

Rapid Paper

Reduction of Ovomucoid Immunogenic Activity on Peptic Fragmentation and Heat Denaturation

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Three fragments with molecular weights of about 25,000, 18,000 and 13,000 daltons, respectively, were prepared from chicken egg white ovomucoid (MW, 28,000) by peptic limited hydrolysis, and denatured ovomucoid without antigenicity toward anti-ovomucoid antibody was prepared by heating (100°C, 30 min, pH 9.0). Mice were immunized by intraperitoneal injection of each preparation in Freund's complete adjuvant, and later they were given 2 booster injections of the antigen mixed with $Al(OH)_3$. The IgE response to each preparation was assayed by the heterologous passive cutaneous anaphylaxis (PCA) test. The antibody response to fragments or denatured protein was much weaker than that to native ovomucoid, suggesting that either the slight decrease in molecular size or heat denaturation effectively reduces the ovomucoid immunogenic activity.

Chicken egg white is known to cause several types of allergic reactions, and ovomucoid (MW, 28,000), which accounts for about 10% of the egg white protein,¹⁾ has been reported to be one of the typical allergenic proteins in egg white.^{2,3)}

Heat treatment, 100°C, caused ovomucoid to lose its ability to precipitate with specific antibody^{4,5)} and the reduction of disulfide bonds in ovomucoid markedly altered its immunochemical properties.⁶⁾ Ovomucoid fragments obtained by enzymatic or chemical cleavage retained most of the antigenic activity of the parent molecule.^{7~10)}

Thus immunochemical studies on ovomucoid have been focussed mainly on its antigenic properties, while little attention has been paid to its immunogenic properties. However,

immunogenic activity would be an important property, when protein antigens are investigated as food allergens. Hence, it is practically interesting to investigate the immunogenic properties of ovomucoid enzymatic hydrolysates and heat-denatured ovomucoid.

In the present study, the IgE antibody response in mice to ovomucoid fragments and denatured ovomucoid was investigated to determine whether enzymatic fragmentation and heat denaturation reduce the ovomucoid immunogenic activity.

MATERIALS AND METHODS

Ovomucoid preparation. Ovomucoid was isolated from egg white of White Leghorn hens by means of SP-Sephadex C-50 column chromatography as described by Waheed and Salahuddin.¹¹⁾

Preparation of ovomucoid peptic fragments and denatured ovomucoid. Two mg of pepsinogen (Sigma Chemical Company, USA) was added to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) suspended in coupling buffer (0.1 M sodium carbonate buffer, pH 8.3, containing 0.5 M NaCl) followed by shaking for 2 hr at room temperature. After treatment with 0.2 M glycine solution, pH 9.0, the pepsinogen coupled to Sepharose 4B was activated by incubation with 100 µg of pepsin at 37°C for 10 min in 10 mM HCl. The free pepsin was removed completely from the immobilized pepsin by washing with 10 mM HCl. The pepsin-coupled Sepharose 4B was added to the ovomucoid solution (50 mg in 50 ml of 10 mM HCl) and the mixture was incubated at 37°C for 3 hr. After removing the pepsin-coupled Sepharose 4B, the pH of the solution was adjusted to 7.0 with dil. NaOH followed by freeze-drying. The peptic hydrolyzed ovomucoid was dissolved in 10 mM phosphate buffer, pH 7.2/0.15 M NaCl (PBS), and applied to a column of Bio Gel P-30 equilibrated with PBS for the fractionation of the individual fragments.

The ovomucoid solution (1 mg/ml, pH 9.0, adjusted with dil. NaOH) was heated at 100°C for 30 min and then cooled in ice water. The solution was dialyzed against H_2O and freeze-dried.

Mouse immunization. AKR mice were used in the present study, because of their high immune response to a low dose of ovomucoid.¹²⁾ Three mice (6- to 8-week-old, female) were immunized by intraperitoneal injection of 1 µg of ovomucoid or each sample preparation in 100 µl of PBS with Freund's complete adjuvant at a water to oil ratio of 1:1. The mice received 2 booster injections of 1 µg of the antigen mixed with 1 mg of $Al(OH)_3$ in 500 µl of PBS, 5 and 29 days after the first immunization. Blood was

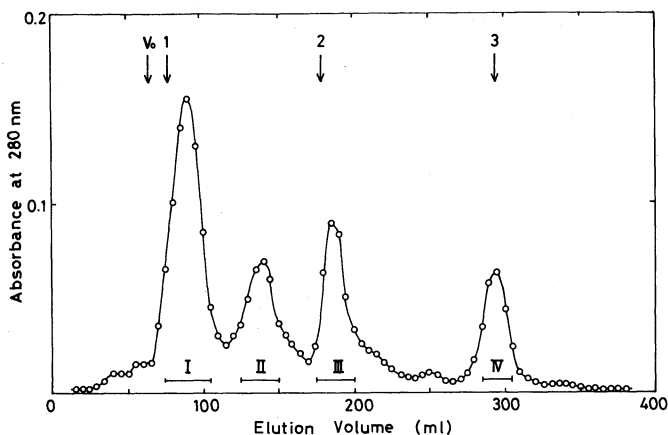


FIG. 1. Gel Filtration of Peptic-digested Ovomucoid on a Column of Bio Gel P-30.

Peptic-digested ovomucoid (50 mg) was applied to the column (2×95 cm) and eluted with PBS. Fractions under the peaks were pooled separately as indicated by bars (fractions I~IV). The void volume, V_o , was determined with blue dextran, and the elution positions of standards are indicated by arrows. 1, native ovomucoid (MW, 28,000); 2, α -lactalbumin (MW, 14,400); 3, tyrosine (MW, 181).

collected 4 days after each booster injection by puncturing of the ophthalmic retro-orbital venous plexus. The serum was separated from the pooled blood of three mice, and stored at -80°C before use.

Passive cutaneous anaphylaxis (PCA) test. The specific IgE antibody titer was determined from the heterologous PCA reaction in the dorsal skin of male Sprague-Dawley rats.¹³⁾ One-tenth of a milliliter of the pooled serum serially diluted with PBS was injected intradermally into the shaved back of an SD rat. After 3 hr, the rat was challenged by intravenous injection of 1 mg of antigen in 1 ml of 0.5% Evans blue dye dissolved in PBS. After 30 min the rat was decapitated and the reaction was observed from the inside of the dorsal skin. The antibody titer was expressed as the reciprocal of the highest dilution yielding a positive-reaction blue spot. Each sample was tested in duplicate.

Polyacrylamide gel electrophoresis. Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (15% acrylamide) was performed according to the method of Laemmli.¹⁴⁾ The gel sheets were stained with Coomassie brilliant blue R-250 and destained with 7% acetic acid containing 10% methanol.

Protein determination. Protein concentration was determined by the method of Lowry *et al.*¹⁵⁾

RESULTS

Ovomucoid treated with pepsin coupled to Sepharose 4B was fractionated by gel filtration

on Bio Gel P-30. Figure 1 shows the elution profile from the column. From the elution volumes of ovomucoid, α -lactalbumin and tyrosine, the molecular weights of peaks I, II, III and IV were estimated to be about 25,000, 18,000, 13,000 and less than 2,000 daltons, respectively. Fractions under the first 3 peaks were pooled separately and rechromatographed on the same column. Fraction IV was so small that it passed through the dialytic membrane tubing (molecular weight cut-off of about 3500 daltons; Spectrapor 3, Spectrum Medical Industries, Inc., U.S.A.), so it was ignored in the following immunochemical experiments. Peptide fragments with such a low molecular weight would not be immunogenic.

The electrophoretogram of fractions I, II, III and ovomucoid is shown Fig. 2. Each fraction was not contaminated by intact ovomucoid or fragments of other fractions. The fragments in each fraction were named fragments I, II and III (FI, FII and FIIL), respectively, and used as immunogens against mice.

The IgE antibody responses to ovomucoid and each fragment are shown in Fig. 3. Four days after the first booster injection, the PCA

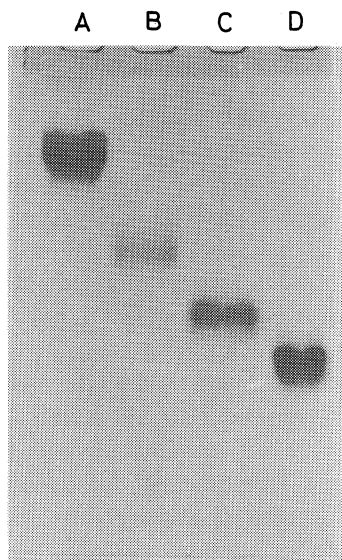


FIG. 2. Polyacrylamide Gel Electrophoresis of Fractions I, II, III and Ovomuroid.

A, native ovomucoid; B, fraction I; C, fraction II; D, fraction III.

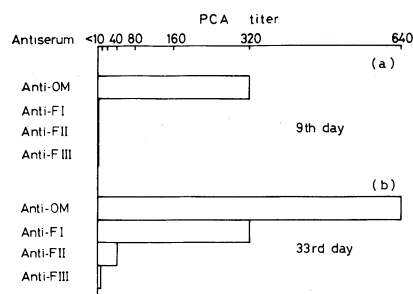


FIG. 3. IgE Antibody Response to Ovomuroid and Its Peptic Fragments.

AKR mice were immunized with FI, FII, FIII and ovomucoid, and the specific antibody to each immunogen was assayed on the 9th (a) and 33rd (b) days.

titer against ovomucoid was no less than 320, whereas no positive PCA reaction was detected against either fragment under these experimental conditions. Even after the second booster injection, the antibody responses to the fragments were remarkably weaker than that to intact ovomucoid.

The reactivity of anti-fragment IgE antibodies toward intact ovomucoid was examined by using sera collected on the 33rd day. Figure 4 shows the PCA titers of rats passively sensi-

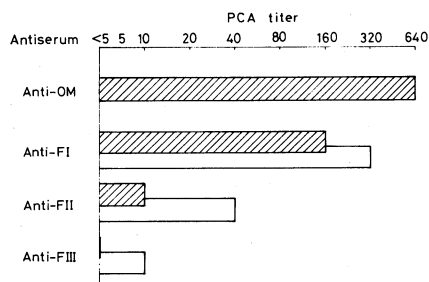


FIG. 4. Reactivity of anti-fragment IgE antibodies with intact ovomucoid.

A rat sensitized with an anti-fragment serum (33rd day-serum) was challenged with ovomucoid (▨), and another rat sensitized similarly was challenged with the fragment itself (□).

tized with anti-fragment sera and then challenged with each fragment or intact ovomucoid. A PCA reaction in a rat sensitized with anti-F I or anti-F II serum was elicited by challenging it with not only the fragment itself but also ovomucoid, though the titer of the reaction caused by ovomucoid was lower than in the cases of the fragments. When a rat sensitized with anti-F III serum was challenged with intact ovomucoid, a PCA reaction was not detected.

Denatured ovomucoid was prepared by heating in an alkaline solution, and the antigenic activity of the denatured ovomucoid was tested by the Ouchterlony immunodiffusion¹⁶⁾ and PCA methods. Immunoreactivity of the denatured ovomucoid against antibody to the native protein was not at all detected in either test.

The IgE antibody responses to native and heat-denatured ovomucoids are shown in Fig. 5. The PCA titer of sera against denatured ovomucoid was much lower than that in the case of the native protein; the titer of anti-denatured ovomucoid was about 1/16~1/32 of that of the native protein after the first or second booster injection. The reactivity of anti-denatured ovomucoid antibody toward native ovomucoid was examined by using sera collected on the 33rd day; rats were sensitized with the anti-denatured ovomucoid serum and then challenged with native ovomucoid.

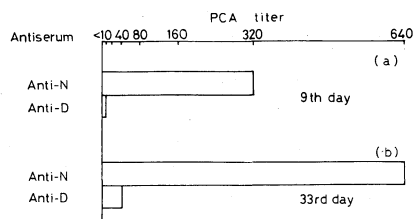


FIG. 5. IgE Antibody Response to Native and Heat-denatured Ovomuroids.

The specific antibody to native (N) or denatured (D) ovomucoid was assayed on the 9th (a) and 33rd (b) days.

However, no PCA reaction was detected.

DISCUSSION

To prepare ovomucoid fragments as immunogens, pepsin coupled Sepharose 4B was used in the present study. If conventional hydrolysis with pepsin had been used, it would have been troublesome to fractionate the ovomucoid peptic fragments because of the contamination by pepsin and its autolysates.

The IgE antibody response to ovomucoid peptic fragments indicated that the immunogenic activity of ovomucoid was remarkably but not completely lost on peptic fragmentation (Fig. 3). In general, the antibody response to small antigens is weaker than that to larger ones.¹⁷⁾ In the case of ovomucoid, fragmentation would be especially effective in reducing the immunogenic activity, for it seems that the slight decrease in molecular size caused peptic limited hydrolysis induced a great decrease in ovomucoid immunogenic activity (Figs. 1 and 3).

Ovomucoid would not be denatured by incubation in 10 mM HCl at 37°C for 3 hr, because ovomucoid is highly resistant to extreme pHs such as in trichloroacetic acid treatment.¹⁸⁾ In fact, ovomucoid retained almost completely its trypsin inhibitory activity and antigenic activity even after incubation in 10 mM HCl at 37°C for 24 hr without pepsin (data not shown).

An ovomucoid molecule contains 3 isoleucine and 2 methionine residues; the former and latter are present only in the first and

second domains, respectively.¹⁹⁾ By amino acid analysis of each fragment, it was found that F-I contained both isoleucine and methionine residues, whereas F-II and F-III contained neither of them (data not shown). Hence, F-I would be derived mainly from the first and second domains, and F-II and F-III from the third domain of ovomucoid.

The antibody against fragments with lower molecular weights seemed to have a lower reactivity with intact ovomucoid (Fig. 4). Immunodiffusion analysis by the Ouchterlony method¹⁶⁾ showed that all of the fragments reacted with anti-ovomucoid antibody (data not shown). This indicated that the ovomucoid antigenic structure was not greatly destroyed on peptic fragmentation. Hence, the non-reactivity of anti-F III antibody with intact ovomucoid might be due to the difference in the regions recognized as antigenic determinants. In mice immunized with F III, some regions which are buried inside an intact molecule might be recognized as antigenic determinants.

It is well known that protein antigenic activity can be lost on heat denaturation. There was some suspicion that denatured proteins might become alternative immunogens. In the present study, however, denatured ovomucoid without immunoreactivity toward anti-native ovomucoid antibody was demonstrated to be much weaker immunogen than native ovomucoid. The conformation of randomly denatured ovomucoid would differ from molecule to molecule. It is uncertain why denatured ovomucoid showed weaker immunogenicity, but the heterogeneity in molecular conformation of the denatured protein might contribute to the low immunogenic activity. Nevertheless, it can be said that heat denaturation reduced not only the antigenic activity but also the immunogenic activity of ovomucoid.

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