

Immunoreactive Glycopeptides Separated from Peptic Hydrolysate of Chicken Egg White Ovomucoid

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ABSTRACT

Chicken egg white ovomucoid was hydrolyzed with pepsin, fractionated by gel filtration, and the reactivity to anti-ovomucoid antibody was detected in the two carbohydrate-containing fractions. Immunoreactive fragments separated from these fractions by immunoaffinity chromatography were positive to both protein and carbohydrate staining, i.e., glycopeptides. Ovomucoid glycopeptides prepared by pronase-hydrolysis did not have this immunoreactivity. These results show that some glycopeptides with immunoreactivity remained after peptic hydrolysis, suggesting that the carbohydrate moieties of ovomucoid exert a protective effect against peptic hydrolysis.

INTRODUCTION

DIETARY EGG WHITE is known to cause allergic reactions (Taylor, 1980), and ovomucoid has been reported to be one of the typical allergenic proteins in egg white (Hoffman, 1983). Chicken egg white ovomucoid is a glycoprotein with a molecular weight of 28,000 daltons, and carbohydrate accounts for about 25% of the whole molecule (Liu and Feeney, 1972). The amino acid sequence of chicken ovomucoid has already been determined, and the attachment sites for the carbohydrate chains were reported to be 5 asparagine residues (Kato et al., 1978). Though some immunochemical investigations on ovomucoid or its chemical or enzymatic fragments have been done (Morton and Deutsch, 1961; Beeley, 1971), it is still uncertain whether the carbohydrate chains participate in ovomucoid immunoreactivity. Nevertheless, it has been considered that the carbohydrate chain itself has weak or no contribution to the immunoreactivity, because ovomucoid immunoreactivity was lost by reduction of the disulfide bonds or heat denaturation (Beeley, 1971; Matsuda et al., 1982). On the other hand, the carbohydrate chains of glycoproteins have been considered to exert protective effects against proteolytic enzymes. In fact, the hydrolysis of ovomucoid with pepsin (5% of substrate weight) for 60 min did not destroy ovomucoid immunoreactivity, and some immunoreactive fragments with carbohydrate chains were produced (Matsuda et al., 1983).

This study was initiated to investigate the effect of drastic hydrolysis with pepsin on the ovomucoid immunoreactivity and the contribution of carbohydrate moiety to the ovomucoid immunoreactivity.

MATERIALS & METHODS

OVOMUCOID was isolated from egg white of White Leghorn hens according to the method of Waheed and Salahuddin (1975). Porcine pepsin and pronase were obtained from Worthington Biochemical Corp. and Kaken Kagaku (Tokyo), respectively. Rabbit and mouse anti-ovomucoid antisera were prepared as described previously (Matsuda et al., 1982, 1983).

Hydrolysis with pepsin

Ovomucoid (200 mg) was dissolved in 10 mL of 10 mM HCl and pH of the solution was adjusted to 2.0 with dil. HCl. Five mg of pepsin (2585 units/mg) were added to the ovomucoid solution every 6 hr over a 24-hr period. The reaction mixture was incubated at 37°C for 24 hr under toluene. The pH of the solution was readjusted to 2.0 with dil. HCl each time pepsin was added. The reaction was stopped by raising the pH to 8.0 with dil. NaOH. The hydrolysate was kept for 4 hr at 25°C to inactivate pepsin and applied to gel filtration on a Sephadex G-50 column equilibrated with 0.1 M CH₃COOH.

Hydrolysis with Pronase

Ovomucoid (200 mg) was dissolved in 10 ml of 0.1 M borate buffer, pH 8.0, containing 10 mM calcium acetate. Two mg of Pronase (750 units/mg) were added to the ovomucoid solution every 24 hr over a 120-hr period. The reaction mixture was incubated at 37°C for 120 hr under toluene. The hydrolysate was applied to the gel filtration of a Sephadex G-50 column equilibrated with 0.1 M CH₃COOH.

Immunodiffusion

Rabbit anti-ovomucoid serum was diffused against test samples in 1.0% agarose gel containing 0.1% NaN₃ according to the method of Ouchterlony (1952). Samples (8-10 µg) to be tested were applied to each well of the plates, and the plates were incubated at 25°C for 15 hr.

Passive cutaneous anaphylaxis (PCA) test

The heterologous PCA test was performed as described by Mota and Wong (1969). One-tenth of a milliliter of mouse anti-ovomucoid serum serially diluted with 0.15M NaCl/0.01M phosphate buffer, pH 7.2 (PBS) was injected intradermally into the shaved back of a Sprague-Dawley rat. After 2 hr, the rat was challenged by intravenous injection of 1 mg of a test sample in 1 mL of 0.5% Evans Blue dye dissolved in PBS. The rat was decapitated after 30 min and the skin was removed from the rat and observed from the inside. Each sample was tested in duplicate.

Preparation of immunoreactive fragments

Immunoreactive fragments were separated from the carbohydrate containing fractions of ovomucoid peptic hydrolysate by immunoaffinity chromatography on antibody-Sepharose 4B. 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). Each fraction (2 mg) was incubated with the antibody-Sepharose 4B in PBS at 37°C for 4 hr and, then, at 4°C overnight. The antibody-Sepharose 4B was packed in a column and washed with PBS thoroughly. After washing out the PBS with a little distilled water, the absorbed fraction was eluted with 0.1M CH₃COOH. The fractions eluted with 0.1M CH₃COOH were pooled and freeze-dried.

Electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (20% acrylamide) was performed according to the method of Laemmli (1970), except that the sample buffer was prepared without 2-mercaptoethanol. One slab gel was stained with 0.2% Coomassie Brilliant Blue R-250 and destained with 7% CH₃COOH containing 10% methanol. For carbohydrate staining, another gel slab was treated with periodic acid-Schiff reagent (PAS) (Zacharius et al., 1969).

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Measurement of trypsin activity

Trypsin activity was assayed by measuring the initial rate of increase in absorbance at 420 nm with α -N-benzoyl-L-arginine-p-nitroanilide (Waheed and Salajuddin, 1975).

Analytical method

Amino acid analysis was performed with JLC-6AH amino acid analyser (JEOL, Tokyo) after hydrolysis at 110°C for 24 hr in 6N HCl in evacuated, sealed tubes. Neutral hexose was determined by the phenol-sulfuric acid method (Dubois et al., 1956). The concentration of ovomucoid and glycopeptides was determined by measuring sample weight after drying in an air oven.

RESULTS

THE PEPTIC HYDROLYZED OVOMUCOID was fractionated by gel filtration on Sephadex G-50. Fig. 1 shows the elution profile as measured by the phenol-sulfuric method and ultraviolet absorbance. The carbohydrate determination gave two peaks and one small shoulder. The fractions under the carbohydrate peaks were pooled separately and freeze-dried. The two fractions were further purified by gel filtration on Bio-Gel P-10 (superfine, Bio-Rad Laboratories). The samples were eluted with 0.1M CH_3COOH , and a carbohydrate peak was pooled and freeze-dried. These two fractions under the first and the second peaks shown in Fig. 1 were named PEP-I and PEP-II, respectively.

The reactivity of PEP-I and PEP-II to rabbit anti-ovomucoid antibody was tested by Ouchterlony immunodiffusion. As shown in Fig. 2, both PEP-I and PEP-II formed a weak and single precipitin line with antiserum. Though it could not clearly be defined, each precipitin line of PEP-I and PEP-II appeared to fuse. Hence, PEP-I and PEP-II might contain overlapping fragments with immunoreactivity. The peptide fractions without carbohydrate obtained by the above described gel filtration (Fig. 1) also were applied to the immunodiffusion analysis, but no fractions produced a precipitin line with anti-ovomucoid serum in this experimental condition.

The immunoreactivity of PEP-I and PEP-II to mouse IgE antibody directed against ovomucoid was examined by the PCA test. Both PEP-I and PEP-II gave a positive PCA reaction, and the highest antiserum dilution giving a positive reaction was 1/15 for each sample. But the density of the

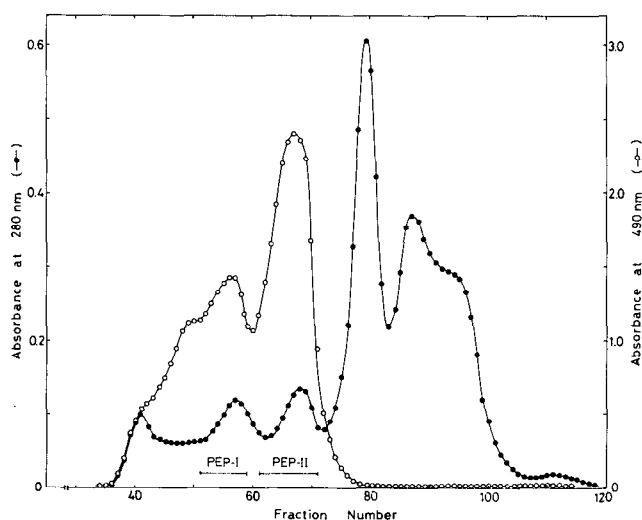


Fig. 1—Chromatography of pepsin-hydrolyzed ovomucoid on a column (3 x 80 cm) of Sephadex G-50. Ovomucoid hydrolyzed with pepsin was applied to the column and eluted with 0.1M CH_3COOH . The elution profile was monitored by the determination of hexose content (A_{490} , $\circ-\circ$) and UV absorbance (A_{280} , $\bullet-\bullet$). The fractions under peaks were pooled separately as indicated by bars (PEP-I and PEP-II), and freeze-dried.

blue spot of PEP-II was somewhat stronger than that of PEP-I.

To examine whether glycopeptide fractions contained traces of intact ovomucoid as an impurity, the trypsin inhibitory activity of PEP-I and PEP-II was measured. None of the glycopeptide fractions inhibited trypsin activity. One μg of ovomucoid gave about 100% inhibition, whereas 100 μg of each glycopeptide fraction gave no inhibition.

Immunoreactive fragments were separated from PEP-I and PEP-II by immunoaffinity chromatography. The fraction eluted with 0.1M CH_3COOH was freeze-dried and applied to SDS polyacrylamide gel electrophoresis without 2-mercaptoethanol treatment. As shown in Fig. 3, the immunoreactive fragments were positive to both carbohydrate and protein staining. The stained regions appeared to be broad or to consist of several bands.

The ovomucoid hydrolyzed with Pronase was fractionated by gel filtration of the same column, and elution profile is shown in Fig. 4. One carbohydrate peak with little absorbance at 280 nm was found, and the fractions under this peak were pooled, freeze-dried (PRO-I). Most of PRO-I was carbohydrate but a small quantity of amino acids (Asp and Thr) also was present. The immunoreactivity of PRO-I was tested by Ouchterlony Immunodiffusion and PCA tests in the similar manner as above. However, neither precipitin lines nor positive skin reactions were detected.

DISCUSSION

THE REACTIVITY of PEP-I and PEP-II to anti-ovomucoid antibody was demonstrated by the immunodiffusion and PCA tests. However, the precipitin line intensity and PCA titer of PEP-I and PEP-II were much weaker than those of ovomucoid. Hence, either some fragments in PEP-I and PEP-II have immunoreactivity or they react only partially with anti-ovomucoid antibodies.

Both PEP-I and PEP-II were the carbohydrate-containing fractions in the gel filtration chromatography. However, there were some doubts as to whether the immunological activities observed arose from contaminating peptides without carbohydrate. Hence, immunoreactive fragments were

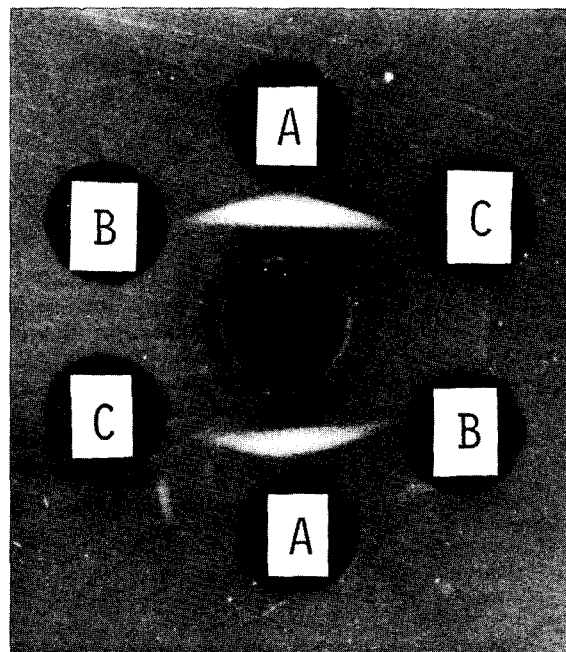


Fig. 2—Double immunodiffusion of carbohydrate-containing fractions (PEP-I and PEP-II) of ovomucoid peptic hydrolysate. Rabbit anti-ovomucoid serum was diffused from the center well against ovomucoid (A), PEP-I (B) and PEP-II (C).

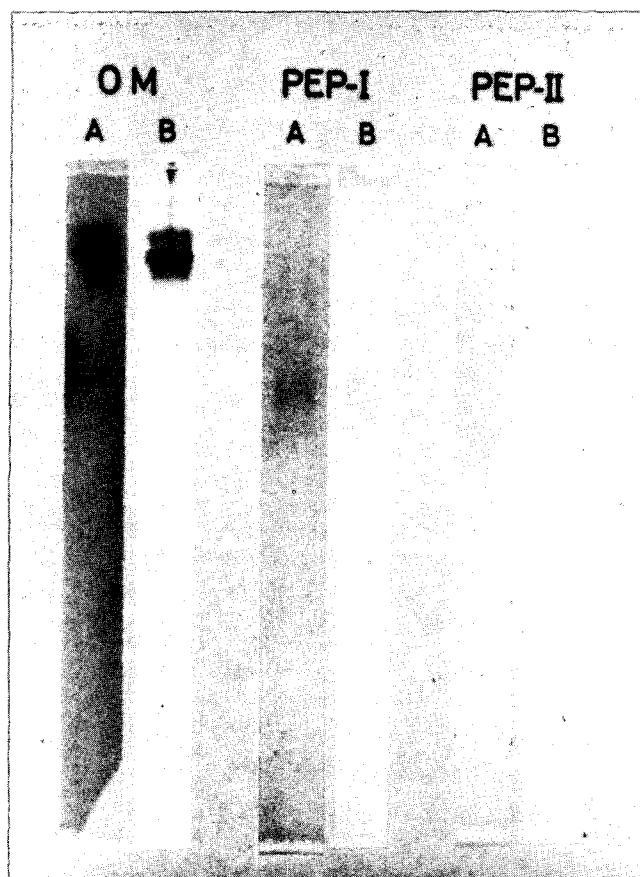


Fig. 3—SDS polyacrylamide gel electrophoresis of immunoreactive fragments prepared from the carbohydrate-containing fractions (PEP-I and PEP-II) by the immunoaffinity chromatography. Ovomucoid (OM) and the immunoreactive fragments prepared from PEP-I and PEP-II were applied to the gel, and the gel slabs were stained with Coomassie Brilliant Blue R-250 (A) or PAS reagent (B).

separated from PEP-I and PEP-II by the immunoaffinity chromatography. The positive reactions of the immunoreactive fragments to both protein and carbohydrate staining in the electrophoretogram (Fig. 3) indicated that these fragments did not consist of the mixture of peptide fragments and carbohydrate chains but that they were glycopeptides. When 2-mercaptoethanol was added to the sample buffer for electrophoresis, only slight bands were detected by either staining in the electrophoretogram (data not shown). This suggested that some peptides were attached to the glycopeptides through disulfide bonds (ovomucoid contain 9 disulfide bonds per molecule).

PRO-I, which seemed to be comprised almost entirely of carbohydrates, did not produce precipitin lines in the immunodiffusion assay and did not cause a PCA reaction. These results indicated that the peptide portion of the glycopeptides was essential to precipitin line formation and the positive PCA reaction. Tertiary conformation of peptide moieties crosslinked by disulfide bonds might convey the glycopeptide immunoreactivity.

The glycopeptide fragments remained with immunoreactivity even after ovomucoid was hydrolyzed for longer periods of time with a larger amount of pepsin, whereas other fractions without carbohydrate eluted from the gel filtration column had no immunoreactivity. It appears that polypeptide antigenic sites near carbohydrate attachment sites are not attacked easily by proteases like pepsin. This might be due to the protective effect of carbohydrate chains against proteolytic attack. Ovomucoid is a proteinase inhibitor and contains no fewer than 9 disulfide bonds. Hence, ovomucoid might ordinarily possess a tight and

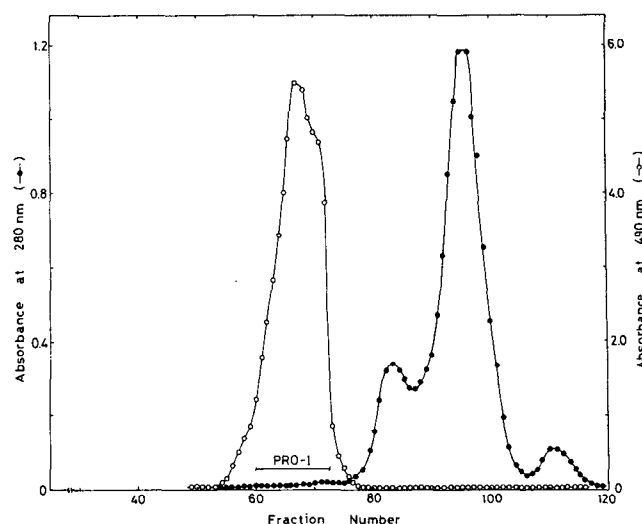


Fig. 4—Chromatography of pronase-hydrolyzed ovomucoid on a column (3 x 80 cm) of Sephadex G-50. Ovomucoid hydrolyzed with pronase was applied to the column and eluted with 0.1M CH_3COOH . The elution profile was monitored by the determination of hexose content (A_{490} , -○-) and UV absorbance (A_{280} , -●-). The fractions under the carbohydrate peak were pooled as indicated by bar, and freeze-dried (PRO-I).

proteolytically inaccessible conformation maintained by the high content of disulfide bonds.

In the present study, the immunoreactivity of ovomucoid glycopeptides to rabbit IgG and mouse IgE antibodies was demonstrated. However, the allergenic response in humans based on IgE antibody may be quite different in terms of molecular recognition sites from the antibody response in animals. Studies on the ovomucoid immunoreactivity to human antibody are now in progress, using the human sera from patients with known clinical histories of allergy to egg white.

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