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Full Length Research Paper

A kinetic Study in the changes of SDS-PAGE profile of whey proteins during storage at different temperatures

Tahmasebi E., Moein Bozorgi and Keyvan Khukhan

School of Health, Environmental Health Research Center, Islamic Azad University, Tehran, Iran.

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Whey is the soluble fraction of milk separated from the caseins during manufacture of rennet-coagulated cheese, (sweet whey) or use of acids (acid whey). Whey proteins are widely used in a variety of foods, primarily for their superior functional properties such as gelling and emulsification. Solubility is the major protein attribute affecting all functional properties. In this study, the effect of storage at different temperatures on sweet and acid whey proteins solubility and gel electrophoresis pattern was investigated. Acid whey was produced by adjusting the pH of pasteurized milk to 4.6 and sweet whey was produced by adding microbial rennet to the pasteurized milk held at 37°C for 1 h. Whey samples were stored at -18, 4, 25, 40 and 60°C. At different time intervals, samples were removed and used for protein solubility determination and SDS-PAGE. Results showed that the solubility of proteins of both whey types stored at -18 and 60°C significantly decreased, such that at -18^{O}C, 50 and 61% decrease for sweet and acid whey protein solubility, respectively, occurred after 6 weeks. These decreases were 44 and 55% for samples stored at 60°C for 5 days. The decrease in solubility at 4, 25 and 40°C was less drastic. At all temperatures, decrease in protein solubility followed a first order kinetic. The activation energies for loss of protein solubility were 6.3 to 6.4 kcal/mol for sweet and acid whey proteins. The SDS-PAGE patterns indicated significant denaturation and aggregation of major whey proteins during storage.

Key words: Whey protein, solubility, kinetics, SDS-PAGE, activation energy.

INTRODUCTION

Whey is the soluble fraction of milk, rich in proteins, minerals and lactose, that is separated from the casein during manufacture of rennet-coagulated cheese or by adding acid to milk. The whey produced from rennet-coagulated casein or cheese is referred to as sweet whey whereas that from mineral or lactic acid-coagulated casein is called acid whey (Corredig and Dalgleish, 1996). Whey proteins are globular proteins with molar mass ranging from 14 to 1000 kDa and are composed of 60% β-lactoglobulin β-Lg, 22% α-lactoalbumin α-La, 5.5% bovine serum albumin (BSA) and 9% immunoglobulins ((Ig's) (Cavallieri et al., 2007). The isoelectric points of these proteins are 5.2 for β-Lg, 4.2 to 4.5 for α-La, 4.7 to 4.9 for BSA and 5.5 to 6.8 for Ig's (Bryant and McClements, 1998). Heat-induced aggregation and gelation of whey proteins have been extensively studied (Corredig and Dalgleish, 1996; Bryant and McClements, 1999; Livney et al., 2003). It has long been known that when temperatures are elevated above room temperature, β-Lg dissociates from a dimer to a monomer, exposing its thiol group and interior hydrophobic residues, enabling thiol/disulfide exchange reactions. The hydrophobic, thiol-disulfide and electrostatic interactions induced by thermal aggregation have since been examined under various conditions for β-Lg and α-La (Alting et al., 2003; Havea et al., 2004). It has been reported that the molecular masses of aggregates are dependent on concentration, pH, ionic strength and temperature of heating (Kazmerski et al., 2003). However, the complete mechanism of heat denaturation and aggregation of the isolated proteins is not entirely understood (Kazmierski and Corredig,

*Corresponding author.E mail: mesabi_ahmed@yahoo.com

2003). Whey proteins are valuable by-products from the cheese industry. They are used widely in a variety of foods primarily for their superior gelling and emulsification properties (Solar and Gunasekaran, 2010). In addition, whey proteins have the advantage of their high nutritive value and are generally recognized as safe (GRAS) (Kresic et al., 2006; Herceg et al., 2007). The physicochemical properties of whey proteins suggest that they may be suitable for other novel food and non-food applications (Sun and Gunasekaran, 2010). Whey proteins are used as functional ingredients in a wide variety of food products because of their ability to modify the texture and stability of emulsions, foams and gels ((Bryant and McClements, 1998, 1999; Cavallieri et al., 2007; Solar and Gunasekaran, 2010; Herceg et al., 2007). The development of foods with improved quality depends on improving our understanding of the molecular basis of protein functionality.

One important functional property of proteins is their water solubility. The protein solubility is a function of many factors, such as the native or denatured state and environmental factors (that is pH, temperature) (Lieske and Konrad, 1996; Guyomarc et al., 2003; Floris et al., 2008; Damodaran, 2008). The pH of the solution affects the nature and the distribution of the protein's net charge. Generally, the proteins are more soluble at low (acids) or high (alkaline) pH values because of the excess of charges of the same sign, causing repulsion among the molecules and consequently, contributing to its largest solubility. Temperature is also a factor that influences protein solubility. Protein solubility increases with temperature between 40 and 50°C. When the temperature of the solution is raised high enough for a given time, the protein is denatured (Pelegrine and Gasparetto, 2005; Damodaran, 2008). Majority of studies on whey protein solubility and functionality are focused on elevated temperatures. Little data is available on the behavior of whey proteins at low temperatures.

In this study, the changes in solubility and SDS-PAGE patterns of sweet and acid whey proteins during storage at low and moderately high temperatures was investigated. The kinetics of solubility loss was also studied.

MATERIALS AND METHODS

Preparation of sweet and acid whey

Fresh milk was obtained from a local animal husbandry center and pasteurized at 63°C for 30 min using a batch pasteurizer. Acid whey was produced as follows: the pH of pasteurized milk was adjusted to 4.6 with lactic acid at 25°C. After 1 h the whey was pressed in a cheese cloth. Whey samples were aseptically transferred to 45 ml plastic tubes and stored at -18, 4, 25, 40 and 60°C. At different time intervals, samples were analyzed for protein solubility and SDS-PAGE. Sweet whey was manufactured using rennet (Microbial METIO rennet, METIO SANGYO Co., LTD, Japan), according to the manufacturer's recommendations. The enzyme was added to pasteurized milk at 37°C and the milk was incubated at 37°C for 1 h. The coagulated casein was cut into 1 cm^3 cubes and the whey was treated as before. Samples were stored at different temperatures, as described earlier. Time of sampling was, for both acid and sweet whey, every week for samples incubated at -18°C, every 4 days for samples at 4°C, and every day for samples stored at 25, 40 and 60°C.

Solubility measurement

Protein solubility was determined by the method of Inklaar and Fortin (1969) with some modification. Total nitrogen in each sample was determined by the standard micro–Kjeldahl procedure (AOAC, 2002). Each sample was centrifuged using International Centrifuge Universal, Model UV, USA, at 2700·g for 10 min. The nitrogen content of the supernatant was measured and the solubility was calculated as:

$$\% \text{ Protein Solubility} = \frac{\text{Nitrogen content of the supernatant}}{\text{Total nitrogen in the whey sample}} \times 100$$

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS– PAGE)

The supernatants which were used for solubility measurement were also used for SDS–PAGE studies. Slabs for a 10% SDS-PAGE were formed according to the discontinuous buffer system of Laemmli (1970). Each sample was diluted with sample buffer to give a final concentration of 1 mg ml^{-1} protein, 0.01 mol L^{-1} Tris-HCl, pH 6.8, 0.4% SDS, 10% glycerol, and 0.004% bromophenol blue. The running gel was made of 5 to 20% gradient acrylamide in 1.2 mol L^{-1} Tris-HCl, pH 8.8 and 0.3% SDS. The stacking gel contained 3% acrylamide in 0.25 mol L^{-1} Tris-HCl, pH 6.8 and 0.2% SDS. Samples were heated in boiling water for 10 min and 50 µl was applied to each well. The electrode buffer comprised 0.025 mol L^{-1} Tris-HCl, 0.192 mol L^{-1} glycine and 0.1% SDS at pH 8.16. Electrophoresis was performed at constant 25 mA. Gels were stained with 0.25% Coomassie Brilliant Blue in 50% acetic acid/25% methanol and destained with a 10% acetic acid/7.0% methanol solution.

Statistical analysis

The protein solubility data was statistically analyzed by SPSS/PC software (Version 17). One–way ANOVA, independent–sample and paired Student's t tests were used for comparison of the means. The significant differences were set at P=0.05.

RESULTS AND DISCUSSION

The functional properties of proteins are often affected by protein solubility, and those most affected are thickening, foaming, emulsifying, and gelling. Insoluble proteins have very limited use in food. The solubility of a protein is the thermodynamic manifestation of the equilibrium between protein-protein and protein-solvent interactions (Damodaran, 2008). The changes in the solubility of acid and sweet whey samples are shown in Figure 1. The significant ($P<0.05$) decreases in the protein solubility values of enzymatically-prepared whey samples stored

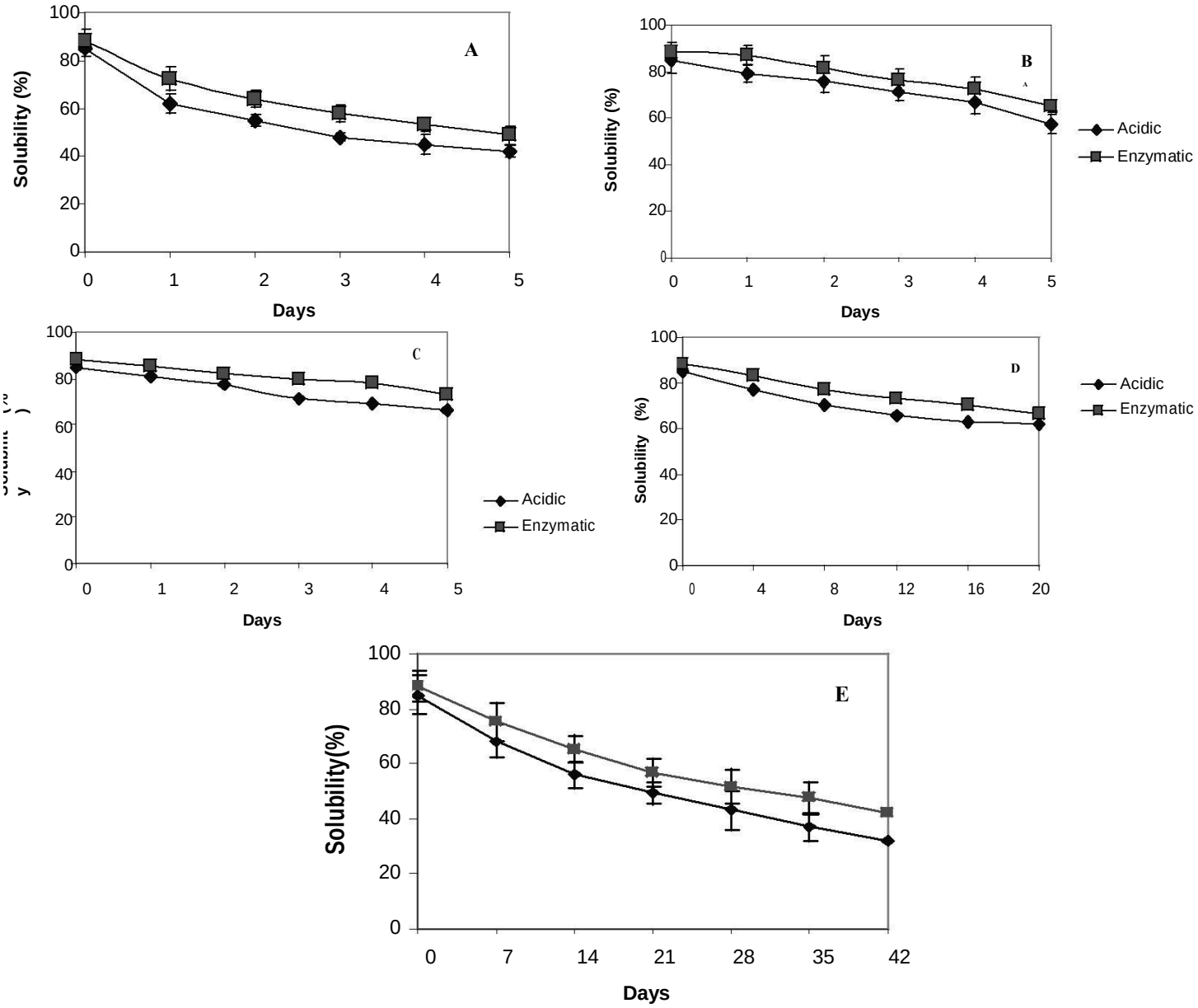

Figure 1. Changes in the solubility of whey proteins stored at a) 60°C, b) 40°C, c) 25°C, d) 4°C and e) -18°C. Acid whey Sweet whey .

for 5 days at 25, 40 and 60°C were 11, 28 and 44%, respectively. Corresponding values for acid-prepared whey samples were 22, 39 and 55% (Figure 1), indicating higher solubility of rennet whey proteins than those of acid whey samples. For samples stored under refrigeration condition (4°C) after 20 days storage, solubility significantly ($P<0.05$) dropped by 11.5 and 22.3% for sweet and acid whey proteins, respectively, (Figure 1). A drastic decrease in solubility occurred at -18°C such that at the end of the study (6 weeks) a 50 and 61% reduction in solubility was determined for sweet and acid whey proteins, respectively (Figure 1). Examination of the data of Figure 1, indicates that loss of solubility (and consequently) aggregation of whey proteins, followed a first order kinetics which is described by conventional first order kinetics according to the following equation (Tsoublli and Labuza, 1991):

$$\ln (Rsol) = kt$$

Where Rsol is the relative residual solubility (solubility at any time relative to time zero of storage) and k is the first order insolubilization rate constant which can be obtained from the slope of the regression of line of ln (Rsol)

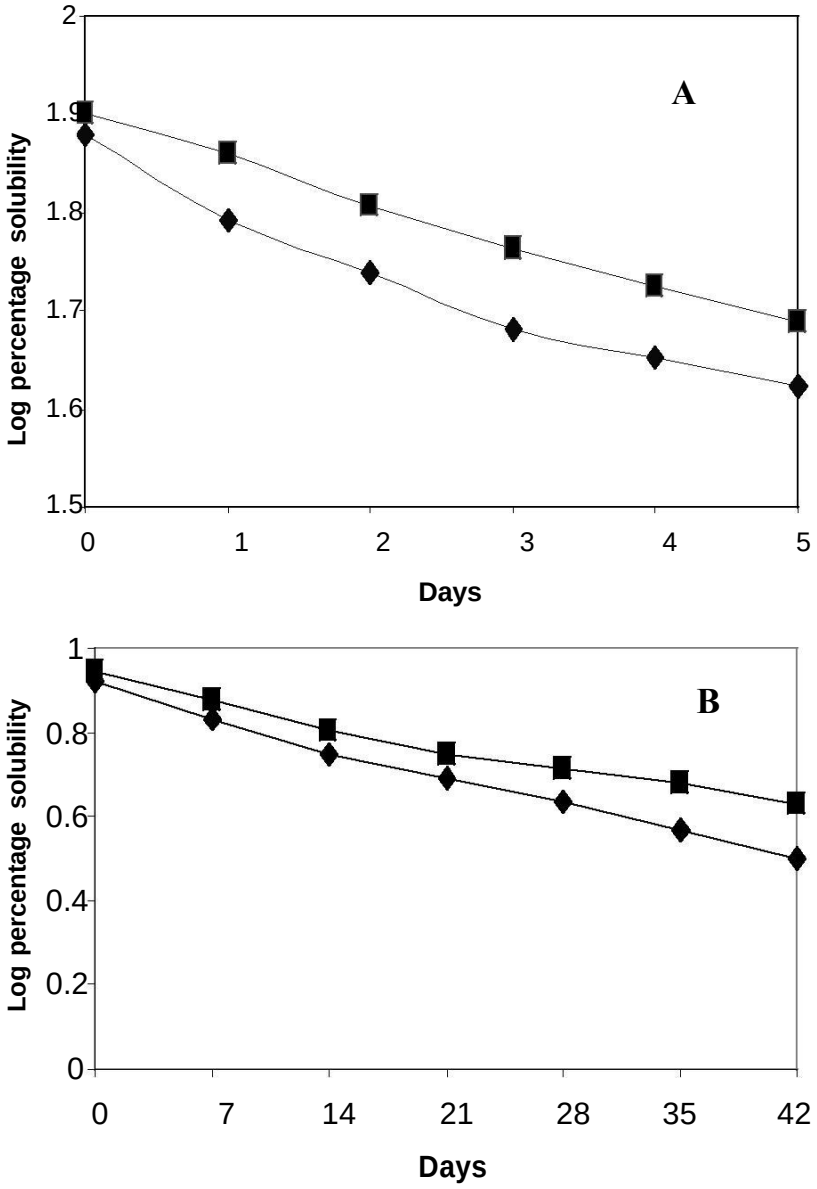

Figure 2. Semi-log plot of changes in the solubility of whey proteins stored at a) 60°C and b) -18°C. Acid whey , Sweet whey .

versus time. Figure 2 depicts semilog plots of data of Figure 1 (shown only for data at 60 and -18°C). From the slope of these curves, the rate constants at different temperatures can be calculated (Table 1). The temperature dependence of the rate of insolubilization can be best represented by the Arrhenius plot (Tsoublli and Labuza, 1991). Activation energy was determined using the slope of straight line obtained by plotting log k against 1/T (Figure 4). From this plot, Ea for 25 to 60°C was calculated to be about 6.3 kcalmol^{-1}, for the sweet whey proteins and 6.4 for acid whey proteins in the temperature range studied. These values are significantly lower than ~ 60 kcalmol^{-1} reported earlier for β-Lb in the temperature range of 75 to 120°C (Galani and Apenten, 1999). It can be seen in Figure 4 that a biphasic situation exists and the Ea at 4 and -18°C is very small. Several authors have reported such biphasic Arrhenius plot for β-Lg aggregation rates, and is believed to be the consequence of there being two distinct rate determining steps (Anema and McKenna, 1996; Galani and Apenten, 1997, 1999). These Ea low values obtained in our study indicate low sensitivity of the rate of insolubilization and aggregation of whey proteins to temperature under the conditions of this study. The reason for this difference is not known at present, but might be related to the difference in the temperature ranges used or possible

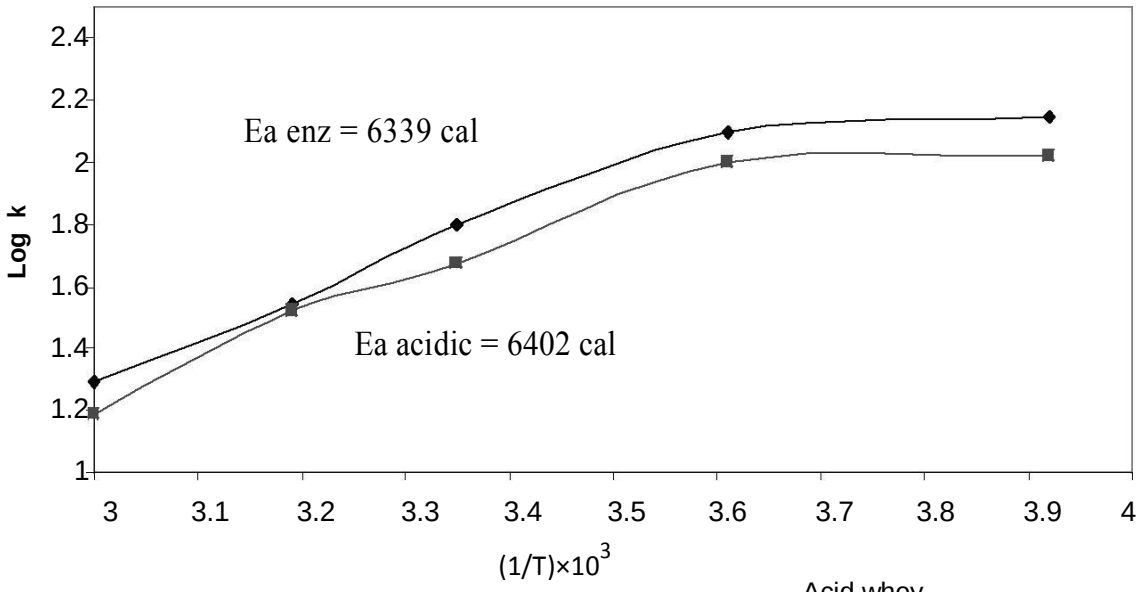

Figure 4. The Arrhenius plot for the insolubilization of sweet and acid whey proteins, Acid whey, Sweet whey.

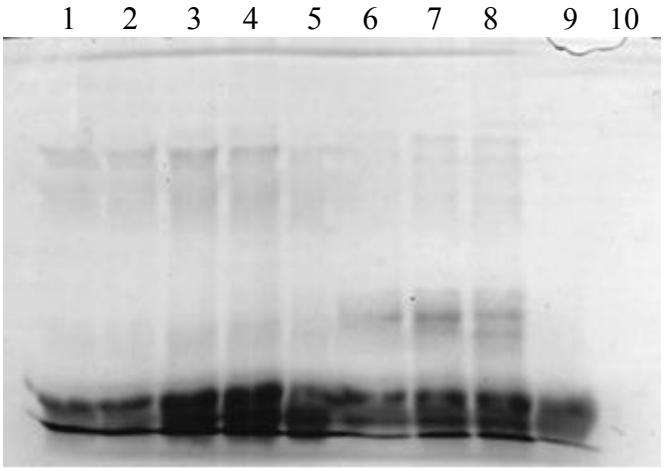

Figure 3. SDS-PAGE profile of whey proteins at 25°C (10%). 1-5: Enzymaticly prepared whey: 1 Day 1; 2 Day 2; 3 Day 3; 4 Day 4; 5 Day 5. 6-10: Acid prepared whey: 6 Day 1; 7 Day 2; 8 Day 3; 9 Day 4; and 10 Day 5.

changes in the aggregation mechanisms operating at freezing temperature (Anema and McKenna, 1996; Fennema et al., 2008).

Results of (Figure 3) SDS-PAGE for whey samples stored at 25°C are shown in Figure 2. Similar results were observed for other temperatures. Significant differences were seen between sweet and acid whey samples. Slight differences among SDS-PAGE patterns in each whey type were observed during storage. This result seems to contradict the results of NSI which indicated loss of protein solubility during prolonged storage. As explained under materials and methods, electrophoresis was performed on the supernatants of samples after centrifugation. At this stage, it is possible that some proteins precipitated due to centrifugation and did not appear in the supernatant. The fact that SDS-PAGE results show little significant difference between samples is probably related to extraction method used for preparation of samples for electrophoresis.

In this procedure ~50 µg protein was loaded into each well, therefore the concentration of protein in all wells was similar. When stored at 60°C several high molecular

weight bands started appearing from the third day of storage of the enzymatically prepared whey samples. In contrast, in acid prepared whey samples, several of the bands were lost and diffused. Low molecular weight protein bands also began to appear, the intensity of which increased as the storage time increased from 1 to 5 days. The main proteins affected by heating at 60°C are β-Lg and α-La, with approximate molecular weights of 18 and 14 kDa, respectively (Farrel, 1988). Similar results, but less drastic, were observed for samples stored at -18, 4, 25, and 40°C. Other investigators have reported similar results, but more significantly occurring at higher temperatures (Bryant and McClements, 1998; de Kruif and Tuinier, 1999; Sava et al., 2005; Cavallieri et al., 2007; Jovanovic et al., 2007).

The combined effects of thermal treatment and pH have profound effect on whey protein solubility. While native whey proteins resist aggregation at ambient temperatures for a wide range of pH's, slight heating at 70°C at various time intervals indicate a minimum solubility at isoelectric pH (~4.5) (Zhu and Damodaran, 1994). Drastic drop in solubility of whey protein during frozen storage as observed in this study could produce profound effects on the structural and chemical properties of proteins. Several mechanisms are believed to be responsible in altering the functional properties of proteins, including acceleration and formation of ice crystals (Bhargava and Jele, 1998; Damodaran, 2008; Strasburg et al., 2008), oxidation of sulfhydryl groups to disulfide bonds (Shenouda, 1980; Lee et al., 1992), and increase in the rate of chemical reactions due to the concentration effects of freezing, that is, macro-molecoules are forced together, making interactions more probable with resultant denaturation, insolubilization, aggregation, and precipitation of proteins. Taken together, the results of the solubility studies and SDS-PAGE indicate decrease in solubility of whey proteins during storage, even on refrigeration (4°C), ambient and moderately high (40 to 60°C) temperatures. Changes in solubility at these temperatures are probably the result of several types of interactions, especially electrostatic and hydrophobic interactions between whey protein molecules, the latter playing a more important role (Farrel, 1980; Corredi and Dalgleish, 1996; Sava et al., 2005). β-Lg is the main protein involved in these interactions fundamentally due to its high concentration (> 50% of whey proteins) (Galani and Apenten, 1999; Cavallieri et al., 2007), and its rather unique self asso-ciation to form dimers or higher ordered polymers at reduced temperatures (Farrel, 1988).

At higher temperatures, the sulfhydryl-disulfide exchange reactions are responsible for decrease in solubility, aggregation and precipitation of these proteins. Also, α-La might participate in these interactions to produce high MW co-aggregates (Farrel, 1988; de Kruif, 1999; Cavallieri et al., 2007). Upon heating β-Lg at neutral pH, the native dimers start to dissociate into monomer, leading to the exposure of the previously buried hydrophobic amino acids and the single free thiol group. Above 78°C, this is accompanied by the aggregation of the β-L molecules as a result of hydrophobic interactions, with consequent decrease in protein solubility (Sava et al., 2005). At the temperatures used, this phenomena does occur leading to some degree of decrease in solubility which is expected to affect the function of whey proteins in any food system.

In conclusion, the result of this study indicates that whey protein solubility is affected by storing at different temperatures, however the degree of temperature dependence of this property seems to be low. Since whey proteins are routinely used in different food systems, some degree of insolubilization is expected to occur during storage, particularly when frozen or stored for a prolonged time. It has been suggested that disaccharides and polymers (such as polysaccharides and polyethylene glycol) can stabilize proteins and protect them against denaturation (Srirangsan et al., 2010). Use of these additives for protecting whey proteins against insolubilization can be considered in future studies on whey protein properties.

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REFERENCES

Alting AC, Hamer RJ, de Kruif G, Paques M, Visschers RW (2003). Number of thiol groups rather than the size of the aggregates determines the hardness of cold set whey protein gels, Food Hydrocoll., 17: 469-479.

Anema SG, McKenna AB (1996). Reaction kinetics of thermal denaturation of whey proteins in heated reconstituted whole milk. J. Agric. Food Chem., 44: 422-428.

AOAC (2002). Official methods of analysis. Association of Official Analytical Chemistry. Airlington.

Bhargava A, Jele P (1995). Freezing of whey protein concentrate solutions and its effect on protein functionality indicators. Int. Dairy J., 5: 533-541.

Bryant CM, McClements DJ (1999). Ultrasonic spectroscopy study of relaxation and scattering in whey protein solutions. J. Sci. Food Agric., 79: 1754-1760.

Bryant CM, McClements DJ (1998). Molecular basis of protein functionality with special consideration of cold-set gels derived from heat-denatured whey. Trends Food Sci. Technol., 9: 143-151.

Cavallieri ALF, Costa-Netto AP, Menossi M, Da Cunha RL (2007). Whey protein interactions in acidic cold-set gels at different pH values. Le-Lait, 87: 535-554.

Corredig M, Dalgleish DG (1996). Effect of temperature and pH on the interactions of whey proteins with casein micelles in skim milk. Food Res. Int., 29: 49-55.

Damodaran S (2008). Amino, acids, peptides and proteins, in Fennema O.R. (Eds). Food Chemistry, 3rd edn, Marcel Dekker, Inc. New York, USA, pp. 217-329.

de Kruif KG, Tuinier R (1999). Whey protein aggregates and their interaction with exo-polysaccharides. Int. J. Food Sci. Technol., 34: 487-492.

Farrel HM, Wong NP, Jennes R, Keeney M, Marth EH (1988). Physical

equilibria: Proteins, in:, (Eds). Fundamentals of Dairy Chemistry 3rd edn,. AVI PUB., New York, pp. 481-510.

Fennema OR Damodaran S, Parkin KL **(**2008). Introduction to Food Chemistry. In: "Food Chemistry" 4th ed. CRC Press, Taylor and Francis Group, New York, pp. 1-16.

Floris R, Bodnar I, Weinbreck F, Alting AC (2008). Dynamic rearrangement of disulfide bridges influences solubility of whey protein coatings. Int. Dairy J., 18: 566-573.

Galani D, Apenten RKO (1999). Heat-induced denaturation and aggregation of β-Lactoglobulin: kinetics of formation of hydrophobic and disulphide-linked aggregates. Int. J. Food Sci. Technol., 34: 467-476.

Galani D, Apenten RKO (1997). The comparative heat stability of bovine β-The comparative heat stability of bovine β-lactoglobulin in buffer and more complex heating media. J. Sci. Food Agric. 74: 89– 98.

Guyomarc ZF, Law AJR, Dalgleish DG (2003). Formation of soluble and micelle-bound protein aggregates in heated milk. J. Agric. Food Chem., 51: 4652-4660.

Havea P, Carr AJ, Creamer LK (2004). The roles of disulphide and non-covalent bonding in the functional properties of heat-induced whey protein gels. J Dairy Res., 71: 330-339.

Herceg Z, Režek A, Lelas V, Kresic G, Franetovic M (2007). Effect of carbohydrates on the emulsifying, foaming and freezing properties of whey protein suspensions. J. Food Eng., 79: 279-286.

Inklaar PA, Fortin J (1969). Determining the emulsifying and emulsion stabilizing capacity of protein beef additives. Food Technol., 23: 103-107.

Jovanovic S, Barac M, Macej O, Vucic T, Lacnjevac C (2007). SDS-PAGE analysis of soluble proteins in reconstituted milk exposed to different heat treatments. Sensors 7: 371-383.

Kazmerski M, Agboola S, Corredig M (2003). Optimizing stability of orange juice fortified with whey protein at low pH values .J. Food Quality. 26: 337–352.

Kazmierski M, Corredig M (2003). Characterization of soluble aggregates from whey protein isolate. Food Hydrocoll., 17: 685-692.

Kresic G, Lelas V, Herceg Z, Anet RA (2006). Effects of high pressure on functionality of whey protein concentrate and whey protein isolate**.** Le Lait, 86: 303-315.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriofage T_4. Nature, 227: 680-685.

Lee SY, Morr CV, Ha EYW (1992). Structural and functional properties of caseinate and whey protein isolate as affected by temperature and pH. J. Food Sci., 57: 1210-1229.

Lieske B, Konrad G (1996). Microparticulation of whey protein: related factors affecting the solubility. Zeitsch Leb Forsch A. 199: 289-293.

Livney YD, Corredig M, Dalgleish DJ (2003). Influence of thermal processing on the properties of dairy colloids, Curr. Opin. Colloid Interface Sci. 8: 359-364.

Pelegrine DHG, Gasparetto CA (2005). Whey proteins solubility as function of temperature and pH. LebensmWiss u Technol., 38: 77-80.

Sava N, Rotaru G, Hendrickx M (2005). Heat-induced changes in solubility and surface hydrophobicity of β-lactoglobulin. Agroalim. Processes Technol., 1: 41-48.

Shenouda SYK (1980). Theories of protein denaturation during frozen storage of fish flesh. Adv. Food Res., 26: 275-2311.

Solar O, Gunasekaran S (2010). Rheological properties of rennet casein-whey protein gels prepared at different mixing speeds. J. Food Eng., 99: 338-343.

Srirangsan P, Kawai K, Hamada-Sato N, Watanabe M, Suzuki T (2010). Improvement in the remaining activity of freeze-dried xanthine oxidase with the addition of a disaccharide–polymer mixture. Food Chem., 119: 209-213.

Strasburg G, Xiong XL, Chiang W (2008). Physiology and chemistry of edible muscle tissues. in: Fennema, D.R. (Ed}. Food Chemistry, 4th ed, Marcell Dekker, New York, pp. 923-73.

Sun C, Gunasekaran S (2010). Rheology and oxidative stability of whey protein isolate-stabilized Menhaden oil-in-water emulsions as a function of heat treatment J. Food Sci., 75: C1-C8.

Tsoublli MN, Labuza TP (1991). Accelerated kinetic study of aspartame degradation in the neutral pH range. J. Food. Sci. 56: 1671-1675.

Zhu H, Damodara S (1994). Heat-induced conformational changes in whey protein isolate and its relation to foaming properties. J. Agric. Food Chem., 42: 846-855.