Immunochemical and Physical Properties of Peptic-Digested Ovomucoid

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Ovomucoid was digested by pepsin (5% of substrate weight), and the immunochemical and physical properties of the peptic-digested ovomucoid were investigated. The peptic digestion induced conformational changes and decreased the trypsin inhibitory activity of ovomucoid. In the quantitative precipitation experiment, the maximum immunoprecipitation yielded by the peptic-digested ovomucoid was only 30% of that by native ovomucoid. However, the digested protein inhibited about 70% of the specific immunoprecipitation reaction of native ovomucoid and also inhibited the rat passive cutaneous anaphylaxis reaction. The immunoelectrophoresis of peptic-digested ovomucoid and the immunodiffusion of peptic fragments fractionated by gel filtration demonstrated that some of the ovomucoid peptic fragments retained their antigenicity. The circular dichroism analysis of antigenic fragments prepared by the immunoaffinity chromatography of antibody-conjugated Sepharose 4B suggested that peptic fragments with antigenicity contained some ordered secondary structure. These results indicated that the peptic digestion did not completely destroy ovomucoid antigenic sites. Thus, some antigenic fragments were produced.

Chicken egg white has been found to cause allergic reactions frequently at the clinical level (Breneman, 1978). It has also been reported that most active allergen in egg white is a heat-stable protein, ovomucoid (Bleumink and Young, 1969), though allergic reactions to ovalbumin and lysozyme have been noted only to a lesser extent (Virtue and Wittig, 1970). The allergenicity of ovomucoid is partly associated with its heat stability, because heat-stable proteins can survive certain types of processing (Taylor, 1980). In fact, many investigators have reported on the heat stability of ovomucoid (Fredericq and Deutsch, 1949; Stevens and Feeney, 1963) and the antigenicity of heat-denatured ovomucoid (Deutsch and Morton, 1956; Matsuda et al., 1982).

Another important factor in the allergenicity of food proteins is the effect of proteolytic digestion on protein immunoactivities. β-Lactoglobulin, a milk protein possessing allergic activity, was split by peptic digestion into fragments with a molecular weight of 12000 or less, which had both antigenicity and immunogenicity (Spies et al., 1970; Haddad et al., 1979). It was also reported that the enzymatic hydrolysate of bovine casein with a molecular weight of less than 1000 did not show antigenic activity against anti-casein antiserum (Takase et al., 1979). Though many such studies on milk protein have been reported, the antigenicity of proteolytic hydrolysates of other food proteins is still not well investigated.

Ovomucoid is found in the egg white of avian species and comprises about 10% of the egg white proteins (Rhodes et al., 1960). Chicken ovomucoid is a trypsin inhibitor and is thus resistant to tryptic digestion but does not inhibit human trypsin (Buck et al., 1962). Ovomucoid had a characteristic molecular structure (i.e., it consists of three well-separated domains in its amino acid sequence (Kato et al., 1978), and the three domains were structurally independent (Matsuda et al., 1981)). Like some proteins such as myoglobin (Atassi et al., 1973), lysozyme, and serum albumin (Atassi, 1975), relatively larger fragments produced by limiting hydrolysis (Morton and Deutsch, 1961) or CNBr cleavage (Beeley, 1976) of ovomucoid have been reported to show almost as much antigenicity as the parent molecule.

In the present study, the effect of peptic digestion on the immunochemical and physical properties of ovomucoid was investigated by several immunochemical methods, trypsin-inhibitory activity measurement, and circular dichroism analysis.

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MATERIALS AND METHODS

Ovomucoid was isolated from egg white of White Leghorn hens by means of the trichloroacetic acid method and SP-Sephadex C-50 column chromatography as described by Waheed and Salahuddin (1975). All fractions with trypsin inhibitory activity recovered from the column chromatography were pooled, dialyzed, and lyophilized. Porcine pepsin was obtained from Worthington Biochemical Corp. and used without further purification.

Peptic Digestion. Ovomucoid was digested by pepsin (5% of substrate weight). Twenty milligrams of ovomucoid was dissolved in 10 mL of distilled water and pH of the solution was adjusted to 2.0 with HCl. One milliliter of the ovomucoid solution was placed in test tubes and 100 μL of pepsin solution (1 mg/mL in 10 mM HCl) was added to each tube. The solutions were incubated at 25 °C for 2, 4, 10, 20, and 60 min. The enzyme reaction was terminated by adding 51 μL of 0.2 M NaOH. NaOH was added to the sample of zero-time incubation before addition of the pepsin solution. Some of each solution was used for electrophoresis, and the remainder was diluted 10-fold with 0.15 M NaCl–0.01 M phosphate buffer, pH 7.2 (PBS), and used for the immunodiffusion, immunoprecipitation, and PCA experiments.

Polyacrylamide Gel Electrophoresis. Slab gels of 10% acrylamide and electrophoresis buffer of Tris–glycine were prepared as described by Davis (1964). Sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis (15% acrylamide) was performed according to the method of Laemmli (1970). Gel sheets were stained with a solution of 0.2% Coomassie Brilliant Blue R-250 in water–2-propanol–acetic acid (5:5:1 v/v/v) and destained with 7% acetic acid containing 10% methanol.

Trypsin Inhibitory Activity. Trypsin inhibitory activity was assayed by measuring the initial rate of increase in absorbance at 420 nm with N^{α}-benzoyl-L-arginine-*p*-nitroanilide as described by Waheed and Salahuddin (1975).

Circular Dichroism (CD) Analysis. CD spectra of samples were measured at 25 °C with a Jasco J-40CS spectropolarimeter; the mean residue ellipticities, [Θ], of the spectra were corrected by subtracting the spectra of the carbohydrate moiety (Watanabe et al., 1981).

Preparation of Antisera. Rabbit antiserum to ovomucoid was prepared as described previously (Matsuda et al., 1982). Mouse antisera to ovomucoid were prepared

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from a 6- to 8-week-old male mouse of the AKR, inbred strain, which was chosen because of its high immune response to low doses of ovomucoid (Vaz et al., 1971). The mice were immunized by intraperitoneal injection of 1 μg of ovomucoid in 0.1 mL of PBS with Freund's complete adjuvant in a water:oil ratio of 1:1. The mice received two booster injections of 1 μg of antigen mixed with 1 mg of $Al(OH)_3$ in 0.5 mL of PBS, 4 and 28 days after the first immunization. They were bred 5 days after the last booster injection, and the antisera were pooled and frozen at −80 °C in several batches that were allowed to thaw at 20 °C immediately before use.

Immunodiffusion. Ouchterlony analysis (Ouchterlony, 1949) was performed in 0.1% agarose in PBS.

Immunoelectrophoresis. Samples to be tested were developed by means of slab gel electrophoresis of polyacrylamide in the manner described above. The gel slab was cut into strips (6–8 mm wide) along the axis of the electric current, and the strips were buried in agarose gel plates (1.0% agarose in PBS). The rabbit antiserum was allowed to diffuse from the trough between the gel strips. After 24 h, the plates were washed with PBS for 3 days, stained with 0.5% Amide Black 10B in methanol–acetic acid (9:1 v/v), and destained with 2% acetic acid.

Quantitative Immunoprecipitation and Immunoprecipitation Inhibition. Quantitative immunoprecipitation was performed by incubating several amounts of antigen with rabbit antiserum and measuring the precipitation of immune complex. The precipitate was dissolved completely in 0.05 M NaOH and protein concentration was measured according to the method of Lowry et al. (1951) with rabbit IgG ($E^{1\%}_{1cm}$ = 14.0) as the standard. Immunoprecipitation inhibition was also carried out by using the supernatant solution separated in the quantitative immunoprecipitation experiment (Matsuda et al., 1982).

Passive Cutaneous Anaphylaxis (PCA) Test. PCA test was performed in the dosal skin of male Sprague-Dawley rats. Sample solutions containing various amounts of native or peptic digested ovomucoid were added to 20 μL of mouse antisera. The mixtures were filled with up to 200 μL of PBS and then incubated for 1 h at 25 °C. One-tenth of a milliliter of the incubated mouse antisera was injected intradermally into the shaved back of an SD rat. After 2 h, the rat was challenged by intraveneous injection of 1 mg of ovomucoid in 1 mL of 0.5% Evans blue dye dissolved in PBS. The rat was decapitated after 30 min and the skin was flayed.

Preparation of Antigenic Fragments. Ovomucoid (20 mg) was digested by pepsin (1 mg) in the manner as described above, and the digest was used for the gel filtration described below. Fractions I, II, and III obtained by the gel filtration were incubated with the anti-ovomucoid antibody conjugated Sepharose 4B at 37 °C for 1 h and then at 4 °C for 24 h. The resins were packed in columns and washed with PBS. Then the elution buffer was changed to 0.17 M glycine–HCl buffer, pH 2.5. The eluate was collected by 600 μL in the test tubes containing 60 μL of 2 M Tris–HCl buffer, pH 8.5. The fractions eluted with the glycine buffer were pooled, dialyzed against distilled water with membrane tubing (Spectrapor 3, Spectrum Medical Industries, Inc.), and lyophilized. The antibody-conjugated Sepharose 4B was prepared by incubating the globulin fraction of anti-ovomucoid serum (rabbit) with CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) as described previously (Matsuda et al., 1982).

RESULTS

Electrophoresis. Polyacrylamide gel electrophoretic patterns of peptic-digested ovomucoid are shown in Figure 1. The band intensity of intact ovomucoid was decreased by increasing the incubation time, and several new bands with large electrophoretic mobilities were observed in the patterns of samples incubated for more than 4 min. In the electrophoretic pattern of 60 min incubated ovomucoid, the dye intensities were generally weaker and the bands with large mobilities were not clearly detected. This was probably because small peptides were washed out of the gel during the destaining treatment. The samples were treated with 2-mercaptoethanol and $NaDodSO_4$ and used for the $NaDodSO_4$ electrophoresis. The electrophoretic patterns were also shown in Figure 1. As the incubation time increased, both the number and the intensity of stained bands with larger mobility increased more remarkably than those in the pattern of electrophoresis without $NaDodSO_4$ (Figure 1, upper). The pattern of the sample incubated for 60 min revealed no stained band of intact ovomucoid.

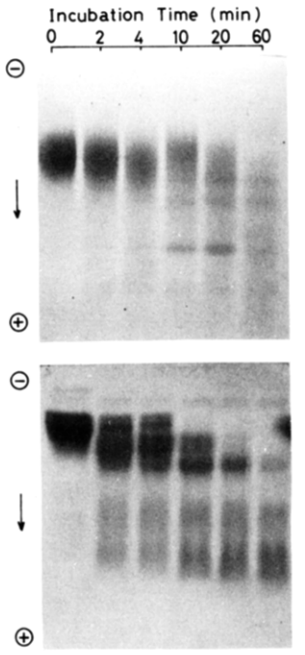

Figure 1. Polyacrylamide gel electrophoresis (upper) and $NaDodSO_4$–polyacrylamide gel electrophoresis (lower) of peptic-digested ovomucoids for 0–60 min.

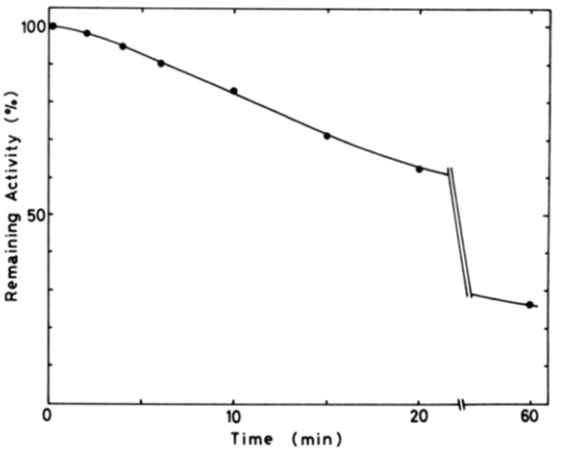

Figure 2. Effect of peptic digestion on the trypsin inhibitory activity of ovomucoid.

Trypsin Inhibitory Activity. The effect of peptic digestion on the trypsin inhibitory activity of ovomucoid is shown in Figure 2. The activity is given as a percentage of the native ovomucoid activity. It slowly diminished by

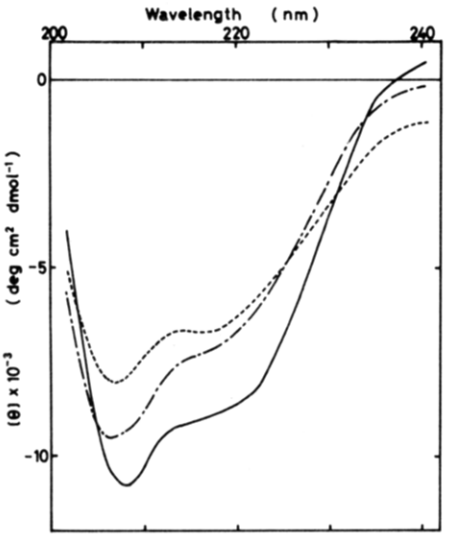

Figure 3. CD spectra of peptic digested ovomucoids for 20 (---) and 60 min (---) and native ovomucoid (—).

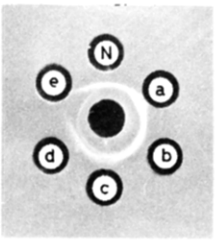

Figure 4. Immunodiffusion of peptic-digested ovomucoids for various periods, (a) 2, (b) 4, (c) 10, (d) 20, and (e) 60 min, and native (N) ovomucoid.

increasing incubation time, and the digestion for 60 min induced a considerable inactivation of ovomucoid.

CD Analysis. The CD spectra of ovomucoid digested for 20 and 60 min are shown in Figure 3. The negative ellipticities of the CD were gradually diminished by increasing incubation time. The spectra of digested ovomucoid were obviously different in their shape and intensity from that of native ovomucoid.

Immunodiffusion and Immunoelectrophoresis. Figure 4 shows the immunodiffusion of native and peptic-digested ovomucoids against rabbit anti-ovomucoid serum. A single and sharp precipitin arc was formed against native ovomucoid, whereas peptic-digested ovomucoid for 2 or 4 min revealed a broad precipitin arc, and a spur was formed by the precipitin arcs of native and 60 min digested ovomucoids.

After slab gel electrophoresis, native and peptic-digested ovomucoids were allowed to diffuse against rabbit antiserum. The immunoelectrophoretic pattern is shown in Figure 5. Whereas the native ovomucoid gave single precipitin band, peptic-digested ovomucoid yielded a band with what appeared to be at least two distinct, continuous arcs. Thus, the peptic fragments of ovomucoid with large electrophoretic mobilities formed precipitin arcs against anti-ovomucoid serum.

Quantitative Immunoprecipitation. As seen in Figure 6, native ovomucoid showed a precipitin curve with maximum precipitation at about 6 μg of antigen addition, whereas peptic-digested ovomucoid yielded a maximum precipitation only 30% of that with native ovomucoid, at about 4 μg of antigen addition.

The immunoprecipitation inhibition of native and peptic-digested ovomucoids was tested by adding native ovomucoid (3.8 μg in 10 μL of PBS) to the supernatant

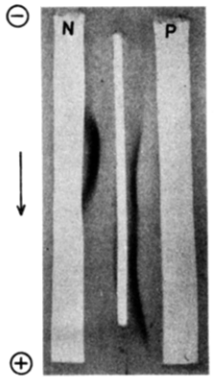

Figure 5. Immunoelectrophoresis of peptic-digested ovomucoid for 60 min (P) and native ovomucoid (N).

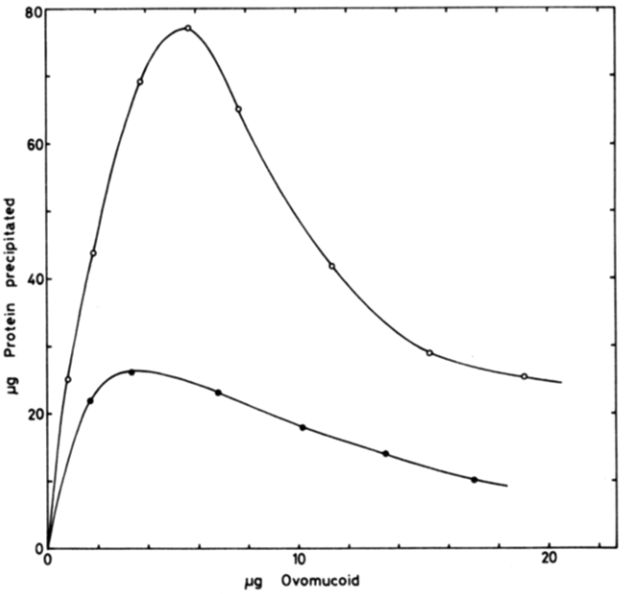

Figure 6. Quantitative immunoprecipitation of peptic-digested ovomucoid for 60 min (●) and native ovomucoid (○).

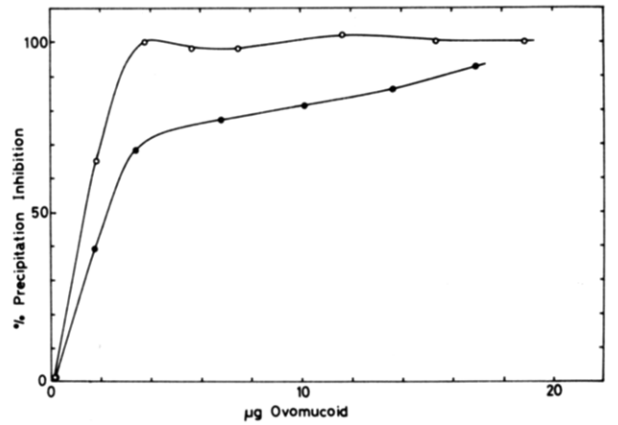

Figure 7. Inhibition of native ovomucoid immunoprecipitation by peptic-digested ovomucoid for 60 min (●) and by native ovomucoid (○). Various amounts of native or peptic-digested ovomucoid were preincubated with antiserum, and the inhibition of native ovomucoid reaction with specific antibody was measured.

solution (150 μL) separated in the immumoprecipitation experiment, and the inhibition profiles are shown in Figure 7. Native ovomucoid attained 100% inhibition with addition of about 4 μg of antigen. Peptic-digested ovomucoid also inhibited the specific immunoprecipitation reaction between native ovomucoid and antibody and attained 70% inhibition at a 4 μg of antigen addition. The inhibition

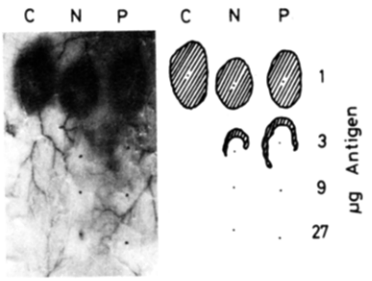

Figure 8. Rat PCA inhibition test of peptic-digested ovomucoid for 60 min (P) and native ovomucoid (N). Mouse antiserum control (C) was also injected for comparison.

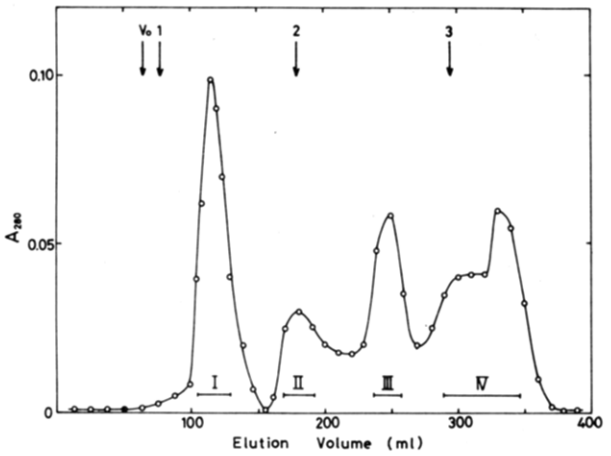

Figure 9. Gel filtration of peptic-digested ovomucoid. The ovomucoid (20 mg), digested for 60 min, was applied to a Bio-Gel P-30 column (2 × 95 cm) and eluted with PBS. Fractions under peaks were pooled separately as indicated by bars (fractions I–IV). The void volume, V_0, was determined with blue dextran, and elution positions of standard are indicated by arrows. 1, native ovomucoid (M_r 28 000); 2, α-lactalbumin (M_r 14 400); 3, tyrosine (M_r 181).

curve of peptic-digested ovomucoid showed a clear bent at about 4 μg of antigen addition, and the inhibition was gradually increased by increasing the antigen addition.

PCA Inhibition Test. To investigate the antigenicity of native and peptic-digested ovomucoids against mouse immunoglobulin, IgE, the inhibition of rat PCA was tested. The result is shown in Figure 8. The mouse antiserum preincubated with 3 μg of native ovomucoid yielded a small and weak blue spot, but the serum was neutralized thoroughly by preincubation with more than 9 μg of native ovomucoid. Peptic-digested ovomucoid also inhibited the PCA reaction, but the blue spot produced by the antiserum preincubated with 3 μg of peptic-digested ovomucoid was a little stronger than that produced by the antiserum preincubated with the same amount of native ovomucoid.

Gel Filtration. The ovomucoid (20 mg) digested for 60 min was fractionated by gel filtration with Bio-Gel P-30 as shown in Figure 9. The peptic-digested ovomucoid appeared to contain a trace amount of nondigested ovomucoid, but a large amount of ovomucoid was hydrolyzed into several peptide fragments with lower molecular weights. The fractions under the peaks were pooled separately and concentrated with an ultrafiltrate membrane (Diaflo, UM-2; Amicon Corp.). The carbohydrate content of each fraction was measured by the phenol–H_2SO_4 method (Dubois et al., 1956). Fractions I and II contained carbohydrate.

Antigenicity of Ovomucoid Peptic Fragments. Figure 10 shows the immunodiffusion analysis of ovomucoid peptic fragments. Fractions I–III formed precipitin arcs against anti-ovomucoid serum, but fraction IV did not.

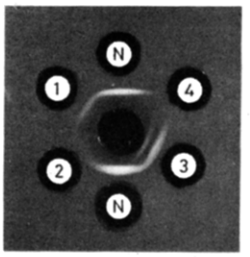

Figure 10. Immunodiffusion of ovomucoid peptic fragments fractionated by gel filtration. Antiserum was allowed to diffuse from the center well against fraction I (1), II (2), III (3), and IV (4) and native ovomucoid (N).

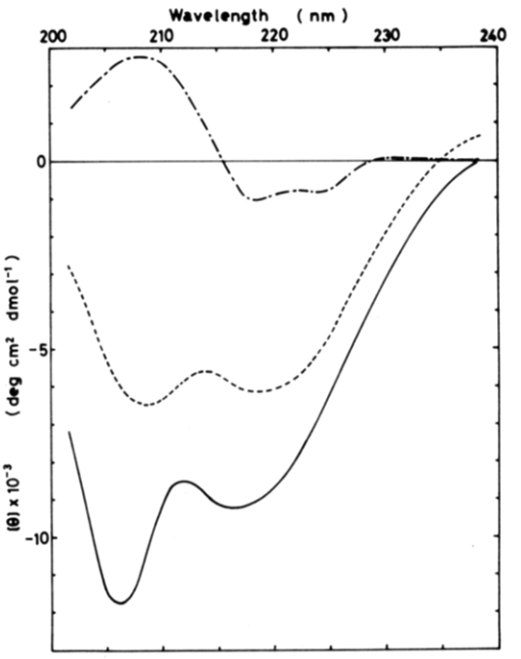

Figure 11. CD spectra of ovomucoid antigenic fragments. The antigenic fragments obtained from the fractions I (—), II (- - -), and III (- · -) were dissolved in PBS, and the spectra were measured at 25 °C.

The arc of fraction II was rather weak, and two precipitin arcs were observed between fraction III and the antiserum.

CD Analysis of Antigenic Fragments. The antigenic fragments obtained by immunoaffinity chromatography were used for CD analysis. Figure 11 shows the CD spectra of antigenic fragments obtained from fractions I–III. No carbohydrate correction of the spectra was made. The fragments from fractions I and III showed the spectra with two negative maximum at about 208 and 218 nm, whereas the ellipticity of fragments from fraction II was rather weak.

DISCUSSION

Ovomucoid was appreciably digested by incubation with 1:20 (w/w) of pepsin for 60 min and split into various kinds of peptide fragments. More stained bands corresponding to hydrolysates were observed in $NaDodSO_4$ electrophoresis after 2-mercaptoethanol and $NaDodSO_4$ treatment. This indicates that several fragments produced by peptic digestion would be linked by the nine disulfide bonds in an ovomucoid molecule. Each domain of ovomucoid contains three intradomain and no interdomain disulfide bridges (Kato et al., 1978). Hence, it is probable that intradomain peptide bonds, as well as interdomain ones, were cleaved by the peptic digestion.

The conformational changes of ovomucoid induced by peptic digestion were monitored by means of CD analysis.

The CD spectra of peptic-digested ovomucoid indicated that peptic digestion induced remarkable conformational changes in ovomucoid. The present authors have already reported that the ovomucoid secondary structure was little affected by only cutting ovomucoid domains apart (Watanabe et al., 1981). Hence, the peptic digestion of this experiment would cleave some peptide bonds other than those of the connecting peptides between the domains.

The antigenicity of peptic-digested ovomucoid was analyzed by immunodiffusion and immunoelectrophoresis methods (Figures 4 and 5). Two precipitin arcs formed between ovomucoid digested for 20 min and antiserum indicated that some ovomucoid fragments produced by the peptic digestion retained their antigenicity. Moreover, the antigenicity of peptic fragments was demonstrated clearly by immunoelectrophoresis.

To quantitate the antigenicity of peptic-digested ovomucoid, immunoprecipitation analysis was performed in the PBS system (Figure 6). The remarkable decrease of immunoprecipitation by peptic digestion is well explained by one or both of the following hypotheses. Some of the ovomucoid antigenic determinants were destroyed by the peptic digestion and/or ovomucoid antigenic sites were separated by the peptic fragmentation. Therefore, nonprecipitating antigens with only one or two antigenic sites were produced (lattice formation and immunoprecipitation are said to require at least three determinant groups per antigenic unit). The results of immunoprecipitation inhibition analysis (Figure 7) strongly supported the latter hypothesis rather than the former one. The inhibition (about 70%) by the peptic-digested ovomucoid indicated that a considerable amount of nonprecipitating antigen was produced. Moreover, the rapid increase up to 70% in the inhibition of peptic-digested ovomucoid suggested that about 70% of the antigenic sites in a ovomucoid molecule remain intact in most molecules after the digestion.

The peptic-digested ovomucoid inhibited the PCA reaction to the same degree as native ovomucoid, indicating that most of ovomucoid antigenic sites retained their binding capacity with mouse IgE antibody. Human IgE antibody is known to induce an immediate type of food allergy called the Gel-Coombs Type I reaction (Breneman, 1978). Therefore, it is probable that peptic-digested ovomucoid will cause allergic symptoms in a patient already sensitized to ovomucoid.

The immunodiffusion analysis of fractions prepared by the gel filtration suggested that at least three kinds of fragments with antigenicity were produced by the peptic digestion. The antigenic fragments from fractions I and III seemed to contain some ordered secondary structure (helix and β-sheet), when the CD spectra of the fragments were compared with the standard spectra of helix and β-sheet (Chang et al., 1978). The weak ellipticity of the antigenic fragments from fraction II may be due to structural destruction by peptic fragmentation and/or by treatments during sample preparation.

Ovomucoid contains no less than about 25% carbohydrate. From the present study, it is uncertain whether the carbohydrate groups contributed in any way to the antigenicity of the ovomucoid. The carbohydrate groups may have something to do with resistance of antigenic sites to peptic hydrolysis. However, the carbohydrate groups are considered to make less or no contribution to the antigenicity itself, because denatured ovomucoid was found to have no antigenicity to anti-native ovomucoid antibody (Morton and Deutsch, 1961; Beeley, 1976).

It is quite predictable from the above-described molecular characteristics of ovomucoid that ovomucoid fragments produced by limiting hydrolysis of interdomain connecting peptides would retain their antigenicity. However, the peptic treatment of this study seemed to cleave not only the interdomain connecting peptides but also intradomain peptide bonds. It is not clear why the ovomucoid antigenicity was only slightly diminished by the appreciable peptic hydrolysis. Maintenance of structure after peptide-chain cleavage may be aided by the presence of a high proportion of disulfide bonds in each of the fragments (Beeley, 1976). The isolation and characterization of antigenic fragments from the ovomucoid proteolytic hydrolysate are now in progress.

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