

Review Article

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## Different Approaches to Improve Thermostability of Whey Proteins: A Review

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In the dairy industry the stability of dairy proteins toward heat treatment is a major processing issue. Heat treatment of whey proteins to temperatures above 70°C causes denaturation, which in turn leads to protein aggregation. This can result in excessive thickening or gelling during processing of the dairy product and later, upon storage. Whey proteins (WPs) have distinctive nutritional and functional properties that make them unique food ingredients. It is widely used as an ingredient in many traditional and novel food products primarily because of its high nutritional value and some desirable functional properties in whey protein products. This paper reviews the several approaches such as application of transglutaminase, addition of chelating agents, carbohydrates, hydrophobic compounds, H<sub>2</sub>O<sub>2</sub>, chitosan and novel methods such as high hydrostatic pressure, ultrasonication to reduce denaturation and aggregation of whey proteins during heating and to produce heat stable whey protein products.

### Introduction

Whey proteins constitute 20% of the proteins in milk and include  $\beta$ -lactoglobulin ( $\beta$ -Lg; approximately 3.2 g/L),  $\alpha$ - lactalbumin ( $\alpha$ -La; approximately 1.2 g/L), bovine serum albumin (BSA; approximately 0.4 g/L), and immunoglobulins (approximately 0.7 g/L) (Raikos, 2010). Whey protein is considered as high quality protein because of its branch chain amino acid. Growing awareness about the nutritional benefits of whey proteins and technological advancements in improving functional properties has led to an ever

increasing demand of whey proteins in the form of commercial whey protein ingredients, such as whey protein concentrates (WPC) and whey protein isolates (WPI). Whey protein ingredients are widely used in a variety of products, such as whey protein fortified sports beverages, nutritional and meal replacement as well as medical and clinical nutrition beverages (Suresh and Hasmukh, 2017). Whey protein isolates (WPI) and whey protein concentrate (WPC) are used as food ingredients due to their commercially important functional properties such as solubility, viscosity, water-holding capacity,

gelation, adhesion, emulsification and foaming. As foodstuffs they are applied not only because of their functional properties, but also because of their high nutritive value, reasonable cost and GRAS status (Greta *et al.*, 2006).

Heat treatment of whey protein solutions above 85°C leads to denaturation and aggregation of whey proteins and at a sufficiently high protein concentration (typically beyond 8–10% proteins) they tend to form heat-induced gels. Denaturation of whey proteins is accompanied by release of small sulfur-containing compounds such as hydrogen sulfide and methanethiol which are highly flavor some compounds and cause cooked flavors in heated milk (Alattabi *et al.*, 2009). In general, whey protein aggregation involves the interaction of a free –SH group with the S–S bond of cystine-containing proteins such as  $\beta$ -Lg,  $\kappa$ -casein ( $\kappa$ -Csn),  $\alpha$ -La, and BSA via –SH/S–S interchange reactions (Considine *et al.*, 2007). These protein–protein interactions lead to irreversible aggregation of proteins into protein complexes of varying molecular size depending on the heating conditions and protein composition as depicted in figure 1. Knowledge of ways of inhibiting the formation of these protein complexes is needed in order to minimize the negative practical consequences that may arise.

### **Whey protein fractions in bovine milk**

$\beta$  – Lactoglobulin ( $\beta$  – Lg) is the most abundant of the whey proteins in ruminant milks and comprises upto 50% of the total whey protein in bovine milk. It has molecular weight of 18.3KDa (monomeric form).  $\alpha$  - Lactalbumin is the second major bovine whey protein, compact globular protein of relatively low molecular weight (14 KDa). The whey protein profile of bovine milk is presented in table 1.

### **Approaches to improve thermostability of whey proteins**

Whey protein is a valuable by-product of cheese manufacturing process and widely used as an ingredient in many traditional and novel food products primarily because of its high nutritional value and some desirable functional properties, such as gelation, emulsification, foaming, flavor binding properties (Smithers, 2015). Whey protein isolate (WPI) products refer to commercially available products purified to protein content higher than 90% (Foegeding *et al.*, 2002).

### **Application of transglutaminase**

Transglutaminase (TGase) (EC 2.3.2.13) is an enzyme that catalyzes an acyl transfer reaction between  $\gamma$ -carboxyamide moiety of protein bound glutamine residue (acyl donor) and a primary amine (acyl acceptor). When lysine residues act as acyl acceptors,  $\epsilon$ -( $\gamma$ -glutamyl) lysine “isopeptide” covalent bonds are formed in proteins, leading to intra- and inter-molecular cross-linking. The enzyme also catalyzes hydrolysis of the  $\gamma$ -carboxyamide group of glutaminyl residues, resulting in deamidation (Yokoyama *et al.*, 2004), which may greatly alter the electrostatic properties of the protein as glutamine residues are abundantly present in most proteins. Whey proteins become more susceptible to crosslinking by transglutaminase in the presence of reducing agents (Kuraishi *et al.*, 2001), increasing enzyme access to NH<sub>2</sub> groups by maintaining the active site (sulphydryl) in the reduced state.

Denaturation of whey proteins by heat or addition of a reducing agent, such as DTT or cysteine, before incubation with TGase can enhance the cross-linking (Tang and Ma 2007). The conformational changes of proteins due to denaturation or reduction by

reducing agents would favor the exposure of the enzyme-targeted sites, namely reactive lysine and glutamyl residues.

The effects of preheating TGase treated whey protein solutions on the heat stability of proteins (Zhong *et al.*, 2013). Preheating of 5% (w/v) WPI (pH 7.0, 80°C for 15 min) before various treatments with TGase (at 50 °C for various times) remarkably improved its heat resistance to thermal denaturation. Although NaCl (50 mM) was added to the 5% WPI solutions and heated to 138°C for 5 min, the WPI dispersions remained transparent. This suggests the feasible application of such conditions to the development of food products with a high whey protein concentration (5% w/v) such as sterilized beverage products as shown in figure 2. Srinivasan and Kingsley, (2013) reported that heat shocking at 70°C/10 min of WPI dispersions (5% protein, pH 7.0) and treated with TGase increased the thermal denaturation temperature ( $T_d$ ) of  $\beta$ -Lactoglobulin by about 1.5°C. MTGase treatment (30 h, 37°C) of the heat-shocked WPI increased the  $T_d$  of  $\beta$ -lactoglobulin by about 6.3-7.3°C when compared with heat-shocked only WPI at pH 7.0. Despite the potential beverage applications of TGase at neutral pH, TGase has been found to markedly reduce the heat stability of whey proteins at acidic pH (pH 4.0 to 4.5). Therefore, TGase application is not recommended for acidic beverage applications. Generally, whey proteins are stable to heating at acidic pH. However, TGase-treated WPI (4% w/v) at pH 4.5 showed decreased heat stability. The decreased stability of the TGase-treated whey proteins at pH 4 to 4.5 was attributed to partial loss of positive charges on lysine residues, which reduced the hydrophilic-hydrophobic balance on the protein surface (Agyare and Damodaran, 2010).

### Addition of additives/ compounds

#### Addition of carbohydrates

Improving the heat stability of a whey protein-containing nutritional beverage by adding various sugars is patented by Smulders and Somers (2012). Beverages containing 5% to 12% w/w of whey protein and 4% to 16% w/w of carbohydrate (mono-, oligo-, or polysaccharide) were used. It shows that the heat-stabilizing effect is dependent on the type and concentration of carbohydrate as well as pH. The beverage consisting of 10% WPC (8% protein) heated at 90 °C for 5 min was stable when it contained 6% to 10% sucrose or lactose at pH 7.0, or 8% to 10% fructo-oligosaccharides or 10% inulin at pH 7.5; lower concentrations of each of these carbohydrates resulted in formation of a gel rather than remaining liquid. The amount of sucrose has been shown to strongly affect its stabilizing behavior on heated whey proteins (Kulmyrzaev *et al.*, 2000). Adding sucrose at low (0% to 10% w/w) concentration to WPI solutions (10% w/w, pH 7, 15 mM CaCl<sub>2</sub>, heated at 75 °C for 15 min) decreased the rate of whey protein aggregation and gelation. In contrast, the addition of sucrose at high (10% to 30% w/w) concentration increased protein-protein interactions and enhanced protein aggregation. Lactose and ribose stabilized WPI solutions (13.5% to 15% w/v, pH 6 to 9), shown increases in gelation temperature (by 7 and 3 °C, respectively) whereas, ribose enhanced the Maillard reaction and covalent cross-linking of proteins. Browning was observed in the ribose-containing gels but no discoloration was observed in the lactose-containing gels. Pentose sugars (such as ribose) react more readily with proteins than hexoses (such as glucose) and disaccharides (such as lactose) (Rich and Foegeding, 2000).

Zhang *et al.*, (2012) reported that, heat-induced aggregation of WPI in the presence

of low methoxyl pectin at near neutral pH. As for the WPI-dextran conjugate, the heat stability of the WPI-pectin conjugate depended on the protein: pectin ratio, as well as on the pH. At a high concentration of pectin (pectin: protein weight ratio >0.2) at pH 6.0 and 6.2 (values above pI but below 6.4), the turbidity and particle size of the mixture decreased during heating due to increased interaction between the negatively charged pectin and positively charged domains of the proteins, which limits the protein-protein interactions. However, at low concentrations of pectin (pectin: protein weight ratio <0.2) at pH 6.4, the turbidity and particle size of whey proteins increased during heating resulting in phase separation and formation of large protein aggregates. The results described here indicate that optimized conditions of interactions between polysaccharides and whey proteins must be achieved in order to successfully improve the heat stability of the whey proteins.

### Addition of chelating agents

Keowmaneechai and McClements (2006) investigated the effect of adding EDTA and trisodium citrate to whey protein-stabilized emulsions containing 10mM CaCl<sub>2</sub>. These emulsions, which contained 6.94% soybean oil and 0.02% WPI, are similar to those used in some commercial nutritional beverages. Without the chelating agents, the emulsions were unstable to heating at 90 °C but were stable in the presence of EDTA at a molar ratio to calcium of ≥1:1, or of citrate at a molar ratio to calcium of ≥1.5:1. The improved heat stability was shown by decreased particle size and emulsion viscosity. The improved stability of the whey protein stabilized emulsions was due to the ability of the chelating agents to bind free calcium ions, hence reducing droplet aggregation caused by calcium-induced whey protein denaturation and aggregation

(Keowmaneechai and McClements, 2002). Citrate was less effective than EDTA in preventing aggregation because of its lower binding constant for calcium. The presence of chelating agents did not protect the emulsions from gelation at 120 °C (Keowmaneechai and McClements, 2006).

### Addition of hydrophobic compounds

β-Lg is known to have high affinity toward a wide range of hydrophobic compounds (Loch *et al.*, 2011). The hydrophobic compounds include hydrolyzed/hydroxylated lecithin, and saturated/unsaturated fatty acids that have hydrocarbon chain lengths of 12 to 20 or 5 to 9 carbon atoms. Tran *et al.*, (2007) studied, the effectiveness of lecithin as a hydrophobic agent for inhibiting aggregation. They reported that native, hydrolyzed, and hydroxylated lecithin, when added to a WPI/micellar casein mixture (2.75%/5.5% w/v, pH 6.5, 80 °C for 5 min), had different effects on aggregate formation. Hydrolyzed and hydroxylated lecithins were more effective than native lecithin in reducing aggregate formation. It was postulated that the difference was due to the degree of hydrophobicity, since the native lecithin has the lowest hydrophobicity of the 3 types of lecithin. A mechanism of inhibition by lecithin was proposed whereby hydrolyzed lecithin binds to the exposed hydrophobic and -SH groups of heated whey protein before the denatured whey proteins interact with the casein micelles. Subsequently, the unfolded state of the denatured whey protein is stabilized and complex formation with casein is minimized. Lysolecithin (1% w/v) was found to partially prevent aggregation in heated WPI (2.75% w/v, <80 °C) (Le *et al.*, 2011). Upon heating to 80 °C for 15 min, lysolecithin significantly reduced the gel strength. Using nuclear magnetic resonance, Le *et al.*, (2011) proved that lysolecithin had interacted with the whey protein before the

heat treatment and the interaction was greater during heating. Their results further support the protective mechanism of lecithin postulated earlier by them (Tran *et al.*, 2007). These results indicate that lecithins exhibit protective behavior toward whey proteins during heat treatment, but the effect is strongly dependent upon the type of lecithin used and the experimental conditions.

The addition of conjugated linoleic acid (CLA) or myristic acid to a  $\beta$ -Lg B solution (molar ratio of 1.1:1) prior to heat treatment (40 to 93 °C for 12 min) delayed aggregation during heating (Considine *et al.*, 2007). However, polymerization reactions were prevented in the presence of CLA but not myristic acid. The authors postulated that the ability of CLA and myristic acid to protect  $\beta$ -Lg during heating was due to their high affinity toward  $\beta$ -Lg.

### Addition of H<sub>2</sub>O<sub>2</sub>

Recently, the effect of hydrogen peroxide treatment of whey proteins on denaturation, aggregation, gelation to improve the heat stability of whey protein isolate (WPI) solution (12–14% w/w protein) was investigated by Suresh and Hasmukh (2017). A concentration of H<sub>2</sub>O<sub>2</sub> in the range of 0–0.144 H<sub>2</sub>O<sub>2</sub> to protein ratios (HTPR) was added. The lower level of addition of H<sub>2</sub>O<sub>2</sub> (0.0014 and 0.0029 HTPR) to whey protein solution was not effective in decreasing denaturation of  $\beta$ -LG and, therefore, it did not improve heat stability. The samples treated with high levels of H<sub>2</sub>O<sub>2</sub> (0.072 and 0.144 HTPR) shown reduction in denaturation of  $\beta$ -LG and led to a dramatic improvement in the heat stability of whey proteins. It is proposed that treatment of whey proteins with sufficient level of H<sub>2</sub>O<sub>2</sub> lead to interaction of H<sub>2</sub>O<sub>2</sub> with free SH group of whey protein, which converted free thiol of Cys121 to a stable

form (R- SO<sub>3</sub>H) that does not promote intermolecular sulfhydryl-disulfide interchange. These mechanisms resulted in the prevention of whey protein denaturation and aggregation, when proteins are heated in the presence of sufficient level of H<sub>2</sub>O<sub>2</sub>.

### Addition of chitosan

Zhengtao and Qian (2017) studied the effect of chitosan on the heat stability of whey protein solution at pH 4.0–6.0. At pH 4.0, a small amount chitosan was able to prevent the heat-induced denaturation and aggregation of whey protein molecules. At higher pH values (5.5 and 6.0), whey proteins complexed with chitosan through electrostatic attraction. The formation of chitosan – whey protein complexes at pH 5.5 improved the heat stability of dispersions and no precipitation could be detected up to 20 days.

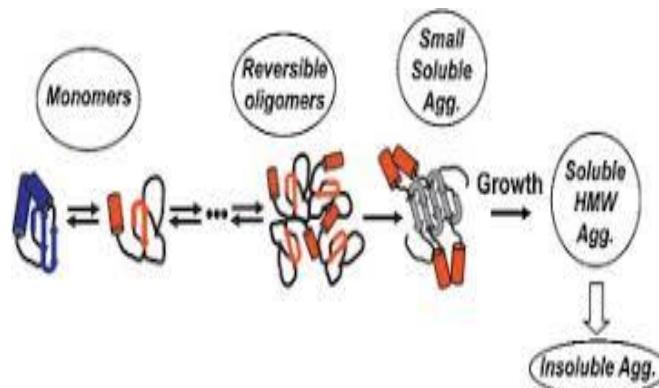
### High hydrostatic pressure

The increased interest in novel technologies using mild treatments and without addition of chemicals is nowadays very much in demand. One of the important aspects of pressure treatment is that food can be processed with minimal effect on the natural colour, flavour, taste and texture with little or no loss of vitamins. Pressure acts as a physicochemical parameter that alters the balance of intramolecular and solvent-protein interactions. Low protein concentrations and pressures up to 200 MPa usually result in reversible pressure-induced denaturation. Higher pressures (above 300 MPa) have irreversible and extensive effects on proteins, including denaturation due to unfolding of monomers, aggregation and formation of gel structures. The extent of high-pressure-induced denaturation of whey proteins increases with treatment time (Huppertz *et al.*, 2004).

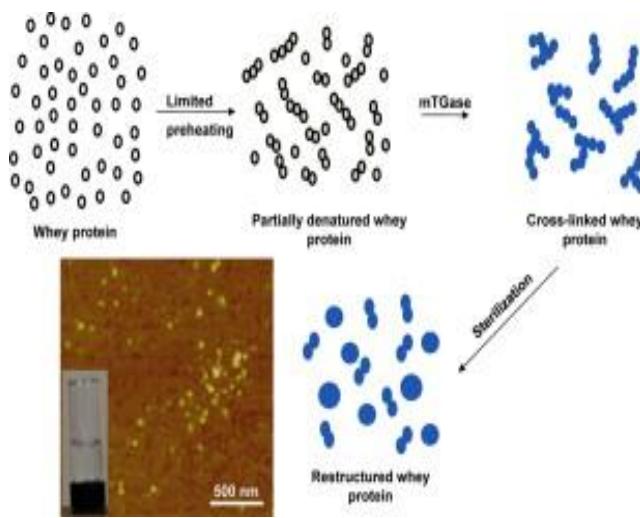
**Table.1** Profile of whey protein in bovine milk (Madureira *et al.*, 2007)

Whey protein fractions	Concentration (g/Lt)	Molecular weight (kDa)	Number of amino acids residues
<b><math>\beta</math>-Lactoglobulin</b>	1.3	18277	162
<b><math>\alpha</math>-Lactalbumin</b>	1.2	14175	123
<b>GMP</b>	1.2	6700	64
<b>Immunoglobulins</b>	0.7	Long chain:25000 Heavy chain: 50000-70000	-
<b>Bovine serum albumin</b>	0.4	66267	582
<b>Lactoferrin</b>	0.3	80000	700
<b>Lactoperoxidase</b>	0.01	70000	612

**Fig.1** Effect of heat treatment on whey protein



**Fig.2** Cross linking of whey protein by Transglutaminase



The application of pressure has a disruptive effect on intramolecular hydrophobic and electrostatic interactions. As hydrogen bonding interactions are relatively insensitive to pressure, high pressure disrupts the quaternary and tertiary structure of globular proteins with relatively little influence on their secondary structure.

Greta *et al.*, (2006) studied that, influence of high-pressure treatments on the solubility, surface hydrophobicity, foaming and emulsifying ability of whey protein concentrate (WPC) and whey protein isolate (WPI). Dispersions of WPC and WPI powders [10% (w/w)] was processed at 300 MPa and 600 MPa, for 5 and 10 min at  $40 \pm 2$  °C. They found that significant modification of solubility and surface hydrophobicity with increasing intensity and duration of applied pressure, indicating partial denaturation and aggregation of proteins. Influence of high pressure on pure  $\beta$ -lactoglobulin showed a notable effect on its conformational and aggregation properties, affecting its functionality. However, functionality of WPC and WPI modified by high pressure still does not meet the expectations which could realise the full potential of these food ingredients in industrial application (Huppertz *et al.*, 2006).

### **Ultrasonication**

High-intensity (10- 1000 W cm<sup>2</sup>) ultrasound has gained particular attention for utilisation within the food industry. Besides microbiological destruction effect, high intensity ultrasound technology has been used to enhance food quality in recent years. High-intensity ultrasound may exert effects on the physical, chemical or biochemical properties of foods through acoustic cavitation, which can release high amounts of highly localized energy by collapse of cavitation bubbles in liquids (Gallego *et al.*, 2010). Acoustic cavitation in liquids can induce chemical and

physical changes in foodstuffs. These bubbles undergo a formation, growth and implosive process. High temperatures within the bubbles are produced at the moment of the collapse of cavitation bubbles, which is along with emission of light (sonoluminescence). Shockwave, turbulent motion of the liquid and radicals are also produced by acoustic cavitation in liquids (Ashokkumar, 2011).

Ashokkumar *et al.*, (2009) observed short preheat treatment followed by sonication of the whey protein solution for a short time at 20 kHz increased the heat stability. An aqueous solution containing 4 to 15% total protein (by weight) was preheated to 80°C and post-heat-treated to 85°C in an water bath held for 1 min and 20 min. The preheated solution was then subjected to high-intensity, low-frequency ultrasound for less than 5 min. They found that the effects on solution viscosity for a 6.4% protein (by weight) of preheated sample increased due to heat-induced aggregation of whey proteins but when subjected to sonication for even 5 s shown a significant decrease in viscosity due to the shear forces generated by acoustic cavitation disrupt the hydrophobic interactions or the intermolecular disulfide bonds. At higher protein concentrations, these heat treatments can lead to gel formation. Further it is postulated that aggregation of protein particles occurs to a much lesser extent during the post-heat-treatment process, functional groups responsible for such inter particle interactions such as free thiols are also deactivated during the heat treatment and sonication sequence. The high intensity ultrasound on physicochemical and emulsifying properties of thermally aggregated whey proteins was studied by Xue *et al.*, (2017). Whey protein isolate (WPI) solutions was sonicated for 20 min using an ultrasonic probe (frequency: 20 kHz; amplitude: 20%) pre- and post thermal treatment (85°C for 30 min). They observed

that, no significant changes in zeta-potential, total free sulphydryl group by ultrasound either pre-or post thermal aggregation and concluded that ultrasound treatment on post-thermal aggregation has improving effect on physicochemical and emulsifying properties of whey protein soluble aggregates for potential industrial applications.

Whey proteins (WPs) have distinctive nutritional and functional properties that make them unique food ingredients. Different attractive and repulsive molecular forces, involved in the stability of unique three-dimensional structure of proteins. A drawback of whey proteins is their instability to thermal processing, which leads to their denaturation, aggregation, and, under some conditions, gelation.

Denaturation of WPs results in unfolding of the compact structures, which subsequently causes aggregation mainly due to the exposure of previously buried apolar groups and occurrence of sulphydryl/disulfide exchange chain reactions via activated thiol groups. Several approaches such as application of protein – CHO conjugation, transglutaminase, addition of chelating agents, carbohydrates, polysaccharides, hydrophobic compounds, H<sub>2</sub>O<sub>2</sub>, chitosan and novel methods such as HPP, ultrasonication have been used to reduce denaturation and aggregation of whey proteins during heating and to produce heat stable whey protein products.

The choice of the most suitable approach will ultimately depend on the final purpose of the product and whether it is important to retain the whey proteins in their native form or it is acceptable for the proteins to be in a modified (chemically or physically) heat-stable form which is convenient to use and has desirable functional and/or nutritional properties.

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