes in pH, temperature and shear, and these changes substantially influenced its antigenic property. None of the combinations with different pH, heat and shear could totally abolish the antigenic capacity of gluten. Overall, at pH 3, up to 90 °C, the antigenicity was reduced by 30% in comparison to the control sample. Further heating to 100 °C increased antigenicity likely as a consequence of exposure of some new epitopes. Unlike pH 3, at pH 5 and 7, the antigenicity was as high as the control sample; and heating reduced the antigenic response. High shearing rate (1000 or 1500 s^{-1}) with high temperature (100 °C) at pH 7 synergistically enhanced ($P < 0.05$) antigenicity. However, it is subject of further investigation to understand how these changes affect digestibility, availability and characterisation of potential antigenic fragments post digestion and the reactivity of the digested product in gluten sensitive patients at cellular level.

Conflict of interest

The authors declare that there is no conflict of interest with regards to publication of this research work.

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Chapter 6

Effect of heat, pH and shear on digestibility and antigenic characteristics of wheat gluten

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PART B:

DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS BY PUBLICATION

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

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Signature: [redacted]

Date: 30/03/2016

Paper Title:

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In the case of the above publication, the following authors contributed to the work as follows:

Name	Contribution %	Nature of Contribution
Md. Toheder Rahaman	80	Designing and conducting the experiment, data collection and manuscript writing
Todor Vasiljevic	10	Concept development, experiment designing, data analysis and manuscript editing
Lata Ramchandran	10	Concept development, experiment designing, manuscript editing and submission for publication

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ORIGINAL PAPER

Effect of heat, pH and shear on digestibility and antigenic characteristics of wheat gluten

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Abstract Processing-induced conformational changes may affect digestibility and antigenic potential of food proteins. In vitro gastrointestinal digestibility of gluten proteins was established after treatment at various pH (3 or 7), temperature (room temperature or 100 °C) and shear (0 or 1500 s^{-1}). Electrophoretic patterns (SDS-PAGE) of the resulting hydrolysates and antigenicity (ELISA and immunoblotting) of gliadin fraction of the proteins were also studied. Digestibility was positively correlated with α-helix/β-sheet ratio. Lower antigenic reaction shown by digested hydrolysate of gluten samples treated at pH 3 and room temperature was ascribed to its acidic deamidation and improved digestibility. Further heating to 100 °C slightly increased antigenicity. In contrast, hydrolysate of gluten at pH 7 and room temperature exhibited highest antigenicity, attributed to partial resistance of α-/β-gliadin to digestion and appearance of some new potentially antigenic polypeptides. However, heating at 100 °C caused heat-induced protein aggregation, consequently lowered digestibility and availability of antigenic components resulting in a minimum (60 % reduction) antigenicity. Overall, shear had no effect on digestibility and antigenicity irrespective of pH and temperature. Thus, antigenic potential of gliadins can be minimized by selecting appropriate parameters during processing.

Keywords Antigenicity · Digestibility · Gluten · Heat · Shear · pH

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Introduction

Wheat is a staple food in many parts of the world which is consumed as various products. Extensive food use of wheat is attributed to viscoelastic properties of its gluten protein. Gluten is a major seed storage protein comprising 80 % of the total wheat protein. It consists of alcohol-soluble monomeric gliadins (α-/β-, γ- and ω-gliadins) and polymeric glutenin. Glutenin consists of a high molecular weight glutenin subunit (HMW-GS) and a low molecular weight glutenin subunit (LMW-GS), which are interlinked by disulphide (SS) bonds. Due to their unique physico-chemical and nutritional properties, availability and low cost, gluten is used not only in cereal products but also in other foods such as sausages, ice cream and even some drugs [1]. Despite its wide use in food products, gluten creates adverse immune reactions in some individuals. The most prevalent condition is coeliac disease, which is an autoimmune disorder of small intestine triggered by ingestion of gliadin and characterized by mucosal inflammation, villous atrophy and consequent malabsorption of various nutrients [2]. Some IgE-mediated reactions such as baker's asthma, exercise-induced anaphylaxis and atopic dermatitis are also caused by gluten [3].

Such allergic reactions to food occur either by primary contact of food protein antigens to components of the immune system and initial sensitization or through later repetitive exposure to the same antigens and elicitation of allergenic reaction. Since most of the immune sensitization to antigens takes place via gastrointestinal tract, digestibility of food protein antigens and their permeability into the immune system are key factors that may affect their antigenic potential [4]. A characteristic feature of most food antigens is their resistance to gastrointestinal digestibility allowing the preservation of sufficient intact epitopes

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to bind with a specific antibody. Therefore, understanding digestibility of food protein antigens and their post-digestion antigenic potential is fundamental to managing food allergenicity.

Foods undergo various processing to produce diversified end products that affect conformation of food proteins and, in turn, their susceptibility to gastrointestinal digestion [5]. Processing may increase [6, 7] or decrease [8] digestive susceptibility of food proteins which may, in turn, affect antigenic consequences. Wheat is always consumed after some degree of processing, e.g. baking, cooking, extrusion , and it is important to know how these processes affect the digestibility and antigenicity of gluten proteins. Some studies [9, 10] have shown that in vitro digestion of unheated bread dough showed almost complete disappearance of IgE-binding protein which persisted in bread crumb and crust. Heat-induced covalent protein aggregation during baking rendered the proteins less susceptible to proteolysis, thereby allowing passage of large IgE-reactive fragments through gastrointestinal tract where they could elicit more allergic reaction. Another important processing factor is pH, which can significantly affect the physico-chemical properties of gluten [11, 12] in products such as sourdough bread (pH < 5) [13]. Such variations in pH may affect digestive stability and consequently antigenicity of gluten proteins. Shear is another processing factor usually embedded in processes such as mixing, centrifugation and ultrafiltration that can potentially affect the physico-chemical properties of gluten [14]. However, shear alone may have no effect on the conformational and antigenic features of gluten proteins, but could have an influence in combination with particular pH and temperature [15]. To the best of our knowledge, no study has focused on the effect of different pH and shear in combination with different temperatures on the digestibility as well as antigenic potential of digested gluten proteins and, therefore, this work was aimed at addressing this gap.

Materials and methods

Preparation and selection of samples

Extracted gluten from plain wheat flour [16] was freeze-dried and ground to powder using a mortar and a pestle and sieved (no. 50) to obtain a fine powder. Exactly 8 % (w/w) gluten dispersion (concentration chosen on the basis of average gluten content in wheat flour [17]) was prepared in a citric acid-phosphate buffer at different pH values (3, 5 or 7) and then treated at different shear rates (500, 1000 or 1500 s^{-1}) and temperatures (80, 90 or 100 °C) in a pressure cell (CC25/PR-150) of a rheometer (Physica MCR 301 series, Anton Paar, GmbH, Graz, Austria) as described previously [18]. Gluten dispersion in Milli-Q water (pH 6.6) without any treatment (shear and heat) served as a control sample. Selected treatments (pH 3, room temperature and 0 s^{-1}; pH 3, 100 °C and 0 s^{-1}; pH 7, room temperature and 0 s^{-1}; pH 7, 100 °C and 0 s^{-1}; and pH 7, 100 °C and 1500 s^{-1}) were chosen based on the results of our reported work [15]. These conditions of high and low antigenicity were selected for further investigation into their digestibility and consequent impact on antigenicity.

Quantification of α-helix and β-sheet ratio (α/β)

Fourier transform infrared spectroscopy (FTIR) of both treated and control samples was performed by using a FTIR spectrometer (IRAffinity-1, Shimadzu Corp., Kyoto, Japan) as described previously [15]. Briefly, gluten powder (4 mg) was mixed with spectroscopy-quality KBr (60 mg) and homogenized with mortar and pestle. The mixture was pressed into pellets (1- to 2-mm-thick films) with a 10-ton hydraulic press. FTIR spectra of the pellets were collected between the wavelengths of 400–4000 cm^{-1}, with 20 scans at a resolution of 4 cm^{-1} using Happ-Genzel apodization. After smoothing the spectra (7 points), the bands for α-helix and β-sheet in amide I region (1600–1700 cm^{-1}) were identified [19, 20] and quantification of α-helix/β-sheet was achieved by estimating respective peak areas from the peak table of the spectra obtained using IRsolution software (Shimadzu Corp.).

Simulation of gastrointestinal digestion

In vitro digestion of all gluten samples was performed as described before [9] with some modifications. Briefly, exactly 120 mg of sample was suspended in 8 ml of 0.2 N HCl (pH 2.2) containing 0.05 mg/ml of pepsin. One hour after peptic digestion at 37 °C in a shaking water bath, mixture of 2.30 ml of 1 M boric acid and 0.5 N NaOH containing pancreatin (0.25 mg/ml) was added. The pH of the mixture was finally adjusted to 7.6 with 1 M NaOH. After 3 h of pancreatic digestion, the hydrolysate thus obtained was heated in boiling water bath for 10 min to inactivate the enzyme and then centrifuged (3000*g*) in a bench-top centrifuge (RT7, Sorvall, Newtown, CT). The resultant supernatant was stored at −80 °C for further analysis.

Determination of degree of hydrolysis

The degree of hydrolysis (DH) of the digested gluten samples was determined as the ratio of the percentage of 10 % trichloroacetic acid (TCA)-soluble nitrogen to total nitrogen in the sample [21]. To 5 ml of hydrolysate removed at the end of pancreatic digestion, an equal volume of 20 % TCA was added so as to obtain 10 % TCA-soluble and

TCA-insoluble fractions. After standing for 1 h, the mixture was centrifuged at 3000*g* using a bench-top centrifuge (RT7, Sorvall) at room temperature (20 °C). The supernatant was then analysed to determine nitrogen content by Kjeldahl method [22]. The DH was calculated as:

$$\mathrm{DH} = \frac{(\text{Soluble nitrogen in 10 \% TCA in hydrolysate} - \text{oluble nitrogen in 10 \% TCA in sample without hydrolysate}) \times 100\,\%}{\text{Total nitrogen in dispersion}}$$

Enzyme-linked immunosorbent assay

Antigenicity of the hydrolysates obtained was established by sandwich enzyme-linked immunosorbent assay (ELISA) using Ridascreen® Gliadin ELISA kit (R-Biopharm AG Darmstadt, Germany). An aliquot of 100 µl of supernatant from the hydrolysates was diluted with 1 ml of sample diluent. Exactly 100 µl of diluted samples was added to each separate duplicate well pre-coated with gliadin antibody. After 30-min incubation at room temperature (20 °C), the liquid was poured out and washed three times with a washing buffer (R-Biopharm AG Darmstadt, Germany) to remove unbound antigens. The plate was then tapped against absorbent paper to ensure complete removal of liquid from wells. After that, exactly 100 µl of diluted horse radish peroxidase (HRP)-conjugated detecting antibody was added to each well and incubated for 30 min at room temperature. Thereafter, the plate was washed three times and again tapped upside down against absorbent paper. Precisely, 50 µl of the substrate and 50 µl of the chromogen [3,3′, 5,5′-tetramethylbenzidine (TMB)] were added to each well followed by 30-min incubation at room temperature in dark to allow for colour development. Finally, 100 µl of stop reagent (1 N H_2SO_4) was added to each well, and the absorbance was measured at 450 nm using a microplate reader (iMarkTM, Bio-Rad Laboratories, NSW, Australia). The antigenicity was calculated from the standard curve and expressed as equivalent of mg of gliadin/kg of sample.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Reducing SDS-PAGE of hydrolysates from various treated gluten samples, control as well as an unhydrolysed gluten standard was employed in a study of the electrophoretic patterns. Protein fractions from supernatants of each hydrolysates were extracted by chloroform–methanol precipitation method [23]. Briefly, 100 µl of protein samples was mixed with 400 µl of methanol in a 1.5-ml Eppendorf tube and vortexed and then 100 µl of chloroform and 300 µl of distilled water were added and additionally vortexed. After centrifugation at 14,000*g*, the aqueous supernatant was discarded. Again 400 µl of methanol was added, mixed thoroughly and centrifuged at 14,000*g*. Finally, the methanol was discarded leaving the protein pellets at the bottom. The pellets were air-dried and solubilized in 20 µl of Tris-Tricine sample buffer (200 mM Tris–Hcl, pH 6.8, 2 % SDS, 40 % glycerol, 0.04 % Coomassie Brilliant Blue G-250, 2 % β-mercaptoethanol). Precisely, 1 mg of gluten was suspended in 200 µl sample buffer and served as a standard. Exactly, 20 µl of samples, gluten standard and molecular weight marker were loaded onto a 16.5 % Mini-PROTEAN® Tris-Tricine Gel (Bio-Rad Laboratories, NSW, and Australia). Electrophoresis was performed at a constant voltage of 100 V for 100 min. Then, the gels were immersed overnight in the staining solution composed of 0.025 % Coomassie brilliant blue, 40 % methanol, 7 % acetic acid and 50 % distilled water. Thereafter, the gel was destained with a destaining solution containing 40 % methanol, 7 % acetic acid and 53 % distilled water for 2 h followed by immersing in distilled water until the background became clear. Gel images were taken using a ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories, NSW, Australia).

Immunoblot analysis

Immunoblotting of different hydrolysates was carried out following a previously reported method [24] with some modifications. After electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane (0.2 µm) using a Mini Trans-Blot® cell (Bio-Rad Laboratories, NSW, Australia) and a Tris–glycine transfer buffer system (25 mM Tris, 192 mM glycine and 20 % (v/v) methanol, pH 8.3). The membrane was washed three times with Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.4) and then incubated in a blocking solution (1 % casein) for 1 h at room temperature (20 °C). The washed and treated membrane was transferred to a diluted solution (1:1000 in the blocking buffer) of an anti-gliadin primary antibody (IgG) (Antibodies-online GmbH, Georgia, USA) for 1 h at room temperature. Thereafter, the blot was washed five times with Tris-buffered saline containing 0.1 % Tween 20 (TTBS) with gentle agitation. The membrane was then incubated in secondary antibody (goat anti-rabbit IgG) (Biorbyt Ltd, Cambridgeshire, UK) conjugated with horseradish peroxidase (HRP) at a dilution of 1:3000 in TTBS for 1 h at room temperature. The blot was then washed five times in TTBS. Antibody complexes captured by the immobilized target protein were detected by

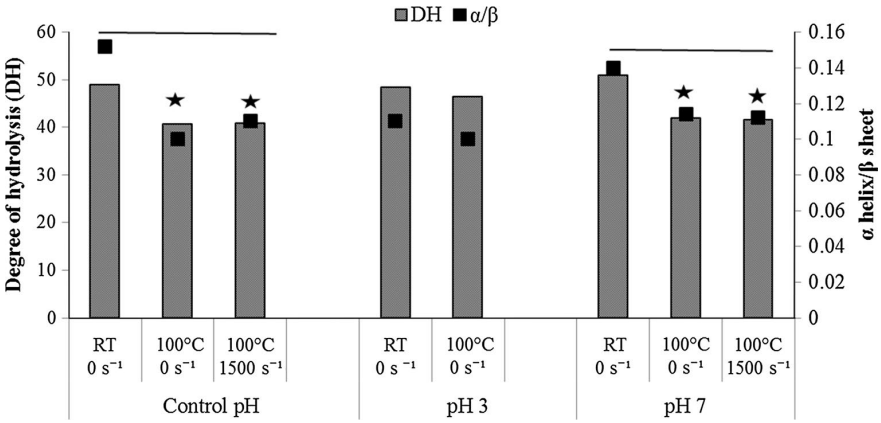

Fig. 1 Degree of hydrolysis (DH) of control and treated gluten samples. The values in this figure are the mean of triplicates. The *lines* above signify the differences (*asterisk*) at $P > 0.05$. *RT* room temperature (~20 °C)

enhanced chemiluminescence detection system using Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories, NSW, Australia). Images were taken using a Chemi-Doc imager (Chemidoc MP, Bio-Rad Laboratories, NSW, Australia).

Statistical analysis

The experiment was designed as a randomized block, full factorial design with pH as the main plot and shear and temperature as the subplots. This block was replicated with three subsampling resulting in at least six independent observations ($n \geq 6$). The results of the various determinations were analysed using the general linear model (GLM) procedure of SAS (SAS, 1996). The level of significance was set at $P = 0.05$.

Results and discussion

Digestibility of gluten treated at different pH, shear and heat

In vitro digestion of both control and treated gluten samples was performed to study the effect of various pH, temperature and shear on the extent of proteolysis of gluten proteins. Degree of hydrolysis (DH) was measured as 10 % TCA-soluble nitrogen content, which consisted of partially digested gliadins as well as small and/or large polypeptides released as products of enzymatic breakdown. Temperature significantly ($P < 0.05$) affected DH of gluten, whereas pH and shear had no remarkable effect (Fig. 1). At pH 7, room temperature and 0 $s-^{1}$ gluten had as high DH as in control sample, and heating to 100 °C caused significant ($P < 0.05$) reduction in DH, which could be attributed to heat-induced physico-chemical changes [9, 15, 25]. One such a change involves variations in conformations of the secondary structure (α-helix and β-sheet) of the protein. Quantification of α-helix and β-sheet components from the FTIR spectra showed that at both, control pH (6.6) and pH 7, and room temperature, gluten had a higher α-helix content. Increasing the temperature to 100 °C brought about a decrease in α-helix content at the expense of the β-sheet conformation (reduction of α/β ratio) suggesting a positive correlation between DH and α/β (Fig. 1). This could be attributed to the comparatively more efficient access by digestive enzymes to flexible helical conformation of gluten than the β-sheet structure. Similar findings have also been reported previously [26]. Such decreased accessibility of digestive enzymes to the β-sheet conformation can also be ascribed to the presence of β-branched amino acids (threonine, valine and isoleucine) in gluten, which are not preferable sites of action for trypsin [27]. Another possible reason for reduced digestibility at high temperature could be due to lowered surface hydrophobicity, as was reported in our earlier work [15], which are the active sites for peptic and chymotryptic digestion [28]. Moreover, at high temperature gluten proteins undergo disulphide-bonded aggregation as evident in our previous study [15], which provides the protein with a more compact structure that could have rendered it partly inaccessible to digestive enzymes [25, 29].

On the other hand, DH of sample at pH 3, room temperature and 0 s^{-1} was as high as control (Fig. 1) although the α/β ratio was not as high. Such higher DH at pH 3 without a substantial change of α/β ratio could be ascribed mainly to the effect of deamidation. It is well known [15, 30, 31] that gluten undergoes acidic deamidation at low pH with a concomitant partial hydrolysis of peptide bonds and such a deamidation results in improved digestibility [26, 32]. However, further increase of temperature to 100 °C caused slight decrease ($P > 0.05$) of DH that could be associated with covalent cross-linking among the gluten proteins that in turn reduced its accessibility to digestive enzymes.

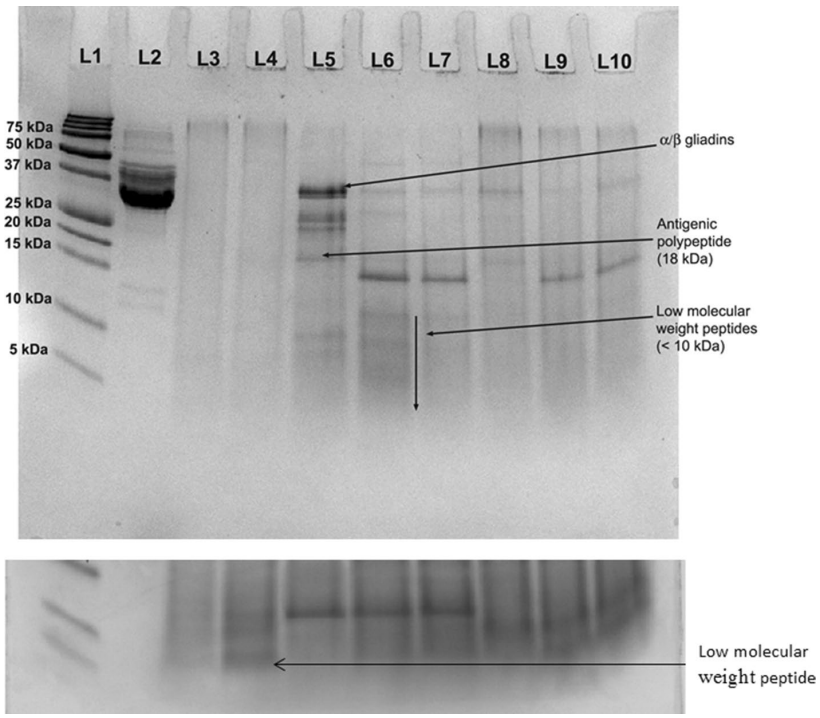

Fig. 2 Reducing SDS-PAGE pattern (after in vitro digestion) of gluten proteins subjected to different pH and temperatures and shear. *L1* molecular marker, *L2* undigested gluten standard, *L3* pH 3 room temp. 0 s^{-1}, *L4* pH 3 100 °C 0 s^{-1}, *L5* pH 7 room temp. 0 s^{-1}, *L6* pH 7 100 °C 0 s^{-1}, *L7* pH 7 100 °C 1500 s^{-1}, *L8* control, *L9* control pH 100 °C 0 s^{-1}, *L10* control pH 100 °C 1500 s^{-1}

Gel electrophoretic analysis

Electrophoretic patterns of hydrolysed gluten samples obtained by reducing SDS-PAGE (Fig. 2) varied as did the DH (Fig. 1) as a consequence of the various treatment conditions. Out of all main factors, pH and temperature influenced the gel electrophoresis patterns greatly, while shear did not have any effect. Undigested gluten (standard) showed typical bands for gluten proteins—α-/β-gliadins (MW 28–35 kDa), γ-gliadins (MW 35 kDa), ω1,2-gliadins (MW 45 kDa), ω-5gliadins (MW 55–60 kDa), HMW-GS (MW 65–90 kDa) and LMW-GS (MW 30–45 kDa). Similar to the control sample (Lane 8), hydrolysis of gluten at pH 7, room temperature (20 °C) and 0 s^{-1} (Lane 5) produced polypeptides corresponding to 25, 18 kDa and some low molecular weight (<10 kDa) fragments. However, the S-rich prolamins, α-/β-gliadins underwent a partial hydrolysis (Fig. 2), which could be attributed to creation of intramolecular disulphide bonds, which rendered their globular structure partly inaccessible to proteases. A similar finding has been reported in another study [9]. In contrast, ω-gliadins, lacking cysteine residues and intramolecular S–S bonds, were more susceptible to proteolysis and were completely hydrolysed as evident by the absence of corresponding bands in the gel (Fig. 2). However, electrophoretic patterns of the hydrolysates from heated samples at pH 7 (Lanes 6 and 7) and control pH (Lanes 9 and 10) were considerably different to those obtained from the corresponding unheated samples (Fig. 2). Digestion of heat-treated samples resulted in the formation of another prominent band at 13 kDa representing an intermediate polypeptide, which was absent/very faint in unheated samples. These additional polypeptides could be a result of partial proteolysis of any of the gluten component, which could otherwise be completely hydrolysed into low molecular weight fractions (<10 kDa) as observed in unheated samples (Lane 5 for pH 7 and Lane 8 for control). Such an incomplete proteolysis of heated samples was also supported by their lower DH value (Fig. 1) and could be attributed to heat-induced conformational modifications such as covalent aggregation and Maillard reactions [10].

Interestingly, gel electrophoretic patterns of samples adjusted to pH 3 were different from those of pH 7 and control pH. Unheated samples at pH 3 underwent extensive hydrolysis to very low molecular weight peptides, which were not apparently retained in the gel. Moreover, contrasting to other pH, all the gliadins (including S-rich components) were completely hydrolysed in the samples at pH 3. Such an extensive hydrolysis that yielded very low molecular fragments in samples at pH 3 could be attributed not only to enzymatic breakdown but also to the effect of acidic deamidation. Our earlier work [15] showed that at low pH gliadins underwent acidic deamidation with partial

Table 1 Antigenicity of in vitro digested gluten treated at different temperature, pH and shear rate

pH	Temperature	Gliadin antigenicity equivalent (mg/kg)	
		Shear (s^{-1})	
		0	1500
Control (6.6)	RT	18.8[a]	–
	100	11.47[b]	10.63[a]
3	RT	12.46[b]	–
	100	16.12[c]	–
7	RT	19.69[a]	–
	100	8.90[d]	9.32[b]
SEM	0.44		

All values presented are the means of six independent observations ($n = 6$)

Significant differences ($P < 0.05$) between means across the same column are indicated by different letters

SEM pooled standard error of the mean, *RT* room temperature (~22 °C)

hydrolysis of peptide bonds into very low molecular peptides. Deamidation is a post-translational modification of a protein resulting in conversion of glutamine (Gln) and asparagine (Asn) to glutamic acid (Glu) and aspartic acid (Asp), respectively, by the removal of the amide functional group [30, 33]. Thus, acidic deamidation and enzymatic breakdown synergistically degraded the gluten proteins almost completely to produce only very low molecular weight peptides.

Antigenicity of hydrolysed gluten

Antigenic prospective of gliadins in the hydrolysed gluten samples was assessed by ELISA (Table 1) and immunoblotting (Fig. 3) against anti-gliadin antibody, which, in general, varied with different pH and temperature, while shear had no effect. Highest antigenic value determined by ELISA was recorded for hydrolysates from gluten samples at room temperature, 0 s^{-1} and pH 7 that was similar to the control (pH 6.6). Immunoblotting study (Fig. 3, Lanes 5 and 8) indicated that two components, viz. at 28–35 and 18 kDa molecular weight, bind with antibody, contributing to such high antigenic value. Components around 28–35 kDa corresponded to α-/β-gliadins (S-rich prolamins), which persisted during in vitro digestion (Fig. 1). A polypeptide observed around 18 kDa likely resulted from hydrolysis of gliadins preserving sufficient epitopes to react with antibody. However, both these reactive components showed lower band intensity (Fig. 3, Lanes 6, 7, 9 and 10) (Table 2) for hydrolysates of gluten at 100 °C, which resulted in a 60 % reduction of antigenic value (Table 1) in comparison with that at room temperature. At higher temperature (100 °C), gliadins undergo covalent aggregation with glutenin as shown in our previous work [15] as well as other studies [34, 35] that might have caused lower availability of α-/β-gliadins to react with the anti-gliadin antibody. Moreover, such heat-induced changes could have also lowered digestibility (Fig. 1) of gliadins resulting in a lower yield of the potentially antigenic polypeptide fraction (MW 18 kDa).

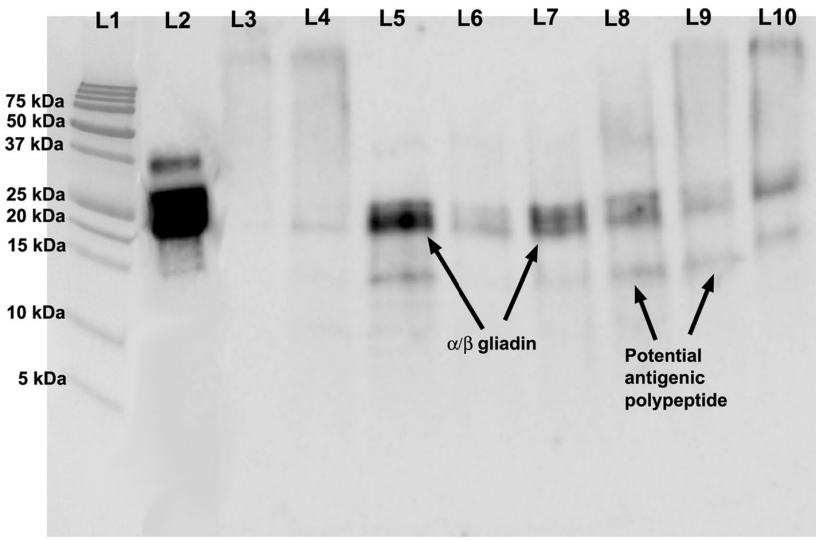

Fig. 3 Immunoblotting showing the binding of anti-gliadin antibodies to the reactive gliadin polypeptides obtained from enzymatically hydrolysed gluten. *L1* molecular marker, *L2* undigested gluten standard, *L3* pH 3 room temp. 0 s^{-1}, *L4* pH 3 100 °C 0 s^{-1}, *L5* pH 7 room temp. 0 s^{-1}, *L6* pH 7 100 °C 0 s^{-1}, *L7* pH 7 100 °C 1500 s^{-1}, *L8* control, *L9* control pH 100 °C 0 s^{-1}, *L10* control pH 100 °C 1500 s^{-1}

Table 2 Band intensities of potentially antigenic fractions from different gluten hydrolysates, due to binding with anti-gliadin antibody in immunoblotting represented in Fig. 2

Band	Band volume (intensity)								
	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
α-/β-Gliadin (MW 28–35 kDa)	14,692,500	0	104,000	3,534,208	674,848	2,090,192	1,392,848	472,320	788,928
Intermediate antigenic polypeptide (18 kDa)	0	0	0	376,112	0	129,424	319,824	300,088	232,768

L2, undigested gliadins standard; L3, pH 3 room temp. 0 s^{-1} ; L4, pH 3 100 °C 0 s^{-1}; L5, pH 7 room temp. 0 s^{-1}; L6, pH 7 100 °C 0 s^{-1}; L7, pH 7 100 °C 1500 s^{-1}; L8, control pH room temp. 0 s^{-1}; L9, control pH 100 °C 0 s^{-1}; L10, control pH 100 °C 1500 s^{-1}

On the other hand, changes in antigenic potential of hydrolysates of gluten at pH 3 were different to those observed at other pH (Table 1) (Fig. 3). A digest from gluten at pH 3, room temperature and 0 s^{-1} had a significantly ($P < 0.05$) lower antigenic value (Table 1) than that of the control sample. Such a lower antigenicity at pH 3 could be attributed to the absence of two antigenic components (α-/β-gliadins, MW 28–35 kDa and a polypeptide, MW 18 kDa; Fig. 3, Lanes 3 and 4) which were present at both pH 7 and control pH (Fig. 3, Lanes 5 and 8). As discussed above, a synergistic effect of acidic deamidation and enzymatic breakdown at pH 3 might have caused an extensive degradation of gluten proteins into predominantly very low molecular weight fragments. These fragments may be too small to be retained in the gel during SDS-PAGE and subsequent transfer to the membrane for immunoblotting although they might have some epitopes to show positive reaction in ELISA. Similar phenomenon was reported in other studies [26] where it has been shown that deamidated gluten became more susceptible to pancreatic digestion and produced very low molecular weight peptides (<5 kDa). However, hydrolysate of heated (100 °C) gluten at pH 3 showed slightly higher ($P < 0.05$) antigenicity (Table 1). Such an increase could have originated from a residual antigenicity of smaller peptides (<5 kDa) (Fig. 2), resulting from limited hydrolysis of gliadins as evident by reduced DH (Fig. 1) due to heat-induced aggregation. However, such smaller peptides were absent in case of the unheated sample at pH 3. Moreover, such heat-induced aggregation left a small portion of α-/β-gliadins undigested which exhibited binding reaction with antibodies during immunoblotting (Fig. 3).

Conclusions

Different processing conditions caused varied effects on physico-chemical properties of gluten, which consequently affected its digestive stability as well as antigenic potential of its gliadin fractions. The results indicated that pH and temperature influence the digestibility and thereby the antigenic characteristics of the resulting hydrolysates, while shear alone had no effect. At low pH (pH 3), gliadins underwent acidic deamidation with enhanced hydrolysis into very low molecular weight fractions resulting in lower antigenicity. At high temperature (100 °C), due to protein aggregation, partial resistance of some gliadins to hydrolysis resulted in slight increase in antigenicity. Nevertheless, at neutral pH (pH 7), similar to control (pH 6.6), gluten had high digestibility yielding some potentially antigenic fractions that produced in maximum antigenicity values. Heating to 100 °C at both pH 7 and control pH (6.6) induced covalent protein aggregation that resulted in decreased digestibility and less availability of antigenic components and therefore minimum antigenicity. Thus, although no treatment can totally abolish the antigenic potential of gliadins in gluten post-digestion, heating samples at neutral pH (pH 7) to 100 °C could remarkably reduce the intensity of sensitization. However, the results need to be further investigated in vivo.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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Chapter 7

Overall conclusions and future research direction

7.1 Conclusion

Different processing conditions affect physico-chemical properties of food proteins in different ways which in turn affect their digestive stability and antigenicity depending on the inherent molecular characteristics of proteins and type and extent of the treatment conditions. Heating β-lg at low pH (pH 3) caused unfolding of protein molecule, increased surface hydrophobicity and β-sheet components and partial acid hydrolysis which led to exposure of some hidden antigenic epitopes resulting in significant increase in antigenicity. In contrast, heating at pH 5 and 7 above 80°C caused disulphide bonded β-lg aggregation with decreased hydrophobicity contributing to destruction and/or masking of conformational epitopes accounting for reduced antigenicity and the rate of reduction was greater at pH 5 than pH 7. Shear rate correlated negatively with antigenicity at pH 3 and 5 whereas at neutral pH (pH 7) the effect was not significant. However, the lowest antigenicity was recorded for the β-lg heated to 120°C at pH 5 and high shear rate (1000 s-1).

Selected processing conditions including pH, temperature and shear mediated conformational changes of β-lg influenced its digestive susceptibility and antigenicity of the resulting digests. Unheated β-lg, regardless of pH, was hardly digested by pepsin and showed high antigenicity as undigested sample while fairly susceptible to pancreatin with considerable reduction in antigenicity. High shear (1000 s-1) slightly reduced digestibility of β-lg and thus enhanced antigenic potential in unheated samples at pH 5 and 7 whereas no effect was noted at pH 3. Heating to 120°C significantly enhanced digestibility of β-lg at all pH with much less antigenicity than the unheated samples. At pH 3 and 7, heated β-lg underwent complete hydrolysis into very low molecular weight fractions <10 kDa which might still possess sequential epitopes with residual antigenicity. However, lowest antigenicity was shown by digest from β-lg

treated at pH 5 and 120°C although it was not completely hydrolysed as in pH 3 and 5. Thus, the reduction of antigenicity is positively correlated with degree of hydrolysis (DH) and such reduction is not only dependent on DH but also on relative susceptibility of peptide bonds involved in integrity of antigenic epitopes to digestive enzymes which might vary according to treatment conditions.

Similar to β-lg, gluten also experienced conformational changes with different pH, temperature and shear and such changes affected its antigenicity considerably. At low pH, deamidation of gliadin fraction (mostly ω-gliadin) contributed to the modification of epitopes resulting in lower antigenicity and at high temperature (100ºC), the antigenicity substantially increased possibly due to exposure of some new epitopes. In contrast, heating at pH 5 and 7 up to 90ºC did not exert any significant change in its antigenicity and further heating to 100ºC induced disulfide linked aggregation between glutenin and gliadins resulting in masking and/or destruction of some conformational epitopes with the consequence of significant reduction of antigenicity. High rate shearing (100 or 1500 s^{-1}) at neutral pH (pH 7) and high temperature (100°C) could slightly enhance the antigenic potential.

Such processing induced changes considerably affected digestibility of gluten and its post digestion antigenicity. Deamidation of gluten at low pH significantly improved its *in vitro* digestibility causing complete hydrolysis of α/β gliadins into very low molecular weight peptides resulting in lower antigenicity and heating at 100ºC slightly reduced its digestibility with little increase in antigenicity. Contrary to pH 3, digestion of unheated gluten at neutral pH (pH 7) yielded a new potential antigenic fraction resulting in maximum antigenicity. Heating to 100°C induced covalent protein aggregation which resulted in decreased digestibility and less availability of antigenic components and therefore minimum antigenicity.

Overall, effect of processing on antigenicity of food proteins is not straight forward; depending on type and extent of treatment and molecular characteristic of the protein. Moreover, although none of the selected conditions could totally abolish antigenic potential of both β-lg and gluten, severity of the sensitization could be minimized by selecting appropriate conditions. Again, challenging the sensitive individual with low antigenic processed food to develop the tolerance could be an alternative therapeutic approach.

7.2 Future research direction

This work summarized the effect of selective pH, temperature and shear on structural changes of purified β-lg and gluten related to their antigenicity. In the real food systems, other ingredients are also present which might affect processing induced conformational changes of β-lactoglobulin and gluten and thereby their antigenic potentials? Therefore, further investigation on the effect of selective treatments on the antigenicity of whole food is required to obtain a more holistic evaluation for their application in food manufacturing.

β-lg and gluten proteins are extensively used in wide range of food products due to their excellent functional properties. Parallel to effect on antigenicity, how does applied processing induced conformational changes affect the functional properties of these two proteins? Due to time constraint this question has not been addressed. Thus, future study on influence of different treatment conditions on the functional properties of β-lg and gluten proteins could settle the suitability of application of the selective parameters in food industries.

In this study antigenicity was assessed on the basis of binding affinity with commercial antibody by ELISA and Western Blotting. To obtain a greater insight at

physiological level, a subsequent work on binding capability of processed β-lg and gluten with serum IgE from known allergic patients is required. Moreover, such serum IgE reactivity of food proteins may not always translate to clinical allergic symptoms. Thus, other assessments such as oral challenge and skin prick test also need to be performed before concluding the effect of any processing method on allergenic potential of any food protein.

Effect of selective factors on the digestive pattern of β-lg and gluten was assessed by *in vitro* digestion model. Sometimes results of *in vitro* digestion are different to those found in *in vivo* models because of difficulties in accurately simulating highly complex physico-chemical and physiological events occurring in animal and human digestive tract. Thus, *in vivo* digestion of processed antigens in animal model, collecting the gastric and intestinal contents and electrophoretic analysis is further recommended to compare with *in vitro* results.

After *in vitro* digestion, some components were identified to show antigenic reaction. Separation of such fractions through HPLC or FPLC and determination of their amino acid sequence could give in-depth knowledge of stability of sequential epitopes in response to applied treatment conditions. Again, measuring the antigenic potential of isolated fraction by assessing their ability to release allergenic mediators such as histamines and cytokines in peripheral blood mononuclear cell (PBMC) could provide more insight at physiological level.

Although many hypoallergenic partial and complete milk hydrolysate baby formulas are available in the market, many of allergic children cannot tolerate such commercial formulas. The β-lg digest in this study from selected treatment condition with lowest antigenicity could be incorporated into commercial milk formulas and

assessing their antigenic response could be a solution for the children with sensitivity to hydrolysed market formulas.

Chapter 8

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