Influence of pH and Ionic Environment on Thermal Aggregation of Whey Proteins†

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A solution of whey protein isolate (WPI) was heated at 1.6 °C/min from 25 to 96 °C to monitor protein-protein aggregation by turbidity measurement. Protein aggregation showed a single transition (71 °C) at pH 5.5, two transitions (76 and 88 °C) at pH 6.0, and no transition above pH 6.5. The addition of 0.6 M NaCl induced a second transition (77 °C) at pH 5.5 and promoted protein-protein interaction above pH 6.5. The aggregation temperatures decreased to a minimum by 20 mM NaCl and 5-10 mM $CaCl_2$. Protein-protein interaction was suppressed at 5 mM sodium phosphate and gradually recovered at increasing sodium phosphate concentrations. In conclusion, low ionic strengths of cations and low pH facilitated protein-protein association, while phosphate promoted protein aggregation only in conjunction with NaCl.

INTRODUCTION

Because of its high nutritional value and functionality, whey protein concentrate (WPC) obtained from dairy processing has become an important source of functional ingredients used in many formulated foods, including processed meat, bakery, and dairy products (Kinsella and Whitehead, 1989). However, current commercial utilization of WPC is still limited partially because of the great variability in its functional properties due to differences in composition and processing treatments. A recent survey by Morr and Foegeding (1990) showed that WPC obtained from different commercial sources had considerable variations in mineral contents, including calcium, sodium, and phosphorus. Hence, improved understanding of mineral effects on whey protein functionality is needed for expanding the utilization of commercial whey products.

Whey protein gelling properties have been shown to vary greatly with $CaCl_2$ and NaCl (Schmidt et al., 1984; Kuhn and Foegeding, 1991a). Replacement of calcium with sodium ion improved whey protein gelling properties (Johns and Ennis, 1981). Demineralization and electrodialysis of WPC or whey protein isolate (WPI) enabled the formation of stronger and more elastic gels than nondemineralized WPC or WPI (Schmidt et al., 1984; Kuhn and Foegeding, 1991b), indicating a deleterious effect of minerals on whey protein gel structure. Nevertheless, low levels of calcium ion (5-10 mM) have been found to facilitate whey protein gelation (Schmidt, 1981; Mulvihill and Kinsella, 1988; Kuhn and Foegeding, 1991a). The effect of calcium on whey protein foaming and emulsification has also been reported; $CaCl_2$ was generally found to be detrimental to WPC foamability and emulsion stability (Schmidt et al., 1984).

To exploit whey protein functionality, interactions between protein molecules in the presence of various ionic minerals and under different processing conditions must be elucidated. Protein-protein interaction is a general term referring to protein association, aggregation, and polymerization (Reithel, 1963). Protein functionality is imparted via interactions between protein molecules and solvent (water), salts (ions), and other food components. The structure development of protein gels and the formation of stable emulsions and foams involve protein-protein interaction. The rate and extent of protein-protein interaction could affect the performance of whey proteins in immobilizing water and stabilizing fat and, ultimately, the textural characteristics of final products. Furthermore, the relationship between heating process and whey protein denaturation/molecular interaction needs to be established for WPC or WPI to be compatibly used in nondairy products.

Protein-protein interaction in an acid whey has been studied by Morr and Josephson (1968) using differential ultracentrifugation and gel filtration. They showed that calcium facilitated protein aggregation and formation of large particles in heated whey. Deng et al. (1976) introduced a spectrophotometric technique to study the dynamics of protein aggregation. In this method, aggregation due to protein-protein interaction was determined by measuring turbidity changes attributed to light scattering at the aggregated particles or polymers (Clark and Ross-Murphy, 1987). Using this technique, Foegeding et al. (1986) and Xiong and Brekke (1990) showed good correlations between aggregation and gelation of muscle proteins; molecular interaction generally preceded gelation. The derivatives (dA/dT, i.e., differential change in optical density as a function of temperature), calculated from the turbidity-temperature curve, are especially useful since they reveal thermal transitions involved in the associations of different protein components (Xiong and Brekke, 1990). The objective of this study was to characterize the molecular interactions of whey proteins in light of the effects of pH and ionic environments.

MATERIALS AND METHODS

Reagents. Chemical reagents used in this study, all of reagent grade, were obtained from Fisher Scientific (Springfield, NJ).

Protein Samples. Whey protein isolate (WPI) was used in this study because WPI contains very little nonprotein substances, which can be readily removed. WPI (>95% protein) prepared by ion-exchange chromatography was obtained from Le Sueur Isolates (Le Sueur, MN). WPI was suspended in distilled and deionized water to give a 10% solids concentration. To remove calcium and small molecules, WPI solution was dialyzed, using a 10 000 molecular weight cutoff cellulose dialysis tube, against 50 volumes of distilled water containing 0.02% EDTA and 0.02% NaN_3 at 5 °C. The dialysis solution was changed two times at 12-h intervals between changes. WPI was further purified by

† Published with the approval of the Director of the Kentucky Agricultural Experiment Station as Journal Article No. 91-5-154.

0021-8561/92/1440-0380$03.00/0

dialysis against pure deionized water (two water changes) and lyophilized. The purified WPI contained neligible amounts of minerals (calcium, 0.4 mg/g; sodium, 2.8 mg/g) as analyzed by atomic absorption.

Pure, lyophilized β-lactoglobulin was purchased from Sigma Chemical Co. (St. Louis, MO). SDS gel electrophoresis (without a reducing agent) showed essentially only a single band (β-lactoglobulin monomer). Atomic absorption indicated negligible amounts of minerals (calcium, 0.15 mg/g; sodium, 2.8 mg/g) in the lyophilized β-lactoglobulin.

Protein Solutions. Purified WPI was suspended to a 1.2 mg/mL protein concentration in different solutions containing the following salts (treatments): (1) 0–0.6 M NaCl, with or without 50 mM monobasic sodium phosphate (NaH_2PO_4); (2) 0–50 mM $CaCl_2$; (3) 0–50 mM NaH_2PO_4. Protein solutions were adjusted to pH 5.50, 6.00, 6.50, 7.00, and 7.50 (±0.02) using 0.05 N NaOH/HCl. In the absence of sodium phosphate, very little titrates (NaOH or HCl, contributing to <1 mM in pH-adjusted protein solutions) were needed to adjust protein solutions to specific values. Sodium phosphate within the pH range 5.50–7.50 existed as a mixture of most mono- (low pH) and dibasic (high pH) salts; thus, the ionic strengths of phosphate solutions at various pH values may slightly differ. However, this difference was not adjusted in this study.

Protein–Protein Interaction. The method described by Deng et al. (1976) and outlined by Xiong and Brekke (1990) was used to determine thermally induced protein–protein interaction. Protein solutions were placed in 500-μL quartz cuvettes (1-cm path length) with stoppers and heated at 1.6 °C/min from 25 to 96 °C in a temperature-controlled thermal unit attached to a Response II UV–vis spectrophotometer (Ciba Corning Diagnostics Corp., Oberlin, OH). The increase in turbidity resulting from protein aggregation was recorded every 1 °C increment by measuring changes in optical density at 320 nm. The differential change in optical density as a function of temperature (first derivative, dA_{320}/dT) was calculated to determine rates and transition temperatures of protein–protein association.

Statistical Analysis. The turbidity measurement for each treatment was replicated two to four times. In each replication, freshly prepared protein solutions (from purified WPI) were used. Data were analyzed using analysis of variance with the general linear model procedure (SAS, 1982). Differences between means were determined using predicted difference procedures.

RESULTS AND DISCUSSION

Ionic Species. Whey proteins at pH 6.0 started to aggregate at 67 °C and showed two transitions with temperature maxima (T_m) at 76 and 88 °C (Figure 1). Based on the relative thermal stability (De Wit and Klarenbeek, 1984) and the abundance of the individual whey proteins, i.e., mainly β-lactoglobulin, α-lactalbumin, immunoglobulin G and serum albumin (Kinsella and Whitehead, 1989), the two transitions of WPI may be attributed largely to the intermolecular association of α-lactalbumin (76 °C) and β-lactoglobulin (88 °C), respectively. At pH 6.0 and a zero ionic strength, α-lactalbumin and β-lactoglobulin denatured at 63 and 78 °C, respectively (De Wit and Klarenbeek, 1984). Presumably, protein structure unfolding (denaturation) (which caused the exposure of reactive groups) would precede protein–protein association to form large protein aggregates (Schmidt, 1981). The maximum transition of pure β-lactoglobulin occurred at 83 °C (Figure 1, dashed line), apparently not matching the second peak in WPI. A plausible explanation would be that the denatured and polymerized α-lactalbumin, along with other whey protein components, influenced the association of β-lactoglobulin molecules in WPI. It is also possible that the different milk/protein sources and the procedures for protein preparations may contribute to the discrepancy (T_m).

Protein–protein aggregation was affected by the ionic environment. The addition of NaCl to 20 mM reduced

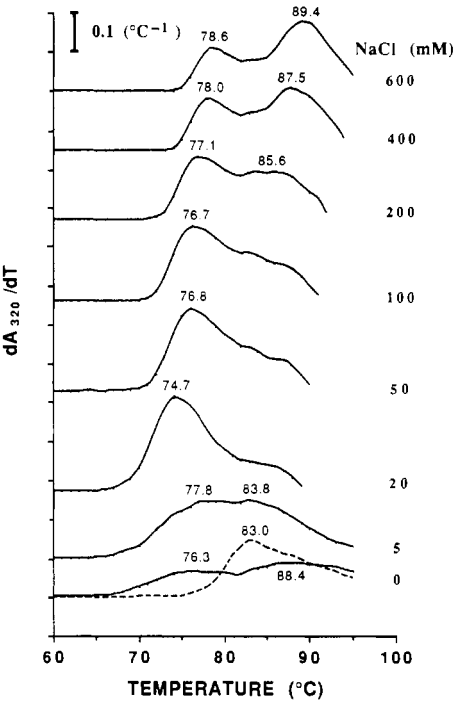

Figure 1. Influence of NaCl at pH 6.00 on thermal aggregation of whey protein isolate. dA_{320}/dT is the differential change in optical density.

the number of transitions, decreased ($P < 0.05$) aggregation temperature, but increased peak height (dA_{320}/dT) (Figure 1). An increase in peak height would suggest accelerated molecular association which may involve interactions between α-lactalbumin and β-lactoglobulin. A further increase in NaCl concentration caused a significant ($P < 0.05$) upward shift in T_m from 74.7 (20 mM NaCl) to 78.6 °C (600 mM NaCl) and attenuation in peak size. Concomitantly, a second transition at higher ($P < 0.05$) temperatures emerged. It is possible that at low NaCl concentrations (low ionic strengths) intramolecular electrostatic forces were modified to facilitate molecular association among WPI components. Hermansson (1979) indicated that at an ionic strength of ≤0.1 univalent ions could significantly alter the electric double layer surrounding a protein molecule. High ionic strengths apparently increased protein stability, probably by increasing the hydration (solubility) of proteins. Increasing the ionic strength suppressed the first transition (presumably α-lactalbumin) but promoted the second transition (β-lactoglobulin) in WPI. This may reflect the difference in tertiary and quaternary structure of the two proteins (Kinsella and Whitehead, 1989).

These results can be correlated to gelation characteristics of whey proteins. For instance, Mulvihill and Kinsella (1988) observed a maximum in gel strength (compression stress) at 100 mM NaCl for β-lactoglobulin suspended in NaCl solutions. Kuhn and Foegeding (1991a) reported that WPI gels formed in NaCl had a maximum shear stress (at failure) at 50–75 mM NaCl. Correspondingly, protein–protein interactions exhibited a major transition within a similar NaCl concentration range (25–100 mM) (Figure 1). Clark and Ross-Murphy (1987) has indicated stress being a measure of protein network strength which is dependent on the manner of intermolecular cross-linking (aggregating). Hence, the effect of NaCl on whey protein gel properties can be accounted for by protein molecular association, i.e., how proteins polymerize (aggregate) prior to forming infinite networks (gel), in the presence of NaCl;

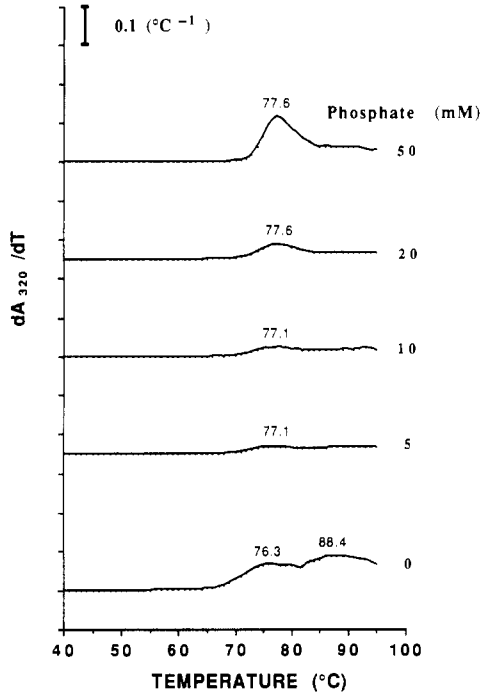

Figure 2. Influence of sodium phosphate at pH 6.00 on thermal aggregation of whey protein isolate. dA_{320}/dT is the differential change in optical density.

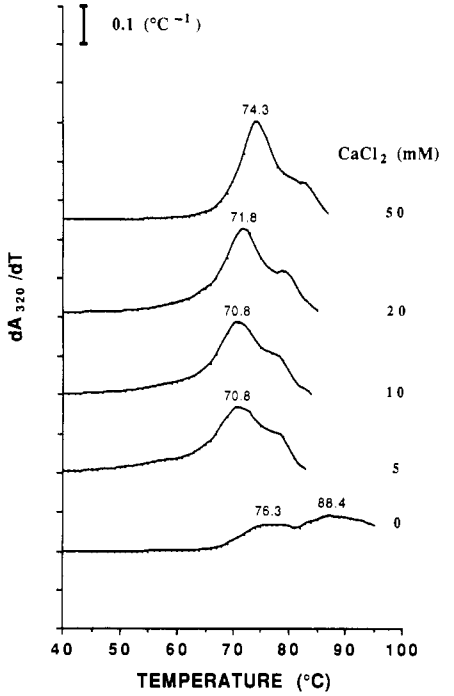

Figure 3. Influence of $CaCl_2$ at pH 6.00 on thermal aggregation of whey protein isolate. dA_{320}/dT is the differential change in optical density.

and the NaCl effect on whey protein gelation can be predicted from the thermal curves shown in this study.

Low concentrations of sodium phosphate (most in the form of NaH_2PO_4 at pH 6.0) greatly suppressed protein chain association. At as little as 5 mM phosphate, the initial two transitions of whey protein converged to a small peak at 77 °C (Figure 2). The T_m value of this transition did not change ($P > 0.05$) with increased phosphate concentrations. However, from 20 to 50 mM NaH_2PO_4, the peak area was considerably enlarged. The role of NaH_2PO_4 in protein aggregation obviously differed from that of NaCl. For instance, at an approximately equal ionic strength of 0.02 (20 mM NaH_2PO_4 or NaCl) at pH 6.0, the phosphate had a marked inhibitory effect on protein molecular association, in contrast to NaCl (Figure 1). This difference can be ascribed to the anions. Phosphoric anions may bind to the oppositely charged amino groups in protein and cause repulsion between peptide chains by increasing electronegativity of the protein.

While the coagulation effect of calcium on whey proteins is widely observed (Morr and Josephson, 1968; Schmidt et al., 1984), the dynamical interactions of protein molecules in the presence of calcium have not been explored. Generally, calcium facilitated protein aggregation by decreasing the aggregation temperatures and increasing the aggregation rates (Figure 3). At 5–10 mM, $CaCl_2$ caused the greatest reduction ($P < 0.05$) in T_m, while at higher $CaCl_2$ concentrations the rate (peak height) of protein–protein interaction was accelerated. Comparisons between Figure 3 and Figure 1 suggest that the effect of $CaCl_2$ differed (e.g., T_m) from that of NaCl, reflecting a functional difference between calcium and sodium ions. Being a divalent cation, calcium is capable of forming an ionic bridge between two adjacent carboxyl groups from different peptide chains, whereas sodium cannot. These results were consistent with the findings from whey protein gelation studies reported elsewhere (Schmidt et al., 1981; Mulvihill and Kinsella, 1988; Kuhn and Foegeding, 1991a). In these gelation studies, an increased whey protein gel strength was observed at 5–10 mM $CaCl_2$, compared with

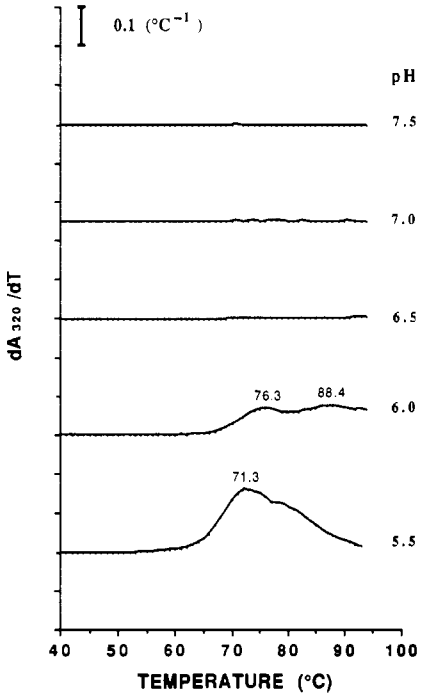

Figure 4. Effect of pH on thermal aggregation of whey protein isolate suspended in distilled water. dA_{320}/dT is the differential change in optical density.

0 or ≥20 mM $CaCl_2$. The increase ($P < 0.05$) in T_m in response to the change in calcium concentration from 10 to 50 mM suggests that whey protein conformations were less destabilized at increasing calcium concentrations.

pH. Electrostatic interactions contribute to protein–protein association in most biological systems, and they can be altered by changes in pH. Therefore, the dynamic aggregation of WPI was monitored under different acidic conditions. Major aggregation at pH 5.5 occurred around 71 °C (Figure 4). A large transition at pH 5.5 is expected because pH 5.5 is close to the isoelectric pH (4.8–5.3) of β-lactoglobulin and α-lactalbumin (Kinsella and White-

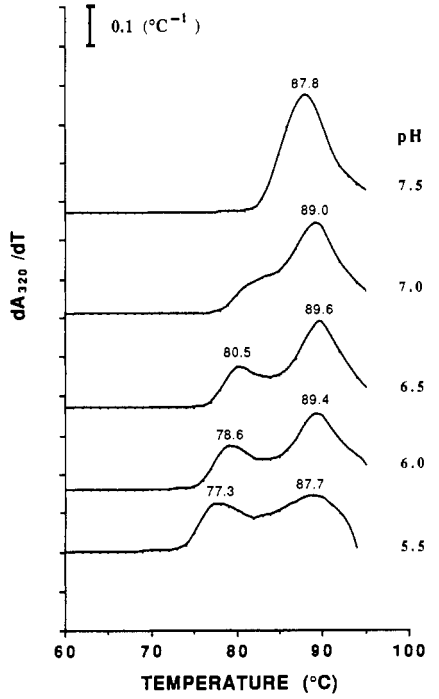

Figure 5. Effect of pH on thermal aggregation of whey protein isolate suspended in 0.6 M NaCl. dA_{320}/dT is the differential change in optical density.

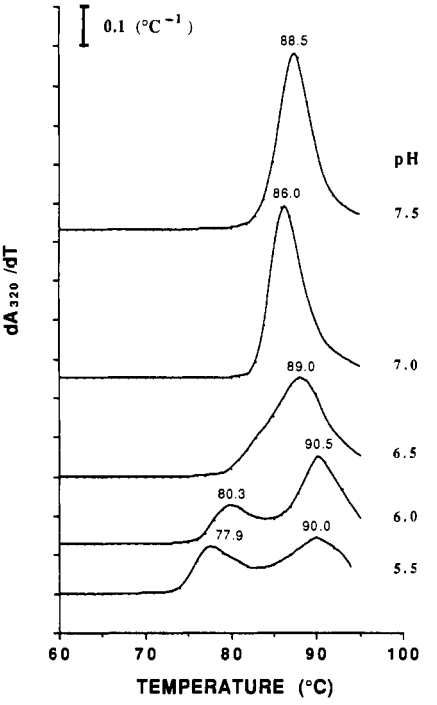

Figure 6. Effect of pH on thermal aggregation of whey protein isolate suspended in 0.6 M NaCl and 50 mM sodium phosphate. dA_{320}/dT is the differential change in optical density.

head, 1989). At pH 5.5, both proteins may substantially reduce their net charges, thereby facilitating intermolecular interaction via hydrophobic and van der Waals bonds. At pH 6.0, two separated transitions were observed; presumably they were derived from α-lactalbumin and β-lactoglobulin. Further increases in pH to 6.5 and above completely inhibited transitions. The exact cause is not clear, but it may be related to the increased intermolecular electrostatic repulsing forces and/or insensitivity of the instrument to detect low-scale protein aggregation. In a previous study, Hermansson (1979) observed no aggregation (turbidity) of WPC suspended (9 mg/mL) in distilled water at pH ≥8.0. Both β-lactoglobulin and mixed whey proteins showed a decreased stability (a decrease in T_m and enthalpy) as the pH was increased from 6.0 to 8.0 (Hermansson, 1979; Hegg, 1980). However, such a destabilization by an increased pH did not facilitate protein–protein interaction.

The mechanism of protein–protein aggregation seemed to differ below and above pH 6.5. It has been reported that whey protein gels formed below pH 6.5 were opaque, whereas gels prepared above pH 6.5 were translucent (Zirbel and Kinsella, 1988). This difference has been inferred from the increased intermolecular disulfide bonds formed at increased pH (Hillier et al., 1980). Between pH 6.0 and 10.0, whey protein gel strength was inversely related to the pH (Hillier at al., 1980; Zirbel and Kinsella, 1988; Xiong and Kinsella, 1990). These results were consistent with the present findings in which decreased intermolecular interactions were associated with a higher pH (Figure 4).

Because NaCl altered whey protein aggregation patterns (Figure 1), we speculated that the pH dependence of WPI (zero ionic strength) transitions would change when NaCl was added to the WPI solution. Therefore, an experiment was designed in which WPI was heated in the presence of 0.6 M NaCl at various pHs. Comparisons between Figure 4 and Figure 5 show marked differences caused by NaCl. The ion-free WPI at pH 5.5 was readily coagulated, showing a single transition at 71 °C (Figure 4); in 0.6 M NaCl, this transition was resolved into two separate ones (Figure 5). Furthermore, at pH ≥6.5, where no protein–protein interaction was detected for WPI suspended in distilled water (Figure 4), one or two major transitions were produced for WPI suspended in 0.6 M NaCl (Figure 5). Ostensibly, NaCl modified protein charges probably by masking some exposed ionic groups, enabling favorable chain association between polypeptides. These results may be important for defining suitable conditions for the utilization of whey proteins as ingredients in formulated products. For instance, processed meat products generally contain 0.6 M NaCl, which may be desirable for WPI to form structured binding materials (gels). From a practical view, the effect of minerals and pH on protein–protein aggregation could be further explored at a higher protein concentration (than 1.2 mg/mL, which was employed in the present study), because whey proteins are often used at much higher concentrations (e.g., 100 mg/mL) for forming gels and other structured products.

The combination of 0.6 M NaCl and 50 mM sodium phosphate showed a complex effect on protein aggregation. At pH 6.0, 50 mM sodium phosphate alone allowed a single and appreciable WPI transition (77 °C) (Figure 2). This transition did not recur when 0.6 M NaCl was also present (Figure 6). It is possible that protein–protein aggregation at pH 6.0 was most sensitive to sodium phosphate in a low ionic strength environment and/or that at pH 6.0 NaCl had a dominant effect. At pH ≥6.5, however, sodium phosphate caused considerable deviation in transition patterns from that of the WPI solution which only contained 0.6 M NaCl. This was evidenced from the reduced number of transitions in 0.6 M NaCl and 50 mM phosphate solution (Figure 6), compared with 0.6 M NaCl solution (Figure 5). It also can be noted that at pH ≥7.0 protein aggregation in the presence of sodium phosphate was accelerated, i.e., a maximum increase (dA_{320}/dT) was 0.49 and 0.28 °C^{-1} for WPI with (Figure 6) or without (Figure 5) 50 mM sodium phosphate, respectively. This may be explained because at pH ≥7.0 phosphate was more ionizable; the increased charges ($H_2PO_4^- \rightarrow HPO_4^{2-}$) and ionic strength ($Na^+$, $H_2PO_4^- \rightarrow 2Na^+$, HPO_4^{2-}, resulting

from adjusting the protein solution to a higher pH) may impart additional balance between protein molecules, rendering a more favorable ionic environment for protein–protein association. Furthermore, the T_m at pH 7.0 was significantly ($P < 0.05$) less than the T_m at pH 6.5 or 7.5. No exact cause is known, but it may be related to the combined effects of phosphorus and imidazole ionizations.

Conclusions. Thermal aggregation of dialyzed WPI was greatly influenced by the ionic strength (salt concentration), ionic species, and pH of the protein solution. Calcium ion, notably at 5–10 mM, was the most effective to initiate protein coagulation. At pH ≥6.5 and a zero ionic strength, protein–protein interaction was largely suppressed. However, the addition of NaCl, and NaCl in conjunction with sodium phosphate, promoted protein–protein interaction. Since WPI and especially WPC from different commercial sources vary greatly in the contents of these minerals, a standardizing procedure by which the mineral contents in different whey protein sources are adjusted to specific levels would allow for a controlled whey protein coagulation or aggregation process. Because the temperature and patterns for protein aggregation can be predicted from a knowledge of the mineral concentration in WPC or WPI solution, it is possible, by manipulating the WPC or WPI composition, to form specific protein products (e.g., gels) with a range of functional and textural properties.

ACKNOWLEDGMENT

The technical support from Dr. Karl Dawson is greatly appreciated.

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Received for review August 20, 1991. Revised manuscript received December 9, 1991. Accepted December 20, 1991.