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Quantitative Assessment of Thermal Denaturation of Bovine α-Lactalbumin via Low-Intensity Ultrasound, HPLC, and DSC

QIN WANG,* ALEXANDER TOLKACH, AND ULRICH KULOZIK

Chair for Food Process Engineering and Dairy Technology, Technische Universität München, 85354 Freising-Weihenstephan, Germany

The degree of irreversible aggregation and the aggregation velocity constant of α-lactalbumin (α-la) were determined by three methods based on different principles: low-intensity ultrasound as a novel method for this purpose, DSC, and HPLC. The denaturation process of α-la causes a decrease in the ultrasonic velocity due to the conformation change of α-la molecules. This decrease is a function of the concentration of native α-la in the sample. A linear correlation was found between the degree of aggregation of α-la determined by these three methods. There is no significant difference between the aggregation velocity constants determined by the three methods. The results show that the ultrasonic method is capable of quantifying the degree of aggregation of a protein, offering an alternative method.

KEYWORDS: Protein denaturation; α-lactalbumin; degree of aggregation; ultrasound; DSC; HPLC

INTRODUCTION

α-Lactalbumin (α-la) is a small, Ca^{2+}-binding whey protein with a molecular mass of 14.2 kDa. It is a globular protein with a secondary structure composed of 26% α-helix, 14% *β*-sheet, and 60% irregular structure (*1*). α-la contains four disulfide bonds and no free thiol group. It has a strong Ca^{2+} binding site. The Ca^{2+} binding stabilizes the conformation of α-lactalbumin and increases its thermal stability against denaturation. The secondary structure of α-lactalbumin molecules can be easily changed by heating, but the α-la molecule is capable of refolding to a conformation similar to that of its native state in the presence of Ca^{2+} (*2*, *3*). An additional reason for the high thermal stability of α-la is the absence of a free thiol group in the molecule (*4*).

The thermal denaturation of globular proteins often involves the unfolding of the protein molecules and the following irreversible aggregation of the unfolded molecules. If α-la is heated alone, aggregates via disulfide bonds can only be formed at extreme time and temperature combinations (*4*). In the presence of *β*-lactoglobulin (*β*-lg), the thermal stability of α-la decreases because *β*-lg, with its free thiol group, acts as a catalyst (promoter) to open the disulfide group in α-la, thus enabling the formation of intermolecular disulfide bonds (*5*).

An established method for determining the degree of irreversible aggregated proteins is reversed phase high-performance liquid chromatography (RP-HPLC). RP-HPLC separates different proteins on the basis of their different molecular hydrophobicities (*6*). By measuring the difference between the native α-la concentration in unheated and heated samples, we can determine the level of irreversibly aggregated α-la during thermal denaturation.

Another method for investigating protein denaturation is calorimetry (*7*). Differential scanning calorimetry (DSC) is a valuable tool for studying thermally induced changes in a protein. In this method, the change in thermal energy in a sample during heating or cooling is measured, which results in a heat flux or a change in the thermal energy. Denaturation of protein causes an endothermic peak in the thermogram (*8*). According to the area of the endothermic peak, the denaturation of α-la can be carried out quantitatively (*7*).

It is well-known that the compressibility of proteins in solution can be measured by measuring the ultrasonic velocity. The compressibility of proteins is composed of the intrinsic compressibility, the hydration compressibility, and the compressibility related to relaxation processes (*9*). The intrinsic compressibility is the compressibility due to imperfect packing of the molecule. The hydration compressibility is the change in solvent compressibility due to the interactions of solvent molecules with the solvent accessible atomic groups of the protein. The hydration compressibility is usually negative since water in the hydration shell is less compressible than bulk water (*10*, *11*). The presence of relaxation increases the compressibility, while an increased level of hydration causes a decrease in compressibility (*12*). For fluids, the following relationship among the ultrasonic velocity (v), the compressibility (κ), and the density (ρ) applies:

$$v = \sqrt{\frac{1}{\kappa\rho}} \tag{1}$$

During denaturation, the globular protein molecules unfold, which leads to changes in the compactness of the interior of

* To whom correspondence should be addressed. Telephone: +49(0)-8161715056. Fax: +49(0)8161714384. E-mail: qin.wang@wzw.tum.de.

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the protein molecules and changes in the hydration state of the molecules due to the enlarged surface area that is accessible to the solvent. Furthermore, protein unfolding causes an increased relaxation contribution due to conformational relaxation or an increased number of proton exchanges due to a higher degree of exposure of side chains to solvent (*13*). These modifications lead to changes in the ultrasonic velocity in the protein solution. Many researchers have investigated conformational changes in protein molecules due to chemical denaturation using ultrasonic velocimetry (*13*−*15*).

However, until a few years ago, there was no high-resolution ultrasonic instrument with temperature scanning capability available. Therefore, temperature-dependent changes in ultrasonic properties of proteins were barely investigated. Corredig, Verespej, and Dalgleish (*16*) first investigated the in situ thermal denaturation of whey protein isolate (WPI) and β-lg using a high-resolution ultrasonic instrument. Their investigation focused on a description of the course of ultrasonic properties of WPI and β-lg over a temperature range of 20−80 °C and a comparison of WPI and β-lg with regard to their ultrasonic properties.

In our work, we focused on the quantitative assessment of the thermal denaturation of α-la. The purpose of this work was to acquire the ultrasonic velocity in α-la solutions during the thermally induced protein denaturation process and to investigate whether the degree of aggregation of this protein can be determined via ultrasonic measurements and how the results correlate with those of established methods, i.e., HPLC and DSC. Thereby, whether the ultrasonic method can provide additional information about the denaturation compared to the established methods was of interest.

MATERIALS AND METHODS

α-Lactalbumin. α-la used was produced from whey protein concentrate by means of micro- and ultrafiltration at adjusted pHs and whey protein, calcium, and lactose concentrations and with subsequent thermal denaturation. The details have been described by Tolkach, Steinle, and Kulozik (*17*). The obtained solution of native α-la was freeze-dried and stored prior to use. The α-la/β-lg ratio in the powder was ∼10/1 according to the HPLC analysis.

To acquire the general course of ultrasonic velocity in an α-la solution with a variation in temperature, a solution containing 40 mg/mL native α-la was used. To achieve different degrees of irreversible α-la aggregation for the measurements, solutions containing 60 and 100 mg/mL native α-la were preheated at 90 °C for varying times. The high concentrations were chosen to avoid overly low native α-la concentrations for measurements in samples with high degrees of aggregation, which could be under the detection limit of the methods applied.

To obtain the α-la solutions of different concentrations, the α-la powder was dissolved in milk serum (UF-permeate) produced by ultra- and diafiltration of milk using a membrane with a nominal cutoff value of 25 000 Da. The UF-permeate has the same composition of lactose and minerals as milk, but casein and whey proteins were excluded. The exact α-la concentrations in the samples were determined by HPLC.

Preheating Experiments. The pH value of the solution was adjusted to 6.5 with NaOH and HCl solutions (Sigma, Taufkirchen, Germany). The solutions were added to small stainless steel tubes with an inner diameter of 4 mm, a length of 260 mm, and a volume capacity of 3.3 mL. The tubes with samples were heated in a water bath at 90 °C. Different degrees of aggregation of α-la were obtained by varying the heating time.

HPLC Method. The concentration of native α-la was determined by reversed phase HPLC (RP-HPLC) according to a method described in detail by Kessler and Beyer (*18*) as well as Tolkach and Kulozik (*19*). According to this method, the pH value of the samples was adjusted to 4.6 with a HCl solution. The irreversibly aggregated α-la coagulates. The aggregates were separated using a membrane filter (ø 45 μm, Chromafil RC-45/25, Macherey-Nagel, Düren, Germany). The concentration of native α-la in the filtrate was determined by RP-HPLC. A PLRP-S 8 μm, 300 Å column from Latek (Eppelheim, Germany) was used. Elution was performed by using a gradient from a mixture of 57% eluent A [0.1% trifluoroacetic acid (TFA) in water] and 43% eluent B (80% acetonitrile, 19.1445% water, and 0.0555% TFA) to 100% eluent B in 23 min. The temperature of the column was kept at 40 °C. The flow rate was 1.0 mL/min. The absorbance was recorded with a UV detector at 226 nm.

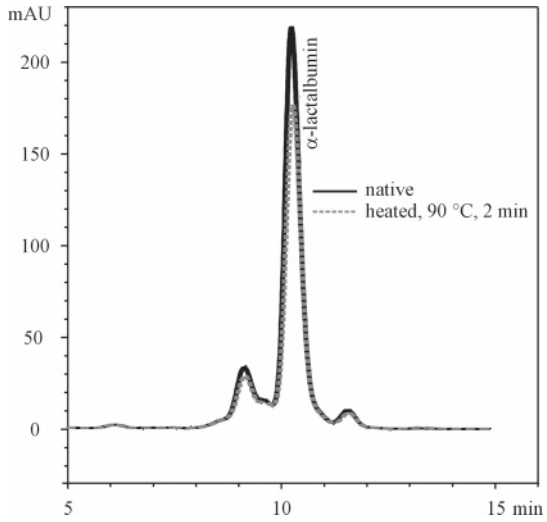

Figure 1. HPLC chromatograms of a solution containing 60 mg/mL α-la, native and preheated at 90 °C for 2 min.

Figure 1 shows the HPLC chromatograms of a solution containing 60 mg/mL α-la, unheated and preheated at 90 °C for 2 min. The difference in the α-la absorption peak areas between the unheated and preheated sample correlates with the amount of the irreversibly aggregated α-la.

The degree of irreversible aggregation of α-la molecules (DA) in preheated samples was calculated from the native α-la concentration in the preheated and unheated sample by the following equation:

$$DA_{HPLC} = (1 - C_{preheated}/C_{unheated}) \times 100\% \quad (2)$$

DSC Method. The DSC measurement was carried out using the DSC Q1000 instrument from TA Instruments (Alzenau, Germany). The pans used for the DSC measurements were high-volume pans made of stainless steel with a sample volume of 75 mg. The reference sample was UF-permeate. The heating rate was 300 mK/min, the same as that in the ultrasonic measurements. The small heating and cooling rate was chosen so that DSC and ultrasonic methods could be compared on the one side and so that a better thermal equilibrium within the sample on the other side could be achieved. The heat flow from the DSC device to the sample was measured as watts per gram of sample. The denaturation enthalpy (ΔH) was calculated by integrating the areas of the endothermic peaks obtained from heated and unheated samples. **Figure 2** shows the DSC thermograms of a solution containing 60 mg/mL α-la, unheated and preheated at 90 °C for 2 min.

The degree of irreversible aggregation in heated samples was calculated from the denaturation enthalpy (peak area) of the preheated and unheated samples during heating:

$$DA_{DSC} = (1 - \Delta H_{DSC,preheated}/\Delta H_{DSC,unheated}) \times 100\% \quad (3)$$

Ultrasonic Method. The ultrasonic measurements were performed using the ResoScan ultrasonic device from TF Instruments GmbH (Heidelberg, Germany). The ResoScan system measures ultrasonic velocity v and attenuation α/f^2, where α is the attenuation coefficient of the sample and f the frequency of the ultrasonic wave. In this work, we consider the ultrasonic velocity only, because it was found to be

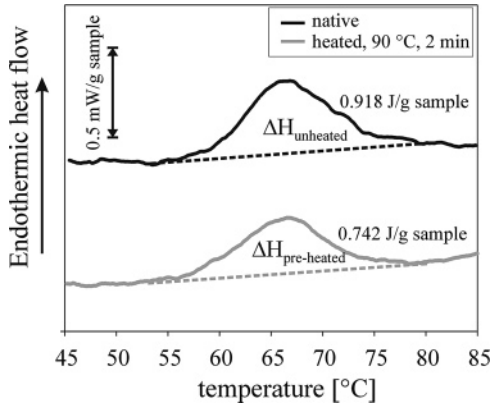

Figure 2. DSC thermograms of a solution containing 60 mg/mL α-la, native and preheated at 90 °C for 2 min.

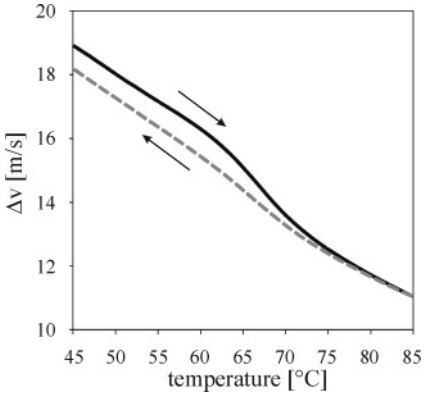

Figure 3. Effect of temperature on Δv in a solution containing 40 mg/mL native α-la at pH 6.5 with heating and cooling.

more sensitive. The ResoScan system is based on the resonance technique. The sample cells are constructed as ultrasonic resonators in which a standing wave is stimulated. During the initialization, a frequency range of ~7−9 MHz is scanned. By locating the resonance frequencies within this range, we can calculate the order of the resonance peaks. The system automatically selects an optimal resonance peak (the master peak) for the measurement. Using this peak, the ultrasonic velocity and attenuation are calculated.

The ResoScan system uses special intrinsic procedures to calculate the parameters velocity and attenuation. Ultrasonic velocity v is calculated from fundamental frequency f_1 and the path length of sample cells (d):

$$v = 2df_1 \tag{4}$$

Fundamental frequency f_1 is indirectly obtained by measuring the frequency at the maximum of the master resonance peak f_n, from which f_1 can be determined. In the ideal model of the ultrasonic resonator, the resonances are described by harmonic overtones of the fundamental frequency. Hence, f_n should be an integer multiple of fundamental frequency f_1. In the real ultrasonic resonator, however, the measured frequencies differ somewhat from the theoretical resonance frequencies. These deviations are used to evaluate the series of real resonances by system specific mathematical correction routines integrated in the software of the ResoScan system (*20*).

The ResoScan system has two closed sample cells with a path length of 7.0 mm. Evaporation of the sample is prevented by the closed lids. In the cells, the samples can be heated or cooled with a rate of 100−350 mK/min via Peltier elements. The absolute accuracy of the thermostat temperature is 10 mK. The resolution of the ultrasonic velocity is 0.001 m/s. The repeatability of absolute velocity after automatic reinitialization is ±0.01 m/s.

All experiments were carried out by measuring the ultrasonic velocity in the α-la solution against that in the reference UF-permeate. The reference UF-permeate and the α-la solutions were added to cells 1 and 2, respectively. The samples were equilibrated at 45 °C before the initialization process of the ResoScan unit. In the initialization process, a resonance peak at 7.8 MHz was chosen for the measurement. The sample was heated from 45 to 85 °C at a rate of 300 mK/min. The ultrasonic velocity in cells 1 and 2 during heating was measured. To determine the effect of heat on α-la alone, the difference between the velocity in an α-la solution and that in reference Δv was used for data analysis.

Calculation of the Velocity Constant of Irreversible α-la Aggregation. Many researchers found that the denaturation of α-la follows a reaction order of 1 (*21*, *22*). For a first-order reaction, the following relationships applies:

$$\ln\frac{C_t}{C_0} = -kt \tag{5}$$

with C_t is the concentration of the reactant (here, native α-la) at time t, C_0 the concentration at time zero, and k the velocity (rate) constant of the reaction.

The velocity constant of irreversible α-la denaturation (i.e., aggregation), k, was determined according to eq 5. The decrease in the native α-la concentration was calculated from the degrees of aggregation determined by the respective method:

$$(C_t/C_0) = 1 - \mathrm{DA}_t \tag{6}$$

where DA_t is the aggregation degree at time t.

Data Analysis. The differentiation of Δv versus time [i.e., $\mathrm{d}(\Delta v)/\mathrm{d}t$] was calculated by using Mathcad 2001i Professional from MathSoft Engineering & Education, Inc. (Cambridge, MA). A smoothed curve was created by calculating an average value of every 25 consecutive data points. For the data smoothing, the following equations were applied:

$$\left\{\left[\frac{\mathrm{d}(\Delta v)}{\mathrm{d}t}\right]_m\right\}_j = \frac{\sum_{i=j}^{j+p-1}\left(\frac{\Delta v_{i+1} - \Delta v_i}{t_{i+1} - t_i}\right)}{p} \tag{7}$$

$$(T_m)_j = \frac{\sum_{i=j}^{j+p-1}\left(\frac{T_{i+1} + T_i}{2}\right)}{p} \tag{8}$$

i is the numbering of the raw data rows ($i = 1, 2, 3, \ldots n$, where n is the total number of data rows), j is the numbering of the smoothed data rows, p is the number of data points included to calculate an average value (i.e., $p = 25$ for this work), $[\mathrm{d}(\Delta v)/\mathrm{d}t]_m$ is the average $\mathrm{d}(\Delta v)/\mathrm{d}t$ calculated from p consecutive data points, and T_m is the average temperature of p consecutive data points.

To compare the velocity constants of α-la aggregation determined by ultrasound, DSC, and HPLC, an ANOVA test was performed using Statgraphics Centurion XV (StatPoint, Inc., Herndon, VA). A P value α of 0.05 was used as a threshold of statistical significance. The velocity constants (k) for all 24 measuring points for the samples containing 60 mg/mL α-la (eight points per method) were calculated using eqs 5 and 6.

RESULTS AND DISCUSSION

Change in Ultrasonic Velocity Depending on Temperature in α-la. Figure 3 shows the dependence of Δv in the solution containing 40 mg/mL α-la on temperature during heating and subsequently cooling. It can be seen that upon heating at first a linear decrease in Δv at temperatures between 45 and 54 °C can be observed. The ultrasonic velocity is related to the compressibility of the medium. The decrease in ultrasonic velocity indicates an increase in protein compressibility with an increase in temperature (eq 1). This increase in compress-

ibility may have been caused by an increasing conformational change and hydrophobic interaction of α-la molecules with an increase in temperature (*16*). Furthermore, the degree of hydration of molecules decreases generally with an increase in temperature due to the higher kinetic energy of water molecules at higher temperatures which induces an increase in the compressibility of the α-la solution. From a temperature of ∼54 °C, the curve is no more linear. It becomes steeper. This indicates changes in α-la molecules during denaturation. Gast et al. (*23*) found that the linear dimensions of the molten globule state and the unfolded state of α-la are longer than those of the native state. This leads to a decreased compressibility and increased ultrasonic velocity due to an enlarged hydration shell. However, the decrease in the ultrasonic velocity correlates with an increase in the overall compressibility. It may be caused by the decreased compactness of the unfolded protein, which leads to increased intrinsic compressibility, and by the conformational and chemical relaxation processes (*15*). During the unfolding, the atomic groups of the side chains are exposed to the solvent. The relaxation contribution increases due to an increase in the conformational flexibility of the protein molecules and the accelerated proton exchange process (*12*). The relaxation decreases the compressibility. The negative contribution of intrinsic compressibility and relaxation to the ultrasonic velocity may be greater than the positive contribution of the hydration. This may be the reason for the decrease in the ultrasonic velocity during the thermal denaturation of α-la. After an inflection point, the curve becomes flatter again. Above approximately 80 °C, the decrease in Δv with an increase in temperature is linear again, which indicates the completion of the conformational change.

To follow the changes in ultrasonic velocity as a function of temperature increase more clearly, the first derivative of Δv [i.e., $d(\Delta v)/dt$] was calculated and plotted against temperature. **Figure 4** shows $d(\Delta v)/dt$ versus temperature in the solution containing 40 mg/mL native α-la. The bottom curve depicts $d(\Delta v)/dt$ as a function of increasing temperature and the top curve $d(\Delta v)/dt$ as a function of decreasing temperature following the upward ramp. Both curves show peaks in the temperature region of 54−80 °C. However, the peak area during cooling is smaller than that during heating. This indicates that the molecular changes detected by ultrasound were partially reversible. This complies with the description of α-la denaturation in the literature (*2*, *3*). The partial reversibility of α-la denaturation can be described by the following reaction scheme:

native α-la $\xrightleftharpoons{\text{partially reversible}}$ partially unfolded α-la (thermally induced molten globule state) $\xrightarrow{\text{irreversible}}$ aggregated α-la

The temperature increase over 60 °C leads to a partial unfolding and formation of the thermally induced molten globule state of α-la. This partially unfolded state exhibits an increased linear dimension and hydrophobity as well as better accessibility of the disulfide bonds to a thiol exchange reaction (*5*). Thus, it can participate in the following intermolecular aggregation, especially in the presence of the traces of *β*-lg. Once the partially unfolded α-la molecules are aggregated, they are not able to refold during cooling. In contrast, the unaggregated molecules refold during cooling. The downward peak in **Figure 4** describes the denaturation (unfolding and aggregation) process of α-la, while the upward peak describes the refolding of the unaggregated α-la. The areas (A_{US}) of the downward and upward peak correspond to the overall change in Δv during denaturation and refolding, respectively. The peak maxima correlate with the maximal rates of change in ultrasonic velocity. The temperatures at the peak maxima were ∼66 °C for both the heating and cooling curves. This agrees with the DSC measurement, which shows that the temperature at the maxima of the DSC endothermic peak is 65.8 ± 0.2 °C.

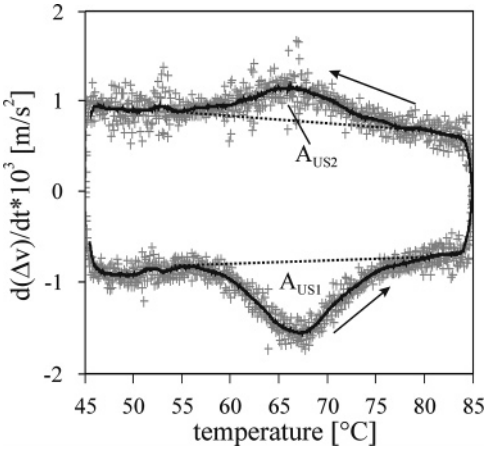

Figure 4. Effect of temperature on $d(\Delta v)/dt$ in a solution containing 40 mg/mL native α-la at pH 6.5 with heating and cooling. The crosses are all measured points. The solid line is a smoothed curve derived from the data points.

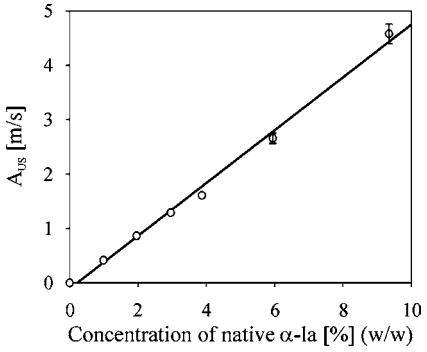

Figure 5. Dependence of peak area A_{US} on the native α-lactalbumin concentration in ultrasonic measurements. Error bars represent the standard deviation. $r^2 = 0.9946$.

According to the results given above, we suspect that peak area A_{US} may be proportional to the native α-la concentration in the sample. To verify this, α-la solutions at different concentrations were investigated by the ultrasonic method. A_{US} peak areas are plotted against the native α-la concentrations in the samples in **Figure 5**. It shows a linear correlation between peak area A_{US} and the native α-la concentration in the sample. The coefficient of determination (r^2) was 0.9946. This confirms that the overall change in Δv during thermal denaturation of α-lactalbumin linearly correlates with the native α-la concentration in the sample.

Figure 6 shows the dependence of $d(\Delta v)/dt$ on the temperature in an unheated and a preheated (90 °C for 2 min) solution containing 60 mg/mL α-la. According to the linear correlation in **Figure 5**, the difference between the peak areas of the unheated and preheated samples is proportional to the amount of irreversibly aggregated α-la. Therefore, the degree of aggregation in the preheated sample can be calculated from the A_{US} of the preheated and unheated samples as follows:

$$DA_{US} = (1 - A_{US,preheated}/A_{US,unheated}) \times 100\% \qquad (9)$$

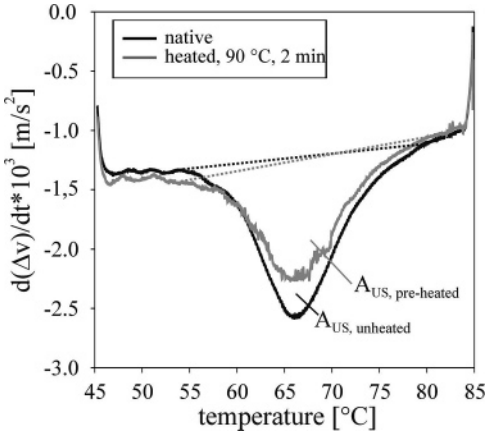

Figure 6. Effect of heat treatment on the temperature dependence of the ultrasonic velocity in the samples. Dependence of $d(\Delta v)/dt$ on the temperature in a solution containing 60 mg/mL α-la, native and preheated at 90 °C for 2 min.

Table 1. Degrees of Aggregation of α-la in a Solution Containing 60 mg/mL α-la Depending on the Heating Time at 90 °C Determined by HPLC (DA_{HPLC}), DSC (DA_{DSC}), and Ultrasound (DA_{US})

heating time (min)	DA_{HPLC} (%)	DA_{DSC} (%)	DA_{US} (%)
2	15.1	19.2	17.4
3	14.1	22.0	17.4
6	19.9	31.0	23.5
10	28.9	34.5	35.0
20	37.4	38.0	37.5
25	47.9	43.5	51.2
44	73.8	77.8	78.9
55	87.1	91.1	89.6

Kinetics of the Thermal Aggregation of α-la Determined by HPLC, DSC, and Ultrasound. To compare the ultrasonic method with HPLC and DSC methods for quantitative determination of α-la aggregation over a wide range of aggregation degrees, we assessed preheated α-la solutions with different degrees of aggregation by HPLC, DSC, and ultrasound. The degrees of aggregation of α-la determined by these three methods in solutions containing 60 mg/mL α-la with different heating times are listed in **Table 1**. The degree of aggregation of α-la increases with an increase in heating time.

In **Figure 7**, the degrees of aggregation of the preheated samples containing 60 mg/mL α-la (presented in **Table 1**) and those of the preheated samples containing 100 mg/mL α-la determined by HPLC, ultrasonic, and DSC methods are plotted against each other. The solution containing 100 mg/mL α-la was additionally used to show the influence of the concentration on the determination of the degree of α-la aggregation. To acquire the reproducibility of the methods, two of the samples containing 6 mg/mL α-la were measured three times each. The standard deviations are plotted as error bars. Independent of the α-la concentration in the samples, linear correlations are shown between the degrees of α-la aggregation determined by the three methods that were studied. The DSC method shows the highest standard deviation of these three methods. Manji and Kakuda (*7*) also observed a poor reproducibility of DSC measurements for whey protein denaturation in samples containing 20−70 mg/mL protein. At low protein concentrations, the endothermic heat flow is too small to be accurately detected. Compared to the HPLC method which determines the degree of irreversible aggregation after the heating process, ultrasonic and DSC measurement can be used to follow the conformational change in protein molecules during the heating process.

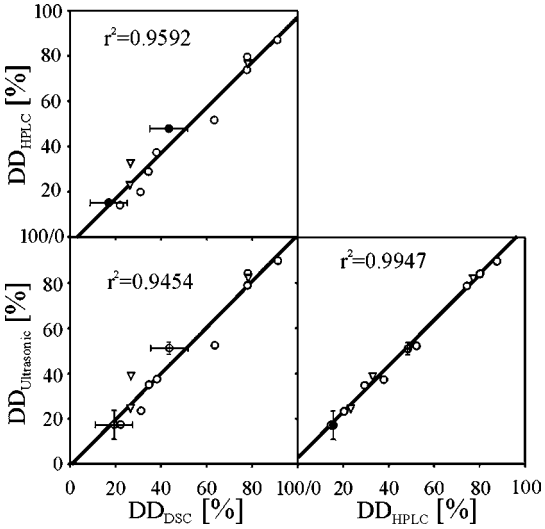

Figure 7. Correlation between the degrees of irreversible aggregation of α-lactalbumin in solutions containing 60 (○) and 100 mg/mL (▽) α-lactalbumin after heating at 90 °C determined by three different methods. Error bars represent the standard deviation.

The velocity constants of α-la aggregation (*k*) calculated from the linear regression of the degree of aggregation depending on heating time were $(5.59 \pm 0.49) \times 10^{-4}$, $(6.19 \pm 0.58) \times 10^{-4}$, and $(6.24 \pm 0.82) \times 10^{-4}$ s^{-1} for HPLC, ultrasound, and DSC, respectively. The *P* value α of the *F* test in the ANOVA to compare *k* values from the three methods was greater than 0.05 ($\alpha = 0.61$); i.e., no significant difference exists between the means of the aggregation velocity constants (*k*) determined by the three methods at a 95% confidence level.

Conclusions. The results of this work show that the thermal denaturation of α-la causes a decrease in the ultrasonic velocity. This decrease linearly correlates with the native α-la concentration in the sample and therefore can be used to quantify the degree of aggregation of α-la. The degrees of aggregation determined by HPLC, DSC, and ultrasound correlate linearly, and they provide similar velocity constants of α-la aggregation without a significant difference, despite the fact that these methods are based on different principles. Both DSC and ultrasonic methods follow heat-induced changes in protein molecules during the heating process, but the ultrasonic method shows a lower standard deviation and a better correlation with the established HPLC method than with the DSC method. Compared to the HPLC method, the DSC and ultrasonic methods are less sensitive for samples with low native protein concentrations, because at low protein concentrations the overall change in thermal energy and compressibility comes close to the detection limit of the methods.

The ability of the ultrasonic method to characterize the denaturation process of α-la allows this method to be applied for most globular proteins, which undergo a change in compressibility due to conformational changes during heat treatment. We also verified the ability of the ultrasonic method to assess the thermal denaturation of egg white and egg yolk proteins in exclusive experiments (results not shown). This method appears to be a useful additional method for investigating the thermal denaturation of globular proteins.

In summary, the HPLC method measures the amount of native α-la which is not irreversibly aggregated during the preheating of the samples, while the DSC and ultrasonic methods measure the thermal and compressional changes in the sample due to the thermal denaturation process, respectively. Therefore, in contrast to the HPLC methods, the DSC and ultrasonic methods

indirectly assess the degree of irreversible aggregation. In the ultrasonic measurements, the structure and size of the protein aggregates, affected by different milieu conditions, may influence the ultrasonic velocity. The larger the aggregates formed during the heating process, the more ultrasound is scattered, which leads to changes in the ultrasonic velocity. Furthermore, the extensive aggregation leads to inhomogeneity of the sample. These changes may affect the determination of the degree of aggregation. The application of the ultrasonic method may be restricted, if large aggregates form in the sample during heat treatment. Further work is required to assess the ultrasonic method in more detail with regard to compositional and processing variables in heating experiments which may lead to different α-la aggregate structures.

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