Effect of Heat Treatments in Very Acidic Conditions on Whey Protein Isolate Properties

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ABSTRACT

The effect of heat treatments of whey protein isolate was studied under very acidic conditions. Dispersions of whey protein isolate in .1 *M* HCl (pH 1.2 to 1.3) were heated at 90°C for 0 to 120 min, adjusted to pH 6.9, dialyzed, and freeze-dried. The resulting isolates were characterized for protein solubility at different pH and for the extent of protein deamidation. Electrophoretic and calorimetric behavior, light transmittance of heated dispersions, and gelling properties were also determined. Thermal treatments produced several protein alterations: protein denaturation, partial deamidation, and a decrease of β-LG, α-LA, and BSA; protein species of low molecular weight and aggregates appeared at the same time. Gels prepared by dispersion of the isolate heated for 2 h in .1 *M* HCl exhibited very low elasticity, firmness, and water-holding capacity. However, texture characteristics of gels of whey protein isolate heated for 0 to 60 min in .1 *M* HCl did not differ in spite of protein changes during treatment.

(**Key words**: heat treatment, acidic conditions, functional properties, whey protein isolate)

Abbreviation key: **DSC** = differential scanning calorimetry, **ME** = 2-mercaptoethanol, **WHC** = water-holding capacity, **WPI** = whey protein isolate.

INTRODUCTION

The effect of heat on whey protein and β-LG at pH between 2.0 and 3.5 has been extensively studied (1, 6, 7, 8, 9, 10, 12, 17, 18, 19). At pH >4, whey proteins coagulated rapidly when heated to more than 70°C; however, at pH <3.5, resistance to coagulation was high (12). Calorimetric studies showed higher thermal stability at pH 3.0 than at neutral pH (3) and a highly soluble whey protein concentrate was obtained by adjustment of the sweet whey to pH 2.5 to 3.5 and heating at 90°C for 15 min (17). Harwalkar (7) found that whey proteins heated (90°C, 30 min) at pH 2.5 remained in solution, exhibiting a partial loss of solubility at pH 4.5. Harwalkar (7) suggested that two fractions of β-LG can occur after the whey has been heated at pH 2.5: the fraction that is insoluble at pH 4.5, which was partially and irreversibly unfolded, and the fraction that is soluble at pH 4.5, which is quite similar to the native β-LG.

However, no information was found to elucidate the effect of the thermal treatment at pH <2.0. Even if undesirable protein modifications were produced during this treatment, the characterization of the protein changes may contribute to an understanding of the effect of heat treatments under very acidic conditions. In the present study, a whey protein isolate (**WPI**) was dispersed in .1 *M* HCl and heated at 90°C for 0 to 120 min; alterations of several parameters, such as denaturation, solubility, and deamidation of the whey proteins and their gelling properties, were studied.

MATERIALS AND METHODS

Materials

The WPI, obtained from Union Laitière Normande (Condé-sur-Vire, France), was prepared on an industrial scale by anion-exchange chromatography of sweet whey on a Spherosil

Received August 16, 1993.
Accepted February 15, 1994.
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QMA column (Rhône-Poulenc, France). After elution from the resin at acidic pH, the eluate was evaporated and spray dried. The WPI contained 6% moisture, 85% protein [(total N – NPN) × 6.38] (dry basis), <1% fat, <1% lactose, and 2.5% ash (dry basis). The WPI also contained 70 g of β-LG (in the dimer form) and 6 g of α-LA/100 g of protein, as determined by gel permeation chromatography. The N solubility index (grams of soluble N per 100 g of total N) at pH 3.75, 4.7, and 7.0 was 83.4, 77.5, and 97.4, respectively. All chemicals were analytical grade.

Heat Treatment. The WPI was dispersed in .1 *M* HCl (200-g dispersion; 5% protein, wt/wt; and pH 1.2 to 1.3) in glass containers (6 cm i.d.). After dispersion, samples were heated in a water bath at 90°C for 0 (control), 15, 30, 60, and 120 min with gentle stirring and then cooled in a water bath to about 15°C. After cooling, the pH was adjusted to 6.9 with 2 *M* NaOH; the resulting samples were dialyzed twice against 2 L of distilled water at 3°C and freeze-dried.

Extent of Deamidation

Amide N was determined as the amount of NH_3 distilled after the sample was refluxed in 2 *M* HCl for 2 or 3 h (2, 14). Results for both times were identical. The extent of deamidation was calculated as 100 × (NH_3 of the unheated sample – NH_3 of the heated sample)/NH_3 of the unheated sample. Each value is the mean (± SD) of at least three determinations.

Protein Solubility

Samples were dispersed in distilled water (about .01 g of protein/10 ml), adjusted to the desired pH with .125 *M* to .0125 *M* NaOH or HCl, slowly stirred for 30 min, and centrifuged at 18,800 × *g* for 15 min. Protein solubility was determined from the protein content of the supernatant and as a percentage of the total protein content. Each value was the mean (± SD) of three independent preparations. Protein concentration was determined by the biuret method (5) using β-LG (Sigma Chemical Co., St. Louis, MO) as a standard.

Electrophoresis

The SDS-PAGE was performed according to the method of Laemmli (13). A linear gradient separating gel (8 to 20% of polyacrylamide) was prepared with an acrylamide to bisacrylamide ratio of 45 to 2. The stacking gel contained 6% polyacrylamide (same ratio). Protein solutions (10 mg/ml) were diluted with an equal volume of .2 *M* Tris·HCl buffer (pH 6.8) containing 30% (vol/vol) glycerol and 4% (wt/vol) SDS with or without 10% (vol/vol) 2-mercaptoethanol (ME) and then heated in a boiling water bath for 4 min. The relative intensity of the bands was determined with a scanning densitometer (GS 300; Hoefer Scientific Instruments, San Francisco, CA) at 590 nm. The molecular mass of each protein species was estimated by comparison with protein standards (Sigma Chemical Co.).

Differential Scanning Calorimetry

For differential scanning calorimetry (DSC) a micro apparatus from Setaram (Lyon, France) was used. Whey protein dispersion samples (850 mg) in distilled water (5% protein, wt/wt; pH 7.0, adjusted with .0125 *M* HCl) were placed in a hastelloy C276 hermetic cell (Setaram). Distilled water was the reference. The sample and the reference were subjected to a linear temperature gradient (20 to 99°C; heating rate, 1°C/min). After cooling, sample and reference were heated a second time under the same conditions. No peaks were obtained during the second heating. The enthalpy of denaturation (Joules per gram of protein) and the apparent transition temperature were computed from the endothermal peak obtained as the difference between the first and the second heatings.

Heating and Gelation of WPI Dispersions

Aqueous dispersions (11% protein, wt/wt) of the freeze-dried samples were adjusted to pH 7.0 with .5 or .125 *M* HCl or NaOH. Dispersions were partially deaerated by centrifugation at 1000 × *g* for 1 min (25), carefully resuspended with a glass rod, and placed in glass tubes (2.2 cm i.d. × 4.5 cm height) with tightly closed stoppers. For gelation, the tubes were heated in a water bath at 87°C for 45 min, as described by Shimada and Cheftel (24). Gel samples were kept at 3°C for 13 h until texture analyses.

Gel Texture

Gels were equilibrated at room temperature (about 20°C) for ≥1 h. The texture analysis was carried out on gel sections (2.2 cm diameter × 2.0 cm height) with a Stevens LFRA Texture Analyzer (St. Albans, England) in a compression mode. Compression was by a cylindrical probe with a flat section (5.1 cm diameter) at a displacement speed of .2 mm/s. Gel firmness was defined as the initial force (Newtons) measured at 20% (4-mm) compression. This compression was then maintained for 10 min, and the force exerted on the probe was measured. Gel elasticity was taken as the force after 10 min divided by initial force. For each type of gel, the mean (± SD) was of single determinations on each of three gels.

Water-Holding Capacity

To determine the water-holding capacity (**WHC**), 1.0 to 1.7 g of gel, equilibrated at room temperature, were placed on a polyethylene net membrane (20-μm pore size; Pharmacia Fine Chemicals, Uppsala, Sweden) maintained in the middle of a 50-ml centrifuge tube. Water loss was determined in duplicate by weighing after centrifugation at 120 × g for 5 min (21). The WHC was expressed as a percentage of the initial gel water remaining in the gel after centrifugation.

Transmittance of Protein Dispersions

Aqueous dispersions (5% protein, wt/wt) of the freeze-dried samples were adjusted to pH 7.0 with .125 or .0125 *M* HCl or NaOH. Heating (87°C, 45 min) was carried out on 5.5 ml of the dispersion sample in a glass tube (10 × 120 mm), according to the method of Shimada and Cheftel (24). Tubes were then cooled rapidly to room temperature in tap water; the gel transmittance was measured at 660 nm against distilled water with a Bausch and Lomb Spectronic 88 colorimeter (Rochester, NY).

RESULTS AND DISCUSSION

Protein Solubility

Figure 1A shows the protein solubility at different pH of WPI heated in .1 *M* HCl (pH 1.2 to 1.3) and subsequently adjusted to pH 6.9, dialyzed, and freeze-dried; Figure 1B depicts protein solubility as a function of heating time in .1 *M* HCl. The shape of the curves of solubility versus pH was similar in all samples with the minimum at about pH 4.7 (Figure 1A).

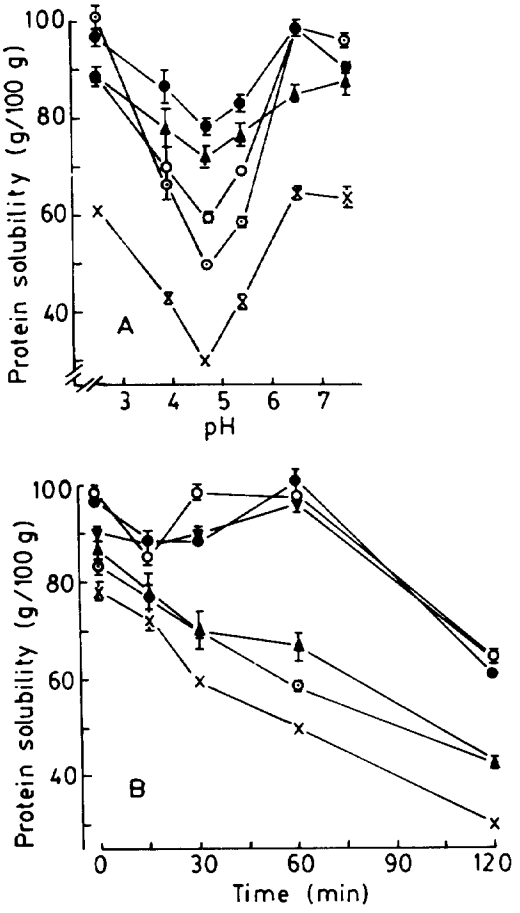

Figure 1. A. Protein solubility of whey protein isolate as a function of pH: control (●), heated in .1 *M* HCl for 15 min (▲), 30 min (○), 60 min (⊙), and 120 min (x). B. Protein solubility as a function of heating time in .1 *M* HCl (pH 1.2 to 1.3): solubility at pH 2.5 (●), pH 3.9 (▲), pH 4.7 (x), pH 5.4(⊙), pH 6.5 (○), and pH 7.5 (▼). Bars represent standard deviations of three determinations.

Protein solubilities were similar at pH 2.5, 6.5, and 7.5 (far from the isoelectric pH): between 85 and 100% in the unheated (control) sample or in samples heated 15 to 60 min in .1 *M* HCl and between 61 and 65% in the sample

heated for 120 min under the same conditions (Figure 1). However, protein solubilities at pH 3.9 to 5.4 (close to the isoelectric pH) decreased as heating time in .1 *M* HCl increased, indicating progressive protein denaturation. Protein solubility was significantly lower ($P < .05$) over the entire pH range for the sample heated for 120 min in .1 *M* HCl than for the samples heated for 0 to 60 min under the same conditions. Also, protein solubilities at pH 4.7 were lower ($P < .05$) in all samples.

Protein solubility in distilled water at pH 4.7 was 59.5% in the sample heated at 90°C for 30 min. Harwalkar (7) found that, when β-LG (about 1% protein, .1 ionic strength, and pH 2.5) was heated at 90°C for 30 min, the protein remained in solution at pH 2.5, but its solubility decreased to 55% at pH 4.5. Harwalkar (7) worked at a less acidic pH, but protein concentration was less than in this study, which would explain the similar protein solubility obtained in both cases. Iung et al. (11) reported that the solubility of β-LG (.5% protein, heated at 90°C during 60 min at pH 2.5) was 38.0% at pH 5.1. In the present work, protein solubility at pH 4.7 was 50.1% in the sample heated at 90°C in .1 *M* HCl (pH 1.2 to 1.3) for 60 min. The lower solubility reported by Iung et al. (11) could be attributed to the lower protein concentration. The WPI heated for 2 h in .1 *M* HCl exhibited the lowest solubility at all pH (Figure 1).

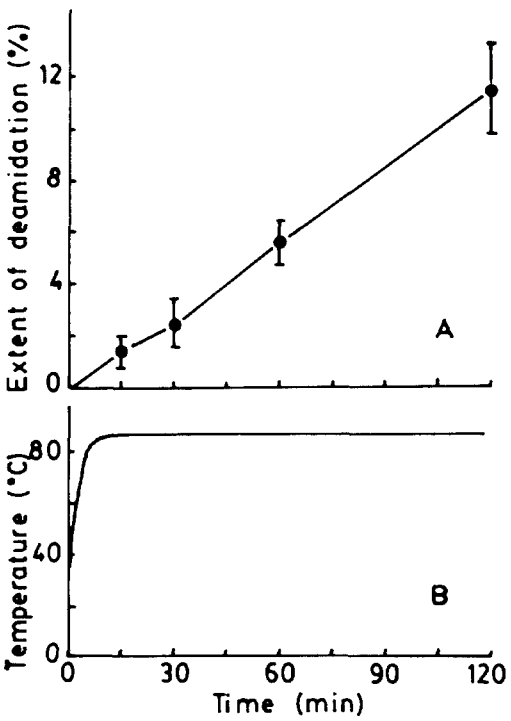

Figure 2. Extent of deamidation as a function of the heating time in .1 *M* HCl, pH 1.2 to 1.3 (A), and thermal history of the samples (B). Bars represent standard deviations of at least three determinations.

Extent of Deamidation

Heat treatments at acidic pH can produce protein deamidation. Figure 2 shows the thermal history and the extent of deamidation of WPI heated in .1 *M* HCl at 90°C for different periods. The extent of deamidation increased linearly as heating time increased, reaching 11% after 2 h of heating.

Popineau et al. (20) found a similar extent of deamidation in gluten heated in .1 *M* HCl at 70°C, and Finley (4) found 10% deamidation in gluten heated in .1 *M* HCl at 75°C for 30 min. Shih and Kalmar (23) reported deamidation of 10 to 20% in cottonseed proteins heated for 1 to 2 h in .2 *M* HCl at 70°C; soy extract heated at about 76°C for 24 h had deamidation of 15 to 20% (22). These results show that deamidation between 10 and 20% is produced by heat treatments in acidic conditions under different proteins and deamidation conditions.

Recently, protein deamidation has been widely investigated because it can effectively improve the functional properties of food proteins, mainly by increasing protein solubility at neutral pH, which in turn can improve other functional properties, such as emulsifying and foaming capacities (20). Undenatured whey proteins present an excellent solubility at all pH, so protein deamidation cannot be expected to improve their functional properties. However, deamidation produces an increase in the negative charge of the protein that must be considered in the analysis of protein modifications by the thermal treatment under acidic conditions.

Mattarella et al. (15) and Mattarella and Richardson (16) studied the changes produced by the amidation of β-LG. The effect was an increase in the positive charge of the protein. These investigators found that the protein (1%, wt/vol, low ionic strength) remained soluble at

pH <8, but solubility decreased to <20% at pH >9, which was near the new isoionic point.

In the present work, solubility curves (Figure 1) indicated that differences between the isoelectric points were not significant before and after deamidation, but solubility decreased from thermal treatment in acidic conditions. Mattarella et al. (15) reported that about 78% of the carboxyl groups (21 to 22 groups) were amidated after the amidation treatment, (β-LG contains 11 Asp and 16 Glu residues). In the present work, deamidation was only 11%, representing about 1.5 groups (β-LG contains 5 Asn and 9 Gln residues), which explains why change in the isoelectric pH was not significant.

Electrophoresis

The electrophoretic patterns of SDS-PAGE of WPI heated in .1 *M* HCl for different periods are shown in Figure 3. Patterns of control WPI show peaks corresponding to α-LA (peak 2), β-LG (peak 4), and a minor peak of BSA (peak 6). These peaks decreased with heating time in .1 *M* HCl; many peaks of low molecular mass appeared at the same time, especially in those samples treated with ME (Figure 3). These protein species of low molecular mass could result from protein hydrolysis.

Harwalkar (6) observed more bands in the electrophoretic pattern of the 2% TCA-insoluble fraction of heated whey. These bands appeared between α-LA and β-LG; some migrated ahead of the α-LA when samples were analyzed by starch-urea gel electrophoresis, which could occur as result of alterations of α-LA, β-LG, or both. At the same time, the BSA band disappeared in whey heated at pH 2.5. Iung et al. (11) also observed by SDS-PAGE many bands in the molecular mass range of 3.5 to 17 kDa after β-LG (.5% in .02 *M* HCl; pH 2.5) was heated at 90°C for 1 h, in agreement with the results in the present study. Iung et al. (11) concluded that the heat treatment in acidic conditions produces partial denaturation and cleavage of peptide bonds, thus obtaining peptides that are more susceptible to tryptic hydrolysis.

Samples treated with ME had electrophoretic patterns with better resolution than the corresponding untreated samples (Figure 3). We observed a high molecular mass peak that increased as heating time in .1 *M* HCl increased in the untreated samples (Figure 3A, zone 7), indicating the presence of aggregates, which was also suggested by decreased protein solubility. Protein aggregation could be expected because of the relatively high protein concentration. Results also suggest that disulfide bonds participate in the formation of the aggregates, in spite of the acidic conditions. Low pH inhibits disulfide interchange reactions; however, some evidence exists for the occurrence of these reactions at pH 2 (1), although the processes were not rapid. Thus, a certain degree of disulfide interchange reactions could be expected, mainly for the samples heated for 1 to 2 h in .1 *M* HCl.

DSC

The enthalpy of denaturation and the apparent transition temperature of WPI heated in .1 *M* HCl for different periods are shown in Table 1. Apparent transition temperatures did not differ. However, the enthalpy of denaturation decreased in samples heated in .1 *M* HCl as a function of the heating time, suggesting that protein denaturation already occurred in samples heated for 15 min under acidic conditions, which correlates with the decrease in protein solubility at pH 3.9 to 5.4.

Harwalkar (7) found that the protein fraction denatured by heating at pH 2.5 and insoluble at pH 4.5 showed no endothermic reaction when it was examined by DSC. This finding indicates that this protein fraction is a product of an irreversible denaturation and that a correlation may exist between solubility and the endothermic reaction.

In the present study, protein solubility at pH 4.7, expressed as a percentage of the solubility of the control at the same pH, was 96 and 76% in the samples heated for 15 and 30 min in .1 *M* HCl, respectively. However, the enthalpies of denaturation were 81 and 64% (expressed as percentages of the control) in the samples heated for 15 and 30 min in .1 *M* HCl, respectively, suggesting that only a part of the denatured protein (observed by DSC) became insoluble.

Texture Characteristics of Gels

Texture characteristics of gels prepared by heating (87°C, 45 min; pH 7.0; 11% protein)

dispersions of the freeze-dried samples are shown in Figure 4. All gels, except those obtained with the WPI heated for 2 h in .1 *M* HCl, presented similar elasticity, firmness, and WHC (Figure 4). Gels prepared with WPI heated in .1 *M* HCl for 2 h had very low

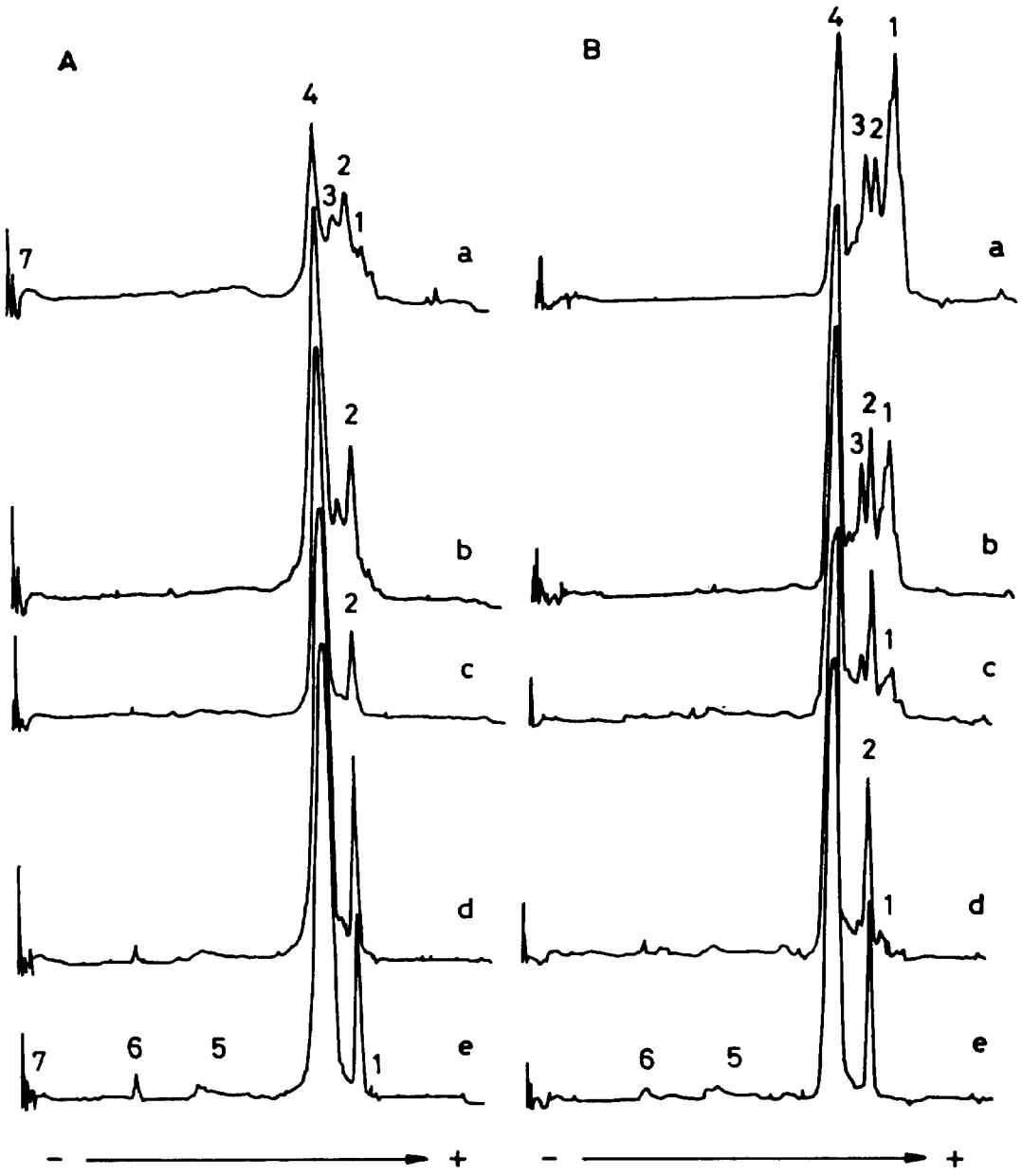

Figure 3. Electrophoretic patterns of whey protein isolate heated in .1 *M* HCl (pH 1.2 to 1.3) for different periods: 120 min (a), 60 min (b), 30 min (c), 15 min (d), and 0 min (e). Samples were either not treated (A) or treated (B) with 2-mercaptoethanol (ME) before electrophoresis. Molecular mass: 10 to 13 kDa (1), 14 kDa (2), 16 to 17 kDa (3), 18 kDa (4), 27 to 58 kDa (5), 67 kDa (6), and 98 kDa (7).

TABLE 1. Enthalpy of denaturation (ΔH) and apparent transition temperature (T_{max}) of whey protein isolate heated in .1 *M* HCl at 90°C for different periods.

Heating in .1 *M* HCl	T_{max}[1]	ΔH
(min)	(°C)	(J/g of protein)
0	73.4–73.6	10.9–11.0
15	72.9–73.9	8.6–9.2
30	72.9–74.0	6.6–7.4

[1]Heating rate: 1°C/min; 5% protein, wt/wt.

elasticity, firmness, and WHC (Figure 4). Gels prepared with WPI heated in .1 *M* HCl for 2 h had very low elasticity, firmness, and WHC. The WPI heated in .1 *M* HCl for 1 or 2 h showed browning that persisted in gels and was more pronounced in WPI heated for 2 h in .1 *M* HCl.

Transmittance of Heated Protein Dispersions

Dispersions of WPI (5% protein, wt/wt), heated under the same conditions (87°C, 45 min; pH 7.0), had similar transmittance at 660 nm (Figure 4A), which decreased slightly as heating time in .1 *M* HCl increased, except for protein dispersion prepared with WPI heated in .1 *M* HCl for 2 h. This sample presented a very low transmittance at 660 nm (Figure 4).

Transmittance of heated protein dispersions and gel elasticity appear to be directly related to protein solubility at neutral pH. Conversely, no relationship was observed between gel characteristics and the loss of solubility of protein constituents in pH 3.9 to 5.4.

CONCLUSIONS

Thermal treatments of WPI in .1 *M* HCl (pH 1.2 to 1.3) at 90°C produced several protein alterations: protein denaturation, as observed by DSC, loss of solubility near the isoelectric pH, partial deamidation, decrease of β-LG, α-LA, and BSA; protein species of low molecular mass and aggregates appeared at the same time.

Gels from WPI at neutral pH, heated for 2 h in these conditions, showed very low elasticity, firmness, and WHC, which correlates with lower protein solubility at neutral pH.

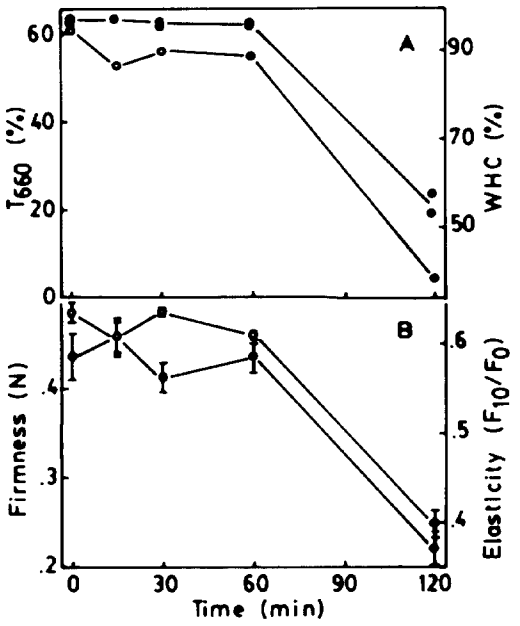

Figure 4. A. Transmittance at 660 nm (T_{660}) of heated dispersions (5% protein, wt/wt) (○) and water-holding capacity (WHC) of heat-induced gels (●). B. Elasticity (○) and firmness (●) of heat-induced gels (11% protein, wt/wt) from whey protein isolate heated in .1 *M* HCl (pH 1.2 to 1.3) for different periods. F_{10}/F_0 = Force after 10 min/initial force.

ACKNOWLEDGMENTS

Thanks are expressed to E. Dumay (Laboratoire de Biochimie et Technologie Alimentaires, Université de Montpellier II) for her advice and help. C. E. Lupano benefited from a scholarship given by the Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina.

REFERENCES

1 Ananthanarayanan, V. S., F. Ahmad, and C. C. Bigelow. 1977. The denaturation of β-lactoglobulin A at pH 2. Biochim. Biophys. Acta 492:194.
2 Darbre, A. 1986. Practical Protein Chemistry—A Handbook. A. Darbre, ed. John Wiley & Sons Ltd., Chichester, England.
3 deWit, J. N., and G. Klarenbeek. 1984. Effects of various heat treatments on structure and solubility of whey proteins. J. Dairy Sci. 67:2701.
4 Finley, J. W. 1975. Deamidated gluten: a potential fortifier for fruit juices. J. Food Sci. 40:1283.
5 Gornall, A., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177:751.
6 Harwalkar, V. R. 1979. Comparison of physico-

chemical properties of different thermally denatured whey proteins. Milchwissenschaft 34:419.
7 Harwalkar, V. R. 1980. Measurement of thermal denaturation of β-lactoglobulin at pH 2.5. J. Dairy Sci. 63:1043.
8 Harwalkar, V. R., and M. Kalab. 1985. Thermal denaturation and aggregation of β-lactoglobulin at pH 2.5. Effect of ionic strength and protein concentration. Milchwissenschaft 40:31.
9 Harwalkar, V. R., and M. Kalab. 1985. Thermal denaturation and aggregation of β-lactoglobulin in solution. Electron microscopic study. Milchwissenschaft 40:65.
10 Harwalkar, V. R., and M. Kalab. 1985. Microstructure of isoelectric precipitates from β-lactoglobulin solutions heated at various pH values. Milchwissenschaft 40:665.
11 Iung, C., D. Paguet, and G. Linden. 1991. Traitements de dénaturation appliqués à la β-lactoglobuline avant hydrolyse trypsique. Lait 71:385.
12 Jelen, P., and W. Buchheim. 1984. Stability of whey protein upon heating in acidic conditions. Milchwissenschaft 39:215.
13 Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (Lond.) 227:680.
14 Ma, C.-Y., B. D. Oomah, and J. Holme. 1986. Effect of deamidation and succinilation on some physicochemical and baking properties of gluten. J. Food Sci. 51:99.
15 Mattarella, N. L., L. K. Creamer, and T. Richardson. 1983. Amidation or esterification of bovine β-lactoglobulin to form positively charged proteins. J. Agric. Food Chem. 31:968.
16 Mattarella, N. L., and T. Richardson. 1983. Physicochemical and functional properties of positively charged derivatives of bovine β-lactoglobulin. J. Agric. Food Chem. 31:972.
17 Modler, H. W., and D. B. Emmons. 1977. Properties of whey protein concentrate prepared by heating under acidic conditions. J. Dairy Sci. 60:177.
18 Modler, H. W., and V. R. Harwalkar. 1981. Whey protein concentrate prepared by heating under acidic conditions. 1. Recovery by ultrafiltration and functional properties. Milchwissenschaft 36:537.
19 Patocka, J., M. Drathen, and P. Jelen. 1987. Heat stability of isolated whey protein fractions in highly acidic conditions. Milchwissenschaft 42:700.
20 Popineau, Y., S. Bollecker, and J.-Y. Thebaudin. 1988. Caractérisation biochimique et fonctionnelle des protéines de gluten désamidées partiellement en conditions ménagées. Sci. Aliments 8:411.
21 Quéguiner, C., E. Dumay, C. Cavalier, and J. C. Cheftel. 1989. Reduction of *Streptococcus thermophilus* in a whey protein isolate by low moisture extrusion cooking without loss of functional properties. Int. J. Food Sci. Technol. 24:601.
22 Shih, F. F. 1987. Deamidation of protein in a soy extract by ion-exchange resin catalysis. J. Food Sci. 52:1529.
23 Shih, F. F., and A. D. Kalmar. 1987. SDS-Catalyzed deamidation of oilseed proteins. J. Agric. Food Chem. 35:672.
24 Shimada, K., and J. C. Cheftel. Texture characteristics, protein solubility, and sulfhydryl group/disulfide bond contents of heat-induced gels of whey protein isolate. J. Agric. Food Chem. 36:1018.
25 Xiong, Y. L., and J. E. Kinsella. 1990. Mechanism of urea-induced whey protein gelation. J. Agric. Food Chem. 38:1887.