Effect of pH and Temperature on Protein Unfolding and Thiol/Disulfide Interchange Reactions during Heat-Induced Gelation of Whey Proteins

Frank J. Monahan,* J. Bruce German, and John E. Kinsella†

Department of Food Science and Technology, University of California, Davis, California 95616

In heat-induced whey protein isolate (WPI) gels, polymerization of the constituent whey proteins, via intermolecular disulfide (S–S) bonding, was dependent on both the pH of the WPI solution and the temperature to which the solution was heated. At pH 9 and 11, polymerization as determined by SDS–PAGE occurred at room temperature (22 °C), while at pH 3, 5, and 7, polymerization was only evident after heating to 85, 75, and 70 °C, respectively. Measurement of total sulfhydryl (SH) group content of gelling WPI solutions at each pH and temperature revealed that in the WPI solutions at pH 9 and 11 significant SH–SH oxidation to S–S occurred even at room temperature. In contrast, the total SH content of WPI solutions at pH 3 and 5 did not change with heating, indicating that polymerization reactions involving SH/S–S interchange rather than SH/SH oxidation predominated. Estimation of the degree of unfolding of the whey proteins by measuring the exposure of hydrophobic amino acid residues showed that at pH 9 and 11 extensive irreversible unfolding of the protein molecules had occurred at room temperature.

Keywords: *Whey protein isolate; pH; temperature; protein unfolding; thiol/disulfide interchange; thiol oxidation; gelation*

INTRODUCTION

The formation of disulfide bonds between protein molecules during food processing contributes to the functionality of the proteins in different systems. Disulfide bond formation leading to polymerization occurs between the glutenins of wheat in the manufacture of bread dough (Ewart, 1968), between soy proteins in tofu manufacture (Saio et al., 1971), and between milk proteins in the formation of whey protein-stabilized emulsions (Dickinson and Matsumura, 1991; Monahan et al., 1993). The desirability of disulfide bond formation depends on the particular food application and the protein functionality sought. For example, in foam and emulsion formation, extensively disulfide-linked proteins which cannot easily unfold, are less surface active than flexible randomly structured proteins (Kim and Kinsella, 1987; Kinsella and Phillips, 1989). The surface activity of such proteins can be improved by controlled reduction of disulfide bonds (Kim and Kinsella, 1987; Klemaszewski and Kinsella, 1991). Once formed, however, film stability may be enhanced by disulfide bond formation (Dickinson, 1986). In the case of heat-induced whey protein gels disulfide-mediated polymerization increases gel firmness and elasticity (Shimada and Cheftel, 1988; Mulvihill and Kinsella, 1988).

In whey, the native globular structures of β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and the immunoglobulins (Ig) are stabilized by intramolecular disulfide bonds, 2, 4, and 17 in the case of β-Lg, α-La, and BSA, respectively. In addition, β-Lg and BSA each have one free sulfhydryl group (Kinsella et al., 1989). Treatments that destabilize native whey protein structure and promote unfolding can facilitate increased protein–protein interactions between whey proteins in solution and lead to intra- or intermolecular thiol/disulfide (SH/S–S) interchange or thiol/thiol (SH/SH) oxidation reactions. Disulfide-mediated polymerization of whey proteins has been shown to occur in response to heating (Dunkerly and Zadow, 1984; To et al., 1985), increasing pH (Huggins et al., 1951), and treatment with urea and reducing agents (Huggins et al., 1951). Controlling the extent of disulfide-mediated polymerization reactions by varying conditions under which polymerization can proceed may be an effective way of manufacturing gels with different rheological properties and food applications.

The objective of the present study was to systematically investigate the pH and temperature dependence of disulfide-mediated polymerization reactions during the gelation of whey proteins. To examine the structural changes that occur as whey proteins approach their gel points, changes in the surface hydrophobicity, sulfhydryl content, and electrophoretic profile of soluble protein in WPI at gelling concentrations were examined.

MATERIALS AND METHODS

Materials. WPI (>97% protein) was obtained from Le Sueur Isolates (Le Sueur, MN) and extensively dialyzed and lyophilized (Yost and Kinsella, 1993). Sigmacote, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 8-anilino-1-naphthalenesulfonic acid (ANS) were obtained from the Sigma Chemical Co., St. Louis, MO.

Gel Preparation. WPI solutions (1 and 13% w/w) were prepared at room temperature by dissolving the dialyzed WPI powder in deionized water, adjusting to the appropriate pH with 0.1 M HCl or NaOH and making up to the final weight with deionized water. Aliquots (2.3 mL) of each solution were dispensed into glass tubes (10 mm i.d. × 75 mm) previously treated with Sigmacote. The contents of each tube were deaerated by placing in a sonicator under vacuum for 30 s. The tubes were capped and placed in a Neslab endocal RTE-9 water bath equipped with a Neslab ETP-3 temperature controller. The samples were equilibrated at 50 °C for 15 min, heated at 1 °C/min to 90 °C, and held for 15 min at 90 °C. The

* Address correspondence to this author at the Department of Food Science, University College Dublin, Belfield, Dublin 4, Ireland.

† Deceased.

0021-8561/95/1443-0046$09.00/0

temperature was monitored by inserting a thermocouple in an extra sample. At each pH, a control sample was maintained at room temperature (22 °C). Tubes were removed from the water bath when the temperature of the gelling solutions had reached 50 °C for 15 min, 60, 65, 70, 75, 80, 85, 90, and 90 °C for 15 min. The tubes were held at 4 °C for 15 h and then tempered at room temperature for 30 min. WPI solutions which did not flow on inversion were termed gels. The contents of each tube was removed for analysis. The experiment was performed in duplicate on a single batch of WPI, and all analyses were performed in triplicate.

SDS–PAGE. Samples (0.02 g) of the WPI solutions or gels at each pH and heat treatment were homogenized in 0.5 mL of 0.0625 M Tris-HCl, pH 6.8, containing 2% SDS (Laemmli, 1970) using five passes of a Potter–Elvehjem homogenizer. Aliquots (0.2 mL) were centrifuged at 10000*g* for 15 min to remove insoluble protein. The protein content of the supernatant was determined by the Lowry procedure (Lowry et al., 1951). The soluble protein contained in the supernatant was analyzed by SDS–PAGE on 12.5% acrylamide gels following the procedure of Laemmli (1970). Comparable quantities of protein were applied to all wells. 2-Mercaptoethanol was excluded from the denaturing buffer to avoid cleavage of intermolecular disulfide bonds formed during polymerization. Gels were run on a Mini PROTEAN II Dual Slab Cell (Bio-Rad Laboratories).

Determination of Sulfhydryl Groups. The SH group content of WPI solutions or gels was determined by the method of Shimada and Cheftel (1989). Solubilization of 0.02 g samples in 2.5 mL of solubilizing buffer (0.086 M Tris, 0.09 M glycine, 4 mM EDTA, 8 M urea, pH 8) was achieved using five passes of a Potter–Elvehjem homogenizer. Aliquots (1.5 mL) were centrifuged at 10000*g* for 15 min to remove insoluble protein. The total SH content of the supernatant was determined by the method of Ellman (1959). To 1 mL aliquots of the supernatant was added 0.01 mL of Ellman's reagent (10 mM DTNB). The absorbance at 412 nm was recorded on a Shimadzu UV-1201 spectrophotometer and the SH concentration was calculated using a molar extinction coefficient of 13 600 M^{-1} cm^{-1}. The protein content of the solubilized protein fractions was determined by the Lowry procedure (Lowry et al., 1951).

Determination of Surface Hydrophobicity. Surface hydrophobicity (H_0) of soluble protein, obtained from gelled and nongelled WPI, was determined by the hydrophobic probe binding method of Creamer et al. (1982). Samples (0.02 g) of WPI solutions or gels in 2.5 mL of 0.1 M phosphate buffer, pH 6.5, were solubilized using five passes of a Potter-Elvehjem homogenizer. Aliquots (1.5 mL) were centrifuged at 10000*g* for 15 min to remove insoluble protein. After protein determination (Lowry et al., 1951), the supernatants were diluted with 0.1 M phosphate buffer to give solutions in the concentration range 0.01–0.1 mg of protein/mL. To 3 mL of each diluted WPI solution was added 200 μL of ANS solution (0.5 mg/mL 0.1 M phosphate buffer, pH 6.5). Ratiometric fluorescence measurements were made in an Aminco-Bowman spectrofluorometer (American Instrument Co. Inc., Silver Spring, MD) using a 1 cm cell, 375 and 485 nm as the excitation and emission wavelengths, respectively, and a 5 nm width of both excitation and emission slits. Measurements were made at room temperature (22 °C). The H_0 of the solubilized WPI solutions was calculated from the slope of the relative fluorescence (R) vs percent (w/v) protein concentration. The relative fluorescence was defined by

$$R = (F - F_0)/F_0$$

where F is the fluorescence reading of the protein–ANS conjugate and F_0 is the reading of the ANS solution without WPI.

RESULTS

Gelation. pH and temperature affected the ability of 13% WPI solutions to gel. On adjusting the WPI solutions to the appropriate pHs at 22 °C, solutions at pH 3, 7, 9, and 11 remained transparent. At pH 11 there was a noticeable increase in viscosity of the WPI solution compared to solutions at lower pHs. At pH 5, close to the isoelectric points of β-Lg (pI = 5.1) and BSA (pI = 4.7–4.9), proteins aggregated and an opaque solution was formed. Following heat treatment, storage at 4 °C for 15 h and tempering at room temperature for 30 min, all solutions at pH 11 (22–90 °C, 15 min) formed transparent gels. WPI solutions at pH 9 (22 °C) were more viscous than solutions at the lower pHs (22 °C) and became increasingly viscous with increasing temperature, but did not gel. At pH 7 and 3 transparent gels were formed only after heating to 80 and 85 °C, respectively. At pH 5 a white coagulum-type gel formed on heating to 75 °C.

SDS–PAGE. SDS–PAGE of untreated whey protein obtained from the manufacturer and comparison with known standards showed bands attributable to the principal whey proteins α-La, β-Lg, BSA, and Ig (Figure 1c, lane 22 °C, bands A, B, D, E). An additional band (Figure 1c, lane 22 °C, band C) was attributed to either covalently bonded dimers of β-Lg or dimers of α-La and β-Lg or both (Xiong and Kinsella, 1990).

The effect of pH and temperature on the extent of disulfide-mediated polymerization in the soluble protein fractions obtained from heat-treated WPI solutions and gels was determined by SDS–PAGE (Figure 1). Polymerization was defined as the appearance of high molecular weight protein bands in the stacking gel and at the top of the resolving gel with a concomitant decrease in the intensity of the monomeric protein bands. In unheated WPI solutions (Figure 1a–e, lane 22 °C), pH had a profound effect on the degree of polymerization of the whey proteins. At pH 11, high molecular weight polymeric proteins appeared at the top of the gel and the relative proportions of monomeric proteins were reduced compared to solutions at lower pHs. At pH 9 polymerization was also evident in unheated solutions (Figure 1d, lane 22 °C) though not as extensive as at pH 11. In solutions at pH 3 and 5 electrophoretic patterns were similar to those of the whey proteins at pH 7 in which polymerization, excluding dimerization, was minimal.

Heat-induced polymerization of whey proteins was influenced by the pH of the WPI solutions. At pH 11, since extensive polymerization had occurred in the unheated solution at 22 °C, further polymerization with heating was minimal (Figure 1e). At pH 9, an increase in the proportion of polymeric WPI occurred with heating to 50 °C and thereafter up to 90 °C (Figure 1d). At the lower pHs the propensity of the WPI solutions to form covalently linked polymers was in the order pH 7 > pH 5 > pH 3. SDS–PAGE showed that the formation of covalently bonded polymers at the expense of β-Lg, α-La and BSA, increased when temperatures of 85, 75, and 70 °C were reached at pH 3, 5, and 7, respectively (Figure 1a–c). At all pHs, the band attributed to BSA diminished rapidly with heating demonstrating that BSA engages readily in polymerization reactions. Compared to BSA, the bands attributed to β-Lg and α-La diminished less quickly. The higher disulfide bond content of BSA compared to β-Lg and α-La may explain its readiness to engage in polymerization reactions during heating.

Inclusion of 2-mercaptoethanol in the denaturing buffer led to a reduction in the intensity of bands attributed to polymers (Figure 2, compare lanes 3, 5,

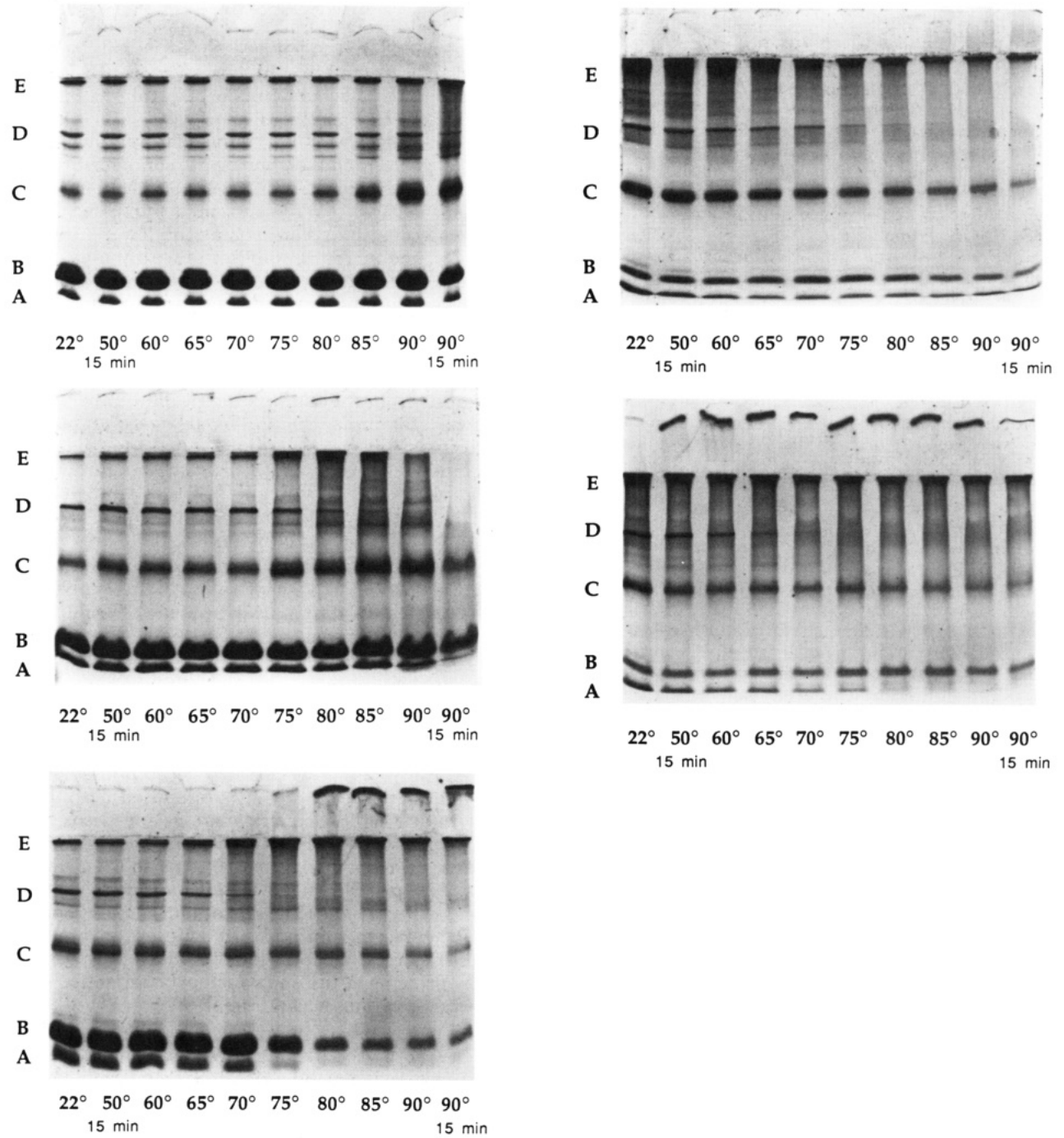

Figure 1. SDS–PAGE gels showing the effect of temperature on polymerization of a 13% whey protein isolate solution at pH 3 (a, top left), pH 5 (b, middle left), pH 7 (c, bottom left), pH 9 (d, top right), and pH 11 (e, middle right). A, α-La (MW, 14 200); B, β-Lg (MW, 18 400); C, dimers of α-La and β-Lg; D, BSA (MW, 66 200); E, Ig (MW, 160 000).

and 7 with lanes 4, 6, and 8). At the same time, the intensity of the monomeric whey protein bands increased in the presence of 2-mercaptoethanol and the banding pattern approached that of the unheated WPI (Figure 2, lane 2). This indicates that intermolecular disulfide bonds formed by thiol disulfide interchange or thiol oxidation reactions were responsible for stabilizing polymerized whey proteins. A noticeable feature in the banding pattern of samples treated with 2-mercaptoethanol was the much reduced intensity of the BSA and Ig bands. The banding pattern of Ig changes in the presence of 2-mercaptoethanol as a result of the dissociation of protein subunits stabilized by disulfide bonds in the native molecule (Hames, 1990). Similarly, the mobility of BSA on SDS–PAGE changes in the presence of 2-mercaptoethanol as a result of the reduction of SH groups in the native molecule (Figure 2, compare lanes 1 and 2).

Sulfhydryl Group Content. The total SH content of solubilized protein fractions obtained from the 13% WPI solutions and gels varied with pH and temperature (Figure 3). The data indicate that in unheated solutions at pH 9 and 11 reduced levels of SH groups were available to react with DTNB compared to solubilized protein fractions obtained at lower pHs. At pH 7 and below, levels of total SH groups are comparable with those reported earlier for whey protein isolate (Shimada and Cheftel, 1989). At pH 3 and 5 the total SH content did not change with heating up to 90 °C. At pH 7 the SH content decreased above 80 °C.

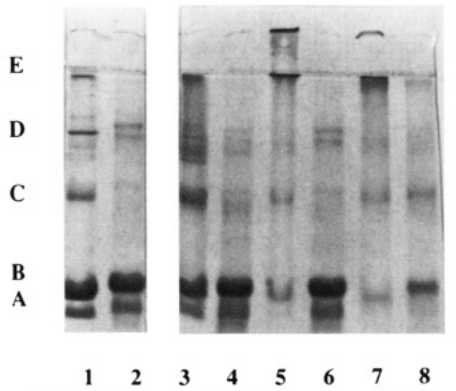

Figure 2. SDS–PAGE gel of WPI samples with (lanes 2, 4, 6, 8) and without (lanes 1, 3, 5, 7) 2-mercaptoethanol in the denaturing buffer. The WPI sample treatments prior to electrophoresis were as follows: lanes 1 and 2, WPI at pH 7 (22 °C); lanes 3 and 4, WPI at pH 3 heated at 90 °C for 15 min; lanes 5 and 6, WPI at pH 7 heated at 90 °C for 15 min; lanes 7 and 8, WPI at pH 11 heated at 90 °C for 15 min. The identities of the principal whey proteins in unheated WPI (lane 1) are as assigned in Figure 1.

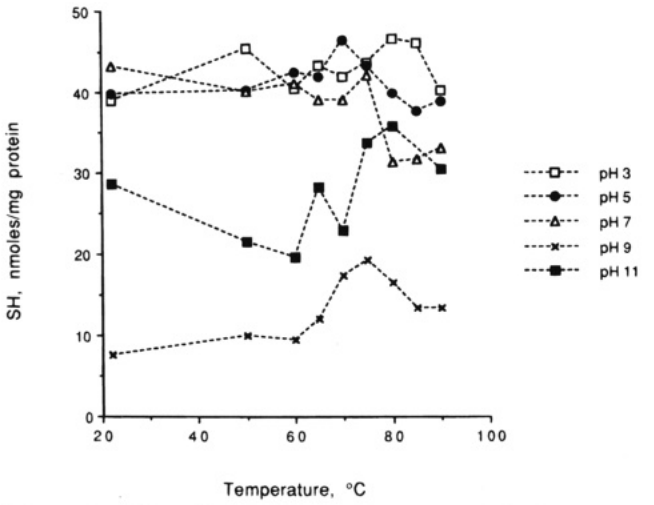

Figure 3. Effect of pH and temperature on sulfhydryl group content of the soluble protein fraction obtained from 13% WPI solutions or gels.

Surface Hydrophobicity. The surface hydrophobicity (H_0) of whey proteins was estimated using the fluorescence probe ANS and was shown to be both pH and temperature dependent (Figure 4). The higher H_0 values in unheated solutions at pH 9 and 11 indicate of exposure of hydrophobic amino acid residues as a result of unfolding of whey proteins. At pH 7 and lower, exposed hydrophobic residues were about 30–35% of those exposed at the alkaline pHs.

The effect of temperature on H_0 values varied with pH. At all pHs changes in the H_0 of the soluble WPI were minimal up to 65 °C. At pH 11 H_0 values of soluble proteins decreased above 65 °C, while at pH 9 values increased with increasing temperature up to 90 °C. At pH 7 H_0 values increased above 65 °C, while at pH 5 they decreased. At pH 3 changes in H_0 values with increasing temperature were slight (Figure 4). In the case of samples that formed gels the surface hydrophobicity measurements relate only to the soluble protein extractable from the gel network. On average only 10–20% of the total whey protein was soluble in samples that formed gels (Figure 5).

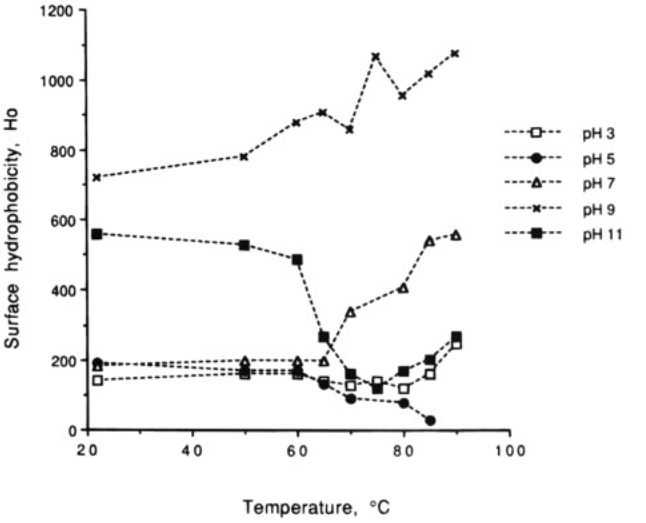

Figure 4. Effect of pH and temperature on the surface hydrophobicity of the soluble protein fraction obtained from 13% WPI solutions or gels.

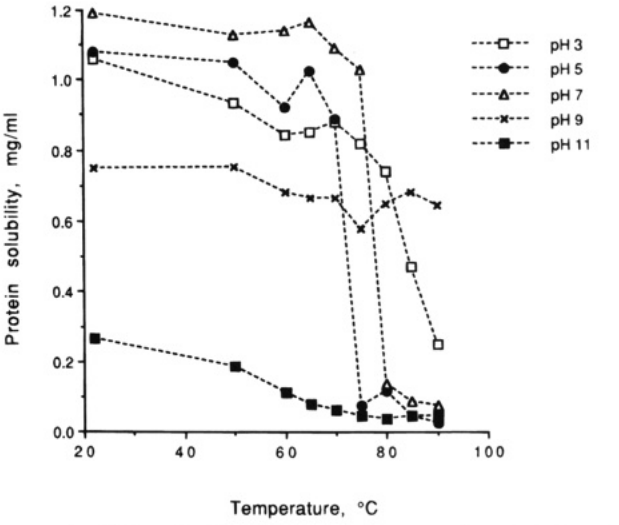

Figure 5. Effect of pH and temperature on the solubility of 13% WPI solutions or gels in 0.1 M phosphate buffer, pH 6.5.

DISCUSSION

The results of SDS–PAGE analysis show that disulfide-mediated polymerization occurs during heat-induced gelation of whey proteins over a wide pH range. However, the extent of polymerization was pH and temperature dependent, occurring in unheated solutions at pH 11 but requiring heating to 85 °C in solutions at pH 3. Previous research with β-Lg (Sawyer, 1967; Matsudomi et al., 1992) and WPI (Schmidt et al., 1978, 1979; Dunkerly and Zadow, 1984; To et al., 1985; Shimada and Cheftel, 1989) showed that disulfide interchange reactions occurred on heating. Disulfide interchange reactions are favoured at high pH (Tanford, 1968). On the other hand, SH/S–S interchange reactions have been shown to be inhibited at low pH (Ryle and Sanger, 1955; McKenzie and Ralston, 1971; McKenzie et al., 1972). For example, after heating a 9% WPI solution for at 90 °C for 45 min at pH 2.5, Shimada and Cheftel (1989) observed some dimerization of β-Lg and α-La but no formation of high molecular weight polymers. Our results showed that dimerization occurred in 13% WPI, pH 3, heated at 90 °C for 15 min. In addition, SDS–PAGE demonstrated that higher molecular weight polymers were formed (Figure 1a), i.e.,

that SH/S−S interchange reactions could occur despite the low pH of solutions.

The observed polymerization reactions were supported by measurements of the degree of unfolding of the proteins (surface hydrophobicity) and estimations of the free SH content. The high H_0 values of unheated WPI solutions at pH 9 and 11 indicate that at alkaline pH significant unfolding of proteins occurred to expose hydrophobic residues (Figure 4). At alkaline pH proteins attain an overall negative charge and repulsion between negatively charge groups promotes unfolding. At pH 3, 5, and 7 in the temperature range 22−65 °C, H_0 values of WPI solutions were lower than those at alkaline pH. Since all measurements were made at pH 6.5 and 22 °C (Creamer et al., 1982), the changes in H_0 detected in whey proteins adjusted to different pHs reflect only irreversible changes. Changes in the overall H_0 of the whey proteins may have occurred at pH 3, for example, yet on adjusting to pH 6.5 for H_0 measurement the proteins may have reverted to a conformation that did not reflect the pH 3 state. Shimizu et al. (1985) reported an increase in surface hydrophobicity of β-Lg on decreasing the pH from 7 to 3. Mills and Creamer (1975) also showed by intrinsic fluorescence and ANS binding that the surface hydrophobicity of β-Lg increased on adjusting the pH from 6.5 to 2 and reverted to a value close to the original value on readjusting the pH to 6.5. However, while these workers found more dramatic changes in the surface hydrophobicity of β-lactoglobulin under acidic conditions compared to alkaline conditions, the changes at acidic pHs were attributed to noncovalent monomer−dimer transitions rather than to substantial changes in protein secondary structure (Mills and Creamer, 1975; Das and Kinsella, 1989; Shimizu et al., 1985). Monomeric β-lactoglobulin is more rigid and resistant to denaturation at pH 3 than at pH 7 (Shimizu et al., 1985; Kella and Kinsella, 1988). The increased propensity of whey proteins to undergo disulfide-mediated polymerization reactions at pH 9 and 11 compared to lower pHs can be attributed to an increase in the frequency of thiol/disulfide interchange reactions in the unfolded proteins at alkaline pH. At pH 3, both the structural stability of monomeric β-Lg, the principal component of whey (Kella and Kinsella, 1988), and the lower reactivity of free thiol groups (Ryle and Sanger, 1955) diminish the likelihood of disulfide-mediated polymerization.

With the onset of polymerization (70 °C at pH 7 and 85 °C at pH 3), as visualized by SDS−PAGE (Figure 1), H_0 values increased, and did not revert to initial 22 °C values when the pH was adjusted to the measurement pH of 6.5, possibly because of constraints to refolding resulting from intermolecular disulfide bond formation. At pH 5, although disulfide-mediated polymerization occurred at 75 °C and above (Figure 1b), isoelectric precipitation and aggregation of the whey proteins preceeded heat-induced disulfide-mediated polymerization. This may have lowered the extent of interaction between ANS and hydrophobic regions on the proteins (Bonomi et al., 1988) and thus account for the lower H_0 values of WPI samples at pH 5.

Thiol oxidation reactions between exposed −SH or S^- groups can occur under alkaline conditions (Watanabe and Klostermeyer, 1976). The lower SH content of WPI solutions at pH 9 and 11 relative to the solutions at lower pHs (Figure 3) suggests that thiol oxidation reactions occur in addition to disulfide interchange reactions at the higher pH values. The decrease in SH content may also be due to degradation reactions. In alkaline media cystine and cysteine residues may be converted to degradation products such as H_2S and dehydroalanine (Nashef et al., 1977; Watanabe and Klostermeyer, 1976). The data in Figure 3 showed that the SH content of WPI samples at pH 9 was lower than that of pH 11 samples, despite evidence of greater polymerization at pH 11 (Figure 1). A possible explanation is that cleavage of intramolecular disulfide bonds is more extensive at pH 11 compared to pH 9 and results in the exposure of more free thiol groups. At the same time, the disulfide bonds that exist at pH 11 may be predominantly of the intermolecular type and account for the high degree of polymerization observed in samples at pH 11 (Figure 1). Watanabe and Klostermeyer (1976) found that total disulfides decreased in heated 1% β-Lg solutions on increasing the pH above 6.9. However, they also showed that total SH content decreased at increasing pH and attributed both observations to the formation of degradation products from cysteine and cystine.

At pH 3, 5, and 7 the polymerization reactions were primarily of the SH/S−S interchange type since the free SH content of solutions or gels at these pHs did not change dramatically from that of untreated WPI dissolved in water (Figure 3). At pH 7 free SH groups decreased after heating to 75 °C, the temperature at which significant polymerization first appeared on SDS−PAGE gels and this suggests that some SH/SH oxidation occurs at pH 7. Shimada and Cheftel (1989) also demonstrated that disulfide-mediated polymerization occurred during heating of 9% WPI at pH 7.5. However, they attributed the polymerization primarily to SH/S−S interchange reactions.

The ability of protein solutions to form self-supporting gels depends on a favorable balance of attractive and repulsive forces between protein molecules. Both noncovalent (e.g., H-bonding, hydrophobic, and electrostatic interactions) and covalent (e.g., disulfide bonds) forces drive the gelation process. Environmental factors such as pH, temperature and ionic strength can enhance protein−protein interactions and thereby favor gelation. In the case of samples that formed gels, the conditions of pH and temperature were sufficient to overcome the stabilizing forces which prevent the native proteins from interacting to form a three-dimensional gel network. However, enhancing one type of interaction may not be sufficient to overcome other forces that collectively prevent gelation. For example, while extensive disulfide bond formation was evident in WPI at pH 9 and samples were visibly more viscous compared to the unheated WPI at pH 7, the overall interactions were insufficient for the proteins to form a gel network.

The temperatures at which WPI solutions formed gels varied with pH. At pH 3, 5, 7, and 11 gels formed at 85, 75, 80, and 22 °C, respectively. At pH 3 electrostatic repulsion between molecules is sufficient to keep the proteins in solution. The temperature at which a transparent gel formed at pH 3 corresponded to that at which disulfide-mediated polymerization became evident (Figure 1). It is tempting to speculate that the onset of gelation was facilitated by the formation of disulfide bonds. Other interactions (e.g., hydrophobic) may be equally important. At pH 3 β-Lg is more resistant to denaturation than at higher pHs (De Wit and Klarenbeek, 1984; Kella and Kinsella, 1988) and thus higher temperatures are required to initiate unfolding and denaturation than at pH 7 and higher

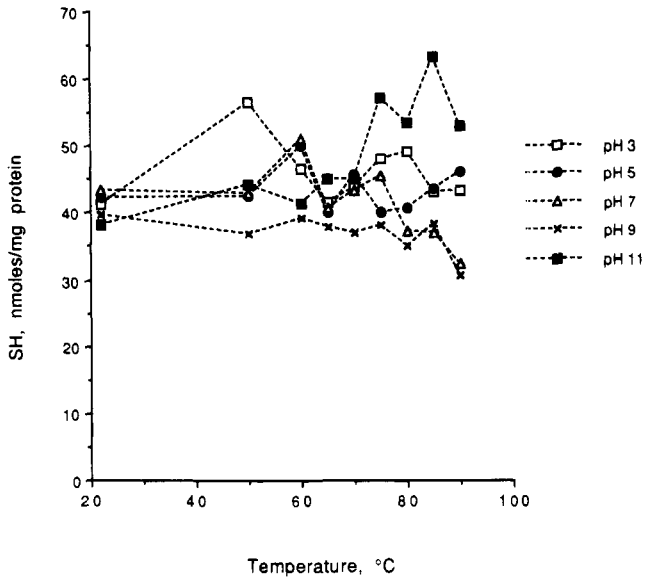

Figure 6. Effect of pH and temperature on sulfhydryl group content of 1% WPI solutions.

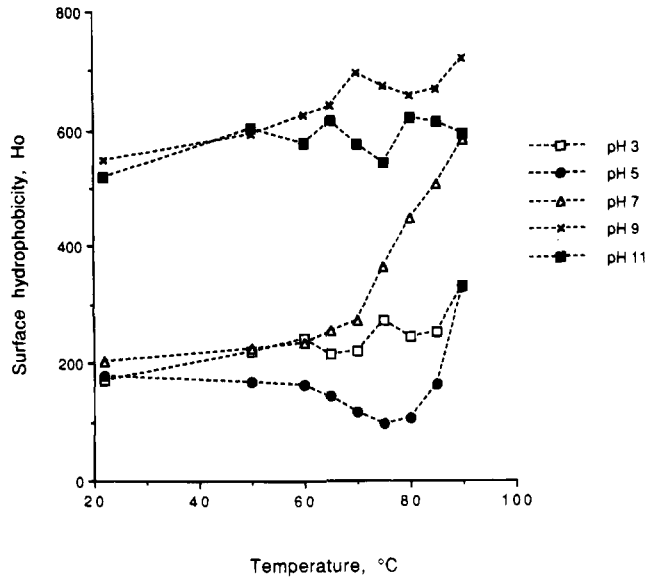

Figure 7. Effect of pH and temperature on protein surface hydrophobicity of 1% WPI solutions.

(Figures 3 and 4). In contrast, at pH 11 samples formed gels at 22 °C. Although proteins at this pH carry a strongly repulsive negative charge, extensive unfolding facilitates sufficient protein–protein interactions, including disulfide bond formation, to bring about gelation. At pH 7, while electrostatic repulsion is lower than at pH 9 or 11, proteins were insufficiently unfolded to form a gel network until a temperature of 80 °C was reached. A denaturation temperature for β-Lg of 65–75 °C at pH 6.8 is reported by De Wit and Klarenbeek (1981). At pH 5 proteins coagulated as a result of excessive attractive forces between the principal whey proteins close to their isoelectric points. A coagulum-type gel formed at 75 °C, the temperature at which disulfide-mediated polymerization also became evident.

To address the difficulty of interpreting results obtained from proteins at concentrations capable of forming a three-dimensional network (13% WPI in the present study), analyses were also undertaken on dilute protein solutions. While a clear pH effect on SH group content was evident at gelling concentrations of WPI (Figure 3), the results were less definitive in 1% solutions (Figure 6). In unheated 1% solutions the initial SH content were not influenced by pH and, while values varied with heating, no consistent trend was evident. It is likely that the same unfolding events at different pH and temperature occur in dilute and concentrated WPI solutions; however, the frequency of interaction between molecules and, hence, the probability of disulfide interchange or thiol oxidation reactions occurring, is lower in more dilute solutions. This may explain why changes in total SH content were less pronounced in 1% WPI solutions compared to 13% solutions.

Measurement of changes in surface hydrophobicity with pH and temperature in 1% WPI solutions (Figure 7) agreed quite closely with those obtained for 13% WPI (Figure 4) at pH 3, 7, and 9. There were some differences, however, in surface hydrophobicity results obtained for 1 and 13% solutions using the hydrophobic ligand probe method (Figures 4 and 7). Notably, in 13% solutions at pH 11 and 5 H_0 values showed an overall decrease with temperature increase while they increased in 1% solutions. The frequency of molecular interactions in 13% solutions compared to 1% solutions may explain the difference. At pH 11 covalent intermolecular interactions are extensive, as shown by SDS–PAGE, and therefore accessibility of the fluorescence probe ANS to exposed hydrophobic amino acid residues may be reduced. Similarly, at pH 5 a 13% solution forms a coagulum-type gel which results from extensive aggregation of the whey protein molecules. Hydrophobic associations between whey proteins in the coagulum may reduce the number of hydrophobic amino acids accessible to ANS (Bonomi et al., 1988). In addition, the proportion of the total protein available for surface hydrophobicity analysis was low in the extensively polymerized 13% WPI at pH 11 and the coagulated 13% WPI at pH 5 (Figure 5), thus making the interpretation of the results difficult for these concentrated protein systems.

CONCLUSIONS

The findings of this study relate to a mixture of milk proteins of which β-Lg is the principal component. In many food applications cost constraints in the production of pure protein fractions dictate that whey proteins are utilized as a mixture of proteins in the form of an isolate or concentrate. Thus, the results of the present study contribute to our understanding of the extent to which disulfide-mediated polymerization occurs in whey proteins under conditions that may arise in food systems. The results indicate that, at pHs close to and lower than the p*I* of whey, thiol oxidation with heating is minimal and disulfide-mediated polymerization is limited, though it does occur. As the pH is increased above the p*I* the propensity of the proteins in whey to undergo irreversible thermal denaturation increases as does the degree of thiol oxidation and polymerization. It is clear that the degree of polymerization may be controlled by the conditions under which whey proteins are processed.

ACKNOWLEDGMENT

The support of the National Dairy Board is greatly appreciated.

LITERATURE CITED

Bonomi, F.; Iametti, S.; Pagliarini, E.; Peri, C. A spectrofluorimetric approach to the estimation of the surface hydro-

phobicity of milk proteins upon thermal treatment. *Milchwissenschaft* **1988**, *43*, 281–285.
Creamer, K. L.; Zoerb, H. F.; Olson, N. F.; Richardson, T. Surface hydrophobicity of α_{s1}-I, α_{s1}-casein A and B and its implications in cheese structure. *J. Dairy Sci.* **1982**, *65*, 902–906.
Das, K. P.; Kinsella, J. E. pH dependent emulsifying properties of β-lactoglobulin. *J. Dispersion Sci. Technol.* **1989**, *10*, 77–102.
De Wit, J. N.; Klarenbeek, G. A differential scanning calorimetric study of the thermal behaviour of bovine β-lactoglobulin at temperatures up to 160 °C. *J. Dairy Res.* **1981**, *48*, 293–302.
De Wit, J. N.; Klarenbeek, G. Effects of various heat treatments on structure and solubility of whey proteins. *J. Dairy Sci.* **1984**, *67*, 2701–2710.
Dickinson, E. Mixed proteinaceous emulsifiers: review of competitive protein adsorption and the relationship to food colloid stabilization. *Food Hydrocolloids* **1986**, *1*, 3–23.
Dickinson, E.; Matsumura, Y. Time-dependent polymerization of β-lactoglobulin through disulfide bonds at the oil-water interface in emulsions. *Int. J. Biol. Macromol.* **1991**, *13*, 26–30.
Dunkerly, J. A.; Zadow, J. G. The effect of calcium and cysteine hydrochloride on the firmness of heat coagula formed from cheddar whey protein concentrates. *Aust. J. Dairy Technol.* **1984**, *39*, 44–47.
Ellman, G. L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
Ewart, J. A. D. A hypothesis for the structure and rheology of glutenin. *J. Sci. Food Agric.* **1968**, *19*, 617–623.
Hames, B. D. One dimensional polyacrylamide gel electrophoresis. In *Gel Electrophoresis of Proteins. A Practical Approach*; Hames, B. D., Rickwood, D., Eds.; Oxford University Press: Oxford, U.K., 1990; pp 1–147.
Huggins, C.; Tapley, D. F.; Jensen, E. V. Sulfhydryl-disulfide relationships in the induction of gels by urea. *Nature* **1951**, *167*, 592–593.
Kella, N. K. D.; Kinsella, J. E. Enhanced thermodynamic stability ot β-lactoglobulin at low pH. A possible mechanism. *Biochem. J.* **1988**, *255*, 113–118.
Kim, S. H.; Kinsella, J. E. Surface active properties of food proteins: effects of reduction of disulfide bonds on film properties and foam stability of glycinin. *J. Food Sci.* **1987**, *52*, 128–131.
Kinsella, J. E.; Phillips, L. G. Structure:function relationships in food proteins, film and foaming behaviour. In *Food Proteins*; Kinsella, J. E., Soucie, W. G., Eds.; American Oil Chemists' Society: Champaign, IL, 1989; pp 53–77.
Kinsella, J. E.; Whitehead, D. M.; Brady, J.; Bringe, N. A. Milk proteins: possible relationships of structure and function. In *Developments in Food Chemistry 4. Functional Milk Proteins*; Fox, P. F., Ed.; Elsevier Applied Science Publishers: London, 1989; pp 55–95.
Klemaszewski, J. L.; Kinsella, J. E. Sulfitolysis of whey proteins: effects on emulsion properties. *J. Agric. Food Chem.* **1991**, *39*, 1033–1036.
Laemmli, U. K. Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature* **1970**, *227*, 680–685.
Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275.
Matsudomi, N.; Oshita, T.; Sasaki, E.; Kobayashi, K. Enhanced heat-induced gelation of β-lactoglobulin by α-lactalbumin. *Biosci., Biotechnol., Biochem.* **1992**, *56*, 1697–1700.
McKenzie, H. A.; Ralston, G. B. The denaturation of protein: Two states? Reversible or irreversible? *Experientia* **1971**, *27*, 617–624.
McKenzie, H. A.; Ralston, G. B.; Shaw, D. C. Location of sulfhydryl and disulfide groups in bovine β-lactoglobulins and effects of urea. *Biochemistry* **1972**, *11*, 4539–4547.
Mills, O. E.; Creamer, L. K. A conformational change in bovine β-lactoglobulin at low pH. *Biochim. Biophys. Acta* **1975**, *379*, 618–626.
Monahan, F. J.; McClements, D. J.; Kinsella, J. E. Polymerization of whey protein in whey protein-stabilized emulsions. *J. Agric. Food Chem.* **1993**, *41*, 1826–1829.
Mulvihill, D. M.; Kinsella, J. E. Gelation of β-lactoglobulin: effects of sodium chloride and calcium chloride on the rheological and structural properties of gels. *J. Food Sci.* **1988**, *53*, 231–236.
Nashef, A. S.; Osuga, D. T.; Lee, H. S.; Ahmed, A. I.; Whitaker, J. R.; Feeney, R. E. Effects of alkali on proteins. Disulfides and their products. *J. Agric. Food Chem.* **1977**, *25*, 245–251.
Ryle, A. P.; Sanger, F. Disulfide interchange reactions. *Biochem. J.* **1955**, *60*, 535–540.
Saio, K.; Kajikawa, M.; Watanabe, T. Food processing characteristics of soybean proteins. Part II. Effect of sulfhydryl groups on physical properties of tofu-gel. *Agric. Biol. Chem.* **1971**, *35* 890–898.
Sawyer, W. H. Heat denaturation of bovine β-lactoglobulins and relevance of disulfide aggregation. *J. Dairy Sci.* **1967**, *51*, 323–329.
Schmidt, R. H.; Illingworth, B. L.; Ahmed, E. M. Heat-induced gelation of peanut protein/whey protein blends. *J. Food Sci.* **1978**, *43*, 613–615.
Schmidt, R. H.; Illingworth, B. L.; Deng, J. C.; Cornell, J. Multiple regression and response surface analysis of the effects of calcium chloride and cysteine on heat-induced whey protein gelation. *J. Agric. Food Chem.* **1979**, *27*, 529–532.
Shimada, K.; Cheftel, J. C. Texture characteristics, protein solubility, and sulfhydryl group/disulfide bond contents of heat-induced gels of whey protein isolate. *J. Agric. Food Chem.* **1988**, *36*, 1018–1025.
Shimada, K.; Cheftel, J. C. Sulfhydryl group/disulfide bond interchange reactions during heat-induced gelation of whey protein isolate. *J. Agric. Food Chem.* **1989**, *37*, 161–168.
Shimizu, M.; Saito, M.; Yamauchi, K. Emulsifying and structural properties of β-lactoglobulin at different pHs. *Agric. Biol. Chem.* **1985**, *49*, 189–194.
Tanford, C. Protein denaturation. *Adv. Protein Chem.* **1968**, *23*, 121–282.
To, B.; Helbig, N. B.; Nakai, S.; Ma, C. Y. Modification of whey protein concentrate to simulate whippability and gelation of egg white. *Can. Inst. Food Sci. Technol. J.* **1985**, *18*, 150–157.
Watanabe, K.; Klostemeyer, H. Heat-induced changes in sulfhydryl and disulfide levels of β-lactoglobulin A and formation of polymers. *J. Dairy Res.* **1976**, *43*, 411–418.
Xiong, Y. L.; Kinsella, J. E. Mechanism of urea-induced whey protein gelation. *J. Agric. Food Chem.* **1990**, *38*, 1887–1891.
Yost, R. A.; Kinsella, J. E. Properties of acidic whey protein gels containing emulsified butterfat. *J. Food Sci.* **1993**, *58*, 158–163.

Received for review December 21, 1993. Revised manuscript received September 20, 1994. Accepted September 28, 1994.[⊗]

[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1994.