Effects of Limited Proteolysis on Functional Properties of Ovalbumin

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We examined the limited proteolysis of ovalbumin by pepsin and its effect on the functional properties of the ovalbumin. Pepsin hydrolyzed only the single peptide bond of ovalbumin between His-22 and Ala-23. This provided a large intermediate (MW 42,500), P-ovalbumin. A P-ovalbumin solution gave a transparent gel when heated. Under the same conditions, an ovalbumin solution gave a turbid gel. We studied the physicochemical properties of P-ovalbumin and the formation of the transparent gel.

Functional properties of food proteins can be improved by chemical modification, but enzymatic modification using proteases, esterases, glycosidases, transglutaminase and so forth are more appropriate for foods from the standpoint of safety. Proteolytic enzymes are among the most useful enzymes for this purpose, as in the use of chymosin for cheese processing. However, most proteolytic enzymes hydrolyze proteins to small peptides and amino acids, and these products generally have poor functional properties as food materials. If we could limit proteolytic enzymes to split a few peptide bonds of the substrate protein, as chymosin (EC 3.4.23.4) does for κ-casein, the enzymes would be more useful for improving the functional properties of proteins.

Here we describe the limited proteolysis of ovalbumin by pepsin and its effect on the gel properties of the products, especially transparency.

Ovalbumin and limited proteolysis. Ovalbumin is a phosphoglycoprotein (1). Its primary structure (2) is shown in Figure 1. Four free sulfhydryl groups are contained in the molecule of 45,000 daltons. These groups do not react with 5,5′dithiobis (2-nitrobenzoic acid) (DTNB) in the native state. Ovalbumin resists proteolytic attack, especially at neutral pH. However, Linderstrom-Lang and Ottesen found that subtilisin (EC 3.4.21.14) splits two or three peptide bonds near the C-terminal end at pH 6.3, producing plakalbumin (3). The sites of splitting are shown in Figure 1. Prolonged enzymatic reaction causes further breakdown of plakalbumin to smaller peptides (4).

Limited proteolysis by pepsin. Pepsin (EC 3.4.23.1) is an acid proteinase very similar to chymosin in its primary structure. Its optimum pH for the hydrolysis of most protein substrates is around 2, but that for small synthetic peptides is around 3 to 4.5, depending on the amino acid sequence of the peptide (5). Therefore, a different reaction pH can result in different products.

Here, ovalbumin was hydrolyzed at pH 2 to 7. α_{s}-Casein was used for comparison. Experimental conditions are given in Figure 2, and the results in Figures 3 and 4. At pH 2, the original band of ovalbumin gradually disappeared. No large intermediate appeared. At pH 4, a new band appeared (MW 42,000). After 12 hr, all of the original band had disappeared, and bands of the large intermediate and a small

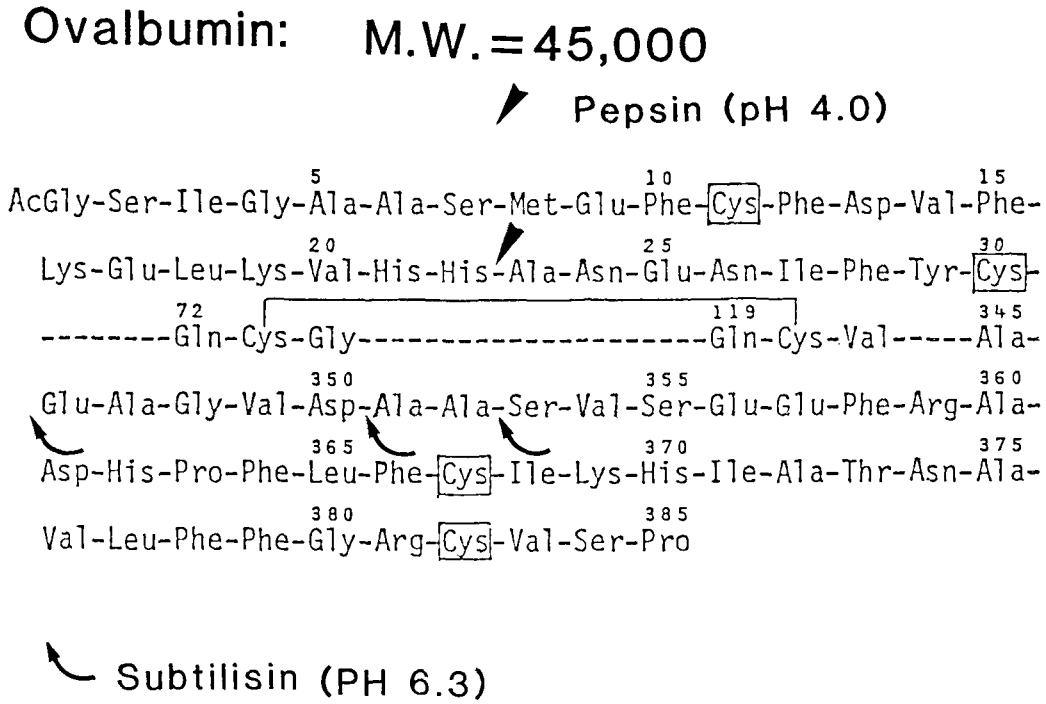

FIG. 1. Primary structure of ovalbumin. The amino acid sequence is cited from Nisbet et al. (2). Curved arrow shows bonds split by subtilisin, and arrowhead shows bond split by pepsin.

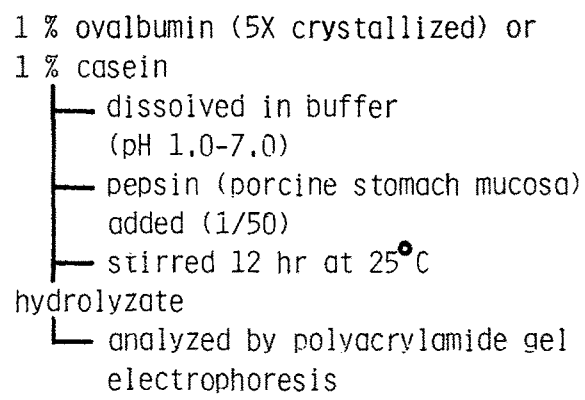

FIG. 2. Peptic hydrolysis of ovalbumin and α_s-casein. Hydrochloric acid-sodium acetate buffer (pH 1-3), sodium acetate buffer (pH 4-5), and sodium phosphate buffer (pH 6-7) were used at 0.1 M concentrations.

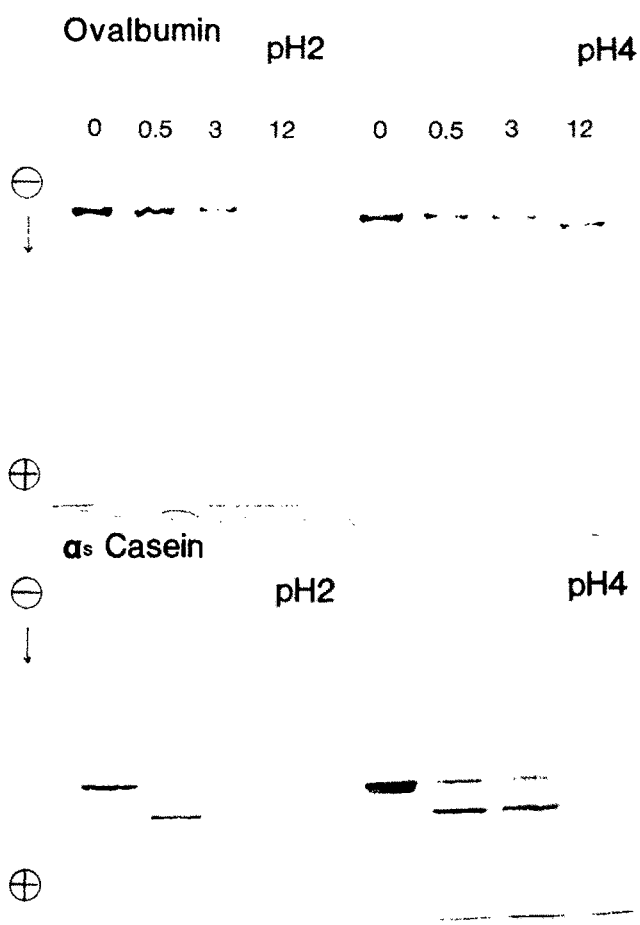

FIG. 3. Sodium dodecylsulfate polyacrylamide gel electrophoresis of the pepsin hydrolyzates of ovalbumin and casein. The enzyme reactions were at 25 C (protein:enzyme, 50:1) at pH 2 and 4. Gel electrophoresis was carried out as described by Laemmli (14), except that 13.5% polyacrylamide was used as a separation gel.

fragment were seen. Similar results were obtained at pH 5 (data not shown), but the large intermediate appeared more slowly. With α_s-casein, a large intermediate was produced at pH 2 and 4, but it was hydrolyzed further after prolonged reaction. The large intermediate produced from ovalbumin did not disappear after 24 hr of reaction. Nor did it disappear when the same amount of pepsin was added again after 12 hr and the reaction continued for another 12 hr at 25 C. That means that the hydrolysis was very limited. The large intermediate and the small fragment were separated by gel filtration (Fig. 5). We then analyzed their amino acid compositions and chemical properties.

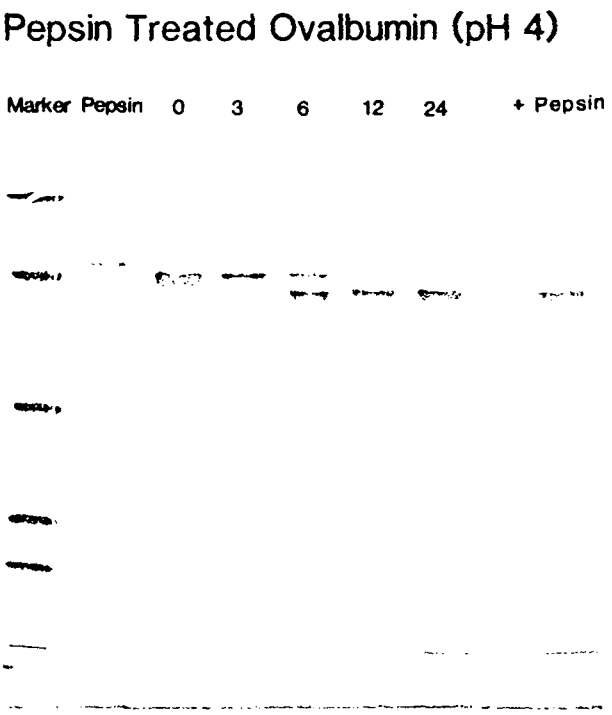

FIG. 4. Sodium dodecylsulfate polyacrylamide gel electrophoresis of limited pepsin proteolyzates of ovalbumin as a function of time at pH 4. Experimental conditions were the same as Fig. 3, except that the reaction proceeded for 24 hr and to one reaction mixture the same amount the enzyme was added after 12 hr, (+pepsin) and left for another 12 hr.

The large intermediate from ovalbumin contained three sulfhydryl groups, one less than the original molecule. Analyses of N-terminal amino acids (not detected for the small fragment, and found to be alanine for the large intermediate) and C-terminal amino acids (histidine for the small fragment and proline for the large intermediate) showed that the split bond was that between His-22 and Ala-23 (Fig. 1). Unlike results reported for subtilisin, pepsin hydrolyzed near the N-terminal end, and only a single peptide bond was split. We named the large intermediate P-ovalbumin.

Reason for limited proteolysis. When we examined the sequence near the N-terminal end of ovalbumin by Kyte and Doolittle's hydropathic index (6), we found that the sequence around the bond hydrolyzed by pepsin was hydrophilic, which means that this portion is exposed on the surface of the protein. The small fragment removed by pepsin is hydrophobic

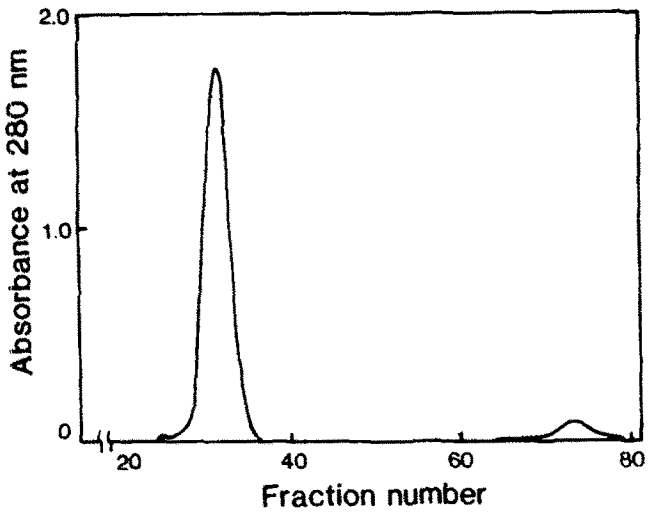

FIG. 5. Separation of the large intermediate and the small fragment from ovalbumin by gel chromatography on a Sephadex G-75 column.

and probably was buried inside the molecule in the native ovalbumin.

We are not sure why only this histidyl-alanine bond was hydrolyzed. One explanation is a change in the substrate specificity of pepsin, as already described. Another explanation is the conformational change in ovalbumin that occurs when the pH changes from 2 to 4. Ovalbumin is relatively stable at acidic pH. The intrinsic viscosity and the circular dichroism (CD) spectrum in the far UV region were the same at pH 2 and 7, which indicates that the conformation of ovalbumin at pH 2 and 7 is not very different. However, the spectra at these pH values were different (Fig. 6). When the absorbance differences at 292 nm are plotted against pH (Fig. 6), the curve is sigmoidal with an inflection point at pH 4. The intensities of fluorescence at 365 nm gave a similar curve when plotted against pH (date not shown). These results suggested that some minor conformational change occurred at acidic pH. The rate of urea denaturation increases at acidic pH, (7) and temperature of heat denaturation decreases at acidic pH (8). Therefore, ovalbumin is more susceptible to denaturation and probably to proteolytic attack at acidic pH. At pH 4 and 5, the molecule is partly altered and thus hydrolyzed by pepsin at only one position.

Physicochemical properties of P-ovalbumin. The molecular weight of P-ovalbumin, 42,500, is 2,500 less than that of ovalbumin. P-ovalbumin has almost the same physiochemical properties as ovalbumin (Table 1). The intrinsic viscosities, CD spectra and difference spectra observed at pH 2 against pH 7 were the same, as were the temperatures of heat denaturation measured by differential scanning calorimetry. P-S-Ovalbumin that had been prepared by alkaline treatment of P-ovalbumin had a heat denaturation temperature close to that of S-ovalbumin. All of these results indicate that P-ovalbumin has almost the same conformation as ovalbumin.

Transparency of P-ovalbumin gel. Some of the functional properties of P-ovalbumin were examined. The stability of the foam from P-ovalbumin was less than that from ovalbumin. A heat-coagulated gel of P-ovalbumin was slightly softer than that of ovalbumin. However, the gel of P-ovalbumin was transparent. The turbidity of heated solutions of ovalbumin and P-ovalbumin was measured by absorbance at 600 nm (Fig. 7) (9). Solutions containing more than 3% protein gave gels after 3 min of heating at 100 C. Even 7% P-ovalbumin gel was transparent (Fig. 8).

Transparent gel made from egg white. We exam-

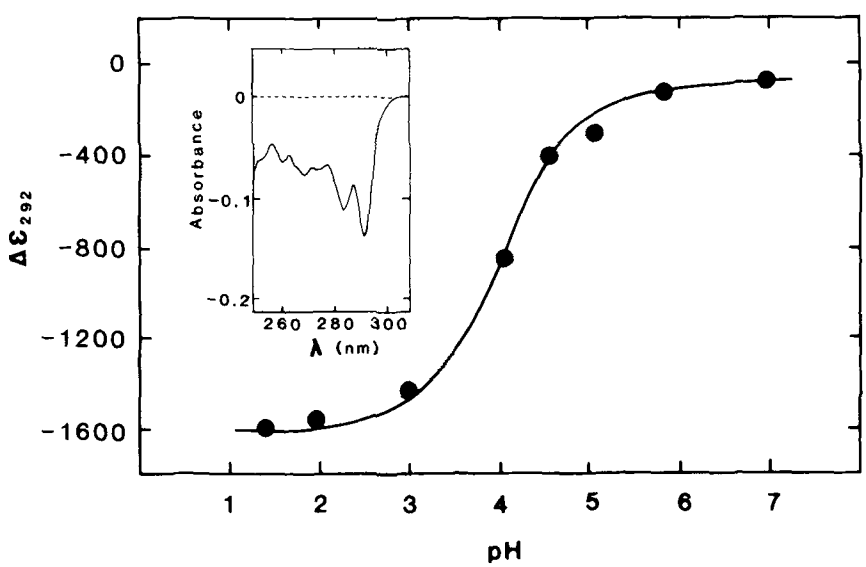

FIG. 6. Differences in absorbance of ovalbumin in acidic and neutral solution at 292 nm plotted against pH. The insert shows the difference spectrum of ovalbumin solution at pH 2 against pH 7.

TABLE 1.

Physicochemical Properties of P-Ovalbumin

	Ovalbumin	P-Ovalbumin
Molecular weight	45,000	42,500
Number of SH groups[a]	4	3
Intrinsic viscosity	4.5 (ml/g)	4.5 (ml/g)
CD spectrum	same	
Denaturation temperature[b]	80 C	79 C
Denaturation temperature of S-ovalbumin[c]	88 C	88 C
Difference spectrum pH 2 *vs* pH 7	same	

[a]SH groups were measured with DTNB at pH 8 after incubation of proteins for 1 hr at 50 C in the presence of 0.5% sodium dodecylsulfate, 8 M urea and 0.1 mM EDTA.
[b]Denaturation temperatures were estimated by differential scanning calorimetry at a heating rate of 1 C/min.
[c]S-Ovalbumin and S-P-ovalbumin were prepared from ovalbumin and P-ovalbumin by incubation of respective protein solution (pH 10) for 16 hr at 55 C.

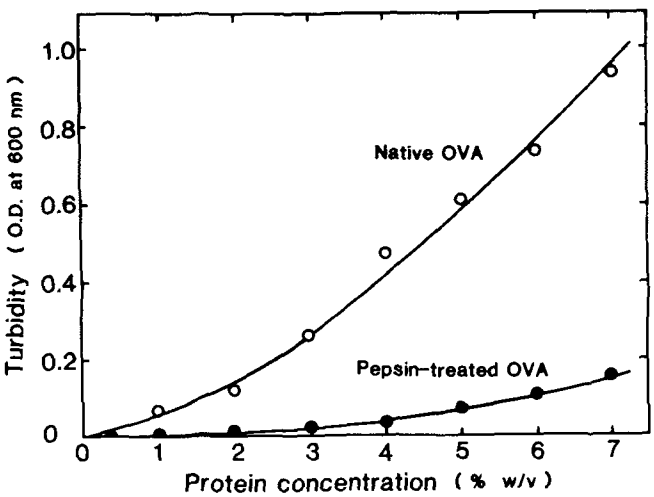

FIG. 7. Turbidity of solutions of ovalbumin (open circles) and P-ovalbumin (closed circles) after being heated 3 min at 100 C. The solutions had been dialyzed against 20 mM sodium phosphate buffer, pH 7.5.

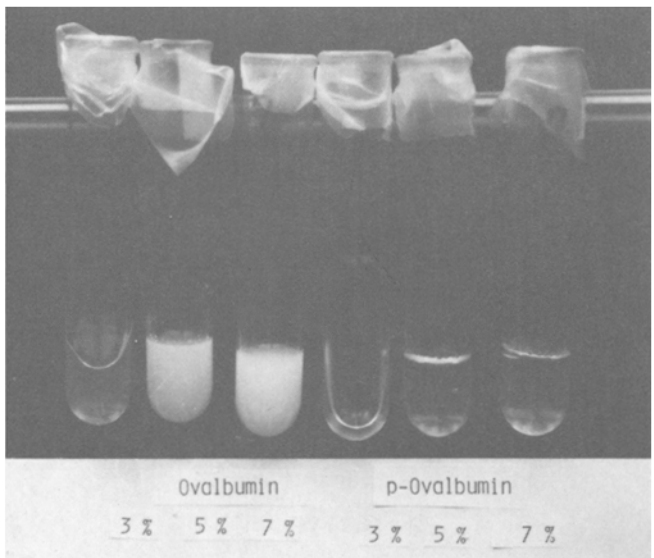

FIG. 8. Appearance of heated ovalbumin and p-ovalbumin solutions. Heating was as in Fig. 7.

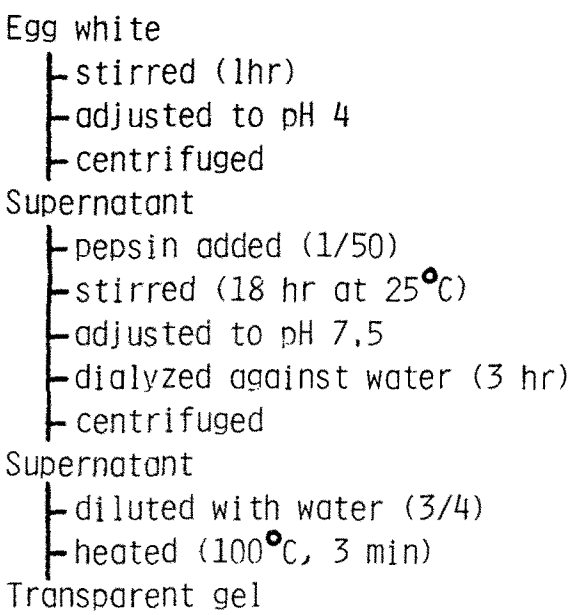

FIG. 9. Preparation of transparent gel from egg white.

ined the transparent gels made from egg white for suitability for practical use in foods (Fig. 9) (9). The egg white was adjusted to pH 4 and the precipitate removed by centrifugation. Pepsin was added and the enzymatic reaction continued for 18 hr at 25 C. At the end of this time, the pH was adjusted to 7.5. After dialysis and appropriate dilution, this solution gave a transparent gel upon heating (Fig. 10). The transparent gels obtained from P-ovalbumin and egg white were stable to heat; they did not melt upon reheating. P-Ovalbumin gel differs from gelatin or agarose gel in this regard. This is a useful property for food gels, because heat sterilization of the gel is possible. The high nutritive value is another advantage of the P-ovalbumin gel over gelatin or agarose gels.

Transparency of gel made from ovalbumin. We noticed that even unmodified ovalbumin gave a transparent gel under certain heating conditions, especially at low ionic strengths (10). A typical example is shown in Figure 11. The photograph under the graph shows the appearance of the gels or solutions. In the presence of 20 mM NaCl, the turbidity of heated ovalbumin solution was highest at around the pH of the isoelectric point (pH 4.7), and decreased on both the acidic and alkaline sides. The hardness of the gel had two maxima at the two pH values where the turbidity decreased. Only around these regions of pH was a turbid or clear gel obtained. At other regions of pH, a clear solution (at pH far from the pI) or a turbid solution or suspension (at pH near the pI) was obtained. P-Ovalbumin solution gave a transparent gel at a relatively wide range of pH under the same ionic conditions.

Recently, Van Kleef has reported that nearly trans-

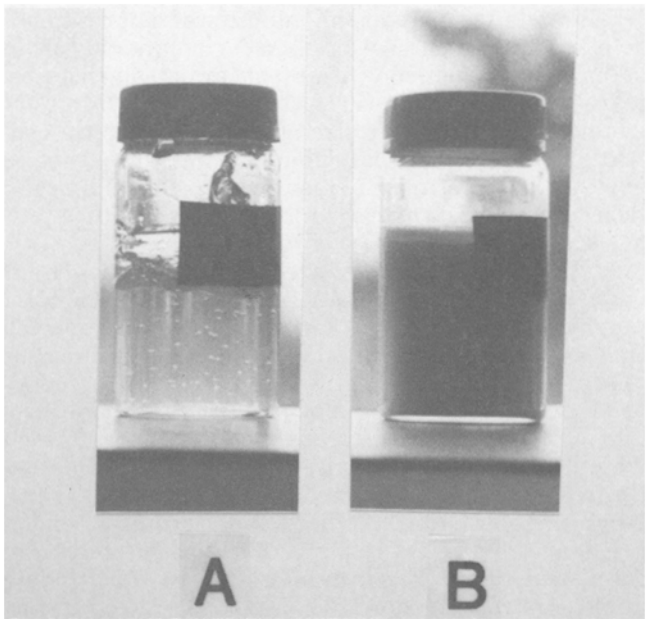

FIG. 10. Appearance of the transparent gels prepared from egg white. A, egg white was treated as in Fig. 9. B, egg white was treated the same way without addition of pepsin.

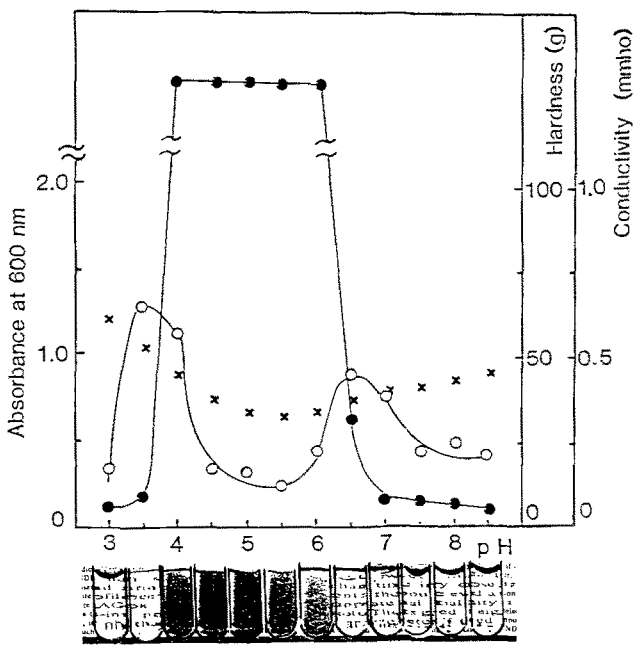

FIG. 11. Hardness and turbidity of heat-induced gel obtained after heating of 5% ovalbumin for 1 hr at 80 C (10). The solutions contained 20mM sodium chloride. ○-○-○, hardness; ●-●-●, turbidity; X X X, electric conductivity.

parent gels are obtained when ovalbumin is heated at pH 10 (12). The results shown in Figure 11 indicate that transparent gels are obtained even at neutral pH, depending on heating conditions.

Conformation of heat-denatured ovalbumin. Until now, the conformational change of ovalbumin arising from heat denaturation was difficult to study because of the turbidity of the gel. By the use of a clear solution, it is possible to examine for conformational change by optical and hydrodynamic methods. Figure 12 shows the CD spectra of native and heat-denatured ovalbumin. The curves are not very different; that is, the secondary structure of heat-denatured ovalbumin was not very different from that of the native molecule. The ovalbumin denatured by 5 M guanidine hydrochloride had a curve typical for random coils. Therefore, the heat-denatured ovalbumin was not a random coil; it still had a globular conformation. Nevertheless, the intrinsic viscosity of the heated ovalbumin solution was very high. Sedimentation and light scattering analyses indicated the presence of polymers of high molecular weights. Transmission electron microscopy of the clear solution obtained by heating of ovalbumin showed the presence of fibrous polymers (Fig. 13). The presence of such fibrous polymers as a unit in the gel matrix for various protein gels has been reported by Clark et al. (11).

Model of gel formation by ovalbumin or P-oval-

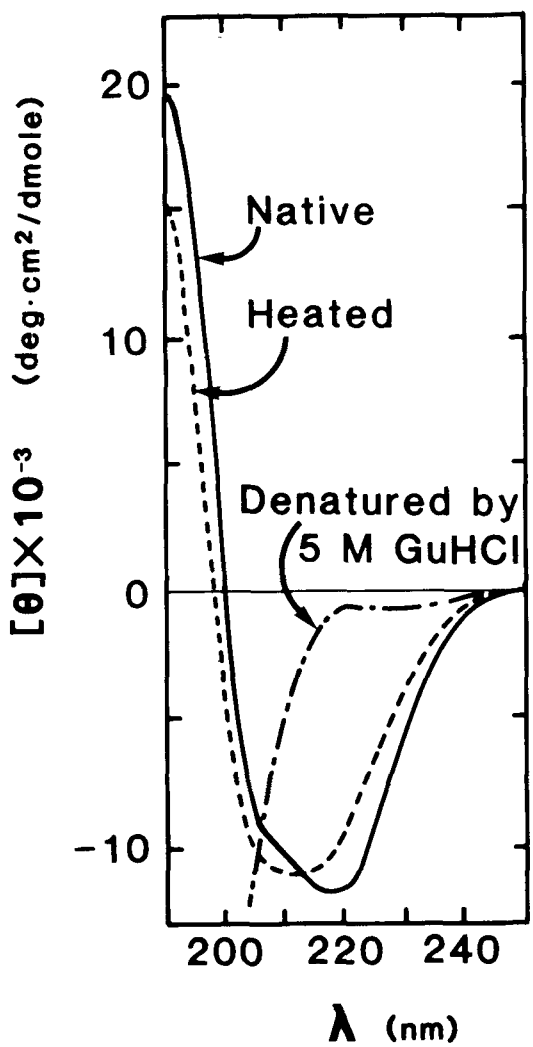

FIG. 12. CD spectra of native ovalbumin and ovalbumin denatured by heat or guanidine hydrochloride.

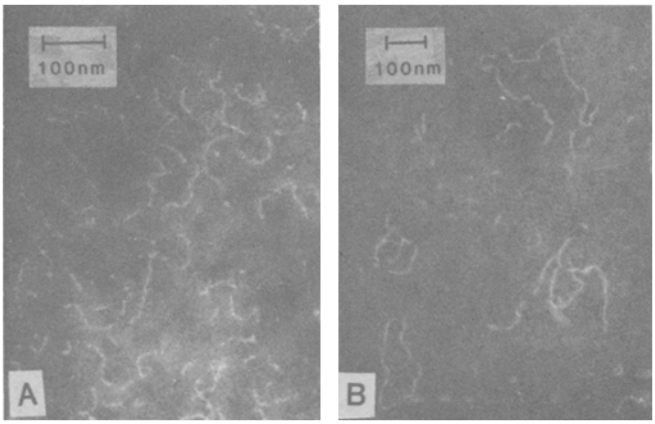

FIG. 13. Transmission electron micrographs of heated ovalbumin solutions. A, 0.5% olvalbumin solution was heated 20 min at 85 C, pH 7.0. B, the same solution was heated 24 hr at 75 C, pH 7.0. The heated solutions were diluted 250-fold, stained with 20% potassium phosphotungstate and viewed under a Hitachi H-700 H transmission electron microscope operating at 100 kV.

bumin. A simplified model of polymerization of heat-denatured ovalbumin and P-ovalbumin is shown in Figure 14. The conformation of the heat-denatured ovalbumin was not very different from that of the native molecule, but some hydrophobic areas that had been buried in the molecule are exposed to the surface of the protein after denaturation. One of the hydrophobic areas is missing in P-ovalbumin. Formation of a linear polymer or aggregate is controlled by the balance of hydrophobic interaction and electrostatic repulsion. We made clear solutions, turbid solutions, suspensions that contained aggregates, transparent gels and turbid gels, using a variety of conditions of pH, ionic strength and protein concentration. A hypothetical model of gel formation to explain the experimental results is given in Figure 15 based on electron micrographs obtained by Clark et al. (11) and by us.

At very low protein concentrations, at a pH far from the pI of the protein and low ionic strength, polymer is not formed. Foster and Rhees (13) reported that aggregation of heated ovalbumin is minimal or absent at pH 2.4 and 9-10. We failed to find monomers at pH 2.4, but they should be present at very low protein concentrations. Fibrous polymers are formed with decreasing electrostatic repulsive force, and then a three-dimensional gel network is formed by intrapolymer interaction. At high ionic strength and a pH near the isoelectric point, proteins aggregate to form a coagulum. Electron micrographs of aggregate at pH 5 have been shown by Van Kleef (12). The presence of such a coagulum interferes with the formation of the gel network and results in the formation of a soft, turbid gel.

The properties of native P-ovalbumin were almost the same as those of ovalbumin, but the hydrophobicity of the surface of the protein in heat-denatured P-ovalbumin is slightly different from that of ovalbumin. The difference in the hydrophobicity of the protein surface might result in the differences in gel hardness and transparency of ovalbumin and P-ovalbumin.

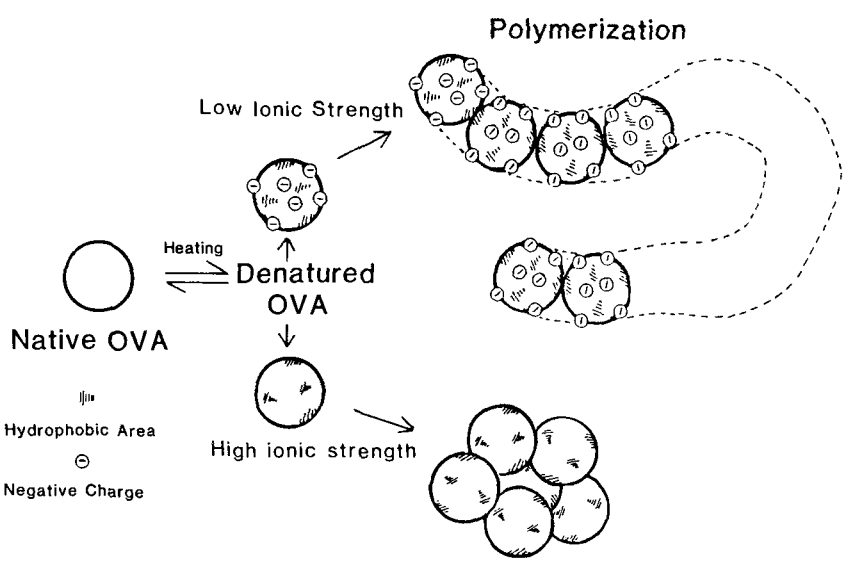

FIG. 14. Model for the heat denaturation and polymerization or aggregation of ovalbumin.

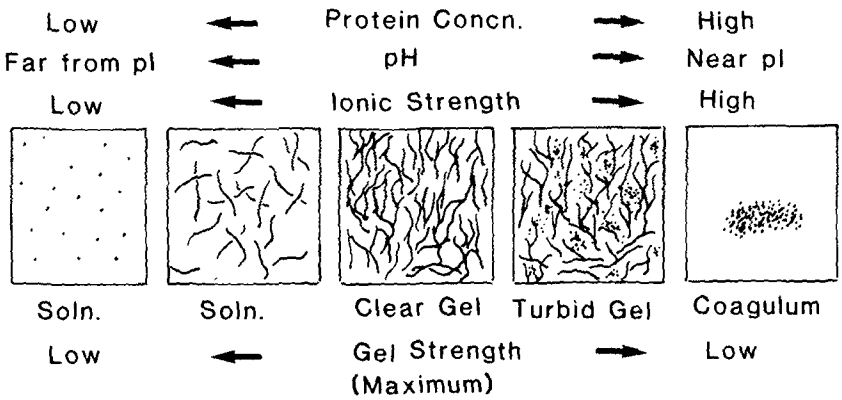

FIG. 15. Model for the formation of gel networks by heated ovalbumin.

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[Received February 19, 1987]