JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Heat-Induced Changes in the Ultrasonic Properties of Whey Proteins

MILENA CORREDIG,* EDITA VERESPEJ, AND DOUGLAS G. DALGLEISH

Department of Food Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

The physical aggregation of commercial whey protein isolate (WPI) and purified β-lactoglobulin was studied by ultrasound spectroscopy. Protein samples were dialyzed to achieve constant ionic strength backgrounds of 0.01 and 0.1 NaCl, and gelation was induced in situ at constant temperatures (from 50 to 75 °C) or with a temperature ramp from 20 to 85 °C. Changes in the ultrasonic properties were shown in the early stages of heating, at temperatures below those reported for protein denaturation. During heating, the relative ultrasound velocity (defined as the difference between sample velocity and reference velocity) decreased continuously with temperature, indicating a rearrangement of the hydration layer of the protein and an increase in compressibility of the protein shell. At temperatures $<$50 °C the ultrasonic attenuation decreased, and $<$65 °C both velocity and attenuation differentials showed increasing values. A sharp decrease in the relative velocity and an increase in the attenuation at 70 °C were indications of "classical" protein denaturation and the formation of a gel network. Values of attenuation were significantly different between samples prepared with 0.01 and 0.1 M NaCl, although no difference was shown in the overall ultrasonic behavior. WPI and β-lactoglobulin showed similar ultrasonic properties during heating, but some differences were noted in the values of attenuation of WPI solutions, which may relate to a less homogeneous distribution of aggregates caused by the presence of α-lactalbumin and other minor proteins in WPI.

KEYWORDS: Ultrasound spectroscopy; whey protein gelation

INTRODUCTION

Whey proteins are often employed as ingredients to improve the texture of food products. For this reason, a better understanding of the reactions leading to the formation of whey protein gels would suggest ways to improve the final structure of food products. During heating, whey proteins modify their structure and interact with one another to form aggregates and gels. Gelation is affected by environmental conditions such as pH, concentration, and ionic strength. In general, under conditions favoring electrostatic repulsions, whey proteins form transparent gels with a fine-stranded structure, while under weak electrostatic repulsion particulate and opaque gels form (*1*). Although many of the details of heat-induced interactions are known, the molecular mechanisms causing the differences in macroscopic properties of whey protein gels are not fully understood and cannot be related to rheological or microstructural observations (*2*).

Different macromolecular whey protein complexes form depending on composition, concentration of protein, and heating temperature (*3*). The most abundant of the whey proteins, β-lactoglobulin, is thought to dominate the overall aggregation behavior. At temperatures below 60 °C, the tertiary structure of β-lactoglobulin changes reversibly (*4*, *5*), and as the temperature increases above 65 °C, these changes become irreversible (*4*, *6*). At temperatures above the maximum transition temperature, β-lactoglobulin aggregates form.

The aggregation behavior of pure β-lactoglobulin at neutral pH has been described as a two-step process: well-defined small aggregate protein clusters form first, and in a second stage these small clusters combine to form larger aggregates (*7*, *8*). These authors reported that the size of the primary aggregates is not affected by concentration, temperature, or ionic strength, at least at the concentration tested (below gelling conditions). In the late stages of the reaction, the intermediate aggregates come together and the final protein complexes have a broad distribution of sizes which depends on heating conditions (*7*). Light-scattering experiments have demonstrated that at neutral pH heating β-lactoglobulin at 65 °C results in aggregates of constant size (*9*), while heating at 75 °C caused protein aggregation via many intermediates, with final aggregates polydisperse in molecular weight (*10*).

The behavior of whey protein isolate (WPI) is less understood than that of β-lactoglobulin in isolation but is of greater interest because of its industrial applications. The aggregation of WPI seems to be governed by β-lactoglobulin; however, the type of aggregate formed is affected by the presence of α-lactalbumin (*11*, *12*).

The molecular aspects of whey protein aggregation with heating have been mostly studied using analytical techniques

* To whom correspondence should be addressed. Phone: 519-824-4120 ext 56101. Fax: 519-824-6631. E-mail: mcorredi@uoguelph.ca.

10.1021/jf0354390 CCC: $27.50 © 2004 American Chemical Society
Published on Web 06/19/2004

that require optically transparent solutions and low concentrations (*7*, *9*, *10*). On the other hand, techniques focusing on the physical properties of the gels formed (for example, rheology) require high concentrations and provide little information on the reactions preceding gelation. To date, the lack of analytical techniques for observing dynamics of molecular interactions has limited our ability to relate these interactions to the macroscopic properties of the gel network.

The objective of this work was to determine the molecular interactions occurring during heating in situ of whey proteins by using high-resolution ultrasound spectroscopy. Ultrasound spectroscopy has been previously employed to observe whey protein aggregates formed after alkali treatment (*13*) as well as to study molecular relaxation, hydration, and conformational changes of molecules (*14*). No studies have been carried out on in situ heat denaturation. Recently, high-resolution ultrasound spectroscopy has been applied to changes occurring during acid gelation of milk (*15*, *16*). In our study, particular attention was focused on the pregelation processes, as the propagation of ultrasound waves is not disruptive and not affected by the optical properties of the samples.

MATERIALS AND METHODS

Whey protein isolate (Alacen 895) was donated by New Zealand Milk Proteins (NZMP, Mississauga, Ontario). Purified β-lactoglobulin was prepared from the same isolate using ion-exchange chromatography on Sepharose Q (Amersham Biosciences, Baie d'Urfé, Quebec), with Tris-HCl buffer at pH 7.0 and a gradient of salt as previously reported (*17*). After purification, the protein was collected and freeze-dried after extensive dialysis.

Whey protein isolate (WPI) was dissolved in 20 mL of MilliQ water, and samples were dialyzed overnight against solutions containing 0.01 or 0.1 M NaCl (100 volumes). The dialyzed protein was centrifuged at 7000*g* for 20 min with a Beckman-Coulter ultracentrifuge (Beckman-Coulter, Mississauga, Ontario) and then filtered through 0.22 μm filters (Millipore nitrocellulose filters, Fisher Scientific, Mississauga, Ontario). After filtration, the final concentration of the samples was adjusted to 10% w/v as defined by UV absorption at 280 nm. Solutions of β-lactoglobulin were also prepared in MilliQ water and equilibrated with 0.01 and 0.1 M NaCl overnight, as for the WPI samples. The β-lactoglobulin solution was filtered through 0.22 μm filters and standardized to 10% w/v concentration. In all experiments a portion of the dialysis solution was also filtered through 0.22 μm filters to eliminate any insoluble material and used as the reference in the ultrasound spectroscopy experiments.

Ultrasound measurements were carried out using a HR−US102 instrument (Ultrasonic-Scientific, Dublin, Ireland) interfaced with the manufacturer's software (version 4-50-25-0, Ultrasonic Scientific). The instrument configuration is described in detail elsewhere (*18*). The equipment passes transverse sound waves through sample and reference cells and measures both the velocity and attenuation of the transmitted sound. WPI and β-lactoglobulin solutions were extensively degassed under vacuum and equilibrated to 20 °C for at least 30 min. Whey protein solutions (1 mL) were loaded in cell 1, while cell 2 contained the filtered, degassed NaCl solution. The instrument was tuned to measure at two frequencies corresponding to 5099 and 7835 kHz for water at 25 °C. The bandwidth and frequency of the sound waves at the two selected frequencies were measured continuously in both the sample and reference cells. Temperature control is a fundamental aspect of ultrasonic velocity measurements, and the internal temperature of the cell was controlled using a programmable Haake F8 water bath (Thermo-Haake, Georgetown, Ontario). Samples were subjected to isothermal runs in the temperature range of 50−75 °C or linear temperature ramps of 20−85 °C at a rate of 0.4 or 0.2 °C/min. The temperature ramps were controlled with the programmable water bath, and the temperature of the spectrometer cell was recorded by a thermocouple installed in the instrument and previously calibrated against the temperature measured by the water bath.

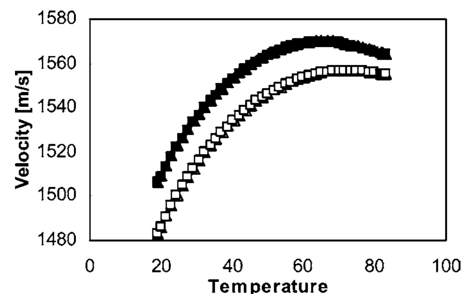

Figure 1. Ultrasonic velocity of 10% WPI in 0.01 M NaCl (solid symbols) and the reference solution (0.01 M NaCl, after dialysis) (open symbols) during heating from 20 to 85 °C (0.4 °C/min) at 5 (●) and 7 (■) MHz. Data are plotted as a function of temperature. Note that symbols overlap at the two frequencies.

At least two independent experiments were carried out in the isothermal runs, while various temperature ramps (at least three) were performed for all samples. All runs were analyzed and plotted using routines written in Microsoft Excel. Details of the changes in ultrasonic velocity and attenuation against temperature were identified by numerically calculating differentials of velocity and attenuation relative to the internal temperature of the cell. The gradient was calculated by separately summing 10 points before and 10 points after a given point, calculating the difference and dividing the result by the temperature gradient in the same data range. Data collected during the isothermal experiments were analyzed statistically using the general linear model procedure (SAS, Cary, NC) to determine if temperature and salt concentration affected the ultrasonic properties of WPI. Results were considered significant at $p < 0.05$.

RESULTS

During heating the ultrasound velocity increased and then decreased with increasing temperature in both the whey protein solutions and the reference salt solution, as indicated in **Figure 1**. This behavior of the velocity of the ultrasonic wave with temperature is typical of aqueous systems and caused by changes in the compressibility of the medium with temperature (*18*). Values of velocity measured at the two frequencies, 5 and 7 MHz, were the same as the velocity of sound is independent of frequency. The high-resolution spectroscopy experiments were always carried out in two identical cells, one containing the whey protein solution and the other containing a reference solution. To determine the effect of heat on the interactions between the proteins alone, the velocity measured in the reference cell was subtracted from the ultrasound velocity measured in the protein samples.

Figure 2 illustrates the changes of the ultrasound wave propagating in whey protein solutions containing 0.1 and 0.01 M NaCl as a function of heating temperature during the running of a temperature ramp. The difference in velocity between the protein sample and the soluble phase, defined as relative velocity, showed a continuous decrease with temperature from 20 to 85 °C (**Figure 2A**). On the other hand, values of attenuation did not show a marked increase until about 50−55 °C was reached (**Figure 2B**). To better distinguish different stages of the aggregation during heating, the rate of change of the ultrasound velocity and attenuation (**Figure 2C**,**2D**, respectively) was calculated as differential of relative velocity or attenuation as a function of temperature.

The relative ultrasonic velocity of whey protein solutions decreased steadily with temperature during heating from 20 to 85 °C, with an apparent change in the behavior at about 70 °C (**Figure 2A**). However, when the differential of the relative velocity was plotted as a function of temperature (**Figure 2C**),

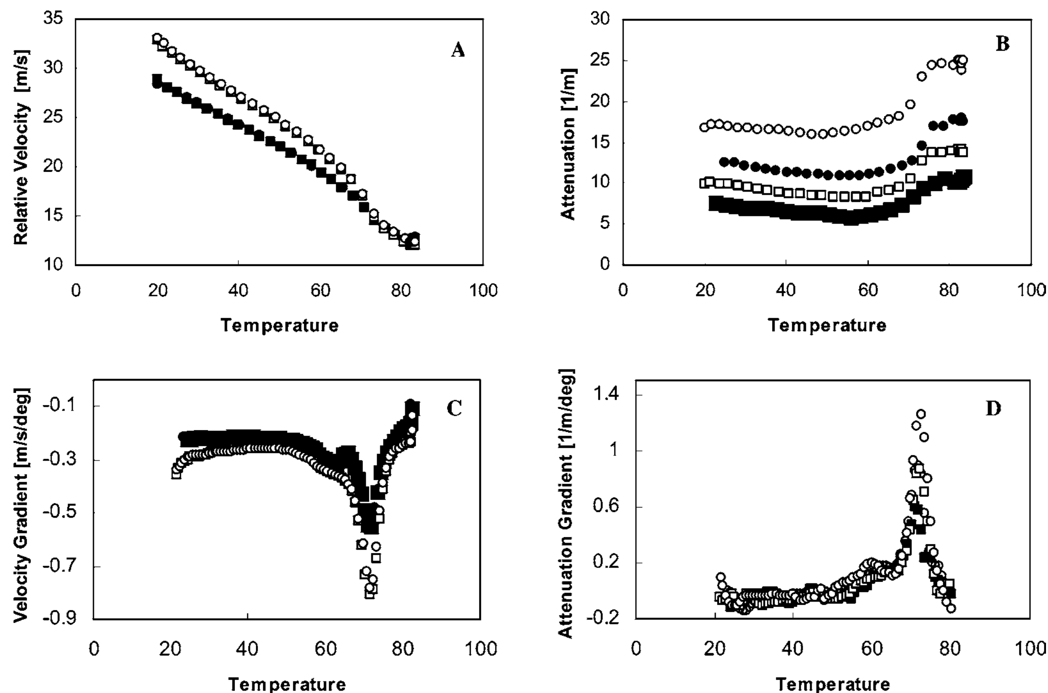

Figure 2. Ultrasonic relative velocity (A) and attenuation (B) for WPI solutions containing 0.01 M NaCl (solid symbols) or 0.1 M NaCl (open symbols). The ultrasonic relative velocity is the difference between the velocity of the WPI solution and the velocity measured in the reference cell. Below are the temperature differentials of the ultrasonic relative velocity [m/s/°C] (C) and the ultrasonic attenuation [1/m/°C] (D) corresponding to A and B. Measurements were perfomed at 5 (●) and 7 (■) MHz.

changes were evident at temperatures as low as 50 °C: a shoulder was shown at 60 °C, and a rapid change at about 70 °C. These suggest the presence of at least two transitions or reactions in the system, the first of which occurred at temperatures lower than those previously reported for whey protein denaturation and aggregation (*3*, *6*). A similar behavior of the ultrasonic velocity was shown for samples containing 0.01 and 0.1 M NaCl, although WPI with 0.1 M NaCl showed a higher peak in the differential velocity, indicating a greater extent of change at high ionic strength compared to low ionic strength.

During heating of WPI solutions, the values of attenuation showed a slight decrease at temperatures between 25 and 60 °C and then an increase at temperatures >60 °C (**Figure 2B**). A second, steeper increase in the values of attenuation was shown at about 70 °C with a plateau at 75 °C. As for the velocity, analysis of the gradient of attenuation facilitated the distinction between different stages in the reactions occurring during heating (**Figure 2D**). The gradient of attenuation started from negative values, indicating the negative rate of change in attenuation at low temperatures, showed a shoulder at about 55 °C, and a peak in the gradient of attenuation at 70 °C, similar to what was observed for the velocity. This peak indicated the onset of gelation of WPI. The gradient of attenuation did not change with frequency, although values of attenuation at 7 MHz are higher than at 5 MHz. As already observed for relative velocity, the behavior of ultrasonic attenuation as a function of temperature did not differ between samples containing different amounts of NaCl, although the peak of the differential attenuation was higher for WPI heated in the presence of 0.1 M NaCl compared to WPI at low ionic strength.

Some differences were shown in the ultrasonic behavior of β-lactoglobulin solutions during heating in the presence 0.01 and 0.1 M NaCl compared to WPI solutions. Results are summarized in **Figure 3**. In agreement with what was described for WPI solutions (**Figure 2**), the relative ultrasound velocity of β-lactoglobulin showed a steady decrease with heating temperature (**Figure 3A**). A change at 70 °C was also noted in the relative velocity of β-lactoglobulin samples, with no difference being seen between 0.1 or 0.01 M NaCl solutions. Overall, the values of relative ultrasonic velocity were lower for β-lactoglobulin than for WPI samples. Plots of the velocity gradient as a function of temperature showed a peak at 70 °C (**Figure 3C**) with a shift to higher temperatures in the presence of 0.1 M NaCl, but the contribution at about 60 °C was absent. In agreement with results described for WPI, protein solutions containing 0.1 M NaCl showed a more rapid and greater extent of change, represented by a higher peak in the velocity gradient, compared to samples heated at low ionic strength.

The changes in ultrasonic attenuation of β-lactoglobulin with temperature were similar to those observed in WPI solutions. Ultrasonic attenuation decreased between 20 and 50 °C much more markedly than it did for WPI and increased at higher temperatures up to a plateau at about 75 °C, similar to that shown in **Figure 1** (**Figure 3B**). Samples containing β-lactoglobulin with 0.1 M NaCl had attenuation values similar to those with 0.01 M NaCl both at 5 and 7 MHz, perhaps indicating the formation of a more homogeneous type of aggregate compared to the aggregates formed in WPI samples. The gradient of attenuation as a function of temperature showed some evidence for a shoulder at about 55 °C and a definite peak at 70 °C (**Figure 3D**). The extent of change at 70 °C was higher for samples containing 0.1 M NaCl than in 0.01 M NaCl solutions. For the solutions of β-lactoglobulin, the peaks in the gradients of attenuation as well as in the gradients of velocity were at different temperatures for the solutions of high and low ionic strength. This was not observed for WPI.

Heating kinetics did not affect the behavior of velocity and attenuation as a function of temperature. Plots of gradient of velocity and attenuation of WPI and β-lactoglobulin heated at 0.2 or 0.4 °C/min were compared, and the parameters measured at 7 MHz are summarized in **Figure 4**. While ionic strength increased the extent of change of the attenuation and relative

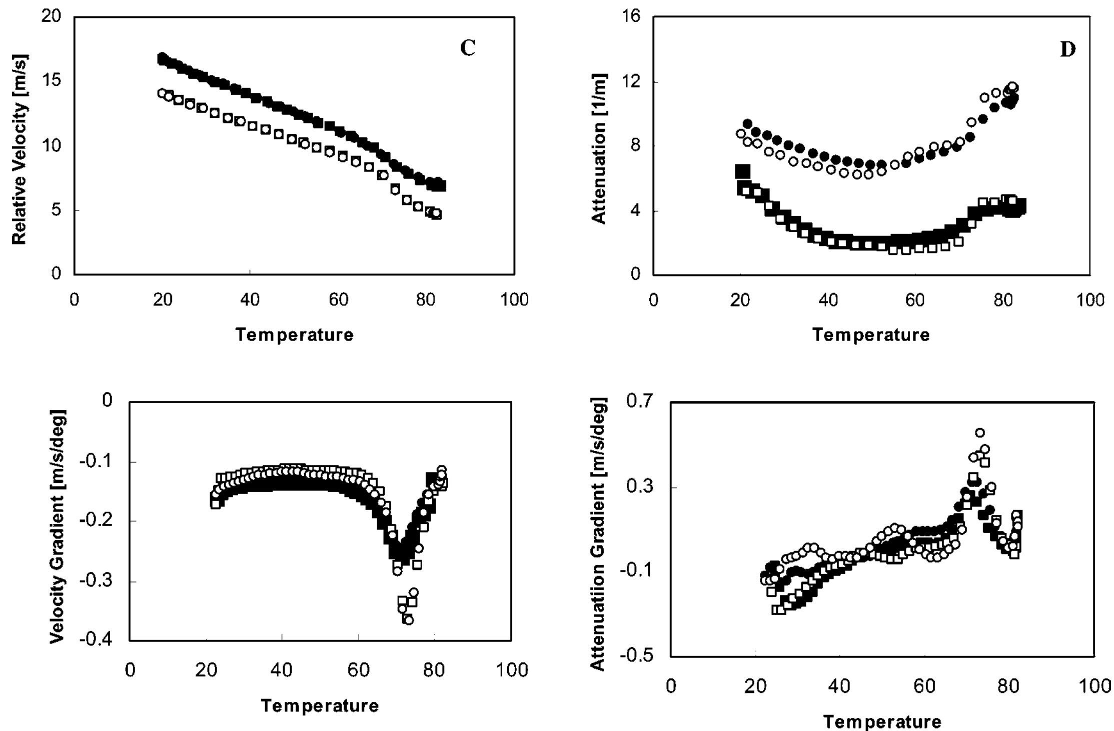

Figure 3. Ultrasonic relative velocity (A) and attenuation (B) for β-lactoglobulin solutions containing 0.01 (solid symbols) or 0.1 M NaCl (open symbols). The ultrasonic relative velocity is the difference between the velocity of the β-lactoglobulin solution and the velocity measured in the reference cell. Below are the temperature differentials of the ultrasonic relative velocity [m/s/°C] (C) and the ultrasonic attenuation [1/m/°C] (D) corresponding to A and B. Measurements were perfomed at 5 (●) and 7 (■) MHz.

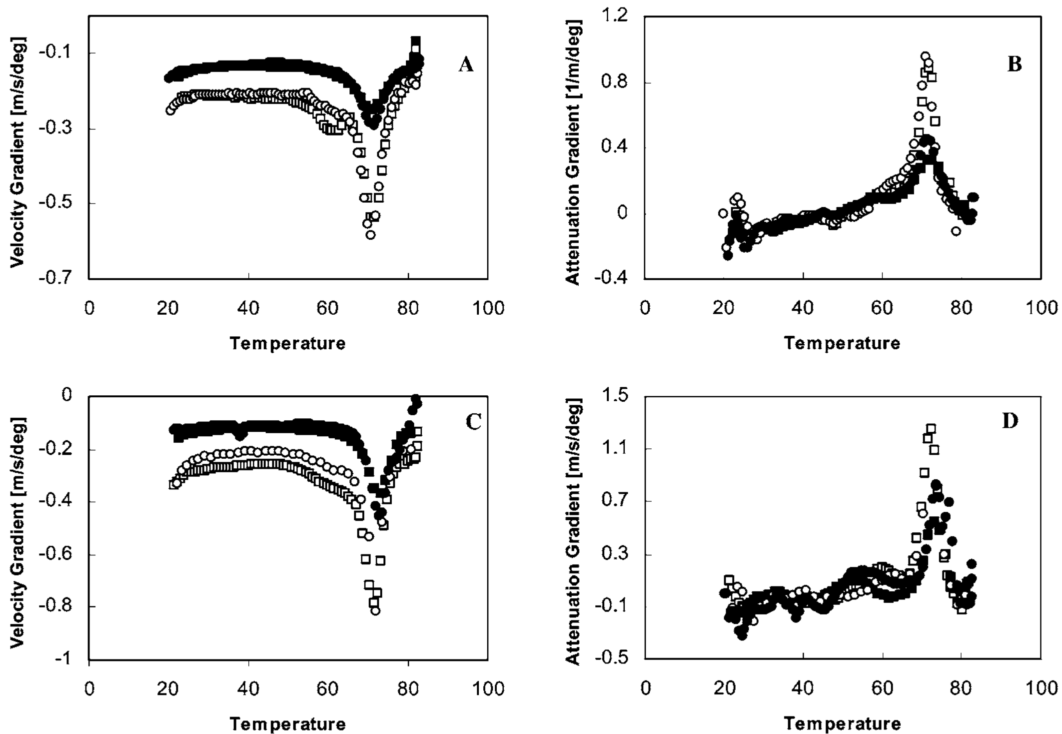

Figure 4. Temperature differentials of the relative ultrasonic velocity (A, C) and attenuation (B, D) for WPI (open symbols) and β-lactoglobulin (solid symbols) as a function of heating temperature for solutions containing 0.01 (A, B) or 0.1 M NaCl (C, D). Heating was carried out from 20 to 85 °C at a rate of 0.4 (●) and 0.2 °C/min (■). Results are measured at 7 MHz.

velocity and a higher peak at 70 °C was shown, heating at a higher rate did not change the temperature of the onset of gelation. **Figure 4C**,**D** also shows the displacement of the peak for β-lactoglobulin at high ionic strength. It was hypothesized that the changes observed by ultrasound spectroscopy were not affected by time during heating and that the ultrasonic param-

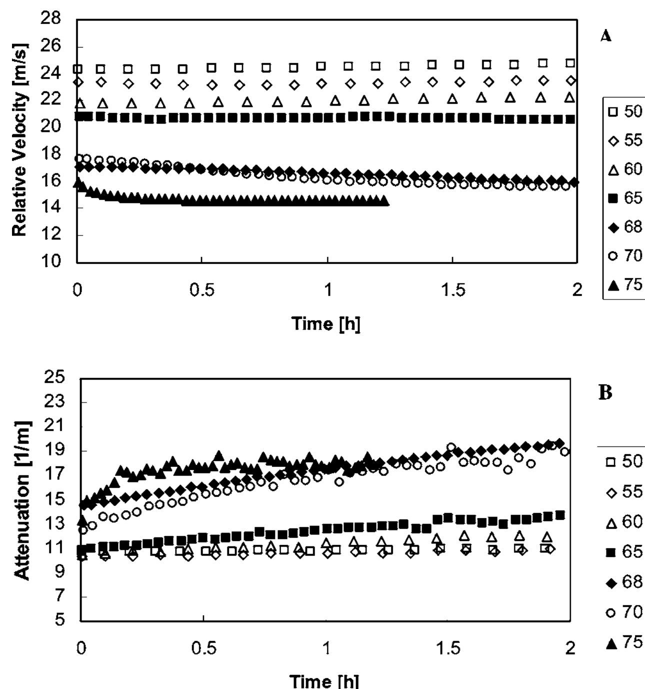

Figure 5. Relative ultrasonic velocity (A) and attenuation (B) measured at 7 MHz for WPI heated at various temperatures [°C] under isothermal conditions for solutions containing 0.1 M NaCl.

eters changed relatively quickly at any given temperature. Because of the high sensitivity of the equipment, only relatively slow heating ramps could be applied. For this reason, to confirm our results, isothermal studies were also performed at temperatures between 50 and 75 °C on WPI solutions.

Figure 5 illustrates the relative ultrasonic velocity and attenuation changes during isothermal heating for WPI solutions containing 0.1 M NaCl. No differences were shown in the relative velocity of WPI heated in the presence of 0.1 M NaCl compared to those in 0.01 M NaCl (results not shown). The initial values of relative velocity decreased with temperature and at temperatures <65 °C showed no changes in the velocity with time of heating (**Figure 5A**). These values of relative velocity at lower temperatures presumably related to the conformational equilibrium of the proteins, and the changes seemed to occur very rapidly. Above 65 °C, the heating temperature significantly affected the relative velocity of the sound propagating in the WPI samples. At temperatures >70 °C, the relative velocity showed a decrease with time of heating at rates which were significantly higher than at 60 and 65 °C. Also during heating at constant temperature, 70 °C appeared to mark the onset of fast whey protein aggregation. Because of instrumental limitations (the setup requires several minutes), it was not possible to make measurements at temperatures >75 °C. Values of attenuation obtained during isothermal runs (**Figure 5B**) also confirmed the results described above with heating at 0.2 and 0.4 °C/min. Values of ultrasonic attenuation were significantly affected by differences in NaCl concentration with higher attenuation values at 0.1 M NaCl compared to 0.01 M NaCl. The ultrasonic attenuation also significantly increased with increasing temperature from 50 to 75 °C. While at low temperature the attenuation did not change with heating time, at higher temperatures the attenuation increased with time up to a plateau value, indicating the occurrence of protein−protein interactions and their dependence on heating time (*14*). The slope of the increase in attenuation was significantly affected by time at temperatures >65 °C.

DISCUSSION

Whey proteins during heating undergo conformational changes, denaturation, and protein−protein interactions, and above a critical concentration these interactions result in a gel network. These reactions were observed by changes in the propagation of the acoustic wave through a whey protein sample during heating in situ. Two ultrasound parameters were measured as a function of time and temperature of heating, velocity, and attenuation. While velocity is related to the compressibility of the medium, attenuation is determined by the absorption and scattering of the ultrasonic wave, the energy of dissipation caused by solute−solute interactions, and the diffusion of particles in the dispersed phase (*18*). By using high-resolution ultrasound spectroscopy it was possible to observe short-term (in the order of minutes) molecular changes occurring with temperature at higher concentrations than those commonly applied by light scattering or size exclusion chromatography (where experimental conditions are kept below a critical concentration to prevent gelation).

The ultrasonic velocity increased with increasing temperature both in the whey protein solutions and in the reference cell because of the increase in compressibility of the medium with temperature (*19*). This is a typical behavior of aqueous systems (*18*). To determine changes in the compressibility of proteins during heating, the velocity of the ultrasonic wave propagating through the reference cell is subtracted from the ultrasonic velocity measured in protein solutions. Velocity values are then related to protein compressibility, caused by the packing of the hydrophobic moieties and the hydrated shell (*20*). In other words, molecular compressibility is a sum of the molar compressibility of the molecule itself and the solute−solvent interactions which characterize the surrounding hydration layer, as water molecules form hydrogen bonds between the various polar groups on the surface of the protein (*19*). The steady decrease in the relative ultrasonic velocity of the protein solution with temperature (see, for example, **Figure 2A**) indicates a general increase in protein compressibility with temperature. The relative ultrasonic velocity change from 20 to 70 °C was about 10 m/s for β-lactoglobulin and 20 m/s for WPI solutions (**Figures 2A** and **3A**). The decrease in the relative velocity may have been caused by an increasing number of proteins participating in conformational changes and an increase in hydrophobic interactions which increased the compressibility of the protein. In general, the changes suggest an overall loosening of the protein structure as the temperature increases.

Molecular interactions are fundamental in understanding the formation of whey protein aggregates. It has been reported that during heating native β-lactoglobulin and α-lactalbumin undergo structural changes which expose their reactive sites, and under certain conditions, conformational changes can be the limiting step of the aggregation, for example, at low concentration or low heating temperature (*3*, *9*). The results presented in **Figure 2A** indicated continuous changes in the protein compressibility with temperature, changes which started at room temperature and preceded the critical temperature of protein denaturation (*6*). Previous authors indicated the presence of a shoulder in the denaturation behavior of β-lactoglobulin at about 60 °C using very low heating rates during differential scanning calorimetry analysis (2 °C/h) (*21*). Values of ultrasonic attenuation confirmed these observations as attenuation also showed changes below the denaturation temperature with a decrease between 20 and 50 °C and an increase at higher temperatures. The decrease in attenuation shown in **Figures 2B** and **3B** may be a result of the change in quaternary structure of β-lactoglobulin,

which at neutral pH changes from dimeric to a monomeric form during heating (*4*). Overall, the ultrasound results confirmed previously reported work on the existence of reversible structural changes of β-lactoglobulin: circular dichroism and nuclear magnetic resonance indicated that at temperatures below 60 °C reversible modifications in the tertiary structure of β-lactoglobulin occur at very short time scales (*4*, *5*). The decrease in ultrasonic attenuation in WPI and β-lactoglobulin solutions at <50 °C was not only shown at 0.4 °C/min but also during heating at 0.2 °C/min. This behavior was less pronounced in WPI systems (**Figure 2B**) than during heating of β-lactoglobulin, possibly because in WPI not only β-lactoglobulin, but also α-lactalbumin and other minor proteins are present.

By calculating the differential of ultrasonic velocity and attenuation as a function of heating temperature, it was possible to distinguish between different stages of the heat-induced aggregation of whey proteins. At temperatures <50 °C, conformational changes occurred which were reflected in the decrease in ultrasonic attenuation. A steady decrease in velocity was also observed starting at 20 °C, indicating a continuous change in the molecular compressibility of the protein. At about 50 °C the steady decrease in ultrasonic velocity deviated from linearity, indicating the beginning of protein−protein interactions or the beginning of some denaturation process. This latter transition is at a much lower temperature than those commonly reported for whey protein denaturation (*6*, *21*, *22*). The process at 50−60 °C was much less marked in β-lactoglobulin than in WPI and may therefore be possibly related to changes in the other proteins in WPI (α-lactalbumin and bovine serum albumin) or some interactions between these proteins and β-lactoglobulin. At temperatures of about 60 °C, a shoulder was seen in the gradient of ultrasonic attenuation for both β-lactoglobulin and WPI. This shoulder may indicate the formation of small aggregates which do not yet cause large changes in compressibility. The fact that the shoulder was apparently lacking in the velocity gradient plots of pure β-lactoglobulin may suggest that the shoulders in velocity and attenuation do not arise from the same cause. At about 70 °C, both differential plots of relative velocity and attenuation indicated a more important transition, which presumably denotes the overall denaturation of the proteins and the onset of gelation. These peaks corresponded to the denaturation temperature commonly reported for β-lactoglobulin (*6*, *23*, *24*). Values of attenuation reached a plateau at 75 °C, perhaps indicating that the formation of the aggregates was completed.

In general, the ultrasonic behavior of β-lactoglobulin and WPI did not show large differences. In addition, similar changes in the ultrasound parameters were measured for protein solutions containing 0.1 and 0.01 M NaCl, i.e., the overall decrease in velocity or increase in attenuation over the main denaturation process did not depend strongly on conditions. However, it was found that the peak in the differential plot was at a slightly higher temperature for pure β-lactoglobulin at 0.1 M NaCl than for the other measurements (**Figure 4C**), in agreement with the reported increase in the temperature of denaturation of β-lactoglobulin with ionic strength (*6*, *22*). Apart from this, varying the ionic strength or the heating kinetics from 0.4 to 0.2 °C/min did not change the onset of whey protein aggregation. This similarity in the overall propagation behavior of the ultrasound wave in whey protein solutions during heating does not show the expected large dependence on ionic strength. It is known that protein−protein interactions, microstructure, and rheology are all affected by ionic strength (*3*, *23*, *25*). It seems reasonable to conclude that ultrasonic parameters measured in whey protein solutions were related to the molecular properties associated with denaturation and aggregation of the proteins rather than to the process of gelation. The changes in relative velocity and attenuation may indicate the formation of aggregates in the stages which preceded gelation, during the process of formation of a space-filling highly hydrated biopolymer network. These intermediate aggregates may not be affected by ionic strength as much as the final gel microstructures, as previous authors hypothesized describing the formation of primary aggregates during heating under dilute conditions (*7*). It must also be remembered that while whey proteins heated at high ionic strength form gels, at low ionic strength they form only particles (*3*, *23*, *24*). Therefore, ultrasonic velocity and attenuation measured during gelation of whey proteins may be less related to the type of interactions which hold the molecules together and the rheology of the gels formed, i.e., we may not be measuring the aggregation or gelation but the molecular changes which make it possible. Peaks in the velocity gradients are higher at higher ionic strengths, showing that the transition is sharper under those conditions. A lack of effect of gelation on ultrasonic parameters has also been noted for acid-induced casein gels: values of attenuation and velocity did not show large differences between heated and unheated milk samples, although the gel strength is known to be much higher for heated milks (*16*).

In WPI solutions, α-lactalbumin forms aggregates with β-lactoglobulin during heating (*11*, *12*). However, the behavior of ultrasound in WPI and β-lactoglobulin solutions was similar, which may confirm that β-lactoglobulin dominates whey protein aggregation. It was noted that not only the values of attenuation but also the extent of change in the gradient of attenuation versus temperature was lower in heated β-lactoglobulin than in WPI, perhaps because of the formation of smaller aggregates with pure β-lactoglobulin compared to those formed in mixed whey protein. This hypothesis is supported by light scattering and microscopy results (*8*, *10*, *12*). However, some of the changes seen in ultrasonic velocity in the WPI solutions suggest that the presence of the other proteins does influence the mechanisms of denaturation and protein interaction.

In conclusion, high-resolution ultrasound spectroscopy allowed the observation of various stages in the heat-induced interactions of whey proteins and particularly showed evidence for stages preceding gelation. It has been previously demonstrated during heating of pure β-lactoglobulin solutions that at temperatures below denaturation reversible changes occur (*4*), and aggregates form between 60 and 65 °C after long heating times (*9*). The observed changes in ultrasonic parameters within a few minutes of heating at temperatures as low as 50 °C may be caused by the high sensitivity of the instrument or the protein concentrations used in the experiments. High-resolution ultrasound spectroscopy using transverse waves did not seem to be effective in determining differences in the strength of the interactions between the aggregates, perhaps because the intermediate aggregates formed during heating at neutral pH are not affected by changes in the environmental conditions as much as in the late stages of the aggregation or perhaps because the ultrasound properties of the aggregates which constitute the network are not distinct from the ultrasound properties of the network itself.

ACKNOWLEDGMENT

The authors would like to acknowledge Dr. Marcela Alexander for numerous discussions on ultrasound spectroscopy.

LITERATURE CITED

(1) Langton, M.; Hermansson, A. M. Fine-stranded and particulate gels of β-lactoglobulin and whey proteins at varying pH. *Food Hydrocolloids* **1992**, *5*, 523−539.

(2) Ikeda, S.; Foegeding, E. A.; Hagiwara, T. Rheological study on the fractal nature of the protein gel structure. *Langmuir* **1999**, *15*, 8584−8589.

(3) De la Fuente, M. A.; Singh, H.; Hemar, Y. Recent advances in the characterization of heat-induced aggregates and intermediates of whey proteins. *Trends Food Sci. Technol.* **2002**, *13*, 262−274.

(4) Iametti, S.; De Gregori, B.; Vecchio, G.; Bonomi, F. Modifications occur at different structural levels during the heat denaturation of β-lactoglobulin. *Eur. J. Biochem*. **1996**, *237*, 106−112.

(5) Li, H.; Harding, C. C.; Foegeding, E. A. NMR studies of thermal denaturation and cation-mediated aggregation of β-lactoglobulin. *J. Agric. Food Chem*. **1994**, *42*, 2411−2420.

(6) Relkin, P. Differential scanning calorimetry: a useful tool for studying protein denaturation. *Thermochim. Acta* **1994**, *246*, 371−386.

(7) Le bon, C.; Nicolai, T.; Durand, D. Growth and structures of aggregates of heat denatured β-lactoglobulin. *Int. J. Food Sci. Technol*. **1999**, *31*, 451−466.

(8) Ikeda, S.; Morris, V. J. Fine stranded and particulate aggregates of heat-denatured whey proteins visualized by atomic force microscopy. *Biomacromolecules* **2002**, *3*, 382−389.

(9) Hoffmann, M. A. M.; Sala, G.; Olieman, C.; de Kruif, K. G. Molecular mass distributions of heat-induced β-lactoglobulin aggregates. *J. Agric. Food Chem*. **1997**, *45*, 2949−2957.

(10) Schokker, E. P.; Sing, H.; Pinder, D. N.; Norris, G. E.; Creamer, L. K. Characterization of intermediates formed during heat-induced aggregation of β-lactoglobulin AB at neutral pH. *Int. Dairy J*. **1999**, *9*, 791−800.

(11) Dalgleish, D. G.; Senaratne, V.; Francois, S. Interactions between α-lactalbumin and β-lactoglobulin in the early stages of heat denaturation. *J. Agric. Food Chem.* **1997**, *45*, 3459−3464.

(12) Schokker, E. P.; Singh, A. M.; Creamer, L. K. Heat-induced aggregation of β-lactoglobulin A and B with α-lactalbumin. *Int. Dairy J*. **2000**, *10*, 843−853.

(13) Bryant, C. M.; McClements, D. J. Ultrasonic spectrometry study of the influence of temperature on whey protein aggregation. *Food Hydrocolloids* **1999**, *13*, 439−444.

(14) O'Brien, W. D.; Dunn, F. Ultrasonic absorption mechanisms in aqueous solutions of bovine haemoglobin. *J. Phys. Chem.* **1972**, *76*, 528−533.

(15) Kudryashov, E.; Smyth, C.; Duffy, G.; Buckin, V. Ultrasonic high-resolution longitudinal and shear wave measurements in food colloids: monitoring of gelation processes and detection of pathogens. *Prog. Colloid Polym. Sci*. **2000**, *115*, 287−294.

(16) Dalgleish, D. G.; Alexander, M.; Corredig, M. Studies of the acid gelation of milk using ultrasonic spectroscopy and diffusing wave spectroscopy. *Food Hydrocolloids* **2004**, in press.

(17) Andrews, A. T.; Taylor, M. D.; Owen, A. J. Rapid analysis of bovine milk proteins by fast protein liquid chromatography. *J. Chromatogr.* **1985**, *348*, 177−185.

(18) Buckin, V.; Smyth, C. High-resolution ultrasonic resonator measurements for analysis of liquids. *Sem. Food Anal.* **1999**, *4*, 113−130.

(19) Buckin, V. A. Hydration of nucleic bases in dilute aqueous solutions. Apparent molar volumes and their temperature slopes at 25 °C. *Biophys. Chem.* **1988**, *29*, 283−292.

(20) Gekko, K.; Noguchi, H. Compressibility of globular proteins in water at 25 °C. *J. Phys. Chem.* **1979**, *83*, 2706−2714.

(21) Hoffmann, M. A. M.; van Miltenburg, J. C.; van Mil, P. J. J. M. The suitability of scanning calorimetry to investigate slow irreversible protein denaturation. *Thermochim. Acta* **1997**, *306*, 45−49.

(22) Boye, J. I.; Alli, I.; Ismail, A. A. Use of differential scanning calorimetry and infrared spectroscopy in the study of thermal and structural stability of α-lactalbumin. *J. Agric. Food Chem.* **1997**, *45*, 1116−1125.

(23) Foegeding, E. A.; Davis, J. P.; Doucet, D.; McGuffey, M. K. Advances in modifying and understanding whey protein functionality. *Trends Food Sci. Technol.* **2002**, *13*, 151−159.

(24) Alting, A. C.; Hamer, R. J.; de Kruif, C. G.; Visschers, R. W. Cold-set globular protein gels: interactions, structure and rheology as a function of protein concentration. *J. Agric. Food Chem*. **2003**, *51*, 3150−3156.

(25) Puyol, P.; Perez, M. D.; Horne, D. S. Heat-induced gelation of whey protein isolate (WPI): effect of NaCl and protein concentration. *Food Hydrocolloids* **2001**, *15*, 233−237.

Received for review December 8, 2003. Revised manuscript received April 12, 2004. Accepted May 6, 2004. The authors would like to acknowledge the Ontario Dairy Council and Natural Sciences and Engineering Research Council for funding this research.

JF0354390