# Thermal Aggregation of Whey Protein Isolate Containing Microparticulated or Hydrolyzed Whey Proteins

C. Sanchez,* M. Pouliot, S. F. Gauthier, and P. Paquin

Centre de Recherches en Sciences et Technologie du Lait (STELA), Faculté des Sciences de l'Agriculture et de l'Alimentation, Université Laval, Pavillon Paul-Comtois, local 1316, Québec, Canada G1K 7P4

Thermal aggregation from 25 to 97 °C (0.8 °C/min heating rate) of diluted whey protein isolate (WPI) containing microparticulated WPI ($\mu$WPI) or specific WPI tryptic hydrolysate was studied at pH 6.0 using a spectrophotometric method. Mixed WPI solutions containing above 3.75% $\mu$WPI displayed faster thermal aggregation at lower heating temperatures of protein species than the control WPI solutions (no $\mu$WPI added), with a shift of aggregation mechanism from predominantly homogeneous to biphasic. Interactions between whey proteins and soluble/insoluble whey protein microparticles were thought to be at the origin of the shift. Results also showed that the presence of 20−40% WPI tryptic hydrolysate into the WPI solutions improved whey protein thermal aggregation at pH 6.0. Such a result could not be ascribed only to protein−peptide interactions because hydrolysate promoted an initial acidification of WPI solutions from 6.9 to 5.4 (20% hydrolysate added) or to 4.6 (20% hydrolysate added), which contributed to whey protein isoelectric precipitation and formation of whey protein aggregates.

**Keywords:** *Whey proteins; thermal aggregation; food microparticles; protein hydrolysates*

## INTRODUCTION

Whey protein concentrate (WPC) and isolate (WPI) are widely used in formulated foods as gelling, surface active, or water-binding agents. Factors affecting the functional properties of whey proteins include the physicochemical properties and structure of proteins (p*I*; surface hydrophobicity and hydrophilic/hydrophobic balance; presence of free thiol groups; size, conformation, and flexibility of macromolecules) and environmental (pH, ionic strength) and compositional (content and relative ratios of the different protein species, minerals, lactose, residual lipids, small peptides) conditions. These factors determine the balance attractive/repulsive forces between the proteins and control the extent of protein−solvent and protein−protein interactions (Kinsella and Whitehead, 1989; Ziegler and Foegeding, 1990).

Heat and enzymatic treatments are currently applied to whey proteins in order to modify their structure and functional properties so as to increase their use in the food industry. Heat aggregation in quiescent conditions of globular proteins such as whey proteins proceeds according to, roughly, three distinct stages: a denaturation stage where native proteins are partially unfolded and expose their reactive nonpolar side chains initially buried inside the macromolecules (and possibly sulfhydryl groups), an initiation stage where two partially unfolded macromolecules interact through noncovalent (e.g., van der Waals and hydrophobic interactions, hydrogen bonds) and covalent (e.g., intermolecular disulfide exchange reactions) interactions to form an aggregate, and finally a propagation stage where newly denatured protein−aggregate and aggregate−aggregate interactions produce polymeric structures (Morr and Josephson, 1968; Hermansson, 1979; Schmidt, 1981; Steventon et al., 1991; McSwiney et al., 1994). Heat denaturation and aggregation of whey proteins produce a heterogeneous population of native soluble proteins and colloidal species that can be defined as soluble aggregated and insoluble aggregated proteins, which relative proportions mainly depend on the composition of WPC or WPI, pH, temperature, and time of heating. Partial heat aggregation of whey proteins ($T$ = 70−80 °C, 15−30 min) at low ionic strength, i.e., the formation of a majority of soluble aggregated proteins, favors their adsorption at the air−water or oil−water interfaces and stabilizes the resulting foams and emulsions through extensive protein−protein interactions and formation of a viscous interfacial film (Beuschel et al., 1992; Britten et al., 1994; Dickinson and Hong, 1994; Zhu and Damodaran, 1994). Partly heat aggregated whey proteins gel at room temperature ($T$ = 25 °C) provided that calcium ions have been added to the protein solution after heating (Barbut and Foegeding, 1993). As well, heat-modified whey proteins gel after acidification (Abd El-Salam and El-Etriby, 1996) or moderated proteolytic hydrolysis at 37 °C by trypsin, pronase, papain, and protease (Sato et al., 1995).

Combined heat treatment (90−120 °C, 5 min−4 s) and intense shear (5000−40000 $s^{-1}$ in scraped-surface heat exchangers or cooking extruders; 750−1000 bars in high-pressure homogenizers) have also been applied to whey proteins to produce microparticles able to match the textural and structural properties of emulsified fat (Singer et al., 1988; Queguiner et al., 1992; Paquin et al., 1993; McCarthy and Maegli, 1994). Thus, a protein microparticle may be defined as an engineered protein aggregate. Similarly to emulsified fat, whey protein microparticles give creamy and smooth texture to a number of food products, including dairy products and food dressings (Sanchez and Paquin, 1997). The main drawbacks of whey protein microparticles lie in their very poor melting properties as compared to animal fat, their inability to impart the taste and flavor of fat in light foods, and their heat sensitivity that causes the

* To whom correspondence should be addressed at Laboratoire de Physicochimie et Génie Alimentaires, ENSAIA-INPL, 2 Avenue de la Forêt-de-Haye, 54500 Vandœuvre-lès-Nancy, France (e-mail sanchez@ensaia.u-nancy.fr).

S0021-8561(97)00061-7 CCC: $14.00 

formation of large aggregates and imparts grittiness to the heated food products (Cheftel and Dumay, 1993). In the present form, whey protein microparticles may be used with a satisfactory efficiency to replace part of the fat in ready-to-eat food products consumed at low temperatures ($T < 10-15$ °C).

Enzyme hydrolysis of proteins results in peptides of smaller molecular sizes and less secondary structure than proteins. Generally, the solubility, water absorption, and emulsifying and foaming capacity are improved by partial enzymatic treatment of proteins (Kuehler and Stine, 1974; Jost and Monti, 1977; Chobert et al., 1988; Turgeon et al., 1991, 1992a,b). Excessive hydrolysis of proteins may alter some functional properties such as viscosity and gelation and reduces foam and emulsion stability (Kuehler and Stine, 1974). The functional properties of hydrolyzed proteins are mainly governed by their molecular weight and hydrophobicity (Jost and Monti, 1982; Chaplin and Andrew, 1989; Turgeon et al., 1992a,b). Although much information is available on the surface properties of hydrolyzed proteins, very few studies have been performed on the existence of protein−peptide interactions and on the effect of such interactions on the functional properties of proteins (Haque et al., 1993).

For purposes of better control of protein microparticle size during manufacturing and more efficient utilization of microparticles in food formulation, it is important to recognize the impact of the different protein fractions present in microparticulated proteins on the thermal behavior of a food protein suspension. In this study, we evaluated the effects of the amount and nature of whey protein microparticles added to WPI solutions on their heat aggregation kinetics. We also performed the same experiment on WPI solutions containing selected $\beta$-lg peptides in order to test the ability of whey protein hydrolysates to modify food protein thermal aggregation.

## MATERIALS AND METHODS

**Preparation of Whey Protein Isolate-Based Microparticles.** Commercial whey protein isolate (WPI) was given by the Protose Co. (Protose Inc., Ottawa, Canada). The WPI was suspended at room temperature in distilled water (5% protein, w/w) under stirring for 90 min. The pH of the WPI suspension was adjusted to 6.00 with 1.0 M HCl, and the suspension was stored overnight at 10 °C. The next day, the pH of the WPI suspension was readjusted to 6.00 with 1.0 M HCl, and the suspension was heat-treated at 85 °C (±1 °C) for 20 s in a laboratory model Spiratherm heat exchanger (Cherry-Burrel, Cedar Rapids, IA). The heat-denatured WPI (dWPI) was concentrated five times, diafiltered with distilled water (1 diavolume) at 25 °C through a hollow-fiber polysulfone membrane (Romicon PM10; Romicon Inc., Woburn, MA), and then spray-dried using a Niro type atomizer (Niro Atomizer, Denmark) with an inlet temperature of 200 °C (±2 °C) and an outlet temperature of 88 °C (±2 °C).

For each protein−protein aggregation experiment, the dWPI was suspended in deionized water (1.0% protein, w/w) under stirring for 30 min, and then the pH (7.3−7.4) was adjusted to 6.00 with 0.1 and 0.05 M HCl. The dWPI suspension was microparticulated at 0.75 kbar (four passes) using a Microfluidizer M-110 Y instrument (Microfluidics Corp., Newton, MA). The reaction chambers of the microfluidizer were previously immersed in an ice bath, and the temperature of the microfluidized suspension was maintained at 20 °C (±3 °C) by a coiled heat exchanger immersed in cold water (20 ± 3 °C) and connected to the outlet of the microfluidization chambers. The microparticulated dWPI ($\mu$WPI) was immediately used without further treatment to make the composite mixtures containing different $\mu$WPI/WPI ratios. The size distribution of WPI microparticles was determined by photon correlation spectroscopy (PCS) on the $\mu$WPI solution after dilution at 0.01% protein (v/v) with deionized water using a Nicomp laser diffractometer (Pacifics, CA) as previously reported (Robin and Paquin, 1991). Typical size distribution obtained is shown in Figure 1. The average diameter of WPI microparticles was 0.33 ± 0.01 $\mu$m as calculated from two experiments (3 assays/experiment), with a size distribution of particles within 0.1−1.0 $\mu$m. The size of protein aggregates from dWPI could not be determined by PCS because of the sedimentation of very large aggregates within a few minutes after the end of the suspension stirring.

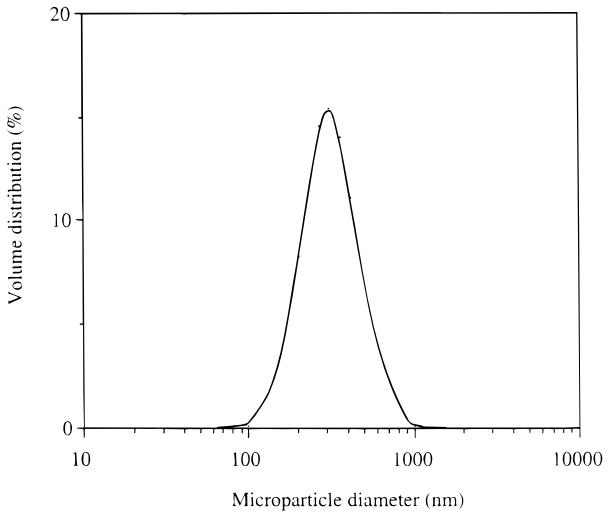


**Figure 1.** Typical size distribution of 1% (w/w) microparticulated whey protein isolate as determined at 23 °C by photon correlation spectroscopy (PCS).

**Preparation and Fractionation of WPI Tryptic Hydrolysate.** The preparation and fractionation of WPI tryptic hydrolysate (TH) were performed by the method of Barbeau (1995). Commercial WPI was obtained from Le Sueur Isolates (Le Sueur, MN). The WPI was rehydrated in deionized water (3.5% protein, w/v) in a stirred fermentor (New Brunswick Scientific Co., Edison, NJ) and the solution was adjusted to pH 8.0 with 2 N NaOH. The mixture was heated to 42 ±1 °C, and the trypsin-TPCK (bovine pancreas, type XIII; Sigma Chemical Co., St. Louis, MO) solution (0.5%, w/v, in 0.001 HCl) was added (enzyme:substrate ratio of 1:200). During hydrolysis, the reaction mixture was maintained at pH 8.0 by addition of 2 N NaOH according to the pH-Stat technique of Adler-Nissen (1977). After 45 min of hydrolysis, the proteolytic products were removed continuously by ultrafiltration using a regenerated cellulose acetate spiral wound membrane with a nominal molecular weight cutoff of 30 000 (S1-Y30, 0.1 $m^2$; Amicon, Danvers, MA). The reaction mixture was concentrated three times at 20 °C under a 149 kPa transmembrane pressure. The permeate, designated as TH, was lyophilized and stored at 0 °C until further analysis and chromatographic fractionation.

The preparative fractionation of TH (600 mg) by hydrophobic interactions chromatography (HIC) was performed using a Michel-Miller column (300 × 24.5 mm i.d.) filled with Vydac $C_{18}$ resin (15−20 $\mu$m; Waters, Canada). The mobile phase was a 5−45% gradient of acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid (TFA). The flow rate was 6 mL/min, and the tryptic hydrolysate was fractionated in four fractions ($F_A-F_D$) using 800 mL of mobile phase. The fraction $F_D$ was collected after 480 mL and lyophilized. Lyophilization was used to dry the peptide fraction $F_D$ instead of atomization because of the small amount of sample available.

**Compositional Analyses.** Moisture, protein, fat, and ash were determined on WPI and dWPI according to AOAC (1984) methods 16.192, 24.038−24.039, 16.199b−16.200, and 16.196, respectively. Lactose content of WPI and dWPI was determined using an enzyme assay (Boehringer-Mannheim, Indianapolis, IN). The calcium and sodium contents of WPI and dWPI were determined by atomic absorption.

**Table 1. Chemical Composition (%, w/w) of Whey Protein Isolate (WPI), Heat-Denatured WPI (dWPI), and Microparticulated WPI (μWPI)[a]**

| protein source | protein | moisture | fat | lactose | ash | $Ca^{2+}$ | $Na^{+}$ | protein fractions | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | NSP | SAP | IAP |
| WPI | 89.1 | 4.9 | 0.4 | 0.3 | 6.7 | 0.12 | 0.63 | 89.7 | 9.3 | 1.0 |
| dWPI | 93.1 | 3.9 | nd | nd | 6.2 | 0.12 | 0.36 | 9.4 | 22.7 | 67.9 |
| μWPI | nd | nd | nd | nd | nd | nd | nd | 10.4 | 30.0 | 59.6 |

[a] Protein solubility was determined after centrifuging 50 mL of 1% (w/w) protein solution at 20000*g* for 15 min (25 °C). nd, not determined; dWPI, heat-denatured whey protein isolate; μWPI, microparticulated whey protein isolate; NSP, native soluble protein; SAP, soluble aggregated protein; IAP, insoluble aggregated protein.

The composition of the fraction $F_D$ obtained by HIC fractionation of WPI trypsin hydrolysate was determined by RP-HPLC following the method developed by Turgeon et al. (1991). The $F_D$ fraction mainly contained two peptides from β-lactoglobulin, namely, the 21−40 (26.6%) and the 41−60 (51.7%) peptides. These peptides demonstrated good oil−water interface activity and an interesting distribution of hydrophobic and hydrophilic amino acid residues in distinct zones (Turgeon et al., 1992b).

**Solubility of the Different Protein Fractions in WPI, dWPI, and μWPI.** The protein solubility of WPI, dWPI, and μWPI was determined at pH 6.0 and 4.6 by centrifuging 50 mL of 1% protein solution (w/w) at 20000*g* for 15 min (25 °C) and measuring the protein content of the supernatant by the macro-Kjeldhal method (N × 6.38). The comparison of protein solubility at pH 4.6 and 6.0 provides a mean to assess the extent of protein heat-denaturation/aggregation during processing of WPI (de Wit and Kessel, 1996). Under our experimental conditions, we defined three main protein fractions in our WPI at pH 6.0: (1) a fraction containing whey proteins in which the structure has not been modified by heat and is defined as native soluble proteins (NSP)

$$\text{NSP (\%)} = \text{protein solubility at pH 4.6}$$

(2) a fraction containing whey proteins in which the structure has been modified by heat so as to form small aggregates without impairing the solubility and is defined as soluble aggregated proteins (SAP)

$$\text{SAP (\%)} = \text{protein insolubility at pH 4.6} - \text{protein insolubility at pH 6.0}$$

(3) a fraction containing whey proteins in which the structure has been markedly modified by heat so as to form large insoluble protein aggregates and is defined as insoluble aggregated proteins (IAP)

$$\text{IAP (\%)} = \text{protein insolubility at pH 6.0}$$

The three protein fractions (NSP, SAP, and IAP) were considered to be representative of the early, intermediate, and final stages, respectively, of whey protein thermal aggregation. It should be stressed that different centrifuging methodology should produce different contents in NSP, SAP, and IAP. The compositions of WPI, dWPI, and μWPI regarding the different protein fractions NSP, SAP, and IAP, as defined above, are given in Table 1.

**Composite Blends Based on WPI and μWPI or WPI and $F_D$.** As previously described, the μWPI suspension was mixed with a defined quantity of WPI powder and deionized water so as to obtain μWPI/WPI ratios in the range 0−0.23 (on a protein basis). At μWPI/WPI ratios higher than 0.23, suspensions were too turbid to provide reliable transmittance monitoring. The soluble protein content of the blends, i.e., NSP + SAP, was fixed at 0.81 mg/mL by considering both the soluble proten content of WPI and μWPI powders. Consequently, the total protein concentration of composite blends varied from 0.82 mg/mL (μWPI/WPI ratio of 0/100) to 0.92 mg/mL (μWPI/WPI ratio of 18.75/81.25). The protein fractions (NSP, SAP, and IAP) of the different blends are given in Table 2. The $F_D$ fraction isolated from WPI tryptic hydrolysate was mixed with the WPI solution at a fixed protein concentration of 0.82 mg of protein/mL so as to obtain $F_D$/WPI ratios within 0−0.4.

**Table 2. Different Protein Fractions NSP, SAP, and IAP (%, w/w) in the μWPI/WPI Composite Suspensions Used in Thermal Aggregation Experiments[a]**

| μWPI/WPI ratio | NSP | SAP | IAP | NSP/SAP + IAP | IAP/SAP |
|---|---|---|---|---|---|
| 0/100 | 89.7 | 9.3 | 1.0 | 0.12 | 0.11 |
| 3.75/96.25 | 86.7 | 10.1 | 3.2 | 0.15 | 0.32 |
| 7.5/92.5 | 83.8 | 10.9 | 5.4 | 0.20 | 0.50 |
| 11.75/88.25 | 80.4 | 11.7 | 7.9 | 0.24 | 0.67 |
| 15/85 | 77.8 | 12.4 | 9.9 | 0.29 | 0.79 |
| 18.75/81.25 | 74.8 | 13.2 | 12.0 | 0.34 | 0.91 |

[a] μWPI, microparticulated whey protein isolate; NSP, native soluble protein; SAP, soluble aggregated protein; IAP, insoluble aggregated protein.

The different composite blends were stirred for 15 min at 22 °C (±2 °C); then the pH of the blends (6.8−6.9) was adjusted to 6.00 using 0.01, 0.05, or 0.1 M HCl. After 30 min of rest, the pH of the blends was readjusted if necessary.

**Protein−Protein Interaction.** The method described by Deng et al. (1976) and used for WPI by Xiong (1992) was used to determine the thermally induced protein−protein association/interaction. Composite blends based on WPI and μWPI, or WPI and the $F_D$ fraction of hydrolyzed WPI, were placed in 3 mL quartz cuvettes (1 cm path length) with stoppers and, after a rest period of 15 min at the initial temperature of the heating bath (25 ± 1 °C), heated at 0.8 °C/min from 25 to 97 °C in a Hewlett-Packard 8451A UV−vis diode array spectrophotometer (Hewlett-Packard, Kirkland, PQ, Canada) equipped with a thermojacketed cell holder. The temperature of samples was regulated by circulating mineral oil from a refrigerating bath through the cell jacket and monitored with a thermocouple. The stoppers of quartz cuvettes were not entirely knocked in order to avoid pressure buildup and breakdown of the cuvettes. The decrease in transmittance, attributable to light scattering and resulting from protein aggregation, was recorded every 1 min increment by measuring changes in transmittance at 320 nm. The differential changes in transmittance as a function of temperature (first derivative $dT_{320}/dT$) were calculated to determine rates and transition temperatures of protein−protein interaction (Xiong, 1992). The results reported are averages for duplicate experiments (2−3 assays/experiment). Typical variations between duplicate experiments were around 5%. It is worth noting that we had chosen to work at pH 6.0 instead of, for instance, pH 7.0 because turbidity of heated whey protein suspensions at pH 7.0 was weak.

**Statistical Analyses.** Maximum first derivative ($dT_{320}/dT$) and transition temperature ($T_m$) calculated from transmittance kinetics were analyzed by ANOVA using StatView 512+ package (BrainPower Inc., Calabasas, CA), and the significance of means was assayed by the Fisher PLSD test at a level of confidence $\alpha = 0.05$.

## RESULTS AND DISCUSSION

**Chemical Composition of WPI, dWPI, and μWPI.** Commercial WPI usually shows high solubility of the proteins over a wide range of pH. From protein

analysis, it was apparent that the WPI we used had very good protein solubility at pH 4.6 (≈90% solubility) and pH 6.0 (≈100% solubility) (Table 1). Under our experimental conditions, and particularly using a centrifugation at 20000*g* for 15 min, we found at pH 6.0 that starting WPI contained 89.7% native soluble proteins (NSP), 9.3% soluble aggregated proteins (SAP), and 1.0% insoluble aggregated proteins (IAP) (Table 1).

Heating whey proteins is known to cause conformational changes of the protein structure and protein−protein interactions mainly mediated by hydrophobic interactions, electrostatic linkages, and disulfide cross-linking (Aguilera, 1995). After heating at 85 °C for 20 s in a heat exchanger, cooling to 25 °C, diafiltration, and spray-drying at 88 °C, the relative proportion of the different protein species in WPI, i.e., NSP, SAP, and IAP, changed (Table 1). The content in NSP drastically dropped from 89.7% to 9.4%. On the other hand, the contents in SAP and IAP increased from 9.3% to 22.7% and from 1.0% to 67.9%, respectively. To summarize, about 90% of the proteins had been aggregated by heat, a large majority (67.9%) of aggregates being insoluble.

Although diafiltration of the heat-treated whey protein suspension removed about 40% of the Na ions, no Ca ions could be eliminated (Table 1). It has been recognized that Ca ions (5−10 mM) improved whey protein aggregation and gelation (Kuhn and Foegeding, 1991; Xiong, 1992;, Foegeding et al., 1992; Matsuura and Manning, 1994) through three possible mechanisms: namely, by stabilizing heat-denatured proteins that are more prone to aggregation, by producing more neutral species which have lower aqueous solubility, or by directly participating in the protein aggregation process through intermolecular Ca cross-bridges (Jeyarajah and Allen, 1994; Li et al., 1994). These mechanisms contributed to the formation of large and insoluble aggregates as previously observed by Morr and Josephson (1968). Moreover, it has been demonstrated that an increasing concentration of Ca ions caused an increasing content of insoluble whey protein aggregates (de Rham and Chanton, 1984; Parris et al., 1993; Hollar et al., 1995). This could explain the invariable Ca ion concentration (≈2 mM) and the high content of IAP that we observed in the dWPI suspension.

The dWPI contained very large aggregates, visible to the naked eye, that rapidly sedimented at room temperature ($T$ = 22 °C). After microfluidization at high pressure ($P$ = 75 MPa, four cycles) of the dWPI, i.e., the microparticulation stage, the average size of whey protein aggregates decreased to 0.33 $\mu$m (see Figure 1). Under the hydrodynamic conditions prevailing into the microfluidization chambers, i.e., turbulence, cavitation and shear, the size reduction of aggregates may be mainly explained by aggregate−aggregate collisions and fragmentation/erosion of the aggregates (Taylor and Fryer, 1994). The mechanical dispersion of aggregates provided at pH 6.0 a 7−8% rise of the whey protein solubility as compared with dWPI (Table 1, SAP = 30% vs 22.7%, IAP = 59.6% vs 67.9%). Thus, microfluidization appears like an effective technology to modify the size of whey protein aggregates and the relative content in the different protein fractions of heat-treated WPI.

**Thermal Aggregation of $\mu$WPI/WPI Composite Blends.** Heat-induced protein−protein interactions may be assessed by recording changes in turbidity of a diluted protein suspension. However it must be remembered that the turbidity of a protein suspension depends on the number, size, and optical properties of the particles in solution (Hermansson, 1978). Thus, it is not possible to differentiate the relative contributions of these parameters from only turbidity changes.

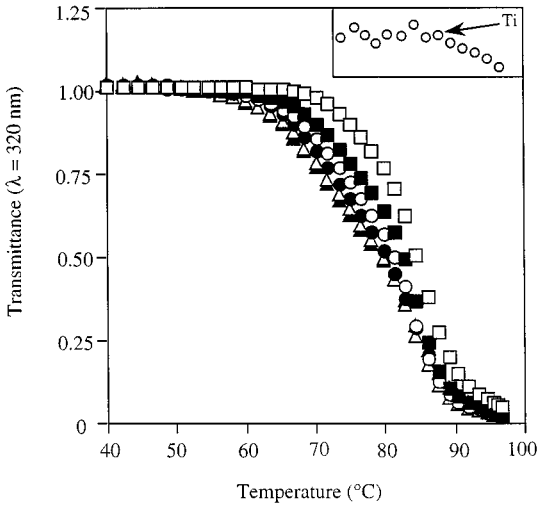


**Figure 2.** Transmittance profiles at $\lambda$ = 320 nm (pH 6.0) of (□) 0/100, (■) 3.75/96.25, (○) 7.5/92.5, (●) 11.25/88.75, (△) 15/85, and (▲) 18.75/81.25 $\mu$WPI/WPI composite suspensions heated from 25 to 97 °C (heating rate = 0.8 °C/min). The soluble protein content was 0.081% (w/w) in all experiments. Inset: Determination of $T_i$ (initiation temperature).

The changes in transmittance ($\lambda$ = 320 nm) of the $\mu$WPI/WPI composite blends as a function of heating temperature (heating rate = 0.8 °C/min) are shown in Figure 2. The changes occurring with the control (no $\mu$WPI added) can be approximately divided into three temperature zones. At temperatures less than 65−70 °C, only minor changes in transmittance were detected indicating little protein−protein interactions. Conversely, the second temperature zone of the control aggregation (70−90 °C) was characterized by a drastic decrease of the transmittance, probably resulting from extensive protein−protein interactions. The last temperature zone (>90 °C) exhibited few transmittance changes of the protein suspension. It will be noted that aggregated whey proteins (SAP + IAP) amounted to 12% in the suspension (IAP/SAP ratio of 0.11).

The presence of $\mu$WPI in WPI suspension did not modify the shape of the transmittance profiles and the number of temperature zones, but a shift of profiles toward lower temperatures was observed with increasing amounts of $\mu$WPI (Figure 2). We determined the temperature from which the relative transmittance did not present random fluctuations and decreased continuously (Figure 2). The temperature was named $T_i$(°C) and is defined as the initiation temperature needed to detect structural changes in the protein suspension according to our spectrophototurbidimetric method. The $T_i$ of the control was 58 °C (Table 3), suggesting that weak protein−protein interactions were initiated at temperatures lower than the denaturation temperature at pH 6.0 of $\beta$-lg (76−78 °C), α-la (61 °C), and BSA (72 °C), the three most abundant whey proteins in whey, as determined by DSC (Bernal and Jelen, 1985). Barbut and Foegeding (1993) found detectable turbidity at 45 °C or lower with heat-treated WPI (4%, w/v, protein) containing 10 mM Ca and assumed that the increase in turbidity at low temperature was caused by the initial formation of protein aggregates. Conformational unfolding of whey proteins, and especially of $\beta$-lg, has been detected from 40 °C heating temperature by RP-HPLC (Parris and Baginski, 1991) and NMR (Li et al., 1994).

**Table 3. Parameters Determined from Transmittance Profiles at $\lambda = 320$ nm of Microparticulated WPI/WPI Composite Blends (0.081%, w/w, Soluble Protein) Heated from 25 to 97 °C (Heating Rate = 0.8 °C/min)[a]**

| $\mu$WPI/WPI ratio | $T_i$ (°C) | $dT_{T_{m1}}/dT$ (°C$^{-1}$) | $T_{m1}$ (°C) | $dT_{T_{m2}}/dT$ (°C$^{-1}$) | $T_{m2}$ (°C) |
|---|---|---|---|---|---|
| 0/100 | 58.3[a] | −0.0355[a] | 77.4[a] | −0.0826[a] | 83.7[a] |
| 3.75/96.25 | 50.7[b] | −0.0329[a] | 77.0[a] | −0.0816[a] | 84.1[a] |
| 7.5/92.5 | 45.9[c] | −0.0316[a] | 73.4[b] | −0.0744[b] | 83.7[a] |
| 11.25/88.75 | 46.6[c] | −0.0335[a] | 72.3[bc] | −0.0565[c] | 83.7[a] |
| 15/85 | 45.2[cd] | −0.0323[a] | 71.4[cd] | −0.0582[cd] | 83.7[a] |
| 18.75/81.25 | 42.3[d] | −0.0335[a] | 69.4[e] | −0.0503[e] | 84.1[a] |

[a] $T_i$, initiation temperature (see text); $T_{m1}$, $T_{m2}$, thermal transitions; $dT_{T_{m1}}$, $dT_{T_{m2}}$, differential changes in transmittance at $T_{m1}$ and $T_{m2}$; $\mu$WPI, microparticulated whey protein isolate. Different superscripts in the same column indicate significant differences at $\alpha = 0.05$ according to the PLSD Fisher test.

Unfolded forms of whey proteins, both induced by heating and existing initially in the protein suspension with small protein aggregates, may interact and contribute to the slight decrease of transmittance that we observed after 58 °C. This suggestion also supported the significant decrease of $T_i$ from 58 to 51 °C after 3.75% $\mu$WPI (SAP + IAP/NSP = 0.15, IAP/SAP = 0.32) was added to the WPI suspension (Table 3). Then, a small quantity of aggregates, here 3% more than for the control, is sufficient to modify $T_i$. An increased number of unfolded proteins and protein aggregates of varying sizes in the initial suspension, which is an increased number of reactive sites available for interaction, may conceivably result in a sooner initial aggregation of the different protein species. With 7.5% $\mu$WPI added to the WPI suspension (SAP + IAP/NSP = 0.2, IAP/SAP = 0.5), the $T_i$ further decreased from 51 to 46 °C, resulting in a 12 °C lowering of $T_i$ as compared to the control WPI suspension. Adding more $\mu$WPI (>7.5%) did not result in a further decrease of $T_i$, suggesting that the amount of added microparticulated whey proteins is not the sole parameter important in the control of initial stages of $\mu$WPI/WPI blend thermal aggregation. Another determining parameter could be the nature of added aggregates, e.g., the AIP/SAP ratio, since aggregates of different structures and sizes may have different effects at different stages of whey protein thermal aggregation.

The differential changes in transmittance as a function of the heating temperature, indicating the rate and extent of protein−protein association/aggregation, showed a very slow aggregation rate for the control WPI suspension until a temperature of about 70 °C was reached (Figure 3). This temperature is close to the denaturation temperatures of α-la, β-lg, and BSA and appeared critical in determining irreversible aggregation and gelation of whey proteins (Matsudomi et al., 1992; Xiong, 1992; Parris et al., 1993; Griffin et al., 1993; Li et al., 1994). Two thermal transitions, $T_{m1}$ and $T_{m2}$, revealing the maximum protein−protein rate of aggregation, were found for the control suspension (Figure 3). Whereas the first transition $T_{m1}$ appeared at 77 °C and corresponded to a small shoulder ($dT_{T_{m1}}/dT$ = −0.036 °C$^{-1}$), the second thermal transition defined a large peak ($dT_{T_{m2}}/dT$ = −0.083 °C$^{-1}$) and appeared at 84 °C (Table 2). By heating a WPI dispersion containing ≈0.8 mM Ca and 12 mM Na and using 1.0 °C/min heating rate, Xiong (1992) found $T_{m1}$ (78 °C) and $T_{m2}$ (84 °C) thermal transitions (at 320 nm wavelength) very close to our results in similar conditions (2 mM Ca, 16 mM Na, heating rate = 0.8 °C/min, $\lambda = 320$ nm). The presence of these two thermal transitions indicated that a fraction of whey proteins present in the WPI suspension aggregated sooner than another one. Clearly, at low temperature of heating and low ionic strength, aggregation at pH 6.0 of whey proteins appears to be slightly heterogeneous. On the basis of the relative thermal stability, as mentioned above, and the abundance of β-lg (≈60−70%), α-la (≈15−25%), and BSA (≈5−10%) in WPI, Xiong (1992) assumed that the two thermal transitions $T_{m1}$ and $T_{m2}$ may be widely attributed to the intermolecular association of α-lactalbumin ($T_{m1}$) and β-lactoglobulin ($T_{m2}$). In our opinion, the situation is probably more complicated because aggregation of whey proteins involves interactions between β-lg, α-la, and BSA, the extent of interactions depending on parameters such as the relative ratio of proteins, the rate of protein aggregation, the temperature and rate of heating, the pH, and the ionic strength (Elfagm and Wheelock, 1978; Parris et al., 1991, 1993; Matsudomi et al., 1992, 1993; Calvo et al., 1993; Hines and Foegeding, 1993; Gezimati et al., 1996). Thus, conclusions of Xiong (1992) could be refined assuming that $T_{m1}$ may be attributed to mixed aggregation of α-la, BSA, and part of β-lg, since $T_{m2}$ may be mainly attributed to β-lg aggregation. It would be instructive to perform similar aggregation experiments with ternary mixtures of β-lg, α-la, and BSA in order to relate the different thermal transitions with the presence of a given protein and the existence of such protein−protein interactions.

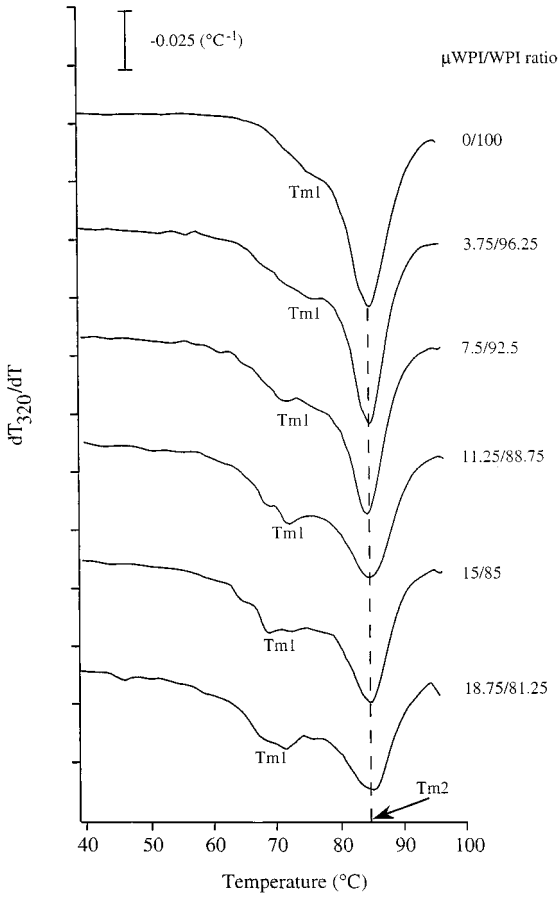


**Figure 3.** Differential changes in transmittance ($dT_{320}/dT$) at $\lambda = 320$ nm (pH 6.0) as a function of the heating temperature of $\mu$WPI/WPI composite suspensions heated from 25 to 97 °C (heating rate = 0.8 °C/min). The soluble protein content was 0.081% (w/w) in all experiments.

The shoulder height at $T_{m1}$ ($dT_{T_{m1}}/dT$) and the $T_{m2}$ value did not change with the addition of $\mu$WPI in the WPI suspension (Table 3). As well, the peak height at

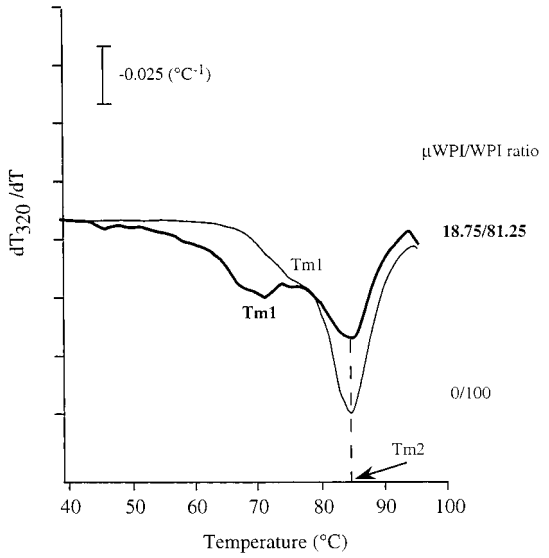


**Figure 4.** Comparison of the differential changes in transmittance ($dT_{320}/dT$) at $\lambda = 320$ nm (pH 6.0) as a function of the heating temperature of 0/100 and 18.75/81.25 $\mu$WPI/WPI composite suspensions heated from 25 to 97 °C (heating rate = 0.8 °C/min). The soluble protein content was 0.081% (w/w) in the two experiments.

$T_{m2}$ ($dT_{T_{m2}}/dT$) and the $T_{m1}$ value were not significantly affected by 3.75% $\mu$WPI added to the WPI suspension. However, as soon as the starting protein suspension contained 7.5% $\mu$WPI, $T_{m1}$ and $dT_{T_{m2}}/dT$ decreased significantly from 77 to 73 °C and from 0.082 to 0.074 °C$^{-1}$ (in absolute value), respectively, revealing that a relative amount of 20% whey protein aggregates (IAP + SAP/NSP = 0.20, IAP/SAP = 0.5) was needed to modify the aggregation kinetics of WPI (Table 3). At 18.75% $\mu$WPI concentration (AIP + ASP/NSP = 0.34, AIP/ASP = 0.91), $T_{m1}$ was 69 °C and $dT_{T_{m2}}/dT$ was −0.05 °C$^{-1}$, i.e., we obtained a 8 °C decrease for $T_{m1}$ and about a 40% peak height decrease at $T_{m2}$. The decrease of the area under $dT_{T_{m2}}/dT$ was compensated by an increase of the area under $dT_{T_{m1}}/dT$ as depicted in Figure 4. One possible explanation is that part of the whey proteins aggregated around $T_{m2}$ = 84 °C in the control WPI suspension have been aggregated at temperatures below 70 °C through interactions with the microparticulated whey proteins, thus resulting in less rapid aggregation around $T_{m2}$ and the decrease of $dT_{T_{m2}}/dT$. Also, whey proteins aggregated around $T_{m1}$ = 77 °C in the control WPI suspension may interact with the added microparticulated proteins and form aggregates that have not contributed to the same extent to whey protein aggregation around $T_{m2}$. It should be noted that no major modifications of the profiles presented in Figure 3 were observed for $\mu$WPI/WPI blends containing more than 11.25% $\mu$WPI (NSP/SAP + IAP = 0.24, IAP/SAP = 0.67), suggesting again that the nature of added aggregates (the AIP/SAP ratio), both with the amount of aggregates, is an important parameter affecting thermal aggregation of whey proteins.

A direct consequence of the increasing content of $\mu$WPI in the WPI suspension was the better resolution of the two thermal transitions as compared with the control (Figure 4). Since the presence of a small shoulder at $T_{m1}$ in the heated WPI suspension indicated local heterogeneity in a mechanism of protein aggregation homogeneous as a whole, the obtention of two well-distinct maxima of aggregation in heated $\mu$WPI/WPI suspension indicated biphasic aggregation of whey proteins. Since it is known that $Na^+$ ions favor whey protein interactions, it is possible that the slight decrease of $Na^+$ concentration in blends, as compared to WPI suspensions, could play a role in the appearance of biphasic aggregation. Such heterogeneous thermal aggregation should produce logically a heterogeneous population of whey protein aggregates, whereas in microparticle-free WPI suspension heating would produce a more uniform population of aggregates. This has been actually observed since the average diameter of whey protein aggregates obtained from the heated WPI diluted dispersion was 0.16 $\mu$m (±0.1) with a narrow size distribution in the range 0.03−0.62 $\mu$m as determined by PCS (results not shown). On the other hand, sedimentation of large aggregates and too heterogeneous size distribution of aggregates did not allow us to determine by PCS reliable sizes of aggregates obtained from the heated $\mu$WPI/WPI suspension. This explains why manufacturing of protein-based microparticles with narrow size distribution needs a starting protein solution not containing more than 5−10% denatured proteins and why protein microparticles are not used as fat replacers in thermally processed food products.

**Thermal Aggregation of $F_D$/WPI Composite Blends.** The following experiments were carried out on blends of WPI and $F_D$ fraction obtained from HIC of WPI tryptic hydrolysates. The $F_D$ fraction mainly contained the 21−40 (26.6%) and 41−60 (51.7%) $\beta$-lg peptides as determined by RP-HPLC, and the remaining fraction (21.7%) consisted in smaller peptides and amino acids. These two peptides have isoelectric points (IEP) of 4.5 (peptide 41−60) and 5.4 (peptide 21−40) and molecular weights around 2000 Da (Turgeon et al., 1992b). The $F_D$ fraction was initially selected because of its ability to improve the thermal stability of $\beta$-lg (Barbeau, 1995). Such behavior was explained in terms of structure stabilization of $\beta$-lg in the early stage of the heat-denaturation through protein−peptide interactions.

The evolution of transmittance as a function of temperature of composite WPI/$F_D$ suspension was similar to those already shown, revealing three distinct temperature zones (Figure 5). The presence in WPI suspensions of 20% or 40% of the fraction $F_D$ ($F_D$/WPI ratio of 0.2 and 0.4) modified the transmittance profiles in the first temperature region (40−65 °C) where the relative transmittance was larger than 1.0. An unexpected result of mixing together diluted WPI suspension and $F_D$ fraction was the pH decrease of the composite $F_D$/WPI suspension. The starting pH was around 6.9 and decreased to pH 5.4 or 4.6 when 20% or 40% of the fraction $F_D$ was added, respectively. The drop in pH was certainly caused by the release of $H^+$ from ionized peptides since, at pH 7.0, 21−40 and 41−60 $\beta$-lg peptides carried −1 and −3 charges, respectively (Turgeon et al., 1992b). In the 4.6−5.4 pH range, whey proteins (IEP ≈ 5) and $\beta$-lg peptides were minimally charged; thus protein−protein, protein−peptide, and maybe peptide−peptide interactions were favored to the detriment of protein−solvent and peptide−solvent interactions. Thereby, a direct consequence of the addition of $F_D$ into the WPI dispersion was the likely formation of whey protein−peptide composite aggregates resulting, as we observed, in a decreased transmittance of the suspension. Aggregation of whey proteins induced by isoelectric precipitation, and especially $\beta$-lg, has been already reported (Stading and Hermansson, 1990). The turbidity did not totally disappear after

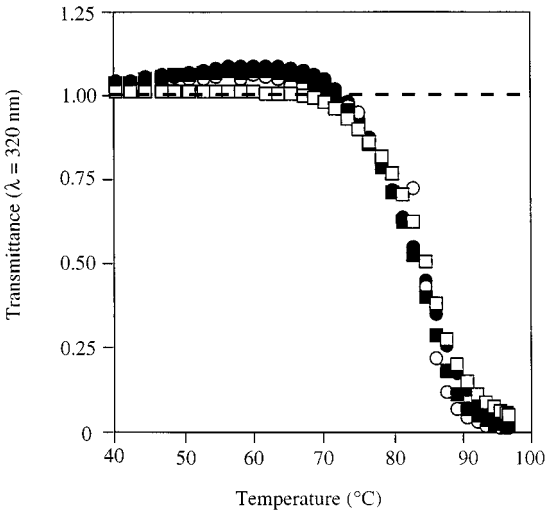


**Figure 5.** Transmittance profiles at $\lambda = 320$ nm (pH 6.0) of (□) WPI control suspension, (■) WPI/20% $F_D$ fraction composite suspension, (○) WPI/40% $F_D$ fraction composite suspension, and (●) WPI suspension in which the pH was decreased from 6.9 to 4.6 with 0.5 M HCl and increased from 4.6 to 6.0 with 0.5 M NaOH, heated from 25 to 97 °C (heating rate = 0.8 °C/min). $F_D$: peptide fraction isolated from HIC of tryptic hydrolysate of WPI and containing mainly 21−40 (26.6%) and 41−60 (51.7%) $\beta$-lg peptides. The WPI concentration was 0.082% (w/w) protein in all experiments.

**Table 4. Parameters Determined from Transmittance Profiles at $\lambda = 320$ nm (pH 6.0) of WPI + 0−40% $F_D$ Fraction Composite Suspensions ($F_D$/WPI Ratio = 0−0.4) Heated from 25 to 97 °C (0.8 °C/min)[a]**

| $F_D$/WPI ratio | $T_i$ (°C) | $dT_{T_{m1}}/dT$ (°C$^{-1}$) | $T_{m1}$ (°C) | $dT_{T_{m2}}/dT$ (°C$^{-1}$) | $T_{m2}$ (°C) |
|---|---|---|---|---|---|
| 0 | 58.3[a] | −0.0355 | 77.4 | −0.0826[a] | 83.7[a] |
| 0.2 | 63.6[b] | | | −0.0787[a] | 82.4[a] |
| 0.4 | 63.6[b] | | | −0.1960[b] | 84.5[a] |
| 0* | 61.9[b] | | | −0.1110[c] | 84.5[a] |

[a] $F_D$, peptide fraction isolated from HIC of tryptic hydrolysate of WPI containing mainly 21−40 (26.6%) and 41−60 (51.7%) $\beta$-lg peptides; $T_i$, initiation temperature (see text); $T_{m1}$, $T_{m2}$, thermal transitions; $dT_{T_{m1}}$, $dT_{T_{m2}}$, differential changes in transmittance at $T_{m1}$ and $T_{m2}$. Different superscripts in the same column indicate significant differences at $\alpha = 0.05$ according to the Fisher PLSD test. *Control WPI suspension in which the pH was decreased to 4.6 using 0.5 M HCl and increased to 6.00 with 0.5 M NaOH.

pH ajustment at 6.0 before performing transmittance measurements but decreased during heating from 25 to ≈65 °C as evidenced by the transmittance value above 1.0 (Figure 5). The lowering of turbidity could be due to one or many of the following factors: sedimentation of the largest aggregates, partial time-dependent recovery at pH 6.0 of the initial structure of whey proteins microcoagulated at pH 4.6 or 5.4, or heat-induced dispersion of aggregates. Additional work is needed to clarify this point. As a consequence, the initiation temperature $T_i$, i.e., the temperature corresponding to the continuous decrease of the relative transmittance as defined in the preceding section, was 5 °C larger for composite suspensions than for the control (Table 4).

The differential changes in transmittance as a function of the heating temperature are depicted in Figure 5. As compared with the control WPI suspension (no $\beta$-lg peptides added), only the second thermal transition $T_{m2}$ was found for WPI/20% $F_D$ suspension. Neither this thermal transition at $T_{m2} = 84$°C nor the peak height ($dT_{T_{m2}}/dT = -0.079$ °C$^{-1}$) were significantly different from those found for the control WPI suspension (Table 4). However, the area under the $T_{m2}$ peak was larger for the WPI/$F_D$ suspension, especially at temperatures around the control $T_{m1}$ ($T \approx 77$ °C), indicating more extensive aggregation of proteins than for the control. The presence of whey protein and composite whey protein/$\beta$-lg peptide aggregates in the WPI suspension was thought to be primarily responsible for the increased protein−protein interactions as revealed by transmittance measurements.

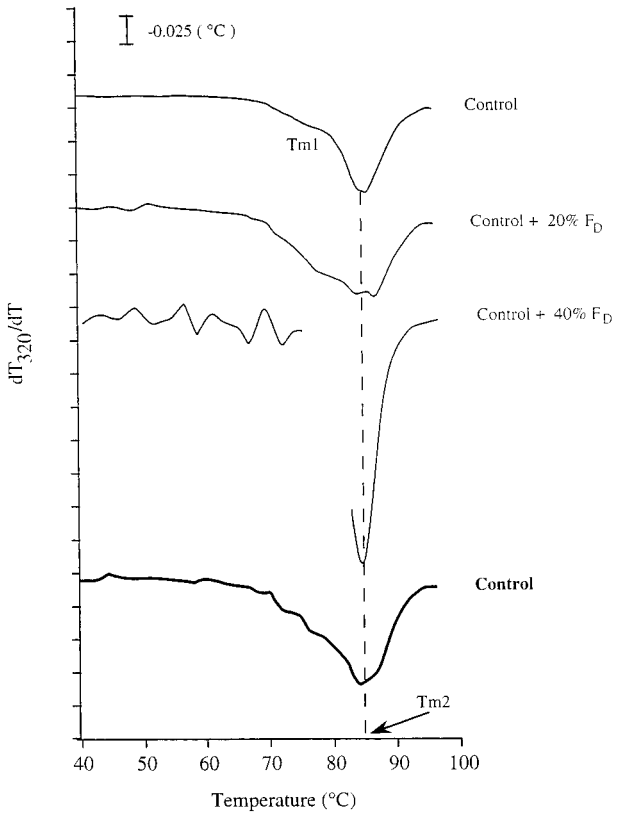


**Figure 6.** Differential changes in transmittance ($dT_{320}/dT$) at $\lambda = 320$ nm (pH 6.0) as a function of the heating temperature of WPI + 0−40% $F_D$ fraction composite suspension heated from 25 to 97 °C (heating rate = 0.8 °C/min). The control was the heated WPI suspension (0% $F_D$ fraction added). The control thick curve represents a WPI suspension in which the pH was decreased from 6.9 to 4.6 with 0.5 M HCl and increased from 4.6 to 6.0 with 0.5 M NaOH. $F_D$: peptide fraction isolated from HIC of tryptic hydrolysate of WPI and containing mainly 21−40 (26.6%) and 41−60 (51.7%) $\beta$-lg peptides. The WPI concentration was 0.082% (w/w) protein in all experiments.

The aggregation profile of WPI/40% $F_D$ suspension was greatly different from those obtained for the control or the WPI/20% $F_D$ suspension (Figure 6). The starting suspension was turbid, as described above, and displayed detectable highly heterogeneous aggregation from 40 °C heating temperature as evidenced by the sinuous shape of $dT_{320}/dT$ curves. From $T \approx 75$ °C, i.e., the temperature both corresponding to the first thermal transition of the control WPI suspension and bringing about a faster aggregation of WPI/20% $F_D$ suspension, error signals were displayed by the spectrophotometer accounting for the discontinuous aggregation profile represented in Figure 6. According to the apparatus specifications, error signals indicated that the reaction rate into quartz cuvettes was faster than the time scale of absorbance measurements ($t = 1$ s). Above $T = 80$ °C, the turbidity of WPI/$F_D$ suspension was recorded again by the spectrophotometer and the $T_{m2}$ thermal transition appeared at $T = 85$ °C. However, the peak

height at $T_{m2}$ was significantly larger than for the control WPI suspension, revealing faster aggregation of whey proteins (Table 4). Moreover, we observed at the end of the heating cycle that phase separation occurred in the quartz cuvette, with a protein-depleted solvent phase in one end and very large protein aggregates ($d \approx 2-8$ mm) in the other. Thus, $\beta$-lg peptide-mediated isoelectric precipitation at pH 5.4, and especially at pH 4.6, of whey protein/$\beta$-lg peptide blends promoted an increase in the extent and rate of whey protein thermal aggregation at pH 6.0.

In order to estimate the role of $\beta$-lg peptides in this improved aggregation of whey proteins, a control WPI suspension was made in which the pH was lowered from pH 6.9 to 4.6 with 0.5 M HCl and then raised to pH 6.0 with 0.5 M NaOH. Due to isoelectric precipitation of whey proteins, the starting suspension was turbid but exhibited a thermal aggregation profile clearly different from that of the WPI/40% $F_D$ suspension (Figure 6). Weak aggregation of whey proteins was observed from 40 °C heating temperature, but no discontinuity in the aggregation profile was recorded and no phase separation occurred in the quartz cuvettes, even if large aggregates were visible. The thermal transition $T_{m2}$ was 85 °C, the same value found for all blends used in this study. The peak height at $T_{m2}$ ($dT_{T_{m2}}/dT = -0.11$ °C$^{-1}$) was significantly larger than that found for the WPI/20% $F_D$ and control suspensions but smaller than the $dT_{T_{m2}}/dT$ of the WPI/40% $F_D$ suspension (Table 4). The last difference was possibly based on different amounts or different structures of composite aggregates in WPI/40% $F_D$ suspension through increased whey protein−41−60 $\beta$-lg peptide interactions at pH 4.6 (41−60 peptide IEP = 4.6). Another contributing parameter could be the different acidification kinetics from $\beta$-lg peptides and 0.5 M hydrochloric acid that probably did not induce at pH 4.6 the same amount of whey protein aggregates and the same AIP/ASP ratio in WPI suspensions.

## CONCLUSION

In conclusion, our results showed that an increasing content of microparticulated whey proteins in WPI suspension resulted in a faster thermal aggregation at lower heating temperatures of protein species, with a shift of aggregation mechanism from essentially homogeneous to completely biphasic. We observed that not only the amount of aggregates but also the soluble/insoluble protein aggregate ratio was important in controlling initial stages of whey protein thermal aggregation.

Further studies are needed to determine more accurately the role of small soluble and large insoluble aggregates in whey protein thermal aggregation, especially whether heated whey proteins interact with the two kinds of aggregates and at what stage of the aggregation, and the part of unfolded whey protein−microparticulated whey protein interactions and microparticulated whey protein−microparticulated whey protein interactions in the kinetics of aggregation.

It emerged also from our study that utilization of specific WPI peptides such as the $F_D$ fraction to produce functional WPI aggregates and modify whey protein thermal aggregation is promising and probably an area of future intensive research. The extent and nature of protein−peptide interactions during isoelectric precipitation or thermal aggregation of whey proteins, as well as the consequences of such interactions on the structure and functional properties of the obtained aggregates, need to be studied. Another development of our study should be to compare the functional properties, particularly heat aggregation and gelation of heat-, HCl isoelectrically-, or $\beta$-lg peptide isoelectrically-induced whey protein microparticles.

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Received for review January 21, 1997. Revised manuscript received April 21, 1997. Accepted April 23, 1997.[⊗] This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC grants) and by the program Synergie (Ministère de l'Enseignement Supérieur et de la Science du Québec).

JF970061P

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.