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Plakalbumin Converts to Heat-Stable Form under the Same Condition as an Ovalbumin–s-Ovalbumin Transformation

Shinji Shitamori and Ryo Nakamura*

A solution of plakalbumin, a protein derived from ovalbumin by mild hydrolysis with subtilisin, was adjusted to pH 9.9 and kept at 55 °C for 16 h. The denaturation temperature of plakalbumin measured by differential scanning calorimetry before and after this treatment was 77.2 and 86.6 °C, respectively. The circular dichroism of both kinds of plakalbumin was almost the same, the isoelectric focusing of plakalbumin became broad, and the surface hydrophobicity of plakalbumin increased slightly with the above treatment. All these results suggest that plakalbumin converts to the heat-stable form (s-plakalbumin) after the manner of an ovalbumin–s-ovalbumin transformation.

Ovalbumin has been shown to change to a heat-stable form (s-ovalbumin) during the storage of shell eggs (Smith, 1964). This conversion also occurs in an isolated ovalbumin solution, the rate increasing with pH and temperature (Smith and Back, 1965). Although slight conformational changes were detected by Raman difference spectroscopy (Kint and Tomimatsu, 1979), the gross conformation of ovalbumin and s-ovalbumin was almost the same (Smith and Back, 1968; Nakamura et al., 1980).

In previous studies (Nakamura et al., 1980, 1981; Nakamura and Ishimaru, 1981), the authors compared the properties of ovalbumin with those of s-ovalbumin and found some differences; the isoelectric focusing of s-ovalbumin became broader than that of ovalbumin whereas their polyacrylamide gel electrophoretic pattern was identical. Also, the intrinsic viscosity of s-ovalbumin was lower than that of ovalbumin, whereas its hydrophobicity was greater. The relationship of these differences between both proteins with the increased thermal stability of s-ovalbumin, however, is not clear.

It is of interest to know whether proteins other than ovalbumin can be converted to the heat-stable form under the same condition as for ovalbumin–s-ovalbumin transformation. Our previous study on several proteins revealed no conversion to the heat-stable form (Nakamura et al., 1980). The present investigation provides results on the conversion of plakalbumin, a protein derived from ovalbumin by mild proteolysis with subtilisin (Linderstrøm-Lang and Ottesen, 1947), to the heat-stable form by heating at 55 °C for 16 h under the pH of 9.9. This form

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of plakalbumin is hereinafter called s-plakalbumin.

MATERIALS AND METHODS

Ovalbumin. Ovalbumin was prepared from fresh egg white by the ammonium sulfate procedure of Sørensen (Marshall and Neuberger, 1972). The ovalbumin was recrystallized 4 times, dialyzed until free of salt, and dried by lyophilization.

Plakalbumin. Plakalbumin was prepared by digestion of ovalbumin with subtilisin which was purchased from Nagase and Company, Ltd., Osaka, as described by Smith (1968). Briefly, 100 mL of 3% ovalbumin solution (0.2 M phosphate buffer, pH 7.2) was incubated with 3 mg of subtilisin at 25 °C for 60 min. The reaction was stopped by bringing the pH to 5.0 and the solution was applied to a column of Sephadex G-75 (3 × 55 cm) equilibrated with a solution of 0.02 M KCl, pH 5.0. On elution with the same solvent the fast-moving band was gathered, dialyzed until free of salt, and dried by lyophilization.

s-Plakalbumin. s-Plakalbumin was prepared from plakalbumin by the same method as for s-ovalbumin (Smith and Back, 1965); a 5% solution of plakalbumin in water was adjusted to pH 9.9 and then heated for 16 h at 55 °C. After the solution was cooled, a small amount of denatured protein (<1–2%) was precipitated at pH 4.7 and the solution dialyzed and lyophilized.

Measurement of Denaturation Temperature. Differential scanning calorimetry (DSC) thermograms were recorded on a Daini Seikosha Model SSC/560 thermal analyzer with a DSC cell programmed at the rate of 1.0 °C/min temperature increase. Samples of 50 μL of 5% protein solutions solubilized in 0.05 M phosphate buffer (pH 6.7) were pressure-sealed in silver pans weighing approximately 1.47 g. A sealed pan which contained a volume of the buffer equal to that of the sample was used as a reference. α-Naphthylamine and palmitic and stearic acids were used as calibrants for temperature and energy (McClain and Wiley, 1972). The denaturation temperature (T_d) was defined as the temperature of the peak maximum in the endotherm. The reproducibility of T_d was ±0.5 °C.

Isoelectric Focusing. Isoelectric focusing was performed in gel sheets (0.1 × 7.5 × 8.1 cm) containing 7.5% acrylamide and 2% carrier ampholyte (Ampholine, pH 4–6.5). Electrophoresis was carried out at a constant voltage of 200 V for 3.5 h at 4 °C. After electrophoresis, gel sheets were washed, fixed by soaking in 3.5% perchloric acid solution overnight, and stained with Coomassie Brillinat Blue G-250.

Circular Dichroism. Circular dichroism (CD) was measured on a Jasco J-40A spectrophotometer at 20 °C. The data were expressed in terms of molar ellipticity, [Θ]. A protein concentration of about 0.07 mg/mL was used measurements with 1.0-mm path length.

Measurement of Surface Hydrophobicity. The surface hydrophobicity of proteins was determined by the method of Kato and Nakai (1980); Parinaric acid–protein conjugates were excited at 325 nm and the relative fluorescence intensity was measured at 420 nm in a Hitachi fluorescence spectrophotometer, Model MPF-2A. The initial slope (S_0) of the fluorescence intensity vs. protein concentration plot was used as an index of protein surface hydrophobicity.

Polyacrylamide Gel Slab Electrophoresis. Gel sheets (0.1 × 7.8 × 8.1 cm) of 7.5% polyacrylamide gel electrophoresis buffer of Tris–glycine were prepared as described by Davis (1964). $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). The gel sheets were stained with a solution of 0.25% Coomassie Brilliant Blue R-250 in water–2-propanol–acetic acid (5:5:1 v/v/v) and destained with 7% acetic acid containing 3% methanol. The molecular weight of the protein band on the $NaDodSO_4$–polyacrylamide gel electrophoretic pattern was determined by using the low molecular weight calibration kit of Pharmacia Fine Chemicals.

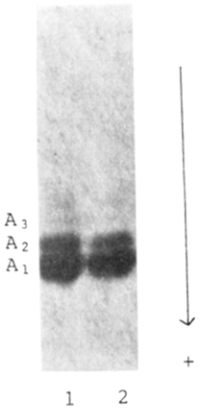

Figure 1. Polyacrylamide gel electrophoresis of ovalbumin and plakalbumin: (1) ovalbumin; (2) plakalbumin.

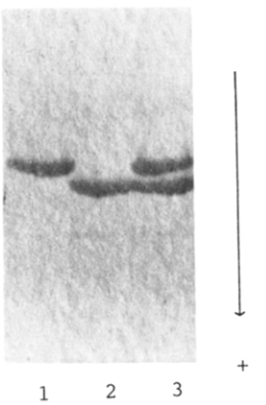

Figure 2. $NaDodSO_4$–polyacrylamide gel electrophoresis of ovalbumin and plakalbumin: (1) ovalbumin; (2) plakalbumin; (3) mixture of ovalbumin and plakalbumin.

Other Analyses. Turbidity of ovalbumin or plakalbumin in $(NH_4)_2SO_4$ solution was measured at 600 nm with a Shimadzu double-beam spectrophotometer, Model CV-200S. Double immunodiffusion was carried out according to Ouchterlony and Nilsson (1978). Rabbit antiserum was raised against ovalbumin.

RESULTS

Identity of Plakalbumin. Figure 1 shows the polyacrylamide gel electrophoretic patterns of ovalbumin and plakalbumin. No noticeable difference could be found between the two proteins; although a thin protein band migrating slower than the A_3 component of ovalbumin was observed in both electrophoretic patterns, there was no contamination of other egg white proteins. This thin band protein could not be identified, but it was assumed to be aggregated portions of either ovalbumin or plakalbumin. Figure 2 shows the $NaDodSO_4$–polyacrylamide gel electrophoretic patterns of ovalbumin and plakalbumin. In this figure, it is clearly seen that plakalbumin gave only one protein band just the same as ovalbumin. Using standard proteins, the molecular weight of this protein band was determined to be approximately 40 000.

As the solubility of plakalbumin in concentrated $(NH_4)_2SO_4$ has been shown to be greater than that of ovalbumin (Linderstrøng-Lang and Ottesen, 1947), turbidity titration of both proteins was carried out. The results are

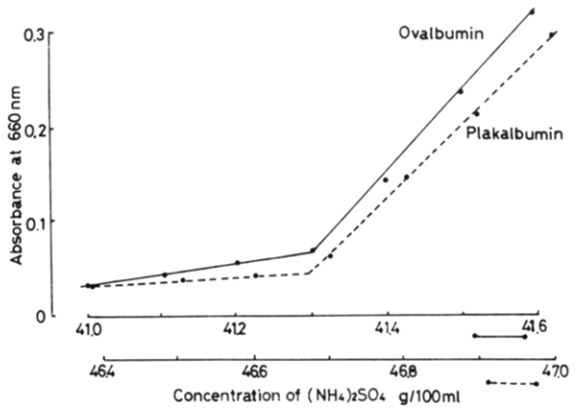

Figure 3. Turbidity titration of ovalbumin and plakalbumin.

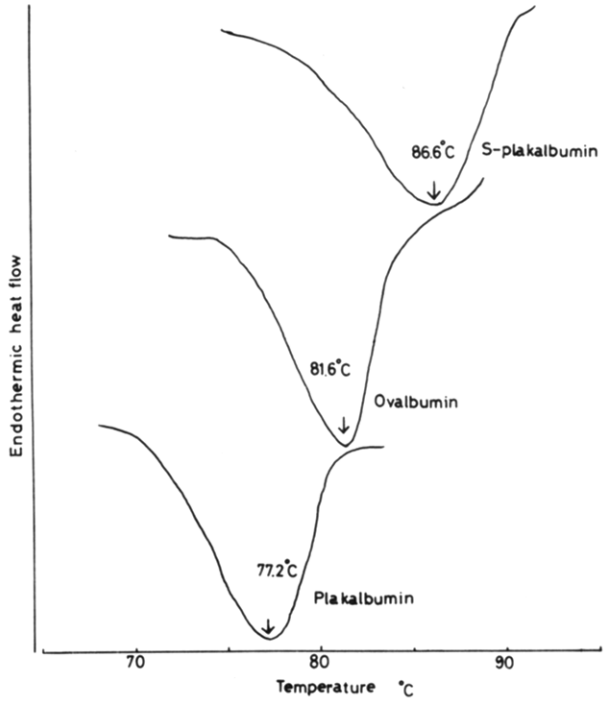

Figure 4. Endotherms of ovalbumin, plakalbumin, and s-plakalbumin. The denaturation temperature of each protein is shown.

shown in Figure 3. In this figure, the turbidity of ovalbumin increased rapidly in the presence of 41.3 g/100 mL $(NH_4)_2SO_4$, but that of plakalbumin increased in the presence of 46.7 g/100 mL $(NH_4)_2SO_4$.

Conversion of Plakalbumin to s-Plakalbumin. Figure 4 shows the thermograms of ovalbumin, plakalbumin, and s-plakalbumin. The denaturation temperature of plakalbumin was lower than that of ovalbumin, but that of s-plakalbumin was higher than that of plakalbumin. The difference of denaturation temperature between plakalbumin and s-plakalbumin is 9.4 °C, which is comparable to the difference of 7.4 °C in denaturation temperature between ovalbumin and s-ovalbumin, obtained by Donovan and Mapes (1976). During the conversion of plakalbumin to s-plakalbumin, no release of peptides or amino acids from the proteins was observed. These results clearly show that plakalbumin can be converted to the heat stable form under the same condition as for ovalbumin–s-ovalbumin transformation.

Properties of Plakalbumin and s-Plakalbumin. As shown in Figure 5, CD spectra of ovalbumin, plakalbumin, and s-plakalbumin were almost the same. No gross structural changes most likely occurred during either the conversion of ovalbumin to plakalbumin or that of plak-

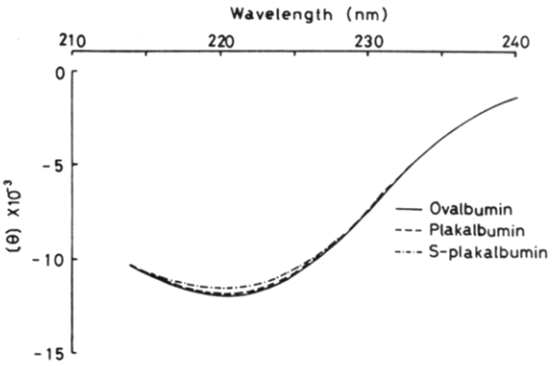

Figure 5. CD spectra of ovalbumin, plakalbumin, and s-plakalbumin.

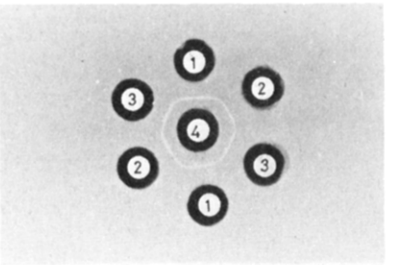

Figure 6. Double immunodiffusion analyses of ovalbumin, plakalbumin, and s-plakalbumin: (1) ovalbumin; (2) plakalbumin; (3) s-plakalbumin; (4) anti-ovalbumin antiserum.

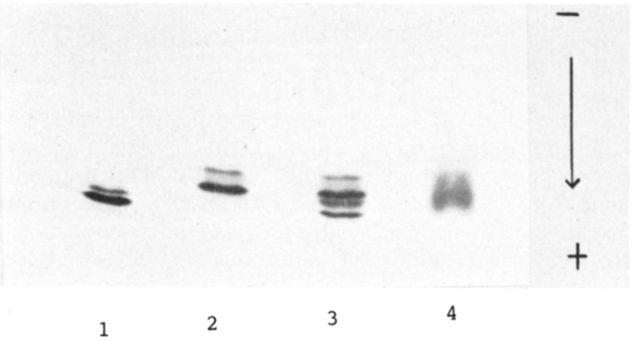

Figure 7. Isoelectric focusing of ovalbumin, plakalbumin, and s-plakalbumin: (1) ovalbumin; (2) plakalbumin; (3) mixture of ovalbumin and plakalbumin; (4) s-plakalbumin.

Table I. Surface Hydrophobicity of Ovalbumin, Plakalbumin, and s-Plakalbumin[a]

ovalbumin	plakalbumin	s-plakalbumin
100	67	100

[a] Values are percent of initial slopes (S_0) of each protein against that of ovalbumin.

albumin to s-plakalbumin. A similar conclusion was obtained from the double immunodiffusion of three kinds of proteins (Figure 6); precipitin lines formed between antibody and either plakalbumin or s-plakalbumin were single and completely fused.

Figure 7 shows the isoelectric focusing of ovalbumin, plakalbumin, and s-plakalbumin. In the isoelectric focusing of ovalbumin two protein bands were noted, the dense band was considered to be A_1 and the thin band was considered to be A_2. A similar situation was also noted in the isoelectric focusing of plakalbumin. These two protein bands of plakalbumin must be P_1 and P_2 of Perlmann (1949). Both P_1 and P_2 bands move slightly to the cathodic side of A_1 and A_2 bands, perhaps due to the release of small peptides containing aspartic acid during the conversion

of ovalbumin to plakalbumin (Ottesen, 1958). There were the broad P_1 and P_2 bands in the isoelectric focusing of s-plakalbumin, just as for s-ovalbumin, as presented in the previous study (Nakamura et al., 1980).

Table I shows the surface hydrophobicity of ovalbumin, plakalbumin and s-plakalbumin. The hydrophobicity of s-plakalbumin was greater than that of plakalbumin, although it was similar to that of ovalbumin.

DISCUSSION

Since studies concerning the preparation and properties of plakalbumin are relatively few, first the properties of plakalbumin found in the present experiment were compared with those of previous studies (Linderstrøm-Lang and Ottesen, 1947; Smith, 1968). Plakalbumin has been shown to release a large peptide of molecular weight 3700, with an N-terminal serine residue, which is firmly bound to the plakalbumin by noncovalent forces and can be isolated by gel filtration in 6 M urea solution (Smith, 1968). This means the molecular weight of denatured plakalbumin is 40 000 or less. In the present experiment, the molecular weight of plakalbumin was measured by using $NaDodSO_4$–polyacrylamide gel electrophoresis in the denatured condition. Actual molecular weight of plakalbumin was about 40 000 and coincided well with the estimated weight. The solubility of plakalbumin in the concentrated $(NH_4)_2SO_4$ increased with the conversion to s-plakalbumin (Figure 3). Although the minimum concentrations of $(NH_4)_2SO_4$ to give the turbidity were slightly different from those cited in the literature (Linderstrøm-Lang and Ottesen, 1947), the ratio of the $(NH_4)_2SO_4$ concentration of ovalbumin to that of plakalbumin (46.7/41.3) was about 1.1, which was almost the same as reported by Linderstrøm-Lang and Ottesen (1947).

The relationship between the properties of plakalbumin and s-plakalbumin is very similar to that of ovalbumin and s-ovalbumin (Nakamura et al., 1980, 1981; Nakamura and Ishimaru, 1981); the gross configuration of s-plakalbumin was almost the same as that of plakalbumin, although the isoelectric focusing of s-plakalbumin gave a broader band and the surface hydrophobicity of s-plakalbumin was greater than that of plakalbumin. All these results suggest that the mechanism of s-plakalbumin formation is the same as that of s-ovalbumin formation from ovalbumin. Since CD spectra of ovalbumin and plakalbumin are almost the same, both small peptides released from ovalbumin during its conversion to plakalbumin and large peptides bound to the plakalbumin by noncovalent forces might not affect the gross configuration of both proteins.

In the previous study (Nakamura and Ishimaru, 1981), the authors showed that the surface hydrophobicity of s-ovalbumin was greater than that of ovalbumin and suggested the exposure of some peptide portions of higher hydrophobic amino acid contents during the ovalbumin–s-ovalbumin transformation. Since the large peptide molecule bound to the plakalbumin by noncovalent forces has large amounts of hydrophobic amino acid (Tompson et al., 1971; Nisbet et al., 1981), a part of the peptide portion might be exposed on the surface of s-plakalbumin during the conversion to s-plakalbumin.

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