Effects of Mechanical Agitation, Heating and pH on the Structure of Bovine Alpha Lactalbumin

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Abstract Functional properties of bovine alpha lactalbumin (α -LA) are related to its structure and are governed by changes in conformation of protein molecules. The effects of mechanical agitation, high temperature and pH on the structural changes of α -LA were examined by circular dichroism (CD), differential scanning calorimetry (DSC), spectrofluorometry, spectrophotometry, and electrical conductivity methods. The α -helix content decreased significantly below pH 5 and above pH 10. The transition enthalpy values at pH 2, 6.7 and 12 were 91.5, 111.6 and 100.3 J/g, respectively. Heating α -LA from 60 to 100°C at pH 6.7, resulted in the increase of absorbance and electrical conductivity and the decrease in fluorescence intensity. Higher speed agitation decreased the α -helix content considerably. It is concluded that α -LA undergoes a conformational change due to strong acid and alkaline pHs, high temperatures and strong agitation.

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Raw milk is pasteurized above 60°C to improve its keeping quality and to destroy the pathogenic microorganisms. During processing time, milk passes through pumps, cream separator, clarifier and homogenizer where every particles are affected with agitation or pressure. High pressure is applied for milk or whey processing by ultrafiltration and reverse osmosis. Heat treatment and application of pressure in milk is essential in the production of certain milk products, affects denaturation of α -lactalbumin (α -LA) and other whey proteins. Depending on the extent and duration of the applied heat and pressure, the yield of the product and the functionality of the constituent proteins may be altered. Thus, fundamental effect of heat and pressure charac-

teristics on the structure of α -LA may provide some insight in designing and optimizing the quality and yield of the milk products for the dairy expert.

Bovine α -LA is a globular whey protein with low molecular weight (14,200). Its physico-chemical properties have been extensively studied because of its importance in milk industry^{1,2,4,5,7,9,13,17,18,26,27,29}. It contains 123 amino acid residues including four tryptophan residues, which account for its high absorbance at 295 nm. Larson and Roller¹⁸ reported that α -LA is the most heat resistant whey protein, as only 50% denaturation occurred upon heating at 77°C for 30 min, and 80% at 85°C for 18 min. On the contrary, Ruegg *et al.*²¹ reported that it is one of the least heat-stable

milk protein as evidenced by calorimetry. Hiraoka *et al.*¹⁰⁾ reported that α -LA is a metalloprotein binding one mole of calcium per mole of protein, which is essential for maintaining the native conformation of the protein. Below pH 4, α -LA undergoes a conformational change, which can be observed by circular dichroism (CD)²⁶⁾ and is accompanied by the release of bound calcium^{15,16)}. Aggregation occurs at pH 2, when its net positive charge is maximum¹⁴⁾. Maes *et al.*¹⁹⁾ reported that α -LA is stabilized by 4 disulfide bonds together with 30–40% α -helix while Herskovits and Mescanti⁹⁾ stated that it contained 26% α -helix, 14% β -conformation and 60% random coils. However, most of the studies on the conformation of α -LA have been concerned with the intact protein molecule. Very few studies have been done yet to investigate the effects of mechanical agitation and high temperature on stability of α -LA at a wide range of pH. In the present study, high speed agitated α -LA at pH 6.7 unheated and heated α -LA from pH 1 to 14 were taken and irreversible conformational change was studied by monitoring the CD and UV spectrum and comparing with that of the intact protein. Changes in the charge density of α -LA solution heated at pH 6.7 were studied by electrical conductivity. The aim of this study was, therefore, to examine the irreversible changes in the structure of α -LA induced by heating and agitation at various pH levels.

Materials and Methods

Materials

All chemicals used were of analytical reagent grade. Lyophilized bovine α -LA was obtained from Sigma Chemical Company (St. Louis, MO, USA).

Preparation of α -LA sample solution

After dissolving α -LA in deionized water, insoluble materials were removed by centrifugation at 20,000 g for 1 h. The supernatant was dialyzed against deionized water at 5°C for 24 h. Concentration of protein in the

supernatant was determined by the method of Gornall *et al.*⁶⁾ using bovine serum albumin as a protein standard and its final concentration was adjusted to 0.1 mg/ml. Purity of α -LA was checked by electrophoresis (SDS-PAGE). pH was adjusted by adding 0.5 M NaOH or HCl, and various buffers such as potassium biphthalate (pH 4), potassium acid phthalate–NaOH (pH 5), and potassium phosphate mono-basic–NaOH (pH 6 to 9) were then added to obtain a final concentration of 0.05 M to maintain pH values. Glycine, HCl and NaOH solution (1M) were used to adjust the pH of α -LA solutions to below pH 4 and above 9, respectively.

Heat treatment of α -LA

Test tubes containing α -LA solution (pH 6.7) were tightly sealed and heated for 15 min at various temperatures ranging from 60 to 100°C, and cooled down to 20°C with ice water. Absorbance, fluorescence intensity, electrical conductivity and circular dichroism of the cooled samples were then measured.

Mechanical agitation

A Labo LP-15 motor (Iwaki Co. Ltd. Tokyo, Japan) was used to agitate the α -LA solution. A beaker containing 300 ml α -LA (0.1 mg of protein/ml) in 0.05 M phosphate buffer (pH 6.7) was placed on a supporting stand. An impeller fitted with sharp blades was immersed in the solution and rotated continuously at 3,200 rpm to develop vigorous agitation of α -LA as it reflected comparable agitation of milk by small capacity milk transporting pumps in dairy plants. Temperature of the solution was maintained at 20°C. Aliquots were taken at an interval of 15 min. Contents were allowed to settle for 5 h to remove air prior to estimating the components of secondary structure. Temperature of α -LA solution was kept at 20°C throughout the experiment. All experiments were repeated at least 5 times.

Absorbance, fluorescence intensity and electrical conductivity (EC)

α -LA solution at pH 6.7 was chosen for

absorbance, fluorescence intensity and electrical conductivity (EC) measurements as it reflected the pH of natural milk.

Absorption spectra of the cooled α -LA samples were measured using a Jasco Ubest-50 UV spectrophotometer (Tokyo, Japan) at 20°C. Maximum absorbance (λ -max) at 280 nm was recorded. Fluorescence spectrophotometry was used to examine conformational changes in the protein. Fluorescence intensity at λ -max (295 nm) was measured using a Jasco FP-770 spectrofluorometer having quartz cells with a path length of 1 cm. Fluorescence emission spectra were recorded over the wavelength range of 280 to 340 nm with excitation at 280 nm.

EC was measured using a Horiba conductivity meter DS-15 (Kyoto, Japan) at 20°C.

Differential scanning calorimetry (DSC)

A Thermoflex differential scanning calorimeter DSC-8240A (Rigaku Denki Co. Ltd., Tokyo, Japan) was used to test the effects of pH on thermodynamic characteristics of α -LA. Hermetically sealed aluminum pans were used to hold 20 mg α -LA solution (5% w/v protein concentration) at pH 2, 4, 6.7, 8 and 12. A pan containing 20 mg deionized water was used as the control. Temperature was raised at 5°C min⁻¹ over the range of 40 to 95°C. At least 5 replicates of each treatment were scanned.

Circular dichroism (CD)

A Jasco J-700 spectropolarimeter (Japan Spectroscopic Co. Ltd. Tokyo, Japan) was used to monitor CD spectra of α -LA solution. The quartz cells with a path length of 2 mm were used. Low concentration of protein (0.1 mg/ml) was chosen to minimize the error in CD measurements. The temperature of α -LA solution was kept at 20±0.1°C by circulating water in a jacket around the cuvette. Molar ellipticity (θ) of the protein was used to estimate α -helix, β -sheet, β -turn and random coil as per Yang *et al.*²⁹⁾. The software of Yang *et al.*²⁹⁾ was considered applicable to estimate the α -helix, β -sheet, β -turn and random coil in

bovine α -LA. Each solution was scanned 3 times.

Results and Discussion

Colloidal suspension-gel transition of a protein depends on the type and concentration of protein, temperature, type and concentration of incorporated mineral ions and pH of the solution. Shukla²⁴⁾ reported that α -LA is the most heat stable whey protein. Intact α -LA in room temperature and near the natural pH of milk exists in rigid globular structure and consists of fixed number of secondary structural components. Heat-induced aggregation is usually preceded by a conformational change in protein molecule. In the case of bovine α -LA heat treatment above 85°C was found critical to its structural changes. The increase in optical density of α -LA solution is thought to be due to the heat aggregation of α -LA. We thought that the heat aggregation of α -LA was caused by the exposure of hydrophobic amino acid residues at higher temperatures. It is now accepted that hydrophobic interactions play an important role in maintaining the conformational integrity of globular proteins, and they also appear to be involved in the formation of complex intramolecular structures, e.g., α -helices⁵⁾. The absorbance of α -LA solution gradually increased with an increase in temperature from 60 to 85°C, and rapidly increased thereafter (Table 1). Low concentration of α -LA did not form visual precipitation upon heating from 60 to 100°C for 15 min. In the case of β -LG, however, visual precipitation occurred at temperatures above 75°C. The variation in response of these two proteins suggests that heat-induced aggregation and conformational changes are not identical. The results were in agreement with UV measurements at 280 nm by Jinnie *et al.*¹²⁾, who reported a sharp increase of absorbance of α -LA heating from 80 to 100°C. We consider that the intact structure balancing internal and external forces of α -LA molecule perhaps became

Table 1. Effect of heating on absorbance and conductivity of bovine α -LA solution

Temperature (°C)	Conductivity (μ S/cm)	Absorbance (280 nm)
20	20.70	0.2490
60	20.81	0.2490
65	21.23	0.2495
70	21.42	0.2500
75	21.61	0.2520
80	21.62	0.2520
85	22.67	0.2540
90	22.72	0.2550
95	22.74	0.2580
100	22.79	0.2620

(0.1 mg/ml in 0.05 M phosphate buffer, pH 6.7).

unbalance at higher temperatures and protein-protein interaction resulted in the aggregates which caused the rise in turbidity of protein solution.

The processing of milk and milk products, in general, involves conditions that favor deamidation. Previous studies have reported that deamidation improved flavor and color of food products due to the release of ammonia, which could then react with sugar via the Maillard reaction^{22, 28)}. Because α -LA contains a significant proportion of glutamine and asparagine which may undergo deamidation, it is important to examine such heat-induced changes. Cecilia³⁾ reported that deamidation increased the negative charge of the protein that must be considered in the analysis of protein modifications by the thermal treatment under acidic conditions. The changes in EC, therefore, reflect changes in charge density that may develop due to deamidation. The increase in EC with an increase in temperature was gradual from 60 to 85°C but it was marginal beyond 85°C (Table 1). Heating of α -LA solution from 60 to 100°C at an interval of 5°C (Table 1) shows a small changes in absorbance and in EC, respectively. These small changes, however, were sufficient to suggest the conformational change of protein molecules.

Conformation and charge density of protein molecule are considered very important properties in gelation and emulsification processes. Upon heating α -LA at pH 6.7 beyond a critical temperature, its characteristic properties were altered significantly and the native state of protein conformation was disrupted. A gradual decrease in α -helix content was noticed with an increase in temperature from 60 to 85°C (Fig. 1). The heated aliquots of α -LA were cooled down to 20°C prior to monitor the CD spectra. The major components of the secondary structure (α -helix, β -sheet and β -turn) started to change above 60°C and critical irreversible changes in secondary structure was observed beyond 85°C. The results thus suggest that gradual transformation of α -helix, β -sheet and β -turn in the direction of random coil occurred at temperatures above 85°C. β -sheet and β -turn of α -LA were more affected by heating at above 85°C.

In big dairy industries high capacity milk pumps, cream separator, clarifier and homogenizer are being used in the processing line of milk and milk products and these equipments exert strong agitation or pressure to every milk particles. Our result showed that continuous and high speed mechanical agitation of α -LA solution at pH 6.7 brought about significant changes in the secondary structure (Fig. 2). The α -helix content decreased gradually with an increase in agitation time, but β -sheet and β -turn components varied at random. The results support the suggestion that the repeated agitation modified the protein network principally by disruption of α -helix²⁵⁾.

The proportion of various components in the secondary structure of intact α -LA was significantly altered by pH, both acidic and alkaline (Fig. 3 A). A complete collapse of the secondary structure occurred at pH 14. In the alkaline range, α -helix content was maximum at pH 10 and minimum at pH 14. In the acid range, it was minimum at pH 2. However, no consistent trend was noticed in the percent of

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Fig. 1. Effect of heating on the secondary structure of α -LA. Bovine α -LA solutions (0.1 mg/ml in 0.05 M phosphate buffer at pH 6.7) were heated at various temperatures for 15 min and cooled down to 20°C. The CD spectra were monitored at 10 nm min⁻¹ in the far UV region (190–270 nm) at 20°C: (□) α -helix; (▲) β -sheet; (■) β -turn; (△) random coil.

Fig. 2. Effect of agitation on the proportion of the secondary structure of bovine α -LA.

α -LA solutions (0.1 mg/ml in 0.05 M phosphate buffer, pH 6.7) were agitated at 3200 rpm in 20°C and the CD spectra were monitored at 10 nm min⁻¹ in the far UV range (190–270 nm) at 20°C. Symbols are the same as in Fig. 1.

β -sheet and β -turn, especially at below pH 5 and above pH 10. There was small changes in α -helix content of the heated α -LA at pH 1 to 4 (Fig. 3B). Significant changes occurred, however, upon heating in the alkaline range. The entire secondary structure collapsed at pH 14, and only β -sheet and random coil remained at pH 13. The heated α -LA thus lost the entire

α -helix and β -turn components at pH above 12. Previous reports indicated that over 50% of the original α -LA remained upon heating at 77°C for 30 min¹⁸⁾ and that α -LA was resistant to heat-induced coagulation below pH 4¹¹⁾. Hiraoka *et al.*¹⁰⁾ has reported that each α -LA molecule can tightly bind one Ca^{2+} ion which is compulsory to maintain its native (N) state.

Fig. 3. Effect of pH on the secondary structure of intact (A) and heated α -LA (B). The CD spectra of bovine α -LA solutions (0.1 mg/ml at pH 1 to 14) were monitored at 10 nm min⁻¹ in the far UV range (190–270 nm) at 20°C. The sample solutions were heated at 85°C for 15 min and cooled down to 20°C. Symbols are the same as in Fig. 1.

Sogami *et al.*²³⁾ used intrinsic fluorescence spectrophotometric method to examine the conformational changes in bovine plasma albumin. Because the fluorescence spectroscopy largely measures changes in the environment of tryptophan and tyrosine residues, the method was considered appropriate for investigations with α -LA, which contains 4 tryptophan and 4 tyrosine residues per molecule. Fluorescence intensity decreased gradually upon heating from 60 to 90°C, perhaps due to the changes in Trp environment of α -LA at moderately high temperatures and remained constant, perhaps due to the critical level of heat-induced denaturation at temperatures above 90°C (Fig. 4). Ptitsyn²⁰⁾ reported that fluorescence intensity increased during the state of molten globule-like structure of the protein molecule and decreased during the collapse phase. Hamada *et al.*⁷⁾ used formic acid to cleavage the peptide bond of α -LA between Asp⁶⁶ and Pro⁶⁷ and reported that the α -helix content was decreased compared to intact α -

LA.

Transition peak temperatures and enthalpy of α -LA varied with the change in pH. The range of denaturation temperature in different pH treatments were, however, mostly similar to those in the control (pH 6.7) (Table 2). The peak denaturation temperatures at pH 2, 4, 6.7, 8 and 12 were 63.3, 67.1, 67.6, 67.3 and 66.1°C, respectively. Differential scanning calorimetry (DSC) of 5% α -LA solutions showed that the denaturation enthalpy of α -LA, which was minimum at pH 2, increased up to pH 6.7 and then declined to an alkaline range. We consider that higher protein concentration (5% w/v) such as the condition of the DSC measurement perhaps enhanced the protein-protein interaction and the heat-induced denaturation was pronounced below 85°C but in the case of CD measurement protein concentration was very low (0.1 mg/ml) and the reaction of intra-protein molecules was weaker at higher temperatures. Therefore, the discrepancy in the DSC and CD results might be caused by the

Fig. 4. Effect of heating on fluorescence intensity of α -LA. Bovine α -LA solutions (0.1 mg/ml in 0.05 M phosphate buffer, pH 6.7) were heated at various temperatures for 15 min and cooled down to 20°C. Maximum fluorescence intensities at 295 nm were plotted against temperatures.

Table 2. Thermodynamic values T_o , T_p , T_c and enthalpy (ΔH) of α -LA at different pHs

pH	T_o (°C)	T_p (°C)	T_c (°C)	Enthalpy (ΔH) (J/g)
2	57.3	63.3	74.1	91.5
4	61.4	67.1	76.2	94.7
6.7	60.6	67.6	77.1	111.6
8	61.1	67.3	76.1	107.6
12	58.4	66.1	73.8	100.3

T_o =Onset temperature; T_p =Peak temperature; T_c =Conclusion temperature.

experimental conditions. Our DSC and CD results were in agreement with Shukla²⁵⁾ who has reported that some 20% of α -LA remained undenatured by a heat treatment at 85°C for 18 min but DSC showed thermal denaturation, enthalpy and entropy changes above 60 to 85°C. Application of low concentration of α -LA in DSC did not allow detection of T_o , T_p , T_c and ΔH , therefore 5% (w/v protein) α -LA was scanned. In a study of structural and conformational changes of β -lactoglobulin B: Hector *et al.*⁸⁾ has reported that after completion of the thermal denaturation of this protein β -sheets and α -helices were still present and the rigid globular structure was not completely unfolded by heat. DSC result suggested that α -LA is more stable to heat near the natural pH of milk in comparison with other pHs.

The results of the present study show the changes of characteristics properties of secondary structure of bovine α -LA at each pH value. The α -helix content gradually changed on heating above 60°C. High speed agitation also led to destroy some helices of α -LA. Heating of α -LA not only changed the components of secondary structure but also increased the charge density. Our findings also suggested that after completion of the thermal denaturation of α -LA, the rigid globular structure was not entirely unfolded by heat.

Conclusions

The results demonstrated that the secondary structure of bovine α -LA was pH dependent and heating above 85°C for 15 min caused rapid alteration. The secondary structure was insignificantly altered by heating at pH 1 to 4, but it was significantly changed at pH above 10. It is suggested that industrial processes, where coagulation of α -LA upon heating is undesirable, must be performed at pH below 4. In order to utilize bovine α -LA as a food ingredient without much loss of its native conformation, it is concluded that α -LA must be processed with slow agitation at temperatures not exceeding 60°C and at pH value near that of natural milk. Thus, it is expected that the findings of this study may be useful for developing new approaches to the utilization of α -LA.

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ウシ α -ラクトアルブミンの構造変化に及ぼす機械的攪拌、 加熱温度および pH の影響

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ウシ α -ラクトアルブミンの機能特性は、その分子構造とその構造変化に関連している。 α -ラクトアルブミンの機械的攪拌や高温処理、pH に対する構造変化を円二色性測定、示差熱走査熱量測定、蛍光強度、紫外吸収および電気伝導度の変化によって調査した。pH 5 以下および 10 以上で α -ヘリックス量は、明らかに減少した。pH 2, 6.7, 12 における変性の吸熱エンタルピーは、それぞれ 91.5, 111.6, 100.3 J/g であった。60°C から 100°C で α -ラクトアルブミンを加熱すると紫外吸収と電気伝導度は増加したが、蛍光強度は減少した。また、機械的攪拌は、 α -ラクトアルブミンの α -ヘリックス量を減少させた。 α -ラクトアルブミンは、強酸や強アルカリ、高温、攪拌によって構造変化を起こした。

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