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REVIEWS OPEN ACCESS

ROLE OF WHEY PROTEIN-CASEIN COMPLEXES ON YOGHURT TEXTURE

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ABSTRACT

Properties of yoghurt extremely depend on the application of heat treatment to milk prior to manufacture. After heating the milk, denatured whey protein and casein makes complexes which dramatically improve the yoghurt texture such as firmness and water holding capacity etc. The formation of complexes, micelle bound or soluble whey protein/κ-casein complexes, and their localization between casein micelles and serum depends on the physical properties (heat treatment, pH, compositions) of milk. We review the formation of these complexes and their effect on the physical, rheological and microstructural properties of acid milk gels. In order to investigate the interactions of denatured whey protein-casein, the formation of covalent and non-covalent bond namely thiol/disulphide, hydrophobic and electrostatic etc. are reviewed, with the emphasis on acid gelation and final yoghurt texture. This review summarizes the different factors and interactions on the formation of the micelle bound complexes or soluble denatured whey protein/κ-casein complexes in heated milk and how these complexes affect the yoghurt properties. This information could be fundamental knowledge for developing a desirable yoghurt texture, may have potential interest for future research.

Keywords: casein micelle, protein complex, whey protein, yoghurt gel.

1. Introduction

Yoghurt is a very popular fermented milk product produced all over the world (Lucey and Singh, 1998; Karam *et al*., 2013), in which starter cultures are used to reduce milk pH resulting to form yoghurt gel (Peng *et al*., 2009). During fermentation, casein becomes unstable and coagulates after reaching the isoelectric point of the casein (pH 4.6), and the casein formed a three dimensional protein networks in which whey is entrapped (Damin *et al*., 2009). Milk heated at temperature higher than 70 °C causes denaturation of whey protein, as a result the disulfide bond is formed between κ-casein and denatured whey proteins (Lucey *et al*., 1998b) which makes a firmer gel. This bond formation between denatured whey protein (mainly β-lactoglobulin) and κ-casein depends on the heating time and temperature, protein concentration and pH (Oldfield *et al*., 1998). Upon milk heating, globular whey protein is unfolded of its tertiary structure resulting exposure of the free thiol group (-SH) of β-lactoglobulin. Consequently, thiol disulphide interchange reactions occur during acidification of heated milk and form covalent bonds that contribute to the strength of yoghurt gel (Matumoto-Pintro *et al*., 2011).

Earlier studies had described widely the heat induced-denaturation and interaction of whey proteins in the skim milk with or without added whey protein (Lucey *et al*., 1999; Karam *et al*., 2013). Heat induced-denatured whey protein are partly associated with casein micelles as micelle-bound complexes occur at low milk pH by forming disulphide bond with κ-casein at the surface of the micelles and partly in serum phase as soluble whey protein/κ-casein complex occur at high milk pH, because of more κ-casein dissociates from casein micelles at these conditions (Anema and Li, 2003; Guyomarc'h *et al*., 2003a; Xu *et al*., 2015). This micelle-bound complex and soluble whey protein/κ-casein complex show distinct properties in the yoghurt gel. Since the formation of the different protein complexes at the various milk conditions prior to heat treatment, may affect the textural properties of yoghurt. In this perspective, the present review will focus on the complex formation between whey proteins and casein in heated milk and their effect on the various physical, rheological and microstructural properties of yoghurt.

This review therefore aims at providing some fundamental knowledge on the effect of micelle bound complex and soluble whey protein/κ-casein complex or whey protein-whey protein aggregation on the properties of yoghurt gel. The present review will first overview on the formation of whey protein-casein complexes in the heated milk (in a first part) and how these complexes alter the yoghurt texture which is discussed in a second part. Further possible research and aspects for improving the

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technological process and modulating the interaction of heated milk to control the serum or micelle bound complexes; this review will provide some extensive information for yoghurt manufacture.

2. Interactions between whey protein and casein in heated milk

Normally all milks used in yoghurt manufacture are subjected to dramatic heat treatment to control the bacterial growth and improve the body structure. Heating milk is one of the most important processing parameters which can enhance the denaturation of whey proteins and their interaction with casein micelles (Lucey and Singh, 1998). In practice, milk is heated at 85 °C for 30 min or 90 - 95 °C for 5-10 min as batch heat treatment in the yoghurt industry, or sometimes very high temperature short time (140 °C for 4 to 16 s) (Sodini *et al*., 2004) which causes denaturation of whey proteins and complex formation between soluble whey protein and κ-casein or between whey protein and κ-casein in casein micelles through disulphide bonding and/or hydrophobic interactions (Singh and Creamer, 1991; Lucey and Singh, 1998; Lakemond and van Vliet, 2008; Lee and Lucey, 2010; Matumoto-Pintro *et al*., 2011). Immediately after heat application, heated milk is cooled to incubation temperature at around 40 - 45 ° C (Sodini *et al*., 2004) before added starter culture (*Streptococcus thermophilus* and *Lactobcillus delbrueckii* ssp. *Bulgaricus*) which convert lactose into lactic acid resulting in decrease of pH (Damin *et al*., 2009; Peng *et al*., 2009). As the milk pH 6.7 is reduced by the action of starter culture and reached to the isoelectric point of casein (pH 4.6), the net negative charge of casein decreases leading to collapsed of hairy layer of κ-casein (De Brabandere and De Baerdemaeker, 1999; Vasbinder and De Kruif, 2003). Consequently it decreases in electrostatic repulsions as well as steric stabilization and increases in casein-casein interactions prompt the formation of a three dimensional protein networks (Lee and Lucey, 2010; Karam *et al*., 2013; Meletharayil *et al*., 2015), as presented in Fig.1.

In heated milk, denatured whey protein interacts with κ -casein in casein micelles or in the serum which act as bridging material to interact with other denatured whey proteins causing the formation of the complex protein networks (Lucey *et al*., 1997; Lucey and Singh, 1998; Corredig *et al*., 2011). By the application of heat treatment in reconstituted skim milks, the covalent bond is formed between denatured β-lactoglobulin and κ-casein through thiol-disulphide exchange reactions (Singh and Fox, 1985; Noh *et al*., 1989; Jang and Swaisgood, 1990) and there is some evidence that α-lactalbumin is also associated in these complexes (Dalgleish, 1990). In addition, few authors (Dalgleish, 1990; Patel *et al*., 2007) have illustrated that bovine serum albumin (BSA), lactoferrin and αs_2-caseins are unfolded upon heat treatment and thereafter they are associated with the β-lactoglobulin/κ-casein complexes via thiol-disulphide exchange reactions. To a minor extent, immunoglobulins are partially involved in these complexes through hydrophobic interactions (Oldfield *et al*., 1998).

Results presented above indicate that all denatured whey proteins are associated with heat induced whey protein/κ-casein complexes either through thiol/disulphide interchange reactions or hydrophobic interactions to form a complex protein networks.

2.1 Covalent bond formation

The globular whey protein is mainly β-lactoglobulin containing two disulphide bonds (Cys106 - Cys119 and Cys66 – Cys160) and one free cysteine (Cys121), exists as a dimer at room temperature and natural milk pH 6.7 but it is converted into monomers at higher temperature (Hoffman and vanMil, 1997; Yuno-Ohta *et al*., 2016). Other whey proteins, for example, α-lactalbumin do not have any free –SH groups but it has 4 S-S bonds (Cys6 - Cys120, Cys28 - Cys111, Cys61 - Cys77, and Cys73 - Cys91), and BSA contains one free –SH group (Cys34) same as β-lactoglobulin as well as 17 S-S bonds (Wijayanti *et al*., 2014). At elevated temperature, the monomer of β-lactoglobulin becomes exposed of their one disulphide bond (S-S) mainly Cys66 - Cys160 disulphide bond, and free cysteine (thiol group, -SH) which initiate thiol/disulphide (SH-SS) interchange reaction (Alting *et al*., 2000; Matumoto-Pintro *et al*., 2011). The covalent intermolecular disulphide bond is formed between Cys160 of β-lactoglobulin with κ-casein on the surface of casein micelle (Henry *et al*., 2002), or κ-casein in the serum phase (Lowe *et al*., 2004), and with other β-lactoglobulin molecules consisting other cysteine rather than intermolecular bound Cys66 (Hoffmann and vanMil, 1997; Livney *et al*., 2003; Donato *et al*., 2007a; Nguyen *et al*., 2013). Exposing the free thiol group of β-lactoglobulin after denaturation of whey protein which is responsible for interaction with the disulphide bonds of other proteins i. e. α-lactalbumin, κ-casein and other β-lactoglobulin through thiol/disulphide interchange reactions to form intermolecular covalent bond (Guyomarc'h *et al*., 2003a; Lowe *et al*., 2004). Similarly, Livney *et al*. (2003) demonstrated that Cys121 of β-lactoglobulin plays a role in initiating the formation of the thiol-disulphide bond between Cys119 and other possible cysteine of both κ-casein and β-lactoglobulin. It is also obvious from the previous description that Cys106 of β-lactoglobulin is less accessible for the formation of the intermolecular disulphide bridge due to remain buried inside the inner parts of the proteins (Hoffmann and vanMil, 1997; Lowe *et al*., 2004).

Moreover, α-lactalbumin containing Cys6, 61, 111, and 120 are involved in disulphide bond formation with β-lactoglobulin through the initial unfolding of the one disulphide bond (Livney *et al*., 2003). It is generally accepted that thiol/disulphide exchange reactions occurred between the free sulfhydryl groups (-SH) of denatured β-lactoglobulin and the disulphide bonds (S-S) of the

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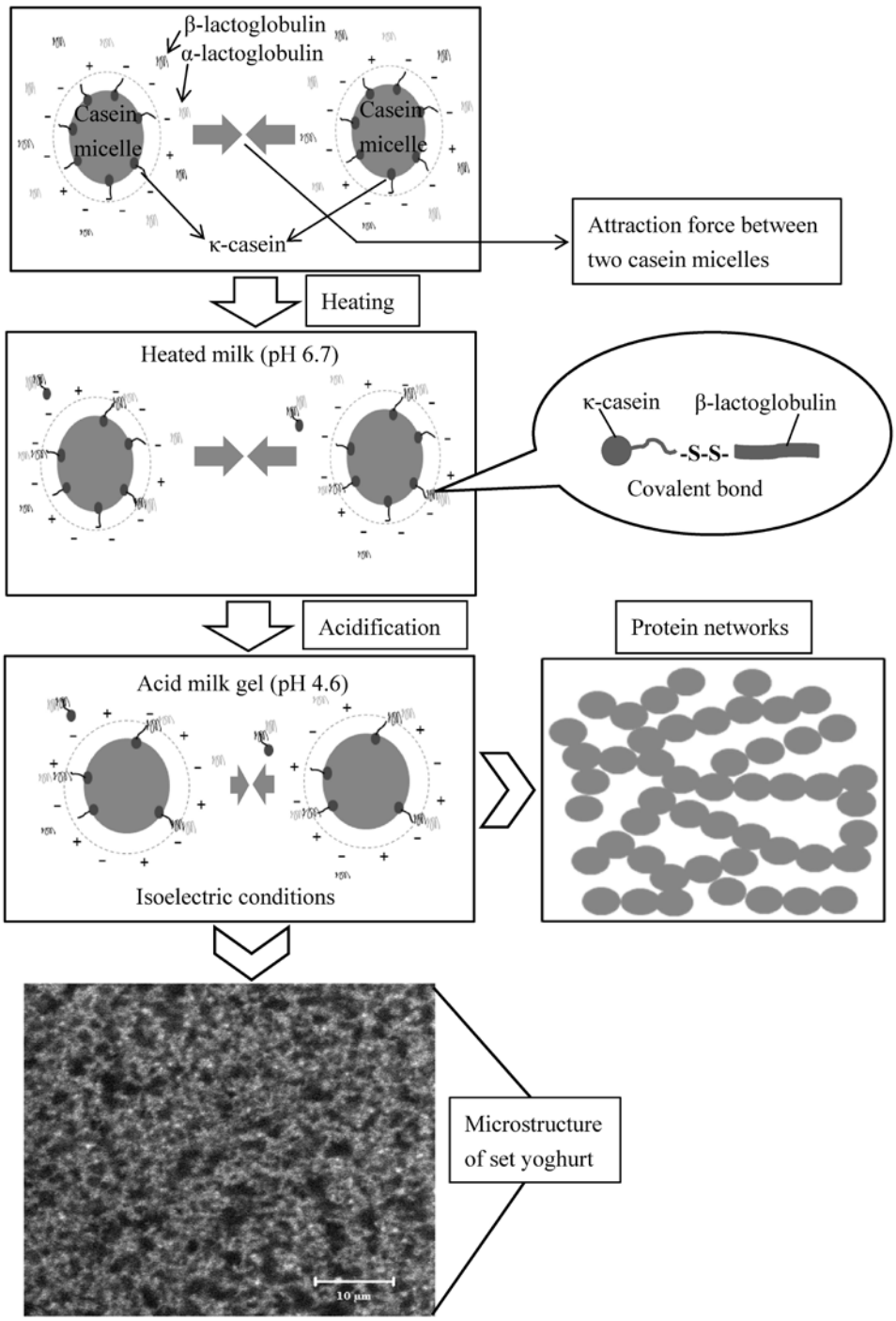

Fig.1. Schematic diagram is presenting the interaction between denatured whey protein and κ-casein as a function of pH and milk heating and the whole fermentation process of yoghurt gel formation (Adapted from De Brabandere and De Baerdemaeker, 1999; Lucey *et al*., 1998b; Anema, 2007).

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κ-caseins (Fig. 1) which remains in the boundary between the para-κ-casein (near micelle core) and the glycomacropeptide region (hairy brush). Due to the interaction of the free sulfhydryl groups of β-lactoglobulin with the disulphide bond of the κ-caseins, β-lactoglobulin penetrate through the hairy layer of glycomacropeptide region (Anema and Li, 2003), leading to the formation of thiol/disulphide exchange reactions.

2.2 Non covalent bonds (Electrostatic, hydrophobic) formation

A number of studies demonstrated that, despite the hindrance to formation of intermolecular disulphide bonds between denatured whey proteins and κ-caseins in heated milks, heat induced non-covalent bonds (hydrophobic, electrostatic and ionic interactions) are formed between denatured whey protein and casein (Hoffmann and vanMil, 1997). Early studies have determined that, when covalent bonds cannot be built during heating, non-covalent bonds developed into heat induced protein aggregations (Hoffmann and vanMil, 1997; Havea *et al*., 2001; Nguyen *et al*., 2014). The extent of the relative importance of noncovalent interactions to the heat induced protein complexes and their contribution to the overall protein aggregations as well as gelation process become of increasing interest in the model systems. According to Havea *et al*. (2001) and Nguyen *et al*. (2014), it has been shown that addition of thiol blocking reagent such as N-ehthylmaleimide (NEM) to block the entire free thiol groups prior to heat treatment, non-covalent bonds could occur followed by hydrophobic interaction and increase the total number of connections between heat induced protein complexes.

Upon heating, denaturation of β-lactoglobulin and their interaction with casein micelles through intermolecular –SH/S-S interchange reactions in milk system followed by hydrophobic interactions. Denatured β-lactoglobulin interact with κ-casein after unfolding its free cysteine (-SH) groups (Cho *et al*., 2003). An early stage of heating, denatured β-lactoglobulin interact with κ-casein via both hydrophobic and S-S interactions but this interactions depend on the protein systems and heating temperature as well as heating time (Cho *et al*., 2003). This agreement is supported by Haque *et al*. (1987), who reported that the interactions between β-lactoglobulin and κ-casein occurs through hydrophobic attractions at early stage and after that covalent bond is formed via –SH/S-S interchange reactions. Furthermore, α-lactalbumin participates to make complex with κ-casein after formation of β-lactoglobulin-α-lactalbumin complex because of unavailable free reactive –SH group containing in α-lactalbumin (Anema, 2009).

Addition to above mentioned interactions, some studies demonstrated that the role of ionic interactions on the formation of β-lactoglobulin/κ-casein complexes is lesser and sometimes questionable cause of influence or hinder to the formation of complexes (Smits and van Brouwershaven, 1980; Doi *et al*., 1983).

However, there is no clear information in such non covalent bond whether hydrophobic or electrostatic interactions are dominant to form the β-lactoglobulin/κ-casein complexes in heated milk systems.

3. Factors affecting the formation of whey protein-casein complexes

Several factors including protein composition, milk pH, temperature, salts and total number of accessible –SH groups that influences the formation of whey protein-casein complexes in milk system. Many studies have been done regarding these factors how they affect the formation of whey protein-casein complexes and their effect on yoghurt texture, which is presented with brief overview in Table 1.

3.1 Milk composition

The protein concentration and total solid content of the heated milk influence the formation of whey protein-casein complexes. Prior to heat treatment, the increase in total protein concentration in skim milk induces the denaturation of whey protein as a result denatured whey protein-casein complexes are formed (Anema *et al*., 2006). On the other hand, a reverse behavior was observed after increasing the total solid content of milk, which hinders the denaturation of the whey proteins because of acting as an obstacle by lactose as well as other soluble non protein substances to unfold the whey proteins (Anema *et al*., 2006).

The original concentrations of the different milk proteins, for examples, β-lactoglobulin, α-lactalbumin of whey protein and κ-casein, α-caseins affect the formation of heat induced aggregation of denatured whey protein with κ-casein. Some authors (Smits and van Brouwershaven, 1980; Noh *et al*., 1989) reported that the addition of high concentration of κ-casein negatively affect the formation of the heat induced whey protein aggregations. Moreover, Zhang *et al*. (2005) investigated that the application of α_s-casein and /or β-casein rather than κ-casein in heated milk which hinder the aggregation of the denatured whey proteins. Their result demonstrated that the disordered caseins carry out a protective "chaperone like" to inhibit on the unfolding globular via phosphoserine residues as well as hydrophobic surface without development of the heat induced complexes. The addition of non-dairy globular proteins including egg proteins or soy proteins and various surface active phospholipids to milk or whey protein solution prevent the formation of heat induced whey protein-casein complexes (Famelart *et al*., 2004; Unterhaslberger *et al*., 2006).

A positive effect on the complex formations was found by increasing the concentration of β-lactoglobulin from 3.8 to 16 mg/mL (Iametti *et al*., 1996). For instance, it was reported that

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Table 1: Factors affecting the formation of the whey protein-casein complexes and their effect on yoghurt texture.

Factors			Predominant protein complexes	Effect on yoghurt texture	References
Milk composition	Milk pH	Heat treatment			
Simulated Milk (Correspondinng to 15% SM)	6.5	20~90 ˚C for 30 min	MBC	Increased gel strength	Schorsch *et al.* 2001
10.45 % SM	6.7	90 ˚C for 10 min	WPA	Increased gel hardness	Vasbinder *et al.* 2003
10-25% SM	6.2 ~ 7.1	80 ˚C for 30 min	MBC	Increased G´, breaking stress	Anema 2008b
SM containing 9.2% protein	6.7	72~90 ˚ C for 7 min	WPA	Highest viscosity	Chever *et al.* 2014
15% SM	6.9	90 ˚ C for 10 min	MBC	Increased gel strength	Meletharayil *et al.* 2015
SM containing 8% protein	~	75-95 ˚C for 5 min	MBC	Increased gel strength	Jørgensen *et al.* 2015
10% SM	6.5~ 7.1	90 ˚ C for 30 min	SDWP	Increased G´	Anema *et al.* 2004
10.45% SM	6.2~ 6.9	80 ˚ C for 10 min	MBC	Highest G´	Lakemond and van Vliet 2008
10.7% SM	6.2~7.2	85˚C for 30 min	MBCor SDWP	Equal effect on yoghurt gel	Ozcan *et al.* 2015
Yak milk	6.6~ 7.4	85 ˚ C for 30 min	SDWP	Increased WHC, firmness & G´	Xu *et al.* 2015
10.7% SM	~	75~90 ˚C for 15 or 30 min	MBC	Increased G´	Lucey *et al.* 1997, 1998b
10% SM	~	90 ˚C for 10 min	MBC	Elastic gel	Guyomarc'h *et al.* 2003b
10% SM	~	90 ˚C for 10 min	MBC	Increased G´	Donato *et al.* 2007b
10% or14% SM	~	90 ˚C for 10 min	MBCor SDWP	Equal effect on milk gel	Guyomarc'h *et al.* 2007, 2009

SM: Skim milk; MBC: Micelle bound complexes; WPA: Whey protein aggregate; SDWP: Soluble denatured whey protein/κ-casein complexes; WHC: Water holding capacity; G´: Storage modulus

intermolecular S-S bridge formation depends on the concentration of β-lactoglobulin at temperature below 75 ˚C, but S-S bridge formation does not depend on the concentration of β-lactoglobulin at temperature higher than 75 ˚C (Iametti *et al.*, 1996). Other researchers (Roefs and De Kruif, 1994; Hoffmann and vanMil, 1997) also found same results upon addition of β-lactoglobulin that the average size of the protein complexes (S-S bridge formation) was increased with increasing the concentration of β-lactoglobulin from 10 to 50 mg/mL during heating at temperature up to 65 ˚C.

3.2 Initial milk pH

The interaction between whey protein and casein in heated milk are affected by a little change in milk pH prior to heat treatment. The effect of pH on the formation of whey protein-casein complexes and on their localization either in colloidal or serum phase was studied by several authors (Law and leaver, 2000; Anema and Li, 2003; Vasbinder and De Kruif, 2003). Milk heated at pH below 6.6, all denatured whey proteins associated to the surface of the casein micelles whereas milk heated at pH higher than 6.6, few amount of denatured whey proteins associated to the surface of the casein micelles and most of the κ-casein is dissociated from casein to form whey protein/κ-casein complexes in serum phase (Anema and Li, 2003; Vasbinder and De Kruif, 2003). From the earlier studies, it has been widely accepted that the formation of heat induced serum complexes and micelle bound complexes depends on the basis of the milk pH, and the milk pH is a key factor to control the formation of both types of complexes (Singh and Fox, 1985; Anema and Li, 2003). In addition, Anema *et al.* (2004) determined that heat induced whey protein-casein complexes and their distribution in both the serum

and the micellar phases of milk depend on the non sedimentable κ-casein and denatured whey proteins through notably related to the pH range from 6.5 to 7.1.

Aside from interactions, denaturation of β-lactoglobulin of whey proteins is also affected by milk pH. In skim milk, it has been reported that the denaturation rate of all whey proteins varied throughout the pH range of 6 to 9 (Law and Leaver, 2000). A report by Hoffmann and van Mill (1997) mentioned that the size of the aggregation of β-lactoglobulin during heating throughout pH 6 - 8, smaller aggregates occurred at pH 8 whereas higher molecular weight aggregates were build up at pH below 7. Alteration of the milk pH influenced on the mineral balance between colloidal and serum phases, availability of free thiol groups, as well as net charge of the proteins. In addition, milk pH has extensive impact on the formation of aggregates, and interaction of whey protein-casein either in serum or colloidal phase.

To best of our knowledge, there is no clear information and chemical investigation how pH regulate the interaction of the whey proteins and casein and their distribution; particularly the size of the complexes and dissociation of κ-casein from casein micelles are related to forming soluble whey protein-casein complexes in the serum phase.

3.3 Heating temperature

Of all extrinsic and intrinsic factors, the effect of heating temperature on the denaturation of whey protein and their interaction with casein has been assessed widely. From the earlier studies, it has been explored that application of heat treatment in the range of 70 to 95 ˚C for a certain time duration (10, 20, or 30 min) increase the denaturation of whey proteins as well as enhanced to the formation of heat-induced serum complexes and micelles bound complexes (Lucey *et al*., 1997; Vasbinder *et al*., 2003). The unfolding behavior of the β-lactoglobulin is depended on the degree of the heating temperature. According to Tolkach and Kulozik (2007), heating at 40 ˚C, the native globular β-lactoglobulin begins to denature into monomers, and the formation of the protein complexes has been occurred but is very limited due to the small concentration of the accessible –SH groups. Upon heating at 60 ˚C, the S-S bond (Cys106 - Cys119) exposed to the surface of the proteins which was buried inside the native dimers, consequently it is capable to react with the other thiol (-SH) groups (Tolkach and Kulozik, 2007).

Additionally, Sava *et al*. (2005) had explained that the exposure of the thiol (–SH) groups to the outer surface was increased with the increasing heating temperature from 67.5 to 80 ˚C. Controversially this surface thiol (–SH) groups was reduced at heating above 80 ˚C because of the formation of the S-S bond. This observation is in agreement with that of Watanabe and Klostermeyer (1976), who investigated the total number of the thiol (–SH) groups in β-lactoglobulin upon the increased temperature from 65 to 145 ˚C. It was observed that the total number of the thiol (–SH) groups were decreased at the higher temperature heating due to the formation of the S-S bond.

However, the denaturation of whey protein and their effect on the formation of whey protein-casein complexes, were extensively assessed by various authors (Lucey *et al*., 1997; Parker *et al*., 2005), mentioning the change in the structure of the whey protein/κ-casein complexes. The size and property of the whey protein/κ-casein complexes in heated skim milk have not been analyzed quantitatively, which would be an interesting research era in near future.

4. Localization of the whey protein-casein complexes in the heated milk

The localization of the heat induced whey protein-casein complexes to the surface of the casein micelles as micelle bound complexes and outside of the casein micelle i.e., serum phase as soluble whey protein/κ-casein complexes, has been reported by many authors (Smits and van Brouwershaven, 1980; Jang and Swaisgood, 1990; Guyomarc'h *et al*., 2003a; Anema *et al*., 2004; Taterka and Castillo, 2015). The interaction of the heat induced whey proteins/κ-caseins complexes and their distribution in either serum phase or micellar phase is regulated by the milk pH and heating temperature. To compare the denatured whey proteins-casein complexes in serum or micellar phase, heated milk was analyzed by using gel electrophoresis or size exclusion chromatography after separation of the serum either by centrifugation or acid precipitation (Anema and Li, 2003; Vasbinder *et al*., 2003).

4.1 Whey protein-casein complexes in micellar phase

It is widely accepted that a greater extent of denaturation, resulting disclose the sulfhydryl groups (-SH) which remained buried inside the protein structure, enhanced the interaction of the denatured whey protein to the surface of the casein micelles. Two mechanisms have been proposed for the association of the denatured whey proteins with κ-casein in casein micelles: the first exposing the sulfhydryl groups (-SH) of β-lactoglobulin and the second, interaction of the sulfhydryl groups with the disulphide bond (S-S) of κ-casein remaining on the surface of the casein micelles (Donato *et al*., 2007a; Taterka and Castillo, 2015). After sulfhydryl-disulphide intermolecular exchange reactions between β-lactoglobulin and α-lactalbumin, it is associated with this complexes in the micelles, which is ascribed by absence of free thiol groups of α-lactalbumin (Corredig and Dalgleish, 1996; Donato and Guyomarc'h, 2009; Wijayanti *et al*., 2014). The interaction of the denatured whey protein with κ-casein in casein micelles was confirmed by measuring the particle size using zetasizer (Chever *et*

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al., 2014; Meletharayil *et al*., 2015) and, by analyzing the proteins complexes in the serum or colloidal phases after centrifugation or acid precipitation using gel electrophoresis (Anema, 2009).

In addition, the complex formation of denatured whey proteins with casein micelles at the surface of the micelles was also visualized by observing their rough surface or appendages on the casein micelle surface using scanning electron microscopy (Jang and Swaisgood, 1990). The size of the casein micelles was increased by the interaction of the whey proteins with casein at the range of heating temperature from 75 to 100 ˚C, which was determined by Anema and Li, (2003). On the other hand, this interaction between the whey proteins and κ-casein in casein micelles is better under slow heating than fast heating. At slow heating, small aggregated species of β-lactoglobulin was formed which is easily penetrate the hairy layer of κ-casein, consequently it leads to enhance better interactions (Oldfield *et al*., 1998).

4.2 Whey protein-casein complexes in the serums phase

The interaction of denatured whey proteins with the soluble κ-casein via intermolecular -SH/S-S interchange reactions occur in the serum phase during heating of milk. There are also two mechanisms involved for the association of the heat-induced whey proteins with the soluble κ-casein in the milk serum. The first mechanism is the dissociation of κ-casein, of key importance to control the formation of the soluble denatured whey protein/κ-casein complexes. At an early stage of heating, the κ-casein dissociate from the casein micelles to the serum phase (Anema, 1997; Guyomarc'h *et al*., 2003a). The second mechanism includes the interaction of denatured whey proteins with the free κ-casein in the serum phase and, subsequently, it forms the soluble denatured whey protein/κ-casein complexes in the heated milk (Corredig *et al*., 2011). It has been exhibited, in general, that the dissociation of the κ-casein from the casein micelles is related to the pH of the heated milk, there is a high proportion of soluble complexes found at alkaline pH values of heated milk (Anema, 2007).

On addition of soluble κ-casein to the skim milk, less denatured whey protein were found on the surface of the casein micelles (Anema, 2008a), or when soluble κ-casein is heated excluding casein micelles, the formation of soluble denatured whey protein/κ-casein complexes occurs in the serum phase (Donato *et al*., 2007a; Guyomarc'h *et al*., 2009). Other studies by Anema (1997; 2007; 2008a) reported that the dissociation of the κ-casein from the casein micelles occurred at lower temperatures than denaturation temperature of whey proteins and also reached its peak prior to the denaturation of the whey proteins. During cooling stage, the soluble κ-caseins which had not associated with the denatured whey proteins in the serum phase, subsequently re-associated with the casein micelles (Anema, 1997; Lucey *et al*., 1998b).

5. Yoghurt texture as a function of whey protein-casein complexes

The role of denatured whey protein coated casein micelles and the soluble denatured whey protein/κ-casein complexes on the acid gelation and, on the yoghurt texture has been documented widely. Conflicting results were observed on the relative role of micelle bound whey protein and the soluble denatured whey protein/κ-casein complexes on the properties of acid gels (Lucey *et al*., 1998b, Guyomarc'h *et al*., 2003a; Anema, 2004). Some reports point out that there is an important role of micelle bound whey proteins on the yoghurt texture (Lucey *et al*., 1998b; Schorsch *et al*., 2001), whereas other reports have highlighted on the significant role of the soluble denatured whey protein/κ-casein complexes (Anema, 2004; Rodriguez and Dalgleish, 2006). Generally, the micelle bound whey protein and/or the soluble denatured whey protein/κ-casein complexes modify the firmness, water holding capacity, whey separation, rheological and microstructural properties of the yoghurt gels. The heat treatment of milk extensively increase the firmness, water holding capacity and rheological properties of yoghurt due to formation of micelle bound whey protein and the soluble denatured whey protein/κ-casein complexes. In this perspective, the effect of whey protein-casein complexes on the physical, rheological and microstructural properties of yoghurt will be reviewed in this section.

5.1 Physical properties (firmness and water holding capacity) of yoghurt

The effect of heating milk prior to fermentation, as compared with unheated milk, heating milk leads to increase the water holding capacity as well as firmness (Lucey *et al*., 1997; Lucey *et al*., 1998b). These positive effects of heated milk on the physical properties is correlated with the denaturation rate of whey protein (Lucey *et al*., 1997) and formation of the micelle bound whey protein and/or the soluble denatured whey protein/κ-casein complexes (Lucey *et al*., 1998b; Vasbinder *et al*., 2003; Anema, 2004). Of two complexes, there are some evidences that the positive effect of the soluble denatured whey protein/κ-casein complexes on the final firmness and water holding capacity of the acid gel (Guyomarc'h *et al*., 2003a). Other researchers (Li *et al*., 2014; Xu et al., 2015) also demonstrated that soluble whey protein/κ-casein complexes were predominant to raise the water holding capacity and firmness of yoghurt gel than whey protein associated casein micelles.

However, Schorsch *et al*. (2001) and Donato *et al*. (2007a) revealed that micelle bound whey protein, rather than the soluble denatured whey protein/κ-casein complexes, are responsible for

increasing the final firmness and also inducing the formation of acid gels. Regarding the role of micelle bound whey protein, Schorsch *et al*. (2001) applied heat treatment on the unheated casein micelles in the presence of soluble denatured whey protein/κ-casein complexes to increase the micelle bound complexes and consequently noticed a firmer gel as well as uniformity of the acid gel. In fact, whey protein-casein micelles induce the lowering syneresis by hindering fusion of aggregated casein micelles or increase water holding capacity due to the high water binding capacity of the unfolded whey proteins (Puvanenthira *et al*., 2002). Other research conversely presented that the above approaches of the micelle bound complexes and the soluble denatured whey protein/κ-casein complexes is not only the key factor to consider for changing the gelation properties and/or textural properties of heated skim milk. The total amount of the denatured whey protein-casein complexes may alter the gelation properties of the heated milk (Vasbinder *et al*., 2003; Donato *et al*., 2007a). In addition to texture development of acid gels, heated milk enhances the involvement of higher number of protein in the gel networks (Guyomarc'h *et al*., 2003a), resulting increase connection and compact network (Lucey *et al*., 1998b) as well as formation of covalent disulphide bonds in the protein network (Lakemond and van Vliet, 2008).

Thus, it is apparent that the role of the denatured whey protein-casein complexes on the final gel firmness and water holding capacity is enhanced through the formation of the higher number of protein networks and through immobilized water inside the protein networks. In conclusion, the final gel strength of acid skim milks depends on the formation of denatured whey protein-casein complexes because of increasing the connectivity between the casein clusters that build the gel network.

5.2 Rheological properties of yoghurt

Formation of the interaction between denatured whey protein and caseins are investigated using large or low amplitude rheology in the most studies of acid milk gels. There are some evidences how to increase storage modulus (G') and reduce tan δ (ratio of loss to the storage modulus) of acid gel during gel formation. Roefs and van Vliet (1990) demonstrated that the acid milk gels are formed by hydrophobic interaction in the protein networks which contribute to increase the final elasticity of acid gel. In other studies, it is believed that the formation of the disulphide covalent bond between denatured whey protein and casein in the heated milk to increase the elastic modulus and lower tan δ (Guyomarc'h *et al*., 2009). This has previously demonstrated by Vasbinder *et al*. (2004) that the formation of the thiol/disulphide exchange reaction in the acid gel during fermentation contribute to rise up the G' of acid gel. Considering the respective role of the protein complexes on the gel strength, it is somewhat complicated to distinguish the specific contribution of the micelle bound complexes and the soluble denatured whey protein/κ-casein complexes.

On the contrary, some researchers (Schorsch *et al*., 2001; Ozcan *et al*., 2015) observed the effect of the micelle bound complexes and the soluble denatured whey protein/κ-casein complexes on the mechanical and structural properties of the yoghurt curd by altering the initial conditions of the milk prior to heat treatment. Some studies focused on the micelle bound complexes by lowering the milk pH below the natural pH (~ 6.7) of milk (Anema and Li, 2003; Lakemond and van Vliet, 2008), which played a significant role on the final elastic properties of acid milk (Lucey *et al*., 1998b; Schorsch *et al*., 2001). Despite the role of the micelle bound complexes, other studies emphasized the important role of the soluble denatured whey protein/κ-casein complexes on the structural properties of acid gels. By changing the pH from natural milk pH to the alkaline conditions, consequently more formation of the soluble denatured whey protein/κ-casein complexes which increase the rheological and physical properties (Vasbinder *et al*., 2003; Anema *et al*., 2004; Rodriguez and Dalgleish, 2006). Possible reasons for the conflicting results of the relative importance of the micelle bound complexes and the soluble denatured whey protein/κ-casein complexes in milk gels is to related to the either methods of acidifications (Lee *et al*., 2003) or the use of the different starting materials (Lucey *et al*., 1998a).

Despite of the type of protein complexes, the nature of the interactions plays a role to modulate the gel structure. For instance, changing the pH of whey protein isolate (WPI) from 6.0 to 8.0 prior heat application, it increases the covalent disulphide bond in the denatured whey protein/κ-casein complexes as result of increase the elastic modulus of the final acid gels (Hoffmann and vanMil, 1999; Anema *et al*., 2004; Donato *et al*., 2007a). Alternatively to blocking the free thiol groups of WPI by using N-ethyl-maleimide (NEM) have been shown to reduce the final storage modulus due to the absence of disulphide bonds (Lucey *et al*., 1998a).

In authors' point of view, formation of the covalent disulphide bonds between denatured whey proteins and caseins are probably the most susceptible factor to control the viscoelastic moduli or the 'solid like' behavior of the yoghurt gels. Further investigation is required to find out the relative importance of the micelle bound complexes and the soluble denatured whey protein/κ-casein complexes on the viscoelastic or the 'solid like' behavior of the acid milk gels.

5.3 Microstructure of yoghurt

The microstructure of yoghurt is crucial for both the texture and the mouth feeling, which are the important parameters for acceptance of the product by the consumer. It is very important to gain an insight in the factors which play a role in the formation

of the microstructure to be able to control the physical properties of existing products, improve or develop new products (Lee and Lucey, 2004). By also applying microscopic techniques, such as confocal laser scanning microscopy (CLSM), transmission (TEM) or scanning electron microscope (SEM) on yoghurt, the microstructure of the gel network can be visualized, as a result of which extra valuable information on the microstructure can be obtained. Generally, different commercially available dyes are used as fluorescent for confocal microscopy on yoghurt, namely Acridine Orange, Fast Green, Rhodamine B, and Rhodamine 6G (Lucey *et al*., 1998b; Lee and Lucey, 2004; Ozcan-Yilsay *et al*., 2007). It is difficult to differentiate the microstructural properties of acid gels containing high level of either the micelle bound complexes and the soluble denatured whey protein/κ-casein complexes. In agreement with the similar findings of Guyomerc'h *et al*. (2009) and Ozcan *et al*. (2015), who reported that the same microstructure were observed at the various level of whey protein-casein complexes. However, the size of the micelle bound complexes were observed in transmission (TEM) or scanning electron microscope (SEM) image after association of the denatured whey protein on the surface of the casein micelles. Subsequently the elongated, a long appendages or round appendages was shown on the casein micelles in heated milk (Kalab *et al*., 1983).

Nevertheless, some studies explain the uniform protein networks remaining a large number of very small pores where casein micelles show a particular entity by using CLSM (Remeuf *et al*., 2003). The micrograph of acid milk gel made from heated milk have been shown a denser network with a large number of protein connection via intermolecular disulphide bonds (Fig. 1) due to the association of the denatured whey protein with casein micelles or serum κ-casein increase the number of the protein bonds (Lucey *et al*., 1998b). The high concentration of protein networks in microstructure is correlated with the higher final G´ values of the acid milk gels (Lucey *et al*., 1998b; Nguyen *et al*., 2013; Chever *et al*., 2014).

To our knowledge, presence of numerous heat induced micelle bound complexes and soluble denatured whey protein/κ-casein complexes lead to increase the concentration and branching of the protein networks, and consequently it shows a strong effect on the gel microstructure. Although micelle bound complexes and soluble denatured whey protein/κ-casein complexes participate in the formation of protein networks, to the author's knowledge no attempt has however yet been undertaken to evaluate which complex is dominant on the formation of protein network of yoghurts.

6. Conclusions

The denatured whey protein-casein complexes have an extensive impact on the properties of yoghurt texture. But the formation of the denatured whey protein-casein complexes was observed by the using only heat treatment rather than other process like using a number of chemicals and biological methods or other technological process such as high pressure treatments. Few studies have focused on the improvement of their functionality as potential ingredients and finds ways to apply other food products than milk gels. Better knowledge to modify the denatured whey proteins-casein complexes and transfer to either micelle bound complexes or soluble denatured whey protein/κ-casein complexes by changing the milk pH, is necessary to upgrade their functionality. Large numbers of studies have investigated the modification protein complexes, whereas only a few studies applied these complexes to observe the gelation behavior and yoghurt texture. However, the role of the micelle bound complexes or soluble denatured whey protein/κ-casein complexes in heated milk gels as a means to altering yoghurt properties may have potential interest for current and future research. Increased understanding the impact of various milk processings on yoghurt texture could be applied at industrial level to develop novel processing strategies targeting to have uniform yoghurt texture whole over the year.

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