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Received for review October 31, 1984. Accepted January 28, 1985. This research was funded in part by U.S. Environmental Protection Agency Grant No. 806421. Paper No. 9549 of the North Carolina Agricultural Research Service, Raleigh, NC 27695-7601.

Effect of $FeCl_3$ on Heat Denaturation of β-Lactoglobulin A in Acid Media

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Effects of $FeCl_3$ on the heat denaturation of β-lactoglobulin A (β-Lg A) in acid media were investigated by circular dichroism (CD) and immunochemical techniques. β-Lg A (0.1% solutions at pH 1.5, 2.0, 2.5, and 3.0) heated (90 °C, 15 min) with $FeCl_3$ ($FeCl_3$ (mole)/β-Lg A (mole) = 5 or 10) was partially and irreversibly unfolded as indicated in the CD spectra, but its partially disordered structure retained some β-Lg A antigenecities. Heating (90 °C, 15 min) of β-Lg A (0.1% at pH 1.5) with $FeCl_3$ ($FeCl_3$ (mole)/β-Lg A (mole) = 10) decreased the maximum immnoprecipitation to about 83% of those by native β-Lg A and heated β-Lg A without $FeCl_3$. Thus, $FeCl_3$ promoted the irreversible denaturation of β-Lg A in combined effect with heating.

Proteins occurring in cheese whey are recovered as precipitate by heating whey solution below the isoelectric point, then cooling it, and adjusting the pH to 4.5. The recovered proteins which were neutralized and spray-dried possessed superior solubility and functional properties (Harwalkar, 1979; Harwalkar and Modler, 1981). It was also demonstrated that the protein fraction denatured by heating at pH 2.5, rendered insoluble at pH 4.5, was unfolded partially and irreversibly (Harwalkar, 1980a). When $FeCl_3$ was added to whole whey, the protein recovery as precipitate was increased whereas the solubility of the protein was reduced (Modler and Emmons, 1977). Amantea et al. (1974) produced an iron-fortified whey protein concentrate by heating concentrated whey at pH 2.5–3.5. The resulting product had an excellent amino acid profile and good functional properties, although high solubility was limited to the neutral pH range.

β-Lg A normally exists at neutral pH as oligomers of the 18400 dalton monomer and the degree of association varies as the pH changes (McKenzie, 1971). At pH 2.0 approximately 90% of the protein has been reported to be present as the monomer (McKenzie and Ralston, 1973; Townend et al., 1960). Because such a low pH would prevent interchange reaction of the protein, heat denaturation of β-Lg at low pH has been studied by several workers (Ananthanarayanan et al., 1977; Ananthanarayanan and Ahmad, 1977; Harwalkar, 1980ab). But none of the earlier investigations was performed on β-Lg A in the presence of $FeCl_3$.

In the present investigation, a comparison has been made of the heat denaturation of β-Lg A with and without $FeCl_3$ in acid media by means of circular dichroism (CD) and immunochemical techniques. The results obtained are expected to provide basic information on the methods of whey protein preparation.

MATERIALS AND METHODS

Preparation of β-Lg A. β-Lg A was prepared from the milk of homozygous cows by using the procedure of Armstrong et al. (1967). The prepared protein which was electrophoretically pure was dialyzed exhaustively in distilled water and lyophilized.

Heat Treatment. The lyophilized β-Lg A was dissolved in aqueous solution up to a final concentration of about 1 mg/mL, and the pH was adjusted to the desired values (pH 1.5–3.0) by addition of 1 N HCl. Heat treatment of β-Lg A was conducted by placing 3 mL of β-Lg A solution in test tubes (1.2 × 14 cm), which were positioned in a rack and immersed in a controlled temperature water bath. The test tubes were shaken gently for 30 s and then kept at 90 °C for 15 min. Strict corrections for temperature lag were not made. Each sample was cooled immediately after heat treatment by placing the tubes in ice water.

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CD Spectra Measurements. CD spectra of samples were measured at 25 °C with a JASCO J-40CS spectropolarimeter, and mean residue ellipticities [θ] were calculated on the basis of an average residue weight of 112.

Thermal Transition Experiments. To monitor the changes in the conformation of β-Lg A, the changes in the CD at 222 nm were measured. β-Lg A solution in a stoppered cuvette was placed in a thermostatically controlled cell holder, and the temperature was varied in steps of 3–10 °C. At each temperature the CD was measured with time until constant values were observed. This usually took 2–5 min. Moreover, the β-Lg A solution, heat treated above, was cooled in steps of 3–10 °C, and the CD was measured in a manner similar to that above to test the reversibility of the thermal transition. During measurements the temperature of the solution was controlled by water through the cell holder jacket from a constant temperature bath and monitored continuously by means of a thermocouple connected to a digital thermometer.

Rabbit Anti-β-Lg A Serum. A rabbit was immunized subcutaneously by the injection with 1 mg of β-Lg A in 1 mL of 0.15 M NaCl/0.01 M phosphate buffer, pH 7.2, emulsified with Freund's complete adjuvant. The rabbit was then given two booster injections of the same antigen 14 and 28 days after the first immunization. Bleeding was performed 10 days after the last booster injection. The serum was separated by centrifugation and stored at –80 °C.

Immunodiffusion. Ouchterlony analyses (Ouchterlony, 1949) were performed in 1.0% agarose in phosphate-buffered saline containing 0.1% NaN_3. The 5-μL samples (100–150 μg of antigen/mL) to be tested were applied to each well of Ouchterlony plates. The plates were incubated at 25 °C for 15 h.

Quantitative Immunoprecipitation. The 50-μL antiserum diluted four times with phosphate-buffered saline was added to solutions containing various amounts of native and heated β-Lg A and made up to 200 μL with phosphate-buffered saline. The solutions were incubated at 37 °C for 60 min and then at 4 °C for 24 h. A correction for nonspecific precipitation was made by analyzing controls containing only antiserum under identical conditions. After centrifugation at 1000*g* for 10 min, the precipitates were washed with cold phosphate-buffered saline and centrifuged again. The supernatants were inverted over filter paper to drain liquid from the precipitates. The precipitates were dissolved completely in 200 μL of 0.05 M NaOH, and protein concentration was measured according to the method of Lowry et al. (1961) with rabbit IgG ($E^{1\%}{}_{1\,cm}$ = 14.0) as standard.

RESULTS

Figure 1 shows the effect of added $FeCl_3$ concentrations on heat-induced conformational changes of β-Lg A which were determined by CD. The conformational changes of β-Lg A in the presence of $FeCl_3$ occurred in equal moles of $FeCl_3$ and β-Lg A and were not promoted by further increases in amounts of $FeCl_3$.

Figure 2 shows the CD spectra of native and heated β-Lg A at pH 1.5 with and without $FeCl_3$. The spectra of native β-Lg A in spite of the presence and absence of $FeCl_3$ were characterized by the negative extreme at 216 nm with ellipticity of about –5750. The spectrum of heated β-Lg A without $FeCl_3$ indicated almost the same pattern as that of an unheated sample. Such patterns were almost identical with those obtained by Sawyer et al. (1971) at pH 6.6. On the other hand, when β-Lg A was heated in the presence of $FeCl_3$, the spectrum was characterized by a change to more negative ellipticity between 230 and 210 nm with the disappearance of the negative maximum at 216 nm.

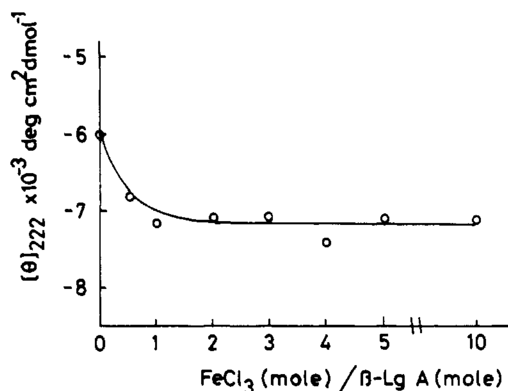

Figure 1. Effect of added $FeCl_3$ concentration on heat-induced conformational changes of β-Lg A. Heating condition: pH 1.5, 90 °C, 15 min. [$FeCl_3$(mole)/β-Lg A(mole) = 10 corresponded to 0.56 mM $FeCl_3$ in the protein solution.]

Effects of pH (2.0, 2.5, and 3.0) at heating on the conformational changes of β-Lg A in the presence and absence of $FeCl_3$ were examined by using the same CD technique as in Figure 2. Every CD spectrum of the acidified samples was not very different from those of the corresponding samples with and without $FeCl_3$ in Figure 2. These results indicated that pH in the acidic side did not induce the great conformational changes under heat treatment at temperatures below 90 °C, whereas $FeCl_3$ promoted them.

The variations in the CD at 222 nm brought about by varying the temperature of β-Lg A with or without $FeCl_3$ are shown in Figure 3. The transition was effected between 67 and 90 °C without $FeCl_3$ and between 67 and 84 °C with $FeCl_3$ and had a midpoint at 78 and 76 °C, respectively. The negative ellipticity at 222 nm in both samples increased slowly with a rise in temperature from 25 to 60 °C, and it varied rapidly, as the temperature was increased from 60 to 98 °C, from –6100 to –7080 in Figure 3-A and from –6100 to –6950 in Figure 3-B.

These changes were not reversible: as the temperature was decreased from 98 to 25 °C, the changes in magnitude of negative ellipticities from –7080 to –6850 in Figure 3-A and from –6950 to –7150 in Figure 3-B could be observed. Actually, negative ellipticity in the sample with $FeCl_3$ was further increased by cooling.

The antigenicity of heated β-Lg A with and without $FeCl_3$ was analyzed with the immunodiffusion method of Ouchterlony. Either native β-Lg A or the acidified and heated β-Lg A without $FeCl_3$ revealed a single precipitin arc. The precipitin arcs of β-Lg A heated with $FeCl_3$ in acid media also fused with each other, and no spur was detected. So, it could be said that even β-Lg A heated with $FeCl_3$ retained its antigenicity. These results were confirmed by the quantitative immunoprecipitation. As seen in Figure 4, native β-Lg A and heated β-Lg A without $FeCl_3$ gave precipitin curves with maximal precipitation at 10 μg of the antigen addition, where the maximum immunoprecipitation of heated β-Lg A with $FeCl_3$ was about 83% of those of the above-mentioned samples.

DISCUSSION

The ellipticity at 222 nm in Figure 2 and 3 was more negative for the heat-denatured state in comparison to the native β-Lg A. This phenomenon has been previously observed (Ananthanarayanan and Ahmad, 1977, Sawyer et al., 1971). Generally, the heat-denatured state is known to have a less ordered structure which shows more positive ellipticity in the vicinity of 222 nm. However, during the transition from the native β-Lg to the guanidine–HCl denatured state, $[\theta]_{220}$ was found to undergo a change

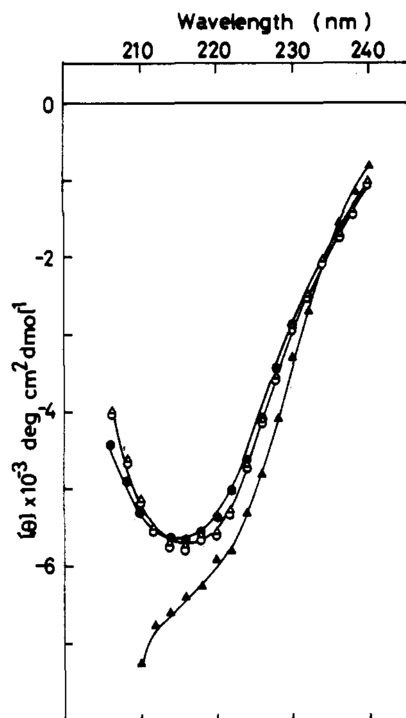

Figure 2. CD spectra of native and heated β-Lg A with and without $FeCl_3$. Native β-Lg A (○); heated β-Lg A (●); native β-Lg A with $FeCl_3$ (△); heated β-Lg A with $FeCl_3$ (▲). Heating condition: pH 1.5, 90 °C, 15 min. $FeCl_3$(mole)/β-Lg A(mole) = 10.

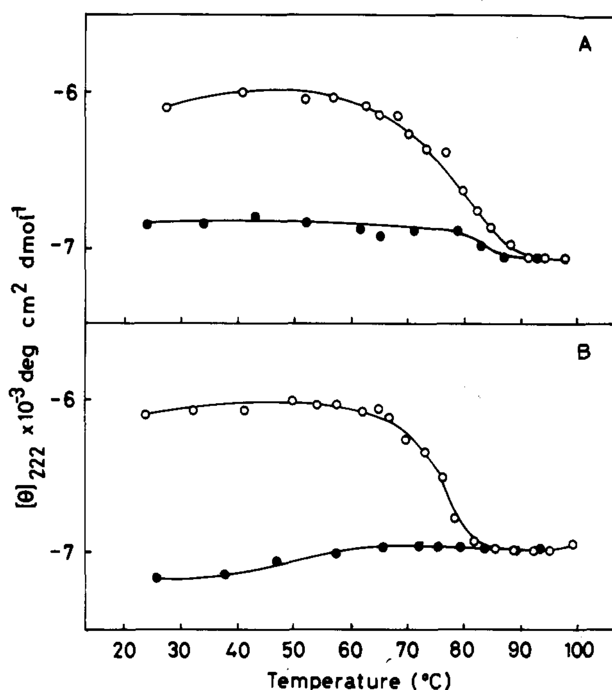

Figure 3. Heat-induced conformational changes of β-Lg A monitored by variation in CD at 222 nm. A: β-Lg A heated without $FeCl_3$ (pH 1.5). B: β-Lg A heated with $FeCl_3$ (pH 1.5). $FeCl_3$(mole)/β-Lg A(mole) = 5. Experimental values were obtained by heating (○) or cooling down (●) protein solutions and keeping them at the temperature indicated until constant values were observed.

toward more negative values at lower concentrations of guanidine–HCl (<2.3 M) before changing to more positive values at higher concentrations of the denaturant (Ananthanarayanan and Ahmad, 1977). Thus, the change to more negative ellipticity of β-Lg during the denaturation has been interpreted in terms of anomalous behavior.

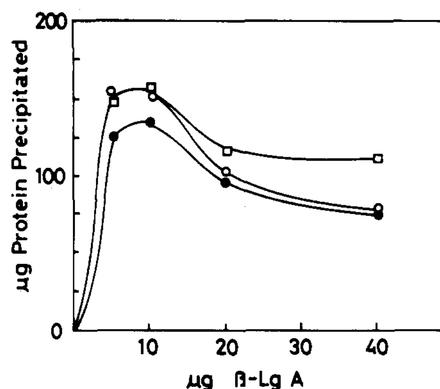

Figure 4. Quantitative immunoprecipitations of native β-Lg A, and heated β-Lg A with and without $FeCl_3$. Native β-Lg A (□); heated β-Lg A (○); heated β-Lg A with $FeCl_3$ (●). $FeCl_3$ (mole)/β-Lg A (mole) = 10. Heating condition: 90 °C, 15 min, pH 1.5.

The recovery of residual protein from cheese whey is important to the dairy industry, and the high-heat, low-pH method of denaturing whey proteins with added $FeCl_3$ has been proved to be a popular and economical means for obtaining high recovery of the proteins as described by Modler and Emmons (1977). The denaturation of proteins prepared by such a procedure is one of the important effecting factors on the functional properties of the preparation. The results reported here show that $FeCl_3$ promoted the partial and irreversible conformational changes of β-Lg A in combined effect with heating in acid media but did not induce considerable denaturation of the protein molecule. Such a denaturation of β-Lg A might result in the increase in recovery and decrease in resolubility of the recovered proteins as precipitate.

Thus, it is necessary to control the partial denaturation for the improvement of functional properties in the preparation of iron-fortified whey protein concentrate. The extent of the partial conformational changes of β-Lg A in the presence of $FeCl_3$ was probably due to the degree of exposure to the high temperature. So, the use of lower temperature should be taken into consideration for the preparation of protein having good functional properties.

Registry No. $FeCl_3$, 7705-08-0.

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Received for review October 15, 1984. Accepted February 1, 1985.