PROCESSING-INDUCED MODIFICATIONS OF NATIVE BOVINE MILK PROTEINS IN RELATION TO IMMUNOGENICITY

A thesis submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

by

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Dedicated to my beloved parents,

Rangani, Vinara and Ryan

Abstract

Bovine milk proteins are a source of high-quality proteins in the human diet. Raw milk is subjected to different processing treatments prior to human consumption to ensure food safety and extend the shelf life. However, the thermal processing including high-temperature short-time (HTST) pasteurization and ultra-high temperature (UHT) treatment and alternative nonthermal methods including application of high pressure (HP) appear to modify the native properties of milk proteins. The processing induced modifications in protein structure, mainly denaturation and aggregation, and associated changes in epitopes can modulate the immunogenicity and potential allergenicity of milk proteins. The severity of some processing conditions appears to alter the native minor proteins including immunoglobulins (Ig), which may otherwise exert immunomodulatory properties in such a way as to prevent occurrence of allergies. Bovine or cow's milk protein allergy (CMPA) is an abnormal immunological reaction to one or more milk proteins and it is the most prevalent food allergy among infants globally. Hence, the overall aim of this study was to identify the modification of native milk proteins induced by selected thermal (heating at 72 for 15 s and 100 °C for 30 s) and nonthermal processing conditions (application of HP at 400, 500, or 600 MPa for 15 min at 30 °C) and to establish their impact on modulation of in vitro immunogenicity as a means of envisaging potential allergenicity.

Processing induced changes in secondary structure of proteins were studied by Fourier transform infrared spectroscopy (FTIR), and protein denaturation and aggregation were mainly examined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC). Changes in antigenicity of milk proteins followed by processing was studied by enzyme-linked immunosorbent assay (ELISA). Modulation of in vitro immunogenicity was assessed based on the concentration of several cytokines secreted

by human peripheral blood mononuclear cells (PBMCs) in response to native and processed milk proteins.

Thermal denaturation of bovine IgG was studied alone and in the presence of major whey proteins. The two heating regimes studied provided the simulated thermal effect compared to HTST (72 °C/ 15 s) and UHT (100 °C/ 30 s equivalent to 140 °C/ 5 s 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 30 s caused formation of covalent complexes of IgG alone, as well as in the mixtures, mainly through thiol-disulfide reactions. Under 100 °C /30 s treatment, bovine serum albumin (BSA) did not interact with IgG through thiol-disulfide reactions in a binary mixture of proteins (IgG+BSA). α-Lactalbumin (ALA) appeared to preferentially lead denaturation of whey proteins over β-lactoglobulin (BLG), in a protein mixture (BLG+ALA+IgG+BSA), 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 for 30 s.

Residual antigenicity of a processed protein is a marker of potential allergenicity. Other milk proteins affect thermal denaturation of bovine BLG and modulate its antigenicity. Denaturation of BLG and altered antigenicity were studied in protein mixtures during 72 °C/ 15 s and 100 °C/ 30 s treatments. BLG denaturation, affected by other proteins, correlated with altered antigenicity. The treatment at 72 °C/ 15 s enhanced antigenicity in BLG+ALA mixture possibly due to exposed epitopes in unfolded structure, while it did not affect other protein mixtures. The treatment at 100 °C/ 30 s resulted in BLG-led protein aggregation by thiol/disulphide interactions and declined antigenicity by fragmentation and masking of epitopes to a different extent depending on the mixture. IgG contributed to diminish antigenicity in BLG+ALA+IgG mixture at 100 °C/ 30 s. The protein denaturation governed by

ALA over BLG in BLG+ALA+IgG+BSA mixture, was possibly catalysed by BSA at 100 °C/30 s, resulting in a higher retention of antigenicity than other mixtures.

In vitro immunogenicity of various native and thermally processed (72 °C / 15 s and 100 °C /30 s) bovine milk protein fractions, their mixtures, whey, and skim milk, was studied by analysing the immune response of T helper (Th) cells in human PBMCs. The secretion of Th types cytokines induced by the protein stimulants was quantified, while determining the heat-induced protein denaturation. Purified whey proteins, caseins and whey fraction, and skim milk, provoked substantial immune responses at various degrees, indicating their potent immunogenicity. The protein mixtures prepared using the fractionated whey proteins with or without caseins appeared less immunogenic in both native and heat-treated forms, implying their potential of producing less immunogenic dairy products. The treatment at 100 °C/ 30 s significantly altered the immunogenicity of most of the potent protein stimulants, which mostly coincided with their levels of protein denaturation. The treatment at 72 °C / 15 s caused least protein denaturation but altered the immunogenicity of several protein stimulants notably including heat-stable caseins and ALA.

High pressure processing (HPP), conducted at 400, 500 or 600 MPa for 15 min at 30 °C, of raw skim milk was studied in comparison to HTST pasteurization (72 °C/ 15 s), considering protein denaturation and in vitro immunogenicity. HTST pasteurization least impacted denaturation of native proteins leading to mostly unchanged milk immunogenicity. HPP resulted in denaturation of whey proteins, mostly BLG and IgG, and disturbed structure of the casein micelle. HPP at 600 MPa caused protein aggregation, involving mainly BLG and κ-casein, through thiol disulphide interactions. ALA was least denatured subjected to all HPP conditions. The balance between expression of Th1 and Th2 type cytokines, which is believed to regulate adverse immune response, was initially shifted toward Th1 with increase in HP, then the immunogenic capacity of milk proteins diminished at 600 MPa. This could be related

to exposure of T cell-specific linear epitopes followed by unfolding of protein structure firstly and masking of them by protein aggregation subsequently with increase in high pressure.

In overall, the conditions applied in raw milk processing should be further optimised in considering modifications of native milk proteins and subsequent modulation of their immunogenicity, in addition to ensuring the food safety, to make the final dairy product both hygienic and hypoallergenic. Mild heat treatments (< 72 °C) or combined mild processing, for instance application of HP below 400 MPa in combination with low temperature (< 50 °C), would be able to fulfil aforementioned requirements.

Certificate

Prof. Todor Vasiljevic, PhD (*UAlberta*)

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This is to certify that the thesis entitled **"PROCESSING-INDUCED MODIFICATIONS OF NATIVE BOVINE MILK PROTEINS IN RELATION TO IMMUNOGENICITY"** submitted by **Dimuthu Kumara Bogahawaththa Hewa Bogahawaththage** in partial fulfilment of the requirement for the award of the Doctor of Philosophy with specialisation in Food Sciences and Technology at Victoria University is a record of bonafide research work carried out by him under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Prof. Todor Vasiljevic

(Principal Supervisor)

Date: 31.08.2018

Declaration

"I, Dimuthu Kumara Bogahawaththa Hewa Bogahawaththage, declare that the PhD thesis by Publication entitled "PROCESSING-INDUCED MODIFICATIONS OF NATIVE BOVINE MILK PROTEINS IN RELATION TO IMMUNOGENICITY" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

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PART A:

DETAILS OF INCLUDED PAPERS: THESIS BY PUBLICATION

Please list details of each Paper included in the thesis submission. Copies of published Papers and submitted and/or final draft Paper manuscripts should also be included in the thesis submission.

Item/ Chapter No.	Paper Title	Authors	Publication Status (e.g. published, accepted for publication, to be revised and resubmitted, currently under review, unsubmitted but proposed to be submitted)	Publication Title and Details (e.g. date published, impact factor etc.)
2A	Modulation of milk immunogenicity by thermal processing	Dimuthu Bogahawaththa, Jayani Chandrapala and Todor Vasiljevic	Published	International Dairy Journal; SJR Q1
3	Thermal denaturation of bovine immunoglobulin G and its association with other whey proteins	Dimuthu Bogahawaththa, Jayani Chandrapala and Todor Vasiljevic	Published	Food Hydrocolloids; SJR Q1
4	Thermal denaturation of bovine β-lactoglobulin in different protein mixtures in relation to antigenicity	Dimuthu Bogahawaththa, Jayani Chandrapala and Todor Vasiljevic	Published	International Dairy Journal; SJR Q1
5	In vitro immunogenicity of various native and thermally processed bovine milk proteins and their mixtures	Dimuthu Bogahawaththa, Rabia Ashraf, Jayani Chandrapala, Osaana Donkor and Todor Vasiljevic	Published	Journal of Dairy Science; SJR Q1
6	Comparison between thermal pasteurization and high pressure processing of bovine skim milk in relation to denaturation and immunogenicity of native milk proteins	Dimuthu Bogahawaththa, Roman Buckow, Jayani Chandrapala and Todor Vasiljevic	Published	Innovative Food Science and Emerging Technologies; SJR Q1

Declaration by Dimuthu Hewa Bogahawaththage: **Signature:** [redacted] **Date:** 15/03/2019

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List of abbreviations and units

˚C = degree Celsius

ALA = alpha-lactalbumin

BLG = beta-lactoglobulin

BSA = bovine serum albumin

CCP = colloidal calcium phosphate

CD = circular dichroism

CMPA = cow's milk protein allergy

CNS = caseins

Cys = cysteine

DSC = differential scanning calorimetry

ELISA = enzyme-linked immunosorbent assay

Fab = facilitated allergen binding

FTIR = fourier transform infrared spectroscopy

g = gram

GLM = general linear model

h = hour

HCl = hydrochloric acid

HP = high pressure

HPP = high pressure processing

HTST = high-temperature short-time

Ig = immunoglobulin

IL= interleukin

kDa = kilodalton

kg = kilogram

L = litre

LF = lactoferrin

MHC = major histocompatibility complex

min = minute

mL = millilitre

mM = millimolar

MPa = mega pascal

MW = molecular weight

ng = nanogram

PAGE = polyacrylamide gel electrophoresis

pH = hydrogen ion concentration

RFI = relative fluorescence intensity

RT = room temperature

s = second

SAS = statistical analysis software

SDS = sodium dodecyl sulphate

SEM = standard error of the mean

SH = thiol group

SS = disulphide

TEM = transmission electron microscopy

UHT = ultra-high temperature

V = volts

v/v = volume per volume

w/w = weight per weight

W = watt

WPC = whey protein concentrate

α = alpha

β = beta

κ = kappa

μ = micro

μg = microgram

μL = microliter

Chapter 1: Introduction

This chapter presents an introduction to the thesis including background information, aim and specific objectives, and structure of the thesis.

1.1 Background

Bovine or cow's milk is considered a balanced and nutritive food in human diet. It is also an important food source for infants, being a substitute for human breast milk (Pereira, 2014). The production of whole fresh cow's milk in the world has been reported to be about 660 million tonnes in 2016 (FAO stat, 2016). Apart from the nutritional importance, epidemiological studies frequently reported the potential contribution of cow's milk consumption in preventing several chronic conditions in human health including cardiovascular diseases, some cancers, obesity, and diabetes. Cow's milk protein allergy (CMPA) and lactose intolerance are the commonly prevalent adverse reactions to cow's milk consumption (Downs, Kabourek, Baumert, & Taylor, 2013; Pereira, 2014).

Cow's milk is a major source of proteins, containing approximately 3-4 % (w/w), which are regarded as high-quality proteins in terms of the human amino acid requirement, digestibility, and bioavailability. The essential amino acid score and protein-digestibility corrected amino acid score frequently classify milk protein as the best source of proteins. Apart from the nutritive value, milk proteins and their peptides perform various favourable biological functions in human body including antibacterial, antiviral, antifungal, anticancer, antioxidant, antihypertensive, and immunomodulatory roles. Caseins and whey proteins are the major proteins fractions present in cow's milk, approximately representing 80 and 20% of total proteins, respectively, which possess different physicochemical properties (Downs et al., 2013; Mills, Ross, Hill, Fitzgerald, & Stanton, 2011; Pereira, 2014; Sah, Vasiljevic, McKechnie, & Donkor, 2015).

Processing of raw milk is a well-established practice in the present dairy industry, as well it is a mandatory requirement of many countries, to ensure the food safety and extend the shelf life prior to human consumption, considering the presence of pathogenic microorganism in raw milk (Claeys et al., 2013; Holsinger, Rajkowski, & Stabel, 1997). Thermal processing,

including pasteurization and ultra-high temperature (UHT) treatment, is the traditional and commonly applied method in processing of raw milk (O'Connell & Fox, 2003). High pressure processing (HPP) is an emerging nonthermal technology, which is also considered an alternative for thermal processing (Georget et al., 2015). However, the processing of raw milk can modify the native properties of milk proteins. In native proteins, depending on the sequence of amino acids and various intramolecular forces (e.g., hydrophobic interactions, hydrogen bonding, electrostatic interactions, and disulfide bonds), primary structure folds into several secondary and tertiary structures and subsequently form a uniquely folded native conformation, which is usually present in a stable form at minimum energy status. However, the processing conditions including application of heat and HP usually create an imbalance among intramolecular forces resulting in structural modifications leading to protein denaturation and aggregation (Bu, Luo, Zheng, & Zheng, 2009; Davis & Williams, 1998; Huppertz, Fox, de Kruif, & Kelly, 2006; Huppertz, Kelly, & Fox, 2002).

The structural modifications of milk proteins induced by heating have been extensively studied especially in considering major proteins such as caseins, BLG, and ALA, as well their interactions. However, the structural modifications of minor proteins including immunoglobulin (Ig) and their association with other major proteins during heating are relatively less researched (Considine, Patel, Anema, Singh, & Creamer, 2007; Huppertz, 2016; Wijayanti, Bansal, & Deeth, 2014). Although several studies have considerably investigated the impact of HPP on milk proteins, their focus were largely on main milk proteins (Huppertz et al., 2006; Patel, Singh, Anema, & Creamer, 2006).

Epitopes are present in the structure of almost all the milk proteins, which render them antigenic and/ or allergenic, or commonly immunogenic (Wal, 2002). Immunogenicity is the ability of a substance to elicit an immune response (Abbas, Pillai, & Lichtman, 2014). Epitope is a portion of the immunogenic molecule, mainly proteins, which can bind with the

complementary site of an antibody (Wal, 2002). CMPA is an abnormal immunological reaction to one or more milk proteins (El-Agamy, 2007). CMPA is the most prevalent food allergy among infants approximately 2-6 % (Host & Halken, 2014; Kim et al., 2011) and milk protein is one of the eight food allergens, which account for about 90% of food allergenic incidents frequently reported in the world (Downs et al., 2013). Although the complete elimination of milk consumption is considered as a reliable approach to reduce the risk of milk allergy, it would result in malnutrition especially in infants. The nutritional quality of the infant formulae (containing hydrolysed milk proteins) recommended for milk allergic infants also appears to be compromised (Clemente, 2000; Crittenden & Bennett, 2005; Hernell & Lönnerdal, 2003; Sathe, Teuber, & Roux, 2005).

Processing induced structural changes in a protein can modify the immunogenic epitopes in many ways including fragmentation, modification, masking, and unmasking, which in turn result in either decreased, increased, or unchanged allergenicity (Rahaman, Vasiljevic, & Ramchandran, 2016). Hence, the investigations are directed toward modulation of the potential allergenicity of milk proteins through the application of various processing methods and conditions (Bu, Luo, Chen, Liu, & Zhu, 2013; Shriver & Yang, 2011; Verhoeckx et al., 2015). For instance, the antigenicity of bovine β-lactoglobulin (BLG), one of the major protein allergen, can be modulated through the application of heat and HP. The antigenicity of BLG was gradually enhanced with increase in temperature up to 80-90 °C and decreased rapidly with further increase in temperature, which corresponded to the unfolding of native conformation initially and subsequent protein aggregation at higher temperatures, respectively (Kleber & Hinrichs, 2007; Kleber, Krause, Illgner, & Hinrichs, 2004). The application of HP resulted in modification of protein structure leading to enhanced antigenicity at 400-600 MPa (Kleber, Maier, & Hinrichs, 2007).

The modification of native proteins and thereby modulation of their immunogenicity and potential allergenicity appear to depend on several factors including the type of processing method (e.g., heating and HP), severity of the treatment, treatment duration, physicochemical properties of the protein, nature of the medium (e.g., milk, whey, and buffer) and other environmental factors (e.g., pH) and importantly influence of the other milk proteins present in the medium (Bu et al., 2013; Rahaman, Vasiljevic, & Ramchandran, 2015; Rahaman et al., 2016; Shriver & Yang, 2011). Although many studies have investigated the processing induced modifications of the major milk proteins (e.g., caseins, BLG, ALA), mostly one at a time, in relation to modulation of antigenicity or immunogenicity (Bloom et al., 2014; Bu et al., 2009; Kleber et al., 2007), most of them have not considered how other milk proteins, especially minor whey proteins including Ig present in the medium, influence these changes. Moreover, the existing literature still fails to establish as to how processing methods and conditions modulate the immunogenicity of various isolated protein fractions and how they can be applied in milk processing for the benefit of milk protein sensitive people (Bu et al., 2013; Shandilya, Kapila, Haq, Kapila, & Kansal, 2013).

Apart from the antigenic and allergenic properties of the milk proteins, cow's milk as a biologically active fluid plays various immunomodulatory roles in human immune system (Cross & Gill, 2000; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007; Perdijk, van Splunter, Savelkoul, van Neerven, & Brugman, 2018), which could, however, alter upon subjecting to different processing conditions including thermal pasteurization (Høst & Samuelsson, 1988; McCarthy, Ross, Fitzgerald, & Stanton, 2015). A several epidemiological studies reported the potential contribution of raw milk (not heated to 72 °C) consumption toward development of protection against allergies and asthma (Loss et al., 2011; MacDonald et al., 2011; Sozanska, Pearce, Dudek, & Cullinan, 2013; Waser et al., 2007). Further studies revealed that the native milk proteins, especially the whey proteins, mainly contribute toward

these positive health outcomes (Loss et al., 2011; van Neerven, Knol, Heck, & Savelkoul, 2012). Moreover, the minor whey proteins including IgG, BSA and lactoferrin (LF) are suggested to play a vital role (Brick et al., 2017; Loss et al., 2011; Perdijk et al., 2018; van Neerven et al., 2012). However, it is not yet clear which protein fraction contributes to what and how since no controlled human studies (in vivo) have been conducted due to ethical concerns in ingestion of raw milk (Perdijk et al., 2018; van Neerven et al., 2012; Verhoeckx et al., 2015). This highlights the necessity of conducting in vitro studies to examine the ability of native and processed milk proteins to modulate the human immune system differently.

1.2 Research aim and objectives

The overall aim of this study was to identify modifications of native milk proteins induced by selected thermal and nonthermal processing methods and to establish their impact on modulation of in vitro immunogenicity.

The specific objectives were:

i. To establish the impact of thermal processing on the modification of native protein fractions and their interactions.
ii. To relate the modification of native protein fractions induced by thermal processing to their antigenicity.
iii. To examine in vitro immunogenicity of various native and thermally processed milk protein fractions and their mixtures in envisaging potential allergenicity.
iv. To assess the HPP as an alternative method for thermal pasteurization in terms of modification of the native milk proteins and modulation of in vitro immunogenicity.

1.3 Thesis outline

This thesis contains 7 chapters. Chapter 1 presents an introduction to the thesis including background information, aim and specific objectives, and structure of the thesis. Chapter 2

critically reviews the literature relating to the study and highlights the fundamental concepts and key research findings. Chapter 3 details the thermal denaturation of bovine IgG and its association with other milk proteins. Chapter 4 explains the effect of other milk proteins on thermal denaturation and antigenicity of bovine BLG. Chapter 5 examines in vitro immunogenicity of various native and thermally processed milk protein fractions and their mixtures. Chapter 6 assesses the HPP as an alternative method for thermal pasteurization based on processing induced changes in native proteins and in vitro immunogenicity. Chapter 7 delivers conclusions of the entire project and the scope for future work.

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Chapter 2: Review of literature

This chapter critically reviews the literature relating to the study and highlights the fundamental concepts and key research findings. This has been divided into two chapters, 2A and 2B.

Chapter 2A: Modulation of milk immunogenicity by thermal processing

This chapter has been published as a review paper entitled “Modulation of milk immunogenicity by thermal processing” by Dimuthu Bogahawaththa, Jayani Chandrapala and Todor Vasiljevic in the peer-reviewed journal, International Dairy Journal, 69 (2017) 23–32. http://dx.doi.org/10.1016/j.idairyj.2017.01.010

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Review

Modulation of milk immunogenicity by thermal processing

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ABSTRACT

Bovine milk proteins, apart from provision of essential amino acids, impact on the human immune system. Heat treatments applied in processing of bovine milk directly influence milk protein structure and changes in associated epitopes, altering the immunogenic and antigenic potential of milk proteins, and thus modulating the human immune system in different ways. The severity of some processing technologies impact on important minor proteins that may exert immunomodulatory properties, in such a way as to prevent occurrence of allergies. In this review, heat-induced modifications of milk protein structure, associated changes in epitopes, alteration of immunomodulatory properties of milk and their effects on immunogenic and antigenic potential of milk proteins are discussed. Thermal processing in the dairy industry could be further optimised in a way to preserve and utilise the favourable immunomodulatory properties of milk, in addition to ensuring food safety, eventually making the final dairy product both hygienic and hypoallergenic.

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Contents

1. Introduction

1.1. The effect of breast-feeding on allergies in infants

Susceptibility to infections and allergies has become a crucial health concern in early life of infants. Globally, around 3 million infants die within the first month of their birth each year; 50% of these of deaths are caused by infectious diseases. Moreover, allergies develop during early childhood in parallel with infectious diseases. Bronchial asthma, allergic rhinitis, atopic dermatitis, food allergy, and acute urticaria are the most prevalent allergic diseases that predominantly affect children, far more than adults. The occurrence of these allergies has been on the rise for the last few decades. The immune deficiencies and defects, especially in mucosal immunity, cause mainly infections and allergies in early life (Hendaus, Jomha, & Ehlayel, 2016; Oza, Lawn, Hogan, Mathers, & Cousens, 2015; Turfkruyer & Verhasselt, 2015).

Breast-feeding is regarded as the perfect and preferred mode of nourishing neonates due to its nutritional, immunological and physiological benefits. Human milk contains not only macro- and

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micro-nutrients but also living cells, antibodies and immunologically active factors, including immunoglobulins (Ig), that potentially compensate for neonatal immune deficiencies and also stimulate maturation of the immune system, leading to immune autonomy after weaning (Friedman & Zeiger, 2005; Hoyt, Medico, & Commins, 2015; Turfkruyer & Verhasselt, 2015). Apart from transferring passive immunity against infections from mother to neonate, mainly through secretary IgA (SIgA) antibodies and lactoferrin, studies have suggested that breast-feeding plays a protective role against the development of childhood allergic diseases, including food allergy, although this impact might be either non-protective or neutral in some instances (Friedman & Zeiger, 2005; Hoyt et al., 2015; Iyengar & Walker, 2012; Turfkruyer & Verhasselt, 2015). These controversial conclusions of different studies can be attributed to the heterogeneity of the study designs, methodological limitations, and complex interactions of breast milk with the infant's immune system (Friedman & Zeiger, 2005; Iyengar & Walker, 2012). Studies have found that postnatal maturation of the gut epithelium is governed by a combination of genetics, microbial colonisation, and nutrition, including consumption of breast milk, which could potentially suppress the allergy development through restricting the permeability of the gut barrier and subsequently regulating passage of antigens. The maturation of gut epithelium also controls the selective transfer of antigens in the small intestine that aid in developing oral tolerance to food antigens (Tawia, 2015; Turfkruyer & Verhasselt, 2015; Udall et al., 1981). Paediatrics associations widely recommend breast-feeding as the best practice for prevention of allergy (Friedman & Zeiger, 2005).

1.2. Cows' milk protein allergenicity

Food allergy is a serious global health concern that affects 4–6% of children and 1–3% of adults. The number of incidents of food allergies has been rising at an alarming rate in the last two decades (Rahaman, Vasiljevic, & Ramchandran, 2016). The prevalence of reported food allergy among children has increased by 18% from 1997 to 2007 in the USA (Branum & Lukacs, 2008). While more than 160 different foods have been reported to be allergic in the medical literature, bovine or cows' milk is one of so called “Big-8” food allergens (hens' eggs, peanuts, tree nuts, fish, crustacean shellfish, soybean and wheat) that cause about 90% of the occurrences of food allergies (Crittenden & Bennett, 2005; Downs, Kabourek, Baumert, & Taylor, 2013). Bovine milk protein allergy, or the more widely accepted term – cows' milk protein allergy (CMPA), is clinically an abnormal immunological reaction to one or more milk proteins (El-Agamy, 2007) that is distinct from intolerances (e.g., lactose intolerance) (Downs et al., 2013). CMPA is the most prevalent food allergy among infants (2–6%) and is rarely found in adults (0.1–0.5%), since tolerance develops with age in most cases (Host & Halken, 2014; Kim et al., 2011). Immunoglobulin E-(IgE-) mediated allergy is a classic immediate allergic reaction, and is comprised of two main phases, called sensitisation and reaction. When an individual is exposed to an offending food, the immune system produces allergen-specific IgE antibodies that attach to the surface of mast cells during the sensitisation phase. On subsequent exposure to the same allergens, IgE cross-linking occurs on the surface of mast cells, resulting in releasing of inflammatory mediators (e.g., histamine and leukotrienes). These mediators cause allergic reactions and manifest symptoms in various parts of the body such as oral allergy syndrome, urticaria, asthma, rhinitis, and generalised symptoms like anaphylactic shock (El-Agamy, 2007; Kaminogawa & Totsuka, 2003). From a clinical point of view, the complete elimination of milk consumption is the only reliable solution currently available to avoid the risk of milk allergy. However, elimination of bovine milk proteins from infants' and children's diets in particular may cause health issues due to malnutrition and poor growth. The wide application of milk proteins as food ingredients also makes complete elimination of these proteins practically difficult. Even though numerous formulae with hydrolysed proteins are recommended as substitutes for infants with cows' milk allergy, certain nutritional issues with such products, such as poor growth and developments of formula fed infants, remain unresolved (Clemente, 2000; Crittenden & Bennett, 2005; Hernell & Lönnerdal, 2003; Sathe, Teuber, & Roux, 2005).

1.3. The impact of thermal processing of bovine milk on development of allergies

A number of epidemiological studies have reported that consumption of raw (unheated) bovine milk may contribute to development of protection against asthma and allergies (Loss et al., 2011; MacDonald et al., 2011; Sozanska, Pearce, Dudek, & Cullinan, 2013). It can thus be reasonably hypothesised that raw or minimally processed bovine milk could also play a similar protective role against the development of allergy that human milk provides, as discussed above, regardless of compositional variations in these types of milk. The various immunomodulatory components present in breast milk, such as Ig, oligosaccharides, and saturated fatty acids, are believed to contribute to developing protection against allergy (Friedman & Zeiger, 2005; Iyengar & Walker, 2012).

In comparison, bovine whey proteins, including Ig, are capable of modulating the immune system in humans in different ways (Madureira, Pereira, Gomes, Pintado, & Malcata, 2007). Application of bovine Ig as a nutraceutical or functional food has been successful for alleviation of a variety of diseases, including rotavirus-induced diarrhoea in infants, chronic diarrhoea in HIV patients, and human dental caries (Bagwe, Tharappel, Kaur, & Buttar, 2015; Hurley & Theil, 2011; Korhonen, Marnila, & Gill, 2000). Though the most abundant Ig types in human and bovine milk are IgA and IgG, respectively, IgG from bovine milk is able to influence immunomodulatory functions in the human body effectively (Gapper, Copestake, Otter, & Indyk, 2007; Hurley & Theil, 2011). Hence, bovine whey proteins, including Ig, may perform an immunomodulatory role against the same milk protein allergens or other potent allergens. Though this mechanism can be hindered by thermal processing either by inactivating or altering immunomodulatory properties of milk (Loss et al., 2011; van Neerven, Knol, Heck, & Savelkoul, 2012), consumption of raw unprocessed bovine milk cannot be recommended, as it may contain pathogens presenting serious human health risks (Claeys et al., 2013). Some historical milestones, such as the commencement of bovine milk consumption by humans (8000 BC), industrial application of pasteurisation (1890) (Holsinger, Rajkowski, & Stabel, 1997), and reported medical evidence of CMPA (1930) (Host & Halken, 2014) may also suggest that thermal processing could be associated with increased prevalence of CMPA in conjunction with other dynamic environmental factors.

It is also well established that heat treatments applied in milk processing can potentially alter protein structures and subsequently modify milk protein immunogenicity, depending on temperature, processing time, native properties of the protein and the properties of the physicochemical environment, such as pH. Such altered immunogenicity could potentially influence the expressed antigenicity and allergenicity differently, such as increasing, decreasing or remaining unchanged (Rahaman et al., 2016; Shriver & Yang, 2011; Verhoeckx et al., 2015; Wal, 2003). Thermal processing has, however, become an essential step in milk processing and milk is mostly subjected to at least one step of heat treatment during processing, regardless of the end product (Holsinger et al.,

1997). Furthermore, pasteurisation has been established as the minimum standard for raw milk processing in the vast majority of countries to improve safety, extend the shelf-life and improve the quality by reducing the pathogen load and microbial and enzymatic activities prior to human consumption (Lewis & Deeth, 2009). In addition, thermal processing methods are also applied to alter the texture, flavour and digestibility of dairy products (Davis & Williams, 1998; Wal, 2003).

Thus far, many review articles have discussed the potential use of various thermal processing methods to modify the milk protein structures purposefully (e.g., denaturation and aggregation) and subsequently achieve a reduced risk of milk allergenicity. This approach is mainly based on the application of heat treatments as a tool to reduce the CMPA, which has not yet been successful (Bu, Luo, Chen, Liu, & Zhu, 2013; Davis & Williams, 1998; Lee, 1992; Sathe & Sharma, 2009; Verhoeckx et al., 2015). It is known that heat treatments applied in milk processing (e.g., pasteurisation) are severe enough to alter the heat-labile immunomodulatory factors (e.g., Ig) (Kummer et al., 1992), but they cannot completely eliminate the immunoreactivity of heat-stable milk protein structures (e.g., linear epitopes of whey proteins and caseins) (Bu et al., 2013), which could potentially progress the protein sensitisation response towards allergenicity, especially in an atopic subject. The present article therefore reviews both structural changes in proteins and alteration of immunomodulatory properties induced by various heat treatments, leading to modified immunogenicity of bovine milk, and its consequences for milk protein allergenicity. This could support consideration of the potential use of appropriate processing methods that would ensure the preservation of native immunomodulatory properties of bovine milk, as a means of reducing the risk of milk protein allergenicity.

2. Allergenic properties of bovine milk proteins

Typical bovine milk contains 3.0–3.5% (w/w) proteins, which are divided into two main categories based on the solubility at pH 4.6. Caseins, the insoluble fraction under such conditions, represent approximately 80% of the total proteins while whey proteins, the soluble fraction, account for the rest of 20%. Both of these fractions are regarded as high-quality proteins in terms of human amino acid requirements, digestibility, and bioavailability. Furthermore, milk proteins and their peptides perform various biological functions that could contribute to protective mechanisms in human health, including antibacterial, antiviral, antioxidant, antihypertensive, and immunomodulatory roles (Pereira, 2014).

Less favourably, many bovine milk proteins are found to be capable of inducing immunological responses, being either antigens or allergens, or frequently both. Allergens are recognised and bound by IgE antibodies, causing allergenicity in atopic individuals, while antigens bind with IgG antibodies, inducing antigenicity (Verhoeckx et al., 2015; Wal, 2002). Although about 20 different milk proteins can potentially be involved in the allergic sensitisation, their degree of involvement differs from one protein fraction to another (Restani, Ballabio, Di Lorenzo, Tripodi, & Fiocchi, 2009). The most abundant protein fractions in bovine milk, such as caseins, β-lactoglobulin (BLG) and α-lactalbumin (ALA), are considered the major allergens. However, bovine serum albumin (BSA), lactoferrin (LF) and immunoglobulins (Igs) also contribute to milk hypersensitivity, although they are present in minor quantities (Monaci, Tregoat, van Hengel, & Anklam, 2006), mostly leading to polysensitisation (Wal, 2004). BLG is usually considered to be the most allergenic protein, probably due to its attributes such as resistance to digestion, stability at low pH, and being a foreign substance to human infants as it is not present in human milk (Kaminogawa & Totsuka, 2003). In contrast, caseins have been identified as the main allergenic and antigenic milk protein by some other investigations (Docena, Fernandez, Chirdo, & Fossati, 1996). Furthermore, α_{S1}-casein, the most abundant casein fraction, showed the highest allergenicity compared with the other caseins (Bernard, Créminon, Yvon, & Wal, 1998). These contradictory results and associated methodologies thus explain the fact that identification of principal protein allergens depends on the method applied and individuals tested (Kaminogawa & Totsuka, 2003). Table 1 summarises the allergenic properties and biological roles of the main milk proteins.

3. Structures of epitopes and heat-induced modifications

Epitopes (antigen determinants) are the portions of immunogenic molecules, mainly proteins, which can bind with a complementary site (paratope) of an antibody. The presence of epitopes in a protein structure renders the protein antigenic and/or allergenic (Wal, 2002). There are two main types of epitopes, which differ in their structure. Continuous or linear epitopes consist of a continuous sequence of amino acid residues based on the primary structure of the protein, while discontinuous or conformational epitopes constitute a discontinuous sequence of amino acid residues that are brought together by folding of the protein due to its native secondary or tertiary structure (Konstantinou & Kim, 2012). Fig. 1 illustrates the basic structures of main two types of epitopes. A protein may contain different or similar kinds of epitopes in several locations in its structure, but only a few are immunodominant, being easily recognised by the immune system (Restani et al., 2004). The structure of an epitope and the physicochemical properties of the constituent amino acids determine the antigenic specificity of that epitope (Konstantinou & Kim, 2012).

Different heat treatments can modify milk protein structure in various ways, which can directly impact on the immunogenicity of the protein through alterations of immunoreactive epitopes present in the protein structure (Verhoeckx et al., 2015). Heat-induced protein structural modifications mostly cause changes in the epitopes such as fragmentation, modification, masking, and unmasking. These changes, occurring in the epitopes, can result in either decreased, increased, or unchanged antigenicity and/or allergenicity of a particular protein (Konstantinou & Kim, 2012). Furthermore, new epitopes (neotopes) can also be formed through these changes in some instances (Davis & Williams, 1998). Generally, conformational epitopes are more susceptible to heat-induced modifications such as denaturation and aggregation than linear epitopes, because of the nature of their structures (Konstantinou & Kim, 2012). However, extreme processing conditions (e.g., Maillard reactions) could modify linear epitopes (Rahaman et al., 2016).

4. Modifications of milk protein structures and associated epitopes and immunogenicity by heat

An immune electrophoretic study found that antigenicity of casein remained unchanged when milk was heated at 120 °C for 15 min (Hanson & Mansson, 1959). Reactivity of IgE to casein was observed after heating milk at 95 °C for 60 min (Bloom et al., 2014). When adults allergic to bovine milk were sensitised to boiled and fresh caseins separately, they showed similar reactions to both forms of caseins through a skin prick test (Norgaard et al., 1996). These findings can be attributed to heat stability and also epitope arrangements of the caseins. Caseins are generally heat-stable due to a unique micellar structure and can be subjected to processes such as heating, freezing, and drying and remain unchanged after reconstitution (Fox & Morrissey, 1977). For instance, caseins did not coagulate after heating at 100 °C for 24 h or at 140 °C for up to 20–25 min (Dissanayake, 2011). Studies have suggested that

Table 1
Allergenic properties and biological functions of the main milk proteins.[a]

Protein	Allergen name	Concentration (g L^{-1})	Percentage of total proteins	Molecular mass (kDa)	Number of amino acids	Biological functions
Caseins						
α_{S1}-Casein	*Bos d 8* α_{S1}	12–15	29	23.6	199	Mineral transport (Ca, PO_4, Fe, Zn, Cu)
α_{S2}-Casein	*Bos d 8* α_{S2}	3–4	8	25.2	207	
β-Casein	*Bos d 8* β	9–11	27	24.0	209	
κ-Casein	*Bos d 8* κ	3–4	10	19.0	169	
Whey proteins						
β-Lactoglobulin	*Bos d 5*	3–4	10	18.3	162	Retinol carrier, fatty acid binding, antioxidant, passive immunity
α-Lactalbumin	*Bos d 4*	1–1.5	5	14.2	123	Immunomodulator, anticarcinogen, Ca transport
Immunoglobulins	*Bos d 6*	0.6–1.0	3	150.0	–	Immunomodulator, passive immunity
Bovine serum albumin	*Bos d 7*	0.1–0.4	1	66.3	582	Immunomodulator, anticarcinogen
Lactoferrin	*Bos d Lactoferrin*	0.09	Traces	80.0	689	Antimicrobial, antioxidant, immunomodulator, iron absorption

[a] Adapted from Pereira (2014) and Restani et al. (2009).

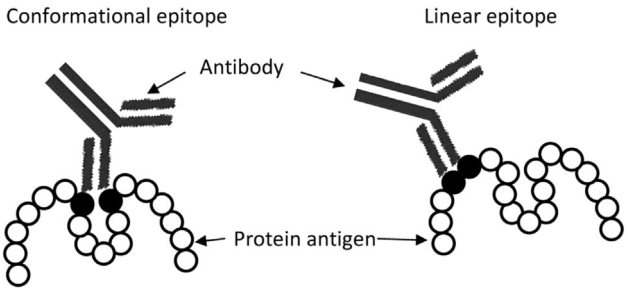

Fig. 1. Epitopes and their basic structures.

caseins contain many linear epitopes and a few conformational epitopes (Spuergin, Mueller, Walter, Schiltz, & Forster, 1996). The presence of numerous linear epitopes and the lack of conformational epitopes may explain the ability of caseins to retain their antigenicity even after severe heat treatment (Mills, Sancho, Rigby, Jenkins, & Mackie, 2009). These characteristics of caseins explain well why hypoallergenic dairy products (containing caseins) could not be produced only by thermal processing (Kilshaw, Heppell, & Ford, 1982; Lee, 1992). It has so far been reported that different casein fractions possess about 60 IgE-binding epitopes, 20, 17, 13 and 10 in α_{S1}-, α_{S2}-, β- and κ-casein, respectively (Matsuo, Yokooji, & Taogoshi, 2015). In terms of α_{S1}-casein, a study revealed that IgE-binding epitopes are structurally identical to IgG-binding epitopes (Spuergin et al., 1996).

In contrast to the caseins, the two main whey protein fractions, BLG and ALA, change their antigenicity in a similar manner when subjected to heating (Bu, Luo, Zheng, & Zheng, 2009b). Each protein increases antigenicity gradually with increasing temperature from 50 to 90 °C and then decreases rapidly between 90 and 120 °C (antigenicity was measured after cooling) (Bu et al., 2009b; Kleber, Krause, Illgner, & Hinrichs, 2004; Rahaman, Vasiljevic, & Ramchandran, 2015; Rahaman et al., 2016). Elevated antigenicity of BLG (from 50 °C to 90 °C) can be attributed to the exposure of epitopes buried in the native protein due to unfolding during the denaturation stage. Furthermore, –SH/S-S exchange reactions potentially promote conformational changes and causes exposure of more antigenic epitopes on the surface of the BLG structure at this stage. Decreasing antigenicity (between 90 and 120 °C) can be ascribed to further denaturation and aggregation of BLG, that could break up or mask the conformational epitopes, followed by S-S mediated aggregations (Bu et al., 2009b; Kleber et al., 2004; Kleber & Hinrichs, 2007;). Generally, a similar pattern of antigenic behaviour can also be observed in ALA with increasing temperature, despite its heat stability. However, the rate and degree of the change in antigenicity with temperature can vary. This could be ascribed to the fact that antigenicity of BLG is associated with both linear and conformational epitopes, whereas antigenicity of ALA is predominantly determined by conformational epitopes (Bu et al., 2009b).

Furthermore, in a protein mixture, BLG and ALA can form aggregates of high molecular mass at temperatures around 80–90 °C, which would result in diminished antigenicity due to masking of antigenic epitopes on the molecular surfaces (Bu et al., 2009b). Moreover, extended high temperature treatments together with presence of lactose could reduce antigenicity, which can be related to initiation of the Maillard reactions and subsequent conformational modifications of the structures of both BLG and ALA, which would obstruct antibodies from accessing antigenic epitopes (Bu, Lu, Zheng, & Luo, 2009a; Bu et al., 2009b; Taheri-Kafrani et al., 2009). In addition, the key findings of several studies conducted on effects of heating on immunogenicity/antigenicity of BLG and ALA are summarised in Table 2, while Fig. 2 elaborates on the common phenomena associated with structural modifications of BLG and ALA, and subsequent changes in antigenicity/allergenicity during heat treatment. The studies have mapped out the amino acid sequences of various IgE-binding epitopes of both BLG and ALA. In principle, each protein contains at around 10 IgE-binding epitopes (Matsuo et al., 2015). In addition, another investigation identified 6 and 3 of IgG-binding regions in BLG and ALA protein structures, respectively, using a peptide synthesis method (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001).

BSA is one of the allergenic minor whey proteins, for which heat-induced modifications of immunogenicity are not well known. It has been reported that BSA subjected to severe heat treatment (100 °C for 5 min) showed a minor allergic sensitisation compared with unheated BSA (Fiocchi et al., 1998). BSA fragmentation, due to reduction of disulphide bonds, prevented reactions with anti-BSA antibodies (Habeeb & Atassi, 1976). Since BSA is arranged as three homologous domains linked by 17 disulphide bridges, the presence of disulphide bonds predominantly determines antigenic epitopes of the native BSA (Monaci et al., 2006; Restani et al., 2004). For this reason, it can be assumed that the decrease in allergenicity is due to break up and/or masking of epitopes followed by cleavage of disulphide bonds and subsequent aggregation. However, the conflicting findings suggest that antigenicity of BSA is only partially associated with its native three-dimensional structure, as the IgE-binding ability of BSA was not affected by either heat treatment or chemical denaturation (sodium dodecylsulphate treatment) (Restani et al., 1998). It appears that

Table 2
Summary of findings on heat-induced changes in antigenicity/immunogenicity of β-lactoglobulin (BLG) and α-lactalbumin (ALA).

Sample type	Treatment	Impact	Reference
BLG as a pure solution and in whole milk	At different temperatures and time periods	Slight decrease in IgE-binding capacity after heating at 74 °C for 15–90 min and significant decrease at 90 °C for 15–90 min.	Ehn, Ekstrand, Bengtsson, and Ahlstedt (2004)
BLG in skim milk and sweet whey	Temperature range from 50 to 150 °C and holding times up to 15 min	Antigenicity increased with increasing temperature. Maximum antigenicity recorded at 80–90 °C, which decreased above 90 °C.	Kleber and Hinrichs (2007)
BLG in an aqueous solution	90 °C for 10 min	New epitopes were found after heating which were not in native BLG.	Davis and Williams (1998)
BLG in citric acid-phosphate buffer	Different pH (3, 5 and 7), temperatures (80, 100 and 120 °C) and shear rates (100, 500, and 1000 s^{-1})	The minimum antigenicity was reported following treatment at 120 °C, pH 5 and 100 −1000 s^{-1}	Rahaman et al. (2015)
BLG	Glycation of BLG with lactose (Maillard reaction) at 65–95 °C for 20 min	High degree of glycation showed significant effect on masking IgE-binding epitopes. Secondary structure of BLG altered and IgE-binding capacity changed between 65 and 75 °C.	Taheri-Kafrani et al. (2009)
ALA in whey protein isolate (WPI)	Conjugation of WPI with glucose at incubation temperature 40–60 °C and time 24–120 h	Minimum antigenicity recorded at 52.8 °C, 78 h and 5.96:1 WPI/glucose weight ratio affected by Maillard reaction	Bu et al. (2009a)
BLG and ALA in WPI	Conjugation of WPI with maltose in different weight ratios and temperature and time combinations	BLG and ALA reduced antigenicity in conjugation with maltose. Temperature affected antigenicity of ALA, while WPI: maltose weight ratio was the main influential factor for BLG.	Li, Luo, and Feng (2011)
BLG and ALA in milk	At different temperatures (60–90 °C) and time (15 s, 60 min) combinations	Immunodetectable levels decreased with severity of treatment. Significant drop in immunoreactivity of ALA and BLG seen at 90 °C for 15 min compared with raw milk	Jedrychowski (1999)
BLG (A and B variants) and ALA in phosphate buffered saline	At different temperatures (30–140 °C) for 10 min	Decreased ALA immunoreactivity at temperatures above 90 °C; both BLG variants showed increased immunoreactivity up to temperature of 80–90 °C and decreased afterwards with increasing temperature.	Karamonova et al. (2003)

BSA contains at least one linear epitope and many conformational epitopes, but these have not been identified yet due to the inconsistency of conditions applied in different studies (Monaci et al., 2006; Restani et al., 2004).

Ig and LF are immunologically important minor whey proteins present in bovine milk (Monaci et al., 2006). However, scientific findings on the impact of heat-induced protein structural modifications on immunogenicity of Ig and LF are less frequently reported. This could probably be due to lower research priority, as they are considered minor allergens, and patients allergic to Ig and LF are also allergic to other major proteins. Furthermore, as bioactive proteins, Ig and LF are mainly assessed in relation to loss of their bioactivity upon exposure to heat treatment, although the heat-induced modification of immunogenicity is also considerably important in terms of CMPA. In addition, it appears that IgE/IgG-binding epitopes of both of these proteins have not been yet fully identified (Chen, Tu, & Chang, 2000; Matsuo et al., 2015; Steijns & Van Hooijdonk, 2000).

Moreover, other covalent modifications of proteins caused by heat treatments, such as reactions with oxidised lipids, disulphide bond scrambling, and deamination of asparagine can also contribute to alteration of antigenicity (Davis, Smales, & James, 2001; Monaci et al., 2006). Besides the effect of proteins, some other milk constituents can also modify the immunogenicity of milk products subjected to heat treatments. For instance, the end products produced through the Maillard reaction, commonly called advanced glycation end products (AGE), lead to generation of AGE-modified protein derivatives containing major immunological epitopes that can cause allergy (Davis et al., 2001; Wal, 2003).

It is thus evident that exposure of milk proteins to different heat treatments can alter their immunogenicity in various ways, while these alterations are interdependent. More comprehensive investigations are required to identify the precise effects of heat treatments on modifications of milk protein antigenicity and expressed allergenicity (Bu et al., 2013; Monaci et al., 2006; Verhoeckx et al., 2015). However, the existing literature can reasonably show that traditional pasteurisation temperatures (70–80 °C) could increase the overall antigenicity of bovine milk by enhancing the antigenicity of major whey proteins such as BLG and ALA.

5. Immunomodulatory properties of milk proteins affected by heat and their consequences for milk protein allergenicity

A systematic review and meta-analysis conducted by MacDonald et al. (2011) found that a few epidemiological studies have established the inverse association between raw bovine milk consumption and development of allergy, which can also be supported by farming-related factors. Another study, however, concluded that this inverse association is independent of the place of residence and farming status (Sozanska et al., 2013). Accordingly, it can be anticipated that consumption of raw bovine milk contributes to development of an immunological phenotype that establishes the protective effect or tolerance in the human body against allergy and asthma (Loss et al., 2011; van Neerven et al., 2012). The GABRIELA study (Loss et al., 2011) examined this protective effect in relation to childhood asthma and atopy, and further looked into the influential milk constituents that potentially contribute to this mechanism. This study found that consumption of raw bovine milk (not heated to 72 °C) was consistently inversely correlated with asthma and food allergen sensitisation, but not with atopy. Early exposure and daily consumption of raw milk complement this effect. Furthermore, variables such as total fat content, total viable bacteria count, total protein content, somatic cell count, and lactose level had no

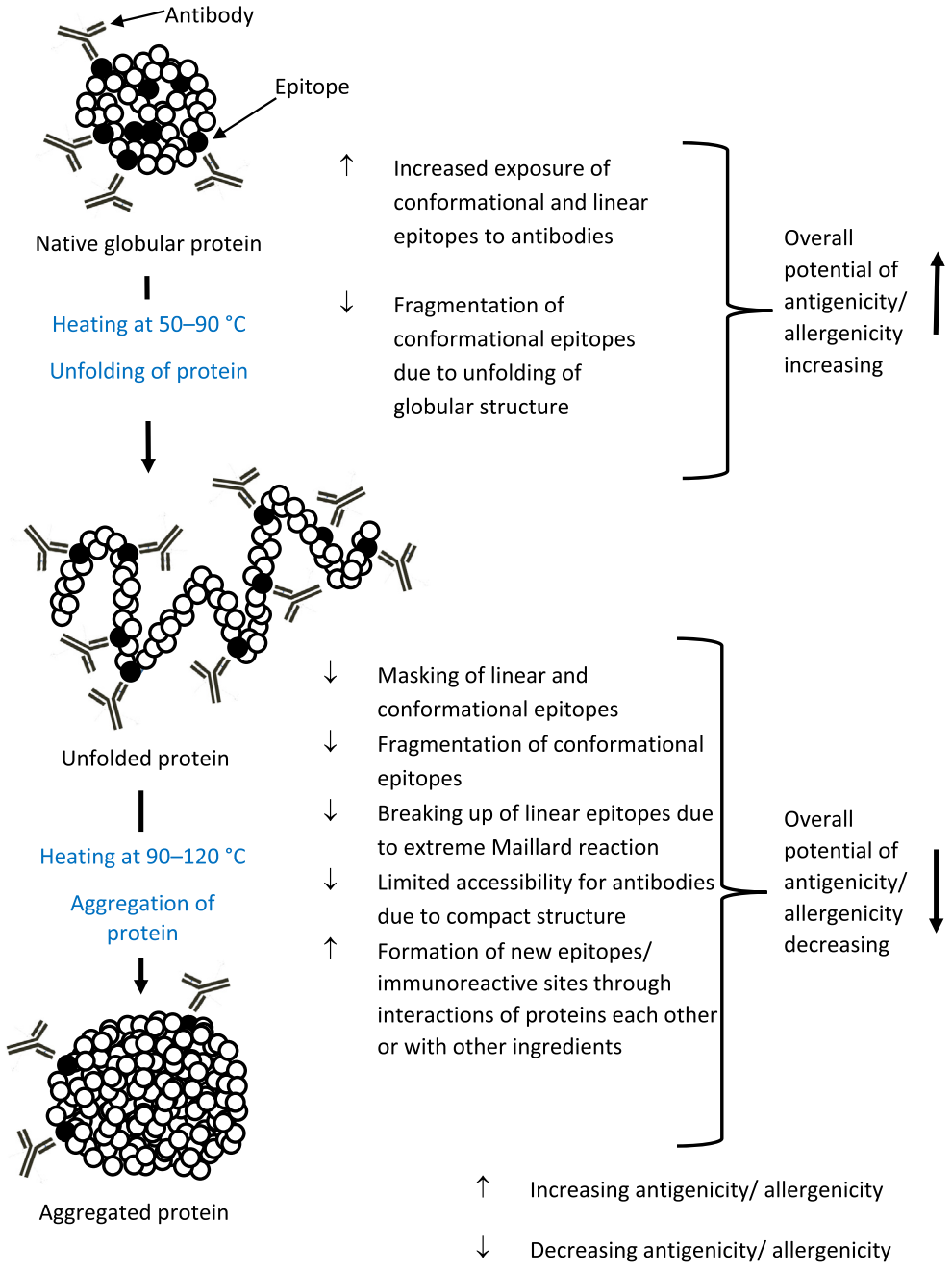

Fig. 2. Schematic model displaying potential structural modifications and epitope changes of major native whey proteins (e.g., BLG and ALA) during heat treatment and their overall impact on the antigenicity/allergenicity.

apparent impact on asthma and allergy, but an increased level of whey proteins was found to be the main contributing factor towards positive health outcomes. Moreover, ALA, BLG, and a high level of BSA showed a statistically significant inverse correlation with asthma and allergy while, Ig and LF showed no significant correlation (Loss et al., 2011). These findings can be ascribed to the ability of bovine milk whey proteins to modulate the human immune system (Table 1), although the underlining mechanisms still remain unclear.

van Neerven et al. (2012) proposed a model to elaborate a relationship between consumption of raw bovine milk and development of protection against allergy in infants. This model hypothesises that most abundant milk proteins, such as caseins, BLG and ALA, could act as the basic protein sources for neonates, while the minor proteins, such as Ig, LF, and cytokines perform immunomodulatory functions against allergy. Though the presence of native proteins in bovine milk correlates with this protective mechanism, commercial milk processing, especially heating, causes denaturation of some of these proteins to varying degrees, which in turn suppresses this mechanism. The GABRIELA study concluded that consumption of heated bovine milk ($\geq$72 °C) had no inverse association with allergy (Loss et al., 2011). An immunological study also found that the immunomodulatory potential of raw milk can significantly be affected by the pasteurisation process, and that raw milk consumption could influence the alteration of allergic sensitisation (McCarthy, Ross, Fitzgerald, & Stanton, 2015). It has also

been reported that the concentration and activity of immunomodulatory and host defence proteins in human milk can be severely affected by pasteurisation (Akinbi et al., 2010; McPherson & Wagner, 2001).

Milk protein sensitisation generally begins with internalising and processing of protein antigens (Ag) by antigen-presenting cells (APCs) (Fig. 3). Upon stimulation by APCs, Th0 (T helper) cells, that are in naive status, can differentiate into two pathways. The pathway towards Th1 phenotype is stimulated by interleukin (IL) 12, with Th1 cells producing characteristic cytokines including IL-2, IL-15 and interferon gamma (IFNγ). The pathway towards the Th2 phenotype is stimulated by IL-4, followed by release of characteristic cytokines, including IL-4, IL-5, IL-10, and IL-13. Most importantly, Th1 and Th2 responses are mutually inhibitory. For example, IL-4 and IL-10 inhibit Th1 responses, while IL-12 and IFNγ inhibit Th2 responses. This cross-regulation can generally result in a mixed phenotype, but could be biased towards one of the pathways in an individual, depending on genetic background, exposure to antigens, and causative environmental factors (Elenkov & Chrousos, 1999; Knopf, 2000; Safri, Lubis, Munasir, & Putra, 2015). In addition to Th1/Th2 cross-regulation, other T cell subsets, such as Treg, Th17 and Th22, also contribute to regulation of the Th1/Th2 pattern (Kiewiet, Gros, van Neerven, Faas, & de Vos, 2015). In terms of immediate milk protein hypersensitivity, the Th2 pathway becomes dominant and IL-4 and IL-13 induce IgE production by plasma cells, which leads to IgE-mediated allergy. In the meantime, IL-5 secreted by Th2 may result in the non-IgE-mediated/cell-mediated allergy due to accumulation and activation of eosinophils (Knopf, 2000; van Neerven et al., 2012).

The tolerance to allergens in a healthy individual is established through the balance between Th cell differentiation and activation of local regulatory network (Fig. 4). The Th2- skewed immune response (Th1 < Th2) is usually modulated by the activation of T cells (e.g., Treg and Th17) and release of associated cytokines (IL-12, IFNγ > IL-10, IL-4), which prevent the initiation of allergic reaction. In an atopic subject, Th0 differentiation skews towards Th2 more than Th1, and the regulatory mechanism is thus hindered, causing allergic reactions (Tsitoura & Tassios, 2006).

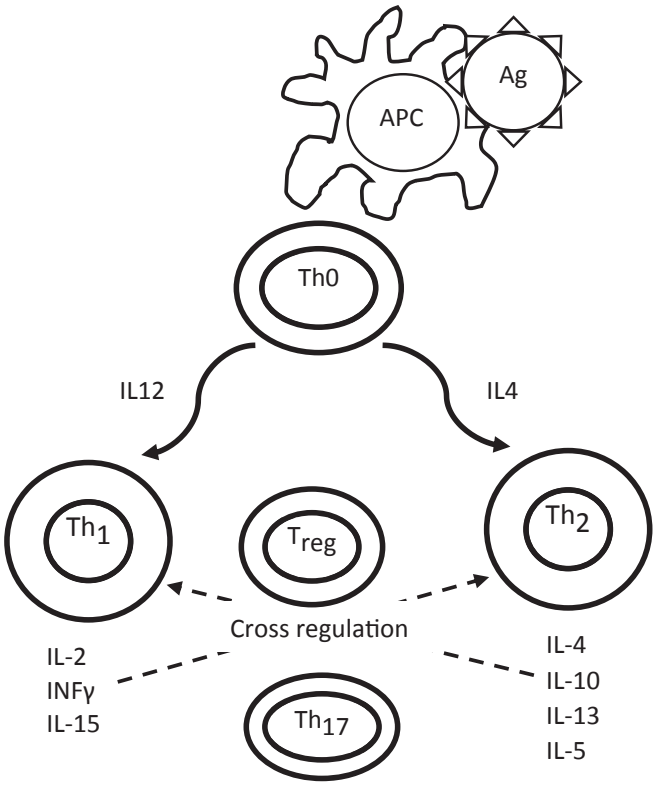

Fig. 3. A cascade of immunological reactions induced by protein antigens.

It has been shown that exposure to raw bovine milk stimulates the immune system in children by elevating the number of Treg cells and could potentially contribute to development of a protective effect against childhood allergic diseases (Lluis et al., 2014). The impact of farm living on allergy development could be attributed to the ability of dendritic cells, major APCs localised at the interface with environment, to produce signals that can differentiate Th0 cells into Th1 and Th2 apart from the signals induced by antigens (von Bubnoff, Geiger, & Bieber, 2001). In contrast, another study suggested that two different phenotypes can be differentiated among children who suffer from IgE-mediated milk allergy. Type I individuals would eventually be able to eliminate Th2 responsiveness and allergic reactions, resulting in a transient milk allergy, whereas type II subjects would not be able to down-regulate Th2 and become persistent allergic patients (Nowak-Wegrzyn et al., 2008).

In addition, a variety of studies have already found various immunomodulatory properties of bovine milk in relation to milk allergenicity and showed how the different thermal processing conditions could alter them. The allergen-specific IgG or IgA in bovine milk can bind to particular allergens and form immune complexes in humans with the contribution of other milk constituents (e.g., vitamins, cytokines, etc.), which can create a regulatory environment, in which IgA and Treg cells are preferentially induced but not IgE (van Neerven et al., 2012). However, bovine IgG begins to lose its immunoreactivity at temperatures ≥72 °C (Li, Bomser, & Zhang, 2005; Li-Chan, Kummer, Losso, Kitts, & Nakai, 1995) with only about 65–79% of active IgG being present in HTST (high-temperature short-time) pasteurised milk compared with that in raw milk and no IgG activity present in either ultra-high temperature (UHT) or evaporated milk (Kummer et al., 1992). Another investigation revealed that immediate skin hypersensitivity of guinea pigs was suppressed by skim milk injected into their skin; this may be related to control of the local histamine secretion by sensitised mast cells due to the influence of LF and κ-casein (Cross & Gill, 2000). However, LF is found to be completely denatured by UHT treatment (135 °C/4 s) whereas pasteurisation (72 °C/15 s) causes a relatively less severe impact (Paulsson, Svensson, Kishore, & Naidu, 1993). A skin prick test (SPT) conducted with children allergic to bovine milk by using raw, pasteurised (75 °C/15 s), and homogenised and pasteurised milk samples showed that both processed milk types gained enhanced potential of provoking detrimental immunological reactions compared with the raw milk (Host & Samuelsson, 1988). Consistent results were generated by another study when rats were immunised intraperitoneally with different bovine milk samples. Significant levels of serum IgG antibodies were detected by ELISA against caseins and whey proteins in rats immunised with pasteurised (74 °C/15 s) and homogenised milk compared with the raw whole or skim milk (Feng & Collins, 1999).

It is also known that the stability to digestion and interaction with intestinal environment can contribute to the allergenic potential of a specific protein (Heyman, 1999). A study conducted with an animal model found that the absorption of ALA and BLG mainly occurs through enterocytes, while caseins are mostly taken up through Peyer's patches. Pasteurisation (at 72 °C for 2 min) of milk proteins caused aggregation of BLG and ALA, which in turn may inhibit uptake of ALA and BLG through enterocytes and redirecting them via Peyer's patches instead. This process significantly promotes Th2 pathway in mice and produces high levels of associated

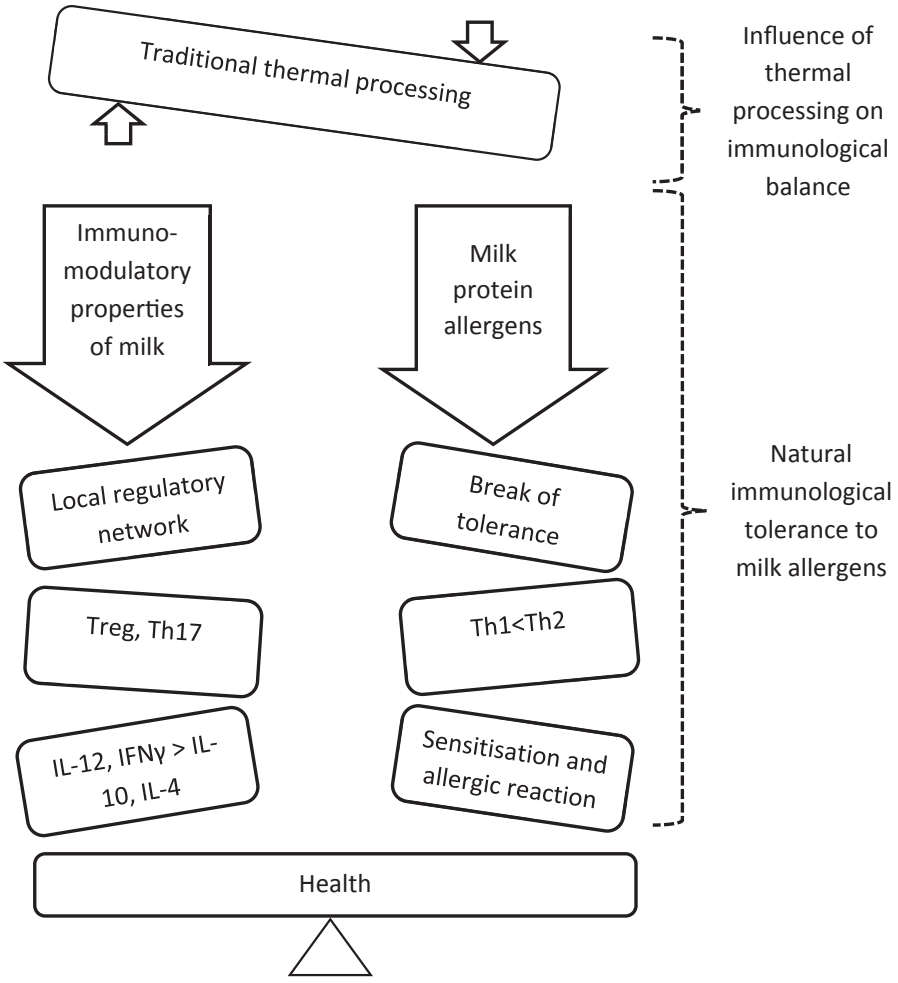

Fig. 4. Mechanism of natural immunological tolerance to allergens.

antibodies and cytokines, compared with native ALA and BLG (Roth-Walter et al., 2008).

Although controlled human studies that assess allergenicity of heated bovine milk in comparison with raw or unheated milk have not been reported yet (Verhoeckx et al., 2015; van Neerven et al., 2012), the findings discussed above potentially demonstrate that traditional heat processing modulates immunomodulatory properties of native milk proteins and interferes with the natural immunological balance, either by inducing Th1 < Th2, or suppressing the modulatory effect of local regulatory network, or both (Fig. 4). This in turn directs the immune response towards overexpression of the phenotype responsible for manifestation of allergies and asthma, which would otherwise be suppressed if these immunomodulatory compounds were retained, for instance by activating cross-regulation and suppressing Th2 functions over Th1 through the mediation of associated cytokines and/or Treg and Th17 cells (Fig. 3).

6. Conclusions

Thermal processing leads to alteration of milk immunogenicity predominantly in two ways: modification of milk protein structures and associated epitopes, and alteration or inactivation of immunomodulatory properties of milk proteins and other milk constituents. The standard pasteurisation conditions (72 °C for 15 s) widely adopted in the dairy industry falls under the temperature limit (<90 °C) that enhances immunogenic potential of major whey proteins in the form of antigenicity and/or allergenicity (e.g., BLG and ALA), and pasteurisation is also performed at a sufficient temperature (≥72 °C) to suppress the immunomodulatory properties of milk, which could potentially develop tolerance against allergy and asthma. Although raw bovine milk contributes to development of this tolerance, consumption of raw milk cannot be recommended as an approach to control CMPA, due to health risks associated with human pathogens. For this reason, an appropriate milk processing method should fulfil two major requirements: inactivation of human pathogens to provide for required level of safety, and preservation of native immunomodulatory properties of milk to maintain its healthy and hypoallergenic status. In this context, traditional milk processing methods, such as HTST pasteurisation and UHT processing, would be challenged and some potential processing techniques include the application of mild heat treatments or equivalent novel processing methods that can meet aforementioned requirements including industrial feasibility.

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Chapter 2B: Supplementary literature review

This chapter provides supplementary literature relating to the study and highlights the fundamental concepts and key research findings.

2B.1 Bovine milk and milk proteins

2B.1.1 Bovine milk

Bovine or cow's milk is a complex biological fluid secreted by the lactating cow, which primarily fulfils the complete nutritional requirements of the calf and also performs various physiological functions, importantly protective roles, mostly by proteins and peptides (Fox, 2003; O'Mahony & Fox, 2014). Milk is mainly composed of water (~ 87%), lactose (~ 4-5%), proteins (~ 3%), fat (~ 3-4%), minerals (~ 0.8%), and vitamins (~ 0.1%) (Pereira, 2014; Walstra, Wouters, & Geurts, 2005). Currently, bovine milk and dairy products become one of the main components of the human diet throughout the world (Fox, 2003; O'Connell & Fox, 2003). The bovine milk proteins can be divided into two main fractions based on their solubility at pH 4.6. The caseins, which are insoluble at pH 4.6, represent around 80% of the total proteins. Whey proteins, the soluble fraction, present the remaining portion of milk proteins (20%). Both fractions contain several individual proteins possessing different physicochemical characteristics (Downs, Kabourek, Baumert, & Taylor, 2013; Huppertz, Fox, de Kruif, & Kelly, 2006; Pereira, 2014).

2B.1.2 Milk proteins

2B.1.2.1 Caseins

Caseins, the major milk protein fraction in bovine milk, are phosphoproteins and can be subdivided into four principal proteins based on the homology of their primary structures. They are amphiphilic nature of monomers known as α_{s1}- (~23.6 kDa), α_{s2}- (~25.2 kDa), β- (~24.0 kDa) and κ-casein (~19.0 kDa) and available in approximate amount of 10, 2.6, 9.3 and 3.3 g/L, respectively. All four types of caseins with the participation of calcium phosphate form aggregates of individual protein molecules naturally in milk, having average diameter of about 150-200 nm, generally termed as casein micelles. The casein micelles are highly hydrated with

around 3.5 g of water per g of caseins. In the micellar structure, almost all κ-casein is present on the surface, β-casein is mostly found in the interior and α_s-caseins exist throughout the structure. The macro-peptide portion (residues 106-169) of κ-casein molecules extend from the micellar surface to form a hairy layer (5-10 nm thick but not so dense) around the micelle, which provides stearic stabilization to the micelle and prevent approaching of micelles each other closely. Furthermore, it avoids movement of individual protein molecules through micellar surface in native caseins (Dalgleish & Corredig, 2012; Fox & Brodkorb, 2008; Horne, 2014; McMahon & Oommen, 2013). Although primary structures of all four types of caseins have already been determined, their full secondary and tertiary structures remain to be revealed (Huppertz, 2013).

2B.1.2.2 Whey proteins

Whey proteins or milk serum proteins are globular proteins with well-defined secondary and tertiary structures (Anema, 2014). Whey proteins possess relatively uniform distribution of hydrophobic and hydrophilic amino acids throughout their primary structure and absence of amphiphilic nature of monomer subunits in comparison to the caseins (Mulvihill & Donovan, 1987). The globular conformation of whey proteins including large volume of helical contents appears to be stabilized by lower content of proline in protein molecules (Hambling, McAlpine, & Sawyer, 1992; O'Mahony & Fox, 2014; Sawyer, 2003). The whey proteins consist of several principal proteins including β-lactoglobulin (BLG; ~ 3.2 g/L), α-lactalbumin (ALA; ~ 1.2 g/L), immunoglobulins (Ig; ~ 0.7 g/L), bovine serum albumin (BSA; ~ 0.4 g/L), and bovine lactoferrin (LF; ~ 0.1 g/L) (Dupont, Croguennec, Brodkorb, & Kouaouci, 2013; Wijayanti, Bansal, & Deeth, 2014).

β-Lactoglobulin (BLG)

BLG is a typical globular protein, having hydrophilic amino acids arranged on the outside and hydrophobic amino acids on the inside of the three-dimensional shape. BLG is the most abundant whey protein present in the whey, approximately 50%. It is a polypeptide chain comprising of 162 amino acid residues and having molecular weight ~18.3 kDa. BLG may appear in either monomeric or dimeric form depending on the pH, ionic strength, and the temperature (Wijayanti et al., 2014; Wong, Camirand, Pavlath, Parris, & Friedman, 1996). The secondary structure of BLG has been characterized by several studies using different methods. For instance, the circular dichroism (CD) spectroscopy has demonstrated that the secondary structure of BLG is composed of 15% of α-helix, 50% of β-sheet and 15-20% of reverse turn (Creamer, Parry, & Malcolm, 1983), while fourier transform infrared (FTIR) spectroscopy determined it as 9-11% of α-helix, 51-55% of β-sheet, 20-27% of β-turn and 9-11% random coil (Dong et al., 1996).

The three-dimensional structure of monomeric BLG (fig. 2B.1) contains nine strands of antiparallel β-sheet, eight of which wrap around to form a β-barrel structure like a flattened cone. The other strand (ninth) extends outside and primarily forms the larger part of the dimer interface (Edwards, Creamer, & Jameson, 2014; Sawyer, 2013; Wong et al., 1996). The hydrophobic, ionic and hydrogen-bond interactions, between peptide chains, are mainly stabilized the structure of BLG monomer, as well two disulfide bridges located at Cys^{106}-Cys^{119} and Cys^{66}-Cys^{160}. BLG monomer also contains a free thiol group (Cys^{121}), which is buried at sheet-helix interface. (De Wit, 2009; Wong et al., 1996). Further, the native structure of BLG mostly depends on temperature, pH, and other environmental factors (Tolkach & Kulozik, 2007).

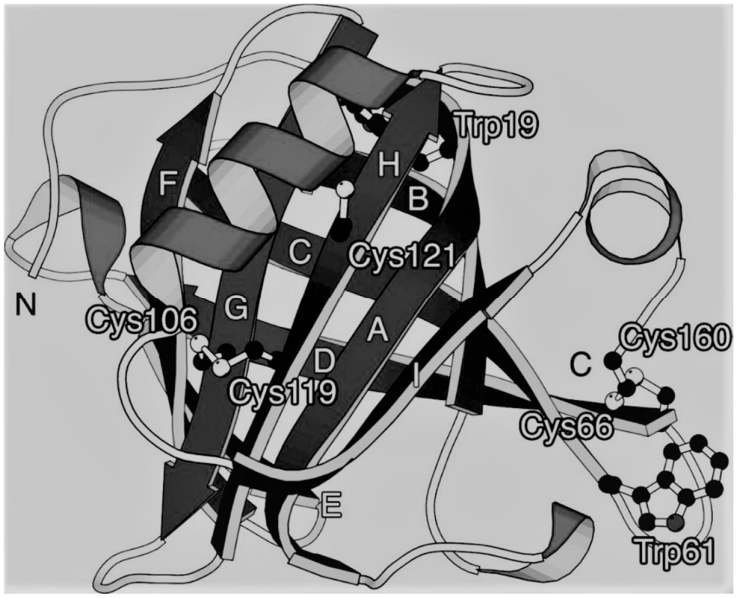

Fig. 2B.1 The crystal structure of bovine BLG (Adapted from Yagi, Sakurai, Kalidas, Batt, & Goto, 2003)

α-Lactalbumin (ALA)

ALA is the 2nd major whey protein present in cow's milk, which consists of 123 amino acid residues having the molecular weight about 14.2 kDa. The secondary structure of ALA has been characterized as 20% of α-helices, 14% of β-sheets, and 60% of unordered structures by CD spectroscopy, which was comparable with the secondary structure determined by X-ray crystallography (Patel, 2007; Robbins & Holmes, 1970). Native ALA occurs as monomeric, compact and globular protein and is basically made up of two lobes. The α-lobe comprises three α-helices and two short 3_{10}-helices, while β-lobe contains a small three-stranded β-sheet and a short 3_{10}-helix. The structure of ALA is mainly stabilized by four disulfide bonds, two of which are in α-lobe (Cys^{6}-Cys^{120} and Cys^{28}-Cys^{111}), one in the β-sheet (Cys^{60}-Cys^{77}), and the other (Cys^{73}-Cys^{90}) binds two lobes together. Besides, there is no free thiol groups in ALA structure (Brew, 2003; Edwards et al., 2014; Patel, 2007; Stanciuc & Râpeanu, 2010).

ALA binds with Ca^{2+} and turns into holo form (Ca^{2+}-loaded), which is more abundant form of ALA in milk than apo form (Ca^{2+}-depleted). It has been reported that the Ca^{2+} is

involved in native folding and disulfide bond formation of ALA structure, as well recovering of native conformation by preventing the unfolding of protein (Brew, Grobler, & Fox, 1992; Dissanayake, 2011). However, the basic structure of apo form is similar to that of holo form except the substantial movement of the Tyr^{103} side chain in the inter-lobe cleft and minor changes around Ca^{2+} binding site (Chrysina, Brew, & Acharya, 2000).

Immunoglobulins (Ig)

Ig are globular whey proteins and antibodies, which can be divided into three major classes namely IgG, IgM and IgA. IgG also contains two subclasses called IgG_1 and IgG_2. IgG_1 represents major part of the total Ig in bovine milk, approximately 80% (Gapper, Copestake, Otter, & Indyk, 2007). The basic structure of Ig molecule (fig. 2B.2) is composed of two identical light chains (~23 kDa) and two identical heavy chains (~53 kDa), which are connected together by disulfide bonds, with a total molecular weight about 160 kDa, regardless the type of Ig (Gapper et al., 2007; Hurley & Theil, 2011).

The number and location of disulfide bridges, which link heavy chains together, vary with a type of Ig. Two identical antigen binding sites are formed in every Ig molecule by the N-terminal part of one light chain and one heavy chain, which appear like Y-shaped molecule. IgG can be observed as monomers in both milk and blood. Structurally, monomeric IgM and IgA are similar to IgG except the presence of a C-terminal octapeptide in heavy chain. Although IgA occurs as monomers and dimers, later it forms a complex called secretory IgA (SIgA) by joining two IgA molecules together through a J-chain and a secretory component. IgM contains five subunits, which are bound together in a circular mode by disulfide bonds and a joining (J) chain (Gapper et al., 2007; Hurley & Theil, 2013; Hurley & Theil, 2011; Korhonen, Marnila, & Gill, 2000).

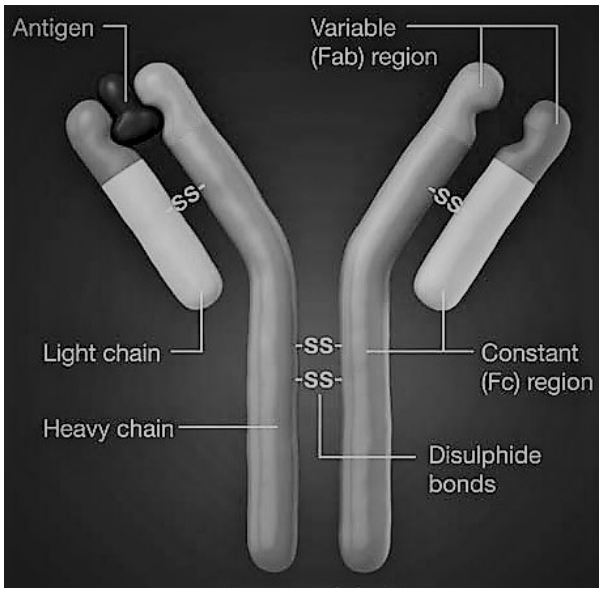

Fig. 2B.2 Schematic of a bovine IgG molecule (Adapted from Gapper et al., 2007)

Bovine Serum Albumin (BSA)

BSA is a single polypeptide comprising 582 amino acid residues and having the molecular weight about 66.3 kDa. BSA molecule is characterized by an overall oblate shape, which contains three domains stabilized by internal network of 17 disulphide bridges. Each domain consists of two large double loops and one small double loop. BSA also contains one free thiol group at Cys^{34}. BSA possesses several (5-12) binding sites at neutral pH, which can mainly bind with fatty acids and cations including Cu^{2+} and Ni^{2+} (Carter & Ho, 1994; Majorek et al., 2012; Patel, 2007). The secondary structure of BSA has been characterized as approximately 66% of helices, 10% of turns, and 23% of extended chains, and specifically no β-sheets (Gelamo, Silva, Imasato, & Tabak, 2002; Gelamo & Tabak, 2000).

Lactoferrin (LF)

LF is a monomeric protein containing 689 amino acid residues with a molecular weight of approximately 80.0 kDa. LF structure contains two globular lobes, with similar amino acid sequence, which are linked by an extended α-helix forming its tertiary structure. LF structure

also contains intramolecular disulfide bonds (16), but no free thiol groups. LF is observed to be a glycoprotein possessing two carbohydrate groups. As well, there are two iron binding sites to attach ferric ions (Fe^{3+}) (Lönnerdal & Suzuki, 2013; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007; Wal, 2002; Wong et al., 1996).

2B.2 Thermal processing of milk and its impact on milk proteins

2B.2.1 Thermal processing

The consumption of bovine milk by humans appeared to commence since about 8000 BC resulting in domestication of cows. Traditionally, people used to preserve the milk by fermentation as fermented milk or cheese and the lipid portion was converted into butter or ghee prior to inventing the thermal processing. In 1860, Louis Pasteur described the concept of microbial destruction through the heat treatment, which was initially adopted in commercial milk pasteurization by mid of 1890. Due to industrialization, manufactures were then able to preserve milk in liquid forms and powders by employing various thermal processing methods (Fox, 2003; O'Connell & Fox, 2003).

Nowadays, thermal processing is an essential step in milk processing and milk is mostly subjected to at least one heat treatment throughout the processing regardless the end product (Fox, 2003; Holsinger, Rajkowski, & Stabel, 1997; O'Connell & Fox, 2003). Table 2B.1 exhibits the types of most commonly applied heat treatments, heating conditions and their purposes. The thermal processing is usually performed in order to enhance the functional, nutritional, and sensory attributes and importantly to ensure the microbiological safety (Wal, 2003).

Table 2B.1 Details of the heat treatments commonly applied in dairy industry (Adapted from O'Connell & Fox, 2003)

Heat treatment	Conditions	Objective
Thermization	65 °C for 15 min	Inactivation of spoilage microbes
Low-temperature long-time (LTLT) pasteurization	63 °C for 30 min	Inactivation of pathogenic microbes
High-temperature short-time (HTST) pasteurization	72 °C for 15 s	Inactivation of pathogenic microbes
Forewarming	90 °C for 2-10 min 120 °C for 20 s	A prior step for sterilization
Ultra-high temperature (UHT) processing	130-140 °C for 3-5 s	Sterilization
In-container sterilization	110-115 °C for 10-20 min	Sterilization

However, it has been well established that the heat treatments applied in milk processing can induce reversible or irreversible modifications of native milk proteins leading to protein denaturation and aggregation (Brick et al., 2017; Considine, Patel, Anema, Singh, & Creamer, 2007; Dissanayake, Ramchandran, Donkor, & Vasiljevic, 2013; Huppertz, 2016; Wijayanti et al., 2014). Any modification in the secondary, tertiary or quaternary conformation of a protein, excluding the rupture of peptide bonds in its primary structure, can be described as protein denaturation, where final conformation after denaturation can correspond to a totally or partially unfolded polypeptide structure (Messens, Van Camp, & Huyghebaert, 1997). As well, the protein aggregation (polymerization, precipitation, coagulation etc.) refers to

unspecified protein-protein interactions leading to formation of large complexes with higher molecular weights (Messens et al., 1997).

Various studies revealed that native food proteins are generally present in a stable form at minimum energy status, which is maintained by hydrophobic interactions, hydrogen bonding, electrostatic interactions, and disulfide bonds. Heat treatments induce the thermal motions of polypeptide chains creating an imbalance among intramolecular forces, which results in reorganization of all levels of the protein structure. Heating thus leads to significant structural modifications in proteins including reversible unfolding or irreversible denaturation and aggregation. Heat induced changes in a globular native protein structure, in general, begin with unfolding of secondary structure (55-70 °C) and proceed to cleavage of disulfide bonds (70-80 °C), formation of new intra- and intermolecular interactions, rearrangement of disulfide bonds (80-90 °C), and finally formation of aggregates (90-100 °C). Apart from physical modifications, chemical changes of the protein may occur at mostly higher temperatures (100-125 °C and above), leading to formation of various adducts through the bonds between proteins and other ingredients such as carbohydrates and lipids in the food matrix (Bu, Luo, Zheng, & Zheng, 2009; Davis & Williams, 1998; Wal, 2003).

2B.2.2 Heat-induced modifications in milk proteins

2B.2.2.1 Caseins

Caseins are remarkably heat stable due to its unique micellar structure. Casein micelle can be subjected to various technical processes including heating, freezing, and drying, but its properties remain mostly unchanged upon reconstitution (De Kruif, 1999; Fox & Morrissey, 1977). Caseins can resist aggregation during the heating at 100 °C for 24 hours or at 140 °C for up to 20-25 min (Fox, 2003). However, the size of the casein micelle appeared to be affected by sever heating such as UHT (ultra-high temperature) treatments. Both increasing of the diameter of casein micelles and reduced range of micellar sizes were observed by electron

microscope when milk was heated at 140 °C for 10 min (Singh, Creamer, & Fox, 1992). Furthermore, the pH dependent disassociation of individual caseins from the casein micelle has been reported, which can also be influenced by heating temperature, treatment time, and composition of the milk (Anema & Klostermeyer, 1997; Singh, 2004).

2B.2.2.2 Whey proteins

In contrast to caseins, the globular whey proteins are heat-labile, and they can experience protein denaturation and aggregation upon heating depending on various factors such as treatment temperature, duration, pH, protein concentration and physicochemical conditions of the protein (Dissanayake, Ramchandran, Donkor, et al., 2013; Dissanayake, Ramchandran, Piyadasa, & Vasiljevic, 2013; Wijayanti et al., 2014; Wong et al., 1996).

The process of heat-induced modification of whey proteins can be either reversible or irreversible. The reversible modifications include partial unfolding of proteins with loss of helical structure, while irreversible changes lead an aggregation process involving thiol (–SH)/ disulfide (S–S) interchange reactions and other intermolecular interactions including hydrophobic and electrostatic interactions. Apart from the interactions between whey proteins (e.g., BLG, ALA, and BSA), they can also interact with cystine-containing caseins, especially κ-casein. Moreover, the evidence points out that the thermal denaturation behavior of individual whey proteins is distinctly different (Anema, 2016; Considine et al., 2007; Wijayanti et al., 2014). Table 2B.2 demonstrates the thermal denaturation temperatures and enthalpies of the main whey proteins.

Table 2B.2 Thermal denaturation temperatures and enthalpies of whey proteins (Representation of the Table 28.2; Singh & Havea, 2003)

Whey protein	Td (°C)	Trt (°C)	ΔH (kJ/mol)
BLG	78	83	311
ALA	62	68	253
Ig	72	89	500
BSA	64	70	803

Td = initial denaturation temperature; Trt = temperature at the DSC (Differential scanning calorimetry) peak maximum; ΔH = enthalpy of denaturation.

β- Lactoglobulin (BLG)

The processes involved in thermal denaturation and aggregation of BLG have been comprehensively studied (Dannenberg & Kessler, 1988; Iametti, Gregori, Vecchio, & Bonomi, 1996; Lefèvre & Subirade, 1999; Sawyer, 2013; Wijayanti et al., 2014; Wong et al., 1996). Native BLG occurs as a mixture of monomers and dimers, which are in equilibrium at room temperature (about 20 °C). However, the dimers disassociate mainly into monomers at temperatures above 30 °C and pH values 6-9. Although the reversible disassociation occurs at low temperatures, BLG loses its native tertiary and secondary structures at higher temperatures and the free thiol group hidden in native conformation can also be exposed. Furthermore, the hydrophobic groups exposed during denaturation lead to formation of hydrophobically associated aggregates. In addition to hydrophobic interactions, thiol/ disulphide interactions can also be induced by heating resulting in aggregation. Depending on the heating temperatures, unfolded BLG can move either toward forming its native conformation or be involved in irreversible aggregation reactions, generally temperatures above 70°C (Dissanayake, 2011; Monaci, Tregoat, van Hengel, & Anklam, 2006; Mulvihill & Donovan, 1987; Wijayanti et al., 2014). The pH value and ionic strength of the protein dispersion also

play a great role in denaturation and aggregation mechanism apart from heating temperature (Oldfield, Singh, Taylor, & Pearce, 1998).

BLG usually denatures at temperatures above 65 ºC at pH 6.7, while critical denaturation temperature has been reported at around 70.4 ºC at the same pH (Wong et al., 1996). It has often been evident that BLG leads whey protein aggregation due to its higher concentration in milk comparing to other types of whey proteins. While heating in progress, BLG can go through various phases such as unfolding, interaction of unfolded BLG with other whey proteins (e.g., ALA and BSA), and also interaction of unfolded BLG with non-whey proteins, for instance κ-casein (Wijayanti et al., 2014).

α-Lactalbumin (ALA)

ALA is more heat-stable in comparison to other whey proteins predominantly due to absence of free thiol group (Calvo, Leaver, & Banks, 1993). ALA did not appear to unfold irreversibly at temperatures ≤ 80 ºC (Dalgleish, Senaratne, & Francois, 1997; Gezimati, Creamer, & Singh, 1997; Hines & Foegeding, 1993), but high temperatures and long holding time (e.g., 95 ºC for 14 min) with absence of Ca resulted in denaturation (Chaplin & Lyster, 1986; Wijayanti et al., 2014). ALA can pass through a few phases during heat induced modification process such as unfolding (mostly reversible) at the temperatures < 90 ºC, irreversible denaturation at > 90 ºC, formation of free thiol and initiating intermolecular thiol/ disulphide interchange reactions, and formation of dimers and large oligomers, which could partially disassociate during cooling (25 ºC) (Chaplin & Lyster, 1986; Hong & Creamer, 2002; McGuffey, Epting, Kelly, & Foegeding, 2005).

Furthermore, it has been reported that ALA tends to form modified monomers and disulphide-linked polymers during heating at 100 ºC for 3 min and they possibly exist in the form of molten globule (Hirose, 1993; Kuwajima, 1989; Wijesinha-Bettoni, Dobson, & Redfield, 2001). Moreover, several studies reveled that ALA does not aggregate tightly but can

only form loosely bound structures under various conditions (Gezimati, Singh, & Creamer, 1995; Hines & Foegeding, 1993; Nonaka, Li-Chan, & Nakai, 1993).

Immunoglobulins (Ig)

Ig are heat-labile proteins/ antibodies, which antigen binding sites are more heat-sensitive than the other areas. Out of different Ig isotypes containing in milk (e.g., IgG, IgA and IgM), IgG is found to be the most heat-stable, while IgM is the most heat-labile (Hurley & Theil, 2013). IgG was not denatured when milk was heated at 62.7 ºC for 30 min. However, only about 59-76% of biologically active IgG was detected in HTST pasteurized milk, while no or little was found in both evaporated canned milk and UHT sterilized milk (Besler, Steinhart, & Paschke, 2001). On the contrary, it was reported that IgG could remain stable without changing its structure during heating at 72 ºC for 15 s (Mainer, Sanchez, Ena, & Calvo, 1997). The denaturation kinetics of IgG in milk has recently been described as a second order reaction (Anema, 2017). The isolated IgG is stable at temperature 37 ºC under pH values 6-7 for several hours but it changes stability intensely at highly acidic ($pH \leq 3$) or alkaline ($pH \geq 10$) conditions (Hurley & Theil, 2013). It has been suggested that the thiol/ disulphide interactions were involved in forming aggregates when IgG was subjected to 95 ºC for 30 s treatment (Oh & Richardson, 1991).

Bovine Serum Albumin (BSA)

BSA is one of the most heat-sensitive whey proteins when it was heated alone at neutral pH (De Wit & Hontelez-Backx, 1984; Takeda, Wada, Yamamoto, Moriyama, & Aoki, 1989; Yamasaki, Yano, & Aoki, 1991). The several studies showed that BSA can resist heat-induced unfolding up to 40 ºC, conformational changes are reversible at 42-50 ºC, unfolding of α-helixes is irreversible at 52-60 ºC, thiol-induced aggregation is initiated at temperatures above 60 ºC, and gel formation occurs when adequate concentration of BSA is heated above 70 ºC.

Furthermore, two distinguished conformational modifications have been observed at 57 and 75 ºC and intermolecular β-sheet formation occurs irreversibly when BSA is heated above 70 ºC (Clark, Judge, Richards, Stubbs, & Suggett, 1981; Considine et al., 2007; Murayama & Tomida, 2004; Takeda et al., 1989).

Lactoferrin (LF)

It has been reported that the iron free LF (apo-LF) are more sensitive to heat-induced denaturation than the iron saturated LF (holo-LF), since iron binding results in conformational changes of LF molecule and it becomes more compact (Paulsson, Svensson, Kishore, & Naidu, 1993; Sánchez et al., 1992). About 30% of holo-LF was denatured when it was heated at 65ºC for 45 min in a phosphate buffer and denaturation processes progressed with increasing temperature and holding time (Sánchez et al., 1992). LF in milk can be completely denatured by 135ºC for 4 s treatment (UHT) but 72ºC for 15 s (HTST) has no considerable impact. Furthermore, LF can interact with caseins and other whey proteins present in the milk during heating (Paulsson et al., 1993).

2B.2.3 Heat-induced interactions between whey proteins

The behavior of thermal denaturation and aggregation of a protein varies depending on whether it is alone or in a protein mixture. For instance, although ALA is considered to be more heat-stable than other whey proteins due to absence of free thiol groups, in a whey protein mixture ALA disappears rapidly as it readily aggregates with other proteins containing free thiol groups (e.g., BLG and BSA) (Havea, Singh, & Creamer, 2001). Whey protein aggregation is generally associated with the heat-stability of BLG, ALA and BSA, while the other whey proteins can also be a part of this aggregation process (Wijayanti et al., 2014).

In a binary mixture of BLG and ALA, ALA can form nonnative monomers, dimers, trimers, and large aggregates with or without the contribution of BLG (homopolymers and heteropolymers) (Calvo et al., 1993; Dalgleish et al., 1997; Gezimati et al., 1997). These

interprotein interactions usually occur through thiol-induced disulphide bond interchange reactions between a free thiol of a nonnative BLG monomer and a cysteine residue located on the surface of ALA molecule (Calvo et al., 1993; Livney, Verespej, & Dalgleish, 2003).

Furthermore, it was observed that the BLG dispersed in a BLG and ALA mixture was completely converted into soluble aggregates through disulphide bonds and hydrophobic interactions during heating (Calvo et al., 1993; Dalgleish et al., 1997; Gezimati et al., 1997; Havea et al., 2001; Oldfield, Singh, Taylor, & Pearce, 2000; Schokker, Singh, & Creamer, 2000). Another study revealed that, in a binary protein mixture of ALA and BLG, denatured BLG initiated interprotein interactions with ALA, which resulted in hydrophobically-linked BLG-ALA aggregates at temperatures < 80 °C, while disulphide-linked BLG-ALA aggregates at temperatures > 80 °C (Oldfield, Singh, Taylor, et al., 1998). The proportion of ALA was found to be smaller in small aggregates while higher in larger aggregates (Hong & Creamer, 2002). BLG has demonstrated mostly a similar thermal behavior regardless whether it was heated alone or in the presence of ALA, implying that BLG leads thermal aggregation of proteins in a binary mixture of BLG and ALA (Hong & Creamer, 2002; Schokker et al., 2000).

Behavior of a BSA pure solution during thermal denaturation and aggregation appears almost similar to BLG, except formation of aggregates by BSA occurs at a lower temperature than BLG (Gezimati, Singh, & Creamer, 1996). Although both BSA and BLG possess a free thiol group (Carter & Ho, 1994), the thermal transition temperature of BSA is reported to be lower than BLG (De Wit & Hontelez-Backx, 1984). Due to lower thermal transition temperature of BSA, it governs the heat-induced aggregation process over BLG in a BSA and BLG mixture at temperatures below 70 °C, whereas when heating at above 75 °C, both BSA and BLG form aggerates at a comparable rate (Gezimati et al., 1995; Matsudomi, Oshita, & Kobayashi, 1994). Although the influence of BLG on heat-induced aggregation of BSA has

not been reported, the aggregation of BLG appeared to be catalyzed by the presence of BSA (Havea et al., 2001; Matsudomi et al., 1994).

When heating a mixture of ALA and BSA at 80 °C for 30 min, the protein aggregates were formed by thiol/ disulphide interchange interactions (Matsudomi, Oshita, Kobayashi, & Kinsella, 1993). BSA was also able to form protein complexes through hydrophobic interactions apart from thiol/ disulphide reactions, where ALA did not participate in formation of hydrophobic aggregates (Havea et al., 2001; Matsudomi et al., 1993). It has been observed that during heating BSA formed disulphide-bonded aggregates, in which thiol groups catalyzed the formation of structurally modified ALA monomers, dimers, polymers and adducts of ALA inclusion of BSA. It was thus suggested that the exposed thiol group of unfolded BSA molecule or BSA aggregate can react with a disulphide of ALA via thiol/ disulphide interchange interactions (Havea, Singh, & Creamer, 2000).

In a protein mixture of BLG, ALA, and BSA, BSA showed a tendency to form polymers earlier than the unfolding of BLG and ALA at 75 ℃ (Gezimati et al., 1997; Gezimati et al., 1996). The formation of interprotein aggregates between these proproteins occurred through both covalent and noncovalent interactions in a whey protein concentrate (WPC) solution (Gezimati et al., 1997; Gezimati et al., 1996). Furthermore, BSA was observed to be more effective than BLG in catalyzing the formation of ALA complexes resulting in greater formation of disulphide-linked aggregates in BSA and ALA mixtures than the BLG and ALA mixtures due to the differences in their thermal transition temperatures (Havea et al., 2001; Wijayanti et al., 2014). However, the role of BSA in heat-induced whey protein aggregation is not fully understood, especially the aggregation behavior between BSA and ALA (Wijayanti et al., 2014).

During heating, Ig appeared to be involved in whey protein aggregation through hydrophobic interactions (Oh & Richardson, 1991), while thiol/ disulfide interactions have also been suggested in forming protein complexes (Anema, 2017; Su & Chiang, 2003).

2B.2.4 Heat-induced interactions between whey proteins and caseins

In comparison to the whey, the milk forms significantly different protein aggregates during heating due to the association of denatured whey proteins with caseins (Donato & Guyomarc'H, 2009). The interactions between whey proteins and caseins during heating of milk have been extensively studied (Anema & Klostermeyer, 1997; Anema & Li, 2003; Anema & McKenna, 1996; Considine et al., 2007; Liyanaarachchi, Ramchandran, & Vasiljevic, 2015; Patel, Anema, Holroyd, Singh, & Creamer, 2007; Patel, Singh, Anema, & Creamer, 2006; Wijayanti et al., 2014). It has been reported that the casein micelle increases its size, during heating at 75-100 °C for up to 60 min, due to interactions of denatured whey proteins with the micelle (Anema & Li, 2003). However, the rate of denaturation of whey proteins appeared to be faster than the rate of their association with casein micelles, which was further influenced by heating temperature and treatment time (Anema & Li, 2003). It has further been reported that the degree of interaction between whey proteins and casein micelle is greater under slow heating conditions (Oldfield, Singh, & Taylor, 1998), which is also influenced by pH, protein concentration, and ionic strength (Anema, 2014).

The interactions between caseins and denatured whey proteins mostly occur through both hydrophobic and disulphide interactions at the early stage of heating, while their mechanisms vastly depend on the protein system and the conditions of heating (Cho, Singh, & Creamer, 2003). BLG usually initiates interactions with κ-casein upon exposure of its free thiol group subjected to thermal denaturation (Cho et al., 2003). ALA possibly interact with κ-casein in comparable to BLG, when it possesses a free reactive thiol group, for instance followed by formation of BLG and ALA complexes during heating (Anema, 2014). Apart from κ-casein,

α_{s2}-casein, possessing disulphide bonds, has shown to interact with denatured whey proteins by thiol/ disulphide interchange reactions. It is, however, less reactive than κ-casein (Horne, 1998; Walstra, 1990). It has also been reported that α_{s1}-and β-casein, having no disulphide bonds, are rarely involved in protein aggregation processes during heating (Corredig & Dalgleish, 1999; Oldfield, Singh, Taylor, et al., 1998).

2B.2.5 Heat-induced interactions between milk proteins and lactose

At high temperatures (>120 °C) with extended holding periods, milk proteins can be subjected to Maillard reaction in the presence of lactose. Primarily, the condensation of lactose with amino groups of proteins (lysine) takes place and produces Schiff base, which leads to formation of Amadori products. The breakdown of Amadori product can result in formation of melanoidins at later stage. Melanoidins usually induce polymerization of proteins and develop brown colour (Dissanayake, 2011). For instance, during Maillard reaction, covalent fixation of lactose on the amino groups of lysyl residues of BLG occurs after condensation and polymerization reactions (Taheri-Kafrani et al., 2009).

2B.3 High pressure processing of milk and its impact on milk proteins

2B.3.1 High pressure processing

It is well-known that thermal processing is an effective, economical and readily available method for food processing. However, it alters sensory and nutritional quality of foods. High pressure processing (HPP) is considered an emerging nonthermal technology, as well an alternative method for traditional thermal processing. HPP can be employed to produce a range of safe and nutritious foods (e.g., fruit juice, jam, jellies, salad dressing, cakes, etc.), while minimizing the impact on their sensory and nutritional quality (Datta & Deeth, 1999; Georget et al., 2015; Liu, Ning, & Clark, 2009; Naik, Sharma, Rajput, & Manju, 2013; Rendueles et al., 2011). Although the HP processed commercial dairy products are not widely

available, the application of HPP has attracted a considerable interest in dairy industry (Huppertz et al., 2002; Patel, 2007). A drinkable commercial bovine milk product (http://www.madebycow.com.au) processed by HP (Cornell, 2017) is currently available in the Australian market.

2B.3.2 High pressure-induced modifications in milk proteins

The Le Chatelier-Braun principle is applicable when studying the effect of HPP on proteins. It explains, whenever a stress is applied to a system in equilibrium, the system will react so as to counteract the applied stress. For instance, the reactions that result in reduced volume are promoted under HP. Such reactions may result in inactivation of microorganisms and or enzymes, denaturation and aggregation of proteins, and textural and functional changes in foods (Balci & Wilbey, 1999; Huppertz et al., 2002; Messens et al., 1997). It has been reported that the covalent bonds are almost unaffected by HP (Mozhaev, Heremans, Frank, Masson, & Balny, 1994), which results in maintaining intact primary structure of proteins. However, the secondary and tertiary structure of proteins can be modified by HP (> 200 MPa) due to rupturing of hydrogen bonds and the changes in hydrophobic and ionic interactions leading to protein denaturation and aggregation (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998; Huppertz et al., 2002).

The effects of HP on proteins basically depend on several factors including type of protein, applied pressure, duration, pressurizing temperature, pH, protein concentration and ionic strength (Huppertz, Fox, & Kelly, 2004b, 2004c; Messens et al., 1997). It has been observed that HP influences various aspects of milk proteins such as modification of the casein micelle, disassociation of the casein micelle, changes in particle size of the proteins, denaturation and aggregation of whey proteins, and interaction of denatured whey proteins with the casein micelle (Huppertz et al., 2002).

2B.3.2.1 Caseins

The studies reported that the high pressurization caused irreversible fragmentation of the casein micelle (150-400 MPa) in milk since hydrophobic and electrostatic interactions were disturbed (Buchheim, Schrader, Frede, Schuett, & Morr, 1996; Mozhaev, Heremans, Frank, Masson, & Balny, 1996) and colloidal calcium phosphate (CCP) was also solubilised (Buchheim et al., 1996; López-fandiño, De la Fuente, Ramos, & Olano, 1998), which in turn changed the size, structure and composition of the casein micelle. Subsequently, the lightness, turbidity, and viscosity of the milk were altered (Garía-Risco, Olano, Ramos, & Lopez-Fandino, 2000; Huppertz et al., 2006; Mussa & Ramaswamy, 1997).

The HP treatment at 250-600 MPa reduced the micelle size by 40-50% in raw (Needs, Stenning, Gill, Ferragut, & Rich, 2000) and reconstituted skim milk (Desobry-Banon, Richard, & Hardy, 1994; Gaucheron et al., 1997), while 150-200 MPa had slight or no apparent effect on it (Gaucheron et al., 1997). The effect of HPP on micellar size was found to be temperature dependant. For instance, when skim milk was pressurized at 250 MPa at 20 °C, micellar size was not substantially affected, but it was increased at 40 °C and decreased at 4 °C (Gaucheron et al., 1997). The increased micellar size at higher temperature (40 °C) could be related to interactions between casein micelles and denatured whey proteins induced by HP (Buchheim et al., 1996; Gaucheron et al., 1997; Schrader & Buchheim, 1998).

During pressurization of the milk, disintegration of casein micelle has been reported, especially disintegration of κ- and β-casein (López-fandiño et al., 1998). It was further observed that the substantial solubilization of α_{s1}- and β-casein in milk occurred when it was pressurized at 100-600 MPa, and it reached the maximum solubility at about 250 MPa (Huppertz, Fox, & Kelly, 2004a). HP-induced disintegration of the caseins occurs in the order β- > κ- > α_{s1}- > α_{s2}-casein (López-fandiño et al., 1998). It was also observed by transmission electron microscopy (TEM) that the HP treatment at 400-600 MPa completely disturbed all

larger micelles into smaller fragments (Needs et al., 2000). Moreover, the disintegration of caseins in HP treated milk was found to be temperature dependant during storage, where it was largely irreversible at 5 °C but mostly reversible at 20 °C (Huppertz et al., 2004a).

2B.3.2.2 Whey proteins

BLG has been reported to govern HP-induced denaturation and aggregation of the entire protein system (Huppertz et al., 2006; Huppertz et al., 2002; Liu et al., 2009; Patel et al., 2006; Ramos, Chiquirrín, García, Condón, & Pérez, 2015). When BLG is pressurized, it can go through various phases including disassociation of dimers into monomers (Iametti et al., 1997), changes in the secondary structure (e.g., loss of α-helix and β-sheet content) (Hayakawa, Linko, & Linko, 1996; Panick, Malessa, & Winter, 1999), and formation of intermolecular disulphide bonds leading to aggregation (Iametti et al., 1997; López-Fandiño, Ramos, & Olano, 1997; Møller, Stapelfeldt, & Skibsted, 1998; Patel et al., 2006; Van Camp, Messens, Clément, & Huyghebaert, 1997).

It was observed that the pressurization of raw milk up to 100 MPa did not affect the denaturation of BLG (López-Fandiño & Olano, 1998; Scollard, Beresford, Needs, Murphy, & Kelly, 2000). However, the increasing HP up to about 400 MPa caused 70-80% denaturation of BLG (Arias, López, & Olano, 2000; Garía-Risco et al., 2000; Moatsou et al., 2008; Ramos et al., 2015; Scollard et al., 2000). Further increasing of HP (400- 800 MPa) resulted in relatively lower rate of denaturation (Scollard et al., 2000). Moreover, the pasteurized milk (Needs et al., 2000) and reconstituted milk powder (Gaucheron et al., 1997), which already contained some denatured proteins prior to HPP, have demonstrated greater extent of denaturation of BLG subjected to HP treatment at 600 MPa. The reaction order of denaturation of BLG induced by HP has been reported as 2.5 (Hinrichs, Rademacher, & Kessler, 1996), signifying this denaturation process is concentration dependant, where a lower initial concentration of native BLG can reduce the extent of denaturation. HP-induced denaturation

of BLG in milk was also reported to depend on pressurization temperature and pH. Almost complete denaturation of BLG in raw milk was reported at 300 MPa at 50-60 °C (temperature in the pressure cell) or at 400 MPa at 40-60 °C (Garía-Risco et al., 2000; López-Fandiño & Olano, 1998). The acidification of natural milk (pH 6.7) to pH 5.5-6, prior to the HP treatment minimized denaturation of BLG, while pH 7 enhanced denaturation process (Arias et al., 2000).

HP-induced aggregation of BLG occurs through the formation of intermolecular disulphide bonds by thiol/disulphide interchange reactions, most likely involving Cys^{66}-Cys^{160} and Cys^{121} (Belloque, López-Fandiño, & Smith, 2000). The inter- and intramolecular reactions of free thiol groups have also shown to be a main interaction in forming BLG aggregates (Tanaka, Tsurui, Kobayashi, & Kunugi, 1996). It has been proposed that during pressurization BLG unfolds resulting in exposure of free thiol group, which can interact with κ-casein, ALA, BLG, or probably α_{s2}-casein via thiol/ disulphide interchange reactions (Huppertz et al., 2004b; Patel et al., 2006). It was observed that the most of the denatured BLG in HP-treated (300-600 MPa) skim milk was associated with casein micelles allowing for sedimentation, while a small amount can remain non-sedimentable either in the form of whey protein aggregates or associated with caseins in forming tiny particles (Huppertz et al., 2004c).

In contrast to BLG, ALA can withstand the HP up to 500 MPa as observed in pressurized in raw milk (Garía-Risco et al., 2000; López-Fandiño & Olano, 1998; Moatsou et al., 2008; Ramos et al., 2015), reconstituted skim milk (Gaucheron et al., 1997), or pasteurized skim milk (Needs et al., 2000). However, the combined effect of HP and the temperature (50-60 °C in the pressure cell) resulted in denaturation of ALA to a greater extent than HP alone (Garía-Risco et al., 2000; López-Fandiño & Olano, 1998). Higher barostability of ALA, in comparison to BLG, is associated with a higher number of intramolecular disulphide bonds (4) and the absence of free thiol groups in ALA structure (Huppertz et al., 2004b, 2004c). Nevertheless, both BLG and ALA showed a substantial stability against HP-induced

denaturation in whey than in milk (Huppertz et al., 2004b). In addition, removal of CCP from milk contributed to reduce the HP-induced denaturation of BLG and ALA (Huppertz et al., 2004b). This indicated that the higher barostability of BLG and ALA in whey than in milk could be ascribed to the absence of casein micelles and CCP in whey (Huppertz et al., 2004b). When ALA was pressurized alone, it resists HP-induced denaturation (Lopez-Fandino, Carrascosa, & Olano, 1996; Scollard et al., 2000) or denatures reversibly (Tanaka & Kunugi, 1996; Tanaka et al., 1996) up to 400 MPa, but forms disulphide-bonded oligomers at about 1000 MPa (Jegouic, Grinberg, Guingant, & Haertlé, 1996).

The investigations on the HP-induced modifications of minor whey proteins are relatively limited (Considine et al., 2007; Huppertz et al., 2006; Huppertz et al., 2002; Patel, 2007). BSA has been reported to resist HP-induced denaturation up to 400 MPa in raw milk (Lopez-Fandino et al., 1996) and in a pure BSA solution (Hayakawa, Kajihara, Morikawa, Oda, & Fujio, 1992). The high barostability of BSA has been proposed to be associated with its rigid molecular structure stabilized by 17 intramolecular disulphide bonds and the availability of several separate domains (Lopez-Fandino et al., 1996). A study reported that the minor whey proteins in skim milk including IgG, BSA, and LF were substantially affected by HP-treatment at 400-800 MPa for 30 min (Patel et al., 2006). The HP-treatment at 600 MPa for 15 min caused denaturation of 44% IgG and 70% LF in raw skim milk (Ramos et al., 2015). The immunological activity of IgG was preserved when the colostrum was treated by HP up to 300 MPa for 10 min (Masuda, Rehinarudo, Suzuki, Sakai, & Morichi, 2000), while the isolated IgG can resist HPP up to 400 MPa (Indyk, Williams, & Patel, 2008).

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Chapter 3: Thermal denaturation of bovine immunoglobulin G and its association with other whey proteins

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

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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.

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1. Introduction

Bovine immunoglobulins (Ig), secreted in colostrum and milk, are a family of globular proteins with a range of protective bioactivities 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 subunits 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

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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 μm 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 × 7.8 mm) Size Exclusion Chromatography (SEC) column (Phenomenex Australia Ply 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 (~250 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 °C/30 s, was quantified and confirmed the effectiveness of the UHT treatment with available literature (Dannenberg & 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 4000–600 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 1600–1700 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 (1600–1700 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 cm^{-1}), intramolecular β-sheet (1636, 1641, 1685 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-helixes (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 °C/15s) 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 (≥70 °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 (~53 kDa) and two identical light chains (~23 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

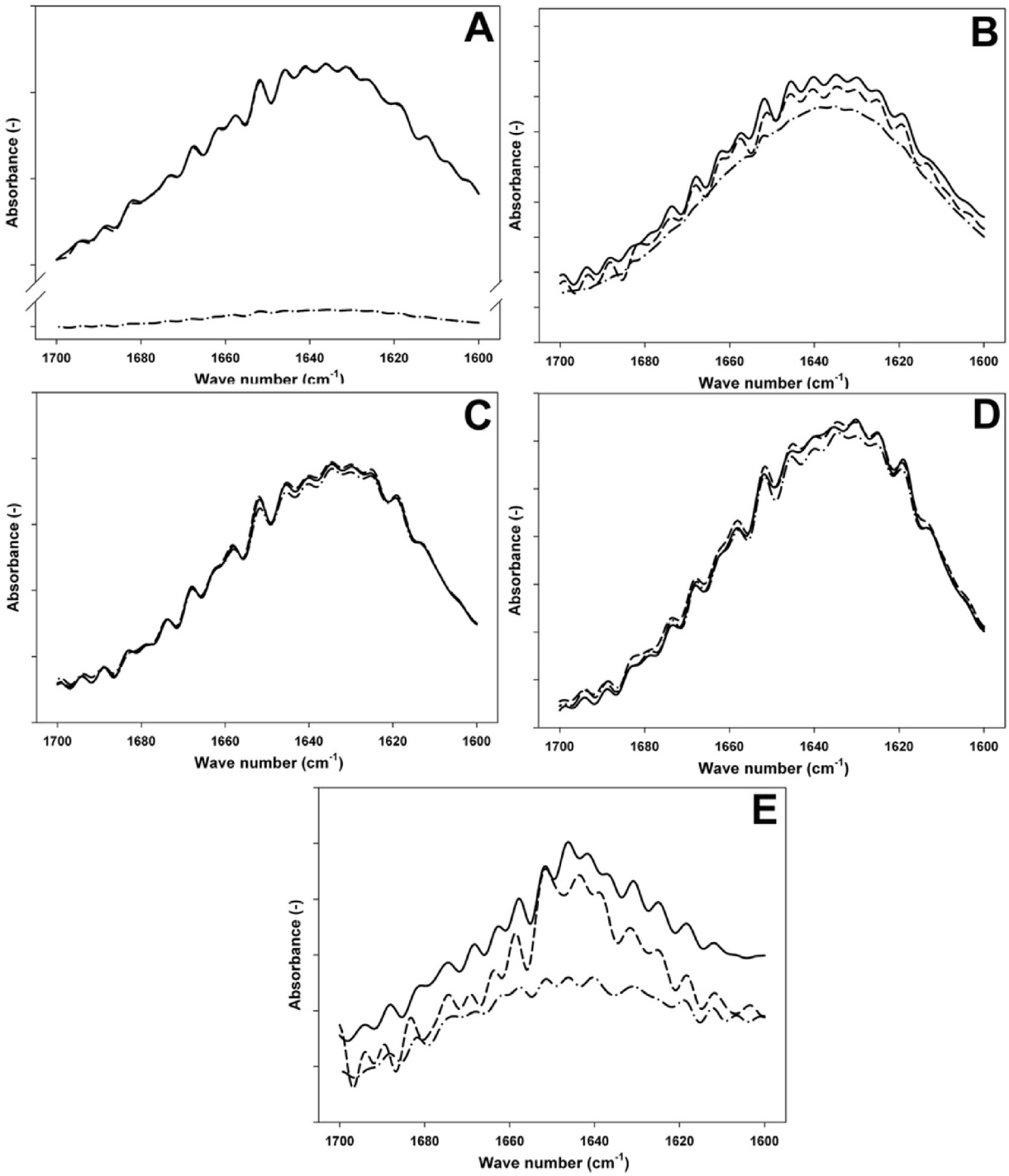

Fig. 1. FTIR spectra of protein/protein mixtures containing (A) IgG, (B) BLG and ALA, (C) BLG, ALA and IgG, (D) BLG, ALA, IgG and BSA, and (E) native whey at their natural concentrations in raw milk subjected to heat treatment at 72 °C (—) or 100 °C (—·—) for 15 s and 30 s, respectively. Untreated samples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

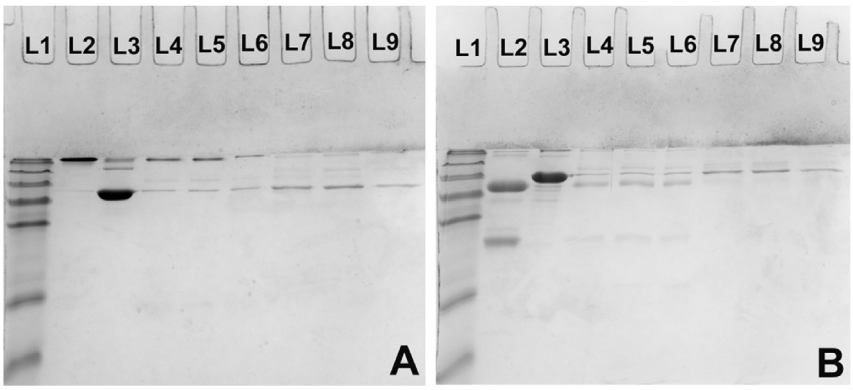

Fig. 2. Non-reducing (A) and reducing (B) SDS-PAGE analysis of protein/protein mixtures. Lanes are designated as: L1 - molecular weight markers; L2 - IgG standard; L3- BSA standard; L4 – IgG control; L5 – IgG 72 °C/15 s treated; L6 - IgG 100 °C/30 s treated; L7-BSA control; L8- BSA 72 °C/15 s treated;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

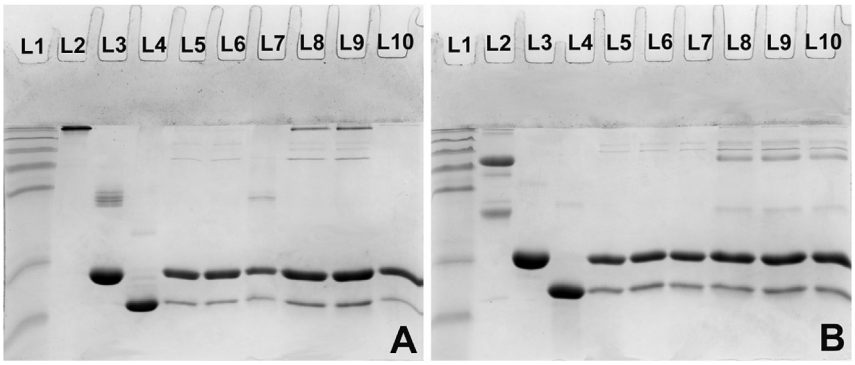

Fig. 3. Non-reducing (A) and reducing (B) SDS-PAGE analysis of protein/protein mixtures. Lanes are designated as: L1 - molecular weight markers; L2 - IgG standard; L3- BLG standard; L4- ALA standard; L5 – BLG and ALA control; L6- BLG and ALA 72 °C/15 s treated; L7 – BLG and ALA 100 °C/30 s treated; L8-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

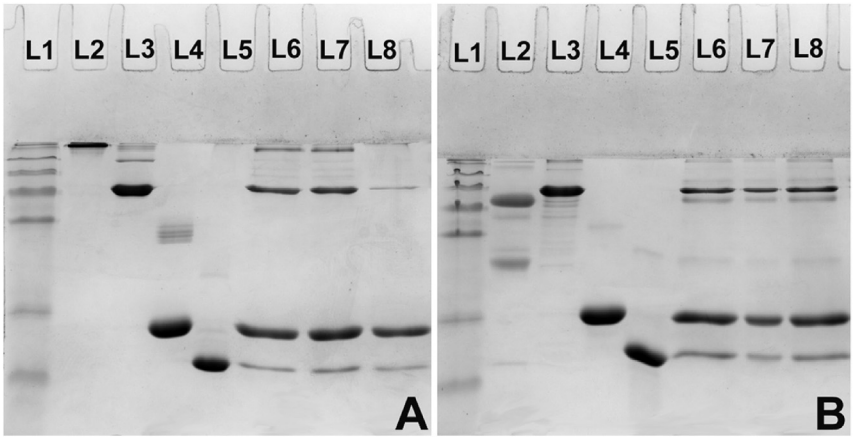

Fig. 4. Non-reducing (A) and reducing (B) SDS-PAGE analysis of protein/protein mixtures. Lanes are designated as: L1 - molecular weight markers; L2 - IgG standard; L3- BSA standard; L4- BLG standard; L5- ALA standard; L6- BLG, ALA, IgG and BSA control; L7 – BLG, ALA, IgG and BSA 72 °C/15 s treated; L8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

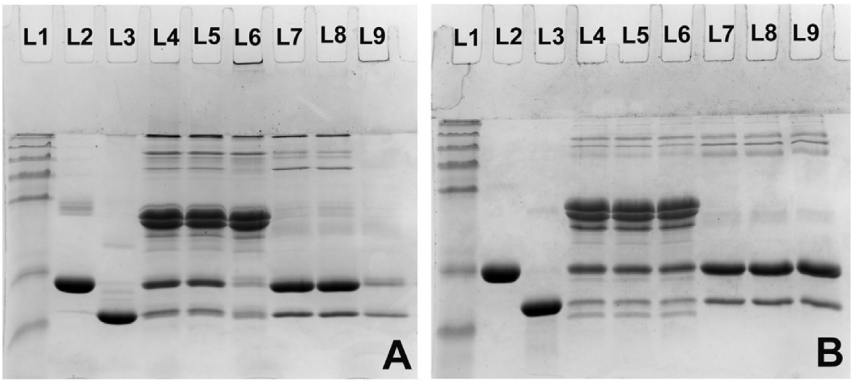

Fig. 5. Non-reducing (A) and reducing (B) SDS-PAGE analysis of skim milk and whey samples. Lanes are designated as: L1 - molecular weight markers; L2- BLG standard; L3- ALA standard; L4-skim milk control; L5 – skim milk 72 °C/15 s treated; L6 – skim milk 100 °C/30 s treated; L7-whey control; L8 – whey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

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Chapter 4: Thermal denaturation of bovine β-lactoglobulin in different protein mixtures in relation to antigenicity

This chapter has been published as a research paper entitled “Thermal denaturation of bovine β-lactoglobulin in different protein mixtures in relation to antigenicity” by Dimuthu Bogahawaththa, Jayani Chandrapala and Todor Vasiljevic in the peer-reviewed journal, International Dairy Journal, 91 (2019) 89-97. https://doi.org/10.1016/j.idairyj.2018.10.004

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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.

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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.

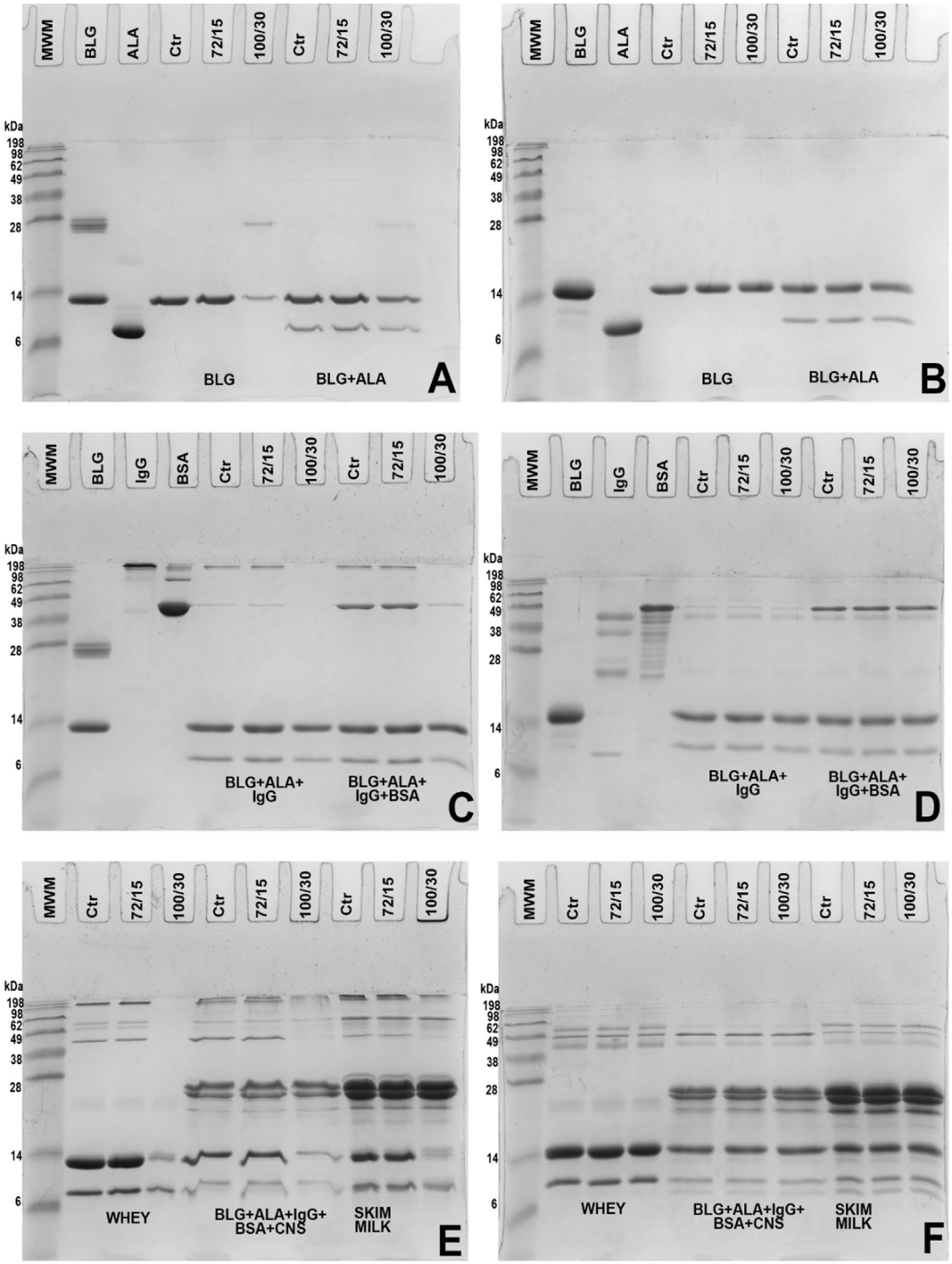

Fig. 1. Non-reducing (A, C, and E) and reducing (B, D, and F) sodium dodecylsulphate-polyacrylamide gel electrophoresis analysis of: (A and B) β-lactoglobulin (BLG) and BLG+α-lactalbumin (ALA); (C and D) BLG + ALA + immunoglobulin G (IgG) and BLG + ALA + IgG + bovine serum albumin (BSA); (E and F) whey, 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

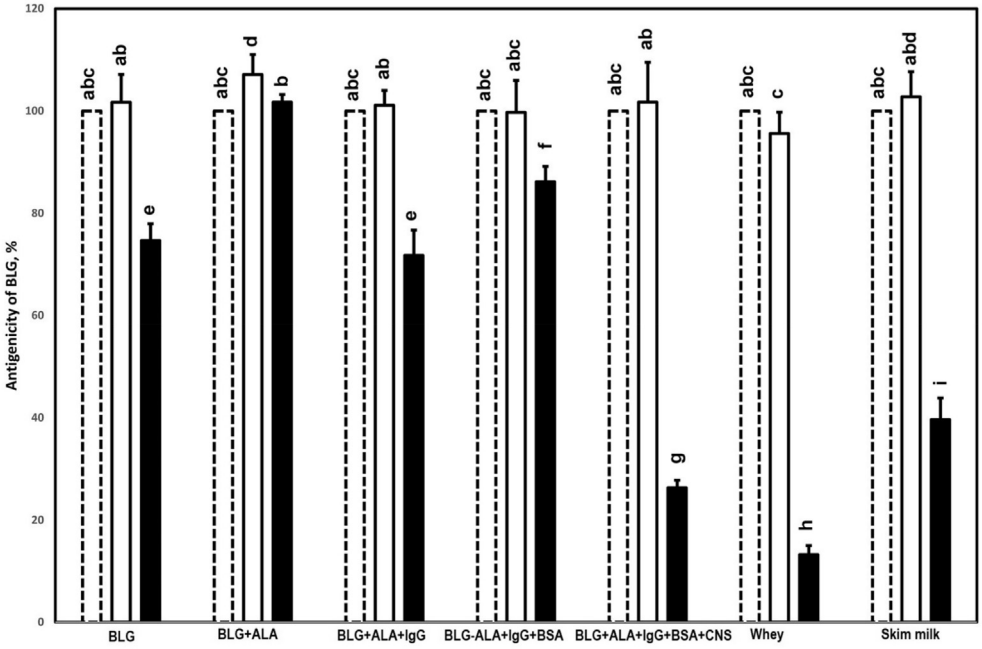

Fig. 2. Percentage (%) change of the antigenicity of β-lactoglobulin (BLG) in various protein samples (ALA, α-lactalbumin; IgG, immunoglobulin G; BSA, bovine serum albumin; CNs, caseins) subjected to 72 °C/15 s (□) and 100 °C/30 s (■) treatments. The control/untreated (⬚) is considered equivalent to 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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Chapter 5: In vitro immunogenicity of various native and thermally processed bovine milk proteins and their mixtures

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In vitro immunogenicity of various native and thermally processed bovine milk proteins and their mixtures

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ABSTRACT

In vitro immunogenicity of various native and thermally processed (72°C/15 s and 100°C/30 s) bovine milk protein fractions, their mixtures, whey, and skim milk, was studied by analyzing the immune response of T helper (Th) cells in human peripheral blood mononuclear cells. The secretion of Th type cytokines induced by the protein stimulants was quantified while determining the heat-induced protein denaturation. Purified whey proteins, caseins and whey fraction, and skim milk provoked substantial immune responses at various degrees, indicating their potent immunogenicity. The protein mixtures prepared using the fractionated whey proteins with or without caseins appeared less immunogenic in both native and heat-treated forms, implying their potential of producing less immunogenic dairy products. The 100°C/30 s treatment significantly altered the immunogenicity of most of the potent protein stimulants, which mostly coincided with their levels of protein denaturation. The 72°C/15 s treatment caused the least protein denaturation but altered the immunogenicity of several protein stimulants notably, including heat-stable caseins and α-lactalbumin.
Key words: bovine milk protein, thermal processing, protein denaturation, immunogenicity

INTRODUCTION

Bovine milk provides high-quality proteins to fulfil the AA requirements of humans, and usually it is the first source of foreign proteins ingested by infants in large quantities (Caira et al., 2012). However, the infant's intestinal system is insufficiently developed to digest bovine milk proteins, and their immune system frequently reacts to milk proteins (Caira et al., 2012). The presence of bovine milk proteins in the breast milk of lactating women and bovine milk protein-specific antibodies in the cord blood demonstrate the early exposure of neonates to bovine milk proteins (Høst et al., 1999).

Immunogenicity is the ability of a substance to elicit an immune response or capacity to provoke a detectable immune response (Bier et al., 1981; Actor, 2014). Generally, different proteins demonstrate either potent or weak immunogenicity. The molecular size and other characteristics, including the nature of AA, which make up immunogenic epitopes and the accessibility to those epitopes, contribute to immunogenic capacity of a specific protein (Bier et al., 1981). Epitopes are the portions of immunogenic molecule, mostly proteins, that can bind with the complementary sites of an antibody or T/B cells (Bogahawaththa et al., 2017a). Moreover, the immunogenicity of a protein fraction can alter when it is alone or in combination with other proteins or in the original source of protein (Cross and Gill, 2000).

Bovine milk proteins, more broadly, are immunogens (e.g., antigens and allergens), which can provoke immune response in human immune system through modulating the functions of immune cells including T cells or binding with antibodies such as IgG or IgE (Cross and Gill, 2000). The bovine β-LG and BSA were reported to induce the activation and proliferation of T helper (**Th**) cells in human peripheral blood mononuclear cells (**PBMC**) and secretion of associated cytokines, where β-LG was more potent than BSA (Vocca et al., 2011). The PBMC from a specific group of infants (suffered from necrotizing enterocolitis) showed significantly elevated production of Th type cytokines in response to bovine β-LG and caseins, where β-LG was more immunogenic than the caseins (Chuang et al., 2009). The Th cells, including Th1 and Th2 subsets, play a key role in mediating the immune defense through the cell- and antibody-mediated immune response, respectively. Moreover, the balance between Th1 and Th2 type cy-

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tokines is believed to determine the appropriateness of the immune response. The overexpression of Th2 type cytokines may contribute to production of IgE antibodies, which can elicit an immediate allergic response, for instance classical milk protein allergy reaction, in an immunocompromised subject (Rengarajan et al., 2000; Donkor et al., 2012).

Most bovine milk proteins can potentially bind with protein-specific antibodies. The ability of protein antigens to bind with IgG antibodies is termed antigenicity, whereas binding with IgE antibodies mostly result in allergenicity in humans. The bovine or cow milk protein allergy is the most prevalent food allergy among infants (2–6%). The caseins and β-LG usually act as the major allergens, whereas other milk proteins such as α-LA, BSA, and immunoglobulins can also be involved in milk protein allergy; however, their degree of involvement differs mostly from one protein fraction to the other (Bogahawaththa et al., 2017a). It is also well established that various heat treatments applied in the thermal processing of milk in the dairy industry can potentially denature native milk proteins, including unfolding and aggregation of native structure, which could subsequently modify the immunogenic epitopes and or their accessibility leading to altered immunogenicity (Bogahawaththa et al., 2017a).

The immunogenicity of native bovine milk proteins, in terms of their ability to provoke T cell-mediated immune response in human, has been studied to a certain extent using some individual protein fractions (e.g., β-LG, BSA; Vocca et al., 2011) and milk protein sources (e.g., skim milk; Opatha Vithana, 2012). However, the immunogenicity of various protein mixtures (e.g., β-LG and α-LA) appears to be unknown. Although several studies reported on the effect of thermal processing on altered antigenicity and allergenicity of bovine milk proteins (Bu et al., 2013), it is largely unknown how thermal processing affects the ability of various bovine milk proteins and their mixtures to provoke T cell-mediated immune responses in relation to protein denaturation. Thus, the present study aimed to examine the immunogenicity (in the form of ability and capacity of provoking Th cell-mediated immune response in vitro) of various native and thermally processed bovine milk protein stimulants (at their natural concentrations), such as fractionated major proteins, their mixtures, native whey, and skim milk, using human PBMC and analyzing the secretion of associated Th type cytokines. We also expected to be able to identify possible associations between the altered immunogenicity affected by thermal processing and the level of protein denaturation.

MATERIALS AND METHODS

Materials and Preparation of Protein Samples

Skim Milk and Native Whey. Murray Goulburn Co-operative (Laverton North, Victoria, Australia) provided raw bovine milk on 2 separate occasions. Upon arrival, the raw milk was skimmed by centrifugation (Avanti J-26XP, Beckman Instrument Australia Pty. Ltd., Gladesville, New South Wales, Australia) at 3,500 × *g* for 20 min at 20°C. An aliquot of skim milk was set aside (−20°C) for further experiments (S1 in Figure 1), and the remaining portion of the skim milk was divided into 2 parts used to prepare the native whey and caseins separately. The outline of the steps followed for the preparation of various fractionated proteins and protein mixtures is shown in Figure 1. The pH of the skim milk was adjusted to 4.6 using 0.1 *M* HCl and then the precipitated caseins were separated from the whey by centrifugation (Avanti J-26XP centrifuge, Beckman Instrument Australia Pty Ltd.) at 30,000 × *g* for 2 h at 20°C as explained previously (Bogahawaththa et al., 2017b). The pH of the resultant whey was readjusted to 6.7 using 0.1 *M* NaOH, which was the pH of the fresh raw milk. An aliquot of purified native whey was stored (−20°C) for further experiments (S2 in Figure 1), whereas the rest was used for the fractionation of native whey proteins as per our previous work (Bogahawaththa et al., 2017b).

Fractionation of Whey Proteins. In brief, 100 μL of the native whey was injected at a time with mobile phase (0.05 *M* sodium phosphate buffer at pH 7, including 0.3 *M* sodium chloride) into a size exclusion chromatography (SEC) column (Biosep SEC-s2000, Phenomenex Australia Ply Ltd., Lane Cove West, New South Wales, Australia), which was mounted to a fast protein liquid chromatography (FPLC) system (GE Healthcare Australia Pty. Ltd., Parramatta, New South Wales, Australia). The different whey proteins, such as IgG, BSA, β-LG, and α-LA, were eluted at different retention times with mobile phase and collected separately using a Frac-950 fraction collector (GE Healthcare Australia Pty. Ltd.). An RVC 2–18 rotational vacuum concentrator (John Morris Scientific, Deepdene, Victoria, Australia) was then used to concentrate the fractionated proteins by evaporating the mobile phase at 30°C.

Caseins. The other portion of skim milk was used to separate the caseins from the whey by ultracentrifugation (Beckman L-70 ultracentrifuge, Beckman Instrument Australia Pty Ltd.) at 100,000 × *g* for 1 h at 22°C without adjusting the pH (O'Mahony and Fox,

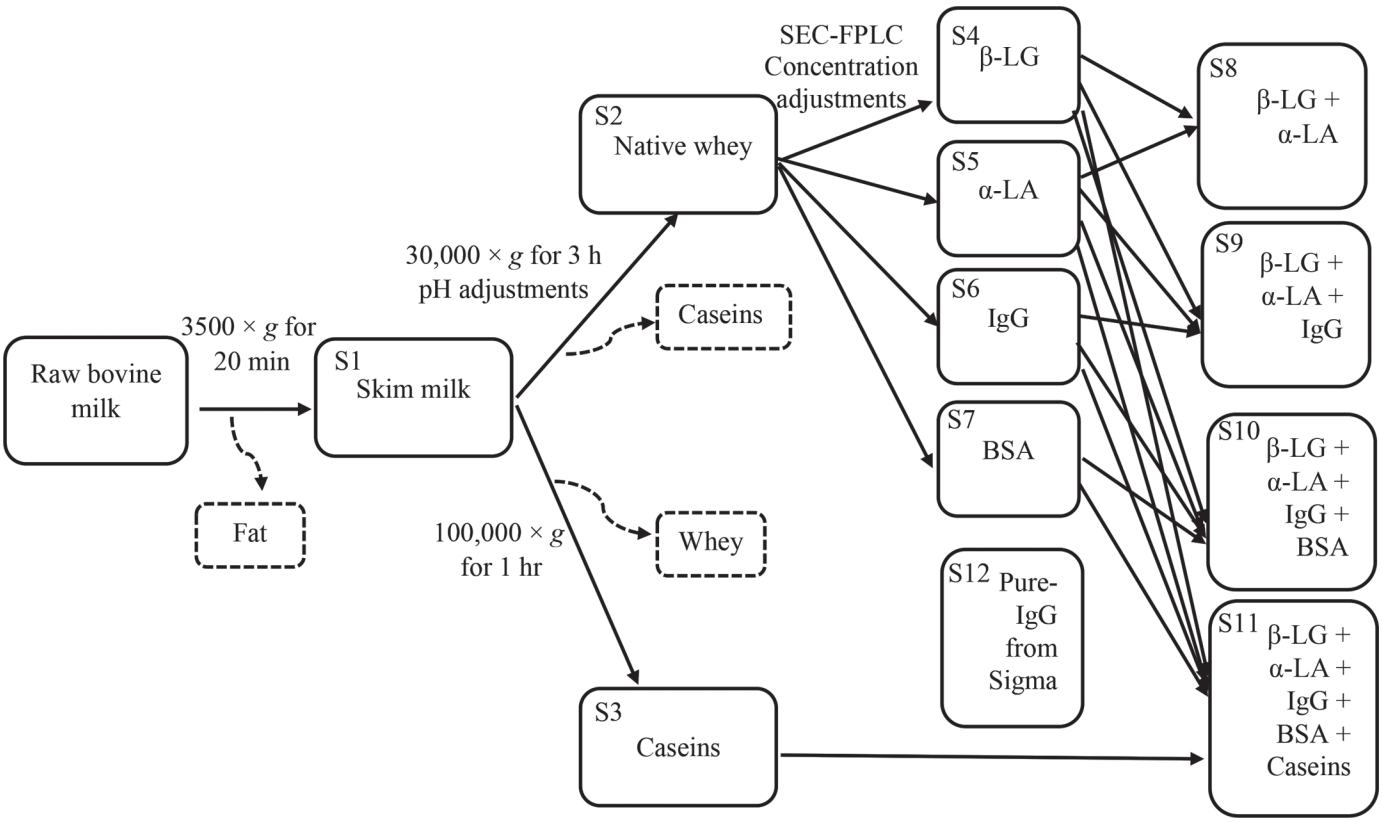

Figure 1. Outline of the steps followed for the fractionation of various bovine milk proteins and preparation of the samples (S1–12). SEC-FPLC = size exclusion chromatography fast protein liquid chromatography; Sigma = Sigma-Aldrich, St. Louis, MO.

2013). The casein pellet was removed from the centrifuge tube followed by 2 washing steps (4,000 × *g* for 10 min at 22°C) with simulated milk ultrafiltrate (**SMUF**; Rosmaninho and Melo, 2006) and then resuspended in SMUF with continuous stirring for 48 h at 4°C.

Preparation of Protein Samples. The 11 protein samples were then prepared including individual proteins alone (5 samples, S3–7: caseins, β-LG, α-LA, IgG, and BSA), by mixing different whey protein fractions together based on the descending order of their concentration in milk (3 samples, S8–10: β-LG + α-LA, β-LG + α-LA + IgG, and β-LG + α-LA + IgG + BSA), a mixture of caseins and 4 different whey protein fractions (1 sample, S11: caseins + β-LG + α-LA + IgG + BSA), and the native whey and skim milk (2 samples, S1–2). The concentration of all the individual protein samples and protein mixtures were already readjusted using SMUF to their corresponding concentrations in skim milk, caseins, β-LG, α-LA, IgG, and BSA at 2.6, 0.32, 0.12, 0.08, and 0.04% (wt/wt), respectively (Dupont et al., 2013), as described previously (Bogahawaththa et al., 2017b). However, the IgG sample fractionated from the native whey was found to contain some BSA (~20% of the total IgG quantified by SDS-PAGE). Thus, the purified bovine IgG was purchased (Sigma-Aldrich, St. Louis, MO) and prepared with another pure IgG sample 0.08% (wt/wt) in SMUF, making the total number of samples 12 (S12 in Figure 1).

Treatment of Samples

All of the samples (12) were divided into 3 aliquots. One aliquot was the control and the other 2 aliquots were subjected to 2 heat treatments separately. The 2 heat treatments applied were 72°C/15 s and 100°C/30 s. The 72°C/15 s is considered HTST, and the effect of 100°C/30 s treatment on the level of denaturation of β-LG in skim is reported to be comparable to UHT processing (Dannenberg and Kessler, 1988). Both treatments were performed in a CS/CR rheometer (MCR 301, Anton Paar GmbH, Ostfildern, Germany), as previously described (Bogahawaththa et al., 2017b), under a constant shear (1,000 s^{-1}) and pressure (~250 kPa).

Fourier Transform Infrared Spectroscopy

Soon after performing each treatment, all the samples including controls were analyzed by a PerkinElmer

Frontier Fourier Transform Infrared (**FTIR**) spectrometer (PerkinElmer, Waltham, MA) with a combined software of IR Solution (version 1.40, Shimadzu Corporation, Kyoto, Japan), as previously described (Bogahawaththa et al., 2017b). The samples spectra (range 4,000–600 cm^{-1}) were obtained in the absorbance mode by subtracting the background. With the aid of the software, the second derivative of every spectrum was obtained to enhance the resolution. The peaks, which corresponded to the protein secondary structure, were studied within broad amide I region (1,600–1700 cm^{-1}).

SDS-PAGE

Both nonreducing and reducing SDS-PAGE were performed as explained previously (Bogahawaththa et al., 2017b) for all treated and control samples, which were already mixed with SDS sample buffer (100 μL of sample in 1 mL of sample buffer). β-Mercaptoethanol was used to reduce the covalent bonds for reducing SDS-PAGE. Commercial whey proteins (β-LG, α-LA, IgG, and BSA) recommended for electrophoresis and the broad-range prestained SDS-PAGE standards (SeeBlue Plus2 Pre-stained Protein Standard, Thermo Fisher Scientific, Scoresby, VIC, Australia) 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).

Isolation of Human PBMC

Human PBMC are widely used to investigate the effect of food bioactives on immune cells in vitro (Eriksen et al., 2008; Kleiveland, 2015). Furthermore, the use of PBMC has been recommended as an effective method, in comparison to the use of T cell lines or clones, for studying the immune response of human T cells in response to bovine milk proteins (Vocca et al., 2011). The ethics application (ID: HRE16–058) for the use of human PBMC in the current study was accepted by the Victoria University Human Research Ethics Committee.

The PBMC were isolated from buffy coats obtained from healthy individuals (Australian Red Cross Blood Services, Melbourne, Australia) following an established method in our laboratory (Donkor et al., 2012; Bogahawaththa et al., 2018) with some modifications. Briefly, about 60 mL of buffy coats were diluted with 1× PBS (1:2 volume ratio) and layered gently on Ficoll-Paque Plus (GE Healthcare). After centrifugation (400 × *g* for 30 min at 18°C with no break; Sorvall-RT7 centrifuge, DuPont, Newtown, CT), the PBMC layer was collected and immediately washed with RPMI-1640 (Sigma-Aldrich, Castle Hill, New South Wales, Australia) at 400 × *g* for 10 min at 18°C with half break. After 2 more washing steps (250 × *g* for 10 min at 4°C with half brake) with RPMI-1640, cells were adjusted to 2.5×10^7 cells/mL in the recovery cell culture freezing medium (Thermo Fisher Scientific Australia Pty Ltd.) and stored in liquid nitrogen until use.

Stimulation of PBMC

The stimulation of PBMC was performed as previously reported (Tafaro et al., 2007; Bogahawaththa et al., 2018) with some modifications. After thawing and removing of cells from freezing medium, the cells were resuspended in RPMI-1640 supplemented with 10% fetal bovine serum, qualified and heat inactivated (Thermo Fisher Scientific Australia Pty Ltd.), and antibiotic-antimycotic solution (Sigma-Aldrich) at 3×10^6 cells/mL. The stimulation of PBMC was conducted in several 24-well flat-bottomed polystyrene microtiter plates with final concentration of 1×10^6 cells/mL in the presence of different protein stimulants (100 μL/mL) at 37°C in 5% CO_2 for 96 h of incubation. Lipopolysaccharide from *Escherichia coli* O111:B4 (Sigma-Aldrich) was used to stimulate PBMC at the concentration of exactly 1 μg/mL as a positive control, whereas unstimulated PBMC in RPMI-1640 were also tested to determine the basal cytokine production. Supernatants of all the wells were collected by centrifugation (at 400 × *g* for 10 min at 18°C) after the incubation period (96 h) and stored at −20°C until the cytokines were quantified.

Cytokine Assays

An ELISA (Thermo Fisher Scientific Australia Pty Ltd.) was used to determine the cytokine concentrations of the supernatants including IL-4, IL-10, IL-12 (p70), IFN-γ, and IL-17A. The assays were performed according to the manufacturer's instructions (https://www.thermofisher.com/elisa/product/).

Statistical Analysis

All the experiments were replicated by obtaining 2 batches of raw bovine milk, leaving a gap of 2 wk. All the samples were also subsampled to obtain at least 4 independent observations ($n \geq 4$). The results were analyzed as a randomized split plot design with protein stimulants as the main plot and heat treatment as a subplot using a General Linear Model of SAS statistical program. The level of significance was preset at $P \leq 0.05$. Tukey's Studentized Range (HSD) test was used for multiple comparisons of means of cytokines secre-

tion. The mean of at least 4 independent observations ($4 \geq$ n) was considered for comparison purposes.

RESULTS AND DISCUSSION

In Vitro Immunogenicity of Bovine Protein Fractions, Whey, and Skim Milk

The bovine milk proteins are capable of provoking protein-specific Th cell responses in the human subjects regardless of health or milk protein sensitivity (Schade et al., 2000; Vocca et al., 2011). The T cells are believed to control the isotype switching of antigen-specific B cells to IgE, which can lead to initiation of allergic reaction as well as induction of the tolerance (Ruiter et al., 2006). In vitro immunogenicity of various bovine milk protein stimulants was studied based on the response of Th cells, including Th1, Th2, and Th17, in human PBMC by analyzing the secretion of associated cytokines. These results could be linked to immune responses induced by proteins including potential allergenicity instead of testing in vitro antigenicity by the ELISA technique as in many studies (Kleber et al., 2004; Rahaman et al., 2015). The protein antigens mostly bind with IgG antibodies (antigenicity) in every individual regardless of their sensitivity to these proteins (Abbas et al., 2014). In addition, analysis of antigenicity does not provide specific information about the nature of immune response elicited, which mainly governs the reaction of human body to a particular protein (Abbas et al., 2014). Furthermore, due to ethical reasons and a greater risk of pathogen presence, the controlled human studies (in vivo) have not been conducted yet (Perdijk et al., 2018) to establish how native proteins or raw milk modulate the human immune system differently in comparison to those of heat-treated proteins. Thus, the in vitro method would be useful to generate background information for conducting in vivo human studies in the future that would potentially examine the inverse association between consumption of raw milk and development of allergy and asthma (Loss et al., 2011).

Out of 5 cytokines quantified, IFN-γ, IL-4, and IL-17 are the signature-cytokines of Th1, Th2, and Th17, respectively (Raphael et al., 2015). Furthermore, expression of IL-12 (p70) and IL-10 were also quantified in relation to Th1 and Th2 cytokines, respectively (Raphael et al., 2015); however, the production of IL-4 and IL-12 (p70) was not at a detectable level in response to all the protein stimulants quantified by ELISA. Comparably, previous studies also did not detect IL-4, which is considered to elicit IgE-mediated allergy, even when the PBMC from milk-allergic subjects were stimulated with various bovine milk proteins (Benlounes et al., 1996; Vocca et al., 2011). The other 3 types of cytokines, such as IFN-γ, IL-10, and IL-17A, were detected in the supernatants of PBMC at various levels in response to all 12 protein stimulants, except IFN-γ in the supernatant stimulated by BSA, as shown in Table 1.

In terms of Th1 or Th2 cytokines (IFN-γ or IL-10), only 5 different protein stimulants, such as skim milk, native whey, caseins, α-LA, and pure-IgG, were able to stimulate significantly ($P < 0.05$) higher mean cytokine production in comparison to the basal cytokine concentration (RPMI). The expression of IL-10 (42.2 pg/mL) in response to β-LG was also substantially higher than the respective basal cytokine production. The mean cytokine concentrations of the unstimulated

Table 1. Cytokine profile secreted by human peripheral blood mononuclear cells (PBMC) in response to various milk protein stimulants and controls[1]

	Cytokine concentration (pg/mL)		
Stimulant	IL-10	IFN-γ	IL-17A
LPS (positive control)	309.4 (±19.5)^{A}	627.4 (±56.4)^{A}	158.6 (±18.8)^{A}
IgG (pure-IgG)	298.4 (±8.2)^{AB}	384.2 (±12.2)^{B}	79.2 (±3.8)^{B}
Caseins	269.9 (±4.1)^{B}	115.0 (±11.5)^{D}	13.8 (±0.2)^{C}
α-LA	268.6 (±14.4)^{B}	216.6 (±4.9)^{C}	69.9 (±26.6)^{B}
Skim milk	179.8 (±4.5)^{C}	46.8 (±0.2)^{E}	6.8 (±5.7)^{C}
Native whey	136.8 (±4.8)^{D}	12.7 (±2.5)^{E}	10.75 (±5.2)^{C}
β-LG	42.2 (±15.9)^{E}	4.1 (±0.9)^{E}	6.25 (±0.9)^{C}
β-LG + α-LA + Ig + BSA	20.8 (±1.2)^{EF}	3.2 (±2.6)^{E}	7.4 (±3.8)^{C}
RPMI (basal cytokine level)	18.5 (±0.2)^{EF}	32.4 (±3.3)^{E}	28.6 (±1.1)^{C}
IgG (contains ∼20% of BSA)	13.8 (±2.6)^{EF}	1.5 (±0.1)^{E}	2.7 (±0.6)^{C}
Caseins + β-LG + α-LA + Ig + BSA	13.2 (±5.2)^{EF}	12.1 (±0.6)^{E}	2.2 (±3.1)^{C}
β-LG + α-LA	11.5 (±2.9)^{EF}	1.0 (±0.5)^{E}	0.75 (±1.1)^{C}
BSA	11.3 (±0)^{E}	0.0 ^{E}	1.2 (±0.8)^{C}
β-LG + α-LA + IgG	5.0 (±1.4)^{F}	5.8 (±0.2)^{E}	4.25 (±5.6)^{C}

$^{A–F}$Means in the same column with different superscripts are significantly different ($P < 0.05$).

[1]Values are presented as the mean cytokine concentration (pg/mL) of at least 4 independent measurements ($4 \geq$ n) plus or minus SD.

supernatant (supernatant of PBMC in RPMI medium alone) were considered the basal cytokine concentrations and were recorded at 18.5, 32.4, and 28.6 pg/mL of IL-10, IFN-γ, and IL-17A, respectively. Thus, only skim milk, native whey, caseins, α-LA, pure-IgG, and β-LG appeared to be the potent immunogens under the current experimental conditions, and were considered for further analysis.

The rest of the stimulants appeared relatively less immunogenic. They were mostly the protein mixtures prepared from the protein fractions (β-LG + α-LA, β-LG + α-LA + IgG, β-LG + α-LA + IgG + BSA, and caseins + β-LG + α-LA + IgG + BSA) and the IgG containing ∼20% of BSA. This generated some interesting information to direct further studies toward the preparation of less immunogenic bovine milk-based products by manipulating the natural composition of the milk proteins, which are suitable for the bovine milk protein-sensitive subjects. A study reported that the modified bovine milk, which possessed the modified caseins to whey proteins ratio of 40:60 (this ratio is usually 80:20 in natural bovine milk and 40:60 in human breast milk), demonstrated potentially fewer allergic properties than the native bovine milk in a murine model of atopy (Lara-Villoslada et al., 2005). This modified casein-to-whey protein ratio is suggested to induce biochemical interactions between caseins and β-LG, which in turn facilitate the digestion of β-LG leading to reduced allergenicity. Another study suggested the possibility of producing hypoallergenic dairy products including only the heat-denatured whey proteins (≥100°C) without any caseins (Kilshaw et al., 1982).

Cross and Gill (2000), in a review, stated that the immunogenicity and immunomodulatory properties of the individual protein fractions become more potent once they are purified from the primary protein source. On the other hand, those effects could be either diminished or remain undetectable when they are present in milk or milk products. Interestingly, in the current study, we also observed some significant ($P < 0.05$) differences between the immunogenicity of the fractionated proteins, their protein mixtures, and their protein sources (Table 1). For instance, the mean cytokine productions of IFN-γ and IL-10 in response to α-LA were significantly ($P < 0.05$) higher than that of β-LG + α-LA. The PBMC stimulated by pure IgG secreted significantly higher amounts of IFN-γ and IL-10 than the IgG containing some BSA. A significantly ($P < 0.05$) higher level of IFN-γ and IL-10 was secreted by PBMC in response to bovine caseins than the caseins with other 4 whey proteins (caseins + β-LG + α-LA + IgG + BSA). On the contrary, the BSA, as a purified protein fraction, could not stimulate a substantial cytokine response in comparison to the basal cytokine levels. Vocca et al. (2011) also reported that the secretion of IFN-γ and IL-10 by human PBMC in response to BSA was either undetectable or nonsignificant.

Although the total protein concentrations were higher in all the protein mixtures than those of individual protein stimulants discussed above, all the protein mixtures showed the diminished immunogenic capacity in comparison to the respective individual proteins. Cross and Gill (1999) reported that the modified bovine whey protein concentrate can modulate the proliferation of murine T and B cells in a dose-dependent manner (1.6 to 1.5 μg/mL), where high whey protein concentrate concentrations (≥0.4 mg/mL) significantly suppressed both T and B cell proliferation. In contrast, in the current study, the secretion of IL-10 was significantly ($P < 0.05$) greater by stimulated PBMC in response to mixture of 4 main whey protein fractions (β-LG + α-LA + IgG + BSA), native whey, and skim milk, in the same order, although the total protein concentration also increased in that order. This highlighted the fact that not only protein concentration, but also the nature and composition of the proteins, can possibly modulate T cell-mediated immune response differently.

The PBMC stimulated with pure IgG and α-LA secreted substantially higher concentrations of all 3 types of cytokines than β-LG and caseins, which are considered the major milk protein antigens or allergens (Lifschitz and Szajewska, 2015). This could be ascribed to the ability of these protein fractions to modulate the proliferation or activation of lymphocytes differently (Reyes-Díaz et al., 2017) apart from their ability to bind with IgG or IgE antibodies. For example, the bovine αs_1-, β-, and κ-CN were capable of inhibiting the proliferative responses of the mouse lymphocytes (Otani and Hata, 1995). The immune responses of Th and B lymphocytes were more enhanced by α-LA than caseins in mice (Bounous and Kongshavn, 1985). The substantially lower concentration of cytokines stimulated by β-LG, in comparison to pure-IgG, α-LA, and caseins observed in our study could be attributed to the considerably lower level of proliferation of immune cells stimulated by β-LG in healthy PBMC (Vocca et al., 2011) and the activation of regulatory T cells and associated suppressive mechanisms in healthy individuals (Akdis et al., 2004) in comparison to milk-allergic subjects. This emphasized the fact that understanding not only the ability of various bovine milk proteins to bind with antibodies (IgG or IgE), but also their ability to modulate the functions of human lymphocytes, would be important when developing hypoimmunogenic milk products.

Bovine milk proteins are known to induce the Th2 polarized (Th1 < Th2) immune response, leading to

IgE-mediated milk allergy in atopic subjects (Tsitoura and Tassios, 2006). In the current study, skim milk, caseins, native whey, α-LA, and β-LG stimulated the PBMC to secrete a substantially lower level of IFN-γ (Th1 type cytokine) than IL-10 (Th2 type cytokine). The expression of IFN-γ or IL-10 in response to skim milk, caseins, native whey, α-LA, and β-LG were reported as 46.8/179.8, 115.0/269.9, 12.7/136.8, 216.6/268.6, and 4.1/42.2 pg/mL, respectively. Only pure IgG stimulated the PBMC to secrete a substantially higher level of IFN-γ (384.2 pg/mL) than IL-10 (298.4 pg/mL). Vocca et al. (2011) observed a higher number of IL-10 producing cells in human PBMC of both milk protein allergic and healthy subjects than the IFN-γ–producing cells in response to β-LG, which was in line with our results.

Only 2 protein stimulants, α-LA and pure IgG, were capable of inducing the PBMC to secrete significantly higher ($P < 0.05$) amounts of IL-17A, recorded at 69.9 and 79.2 pg/mL, respectively, than its basal level (28.6 pg/mL). Dhuban et al. (2013) observed a significantly lower level of IL-17A production in Th17 cells of food allergic subjects than healthy controls in response to various food antigens. A trend toward negative correlation has been reported between the level of IL-17A in whole blood and sensitization to food antigens (Herberth et al., 2010). The ability of bovine IgG in stimulating the human PBMC to express substantially higher level of Th1 and Th17 type cytokines (e.g., IFN-γ and IL-17A, respectively) in the current study hinted at its ability in mediating adverse immune responses relating to milk allergy. However, the real effects of these results on milk protein allergy can only be established through comprehensive in vivo studies in consideration of the fate of bovine milk proteins in human gastrointestinal tract and the complex interplay of immune responses in the atopic subjects.

Effects of Thermal Processing on Immunogenicity of Bovine Milk Proteins in Relation to Protein Denaturation

In the current study, 2 heat treatments (72°C/15 s and 100°C/30 s) were applied to all the 12 protein stimulants, whereas the untreated protein samples at room temperature (~20°C) were considered controls. The 6 protein stimulants, skim milk, native whey, caseins, β-LG, α-LA, and pure IgG, which induced substantially high cytokine profiles, displayed variations in expression of cytokines depending on the different heat treatments, as shown in Figure 2-A, B, C, D and E. The remaining portion of the protein stimulants, which demonstrated relatively less immunogenic capacity, did not display substantial differences in their cytokine profiles even after heat treatments (data not shown). This indicated the potential of applying the traditional thermal methods to process those protein mixtures largely without affecting their immunogenicity.

The skim milk subjected to 100°C/30 s treatment stimulated a significantly ($P < 0.05$) different cytokine profile than that of the control in terms of all 3 different cytokines (IL-10, IFN-γ, and IL-17A). However, the cytokine profile induced by 72°C/15 s treated skim milk appeared mostly similar to that of the control except the lower production of IFN-γ. Comparably, the native whey subjected to 100°C/30 s treatment induced the secretion of IL-10 and IL-17A significantly different than that of the control, whereas the native whey treated by 72°C/15 s demonstrated an almost similar cytokine profile in comparison to its control. These results appeared to coincide with the level of protein denaturation in skim milk and whey, respectively. Our FTIR (data not shown) and SDS-PAGE results (Figure 3) of the current study demonstrated that the 72°C/15 s treatment resulted in no or minimal denaturation of the native milk proteins. Our previous study also reported comparable results in relation to all the major whey proteins (β-LG, α-LA, IgG, and BSA) and native whey (Bogahawaththa et al., 2017b). The mostly unchanged cytokine profiles displayed by 72°C/15 s treated skim milk and whey in comparison to those of controls could be attributed to no or little denaturation of the native proteins affected by that treatment. However, the denaturation of minor whey proteins, including IgG and BSA, to a certain extent has been reported when skim milk was subjected the same treatment elsewhere (Patel et al., 2006). Moreover, other in vivo studies reported the alteration of immunogenicity of bovine milk upon processing by pasteurizing treatments such as 72°C/15 s (Høst and Samuelsson, 1988) and 74°C/15 s (Feng and Collins, 1999). These contradicting results in comparison to the current study could be attributed to the variations in the application of the heat treatments, including variation in heating rates and the methods used for assessing the immunogenicity.

The significantly different cytokine profiles induced by skim milk and whey, after 100°C/30 s treatment in comparison to those of controls, can possibly be related to the denaturation of heat-labile milk proteins affected by that treatment. The SDS-PAGE results of the current study displayed (Figure 3) that 100°C/30s treatment resulted in denaturation of β-LG and further formation of the protein aggregates via covalent interactions with the contribution of other whey proteins and caseins (e.g., κ-CN), where available. These findings on protein denaturation and aggregation agreed with the available literature (Wijayanti et al., 2014; Bogahawaththa et al., 2017b).

The cytokine profiles stimulated by β-LG, subjected to 72°C/15 s and 100°C/30 s treatments, were different from its control only in terms of expressing IL-10 ($P < 0.05$). Denaturation of β-LG was not observed in our results at 72°C/15 s, but treatment at 100°C/30 s resulted in denaturation and covalent aggregation of β-LG, as reported previously (Wijayanti et al., 2014). The pure IgG also demonstrated comparable results. At least 2 different cytokines were significantly different in the cytokine profiles induced by 72°C/15 s and 100°C/30 s treated samples in comparison to that of the control, whereas we found no notable denaturation of IgG affected by 72°C/15 s treatment. On the other hand, the treatment at 100°C/30 s caused denaturation and induced covalent aggregation of IgG, as observed previously (Bogahawaththa et al., 2017b). It is thus unlikely to relate the altered cytokine profiles induced by 72°C/15 s treated β-LG and pure IgG with the denaturation of the respective proteins. However, 72°C appeared to be a critical temperature limit, which would initiate the denaturation of IgG contributing to the altered immunogenicity (Li et al., 2005). The heat-aggregated bovine IgG was previously demonstrated to have altered immunogenicity, in comparison to its native form, with stimulation of human PBMC (Kulczycki et al., 1987).

Moreover, the cytokine profiles induced by α-LA and caseins, which were subjected to 72°C/15 s and 100°C/30 s treatments, were noticeably different from those of the controls. At least the expression of 2 types of cytokines were significantly different ($P < 0.05$) than the control; however, α-LA and caseins are generally more heat-stable than other milk proteins (Wijayanti et al., 2014; Huppertz, 2016). We observed no apparent formation of covalent complexes in our SDS-PAGE results of both α-LA and caseins, even at 100°C for 30s, but the reduction of some secondary structures in α-LA (α-helix at 1,651 cm^{-1} and β-sheets at 1,636 cm^{-1}) was observed by FTIR (data not shown), indicating some unfolding of the protein.

It is established that the heat-induced denaturation of milk proteins can modify IgG- or IgE-binding epitopes, which may result in enhanced, diminished, or unchanged antigenicity or allergenicity (Bogahawaththa et al., 2017a). In parallel, heating may modify the T cell-specific epitopes present in bovine milk proteins

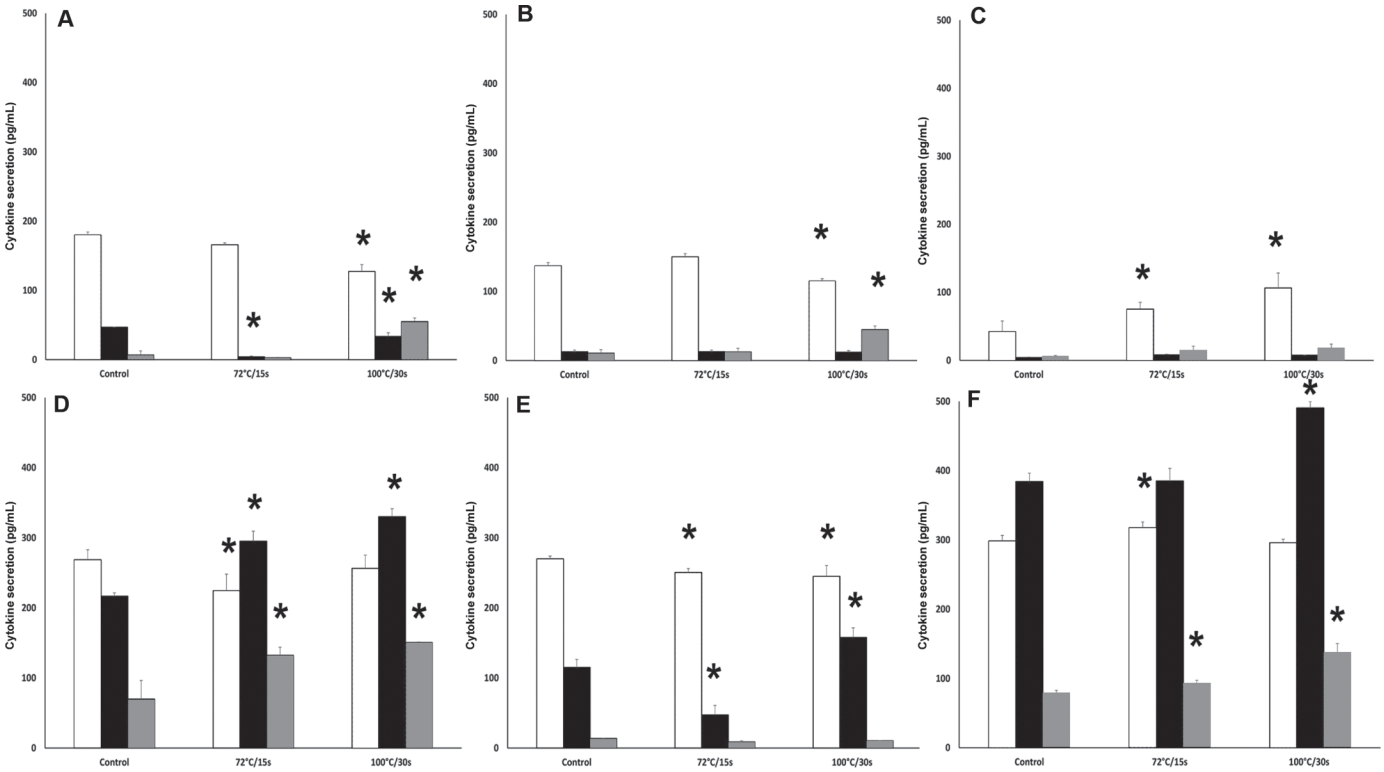

Figure 2. Production of cytokines, IL-10 (white), IFN-γ (black), and IL-17A (gray), by human peripheral blood mononuclear cells (PBMC) stimulated by (A) skim milk, (B) native whey, (C) β-LG, (D) α-LA, (E) caseins, and (F) pure IgG. Control was untreated, and 72°C/15s and 100°C/30s are the 2 heat treatments applied, which appear in the same order. Data are expressed as the mean cytokine secretion (pg/mL) of at least 4 independent measurements ($4 \geq n$) plus or minus SD. *Mean cytokine secretion induced by the treatment is significantly different than that of the control ($P < 0.05$).

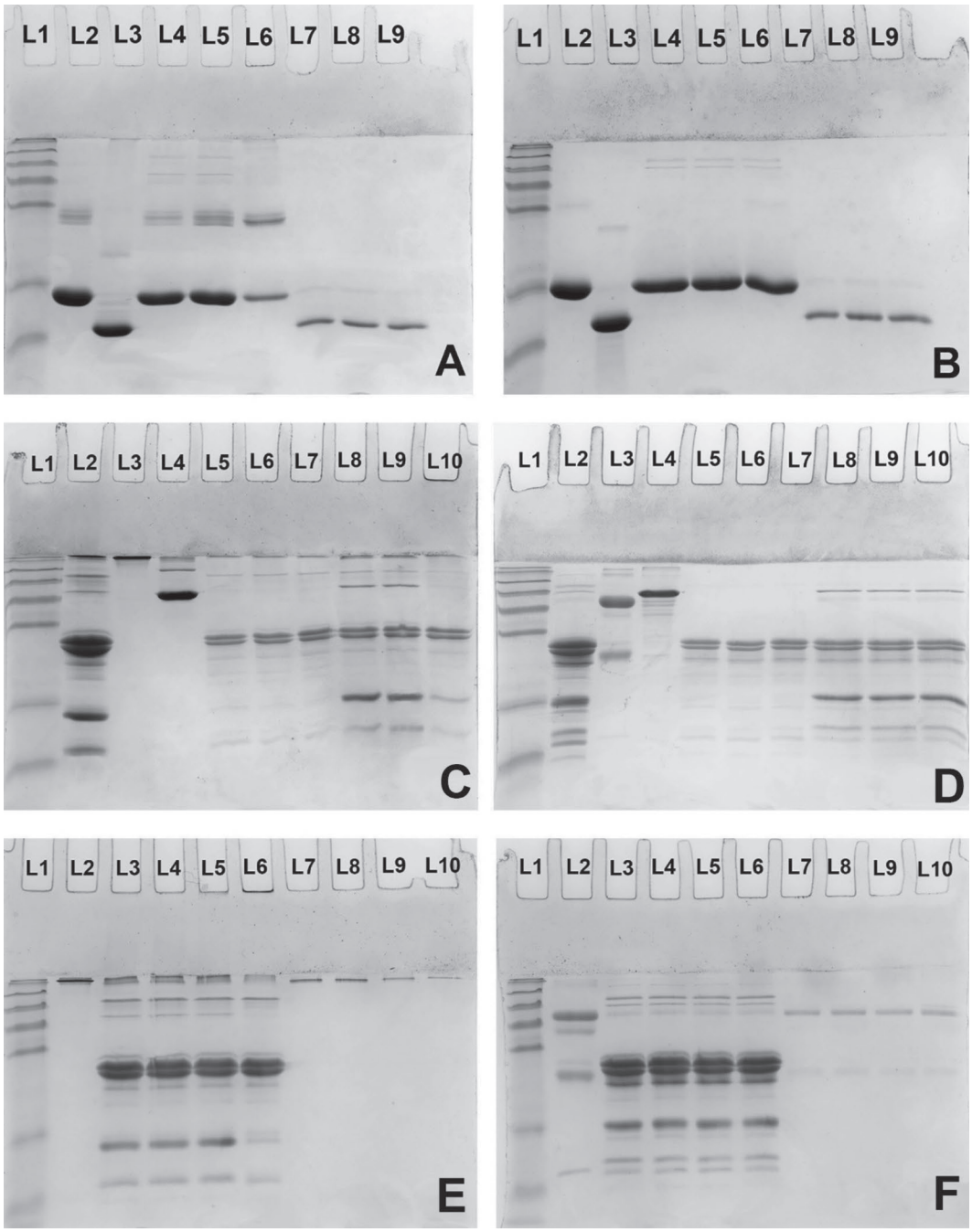

Figure 3. Nonreducing (A) and reducing (B) SDS-PAGE analysis of β-LG and α-LA. Lanes are designated as L1 = molecular weight markers; L2 = β-LG standard; L3 = α-LA standard; L4 = β-LG control; L5 =β-LG 72°C/15 s treated; L6 = β-LG 100°C/30 s treated; L7 = α-LA control; L8 = α-LA 72°C/15 s treated; L9 = α-LA 100°C/30 s treated. Nonreducing (C) and reducing (D) SDS-PAGE analysis of caseins and caseins + β-LG + α-LA + IgG + BSA. Lanes are designated as L1 = molecular weight markers; L2 = skim milk control; L3 = IgG standard; L4 = BSA standard; L5 = caseins control; L6 = caseins 72°C/15 s treated; L7 = caseins 100°C/30 s treated; L8 = caseins + β-LG + α-LA + IgG + BSA control; L9 = caseins + β-LG + α-LA + IgG + BSA 72°C/15 s treated; L10 = caseins + β-LG + α-LA + IgG + BSA 100°C/30 s treated. Nonreducing (E) and reducing (F) SDS-PAGE analysis of skim milk and pure-IgG. Lanes are designated as L1 = molecular weight markers; L2 = IgG standard; L3 = skim milk control; L4 = skim milk 72°C/15 s treated; L5 = skim milk 80°C/2 min treated*; L6 = skim milk 100°C/30 s treated; L7 = IgG control; L8 = IgG 72°C/15 s treated; L9 = IgG 80°C/2 min treated*; L10 = IgG 100°C/30 s treated. *The treatments have not been considered for this study.

and modulate the functions of T cells differently (Ruiter et al., 2006). In general, the altered immunogenicity observed in most of the protein stimulants followed by 100°C/30 s treatment could be related to the effect on T cell epitopes, such as modifications, fragmentation, masking, unmasking, and formation of new epitopes, through the unfolding of native protein structure, inter- or intraprotein aggregation, and probably interactions with other nonprotein constituents (e.g., lactose). We have already reviewed the comparable findings in relation to IgG- or IgE-binding epitopes (Bogahawaththa et al., 2017a). Furthermore, Roth-Walter et al. (2008) reported that the absorption path of heat-aggregated bovine β-LG and α-LA in intestinal epithelium was different from the path of those of the native proteins, which can modulate the functions of T cells differently and provoke Th2-type immune response in mice. However, further investigations are required, in consideration of digestion, absorption, and the complex interplay of immune response, to establish the associations between the protein denaturation and the alteration of immunogenicity and to elucidate how immunogenicity can alter without apparent denaturation of the proteins affected by heating.

CONCLUSIONS

Bovine skim milk, its main protein fractions (e.g., whey and caseins), and purified major whey proteins such as β-LG, α-LA, and IgG are capable of provoking notable immune responses in human PBMC at various capacities. When 2 or more fractionated whey proteins or caseins or both are mixed together, the immunogenic capacity of those protein mixtures appear to be weak and not to be affected by thermal processing. This could be a useful strategy to produce less immunogenic dairy products from the fractionated milk proteins, suitable for people with milk protein sensitivity. The immunogenicity of bovine milk proteins appears to depend on the factors such as the nature of the protein, protein concentration, composition of the protein mixture, and the level of protein denaturation. The altered immunogenicity demonstrated by most of the potent protein stimulants subjected to 100°C/30 s treatment, in comparison to their controls, appears to be associated with the level of protein denaturation, possibly due to the modifications in T cell epitopes. The 72°C/15 s treatment did not seem to denature all the protein stimulants notably, but the immunogenicity appears substantially altered in some of the milk stimulants, including the heat-stable proteins such as α-LA and caseins. Further studies are thus required to elucidate the effect of thermal processing on the altered immunogenicity of bovine milk proteins in relation to protein denaturation and to establish the real effects of native and thermally processed milk protein stimulants on the human immune system in vivo.

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Chapter 6: Comparison between thermal pasteurization and high pressure processing of bovine skim milk in relation to denaturation and immunogenicity of native milk proteins

This chapter has been published as a research paper entitled “Comparison between thermal pasteurization and high pressure processing of bovine skim milk in relation to denaturation and immunogenicity of native milk proteins” by Dimuthu Bogahawaththa, Roman Buckow, Jayani Chandrapala and Todor Vasiljevic in the peer-reviewed journal, Innovative Food Science and Emerging Technologies, 47 (2018) 301–308. https://doi.org/10.1016/j.ifset.2018.03.016

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Comparison between thermal pasteurization and high pressure processing of bovine skim milk in relation to denaturation and immunogenicity of native milk proteins

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ABSTRACT

High pressure processing (HPP), conducted at 400, 500 or 600 MPa for 15 min at 30 °C, of raw skim milk was studied in comparison to high-temperature short-time (HTST) pasteurization (72 °C for 15 s), considering protein denaturation and immunogenicity. HTST pasteurization least impacted denaturation of native proteins leading to mostly unchanged milk immunogenicity. HPP resulted in denaturation of whey proteins, mostly β-lactoglobulin (BLG) and immunoglobulin G (IgG), and disturbed structure of the casein micelle. HPP at 600 MPa caused protein aggregation, involving mainly BLG and κ-casein, through thiol disulphide interactions. α-Lactalbumin (ALA) was least denatured. The balance between expression of T helper (Th)1 and Th2 type cytokines, which is believed to regulate adverse immune response, was initially shifted toward Th1 with increasing high pressure, then the immunogenic capacity of milk proteins diminished at 600 MPa. This could be related to exposure of T cell-specific linear epitopes followed by unfolding of protein structure firstly and masking of them by protein aggregation subsequently with increasing high pressure.

Industrial relevance: HPP of raw milk has been well studied as an alternative process for conventional thermal pasteurization, based on inactivation of microorganisms and extension of shelf life. However, HPP can denature native milk proteins leading to altered immunogenicity. Three recommended HPP conditions and a commercial HPP method were assessed in comparison to HTST pasteurization considering impact on denaturation of milk proteins and milk immunogenicity. Scientific findings of the current study would guide the industry to identify HPP conditions that not only achieve the required level of microbial inactivation and shelf life, but also to establish desired levels of native properties of milk and milk immunogenicity.

1. Introduction

Bovine milk is generally considered a balanced and nutritive food in the human diet. Although the controversies are raised, predominantly due to heterogeneity of the species, the nutritional importance of bovine milk in the human diet has been confirmed (Pereira, 2014). Cow milk protein allergy and lactose intolerance are regarded as common adverse reactions to milk consumption. Milk is one of the essential sources of proteins, which supplies high-quality proteins to humans in terms of their amino acid requirements, digestibility and bioavailability. Furthermore, milk proteins and their peptides perform various important biological roles in human health including immunomodulatory functions (Pereira, 2014).

Even though some health benefits are believed to be associated with the consumption of raw unprocessed milk, this practice is strongly discouraged in many countries due to health risks potentially caused by pathogenic microorganisms in raw milk. Milk is thus processed in the dairy industry mainly by thermal pasteurization in order to ensure the food safety and to extend the shelf life (Claeys et al., 2013). High-temperature short-time (HTST) is the most widely applied commercial thermal pasteurization method, which is usually performed at 72 °C for 15 s. Heat treatments, however, potentially result in denaturation of native milk proteins. HTST pasteurization has usually shown no or minimum impact on major milk proteins such as caseins, β-lactoglobulin (BLG), and α-lactalbumin (ALA) (Bogahawaththa, Chandrapala, & Vasiljevic, 2017b; Patel, Singh, Anema, & Creamer, 2006), while other

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important minor whey proteins such as immunoglobulin G (IgG), lactoferrin (LF), and bovine serum albumin (BSA) can possibly be affected (Patel et al., 2006). Furthermore, studies reported that thermal pasteurization can contribute to enhancement of allergenic properties of milk proteins (Høst & Samuelsson, 1988; Roth-Walter et al., 2008).

High pressure processing (HPP) in the range of 300–600 MPa is considered an alternative nonthermal method for milk pasteurization (Trujillo, Capellas, Saldo, Gervilla, & Guamis, 2002). Moreover, HPP appeared to preserve the native properties of food (e.g. organoleptic and nutritional qualities) and some important proteins (e.g., IgG) in milk or colostrum better than traditional heat treatments. However, HPP can also result in denaturation of native milk proteins, for instance, denaturation of BLG and other minor whey proteins, and changing structure of the casein micelle leading to its disassociation depending on the factors such as pressure levels, treatment time, treatment temperature, and milk pH (Huppertz, Fox, de Kruif, & Kelly, 2006; Indyk, Williams, & Patel, 2008).

HPP also appears to impact milk protein allergenicity and/or antigenicity through alteration of protein structures (IgE/IgG binding epitopes) and protein digestibility (Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015). *In vitro* studies showed that the changes induced by HPP (400 MPa for 30 min at 25 °C) in BLG structure resulted in increased allergenicity (Meng, Bai, Gao, Li, & Chen, 2017), while an *in vivo* study demonstrated the potential of producing hypoallergenic protein hydrolysates under HPP (400 MPa for 20 min at 37 °C) conditions (López-Expósito, Chicón, Belloque, López-Fandiño, & Berin, 2012). Although effects of HPP on allergenicity/antigenicity of individual milk proteins and protein hydrolysates have been reported, the impact of HPP on the immunogenicity of skim milk as a whole appear to be unknown (Huang, Hsu, Yang, & Wang, 2014; Jiménez-Saiz et al., 2015; Vanga, Singh, & Raghavan, 2017).

Immunogenicity is the ability of a substance to elicit an immune response or the capacity to provoke an immune response (Actor, 2014). In the presence of bovine milk proteins, the human lymphocytes, for instance, T helper (Th) cells, provoke specific immune response and secrete Th1 and/or Th2 types of cytokines. It is believed that the Th1/Th2 balance is maintained in a healthy person, which determines suitability of the immune response. The overexpression of one type of cytokines over the other may lead to adverse immune reactions. For instance, overproduction of Th2 type cytokines, resulting in a disturbed Th1 < Th2 balance, may elicit an allergic reaction in immunocompromised subjects (Kiewiet, Gros, van Neerven, Faas, & de Vos, 2015; Rengarajan, Szabo, & Glimcher, 2000; Vocca et al., 2011). Furthermore, it has been proposed that minor whey proteins including IgG may contribute to the regulation of the Th1/Th2 balance or the development of some protective effects against allergy (Bogahawaththa, Chandrapala, & Vasiljevic, 2017a; van Neerven, Knol, Heck, & Savelkoul, 2012). This signifies the importance of studying how the milk immunogenicity is affected by processing.

The current study thus projected to establish the impact of selected HPP conditions, comparable to HTST pasteurization in terms of microbial inactivation and shelf life extension, on the denaturation of native proteins in skim milk and the corresponding *in vitro* immunogenicity using an established cellular model. Furthermore, it was expected to identify potential relationships between the level of protein denaturation and alterations in immunogenicity.

2. Materials and methods

2.1. Materials and treatment of samples

Raw bovine milk was kindly supplied by Murray Goulburn Co-operative (Laverton North, VIC, Australia) at two different time points. Upon arrival, raw milk was defatted by centrifugation (Avanti J-26XP, Beckman Instrument Australia Pty. Ltd, Gladesville, NSW, Australia) at 3500*g* for 20 min at 20 °C. As a reference, a commercial whole milk product processed by a commercial HPP method termed “cold pressed” was purchased from a local organic shop in Victoria (Australia) and defatted as stated above. It is understood that this commercial HPP process applies a double (2×) high pressure treatment at 600 MPa for 90 s as it results in greater reduction of dairy pathogens and longer shelf life (> 42 days at 5 °C) of bovine milk than a single high pressure treatment at 600 MPa for up to 15 min (Cornell, 2017).

Raw skim milk was divided into 5 aliquots, which were then subjected to either a heat treatment or three treatments of HPP. The untreated sample was considered as the control. The defatted HPP commercial milk sample was analysed as it was without further treatment. The heat treatment, HTST pasteurization (72 °C for 15 s), was performed as explained previously (Bogahawaththa et al., 2017b) using a CS/CR rheometer (MCR 301, Anton Paar Germany GmbH, Ostfildern, Germany). Apart from the heating, a constant shear (Σ1000 s^{-1}) and pressure (Σ250 kPa) were also maintained to imitate the industrial processing conditions within the laboratory limitations.

The three HPP conditions were selected in comparable to thermal pasteurization (HTST) in terms of microbial inactivation and shelf life extension as reported previously (Buffa, Guamis, Royo, & Trujillo, 2001; Kolakowski, Reps, Dajnowiec, Szczepek, & Porowski, 1997). Furthermore, the evidence points out that the application of HPP in combination with mild temperature (30–50 °C) is more effective in terms of inhibition and destruction of microorganisms in foods than the pressure alone (Trujillo et al., 2002). Hence, the HPP was performed at 400, 500, and 600 MPa for 15 min and at ~30 °C, respectively. Every sample reached the temperature limit ~30 °C during the treatment time (15 min). HPP was performed using a Stansted ISO-LAB FPG11501 high pressure 3.6 L unit (Stansted Fluid Power Ltd., Stansted, Essex, UK) as described elsewhere (Knoerzer, Buckow, Sanguansri, & Versteeg, 2010). Deionised water and propylene–glycol mixture (40% glycol) was used as pressure-transmitting medium, while the compression and decompression rates were set to 600 and 1200 MPa/min, respectively.

After treatments, all samples were divided into 2 aliquots and one of them was subjected to acid precipitation by adjusting pH to 4.6 using 0.1 M HCl. Most of the caseins and denatured whey proteins were then separated from the soluble proteins 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 supernatant (serum phase) of all milk samples were preserved at −20 °C for further analysis.

2.2. Fourier transform infrared (FTIR) spectroscopy

Soon after completion of each treatment, treated and control milk samples were scanned by a PerkinElmer Frontier FTIR spectrometer (PerkinElmer, MA, USA) using a combined software of IR Solution (Shimadzu Corporation, Kyoto, Japan) version 1.40 as described earlier (Bogahawaththa et al., 2017b).

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

Non-reducing and reducing SDS PAGE were performed for all milk samples and their supernatants as described previously (Bogahawaththa et al., 2017b) using the gels containing 30% acrylamide and 10% SDS. Samples were reduced using β-mercaptoethanol. 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), staining, and de-staining, gel images were captured by ChemiDoc Imaging System (Bio-Rad Laboratories, Australia) for further analysis.

2.4. Size-exclusion chromatography (SEC)

The supernatant (serum phase) of all milk samples was analysed for

the content of soluble protein fractions (e.g. IgG, BSA, ALA and BLG) using a Biosep SEC-s2000 (300 × 7.8 mm) Size Exclusion Chromatography (SEC) column (Phenomenex Australia Ply Ltd., NSW, Australia) with a mobile phase consisting of 0.05 M sodium phosphate buffer (pH 7) including 0.3 M sodium chloride at a constant flow rate of 1.0 mL/min. The eluted peak fractions were collected and then identified by SDS-PAGE as reported previously (Bogahawaththa et al., 2017b).

2.5. Particle size measurements

Average particle size (diameter) of the milk samples was measured after 1000 dilution with simulated milk ultrafiltrate (SMUF) (Rosmaninho & Melo, 2006) using a zetasizer (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK) as described elsewhere (Liyanaarachchi, Ramchandran, & Vasiljevic, 2015). The refractive indices for milk and dispersant were considered 1.35 and 1.33, respectively.

2.6. Isolation of human peripheral blood mononuclear cells (PBMCs)

The ethics application (ID: HRE16-058) for the use of PBMCs in the present study has been accepted and considered meeting the requirements of the National Health and Medical Research Council 'National Statement on Ethical Conduct in Human Research (2007)' by the Victoria University Human Research Ethics Committee.

An established method in our laboratory (Ashraf, Vasiljevic, Day, Smith, & Donkor, 2014; Donkor et al., 2012) with some modifications was used to isolate the PBMCs from buffy coats of healthy donors, which were kindly supplied by the Australian Red Cross Blood Services (Melbourne, Australia). About 60 mL of buffy coats were diluted with 1× phosphate buffered saline-PBS in 1:2 volume ratio and layered gently on Ficoll-Paque Plus (GE Healthcare Pty Ltd., Silverwater, NSW, Australia). After centrifugation (Sorvall-RT7 centrifuge, DuPont, Newtown, USA) at 400*g* for 30 min at 18 °C with no break, PBMCs layer was collected and washed with RPMI-1640 immediately (400*g* for 10 min at 18 °C with half break). Followed by 2 more washing steps with RPMI-1640 (250*g* for 10 min at 4 °C with half break), cells were adjusted to 2.5×10^7 cells/mL in "Recovery" cell culture freezing medium (Thermo Fisher Scientific Australia Pty Ltd, North Ryde, NSW, Australia) and stored in liquid nitrogen until use.

2.7. Stimulation of PBMCs

The stimulation of PBMCs with different milk stimulants was performed as explained previously (Ashraf et al., 2014; Tafaro et al., 2007) and with some modifications. After thawing and removing the freezing medium, the cells were resuspended in a complete cell culture medium containing RPMI-1640 supplemented with 10% fetal bovine serum (FBS) qualified and heat inactivated (Thermo Fisher Scientific Australia Pty Ltd) and antibiotic-antimycotic solution (Sigma Aldrich Pty Ltd., Castle Hill, NSW, Australia) at 3×10^6 cells/mL. Several 24-well flat-bottomed polystyrene microtitre plates were used to stimulate PBMCs with final concentration of 1×10^6 cells/mL in the presence of different milk stimulants (100 µL/mL) at 37 °C in 5% CO_2 for 96 h incubation period. Exactly 1 µg/mL of lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 (Sigma–Aldrich Pty Ltd.) was used to stimulate PBMCs as a positive control, while unstimulated PBMCs in the complete medium (RPMI-1640) were also tested for quantifying basal cytokine production. The supernatants collected (after 96 h) from all the wells were stored at −20 °C for the cytokine assays.

2.8. Cytokine assays

Concentration of different cytokines, such as interleukin (IL)-4, IL-10, IL-12 p70, interferon (IFN)-γ and IL-17A, produced by stimulated PBMCs in the presence of different milk stimulants were quantified by Enzyme-linked immune sorbent assay (ELISA) (Thermo Fisher Scientific Australia Pty Ltd.) according to the manufacturer's instructions.

2.9. Quantification of native bovine immunoglobulin G

The availability of native bovine IgG in control and treated skim milk samples were quantified using bovine IgG ELISA quantification kit (Abcam, Melbourne, Australia) according to the manufacturer's instructions.

2.10. Statistical analysis

Results were analysed based on a randomised split plot design with milk stimulants as the main plot and treatments as sub plot using a General Linear Model of SAS statistical program (SAS1996). The level of significance was pre-set $p \leq 0.05$. Tukey's Studentized Range (HSD) test was used for multiple comparisons of means of cytokines secretion.

3. Results and discussion

3.1. Impact of HPP on the denaturation of native milk proteins in comparison to HTST pasteurization

It is well established that heat and HPP can induce the denaturation of native milk proteins including unfolding of native conformation and formation of inter/intra protein complexes (Considine, Patel, Anema, Singh, & Creamer, 2007; Huppertz, Fox, & Kelly, 2004a). The second derivative of the FTIR interferograms in the amide I absorption region (1600–1700 cm^{-1}) were analysed to study the modifications of the secondary structure of native milk proteins. The SEC technique was employed to examine the level of denaturation of whey proteins, while the formation of protein complexes through thiol/disulphide interactions was investigated using non-reducing and reducing SDS-PAGE method. In addition, the impact of treatments on the average size of particles in milk was assessed by particle size analysis as the HPP is known to alter the size of casein micelle and potentially form protein aggregates in comparison to mild heat treatments (Considine et al., 2007; Huppertz et al., 2006). The availability of native bovine IgG in treated and control milk samples were quantified using an ELISA quantification kit in consideration with its high heat sensitivity and some barostability (Indyk et al., 2008).

As indicated by the FTIR results in Fig. 1, the milk sample treated by

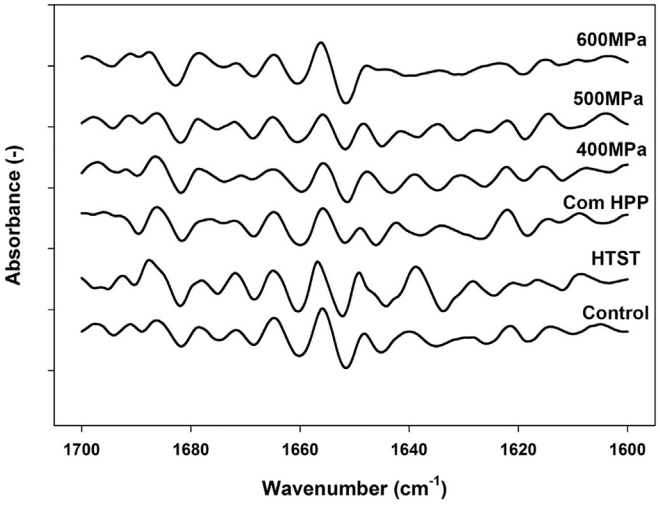

Fig. 1. Second derivative of FTIR spectra of skim milk samples subjected to HPP and HTST pasteurization; (–) control/untreated, (–) HTST treated, (–) 400 MPa treated, (–) 500 MPa treated, (–) 600 MPa treated, and (–) commercial HPP.

HTST pasteurization largely showed a similar pattern of peaks as the control sample indicating that HTST treatment had no or minimal impact on the secondary structure of milk proteins. Both control and HTST pasteurized milk samples showed prominent peaks representing α-helix (~1651 cm^{-1}) and intramolecular β-sheets (~1636 and 1624 cm^{-1}), which are common to the secondary structure of most of the native whey proteins (Bogahawaththa et al., 2017b; Kong & Yu, 2007). Similarly, Qi, Ren, Xiao, and Tomasula (2015) analysed the secondary structure of the whey fraction of skim milk, which was subjected to HTST pasteurization (72 °C for 15 s), and reported that no remarkable reduction of the secondary structure was observed. We reported comparable results when native whey and fractionated whey proteins (IgG, BSA, BLG, and ALA) were subjected to HTST pasteurization (Bogahawaththa et al., 2017b) suggesting that this treatment no or minimally impacts the secondary structure of native milk proteins.

In comparison to the control and HTST pasteurized samples, the FTIR results of high pressure processed milk samples showed a substantial reduction of β-sheets (~1636 and 1624 cm^{-1}) with increasing treatment pressure, while the greatest reduction was observed after treatment at 600 MPa. This can be partly ascribed to the denaturation of BLG with increasing high pressure as the β-sheets are considered its main secondary structural component (Mazri, Sánchez, Ramos, Calvo, & Pérez, 2012; Qi et al., 2015). Only a slight reduction in the α-helix intensity (~1651 cm^{-1}) was visible with increasing high pressure, possibly due to high barostability of ALA, which predominant component of the secondary structure is α-helix (Mazri et al., 2012; Qi et al., 2015). The aggregation of β-sheets was also observed after treatment at 600 MPa by an intensified peak (~1683 cm^{-1}), denoting aggregation of native proteins. The results thus suggest that HPP conditions examined in the current study induced modifications in the secondary structure of native proteins in skim milk resulting in protein denaturation.

Based on our SDS-PAGE results, the skim milk sample treated by HTST pasteurization and its supernatant demonstrated comparable gel band patterns to those of the control samples, implying minimal or absence of formation of protein complexes by thiol/disulphide interactions during HTST pasteurization (Fig. 2A, L4–L5 and C, L4–L5). However, Patel et al. (2006) reported that the pasteurization heat treatment (72 °C for 15 s) caused reduction of the intensity of gel bands to a certain extent corresponding to only minor whey proteins including

Table 1
Average particle size (diameter) and native IgG content in skim milk samples treated by HPP and HTST pasteurization.

Treatment	Average particle size (diameter-nm)[a]	% of native IgG content[b]
Control	154 (± 1.6)^{A}	100^{A}
HTST pasteurization	154 (± 2.0)^{A}	95^{A}
400 MPa for 15 min at 30 °C	121 (± 3.8)^{B}	59^{C}
500 MPa for 15 min at 30 °C	106 (± 1.7)^{C}	24^{D}
600 MPa for 15 min at 30 °C	120 (± 1.5)^{B}	4^{E}
Commercial HPP	107 (± 0.8)^{C}	85^{B}

[a] Values were presented as mean particle diameter (nm) of at least 4 independent measurements (4 ≥ n) plus or minus standard deviation (SD). Means in the same column with different superscripts are significantly different ($p < 0.05$).

[b] Native IgG contents were presented as a percentage (%) in comparison to the control skim milk sample. Values in the same column with different superscripts are significantly different ($p < 0.05$).

IgG, BSA, and LF observed by SDS-PAGE. Our ELISA quantification results showed that the amount of native bovine IgG contained in HTST pasteurized milk was about ~95% in comparison to that of the control sample (Table 1). In contrast, it has been reported that the IgG content (similarly quantified by ELISA) in the commercially homogenized and pasteurized skim milk was about 65–79% (Kummer et al., 1992). Since the immunoreactivity/antigen binding ability of IgG is associated with its native structure (Li, Bomser, & Zhang, 2005), the reduction of IgG detected by ELISA could be related to alteration of its native structure. The inconsistency of the above results could be attributed to the variations in the composition of milk and the method applied for treating milk.

However, in terms of whey protein denaturation and aggregation, the SEC and SDS-PAGE results coincided in the current study. The elution profiles of the supernatants (serum phase) of milk samples obtained by SEC are shown in Fig. 3. The peak patterns of supernatants of the control and HTST treated milk samples eluted by SEC appeared similar, with respect to the peaks corresponding to IgG, BSA/LF, BLG, and ALA, indicating that there was no remarkable denaturation of whey proteins in skim milk after HTST pasteurization.

The intensity of the gel bands corresponding to whey proteins such

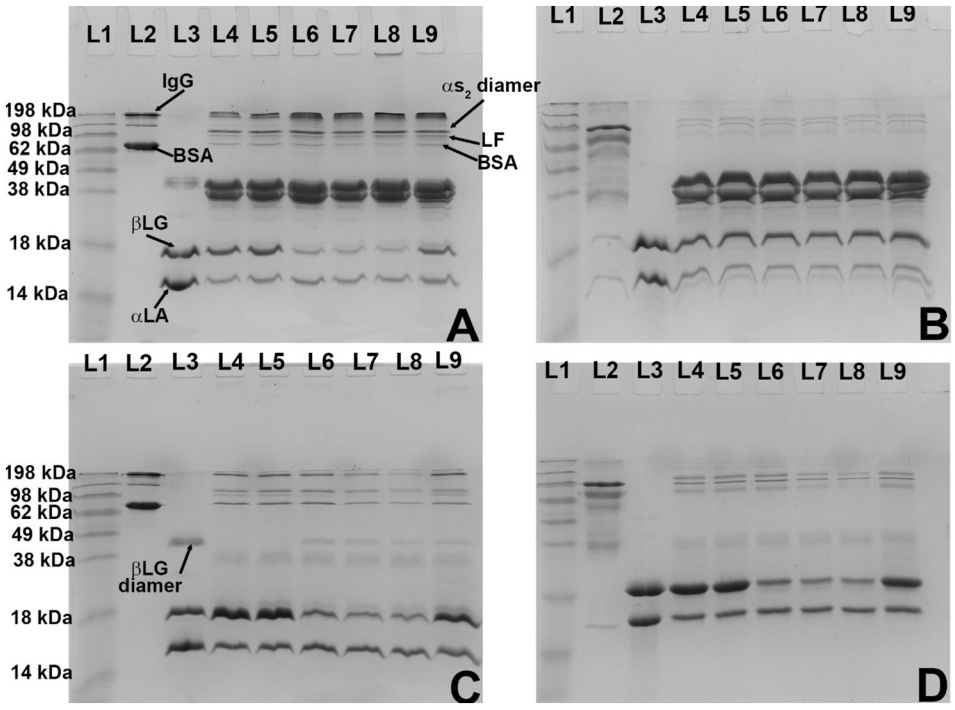

Fig. 2. Non-reducing (A) and reducing (B) SDS-PAGE analysis of skim milk samples subjected to HPP and HTST pasteurization, and non-reducing (C) and reducing (D) of SDS-PAGE analysis of supernatant (serum phase) of those of skim milk samples. Lanes are designated as; L1 - molecular weight markers, L2 - IgG and BSA standards, L3 - BLG and ALA standards, L4 - control/untreated, L5-HTST treated, L6–400 MPa treated, L7–500 MPa treated, L8–600 MPa treated, L9-commercial HPP.

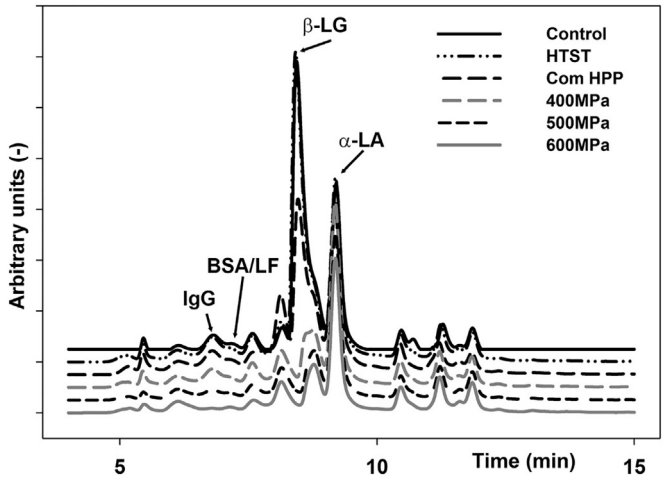

Fig. 3. SEC chromatograms of supernatant (serum phase) of skim milk samples subjected to HPP and HTST pasteurization; (–) control/untreated, (–) HTST treated, (–) commercial HPP, (–) 400 MPa treated, (–) 500 MPa treated, and (–) 600 MPa treated.

as BLG, IgG, LF and BSA reduced substantially with increasing pressure in the HPP milk samples and their supernatants observed by non-reducing SDS-PAGE (Fig. 2A, L6–L8 and C, L6–L8). However, the intensity of the gel band corresponding to ALA was reduced only slightly when increasing treatment pressure. The higher barostability of ALA in comparison to BLG has been frequently reported, which appears to be associated with a higher number of disulphide bonds (4) in ALA structure and also absence of free thiol groups (Huppertz et al., 2004a; Huppertz, Fox, & Kelly, 2004b). Furthermore, large protein aggregates were observed on the stacking gel in the lane corresponding to milk sample treated at 600 MPa (Fig. 2A, L8) under non-reducing conditions.

Upon reduction, the intensity of all gel bands appeared similar regardless of the pressure treatment in all skim milk samples (Fig. 2B, L6–L8), denoting that the protein complexes including large aggregates were mainly formed by thiol/disulphide interactions. Huppertz et al. (2004a) reported that when BLG is subjected to HPP, it unfolds resulting in exposure of its free thiol group, which in turn can react with proteins containing disulphide bonds including BLG, α_{s2}-casein, κ-casein, and ALA through thiol-disulphide interchange reactions. It has also been reported that under HPP conditions, BLG forms aggregates, but most of the denatured BLG interact with casein micelles, which depends on the degree of BLG denaturation (Huppertz et al., 2004b). Patel et al. (2006) also reported the formation of large protein aggregates predominantly between BLG and κ-casein through disulphide-interchange interactions induced by HPP. In addition to BLG, they also suggested the potential contribution of minor whey proteins and ALA (lesser amount) in formation of protein complexes to a certain extent by thiol/disulphide interactions. The minor whey proteins also appeared to contribute to formation of protein complexes under HPP conditions, for instance, the HPP at room temperature for 25 min at or above 400 MPa induced the formation of large protein aggregates of BSA in a phosphate buffer medium via thiol/disulphide interactions (De Maria, Ferrari, & Maresca, 2016).

Moreover, a less intense band corresponding to BLG dimers was observed only in the supernatants of HPP milk samples under non-reducing conditions (Fig. 2C, L6–L9) and they disappeared after reduction, implying formation of BLG dimers through thiol/disulphide interactions. It has been reported that HPP initially induce transient disassociation of BLG dimers into monomers in a BLG solution, which then form covalent complexes irreversibly at 600 MPa for 15 min and < 38 °C (Iametti et al., 1997). Another study reported that skim milk subjected to HPP (≥200 MPa for 30 min at room temperature) can induce formation of BLG dimers by disulphide bonds (Patel et al., 2006).

In the current study, the availability of native IgG quantified by ELISA was 59, 24, and 4% in 400, 500, and 600 MPa treated milk samples (Table 1), respectively, in comparison to the control skim milk sample. Apparently, IgG was denatured significantly by increasing the treatment pressure. This result is in an agreement with other studies, which showed that the denaturation of IgG and loss of its immunoreactivity begins at a pressure of ~300 MPa (Indyk et al., 2008; Masuda, Rehinarudo, Suzuki, Sakai, & Morichi, 2000). Thus, the HPP conditions examined in the current study can be considered severe in terms of denaturing native IgG in milk in comparison to HTST pasteurization.

The peaks pattern, eluted by SEC of the supernatants of HPP samples (Fig. 3), concurred with the bands pattern demonstrated by the SDS-PAGE results of the same samples (Fig. 2C), confirming validity of the results already discussed. The peaks assigned to IgG, BSA/LF, and BLG were intensely reduced by increasing pressure, while the peak representing ALA was slightly affected. Similar results have been reported previously with regard to whey proteins in goat's milk (Felipe, Capellas, & Law, 1997). All the above results demonstrate that the impact of the HPP (400, 500, and 600 MPa for 15 min at 30 °C) on whey protein denaturation, in particular BLG, IgG, BSA, and LF, were substantially greater in comparison to HTST pasteurization.

Furthermore, a distinguished colour change, turning whitish colour into yellowish, was observed before and after HPP regardless the pressure level. This has already been reported for HPP of skim milk for 5 min at 400 MPa (Devi, Buckow, Singh, Hemar, & Kasapis, 2015). The colour of the commercial HPP milk sample appeared similar to those of milk samples processed by HPP in the current study. HTST pasteurization did not appear to change the colour of skim milk (images not shown). The changes in appearance of the milk can be attributed to alteration of the structure of casein micelle affected by HPP (Huppertz, Kelly, & Fox, 2002). The average particle size (diameter) of all the skim milk samples were, therefore, analysed in the current study and recorded as 154, 121, 106, 120, and 107 nm in the control, 400 MPa, 500 MPa, 600 MPa, and commercial HPP milk sample, respectively (Table 1). The average diameter of bovine casein micelles has been reported to be between 130 and 160 nm (McMahon & Oommen, 2013).

The reduction of average particle size of skim milk from control to 400 and 500 MPa treated samples and greatest in 500 MPa treated samples can be related to reduction of the size of the casein micelle affected by HPP. A transmission electron microscopic study reported that the HPP above 400 MPa can disturb the large casein micelles in skim milk into its smaller components (Needs, Stenning, Gill, Ferragut, & Rich, 2000). A notable rise recorded of the average particle size of 600 MPa treated milk could be ascribed to the formation of large protein aggregates, which were observed by non-reducing SDS-PAGE (Fig. 2A, L8). There was no apparent change observed in the particle size after HTST treatment. It is well established that, as a mild heat treatment, HTST pasteurization should not disturb the structure of the casein micelle in milk, which has a high heat stability (Huppertz, 2016).

The analysis of the results of the commercial HPP milk sample was limited due to the absence of a respective control/untreated milk sample and the commercial HPP conditions have been remarkably different. Thus, it was analysed in comparison to the other untreated skim milk of the current study without considering compositional differences of milk. The results of the commercial HPP milk sample showed an apparent denaturation and aggregation of BLG (Figs. 2 and 3), a reduced average size of casein micelles (107 nm), and a distinguished change in colour in comparison to both the control and HTST pasteurized milk samples. The availability of native IgG in the commercial HPP milk sample was about 85% in comparison to untreated milk sample, while HTST pasteurized milk possessed 95%. So, it was apparent that the commercial HPP treatment impacted both on denaturation of whey proteins and the structure of the casein micelle. In general, the commercial HPP milk sample demonstrated that it was subjected to milder HPP conditions in comparison to the other 3 HPP

conditions applied in this current study (400, 500, and 600 MPa for 15 min at 30 °C) but it was still more severe than the HTST pasteurization based on the extent of denaturation of native milk proteins.

3.2. *In vitro immunogenicity of native milk proteins affected by HPP in comparison to HTST pasteurization and in relation to protein denaturation*

It has been well established that processing induces structural modifications in native milk proteins and can modulate the milk immunogenicity leading to altered antigenicity/allergenicity (Bogahawaththa et al., 2017a; Shriver & Yang, 2011) and possibly a differing immune response upon consumption. It was reported that T cell-specific epitopes, the portions of protein molecule that bind with T cell receptors, present in milk proteins can modulate the response of T cells differently (Ruiter et al., 2006). As a means of studying the immunogenicity of skim milk, which were subjected to HPP and HTST pasteurization, the milk protein-specific immune responses of T cells present in human PBMCs were assessed through the quantification of selected cytokines secreted by respective T cell subsets (Tafaro et al., 2007; Vocca et al., 2011). Out of five, three of the cytokines quantified, IFN-γ, IL-4 and IL-17A, are considered the signature-cytokines of Th1, Th2 and Th17 subsets, respectively (Raphael, Nalawade, Eagar, & Forsthuber, 2015).

The secretion of IL-4 and IL-12 (p70) by PBMCs used in the current study, however, was not at a detectable level in response to every milk stimulant. A study reported that PBMCs obtained from milk allergic subjects also did not produce a detectable level of IL-4, though it mainly contributes to allergic reactions (Vocca et al., 2011). The rest of the cytokines (IL-10, IFN-γ and IL-17A) secreted in response to treated and control milk stimulants, as well positive (LPS) and negative (RPMI medium alone) controls, are shown in Fig. 4. The expression of IFN-γ was highest in all the cytokine profiles, while IL-17A was lowest and the level of IL-10 appeared in between. As the production of IL-4 was not detectable, the responses of Th2 cells were determined based on the expression of IL-10 for the current analysis. Vocca et al. (2011) observed a higher production of IL-10 over IFN-γ by PBMCs from both healthy and milk allergic children in response to BLG. These variations in the cytokine profiles can be related to the diversity of the PBMCs and protein stimulants analysed. In our results, PBMCs stimulated with commercial HPP milk stimulant showed a significantly ($p < 0.05$) higher production of IL-10 and IFN-γ over the untreated/control milk sample, displaying a potent immunogenic capacity, possibly due to the variations in the composition of milk proteins rather than the processing conditions.

HTST pasteurized milk did not stimulate a significantly ($p < 0.05$) different cytokine profile in comparison to that of the control milk in terms of all three cytokines, regardless minor differences, implying mostly unchanged immunogenicity. This can be possibly ascribed to the low impact of HTST pasteurization on denaturation of native milk proteins, which was already discussed. However, some substantial impact of HTST pasteurization on milk immunogenicity had been reviewed previously (Bogahawaththa et al., 2017a).

A slight downward trend was observed (Fig. 4) in the expression of IL-10 (Th2 type cytokine) by PBMCs stimulated with the control, 400 and 500 MPa processed milk samples, toward the same order, where the difference was significant ($p < 0.05$) in 500 MPa treated samples but not in 400 MPa, in comparison to the control. Conversely, a higher upward trend line was noticeable in terms of secretion of IFN-γ (Th1 type cytokine) from PBMCs stimulated with the control, 400, and 500 MPa treated milk stimulants, toward the same order, where both 400 and 500 MPa treated milk stimulants were significantly different from the control. Overall, the tendency of shifting Th1/Th2 balance toward Th1 (relatively higher production of IFN-γ) was observed with increasing pressure up to 500 MPa, in comparison to both control and HTST pasteurized milk stimulants. This can be potentially attributed to unfolding of native protein structures with increasing pressure and exposure of T cell-specific epitopes buried in the conformational protein structures, for instance, globular structure of BLG. T cell-specific epitopes of a few milk proteins such as BLG (Meulenbroek et al., 2013), ALA (Meulenbroek et al., 2014), and αs_1-casein (Ruiter et al., 2006) have already been identified mostly in their primary structure, as linear epitopes, which can easily be exposed upon unfolding of native conformation affected by HPP. Kleber, Maier, and Hinrichs (2007) observed comparable results, antigenicity of BLG increased in skim milk with increasing high pressure (200–600 MPa for 10–30 min) due to exposure of antibody-binding linear epitopes by unfolding of native BLG structure. Furthermore, the disruption occurred to Th1/Th2 balance with increasing pressure may be ascribed to the level of denaturation of minor whey proteins (e.g., IgG), which are suggested to contribute in regulating that balance (Bogahawaththa et al., 2017a). The inverse association demonstrated, between the secretion of IFN-γ (Th1-type cytokine) and IL-10 (Th2-type cytokine) in the current study, could also be due to the cross-regulation of Th1 and Th2 responses (Knopf, 2000).

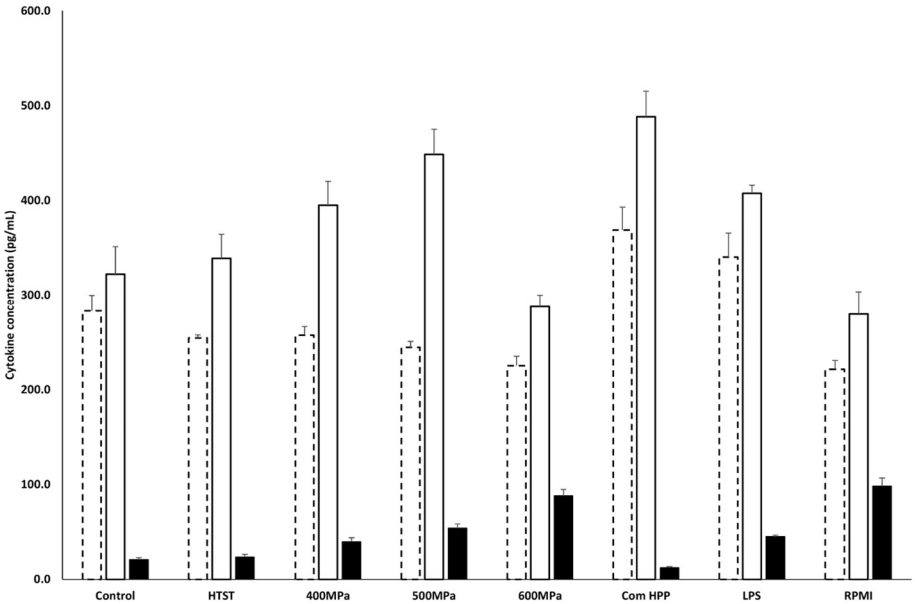

Fig. 4. Production of cytokines; ⬚ IL-10, □ IFN-γ, and ■ IL-17A by human PBMCs stimulated by skim milk stimulants subjected to HPP and HTST pasteurization; control/untreated, HTST treated, 400 MPa treated, 500 MPa treated, 600 MPa treated, and commercial HPP. PBMCs simulated by LPS and without any stimulant (RPMI medium alone) were presented as positive and negative controls, respectively.

The cytokine profile of PBMCs in response to 600 MPa processed milk stimulant was substantially different from that of the control and other two HPP milk stimulants, displaying relatively reduced expression of IL-10 and IFN-γ leading to diminished immunogenic capacity of milk proteins. The substantial denaturation of the native milk proteins and formation of large protein aggregates (e.g., BLG and κ-casein), were also observed at this pressure level (600 MPa) in comparison to the control and other 2 HPP samples. The reduced immunogenic capacity can be potentially ascribed to the masking of T cell-specific linear epitopes due to aggregation of unfolded proteins affected by the increased HPP intensity. This process could restrict the access of T cell receptors to bind with T cell-specific epitopes in milk proteins leading to lowered specific immune responses. Furthermore, the almost complete inactivation of immunoreactive whey proteins, for instance IgG, observed at this pressure level may contribute to this effect. The comparable results have already been reported by others relating to reduced antigenicity followed by processing induced protein aggregation (Bogahawaththa et al., 2017a; Kleber et al., 2007).

The Th1/Th2 balance is considered to mostly determine the appropriateness of immune response with the contribution of other Th subsets including Th17 (Kiewiet et al., 2015; Rengarajan et al., 2000). The trend of shifting the Th1/Th2 balance toward Th1, in the current study with respect to the specific PBMCs analysed, could be utilized for developing a counter effect against allergic reaction, which is usually provoked by Th2 type cytokines in immunocompromised subjects. It has been reported that Th17A cells in food allergic subjects secreted substantially lower level of IL-17A in response to food allergen than healthy people (Dhuban et al., 2013). The expression of IL-17A was always lower than the basal cytokine level (98.3 pg/mL) in the current study. However, further studies, both in vitro and in vivo, are required to elucidate these mechanisms with the consideration of the fate of bovine milk proteins in human gastrointestinal (GI) tract and the complex interplay of immune responses in individuals for optimization of processing conditions.

4. Conclusion

The impact of HTST pasteurization on the denaturation of native milk proteins appeared to be minimal in comparison to HPP at 400 to 600 MPa and 30 °C for 15 min including the HPP method used commercially. HPP resulted in severe denaturation of whey proteins, mainly BLG and other minor whey proteins (e.g., IgG), but not ALA. HPP also disturbed structure of the casein micelles. HPP at 600 MPa induced formation of large protein aggregates, mainly involving BLG and κ-casein and other minor whey proteins by thiol/disulphide interchange interactions. It appeared that the level of denaturation of protein has an association with altered immunogenicity. Minimum denaturation of proteins resulted in mostly unchanged immunogenicity of the HTST pasteurized milk. The unfolding of native protein structures disturbed Th1/Th2 balance with increasing pressure from the control to 500 MPa. However, immunogenic capacity of proteins diminished after HPP at 600 MPa in parallel to further denaturation and aggregation of proteins. Denaturation of immunoreactive minor whey proteins (e.g., IgG) may also affect the Th1/Th2 balance. Further studies (in vitro and in vivo) are paramount to elucidate the real effects of altered immunogenicity on human health. In brief, the HPP conditions studied did not appear to be better alternatives to HTST pasteurization with regard to the impact on native milk proteins and milk immunogenicity. It is advisable to direct future researches toward combined mild processing conditions, for an example HPP at or below 400 MPa in combination with mild temperatures (e.g. < 50 °C), to establish a more appropriate alternative method for milk pasteurization.

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Chapter 7: Conclusions and future directions

This chapter delivers conclusions of the entire project and the scope for future work.

7.1 Overall conclusion

Processing can modulate immunogenicity of native milk proteins predominantly in two ways; changing milk protein structures and associated epitopes resulting in modified immunogenicity and alteration or inactivation of native immunomodulatory properties of milk proteins.

Simulated HTST conditions (72 ℃/ 15 s) appeared to be less detrimental, in comparison to 100 °C/ 30 s 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+BSA) after 100 ℃/ 30 s 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 /30 s condition. The presence of other whey proteins (BLG, ALA, and BSA) had no apparent effect on the thermal stability of IgG at 100 °C.

Thermal denaturation of BLG appeared to be influenced by the other whey proteins (ALA, IgG, and BSA) and caseins, while the modification of the secondary structure and aggregation of this protein were well associated with respective changes in the antigenicity of BLG. The treatment 72 ℃/ 15 s can potentially modify the secondary structure of BLG relating to unfolding of the native structure in a binary mixture of BLG+ALA and skim milk, which in turn may increase the antigenicity due to exposure of epitopes buried in the native conformation. The treatment at 100 °C/ 30 s typically induced formation of protein aggregates via thiol/ disulphide interactions in all the protein mixtures, where BLG mostly played the leading role. This resulted in a loss of BLG antigenicity to a various extent depending on the protein mixture due to fragmentation of conformational epitopes and masking of both conformational and linear epitopes. The native whey showed the greatest reduction of

antigenicity (87%) at 100 °C for 30 s than all the other samples, while no significant reduction of antigenicity was observed in the binary mixture of BLG+ALA in comparison to that of the control. 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.

Bovine skim milk, its main protein fractions (e.g., whey and caseins) and purified major whey proteins such as BLG, ALA, IgG are capable to provoke notable immune responses in human PBMCs. When two or more fractionated proteins are mixed together, the immunogenic capacity of those protein mixtures appear to be weak, and also not to be affected by thermal processing. The immunogenicity of bovine milk proteins appears to depend on the factors such as the nature of the protein, protein concentration, composition of the protein mixture, and the level of protein denaturation. The altered immunogenicity demonstrated by most of the potent protein stimulants subjected to 100 °C/ 30 s treatment, in comparison to their controls, appears to be associated with the level of protein denaturation, possibly due to the modifications in T cell epitopes. Although 72 °C/ 15 s treatment does not cause denaturation of the protein stimulants notably, immunogenicity of some of the proteins including heat-stable ALA and caseins appeared to be affected.

The impact of HTST pasteurization (72 °C/ 15 s) on the denaturation of native milk proteins appeared minimal in comparison to HPP at 400 to 600 MPa and 30 °C for 15 min including the HP processing method used commercially. HP processing resulted in severe denaturation of whey proteins, mainly BLG and other minor whey proteins (e.g., IgG), but not ALA. HP processing also disturbed structure of the casein micelles. HP processing at 600 MPa induced formation of large protein aggregates, mainly involving BLG and κ-casein and other minor whey proteins by thiol/ disulphide interchange interactions. Minimum denaturation of proteins resulted in mostly unchanged immunogenicity of the HTST pasteurized milk. The

unfolding of native protein structures disturbed Th1/Th2 balance with increase in pressure from the control to 500 MPa. However, immunogenic capacity of proteins diminished after HP processing at 600 MPa in parallel to further denaturation and aggregation of proteins. Denaturation of immunoreactive minor whey proteins (e.g., IgG) may also affect the Th1/Th2 balance. In brief, the HP processing conditions studied did not appear to be better alternatives to HTST pasteurization with regard to the impact on native milk proteins and milk immunogenicity.

The overall results highlighted that the processing conditions should be further optimized in considering protein denaturation and associated modulation of immunogenicity in order to produce both hygienic and hypoimmunogenic milk products. Mild heat treatments (< 72 °C) or other mild processing conditions, for instance combined application of HP (< 400 MPa) with low temperature (< 50 °C), could fulfil aforementioned requirements. The impact of other milk proteins on the processing induced modification of a targeted protein (e.g., BLG) could be utilized to modulate its immunogenicity and potential allergenicity favourably. For instance, inclusion of caseins into a whey protein mixture contributed to reduce the antigenicity of BLG after heating.

Although consumption of raw bovine milk appears contributing to the development of tolerance against allergy and asthma, it cannot be recommended as an approach to control the CMPA, due to health risks associated with human pathogens. In this context, the traditional thermal processing methods including HTST pasteurisation and UHT processing should be optimized and or equivalent novel processing method should be introduced in a way to preserve and utilize the health promoting properties of native milk proteins.

7.2 Scope for future research

In the present study, only a few selected processing conditions (heat and HP treatments) were tested to investigate their impact on changes in native proteins and thereby modulation of

immunogenicity and antigenicity. Since the selected conditions appeared to change the native proteins and their immunogenicity substantially, it would be important to study some mild conditions for instances heating < 72 °C and HPP < 400 MPa to establish appropriate conditions.

As well, in vitro immunogenicity and antigenicity of the intact proteins (native and processed) were analysed in this study. However, the milk proteins are usually subjected to a complex digestion process in the human digestive system upon ingestion, which result in hydrolysis of proteins into various peptides. Hence, it would be worthwhile to study as to how the processing-induced modifications of proteins influence the digestion process and how the altered immunogenicity and antigenicity transfer to the respective peptides. Mapping of the fate of epitopes (IgG, IgE, T cell etc.) in native milk proteins followed by processing and digestion would also provide interesting information to be applied in appropriate milk processing.

Furthermore, the methodology used for investigating in vitro immunogenicity cannot be fully comparable with the complex human immune system, especially the immune system of an immunocompromised (e.g., milk protein sensitive) subject to predict the potential allergenicity. In vivo studies are thus recommended for further studies to establish how native and processed proteins modulate the human immune system differently. This will help to incorporate the favourable effects of altered immunogenicity of processed proteins and health promoting properties of native milk proteins in producing milk products for milk protein sensitive subjects.

Moreover, obtaining the comprehensive knowledge on immunomodulatory properties of important minor whey proteins including Ig and LF using an in vivo model and their stability during processing would guide to identify their possible influence on development of tolerance against allergy and asthma, which can be applied in controlling CMPA in future.