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Thermal denaturation of bovine β-lactoglobulin in different protein mixtures in relation to antigenicity

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ABSTRACT

Denaturation of β-lactoglobulin (BLG) was studied in relation to its antigenicity at two heat treatments in several native protein mixtures; allergenicity was determined by enzyme-linked immunosorbent assay based on BLG capacity to bind with immunoglobulin G (IgG) antibodies. The influence of other proteins on BLG denaturation correlated with altered antigenicity. Treatment at 72 °C/15 s enhanced antigenicity in a BLG+α-lactalbumin (ALA) mixture, possibly due to exposed epitopes in the unfolded structure. Treatment at 100 °C/30 s mostly resulted in BLG-led protein aggregation through thiol/disulphide interactions and decreased antigenicity by fragmentation and masking of epitopes, the extent of which was mixture-dependent. The presence of IgG resulted in diminished antigenicity in BLG + ALA + IgG at 100 °C/30 s in comparison with BLG + ALA. ALA governed whey protein denaturation over BLG in BLG + ALA + IgG + bovine serum albumin (BSA), possibly catalysed by BSA at 100 °C/30 s, resulting in a higher retention of antigenicity than in other mixtures.

1. Introduction

Denaturation of bovine β-lactoglobulin (BLG), interaction of denatured BLG with other whey proteins such as α-lactalbumin (ALA) and bovine serum albumin (BSA), and interaction of denatured BLG with caseins (CNs), for instance κ-casein (κ-CN), result in various changes in heated milk (Wijayanti, Bansal, & Deeth, 2014). The denaturation behaviour of BLG induced by heating can vary in different bovine protein mixtures and differ from those of BLG in the whey fraction or in milk. BLG often appears to drive aggregation of whey proteins in milk during heating due to its being at a higher concentration (approximately 3.2 g L^{-1}) than the other whey proteins (Considine, Patel, Anema, Singh, & Creamer, 2007; Patel, 2007; Wijayanti et al., 2014). Conversely, we recently reported a leading role of ALA over BLG in thermal denaturation of whey proteins in the presence of BSA and immunoglobulin G (IgG) (Bogahawaththa, Chandrapala, & Vasiljevic, 2017b).

The antigenic/allergenic potential of native milk proteins is altered when subjected to thermal denaturation and aggregation due to modifications of protein structures and associated antibody binding sites, termed epitopes. Epitopes are specific portions of the protein structure that can bind with the complementary sites of antibodies and provoke immune responses such as antigenic (binding with IgG) and or allergenic (binding with IgE) reactions (Bogahawaththa, Chandrapala, & Vasiljevic, 2017a; Rahaman, Vasiljevic, & Ramchandran, 2016).

There are two main types of epitopes, termed linear and conformational. Linear epitopes are continuous sequences of amino acid residues based on the protein primary structure and conformational epitopes are discontinues sequences of amino acid residues, which are combined by its secondary or tertiary structure. Typically, conformational epitopes are more heat-sensitive than linear epitopes due to their structural features (Bogahawaththa et al., 2017a; Kleber, Krause, Illgner, & Hinrichs, 2004; Konstantinou & Kim, 2012).

Specific secondary and tertiary structures are mainly associated with antigenicity and allergenicity of BLG since both linear and conformational epitopes are spread throughout the BLG structure (Bogahawaththa et al., 2017a; Rahaman, Vasiljevic, & Ramchandran, 2015; Zhong et al., 2012). Several studies have characterised common and prominent antigenic and/or allergenic epitopes in BLG structure, for instance the fragments such as f

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(41–60), f (102–124) and f (149–162) in an intact molecule (Selo et al., 1999; Wal, 2001). The peptide f (41–60) exists as a loop between β-strands C and D on the surface of native BLG molecule and peptide f (102–124) was found to be stabilised by hydrogen and disulphide bonds (Sawyer & Kontopidis, 2000). The other fragment f (149–162) forms a flexible turn at the C-terminal, which is buried in the native conformation, being inaccessible for IgG/IgE antibodies (Kleber et al., 2004). Clement et al. (2002) also reported on the availability of several antigenic epitopes (6 short fragments) in the α-helix and external loop structures of BLG molecule. Antigenicity of BLG can thus be modulated by heat-induced modifications of its native structure. Hence, thermal processing has currently being assessed as a tool to modulate antigenic/allergenic properties of milk proteins including BLG (Bogahawaththa et al., 2017a; Bu, Luo, Chen, Liu, & Zhu, 2013).

Bovine BLG is considered one of the major allergenic proteins. Factors such as resistance to digestion, stability at low pH, and being a completely foreign protein to human infants (BLG is not present in human breast milk) are associated with allergenic properties of BLG (Kaminogawa & Totsuka, 2003). Determination of antigenic response (antigenicity) by enzyme-linked immunosorbent assay (ELISA) is a common method of measuring the residual antigenicity and/or potential allergenicity of a protein source (Jedrychowski, 1999; Kleber et al., 2004; Rahaman et al., 2015). However, almost all protein antigens usually bind with IgG antibodies (antigenicity) in most of human subjects despite their sensitivity to these proteins (Abbas, Pillai, & Lichtman, 2014), which limits the prediction of potential allergenicity based on antigenicity.

In general, the antigenicity of BLG gradually increases with a rise in temperature from 50 to 90 °C and decreases drastically between 90 and 120 °C. This is primarily associated with unfolding of native protein structure initially, and further denaturation and formation of protein aggregates later (Bogahawaththa et al., 2017a; Rahaman et al., 2016). This appears to be a common trend of changing antigenicity of BLG regardless of whether it is in purified form (Rahaman et al., 2015), in a separated whey fraction (whey protein isolate-WPI) (Bu, Luo, Zheng, & Zheng, 2009), or in milk (Kleber & Hinrichs, 2007). However, changes in the antigenicity of BLG in other bovine protein mixtures such as a binary mixture of BLG and ALA, a ternary mixture of BLG, ALA, and IgG, etc., subjected to heating appear unknown (Bogahawaththa et al., 2017a; Bu et al., 2013). In addition, thermal denaturation of whey proteins including IgG and BSA in different protein mixtures has been less researched (Patel, 2007; Wijayanti et al., 2014).

This study investigated the thermal denaturation and aggregation of native BLG in the presence of other native proteins in several bovine milk protein mixtures, whey, and skim milk in comparison with BLG alone and how it affected antigenicity of BLG at two heat treatments (72 °C for 15 s and 100 °C for 30 s). For this work, skim milk, whey, and all the individual protein fractions were derived from the same whole raw milk. The concentration of different protein fractions in protein mixtures was approximately adjusted to their respective concentrations in milk for the purposes of comparison.

2. Materials and methods

2.1. Materials and preparation of samples

2.1.1. Skim milk, whey, and caseins

Whole raw bovine milk, kindly supplied by Murray Goulburn Co-operative (Laverton North, VIC, Australia) on two separate occasions, was used for this experiment. Upon arrival, the raw milk was centrifuged at 3500 ×*g* for 20 min at 20 °C (Avanti J-26XP, Beckman Instrument Australia Pty., Ltd., Gladesville, NSW, Australia) to remove fat. After freezing (−20 °C) an aliquot of the skim milk for further experiments, the remaining portion of the skim milk was divided into two parts that were used to prepare the native whey and caseins (CNs) separately. To separate the whey fraction, pH of the skim milk was adjusted to 4.6 (0.1 M HCl) and then the precipitated CNs were removed by centrifugation (Avanti J-26XP centrifuge) at 30,000 ×*g* for 2 h at 20 °C. The pH of the separated whey fraction was readjusted to 6.7 (0.1 M NaOH), which was the recorded pH of the fresh raw milk. After storing an aliquot of the native whey (−20 °C) for further experiments, the other part of the native whey was used to fractionate individual whey proteins.

The CNs were separated from the other portion of skim milk by ultracentrifugation (Beckman L-70 ultracentrifuge, Beckman Instrument Australia Pty., Ltd.) at 100,000 ×*g* for 1 h at 22 °C without pH adjustment (O'Mahony & Fox, 2013). The CN pellets were washed twice (4000× *g* for 10 min at 22 °C) with simulated milk ultrafiltrate (SMUF) (Rosmaninho & Melo, 2006) before removing them from the centrifuge tubes and then they were resuspended in SMUF with continuous stirring for 48 h at 4 °C.

2.1.2. Fractionation of whey proteins

The whey protein fractionation was performed as per our previous work (Bogahawaththa et al., 2017b) employing a size exclusion chromatography (SEC) column, Biosep SEC-s2000 (Phenomenex Australia Pty., Ltd., Lane Cove West, NSW, Australia), on a fast protein liquid chromatography (FPLC) system (GE Healthcare Australia Pty., Ltd., Parramatta, NSW, Australia). Briefly, about 100 μL of native whey was injected to SEC column at a time with mobile phase of 0.05 M sodium phosphate buffer (pH 7) including 0.3 M sodium chloride. A Frac-950 fraction collector (GE Healthcare Australia Pty., Ltd.), which was attached to the FPLC, collected the different whey protein fractions (IgG, BSA, BLG, and ALA) eluted at different retention times with mobile phase separately. Then, the mobile phase was evaporated and concentrated the separated whey protein fractions using a RVC 2–18 rotational vacuum concentrator (John Morris Scientific, Deepdene, VIC, Australia) at 30 °C.

2.1.3. Preparation of protein samples

Five different protein samples were prepared including BLG alone (1 sample), by mixing different fractionated whey proteins together as per the descending order of their concentration in milk (3 samples; BLG + ALA, BLG + ALA + IgG, and BLG + ALA + IgG + BSA), and by adding fractionated CNs to the mixture of 4 different whey proteins (1 sample; BLG + ALA + IgG + BSA + CNs). Additionally, the native whey and skim milk (2 samples) were also analysed. The concentration of each individual proteins in the samples (except whey and milk) were approximately readjusted to their corresponding concentrations in skim milk; CNs, BLG, ALA, IgG and BSA at 26, 3.2, 1.2, 0.8 and 0.4 mg mL^{-1}, respectively (Dupont, Croguennec, Brodkorb, & Kouaouci, 2013), using simulated milk ultrafiltrate (SMUF). Proximate protein quantification was performed using a highly sensitive spectrophotometer (DeNovix DS-11, Wilmington, DE, USA).

2.2. Heat treatment of samples

After dividing all 7 samples into 3 aliquots, two of them were subjected to two different heat treatments (72 °C/15 s and 100 °C/30 s) separately and each treatment was replicated three times. The 72 °C for 15 s treatment is usually considered as high-temperature short-time pasteurisation (HTST). The effect of heating at 100 °C for 30 s on the level of denaturation of BLG in skim milk was

considered approximately comparable with ultra-high temperature (UHT) processing (Dannenberg & Kessler, 1988). An aliquot of each sample (2.3 mL) was placed in a double gap geometry (DG23.04/Pr/Q1, Anton Paar, GmbH, Ostfildern, Germany) and treated in a pressure cell (CC 25/Pr 150/A1/SS, Anton Paar) attached to a CS/CR rheometer (MCR 301, Anton Paar) and subjected to a constant shear (1000 s^{-1}) and pressure (~250 kPa) (Bogahawaththa et al., 2017b). The untreated aliquots at room temperature (~20 °C) served as the controls.

2.3. Fourier transform infrared spectroscopy

Soon after performing each treatment, Fourier transform infrared (FTIR) spectra of all treated and untreated samples were obtained using a PerkinElmer Frontier FTIR spectrometer (PerkinElmer, MA, USA) in the range of 4000–600 cm^{-1} with background subtraction (16 scans per spectra at a resolution of 4 cm^{-1}). Followed by baseline correction, the spectra were resolved by Fourier self-deconvolution (FSD) using the Origin Pro 2018 software (Origin Lab Corporation, Northampton, MA, USA) and identified the prominent peaks corresponding to the protein secondary structure within broad amid I region of 1700–1600 cm^{-1}. The peak fitting was then performed for all the deconvoluted spectra using a Peak Fitting method with aid of the Gaussian function and the optimum peak fitting was achieved followed by required level of iterations. The areas of all the component peaks assigned to a specific secondary structure were then summed up and divided by the total area. This resulted in identifying five major peak areas corresponding to secondary structure of proteins including side chains (1610–1602 cm^{-1}), β-sheets (1637–1610 cm^{-1} and 1696-1680 cm^{-1}), random coils (1648-1638 cm^{-1}), α-helices (1660–1650 cm^{-1}), and β-turns (1679–1667 cm^{-1}) (Grewal, Huppertz, & Vasiljevic, 2018; Rahaman et al., 2015). The results were then subjected to statistical analysis as per section 2.6.

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

Following heat treatments, all the treated and control samples were mixed with sodium dodecyl sulphate (SDS) sample buffer at 1:25 (v/v) ratio, and preserved at −20 °C until the electrophoresis. Both non-reducing and reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as described previously (Bogahawaththa, Buckow, Chandrapala, & Vasiljevic, 2018). β-Mercaptoethanol was used to reduce covalent bonds for reducing SDS-PAGE. The broad range pre-stained SDS-PAGE standards (SeeBlue Plus2 Pre-stained Protein Standard, Thermo Fisher Scientific, Scoresby, VIC, Australia) and commercial whey proteins recommended for electrophoresis (BLG, ALA, IgG and BSA) were used as protein standards and molecular weight markers, respectively. The gel images were captured by Image Lab 5.1 software (Bio-Rad Laboratories, Galesville, NSW, Australia).

2.5. Determination of antigenicity of β-lactoglobulin

Antigenicity of all the control and treated samples was determined using the bovine BLG enzyme-linked immunosorbent assay (ELISA) quantitation kit (Bethyl Laboratories Inc., Montgomery, TX, USA) according to the manufacturer's instructions (Kleber et al., 2004; Rahaman et al., 2015; Zhong et al., 2012). Briefly, 96-well microtitre plates were coated with capture antibody and incubated for 1 h at room temperature. The capture antibodies were polyclonal rabbit IgG antibodies, which were raised against native bovine BLG. Following washing steps (5 times), the wells were filled with 200 μL of blocking solution for 30 min at room temperature to block residual free binding sites in the wells. After draining the blocking solution and washing steps (5 times), exactly 100 μL of standards and samples diluted in sample diluent were added to each well separately and incubated for 1 h at room temperature. A 1:50,000 (v/v) dilution was performed for all the protein samples to maintain BLG concentration within the concentration limits of standards (1.95–125 ng mL^{-1}). Following steps such as adding BLG-detecting antibody (horseradish peroxidase conjugated rabbit anti-bovine BLG detection antibody) and tetramethylbenzidine (TMB) substrate as recommended, the plates were kept for colour development for 15 min in dark. After ceasing the reaction, the absorbance was measured on a microplate reader (iMark, Bio-Rad Laboratories, Gladesville, NSW, Australia). The antigenicity (mg mL^{-1}) of all the samples was quantified (Table 2) using the standard curve. The percentage (%) of residual antigenicity was also calculated for all treated samples based on the antigenicity of respective untreated/control sample for comparison purpose (Fig. 2).

$$\text{Residual antigenicity } (\%) = \frac{\text{Antigenicity of treated sample}}{\text{Antigenicity of the respective control sample}} \times 100$$

2.6. Statistical analysis

The entire experiment was replicated using the samples prepared from two batches of raw milk. The results were analysed as a randomised split plot design with protein samples as the main plot and heat treatment as a sub plot using a General Linear Model of SAS statistical program (SAS, 1996). This block was replicated with three sub-samplings. Turkey's studentised range test was used for the multiple comparisons of the means, which were comprised of at least 4 independent observations ($4 \geq n$). The level of significance was pre-set at $p \leq 0.05$.

3. Results and discussion

3.1. β-Lactoglobulin

The secondary structure of monomeric BLG includes an α-helix, three short helices, and nine strands of antiparallel β-sheets (De Wit, 2009; Wijayanti et al., 2014; Wong, Camirand, Pavlath, Parris, & Friedman, 1996). FTIR results of the current study (Table 1) determined secondary structure of the native BLG as a combination of mainly β-sheets (54.5%), α-helices (17.6%), β-turns (12.1%), and random coils (10.5%). This result mostly concurred with the previous studies, which characterised the secondary structure of BLG as 50% β-sheets, 15% α-helices, and 15–20% β-turns by circular dichroism (CD) spectroscopy (Creamer, Parry, & Malcolm, 1983) and 51–55% β-sheets, 9–11% α-helices, 20–27% β-turns, and 9–11% random coils by FTIR spectroscopy (Dong et al., 1996).

The secondary and tertiary structures of BLG monomers are mainly stabilised by hydrophobic, ionic and hydrogen-bond interactions between peptide chains, as well importantly by two

Table 1
The areas of different secondary structure as a percentage of the total area measured within broad amide I region (1700–1600 cm^{-1}).[a]

Sample	Band assignment	Control/untreated		72 °C/15 s treatment		100 °C/30 s treatment	
		Band frequency (cm^{-1})	Peak area %	Band frequency (cm^{-1})	Peak area %	Band frequency (cm^{-1})	Peak area %
BLG	β-sheet	1636–1611, 1696–1682	54.5 ± 0.5[a]	1636–1611, 1696–1682	55.1 ± 1.2[a]	1637–1611, 1696–1682	51.3 ± 3.2[b]
	Random coil	1645–1644	10.5 ± 0.6[a]	1645–1644	10.3 ± 0.3[a]	1645–1644	13.2 ± 2.3[b]
	α-helix	1652, 1661–1660	17.6 ± 0.3[a]	1652–1651, 1660	17.6 ± 0.5[a]	1660–1652, 1669–1660	18.5 ± 0.7[a]
	β-turn	1676–1668	12.1 ± 1.4[a]	1681–1668	11.7 ± 1.4[a]	1680–1668	11.6 ± 1.2[a]
BLG + ALA	β-sheet	1636–1611, 1696–1682	54.8 ± 2.4[a]	1635–1611, 1696–1689	47.6 ± 1.9[b]	1639–1611, 1696–1682	56 ± 1.5[a]
	Random coil	1645–1644	10.2 ± 0.8[a]	1644–1643	12.9 ± 2.8[b]	1645–1643	11 ± 0.9[a]
	α-helix	1652, 1660	18.2 ± 0.1[a]	1652–1651, 1660	18.6 ± 0.4[a]	1652, 1660	17.5 ± 1.1[a]
	β-turn	1679–1668	11.6 ± 2.2[a]	1680–1668	15.7 ± 2.1[b]	1675–1667	10.3 ± 0.7[a]
BLG + ALA + IgG	β-sheet	1635–1611, 1682	56.1 ± 0.1[a]	1635–1611, 1682	55.5 ± 0.8[a]	1635–1611, 1696–1682	55.2 ± 0.5[a]
	Random coil	1644	12.5 ± 0[a]	1644	11.4 ± 0.8[a]	1644	12.6 ± 0.2[a]
	α-helix	1652, 1660	18.1 ± 0.2[a]	1652, 1660	17.8 ± 0.5[a]	1652, 1660	18 ± 0.2[a]
	β-turn	1676–1668	8.1 ± 0.1[a]	1675–1668	9.6 ± 1.3[a]	1675–1668	8.2 ± 0.5[a]
BLG + ALA + IgG + BSA	β-sheet	1637–1611, 1696–1682	55.5 ± 1.5[a]	1635–1611, 1693–1682	55 ± 0.4[a]	1636–1611, 1696–1682	55.8 ± 0.3[a]
	Random coil	1645–1644	9.7 ± 0.7[a]	1644	10.9 ± 0.1[a]	1644	11.4 ± 0.6[a]
	α-helix	1652, 1660	17.2 ± 0.5[a]	1652, 1660	17.7 ± 0.1[a]	1652, 1660	17.7 ± 0.3[a]
	β-turn	1680–1668	12 ± 2.5[a]	1675–1668	10.6 ± 0.1[a]	1675–1668	9.5 ± 0.9[b]
BLG + ALA + IgG + BSA + CNs	β-sheet	1636–1611, 1691–1682	56.4 ± 1.1[a]	1635–1611, 1693–1682	55.8 ± 0.6[a]	1636–1611, 1696–1682	55.6 ± 1[a]
	Random coil	1645–1644	11.8 ± 1.3[a]	1644	12.5 ± 0.3[a]	1645–1644	10.9 ± 0.8[a]
	α-helix	1652, 1660	17.8 ± 0.5[a]	1652, 1660	17.7 ± 0.4[a]	1653–1652, 1661–1660	17.2 ± 0.2[a]
	β-turn	1675–1668	8.4 ± 0.5[a]	1675–1668	8.2 ± 0.5[a]	1677–1668	10.7 ± 1.4[b]
Whey	β-sheet	1639–1611, 1696–1682	34.2 ± 2.9[a]	1630–1611, 1692–1682	32.6 ± 3.6[a]	1633–1610, 1697–1682	50.4 ± 0.6[b]
	Random coil	1645–1643	30.6 ± 2.6[a]	1643–1640	28.9 ± 2.3[a]	1645–1640	13 ± 1[b]
	α-helix	1652–1651, 1660	20.3 ± 1[a]	1651–1650, 1660–1659	21 ± 2.5[a]	1650–1649, 1660–1658	18.2 ± 0.7[b]
	β-turn	1680–1668	14.5 ± 0.4[a]	1675–1666	17.5 ± 1.2[b]	1677–1668	18.3 ± 1.2[b]
Skim milk	β-sheet	1635–1611, 1696–1682	45.1 ± 2.1[a]	1638–1612, 1692–1682	47.9 ± 0.7[b]	1638–1610, 1696–1682	55.8 ± 1.5[c]
	Random coil	1644–1642	12.3 ± 1.8[a]	1644	12.1 ± 1.1[a]	1645–1644	10.6 ± 0.5[a]
	α-helix	1652–1650, 1660	23.7 ± 0.5[a]	1653–1652, 1660	21.9 ± 1.6[b]	1652, 1660	18 ± 1.3[c]
	β-turn	1675–1668	16.2 ± 0.8[a]	1676–1668	15.6 ± 0.3[a]	1675–1667	15.2 ± 0.8[a]

[a] Abbreviations are: BLG, β-lactoglobulin; ALA, α-lactalbumin; IgG, immunoglobulin G; BSA, bovine serum albumin; CNs, caseins. Values are presented as mean area percentage of at least 3 independent measurements ($3 \geq n$) plus or minus standard deviation (SD). The means in the same row with different superscripts are significantly different ($p < 0.05$).

Table 2
The antigenicity of β-lactoglobulin (BLG; mg mL^{-1}) in different protein samples.[a]

Sample	Antigenicity of BLG (mg mL^{-1})		
	Control/untreated	Treated	
		72 °C/15 s	100 °C/30 s
BLG	3.1 ± 0.1	3.2 ± 0.2	2.3 ± 0.1
BLG + ALA	2.6 ± 0.1	2.8 ± 0.1	2.7 ± 0.0
BLG + ALA + IgG	3.6 ± 0.2	3.6 ± 0.1	2.6 ± 0.2
BLG + ALA + IgG + BSA	3.0 ± 0.1	3.0 ± 0.2	2.6 ± 0.1
BLG + ALA + IgG + BSA + CNs	3.1 ± 0.1	3.2 ± 0.2	0.8 ± 0.0
Whey	3.6 ± 0.2	3.5 ± 0.2	0.5 ± 0.1
Skim milk	3.0 ± 0.2	3.1 ± 0.1	1.2 ± 0.1

[a] Abbreviations are: ALA, α-lactalbumin; IgG, immunoglobulin G; BSA, bovine serum albumin; CNs, caseins. Values are presented as mean antigenicity of at least 4 independent measurements ($4 \geq n$) plus or minus standard deviation (SD).

disulphide bridges located at Cys^{106}–Cys^{119} and Cys^{66}–Cys^{160} (De Wit, 2009). BLG monomer also contains a free thiol group (Cys^{121}) (De Wit, 2009). The native confirmation of BLG, however, largely depends on temperature, pH, and other environmental factors (Tolkach & Kulozik, 2007). According to the FTIR results, no significant changes ($p > 0.05$) were observed between the control and 72 °C/15 s treated BLG sample. Rahaman et al. (2015) also reported no or minor changes of secondary structure of BLG in an aqueous solution (~3.2 mg mL^{-1}) when heated at 80 °C for 1 min at pH 7.6 as observed by FTIR. However, Qi et al. (1997) reported loss of secondary structure including helical confirmation and some β-sheets of a BLG solution (20–100 mg mL^{-1}) during heating between 60 and 70 °C as determined by CD and IR spectroscopy. Although these results were observed at the same pH (6.7), the protein concentrations (20–100 mg mL^{-1}) were largely different from the current study (~3.2 mg mL^{-1}), which directly influence denaturation of BLG (De Wit, 2009; Lefèvre & Subirade, 1999). Lefèvre and Subirade (1999) reported that no aggregation, but only the unfolding, of structure occurred when the concentration of BLG was set at 2.5–5 mg mL^{-1} during heating at 85 °C for 5 min, as observed by FTIR.

Heating at 100 °C for 30 s, however, affected secondary structure of native BLG. β-Sheets were significantly reduced ($p < 0.05$), while random coil structures increased ($p < 0.05$) indicating substantial modifications of the native conformation. During this treatment, we also observed formation of protein aggregates through SDS-PAGE gel images (Fig. 1A,B). The protein aggregates, present on top of the stacking gel and some BLG dimers which appeared in resolving gel under nonreducing conditions (Fig. 1A), disappeared after the reduction (Fig. 1B), indicating that these protein complexes were formed by thiol/disulphide interactions. Rahaman et al. (2015) reported the comparable results when heating BLG at 100 °C for 1 min at pH 7. BLG usually forms intermolecular protein aggregates through thiol-catalysed disulphide-bond interchange reactions and thiol-thiol oxidation reactions (to a lesser extent), which result in formation of dimers, trimers, tetramers, as well larger aggregates at the temperature >70 °C and natural pH (McSwiney, Singh, & Campanella, 1994; Qi, Brownlow, Holt, & Sellers, 1995).

Changes in specific secondary and tertiary structures (epitopes) modulate antigenicity of BLG (Bogahawaththa et al., 2017a; Rahaman et al., 2015; Zhong et al., 2012). The antigenicity of native BLG did not change significantly followed by 72 °C/15 s treatment (Fig. 2; Table 2), which can be attributed to mostly unchanged structure. Rahaman et al. (2015) reported that BLG antigenicity was not largely affected at 80 °C for 1 min at pH 7.6.

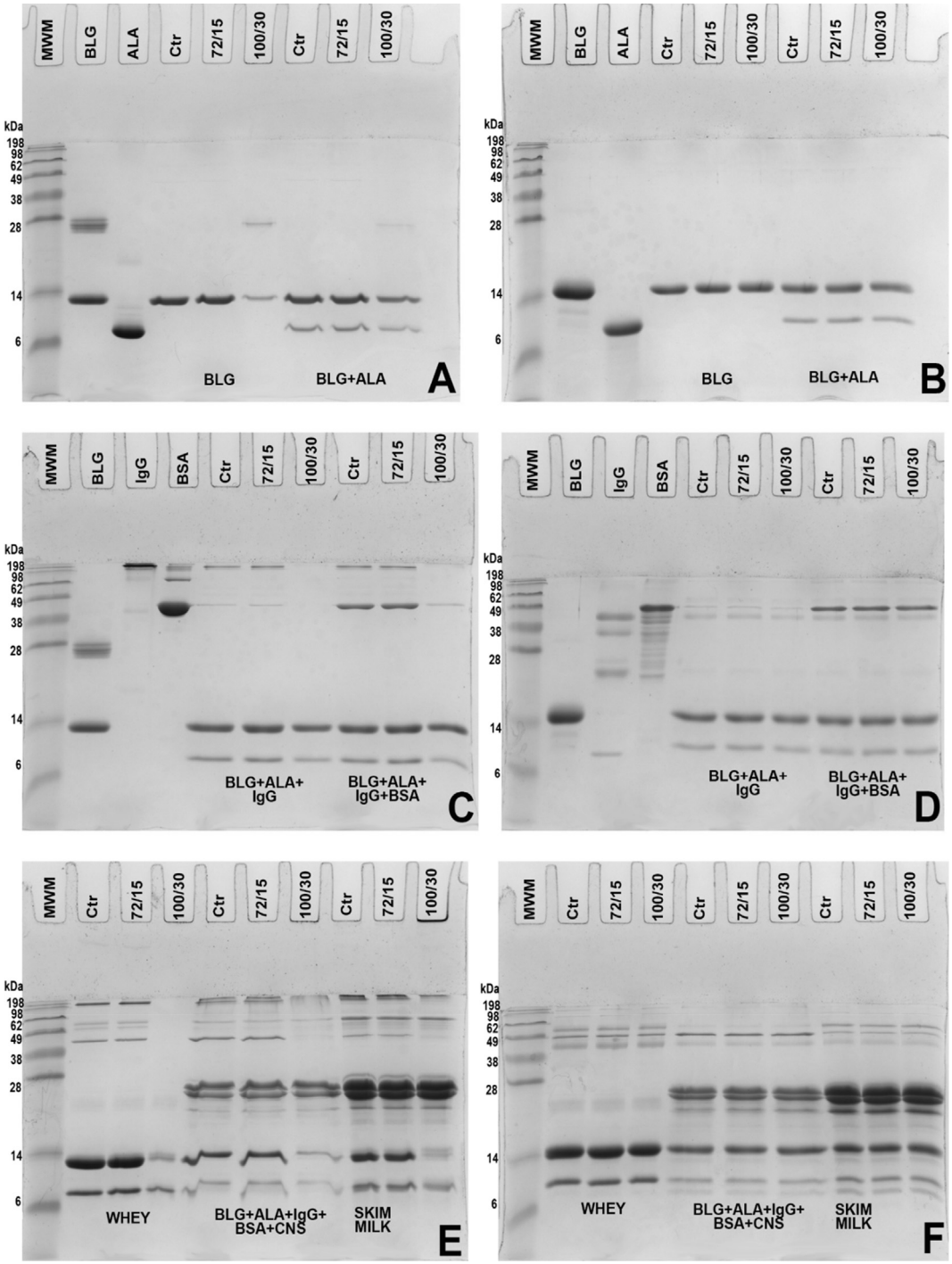

Fig. 1. Non-reducing (A, C, and E) and reducing (B, D, and F) sodium dodecylsulphate-polyacrylamide gel electrophoresis analysis of: (A and B) β-lactoglobulin (BLG) and BLG+α-lactalbumin (ALA); (C and D) BLG + ALA + immunoglobulin G (IgG) and BLG + ALA + IgG + bovine serum albumin (BSA); (E and F) whey, BLG + ALA + IgG + BSA + caseins (CNs), and skim milk samples. Lanes are marked as: MWM, molecular weight markers; BLG, BLG standard; ALA, ALA standard; IgG, IgG standard; BSA, BSA standard; Ctr, control/untreated; 72/15, 72 °C/15 s treatment; 100/30, 100 °C/30 s treatment.

However, antigenicity of BLG treated by 100 °C/30 s significantly decreased (~25%) in comparison with that of the control sample. Rapid unfolding and modification of the native structure usually contribute to fragmentation of conformational epitopes, while aggregation of protein molecules leads to masking of both linear and conformational epitopes, which are then inaccessible for antibodies. Hence, this overall effect leads to loss of antigenicity of BLG at 100 °C/30 s in comparison with the control (Bogahawaththa et al., 2017a; Kleber et al., 2004; Rahaman et al., 2015).

3.2. β-Lactoglobulin and α-lactalbumin

In contrast to BLG, the mixture of BLG + ALA experienced modifications of secondary structure subjected to 72 °C/15 s treatment (Table 1). FTIR results showed a significant ($p < 0.05$) loss of β-sheets and increased content of random coils and β-turns. These modifications could be attributed to unfolding of BLG and or ALA but most of them can be related to BLG in considering its higher concentration (3.2 mg mL^{-1}) than ALA (1.2 mg mL^{-1}) in BLG + ALA sample. No

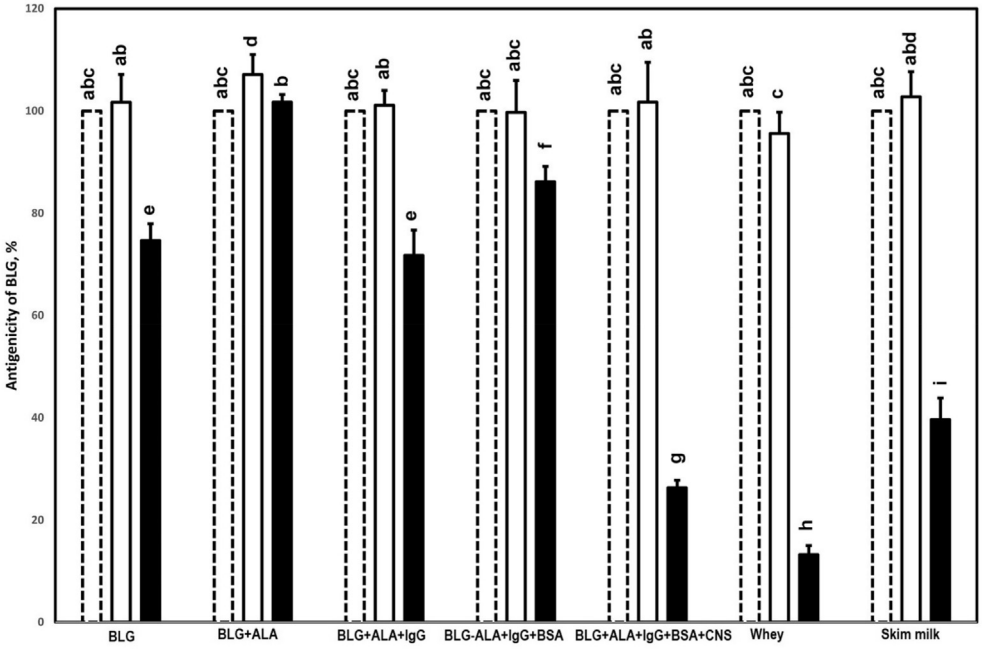

Fig. 2. Percentage (%) change of the antigenicity of β-lactoglobulin (BLG) in various protein samples (ALA, α-lactalbumin; IgG, immunoglobulin G; BSA, bovine serum albumin; CNs, caseins) subjected to 72 °C/15 s (□) and 100 °C/30 s (■) treatments. The control/untreated (⬚) is considered equivalent to 100%. The values are presented as mean antigenicity (%) of at least 4 independent measurements (4 ≥ n) plus or minus standard deviation (SD). The values with different lowercase letters are significantly different ($p < 0.05$).

noticeable protein complexes were formed covalently during this treatment displayed by the SDS-PAGE analysis (Fig. 1A). Although results of the FTIR peak areas analysis did not show any significant changes of secondary structure in BLG + ALA sample subjected to the 100 °C/30 s treatment, noticeable shifting of the peaks occurred. For instance, the peaks corresponding to native β-sheet at 1636–1635 cm^{-1} in the control sample were shifted to 1639–1636 cm^{-1} in the 100 °C/30 s treated sample. Furthermore, a new peak appeared (data not shown) in the range of 1696–1680 cm^{-1} at 100 °C/30 s apart from two in FTIR spectra of the control and 72 °C/15 s treated samples, possibly denoting formation of intermolecular β-sheets aggregates (Rahaman et al., 2015). The protein aggregates were also formed covalently between BLG and ALA, approximately in similar quantities, affected by the same treatment as shown in SDS-PAGE images (Fig. 1A).

ALA, in a pure solution, is considered more heat stable than BLG as it possesses a compact structure stabilised by four disulphide bridges with no free thiol groups (Hong & Creamer, 2002; Schokker, Singh, & Creamer, 2000). When heating a mixture of BLG + ALA, they interact and form large aggregates mainly through disulphide bonds. In the early stage of heating, these aggregates contain more BLG than ALA, but later both BLG and ALA almost equally contribute in protein aggregation (Dalgleish, Senaratne, & Francois, 1997; Hong & Creamer, 2002). Although these findings agreed with our results, the early denaturation of BLG occurred at 72 °C/15 s in BLG + ALA mixture in comparison with BLG alone needs to be further elucidated.

Antigenicity of BLG in BLG + ALA sample, subjected to 72 °C/15 s treatment, significantly ($p < 0.05$) increased by 7% in comparison with that of the control sample (Fig. 2). This can be related to unfolding of BLG structure and exposure of epitopes, which were buried in the native conformation. Unpredictably, antigenicity of BLG in BLG + ALA sample treated by 100 °C/30 s was not significantly different from that of the control sample. It can thus be assumed that antigenicity initially increased with increase in temperature (72 °C) and decreased later at 100 °C. The reduction of antigenicity with further increase in temperature is potentially associated with formation of BLG and ALA aggregates and subsequent masking of epitopes as described above. The gradual increasing of antigenicity with increase in temperature up to 90 °C and then rapid reduction with further increase in temperature is the typical pattern of changing antigenicity of BLG (Bogahawaththa et al., 2017a). However, under these experimental conditions, antigenicity of BLG in 100 °C/30 s treated sample did not decrease below the level of antigenicity in the respective control sample, indicating heat treatments such as 100 °C/30 s and equivalents would not be sufficient to reduce BLG antigenicity in a binary mixture of BLG + ALA.

3.3. β-Lactoglobulin, α-lactalbumin, and immunoglobulin G

In comparison with the BLG sample, BLG + ALA + IgG subjected to 72 °C/15 s treatment did not demonstrate significant changes in secondary structure (Table 1). The 100 °C/30 s treatment, however, resulted in formation of intermolecular β-sheet aggregates displayed at 1696–1690 cm^{-1} (Rahaman et al., 2015). The formation of protein aggregates via thiol/disulphide interactions with the contribution of all three proteins was observed by SDS-PAGE only after 100 °C/30 s treatment (Fig. 1C,D). We reported similar results in our previous work (Bogahawaththa et al., 2017b). These results indicated that the presence of IgG with a small quantity of BSA (BLG + ALA + IgG sample also contained a small amount of BSA as observed in Fig. 1C) can contribute to change in thermal denaturation and aggregation of BLG and ALA in BLG + ALA + IgG sample in comparison with the respective BLG + ALA binary mixture. For instance, contribution of ALA was substantially higher than that of BLG in formation of protein aggregates in BLG + ALA + IgG subjected to 100 °C/30 s treatment (Fig. 1C) while their contribution appeared almost equal in BLG + ALA sample followed by the same treatment (Fig. 1A).

In parallel to the unchanged protein structure, antigenicity of BLG did not change significantly after 72 °C/15 s treatment.

However, it decreased significantly by about 28% after 100 °C/30 s treatment in comparison with that of the control sample, which was also statistically comparable with the BLG sample (25%) subjected to the same treatment. The loss of antigenicity can be related to the fragmentation of conformational epitopes and hiding of both linear and conformational epitopes inside the compact structure followed by unfolding and aggregation of native BLG structure (Bogahawaththa et al., 2017a; Rahaman et al., 2016). These findings suggested that the presence of IgG apart from ALA contributed to further conformational modifications of BLG during thermal denaturation at 100 °C/30 s leading to loss of its antigenicity.

3.4. β-Lactoglobulin, α-lactalbumin, immunoglobulin G, and bovine serum albumin

According to FTIR results, no significant changes ($p > 0.05$) in secondary structure occurred in BLG + ALA + IgG + BSA mixture subjected to 72 °C/15 s treatment, but significant loss of β-turns was observed after the 100 °C/30 s treatment, in comparison with that of the control sample. Moreover, the protein aggregates were formed by thiol/disulphide interactions (Fig. 1C,D) with the contribution of all four different proteins. Importantly, all these results demonstrated that the presence of IgG and BSA can result in changes of thermal denaturation and aggregation of BLG and ALA in a protein mixture in comparison with those of a BLG + ALA binary mixture as discussed above. We have already observed a similar leading role of ALA over BLG in thermal denaturation of a whey protein mixture including IgG and BSA (Bogahawaththa et al., 2017b) and suggested that IgG and/or BSA possibly play a catalytic role on ALA (Havea, Singh, & Creamer, 2001).

In comparison with BLG and BLG + ALA + IgG samples, mostly unchanged antigenicity of BLG was also observed in BLG + ALA + IgG + BSA mixture followed by 72 °C/15 s treatment, while 100 °C/30 s treatment resulted in reduction of antigenicity by around 14%, which was still far below than the respective antigenicity reduction in both BLG (25%) and BLG + ALA + IgG (28%) samples (Fig. 2). The reduction of the antigenicity of BLG in this protein mixture can also be related to the protein denaturation same as the BLG and BLG + ALA + IgG samples above. The degree of loss of BLG antigenicity in BLG + ALA + IgG (28%) and BLG + ALA + IgG + BSA (14%) mixtures subjected to 100 °C/30 s treatment also hinted that IgG and BSA influenced thermal denaturation and aggregation (including structure of protein aggregates) of BLG and ALA differently. Additionally, a potential catalytic role on ALA is mostly performed by BSA rather than IgG. It was already reported that BSA affects thermal behaviour of BLG (Kehoe, Morris, & Brodkorb, 2007) or ALA (Havea et al., 2001) in their binary mixtures (BLG + BSA or ALA + BSA, respectively) and formed protein aggregates through disulphide bonds. Although the influence of BSA on ALA in a heated whey protein mixture had been discussed previously (Bogahawaththa et al., 2017b), further investigations are required to elucidate this observed effect.

3.5. β-Lactoglobulin, α-lactalbumin, immunoglobulin G, bovine serum albumin, and caseins

When BLG + ALA + IgG + BSA + CNs mixture was subjected to 72 °C/15 s treatment, no changes ($p > 0.05$) in secondary structure were observed, but the 100 °C/30 s resulted in significant changes in the content of β-turns (Table 1). Furthermore, the 100 °C/30 s treatment induced aggregation of all four whey proteins (BLG, ALA, IgG, and BSA) and CNs together. They appeared on top of the stacking gel (Fig. 1E) under the nonreducing conditions and disappeared upon reduction (Fig. 1F), indicating that the aggregates were formed by thiol/disulphide interactions as reported previously (Bogahawaththa et al., 2017b; Considine et al., 2007; Wijayanti et al., 2014). In parallel to the unaffected structure of proteins subjected to 72 °C/15 s treatment, antigenicity of BLG was also not affected significantly ($p > 0.05$) by that treatment. The 100 °C/30 s treatment, however, resulted in reduction of BLG antigenicity by 74% in comparison with that of the control sample.

The presence of CNs with whey protein mixture (BLG, ALA, IgG, and BSA) resulted in more progressive denaturation and aggregation of BLG during heating (100 °C/30 s) than when these whey proteins were alone. This could be mainly due to the influence of BLG and κ-casein on heat-induced denaturation and aggregation of proteins (Considine et al., 2007). Nevertheless, ability of CNs to control the heat-induced aggregation of whey proteins was also reported (Kehoe & Foegeding, 2010). Moreover, in this protein mixture BLG showed its usual leading role in thermal denaturation and aggregation of proteins upon inclusion of CNs, which was played by ALA particularly in BLG + ALA + IgG + BSA mixture. The gel images (Fig. 1C) of the nonreducing SDS-PAGE displayed lower reduction of the band intensity corresponding to BLG and greater reduction of the band intensity of ALA in BLG + ALA + IgG + BSA sample subjected to the 100 °C/30 s treatment in comparison with those of the controls. As well, greater reduction of the band intensity of BLG and lower reduction of the band intensity of ALA were observed in BLG + ALA + IgG + BSA + CNs mixture (Fig. 1E) subjected to the same treatment in comparison with that of the control. Results of the BLG antigenicity also aligned with SDS-PAGE results, demonstrating higher residual antigenicity due to lower thermal denaturation and aggregation of BLG in BLG + ALA + IgG + BSA mixture (86%), while lower residual antigenicity due to higher thermal denaturation and aggregation of BLG in BLG + ALA + IgG + BSA + CNs mixture (26%).

3.6. Whey

Following 72 °C/15 s treatment, the whey fraction also did not show major changes in secondary structure except for some significant ($p < 0.05$) modifications in β-turns. However, the 100 °C/30 s treatment caused significant and severe changes including loss of β-sheets, α-helices, and random coils, and enhanced content of β turns. The formation of protein aggregates with the contribution of all whey proteins (BLG, ALA, IgG, BSA, and lactoferrin) via thiol/disulphide interactions was also observed by less intense bands only in the sample subjected to 100 °C/30 s treatment (Fig. 1E,F). These large protein aggregates mostly deposit on the bottom of wells without entering into the stacking gel and thus can be washed away during electrophoresis and staining and destaining of gels. These results agreed with the literature relating to the BLG-led denaturation and aggregation of whey proteins during heating (Bogahawaththa et al., 2017b; Considine et al., 2007; Wijayanti et al., 2014).

The antigenicity of BLG remained unchanged ($p > 0.05$) following 72 °C/15 s treatment. However, 100 °C/30 s treatment resulted in greater reduction ($p < 0.05$) of BLG antigenicity (87%) in comparison with that of the control, which was also reported to be the greatest loss in comparison with all the other protein samples treated similarly. This reduction of antigenicity can be related to fragmentation of conformational epitopes followed by unfolding and rearrangement of BLG structure and masking of conformational and linear epitopes by aggregation of BLG with other whey proteins (Kleber & Hinrichs, 2007). Moreover, the greatest reduction of BLG antigenicity in whey, in comparison with other protein mixtures (lactose-free), can be attributed to the initiation of reactions between proteins and lactose (Maillard reaction) typically at higher temperatures, which in turn lead to further conformational changes that prevent accessing of epitopes by antibodies and

also breaking up of linear epitopes (Bogahawaththa et al., 2017a; Bu et al., 2013; Rahaman et al., 2016).

3.7. Skim milk

When skim milk was heated at 72 °C for 15s, FTIR results showed certain modifications ($p < 0.05$) of secondary structure such as increased content of β-sheets and reduced α-helices in comparison with that of the control, while the 100 °C/30 s treatment resulted in greater impact ($p < 0.05$) on both β-sheets and α-helices (Table 1). According to SDS-PAGE results, 100 °C/30 s treatment caused formation of protein aggregates with the contribution of all whey proteins (BLG, ALA, IgG, BSA, and lactoferrin) and CNs through thiol/disulphide interactions. Patel, Singh, Anema, and Creamer (2006) reported comparable results when skim milk was heated at 100 °C for 100 s.

Antigenicity of BLG in skim milk increased by ~3% following the 72 °C/15 s treatment in comparison with that of the control, while it was statistically comparable with the increased antigenicity of BLG in BLG + ALA sample subjected to the same treatment (Fig. 2). In comparison with the control, the 100 °C/30 s treatment caused a significant reduction (60%) of BLG antigenicity in skim milk. It was reported that the antigenicity of BLG in skim milk increased with rise in temperature up to 80–90 °C due to unfolding of BLG structure and exposure of hidden epitopes. Then, it decreased with greater heat-load because of breaking up and masking of epitopes by protein aggregation (Kleber & Hinrichs, 2007) and Maillard reaction (Rahaman et al., 2016).

4. Conclusions

Thermal denaturation of BLG is influenced by other whey proteins (ALA, IgG, and BSA) and CNs, while modifications of its structure and interactions with other proteins (aggregation) modulate its antigenicity.

Heating at 72 °C for 15 s can be considered a mild treatment, which did not cause denaturation of proteins including BLG in most of the protein mixtures, resulting in mostly unchanged antigenicity. However, the 72 °C/15 s treatment can potentially modify secondary structure of BLG relating to unfolding of its native structure in a binary mixture of BLG + ALA and skim milk, which in turn may increase antigenicity due to exposure of epitopes buried in the native conformation.

The 100 °C/30 s treatment induced formation of protein aggregates via thiol/disulphide interactions led by BLG in most of the protein mixtures. This resulted in a loss of BLG antigenicity, due to fragmentation of conformational epitopes and masking of both conformational and linear epitopes, to a various extent depending on the protein mixture except in the binary mixture of BLG and ALA. Nevertheless, ALA appeared to govern thermal denaturation of whey proteins over BLG in the presence of BSA and IgG during heating at 100 °C for 30 s, where BSA possibly played a catalytic role on ALA. This resulted in a higher retention of BLG antigenicity (86%) in BLG + ALA + IgG + BSA mixture.

Apart from investigating the effects of different heating regimes, it is also worth studying the influence of other milk proteins on thermal denaturation of BLG to modulate BLG antigenicity in dairy products. Further in vitro and in vivo investigations are paramount to see how heat-induced altered antigenicity is affected during digestion and how altered antigenicity could render modified allergenicity.

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