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Study by Differential Scanning Calorimetry of the Thermal Stability of Whey Proteins Concentrate

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Abstract: Many factors who affect the stability of whey proteins treated by heating were studied over 2.5 to 8.5 pH range in differential scanning calorimetry using and heat-induced precipitation. Heating of whey proteins at 95°C for 5 min above pH 3.8 to 3.9 produced extensive of the protein coagulation. The highest denaturation temperature for an acid whey protein concentrate prepared by ultrafiltration was 88°C at pH 3.5, while for an isolated β-lactoglobulin preparation. The highest denaturation temperature, obtained also at pH 3.5 was 81.9°C. Presence of milk sugars (lactose, glucose and galactose) appeared to increase the resistance of β-lactoglobulin to thermal denaturation. Heat stability of α-lactalbumin was lower at pH 3.5 than in pH 6.5 to 4.5 range; at all pH denaturation temperatures of α-lactalbumin (61.5 to 58.6°C) were lower than those for β-lactoglobulin or two serum albumin preparations. Thermal behavior of an ultrafiltration whey protein concentrate appears to be controlled by the dominating β-lactoglobulin fraction.

Key words: Thermal stability, whey proteins concentrate, differential scanning calorimetry

INTRODUCTION

The heat treatment of whey protein products near neutral pH results in considerable loss of protein solubility. At this pH, the heat-denatured proteins interact via irreversible thiol-disulfphide reactions to form protein aggregates, which further group themselves into large sedimentable particles, resulting in precipitation (Theologou *et al*., 2005). This heat-induced protein coagulation constitutes a serious problem in the manufacture of various whey products when thermal processing is carried out under conditions that promote protein aggregation (Dickson and Parkison Emma, 2004). However, whey heating at pH 2.5 produces no precipitation (Sudhir *et al*., 2005).

The precise pH at which whey proteins become resistant to thermal precipitation has not been well established. The effects of heating whey acidified to below pH 4.0 were recently investigated (Anthony *et al*., 2005) using turbidity measurements. The stability of the whey systems at pH 3.7 or lower against visible change upon heating at 92°C for 15 to 30 min was attributed to a higher heat resistance of whey protein. Stability of whey protein was stabilized by oil-in-water emulsions during chilled storage and temperature cycling (Sotirios *et al*., 2004).

However, turbidimetry will not differentiate between protein denaturation and protein aggregation; variations in number, size, or optical properties of the particles in solution can produce changes in turbidity not necessarily related to protein denaturation. Information about heat stability of Whey Protein Concentrates (WPC) at various conditions in formulated foods is important processors contemplating the use of WPC as functional or nutritional ingredients. The specific objectives of this research were) to determine the critical pH point at which transition in the heat aggregation/precipitation stability of the whey proteins occurs; to determine the temperatures of denaturation of an ultrafiltration (UF) WPC and of some individual whey proteins in the pH 2.5 to 6.5 region and to study the influence of certain nonprotein components of whey protein concentrates on the thermal behavior of some of the individual whey proteins.

MATERIALS AND METHODS

The report combines two separate investigations: The study of the effects of acidic pH on heat precipitation of whey proteins from laboratory-prepared acidic whey and Differential Scanning Calorimetry (DSC) determination of protein denaturation temperatures using an UF-produced WPC or individual whey protein fractions.

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Protein precipitation experiments: For the study of heat coagulability of whey protein, acid and whey was prepared by isoelectric precipitation (pH 4.65) of previously skimmed raw milk obtained from the French Milk Company EURIAL. The casein was removed by centrifugation in a Beckman Model J2-21 Centrifuge at 27000xg for 30 min.

The pH of the acid whey samples was adjusted with 2.0 N HCl. After pH adjustment, the samples were centrifuged (5000xg for 20 min) to remove turbidity. The protein content of the surpernatants was determined by the Folin-Lowry method (1951). Samples were then placed in test tubes, which were closed and heated in a water bath to 95 (±0.5) °C and held at this temperature for 5 min. Final desired temperature in the test tubes was reached in less than 2 min. Upon completion of heating, the samples were cooled to 20 to 25°C in an ice bath and the precipitates removed by centrifugation (3000xg for 20 min). The protein content of the supernatants was determined as described and the extent of the protein precipitate was calculated as: % precipitation = (protein in supernatant after heating/protein in supernatant before heating)×100. Two different batches of whey from two different milk lots were used for this study. Each experiment was carried out in triplicate.

Differential scanning calorimetry experiment: Whey protein concentrate was produced in out pilot plant by UF of cottage cheese whey at room temperature in (Franche Comté Serum Company) Laboratory UF module model Lab-20, using GR6P membranes (25 000 MW cut off). The composition of the whey used in this study and the WPC prepared from it by UF are given in Table 1. The whey was obtained from an industrial cottage cheese manufacturer and processed within 24 h. Samples of α-lactabumin (α-la, No. L-4379), β-lactoglobulin (β-lg, No. L-0130) and Serum Albumin (SA) were obtained from EURIAL (French milk company) and used without further purification. Two SA preparations were used: a standard product containing 1.0 to 1.3 mol fatty acids/mol albumin (No. A-7638) and an essentially fatty acid-free preparation (No. A-7511) with less than.005 fatty acids. Each of these proteins was suspended separately in a salt solution known as simulated milk ultrafiltrate (SMUF) prepared according to Jennes and Koops (1962). The protein concentration of final solutions was 100 g L^{-1} although much higher than in whey, this concentration was necessary for a proper DSC response.

The DSC thermograms were recorded in a DuPont thermal analyzer model 990 (DuPont Company; Wilmington, DE). Samples of the protein solutions (20 μL) were sealed into hermetic aluminum pans and heated from 10 to 130°C in the DSC cell. A pan containing 20 μL of distilled water was used as a reference. The heating rates were 2, 5, 10 and 20°C min^{-1}. Each experiment was carried out in triplicate. The temperatures of denaturation were estimated by extrapolation of the temperatures of maximum deflection obtained at different heating rates to a heating rate of 0°C min^{-1}.

The effects of pH on the denaturation temperature of WPC and of isolated α-la, β-lg and SA in SMUF were studied over the pH range of 2.5 to 6.5. The effect of milk sugars on the thermal stability of β-lg over the same pH range was studied by the addition of either 14.6 mM lactose or 14.6 mM glucose and 14.6 mM galactose to a β-lg solution. These sugars were obtained from (French Milk Company EURIAL). To estimate the renaturation of the different proteins, previously denatured samples were cooled down to 10°C in the calorimeter cell at a rate of 10°C min^{-1} and then rescanned. The pH adjustment of the samples was the last step performed prior to recording the DSC thermograms.

Analytical procedures: Total solids of whey and of the WPC produced by UF were determined by overnight evaporation in a vacuum oven at 100°C (kjeldahl method). Determinations were made in triplicate. Protein analyses of the whey and the UF-prepared WPC were carried out according to the Kjeldahl procedure using a conversion factor of 6.37 from percent nitrogen to crude protein. The protein contents of the samples in the precipitation experiments were determined using the Folin-Lowry method. Protein absorbance was recorded at 748 nm in a Beckman DU-8 Spectrophotometer (Beckman Instruments Inc, Irvine, CA). The protein used as reference was β-lactoglobulin from Sigma Chemical CO. (St. Louis, MO).

RESULTS AND DISCUSSION

pH influence on heat precipitation of whey proteins: The Fig. 1 shown the effect of pH on the heat precipitation of whey proteins from acidified cottage cheese whey. The transition from soluble to insoluble or precipitated states occurred within a narrow pH range (3.7 to 3.9). The protein contents of supernatants before heating were not significantly different from the protein content of the original whey (Table 1). Therefore, the differences in protein precipitated among the samples were caused only by the heat treatment. The low amount of protein precipitated below the critical pH region may be the result of high electrostatic repulsion between the denatured protein molecules combined with the absence of disulfide interchange reactions at such a low pH (Harwalkar, 1979). Above pH 3.9, the repulsive forces are not so strong, protein-protein interactions are more likely to occur and heavy protein precipitation is observed. The pH dependance of the dissociation behavior of β-lg, the most abundant protein present in whey, seems to

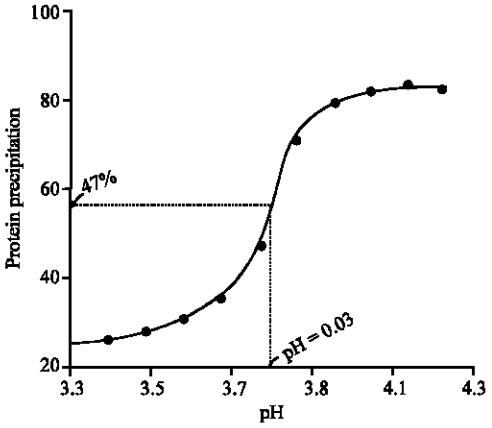

Fig. 1: Effect of pH on the heat precipitation of whey proteins in acid whey heat treatment = 95°C for 5 min

Table 1: Composition of whey and Whey Protein Concentrate (WPC) prepared from it by ultrafiltration

Proteins contents	Protein	Lactose	Total solids
	(g/100 g)		
Whey	0.75	4.9	6.38
WPC	7.50	4.9	13.20

Table 2: Effect of the pH on the denaturation temperature of α-lactalbumin (α-la), β-lactoglobulin (β-lg), Serum Albumin (SA) and Whey Protein Concentrate (WPC). The temperatures were extrapolated to a heating rate of 0°C min^{-1}

	Denaturation temperatures (±5°C)			
pH	β-lg	α-la	SA	WPC
6.5	75.9	61.0	71.9	76.9
5.5	77.8	61.2	72.6	78.8
4.5	81.2	61.5	74.0	82.1
3.5	81.9	58.6	73.5	88.0
2.5	78.7			80.6

support the previous argument. The β-lg dimer reversibly dissociates below pH 3.5 (Makenzie, 1970; Townend *et al*., 1960).

The monomerization of native β-lg molecules is due to repulsive electrostatic forces developed as the pH is decreased (Swaisgood, 1982). The minor difference between the pH of transition to the monomeric state and the critcal pH below which precipitation minimal should not be interpreted so as to consider them totally unrelated phenomena. Bovine β-lg can dissociate to the monomer above pH 3.5; in fact, the dimer is still very weakly dissociated at pH 5.2. However, even at pH 2.7 the monomerization is not complete.

Denaturation of whey proteins in the pH range 2.5 to 6.5: The Table 2 shown the effect of pH on denaturation temperatures of individual whey proteins and UF prepared WPC. The thermal behavior of the WPC seemed to be governed mainly by β-lg; however, the anomalous denaturation temperature (88°C) observed for WPC at pH 3.5 in several repeated experiments could not be explained on the basis of the thermal behavior of β-lg alone. Perhaps a combined stabilizing effect exerted by the total proteins (including immunoglobulins and protease-peptones, which were not studied separately in this study) or by the other constituents of the WPC could have contributed to this seemingly anomalous finding.

The purity of the commercial protein preparations was not ascertained; thus, possible effects of minor contaminants in the experiments with isolated β-lg and other individual protein preparations cannot be dismissed. The overall results prove that although the whey proteins are quite resistant to precipitation below pH 3.7, they are still denatured by heat as applied in this experiment. The temperature range in which a protein is stable depends on the balance among the forces involved in the stabilization of its tertiary structure. Because, some of these forces are pH dependent, it would be reasonable to expect variations in the denaturation temperature of protein at different pH values, reflecting the importance of these forces for the overall stability of the protein (Kinsella, 1981).

Electrostatic interactions within β-lg and perhaps other pH dependent forces seem to play a relatively important role in the stabilization of the three-dimensonal structure of the molecule. Although the denaturation temperature of α-la was also affected by pH, being lower at pH 3.5 than at the pH 4.5 to 6.5 range, a much more important factor for the thermal stability of this protein is the binding of calcium (Bernal and Jelen, 1985). Compared to the β-lg and SA, the α-la showed the lowest heat denaturation temperature at all pH studied. Secondary scanning of the heat-denatured samples produced no evidence of renaturation in any of the proteins except α-la.

Fatty acid content influence on the denaturation temperature of bovine serum albumin: In proteins like SA, which are exposed to a wide range of physiological conditions, non covalent interactions are not the main forces responsible for the stabilization of the tertiary structure. Disulfide bonds, of which bovine SA has (Lowry *et al*., 1951) may play a much more important role. The binding of fatty acids to SA from bovine milk appears to be one of important factors in the stabilization of its structure as shown in Table 3. The stated difference between the two SA preparations was the additional charcoal treatment to remove the fatty acids naturally bound to the protein without altering its native structure. The presence of these fatty acids produced an increase in the denaturation temperatures observed for the protein at all the heating rates used in this study.

An increase in the thermal stability of a protein by ligand binding has been known for some time

Table 3: Effect of the fatty acids content in Serum Albumin (SA), on the maximum deflection temperatures of differential scanning calorimetry thermograms at differing heating rates, pH = 4.5

Heating time (min)	Temperature of maximum Deflection (±5°C)	
	SA	Defacted SA
2	73.0	65.9
5	75.9	66.7
10	77.4	67.2
20	80.0	68.0

Table 4: Effect of milk sugars on the denaturation temperatures of β-lactoglobulin (β-lg) in simulated milk ultrafiltrate

pH	Denaturation temperatures (±5°C)		
	A[2]	B[3]	C[4]
4.5	81.2	82.2	82.4
3.5	81.9	83.1	83.9
2.5	78.7	79.6	80.3

(Donovan and Roo, 1975). A similar stabilization by fatty acid binding in SA was observed by Bernal and Jelen (1985) using DSC. The ability of SA to bind hydrophobic ligands has very important physiological significance in the blood in the transport of such substances between organs and tissues and in the neutralization of the toxic effects of some compounds by this binding. The Table 3 shows also the effect of heating rate on maximum deflection temperatures and the importance of extrapolating to a 0°C min^{-1} heating rate when reporting the denaturation temperature of a protein.

Effects of milk sugars on the denaturation temperature of β-lactoglobulin: The effects of lactose and a mixture of glucose and galactose on the thermal behavior of β-lg are shown in Table 4. The denaturation temperatures over pH range were higher when either lactose or glucose and galactose were added to the β-lg solution. Similar protective effect of lactose on denaturation of β-lg was recently described by (Bouhallab *et al*., 2005), although their denaturation temperatures were higher (85.5 and 84.5°C with and without lactose, respectively). The discrepancy is probably related to the 10°C min^{-1} heating rate, which was used without extrapolation to 0°C min^{-1}. Sugars and polyhydric alcohols are known to stabilize proteins against denaturation (Asylbek *et al*., 2000). These substances tend to maintain or increase the hydration of the protein molecule, enhancing the water structure in its immediate surroundings and contributing to its stability.

The protein is preferentially hydrated in a sugar-containing aqueous system where the macromolecule is stabilized (Arakawa and Timasheff, 1982). While this stabilization takes place at a molecular level, a further increase in the denaturation temperature was observed (Table 4) when the original sugar/protein molar ratio was doubled by the replacement of lactose by glucose and galactose. Although the magnitude of the effect was small, maximum deflection peaks obtained for the β-lg solution in the presence of glucose and galactose were higher than for those obtained in the presence of lactose alone in every single experiment.

CONCLUSIONS

Protein precipitation is the common problem to many industrial applications of WPC. This often undesirable phenomenon could be minimized by controlling the pH of food system (such as a whey beverage) at or below 3.7 prior to thermal processing. Under these conditions, most of the whey proteins were not precipitated after heating at 95°C for 5 min. The thermal behavior of a WPC as well as of the whey proteins in untreated whey seems to be dominated by the behavior of β-lg. Heat denaturation temperatures of the whey protein concentrate and the major isolated whey proteins were pH-dependent and were also affected by some other whey constituents. Thus, bovine SA was protected against heat-denaturation by fatty acid binding. The denaturation temperature of β-lg was increased in the presence of lactose and a slight further increase was obtained when this sugar was replaced by glucose and galactose, as if β-galactosidase had been used to hydrolyze lactose in whey. However, lactose hydrolysis alone would not be suitable to provide protection again heat precipitation of whey protein in industrial whey products.

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