



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.

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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, & Brodtkorb, 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 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

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:

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

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, Brodtkorb, & Kouaoui, 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.

2.4. 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 μ l 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 μ l 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 μ l of diluted horse-radish 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 μ l 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 iMark™ 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 ($n \geq 6$). 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

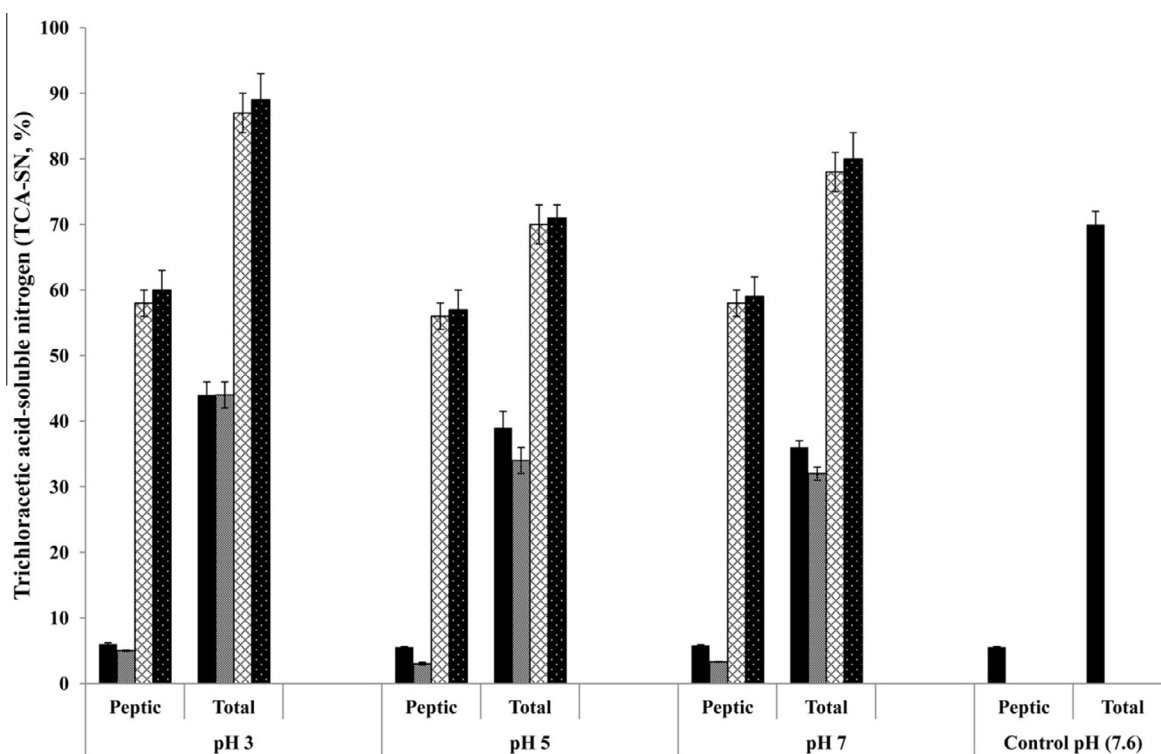


Fig. 1. Trichloroacetic acid-soluble nitrogen (TCA-SN) content (%) after peptic and total (both peptic and pancreatic) digestion of control and treated β -lg at different pH, temperature and shear. The values in this figure are the means of three replications, with ■ representing TCA-SN at RT and 0 s⁻¹, ▨ 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.

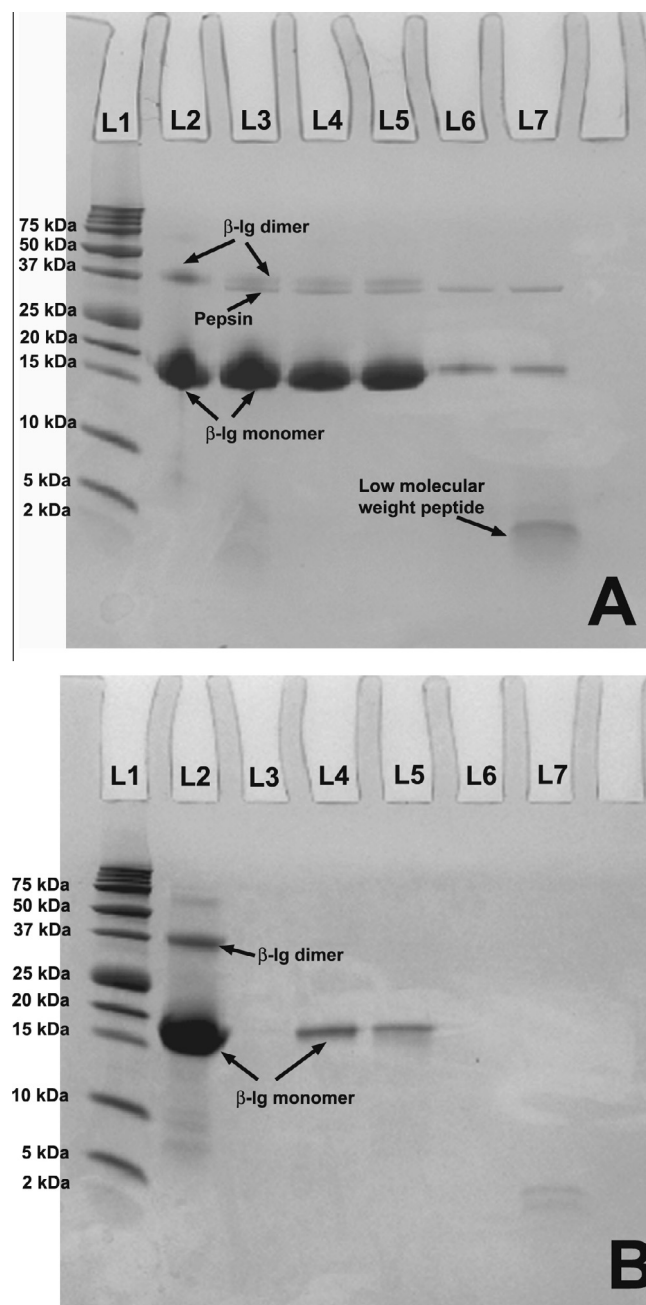


Fig. 2. Non-reducing SDS-PAGE profiles of *in vitro* (A) peptic and (B) total (both peptic and pancreatic) digestion of β -Ig at pH 3. L1 = Molecular marker, L2 = Undigested control, L3 = Digested control, L4 = Room Temperature, 0 s^{-1} ; L5 = Room Temperature, 1000 s^{-1} ; L6 = $120\text{ }^{\circ}\text{C}$ 0 s^{-1} ; L7 = $120\text{ }^{\circ}\text{C}$ 1000 s^{-1} . Control sample was 0.3% β -Ig 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 -Ig 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 -Ig to two pancreatic enzymes (trypsin and chymotrypsin). Heating at $120\text{ }^{\circ}\text{C}$ further

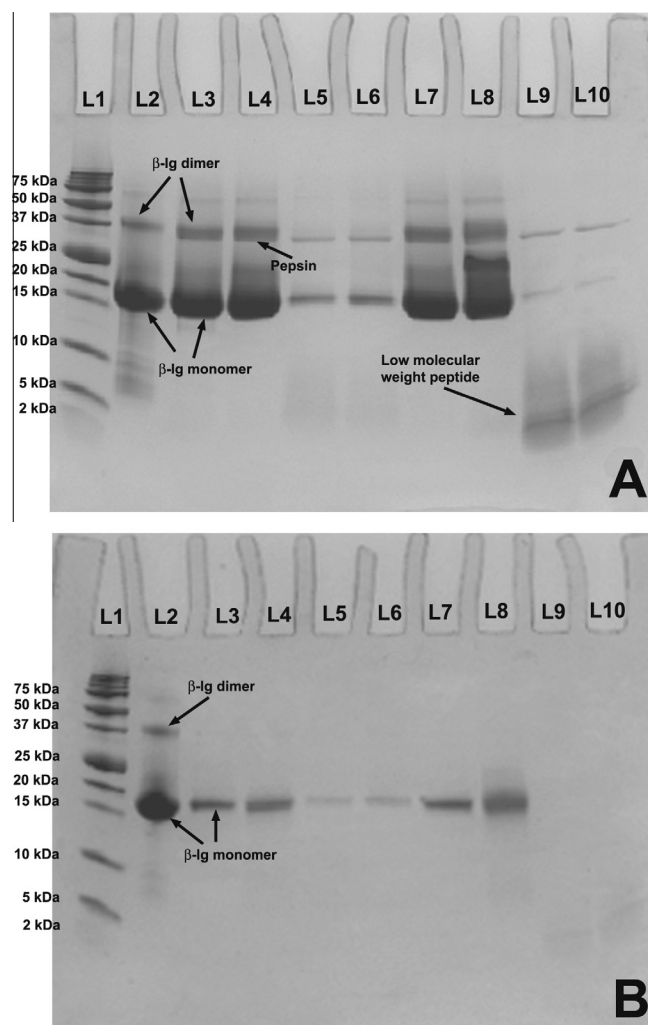


Fig. 3. Non-reducing SDS-PAGE profiles of *in vitro* (A) peptic digestion and (B) total (both peptic and pancreatic) digestion of β -Ig at pH 5 and 7. L1 = Molecular marker, L2 = Undigested control, L3 = pH 5, Room Temperature, 0 s^{-1} ; L4 = pH 5, Room Temperature, 1000 s^{-1} ; L5 = pH 5 $120\text{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

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 -Ig 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 -Ig greatly depended on pH (Fig. 1). After completion of digestion, highest TCA-SN was recorded at pH 3 and $120\text{ }^{\circ}\text{C}$ 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 -Ig heated to $120\text{ }^{\circ}\text{C}$ 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 $120\text{ }^{\circ}\text{C}$.

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

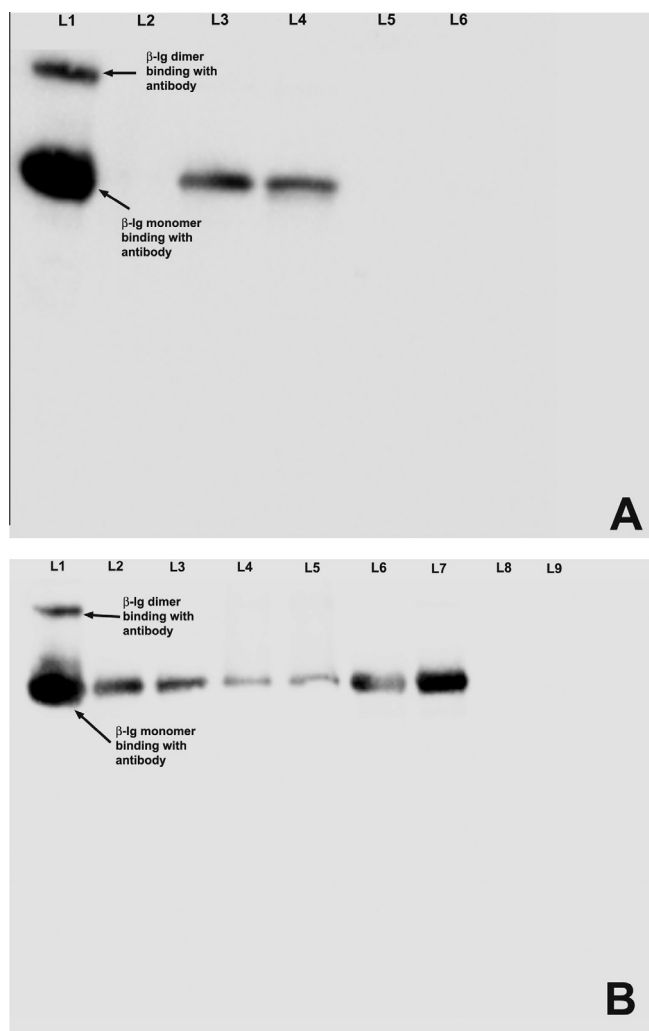


Fig. 4. Western blots depicting binding of anti-β-lg antibody to the reactive sites of digested β-lg treated at selected temperature and shear at (A) pH 3 and B) pH 5 and 7. For (A), L1 = Undigested control, L2 = Digested control, L3 = pH 3, Room Temperature, 0 s⁻¹; L4 = pH 3, Room Temperature, 1000 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 1Antigenicity 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 unheated 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 & Lönnnerdal, 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 unheated 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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