

Thermal denaturation of bovine immunoglobulin G and its association with other whey proteins



Dimuthu Bogahawaththa^a, Jayani Chandrapala^{a, b}, Todor Vasiljevic^{a, *}

^a Advanced Food Systems Research Unit, College of Health and Biomedicine, Victoria University, Werribee Campus, Victoria, 3030, Australia

^b School of Science, RMIT University, Bundoora, Victoria, 3083, Australia

ARTICLE INFO

Article history:

Received 15 May 2017

Received in revised form

13 June 2017

Accepted 16 June 2017

Available online 18 June 2017

Keywords:

Immunoglobulin G

Whey protein

Thermal processing

Denaturation

Covalent-complex

ABSTRACT

Thermal denaturation of bovine immunoglobulin G (IgG) was studied alone and in the presence of major whey proteins. The two heating regimes studied provided the simulated thermal effect compared to high-temperature short-time (HTST, 72 °C/15s) and ultra-high temperature (UHT, 100 °C/30s equivalent to 140 °C/5s in terms of denaturation of β -lactoglobulin) conditions. Simulated HTST conditions least impacted on the secondary structure of IgG and other whey proteins when they were present either alone or in mixtures of whey proteins. The heating at 100 °C for 30s caused formation of covalent-complexes of IgG alone, as well as in the mixtures, mainly through thiol-disulfide reactions. Under 100 °C/30s treatment, bovine serum albumin (BSA) did not interact with IgG through thiol-disulfide reactions in a binary mixture of proteins (IgG and BSA). α -Lactalbumin (ALA) appeared to preferentially lead denaturation of whey proteins over β -lactoglobulin (BLG), in a protein mixture (BLG, ALA, IgG, and BSA), suggesting a possible catalytic role by BSA and/or IgG on ALA, while native whey contains another component that can inhibit this effect. The presence of other whey proteins did not contribute to thermal stability of IgG at 100 °C.

© 2017 Published by Elsevier Ltd.

1. Introduction

Bovine immunoglobulins (Ig), secreted in colostrum and milk, are a family of globular proteins with a range of protective bio-activities that primarily provide immune protection to a newborn calf. Passive transfer of immunity from mother to neonate, in the form of antibodies, is one of the major functions of Ig (Hurley & Theil, 2013, pp. 275–294; Mehra, Marnila, & Korhonen, 2006). Bovine Ig are categorized into three main classes depending on their structure, namely, IgG, IgM and IgA. The monomeric basic structure of all Ig appears similar, which is composed of four sub-units of polypeptides, forming a Y-shaped molecule. IgG occur as monomeric (~160 kDa), while IgA (~370 kDa) and IgM (~1000 kDa) form polymeric Ig. IgG can also be present in two subclasses, called IgG1 and IgG2, in which IgG1 is the mostly abundant Ig isotype in bovine milk and colostrum, approximately 80% in compared to the total Ig content (Gapper, Copestake, Otter, & Indyk, 2007; Hurley & Theil, 2013, pp. 275–294).

Bovine Ig are of immense importance and attract research

interest since they are capable of heterologous transfer of immunity, for instance, utilization of bovine Ig present in milk or colostrum in the form of passive immunity in human and other animals. Moreover, bovine Ig can potentially modulate immune response in human and other animal subjects (Bogahawaththa, Chandrapala, & Vasiljevic, 2017; Hurley & Theil, 2011). The target antibodies contained in immune products, made out from bovine milk, are identified well as promising functional foods, nutraceuticals, or immunological supplements. For example, colostrum-based immune milk demonstrates effective prophylactic role against human rotavirus causing diarrhea (Korhonen, Marnila, & Gill, 2000). Infant formulae and other commercial foods, containing bovine Ig as an immunological supplement, are largely produced and continually penetrate into the world healthcare market due to increasing demand (Gapper et al., 2007; Mehra et al., 2006).

The expression of biological activity of a protein is associated with its three-dimensional conformational structure. Maintenance of this biological integrity is of utmost importance when a particular protein is included in functional foods. Ig are considered relatively heat labile, in which antigen binding sites are more heat sensitive than the other areas. Hence, the exposure of Ig to heat treatments can directly alter the conformational integrity, which in

* Corresponding author.

E-mail address: todor.vasiljevic@vu.edu.au (T. Vasiljevic).

turn influence functional properties and biological activity. It is thus suggested that minimum exposure to heat treatments should be achieved when manufacturing Ig-based functional foods or immune supplements (Gapper et al., 2007; Hurley & Theil, 2013, pp. 275–294).

Evidence point out that structure of Ig and its functions are usually affected by heat treatments approximately above 65 °C (Gapper et al., 2007). IgG tends to lose its immunoreactivity at temperature ≥ 72 °C (Li, Bomser, & Zhang, 2005). Bovine milk subjected to traditional HTST pasteurization contained about 65–79% of active IgG compared to that of raw milk, while little or no any detectable IgG was captured by ELISA (enzyme-linked immunosorbent assay) either in UHT treated or evaporated milk (Kummer et al., 1992). Comparably, concentration and functions of host-defence proteins including Ig in human milk can be adversely affected by standard pasteurization (Akinbi et al., 2010). The studies have revealed that IgG could remain stable without changing its structure subjected to the heat treatment of 72 °C for 15 s (Mainer, Sanchez, Ena, & Calvo, 1997) but the secondary structure was severely altered at 72 °C after 2 min (Li et al., 2005).

Apart from IgG, other globular whey proteins including β -lactoglobulin (BLG), α -lactalbumin (ALA) and bovine serum albumin (BSA) are also heat sensitive and thus thermal denaturation occurs to a various extent depending on the nature of the heat treatment and the physicochemical characteristics of these proteins. Furthermore, in a protein mixture, heat stability of one protein can be influenced by the other proteins, and also interprotein interactions occur generally through the interchange reactions between free sulfhydryl groups (–SH) and disulfide bonds (S–S) of proteins such as BLG, ALA, and BSA (Wijayanti, Bansal, & Deeth, 2014). Likewise, being a protein containing disulfide bonds, IgG form complexes at 95 °C after 20 min in an IgG solution possibly through disulfide-linked interchange interactions (Oh & Richardson, 1991). Human serum IgG showed rapid aggregation and irreversible cluster growth probably through hydrophobic interactions when it was heated above 50 °C (Rosenqvist, Jossang, & Feder, 1987), but the presence of human serum albumin hindered the formation of complexes induced by heat (Soltis, Hasz, Morris, & Wilson, 1979). It has consequently been proposed that BSA might also influence the denaturation of IgG as above in a bovine milk system (Oh & Richardson, 1991). Bovine IgG has demonstrated higher heat stability in milk than in phosphate buffered saline (PBS) (Li-Chan, Kummer, Losso, Kitts, & Nakai, 1995), whereas it was more heat stable in the colostrum than in either whey or PBS (Chen & Chang, 1998). It is believed that most of the constituents present in whey are effective in hindering the heat-induced (≤ 75 °C) denaturation of IgG (Chen & Chang, 1998). Bovine Ig-based products are manufactured by using not only fractionated Ig but also other milk protein sources that contain Ig (e.g. whole colostrum, milk, and whey protein concentrates). Hence, it is vital to understand the heat stability and thermal denaturation behavior of bovine IgG alone and also in the presence of other heat labile whey proteins such as BLG, ALA and BSA.

A few studies have so far looked into the thermal stability of bovine IgG in different milk and model systems (e.g., phosphate buffered saline, whey, milk, and colostrum) (Chen & Chang, 1998; Li-Chan et al., 1995). However, it appears that the studies, which have examined the thermal denaturation of IgG and its association with major whey proteins, have not been reported. Hence, this current study was designed to investigate the thermal denaturation of native bovine IgG and its association with three other native whey proteins (BLG, ALA, and BSA), and also to determine the impact of other whey proteins on thermal stability of IgG, which has not been reported in the literature. Furthermore, two heat regimes selected for this study were designed to achieve thermal

effects of commercial milk processing - HTST pasteurization (72 °C/15s) and UHT processing (100 °C/30s equivalent to 140 °C/5s in terms of denaturation of BLG), that would provide some understanding of the fate of bovine IgG in those commercial dairy products.

2. Materials and methods

2.1. Protein fractionation and sample preparation

Raw bovine milk was kindly provided by Murray Goulburn Co-operative (Laverton North, VIC, Australia) on two separate occasions. Upon the delivery of raw milk, 0.02% (w/w) of sodium azide was added to prevent microbial growth and milk was then centrifuged at 3500 g for 20 min at 20 °C using an Avanti J-26XP centrifuge (Beckman Instrument Australia Pty. Ltd, Gladesville, NSW, Australia) to remove cream and any other coarse particles. The pH of the resultant raw skim milk was adjusted to 4.6 using 0.1 M HCl allowing precipitation of caseins. Most of the caseins was then separated from whey by centrifugation (Avanti J-26XP centrifuge, Beckman Instrument Australia Pty Ltd., Gladesville, NSW, Australia) at 30,000 g for 2 h at 20 °C. The resultant whey was filtered (0.45 μ m pore size) to remove any aggregates and the pH was readjusted to 6.7 using 0.1 M NaOH, which was recorded to be the pH of fresh raw milk. Purified native whey was stored at 4 °C until protein fractionation was conducted.

Whey protein fractionation was performed by a Frac-950 fraction collector (GE Healthcare Australia Pty. Ltd., Parramatta, NSW, Australia) attached to an AKTAmicro fast protein liquid chromatography (FPLC) system (GE Healthcare Australia Pty. Ltd., Parramatta, NSW, Australia). A Biosep SEC-s2000 (300 $\times$ 7.8 mm) Size Exclusion Chromatography (SEC) column (Phenomenex Australia Pty Ltd., Lane Cove West, NSW, Australia) was used with a mobile phase of 0.05 M sodium phosphate buffer (pH 7) including 0.3 M sodium chloride following the method proposed by Kato, Yamasaki, Moriyama, Tokunaga, and Hashimoto (1987) with some modifications, particularly fractionating the native globular whey proteins, which were in the molecular weight range of <300 kDa; IgG (~160 kDa), BSA (~60 kDa), BLG (~18 kDa) and ALA (~14 kDa) into different falcon tubes. The separated protein fractions were then concentrated by evaporating mobile phase at 30 °C using a RVC 2–18 rotational vacuum concentrator (John Morris Scientific, Deepdene, VIC, Australia) and the final concentration of each protein solution were readjusted by adding simulated milk ultrafiltrate (SMUF), which was prepared in accordance with Rosmaninho and Melo (2006), to imitate their corresponding protein concentrations in milk, and also to restore the mineral composition present in raw milk. Hence, the concentration of IgG, BSA, ALA and BLG were maintained at around 0.08, 0.04, 0.12, and 0.32% (w/w), respectively (Dupont, Croguennec, Brodkorb, & Kouaouci, 2013, pp. 87–134). Finally, 8 different protein samples were prepared using individual proteins alone (4 samples: BLG, ALA, Ig and BSA), by mixing different protein fractions together (3 samples: BLG + ALA, BLG + ALA + Ig and BLG + ALA + Ig + BSA) based on the descending order of their concentration, and the native whey as a whole (1 sample).

2.2. Treatment of samples

Each protein sample was divided into 3 aliquots and 2 of them were subjected to two different heat treatments, while keeping the other aliquots as the control. The two heat treatments were 72 °C/15 s (HTST pasteurization) and 100 °C/30 s, which was selected as it was reported to be equivalent to 140 °C/5s (UHT processing) by considering the level of denaturation of BLG (Dannenberg &

Kessler, 1988). All these heat treatments were performed using a CS/CR rheometer (MCR 301, Anton Paar, GmbH, Ostfildern, Germany) following the method of Liyanaarachchi, Ramchandran, and Vasiljevic (2015) with some modifications. An aliquot (2.7 mL) of each sample was placed in a double gap cylinder (DG23.04/Pr/Q1, Anton Paar) and heated in a pressure cell (CC 25/Pr 150/A1/SS, Anton Paar) under a constant shear (1000 s^{-1}) and pressure ($\sim 250 \text{ kPa}$) at all the time. All the conditions were established to provide the simulated thermal effect compared to the industrial processing within the laboratory limitations. The degree of denaturation of BLG (80%) and ALA (32%) in skim milk samples, which were treated at $100^\circ\text{C}/30 \text{ s}$, was quantified and confirmed the effectiveness of the UHT treatment with available literature (Dannenbergh & Kessler, 1988; Datta, Elliott, Perkins, & Deeth, 2002; Oldfield, Singh, Taylor, & Pearce, 1998).

2.3. Fourier transform infrared (FTIR) spectroscopy

Immediately after the treatments, all the samples were analyzed by a PerkinElmer Frontier FTIR spectrometer (PerkinElmer, MA, USA) with a combined software of IR Solution (Shimadzu Corporation, Kyoto, Japan) Version 1.40 following an established method (Nishanthi, Vasiljevic, & Chandrapala, 2017) with some modifications. The samples spectra were obtained in the range of $4000\text{--}600 \text{ cm}^{-1}$ and the absorbance mode after subtracting the background. Each spectrum was a result of an average of 16 scans performed at a resolution of 4 cm^{-1} . Every spectrum was then deconvoluted by the software in order to enhance the resolution and to identify the peaks corresponding to the protein secondary structure within broad amide I region of $1600\text{--}1700 \text{ cm}^{-1}$.

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

Following the treatments, all treated and control samples were mixed with a SDS sample buffer (0.5M Tris-HCl buffer, 0.4% bromophenol blue, glycerol, 10% SDS and milli-Q water) in the ratio of 100 μL of sample to 1 mL of sample buffer and preserved by freezing until required. Both non-reducing and reducing SDS PAGE were then performed for all the samples as described previously (Patel, 2007) using 30% acrylamide gels containing 10% SDS. β -Mercaptoethanol was used as the reducing agent. Commercial BLG, ALA, BSA, and IgG recommended for electrophoresis were used as protein standards, while the broad range pre-stained SDS-PAGE standards (SeeBlue Plus2 Pre-stained Protein Standard, Thermo Fisher Scientific, VIC, Australia) were used as the molecular weight markers. After electrophoresis (210 V, 70 mA, 6.5 W for 60 min), gels were stained with Coomassie Brilliant Blue and then de-stained by a de-staining solution (a mixture of 10% isopropanol and 10% acetic acid). Gel images were obtained by ChemiDoc Imaging System (Bio-Rad Laboratories, Australia) and protein quantification was done for further analysis using Image Lab 5.1 software (Bio-Rad Laboratories, Australia).

3. Results and discussion

3.1. Denaturation of IgG and other whey proteins affected by HTST pasteurization

The globular structure of IgG is directly associated with its heat sensitivity. Conformational modifications occur when IgG molecule is subjected to various heat treatments. The deconvoluted FTIR interferograms, analyzed in this study, identified several prominent peaks in the native (unheated/control) IgG sample, namely at 1636, 1641, 1651, and 1668 cm^{-1} . The amide I absorption region

($1600\text{--}1700 \text{ cm}^{-1}$) selected for this study, primarily represents the C=O stretching vibrations of the peptide bond groups and their frequencies mainly depend on the nature of hydrogen bonding in which C=O groups are involved in different secondary structural components (Buijs, Norde, & Lichtenbelt, 1996). Accordingly, a number of amide I frequency peaks relating to the secondary structure of bovine IgG have been established, such as intermolecular β -sheet ($1625, 1692 \text{ cm}^{-1}$), intramolecular β -sheet ($1636, 1641, 1685 \text{ cm}^{-1}$), α -helix or random coil (1651 cm^{-1}), turns (1670 cm^{-1}), side chain vibration or intramolecular β -sheet (1611 cm^{-1}) (Devi, Coleman, & Truntzer, 2011; Schüle, Frieß, Bechtold-Peters, & Garidel, 2007). Hence, the peaks appeared in native IgG sample can be identified as intramolecular β -sheets (1636 and 1641 cm^{-1}), α -helices (1651 cm^{-1}), and turns (1668 cm^{-1}).

The studies have already suggested that the secondary structure of IgG is predominantly made up of β -sheet structures, whereas α -helix content is comparatively low (Buijs et al., 1996; Gorga et al., 1989), which can be even observed in the crystal structure of intact IgG (Janda, Bowen, Greenspan, & Casadevall, 2016). It has further been reported that the typical β -sheets present in bovine IgG are strongly twisted (Li et al., 2005). The contents of β -sheet and α -helix have been reported to be 76 and 9%, respectively, when IgG was analyzed by FTIR (Buijs et al., 1996). Another FTIR method explained the contents of secondary structure of the IgG as a combination of β -sheet (64%), turns (28%), random (5%) and α -helix (3%) (Gorga et al., 1989). The comparable results, β -sheet (67%), turns (18%), random (12%) and α -helix (3%), were also suggested by the X-ray method (Gorga et al., 1989; Levitt & Greer, 1977).

The peak pattern observed from the IgG sample (Fig. 1A), which was subjected to simulated HTST treatment, was similar to that of the control sample, denoting that the secondary structure of IgG likely remained unaffected by that combination of temperature and holding-time (HTST). Bovine IgG appears to be capable of tolerating HTST ($72^\circ\text{C}/15\text{s}$) treatment, by retaining its native structure, determined by measuring the antigenic response of IgG through single radial immunodiffusion (RID) (Mainer et al., 1997). A double sandwich ELISA method revealed that HTST pasteurized milk contained about 65–79% of IgG compared to that of the raw milk (Kummer et al., 1992). While both of these findings are in a close agreement with the results of the present study, other studies have also suggested that ELISA method detects lower amount of active IgG than RID, which could be attributed to the interference by an unidentified component in milk matrix when performing ELISA (Kummer et al., 1992) and also can be related to the hypothesis, in which during a heat treatment IgG loses its ability to bind with antigens (quantified by ELISA) earlier than its ability to elicit immunological response (determined by RID) (Mainer et al., 1997). The antigen-binding capacity of IgG is associated with its native secondary structure, which changes with increasing temperature ($\geq 70^\circ\text{C}$) would decrease its antigen-binding capacity (Li et al., 2005).

IgG molecule possesses the characteristic Y-shaped structure, which is mainly composed of two identical heavy chains ($\sim 53 \text{ kDa}$) and two identical light chains ($\sim 23 \text{ kDa}$). Both heavy and light chains consist of domains referred to as variable (V_H, V_L) and constant (C_{H1-3}, C_L) regions. Disulfide bonds (S-S) link each heavy and light chain pair and also two heavy chains together. The two antigen binding regions are composed of variable regions (V_H, V_L) of each heavy and light chain, which are the N-terminal portions of IgG molecule. Being an antibody, IgG molecule contains two specific structural areas, called F_{ab} -antigen-specific portion (V_L, C_L) and F_c -cell-binding effector portion (C_{H2}, C_{H3}) (Hurley & Theil, 2013, pp. 275–294; Saito, 2009, p. 347). The CH_1 domain in F_{ab} fragment is the most heat labile component, which unfolds earlier than all the

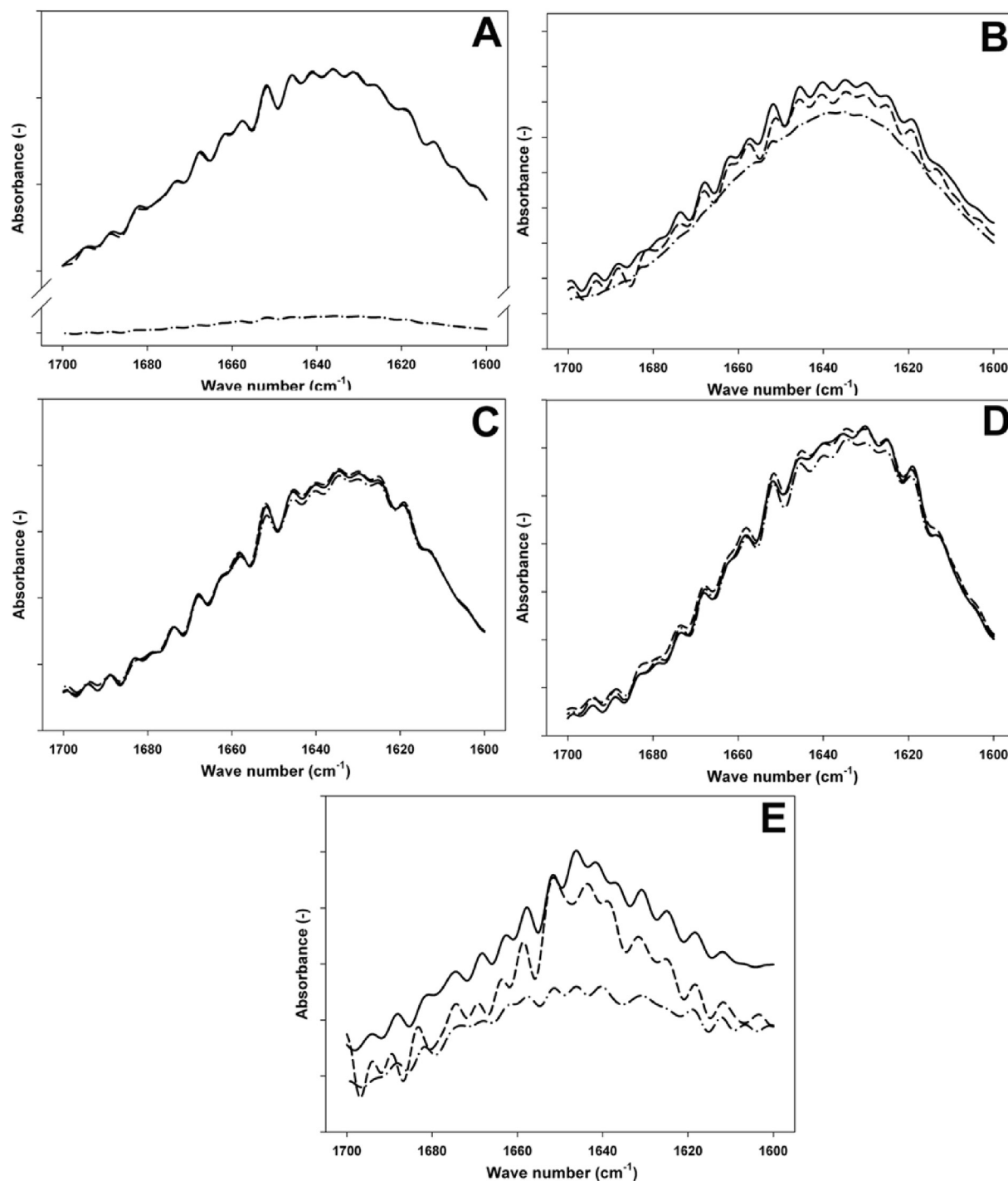


Fig. 1. FTIR spectra of protein/protein mixtures containing (A) IgG, (B) BLG and ALA, (C) BLG, ALA and IgG, (D) BLG, ALA, IgG and BSA, and (E) native whey at their natural concentrations in raw milk subjected to heat treatment at 72 °C (—) or 100 °C (---) for 15 s and 30 s, respectively. Untreated samples served as controls (—).

other regions even at low temperatures (~ 66 °C), for an instance dislocation of peripheral peptide loops exposed to V-C interdomain interface studied by molecular dynamic (MD) simulation (Roterman, Konieczny, Stopa, Rybarska, & Piekarska, 1994). The unfolding of F_{ab} fragment could influence the antigen binding ability of IgG by altering the antigen binding sites (V_H , V_L) (Mainer et al., 1997).

Li et al. (2005) have found that 72 °C was a critical temperature point, at which IgG changes its secondary structure. These changes

(above 72 °C) were irreversible, determined by circular dichroism (CD) spectrometry. These conflicting findings in comparison to the current study could be due to the different holding times applied (Anema, 2017; Mainer et al., 1997), different sample media used (Chen & Chang, 1998; Li-Chan et al., 1995), and different concentrations of IgG treated (Devi et al., 2011; Soltis et al., 1979). Furthermore, IgG investigated in the current study was obtained by fractionation of raw skim milk under mild conditions as opposed to other studies which used commercially available IgG. The native

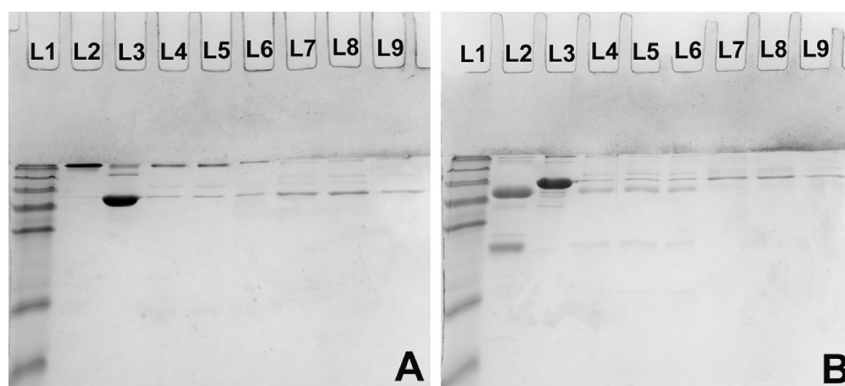


Fig. 2. Non-reducing (A) and reducing (B) SDS-PAGE analysis of protein/protein mixtures. Lanes are designated as: L1 - molecular weight markers; L2 - IgG standard; L3 - BSA standard; L4 - IgG control; L5 - IgG 72 °C/15 s treated; L6 - IgG 100 °C/30 s treated; L7 - BSA control; L8 - BSA 72 °C/15 s treated; L9 - BSA 100 °C/30 s treated.

structure of commercially available IgG could have been possibly altered by some technical processes. Moreover, the inconsistency of research findings on heat stability of IgG during HTST treatment expresses the fact, that the temperature level of HTST (72 °C) has been established in the most critical range (between 70 and 80 °C) (Chen, Tu, & Chang, 2000; Li et al., 2005; Li-Chan et al., 1995), in which IgG tends to unfold its native conformation readily with the changes of other factors including holding-time, concentration of IgG, and the presence of other milk constituents. This can further be supported by the literature since almost all the studies have agreed that no or a very low level of denaturation of IgG took place at temperatures ≤ 65 °C, including low-temperature long-time (LTLT) pasteurization condition (62.5 °C/30 min) (Mainer et al., 1997; Schüle et al., 2007).

The other individual protein samples such as BLG, ALA and BSA also displayed similar results (data not shown), not showing substantial changes of their secondary structures, at HTST conditions as previously reported (Qi, Ren, Xiao, & Tomasula, 2015). In protein mixtures, when the BLG + ALA, BLG + ALA + IgG, and BLG + ALA + IgG + BSA samples were subjected to the same heat treatment (72 °C/15s), both control and treated samples showed mostly similar peaks by FTIR (Fig. 1B, C and D), representing intramolecular β -sheet (~ 1636 cm^{-1}) and α -helix (~ 1651 cm^{-1}), that are common to the secondary structure of all the native proteins (Devi et al., 2011; Rahaman, Vasiljevic, & Ramchandran, 2015; Wong, Camirand, Pavlath, Parris, & Friedman, 1996). It can thus be stated that HTST treatment least impacted on the secondary structure of BLG, ALA, IgG and BSA when they were present either

individually or in their mixtures. Furthermore, the native whey sample showed a parallel pattern of peaks compared with that of the control sample (Fig. 1E), which was subjected to the 72 °C/15s heat treatment. Qi et al. (2015) have also reported that HTST (72 °C/15s) treatment did not change the secondary structure of whey proteins observed by different spectroscopic techniques including FTIR.

The degree and nature of formation of protein complexes through covalent interactions were investigated by non-reducing and reducing SDS-PAGE for all the treated and control samples. The gel bands of both control and the treated samples of all proteins/protein mixtures (BLG, ALA, BSA, IgG, BLG + ALA, BLG + ALA + IgG, BLG + ALA + IgG + BSA, and whey) under non-reducing conditions showed similar patterns (Figs. 2A, 3A and 4A and 5A) denoting the absence of covalent-complexes induced by heat subjected to the simulated HTST conditions. The SDS-PAGE results were thereby in an agreement with FTIR results confirming that HTST pasteurization did not have any major influence on the thermal denaturation of IgG and other whey proteins.

3.2. Denaturation of IgG and its association with other whey proteins affected by heating at 100 °C for 30 s

The IgG sample, which was subjected to 100 °C/30s treatment did not show any prominent native peaks (1636, 1641 and 1651 cm^{-1}) in the FTIR interferograms compared to its control sample (Fig. 1A), signifying the loss of its native conformation. This result agrees well with other studies, which have concluded that no

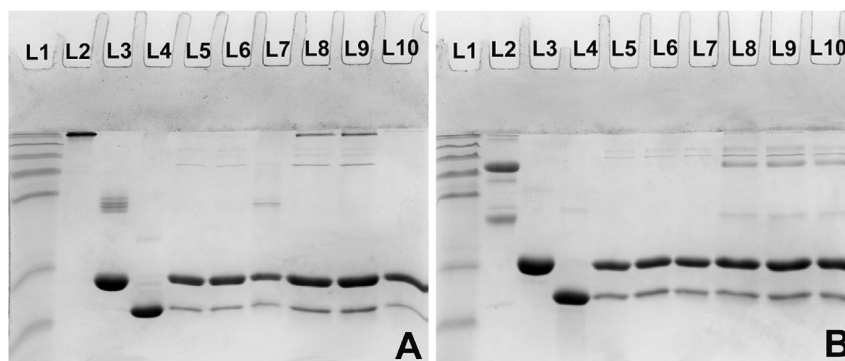


Fig. 3. Non-reducing (A) and reducing (B) SDS-PAGE analysis of protein/protein mixtures. Lanes are designated as: L1 - molecular weight markers; L2 - IgG standard; L3 - BLG standard; L4 - ALA standard; L5 - BLG and ALA control; L6 - BLG and ALA 72 °C/15 s treated; L7 - BLG and ALA 100 °C/30 s treated; L8 - BLG, ALA and IgG control; L9 - BLG, ALA and IgG 72 °C/15 s treated; L10 - BLG, ALA and IgG 100 °C/30 s treated.

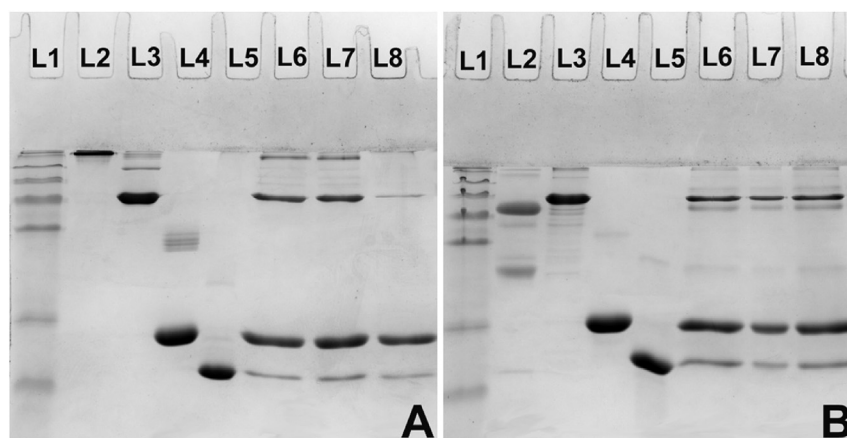


Fig. 4. Non-reducing (A) and reducing (B) SDS-PAGE analysis of protein/protein mixtures. Lanes are designated as: L1 - molecular weight markers; L2 - IgG standard; L3- BSA standard; L4- BLG standard; L5- ALA standard; L6- BLG, ALA, IgG and BSA control; L7 - BLG, ALA, IgG and BSA 72 °C/15 s treated; L8 - BLG, ALA, IgG and BSA 100 °C/30 s treated.

or very little bioactive IgG were detected after the UHT treatment, although they used various methods to capture the bioactive IgG (Kummer et al., 1992; Li-Chan et al., 1995; Yolken, Losonsky, Vonderfecht, Leister, & Wee, 1985). The SDS-PAGE results of the same sample indicated substantial extent of IgG formed protein complexes, which was about 55% of the total IgG (Fig. 2A). This result was in line with the findings that about 30% of IgG formed complexes when IgG was heated alone for 30 s at 95 °C (Oh & Richardson, 1991). A higher rate of formation of complexes in the current study can be attributed to the higher temperature (100 °C) applied as well as shearing. Oh and Richardson (1991) have suggested that IgG form protein complexes induced by heat possibly through thiol-disulfide interactions. This suggestion was confirmed by our results of reducing SDS-PAGE (Fig. 2B). There were no visible IgG aggregates at the top of the stacking gel under the reducing conditions confirming that disulfide-linked covalent interactions were involved in forming protein complexes. Concurrently, all the monomeric IgG were also broken into heavy chains and light chains by the reducing agent as they were linked to each other by disulfide bridges. Hence, all the samples containing IgG appeared as two bands corresponding to the molecular weight of heavy chain (~ 53 kDa) and light chains (~ 23 kDa) under reducing condition (Figs. 2B, 3B and 4B and 5B), which have also been observed by other studies (Anema, 2017; Su & Chiang, 2003).

When BLG + ALA + IgG and BLG + ALA + IgG + BSA samples were subjected to the 100 °C/30s treatment, they showed less

intense peaks in the FTIR results (Fig. 1C and D) in comparison to those of the control samples, corresponding to intramolecular β -sheet and α -helix, indicating unfolding of the native secondary structure. This reduction can primarily be related to the unfolding of β -sheets (1636 cm^{-1}) and α -helices (1651 cm^{-1}) of BLG and ALA, respectively, during a UHT treatment (135 °C/2s), as explained previously (Qi et al., 2015) based on the concentration of each protein (around 50% of BLG and 25% of ALA in the native whey) and their predominant elements of native secondary structure (over 50% of β -sheets in BLG and about 35% of α -helices in ALA). This was observed in our BLG + ALA sample, which was subjected to the same treatment, displaying substantially less intense peaks (intramolecular β -sheet and α -helix) compared to that of the control sample (Fig. 1B).

The appearance of a faint band in the IgG sample (SDS-PAGE), corresponding to the standard band of BSA, indicated the presence of some BSA with IgG (Fig. 2A). However, it was observed that BSA was not involved in forming covalent-complexes with IgG since the intensity of all 3 bands appeared similar even after 100 °C/30s treatment (Fig. 2A). The BSA sample subjected to the same treatment (100 °C/30s) showed formation of protein complexes as observed by others (Yohannes et al., 2010) (Fig. 2A). The impact of the serum albumin on the denaturation of IgG has been investigated by other studies (Oh & Richardson, 1991; Soltis et al., 1979), and an inhibitory effect was suggested. However, in the presence of BLG and ALA (BLG + ALA + IgG + BSA sample) and during 100 °C/

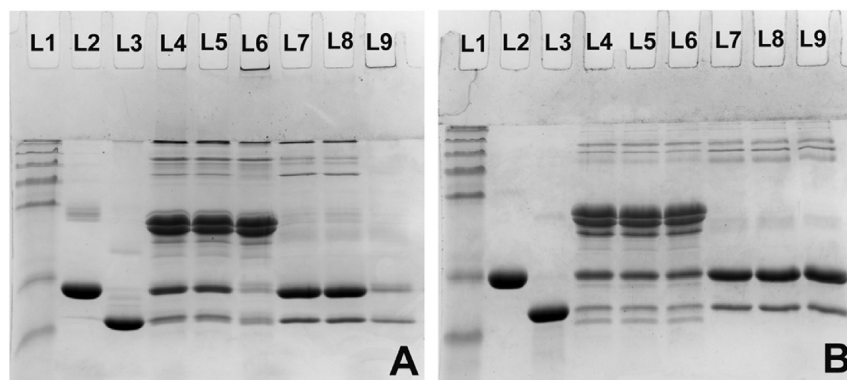


Fig. 5. Non-reducing (A) and reducing (B) SDS-PAGE analysis of skim milk and whey samples. Lanes are designated as: L1 - molecular weight markers; L2- BLG standard; L3- ALA standard; L4-skim milk control; L5 - skim milk 72 °C/15 s treated; L6 - skim milk 100 °C/30 s treated; L7-whey control; L8 - whey 72 °C/15 s treated; L9 -whey 100 °C/30 s treated.

30s treatment, about 85% of BSA was involved in formation of whey proteins complexes (BLG, ALA and IgG) through thiol-disulfide interactions, observed by comparing non-reducing (Fig. 4A) and reducing SDS-PAGE gels (Fig. 4B). When comparing the gel band patterns of the IgG, BLG + ALA + IgG and BLG + ALA + IgG + BSA samples, subjected to the 100 °C/30s treatment, it appeared that the contribution of BSA in heat-induced denaturation of major whey proteins (BLG, ALA, and IgG) depended on the presence of BLG and ALA. This can be partly attributed to the ability of BSA to form heat-induced complexes with BLG and ALA through thiol-disulfide interchange interactions (Considine, Patel, Anema, Singh, & Creamer, 2007) but it needs to be elucidated as to why BSA, having a free thiol group, did not form covalent-complexes with IgG under the same conditions (100 °C/30s) in the absence of BLG and ALA.

During the 100 °C/30s treatment, about 18% of BLG and 48% of ALA in the BLG + ALA + IgG sample (Fig. 3A) and about 19% of BLG and 35% of ALA in the BLG + ALA + IgG + BSA sample (Fig. 4A) contributed to formation of protein complexes as quantified by SDS-PAGE. Disappearance of all these protein complexes under the reducing PAGE condition (Figs. 3B and 4B) indicated that thiol-disulfide interactions were primarily involved in this complex formation. The involvement of ALA (35–48%) in formation of complexes of whey proteins was substantially greater than that of BLG (18–19%), implying that ALA potentially leads heat-induced denaturation of whey proteins in a protein mixture including BLG, IgG and BSA during 100 °C/30s treatment. It is an interesting fact since the BLG was expected to perform such a leading role instead of ALA according to the available literature (Considine et al., 2007; Wijayanti et al., 2014). The above role of ALA was obvious well when the results of BLG + ALA + IgG and BLG + ALA + IgG + BSA samples were compared with BLG + ALA and native whey samples. Around 28% of BLG and 27% of ALA formed covalent-complexes in BLG + ALA sample (Fig. 3A), which was in line with 1:1 ratio of formation of BLG and ALA complexes in a binary mixture observed previously (Hong & Creamer, 2002). The results of the whey sample of the present study also agreed well with results of other studies (Datta et al., 2002; Wijayanti et al., 2014), showing the denaturation of BLG (~74%) was substantially higher than that of ALA (~30%), indicating the leading role of BLG in whey protein denaturation (Fig. 5A). Individual protein samples of BLG and ALA showed formation of covalent-complexes under same (100 °C/30s) conditions as expected (Hong & Creamer, 2002) (data not shown).

Hence, it can be suggested that BSA and/or IgG possibly act as a catalytic agent to induce ALA in participating in heat-induced denaturation of whey proteins over BLG, but another constituent present in native whey could inhibit this effect. It has already been reported that, in a protein mixture (BLG, ALA and BSA), BSA catalyzes the formation of ALA polymers more effectively than BLG (Havea, Singh, & Creamer, 2001). This can be attributed to the ability of BSA to initiate heat-induced unfolding and formation of protein complexes earlier than BLG, and the ability of exposed thiol groups on the BSA molecule, following unfolding, to react with ALA through thiol-disulfide interchange reactions (Considine et al., 2007; Havea, Singh, & Creamer, 2000). The lactose that was present in our native whey sample but was not available in all the other samples (e.g. BLG + ALA + IgG and BLG + ALA + IgG + BSA) could also inhibit the ALA-driven denaturation of whey proteins. The studies have revealed that lactose improved thermal stability of native whey proteins and influenced structural properties of whey protein complexes created by heating (Spiegel, 1999).

This leading role of ALA in denaturation of whey proteins (BLG, IgG and BSA) has also been observed by our FTIR results, as the intensity of the peak corresponding to α -helix (1651 cm^{-1}), which is the most prominent secondary structure of ALA (Qi et al., 2015),

was reduced in the BLG + ALA + IgG and BLG + ALA + IgG + BSA samples (Fig. 2C and D) indicating a higher rate of unfolding of ALA than those of other whey proteins. Although higher thermal stability of IgG has been reported in whey than in PBS, and while the components other than IgG in whey were believed to prevent thermal denaturation of IgG (Chen & Chang, 1998; Chen et al., 2000), our results showed that whey proteins including BLG, ALA, and BSA did not exert such a thermal protective role during 100 °C/30s treatment (Figs. 4A and 5A). This could be due to the high temperature applied (100 °C), that was beyond the temperature limit (~80 °C), at which neither natural nor added thermal protectants can stabilize the IgG effectively (Chen et al., 2000).

4. Conclusion

Simulated HTST conditions appeared to be less detrimental, in comparison to 100 °C/30s treatment, on the changes of the secondary structure of bovine IgG and other whey proteins (BLG, ALA, and BSA) when they were present either alone or in a mixture with other whey proteins. The unfolding of secondary structure and subsequent formation of protein complexes were observed from both fractionated IgG and IgG in the mixtures of other whey proteins (BLG, ALA, and BSA) after 100 °C/30s treatment through thiol-disulfide interchange interactions. BSA did not form covalent-complexes with IgG in the absence of BLG and ALA at the 100 °C/30s condition, which needs to be studied further to understand the real effect of BSA on the thermal stability of IgG. In a whey protein mixture (BLG, ALA, IgG and BSA), ALA unexpectedly led the denaturation of whey proteins subjected to 100 °C/30s treatment. It can thus be suggested that IgG and/or BSA play a catalytic role in inducing ALA to drive the denaturation of whey proteins under these experimental conditions (100 °C/30s). Native whey however contains a component possibly lactose that could inhibit this effect. The other factors including pH and ionic strength of the different media also might contribute to this effect. Further studies are required to elucidate this mechanism. The presence of other whey proteins (BLG, ALA, and BSA) had no apparent effect on the thermal stability of IgG at 100 °C.

Conflict of interest

The authors hereby declare that there is no conflict of interest at all in regard to publication of this research work.

Acknowledgement

The authors sincerely acknowledge the financial and technical support from Victoria University.

References

- Akinbi, H., Meinzen-Derr, J., Auer, C., Ma, Y., Pullum, D., Kusano, R., et al. (2010). Alterations in the host defense properties of human milk following prolonged storage or pasteurization. *Journal of Pediatric Gastroenterology and Nutrition*, 51(3), 347–352.
- Anema, S. G. (2017). A comparison of the kinetics of the thermal denaturation of the immunoglobulins in caprine and bovine skim milk samples. *International Dairy Journal*, 65, 1–4.
- Bogahawaththa, D., Chandrapala, J., & Vasiljevic, T. (2017). Modulation of milk immunogenicity by thermal processing. *International Dairy Journal*, 69, 23–32.
- Buijs, J., Norde, W., & Lichtenbelt, J. W. T. (1996). Changes in the secondary structure of adsorbed IgG and F(ab')₂ studied by FTIR spectroscopy. *Langmuir*, 12(6), 1605–1613.
- Chen, C.-C., & Chang, H.-M. (1998). Effect of thermal protectants on the stability of bovine milk immunoglobulin G. *Journal of Agricultural and Food Chemistry*, 46(9), 3570–3576.
- Chen, C. C., Tu, Y. Y., & Chang, H. M. (2000). Thermal stability of bovine milk immunoglobulin G (IgG) and the effect of added thermal protectants on the stability. *Journal of Food Science*, 65(2), 188–193.

- Considine, T., Patel, H. A., Anema, S. G., Singh, H., & Creamer, L. K. (2007). Interactions of milk proteins during heat and high hydrostatic pressure treatments — a Review. *Innovative Food Science & Emerging Technologies*, 8(1), 1–23.
- Dannenberg, F., & Kessler, H. G. (1988). Reaction kinetics of the denaturation of whey proteins in milk. *Journal of Food Science*, 53(1), 258–263.
- Datta, N., Elliott, A. J., Perkins, M. L., & Deeth, H. C. (2002). Ultra-high-temperature (UHT) treatment of milk: Comparison of direct and indirect modes of heating. *Australian Journal of Dairy Technology*, 57(3), 211.
- Devi, V. S., Coleman, D. R., & Truntzer, J. (2011). Thermal unfolding curves of high concentration bovine IgG measured by FTIR spectroscopy. *The Protein Journal*, 30(6), 395–403.
- Dupont, D., Croguennec, T., Brodkorb, A., & Kouaoui, R. (2013). *Quantitation of proteins in milk and milk products Advanced Dairy Chemistry*. US: Springer.
- Gapper, L. W., Copestake, D. E., Otter, D. E., & Indyk, H. E. (2007). Analysis of bovine immunoglobulin G in milk, colostrum and dietary supplements: A review. *Analytical and Bioanalytical Chemistry*, 389(1), 93–109.
- Gorga, J. C., Dong, A., Manning, M. C., Woody, R. W., Caughey, W. S., & Strominger, J. L. (1989). Comparison of the secondary structures of human class I and class II major histocompatibility complex antigens by Fourier transform infrared and circular dichroism spectroscopy. *Proceedings of the National Academy of Sciences*, 86(7), 2321–2325.
- Havea, P., Singh, H., & Creamer, L. K. (2000). Formation of new protein structures in heated mixtures of BSA and α -lactalbumin. *Journal of Agricultural and Food Chemistry*, 48(5), 1548–1556.
- Havea, P., Singh, H., & Creamer, L. K. (2001). Characterization of heat-induced aggregates of β -lactoglobulin, α -lactalbumin and bovine serum albumin in a whey protein concentrate environment. *Journal of Dairy Research*, 68(03), 483–497.
- Hong, Y.-H., & Creamer, L. K. (2002). Changed protein structures of bovine β -lactoglobulin B and α -lactalbumin as a consequence of heat treatment. *International Dairy Journal*, 12(4), 345–359.
- Hurley, W. L., & Theil, P. K. (2011). Perspectives on immunoglobulins in colostrum and milk. *Nutrients*, 3(4), 442–474.
- Hurley, W., & Theil, P. K. (2013). *Immunoglobulins in mammary secretions Advanced dairy chemistry*. US: Springer.
- Janda, A., Bowen, A., Greenspan, N. S., & Casadevall, A. (2016). Ig constant region effects on variable region structure and function. *Frontiers in Microbiology*, 7.
- Kato, Y., Yamasaki, Y., Moriyama, H., Tokunaga, K., & Hashimoto, T. (1987). New high-performance gel filtration columns for protein separation. *Journal of Chromatography a*, 404, 333–339.
- Korhonen, H., Marnila, P., & Gill, H. (2000). Bovine milk antibodies for health. *British Journal of Nutrition*, 84(S1), 135–146.
- Kummer, A., Kitts, D., Li-Chan, E., Losso, J., Skura, B., & Nakai, S. (1992). Quantification of bovine IgG in milk using enzyme-linked immunosorbent assay. *Food and Agricultural Immunology*, 4(2), 93–102.
- Levitt, M., & Greer, J. (1977). Automatic identification of secondary structure in globular proteins. *Journal of Molecular Biology*, 114(2), 181–239.
- Li-Chan, E., Kummer, A., Losso, J., Kitts, D., & Nakai, S. (1995). Stability of bovine immunoglobulins to thermal treatment and processing. *Food Research International*, 28(1), 9–16.
- Li, S.-Q., Bomser, J. A., & Zhang, Q. H. (2005). Effects of pulsed electric fields and heat treatment on stability and secondary structure of bovine immunoglobulin G. *Journal of Agriculture and Food Chemistry*, 53(3), 663–670.
- Liyanaarachchi, W. S., Ramchandran, L., & Vasiljevic, T. (2015). Controlling heat induced aggregation of whey proteins by casein inclusion in concentrated protein dispersions. *International Dairy Journal*, 44, 21–30.
- Mainer, G., Sanchez, L., Ena, J., & Calvo, M. (1997). Kinetic and thermodynamic parameters for heat denaturation of bovine milk IgG, IgA and IgM. *Journal of Food Science*, 62(5), 1034–1038.
- Mehra, R., Marnila, P., & Korhonen, H. (2006). Milk immunoglobulins for health promotion. *International Dairy Journal*, 16(11), 1262–1271.
- Nishanthi, M., Vasiljevic, T., & Chandrapala, J. (2017). Properties of whey proteins obtained from different whey streams. *International Dairy Journal*, 66, 76–83.
- Oh, S. S., & Richardson, T. (1991). Heat-induced interactions of bovine serum-albumin and immunoglobulin. *Journal of Dairy Science*, 74(6), 1786–1790.
- Oldfield, D. J., Singh, H., Taylor, M. W., & Pearce, K. N. (1998). Kinetics of denaturation and aggregation of whey proteins in skim milk heated in an ultra-high temperature (UHT) pilot plant. *International Dairy Journal*, 8(4), 311–318.
- Patel, H. A. (2007). *Studies on heat-and pressure-induced interactions of milk proteins: A thesis presented in partial fulfilment of the requirements for the degree of doctor of philosophy in food technology at massey university*. Palmerston North, New Zealand.
- Qi, P. X., Ren, D., Xiao, Y., & Tomasula, P. M. (2015). Effect of homogenization and pasteurization on the structure and stability of whey protein in milk. *Journal of Dairy Science*, 98(5), 2884–2897.
- Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2015). Conformational changes of beta-lactoglobulin induced by shear, heat, and pH-Effects on antigenicity. *Journal of Dairy Science*, 98(7), 4255–4265.
- Rosenqvist, E., Jossang, T., & Feder, J. (1987). Thermal properties of human IgG. *Molecular Immunology*, 24(5), 495–501.
- Rosmaninho, R., & Melo, L. F. (2006). The effect of citrate on calcium phosphate deposition from simulated milk ultrafiltrate (SMUF) solution. *Journal of Food Engineering*, 73(4), 379–387.
- Roterman, I., Konieczny, L., Stopa, B., Rybarska, J., & Piekarska, B. (1994). Heat-induced structural changes in the Fab fragment of IgG recognized by molecular dynamics simulation-Implications for signal transduction in antibodies. *Folia Biologica*, 42, 115–128.
- Saito, T. (2009). *Potential for improving Health: Immunomodulation by dairy ingredients*. Bioactive Components in Milk and Dairy Products.
- Schüle, S., Frieß, W., Bechtold-Peters, K., & Garidel, P. (2007). Conformational analysis of protein secondary structure during spray-drying of antibody/mannitol formulations. *European Journal of Pharmaceutics and Biopharmaceutics*, 65(1), 1–9.
- Soltis, R., Hasz, D., Morris, M., & Wilson, I. (1979). The effect of heat inactivation of serum on aggregation of immunoglobulins. *Immunology*, 36(1), 37.
- Spiegel, T. (1999). Whey protein aggregation under shear conditions—effects of lactose and heating temperature on aggregate size and structure. *International Journal of Food Science & Technology*, 34(5–6), 523–531.
- Su, C.-K., & Chiang, B. H. (2003). Extraction of immunoglobulin-G from colostrum whey by reverse micelles. *Journal of Dairy Science*, 86(5), 1639–1645.
- Wijayanti, H. B., Bansal, N., & Deeth, H. C. (2014). Stability of whey proteins during thermal processing: A review. *Comprehensive Reviews in Food Science and Food Safety*, 13(6), 1235–1251.
- Wong, D. W., Camirand, W. M., Pavlath, A. E., Parris, N., & Friedman, M. (1996). Structures and functionalities of milk proteins. *Critical Reviews in Food Science & Nutrition*, 36(8), 807–844.
- Yohannes, G., Wiedmer, S. K., Elomaa, M., Jussila, M., Aseyev, V., & Riekkola, M.-L. (2010). Thermal aggregation of bovine serum albumin studied by asymmetrical flow field-flow fractionation. *Analytica Chimica Acta*, 675(2), 191–198.
- Yolken, R. H., Losonsky, G. A., Vonderfecht, S., Leister, F., & Wee, S.-B. (1985). Antibody to human rotavirus in cow's milk. *New England Journal of Medicine*, 312(10), 605–610.