

Isolation and Properties of An Immunoreactive Glycopeptide Connecting First and Second Domains of Chicken Ovomuroid

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A glycopeptide was isolated from a peptic hydrolysate of chicken ovomucoid using gel filtration after reduction with 2-mercaptoethanol. The glycopeptide was thought to be derived from the sequence Gly⁵¹-Tyr⁷³ of chicken ovomucoid and retained the immunoreactivity to IgG of mouse antiserum and IgE of human serum specific to ovomucoid. About fifty percent of the immunoreactivity to mouse antiserum was inhibited by the sera of patients allergic to ovomucoid and also by the carbohydrate chains on ovomucoid. These results suggested that the glycopeptide, connecting the first domain and second domain of chicken ovomucoid, participated in egg allergy, and the ovomucoid carbohydrate chains contributed to the immunoreactivity of the glycopeptide.

Ovomucoid is a typical allergenic protein in egg white, and antibody specific to ovomucoid is detected frequently in the sera from egg-allergic patients.^{1,2)} Ovomucoid is a protein with M.W. of about 28,000 containing about 25% carbohydrate.³⁾ The attachment sites of carbohydrate chains of ovomucoid were also identified,⁴⁾ and the structure of the carbohydrate chains has been reported by Yamashita *et al.*^{5~7)} In our previous study, some glycopeptides from ovomucoid were found to remain immunoreactive even after drastic hydrolysis with a large amount of pepsin.⁸⁾ It was also reported that IgE antibody in the sera from egg-allergy patients reacted to the carbohydrate-containing third domain of ovomucoid, but not to the carbohydrate-free one.⁹⁾

In this study, a glycopeptide was isolated from the PEP-I fraction of peptic hydrolysate of ovomucoid described previously,³⁾ the amino acid composition was analyzed, and the location of the glycopeptide was estimated based on its amino acid composition and the ovomucoid amino acid sequence. We have characterized the immunoreactivity of the glycopeptide using mouse antisera to ovomucoid by enzyme-linked immunosorbent assay

(ELISA), and the immunoreactivity was inhibited by the sera from allergic patients and the ovomucoid carbohydrate chains.

MATERIALS AND METHODS

Ovomucoid and its glycopeptide. Ovomucoid was isolated from egg white of White Leghorn hens by the procedure of Waheed and Salahuddin.¹⁰⁾ The PEP-I fraction of ovomucoid was prepared from ovomucoid treated with a large amount of pepsin, followed by gel-filtration with Sephadex G-50 as described previously.⁸⁾ The freeze-dried PEP-I fraction (10 mg) was dissolved in 5 ml of 50 mM Tris-HCl buffer, pH 8.6, containing 8 M urea, 0.2% EDTA, and 1.0% 2-mercaptoethanol. The solution was incubated at 25°C for 16 hr and put on a Sephadex G-25 column (70 × 2 cm), equilibrated and eluted with 0.1 M CH₃COOH solution. The fraction positive by the phenol-sulfuric acid assay was pooled and freeze-dried.

Ovomucoid carbohydrate chains. Ovomucoid (100 mg) was dissolved in 5 ml of 0.1 M borate buffer, pH 8.0, containing 10 mM calcium acetate. Two mg of Pronase (750 units/mg) was added to the ovomucoid solution every 24 hr over a 120 hr period under toluene. The hydrolysate was filtered on a Sephadex G-50 column equilibrated and eluted with 0.1 M CH₃COOH solution. Fractions containing carbohydrate were pooled and freeze-dried.

Human sera and mouse antisera. The human sera of allergic patients were obtained from Dr. Shimokata of the

First Department of Internal Medicine of Nagoya University Hospital. The sera with high RAST scores in both IgG and IgE antibody against ovomucoid were selected from sera of patients with known clinical histories of egg allergy. C3H mice (female, about five weeks of age, purchased from Shizuoka Agriculture Cooperative Association for Laboratory Animals, Hamamatsu, Japan) were immunized by the procedure described previously.⁹⁾ The sera were separated from individual blood samples by centrifugation and stored at -80°C before use.

Analytical methods. Amino acids were analyzed with a JLC-6AH amino acid analyser (JEOL, Tokyo) after hydrolysis with 6N HCl at 110°C for 24 hr in evacuated, sealed tubes. Neutral hexose was measured by the phenol-sulfuric acid method.¹¹⁾ The concentration of glycopeptide and carbohydrate chain was measured by sample weight after drying in an air oven. The N-terminal amino acid of the isolated peptide fraction was identified by the dansyl method.¹²⁾ Two hundred μg of dried peptide was dissolved in sodium bicarbonate (0.2 M, 10 μl), dansyl chloride solution (10 μl) added and kept at 37°C for 3 hr. Fifty μl of 6 N HCl was added to the solution and placed at 105°C for 18 hr. The dansyl amino acid was identified by thin-layer chromatography on polyamide sheets.¹³⁾

Enzyme-linked immunosorbent assay (ELISA) and its competitive inhibition analysis. The ELISA procedure was described in our recent study.¹⁴⁾

The immunoreactivity of glycopeptide was also investigated by competitive inhibition of ELISA using mouse IgG antibody to ovomucoid. Flat-bottomed microtiter plates were coated with a glycopeptide solution (5 $\mu\text{g}/\text{ml}$), and 100 μl of competitors (serially diluted allergic patients' sera or ovomucoid carbohydrate chains) were mixed with 50 μl of an antibody solution (1:1000 dilution of the mouse antiserum), and added to the wells. The mouse IgG which reacted with the plate-bound glycopeptide of ovomucoid was determined using a peroxidase-coupled anti-mouse IgG antibody as described previously.⁹⁾

RESULTS

The glycopeptide fraction of PEP-I was separated by gel filtration after the reductive cleavage of disulfide bonds. The elution profile from the gel filtration column is shown in Fig. 1. The first peak was positive to the phenol-sulfuric acid assay, indicating that the fraction under this peak contained carbohydrate. The fractions under this peak (Red-PEP-I, the shaded part in Fig. 1) were collected and characterized by amino acid analysis and ELISA.

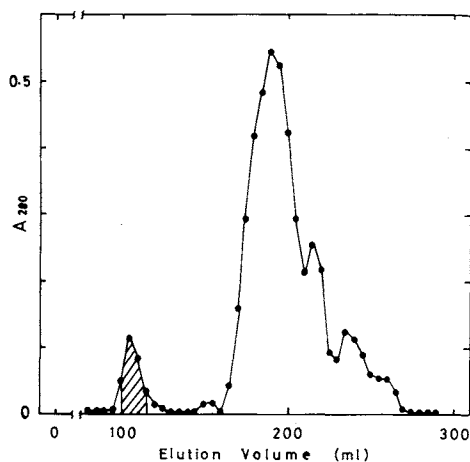


Fig. 1. Gel-filtration of PEP-I Fraction Reduced with 2-Mercaptoethanol on a Column of Sephadex G-25.

The shaded portion positive to phenol-sulfuric acid assay was pooled and freeze-dried.

TABLE I. AMINO ACID COMPOSITION AND N-TERMINAL AMINO ACID OF RED-PEP-I

Amino acid	Red-PEP-I (mol/mol)	Sequence (51~73)
Asx	3.0	3
Thr	2.1	2
Ser	2.8	3
Glx	3.2	3
Pro	N.D.	1
Gly	2.2	2
Val	1.1	1
Met	1.0	1
Ile	1.2	1
Tyr	1.0	1
His	1.2	1
Lys	2.3	2
N-Terminal	Gly	Gly

N.D., not determined.

Both the amino acid composition and N-terminal amino acid of Red-PEP-I are shown in Table I. The amino acid composition of the corresponding sequence (Gly⁵¹-Tyr⁷³) also is shown for comparison. The composition of Red-PEP-I was in good agreement with that of the sequence 51~73 within experimental error. The N-terminal amino acid was identified as Gly by the dansyl method. The location of the Red-PEP-I estimated from its amino acid

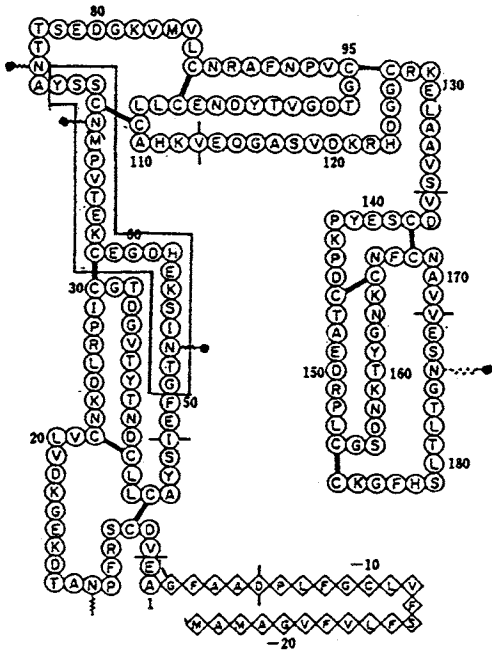


FIG. 2. Estimated Location of Red-PEP-I (Gly⁵¹-Tyr⁷³) Based on the Amino Acid Composition on the Ovomucoid Amino Acid Sequence (Kato *et al.*, 1987).

●—, carbohydrate chain.

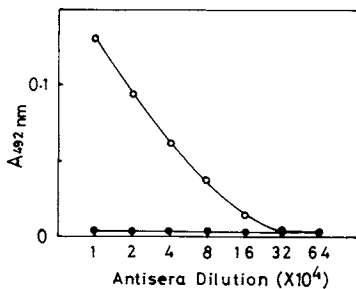


FIG. 3. Reactivity of Red-PEP-I with Mouse Anti-ovomucoid Antiserum (○) and Normal Serum (●) as Measured by ELISA.

ELISA plates were coated with Red-PEP-I. The reactivity of Red-PEP-I against antiserum and normal serum was expressed as peroxidase activity. Each point represents the mean of two measurements.

composition is shown in Fig. 2. The estimated position of Red-PEP-I belonged to the connection peptide between the first and second domain of ovomucoid, and the carbohydrate attachment sites of Asn⁵³ and Asn⁶⁹ residues were contained in this region.

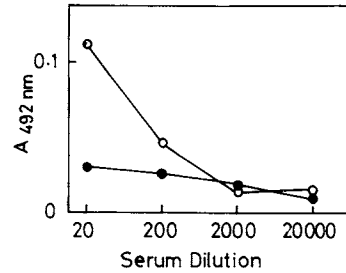


FIG. 4. Reactivity of Red-PEP-I with Patient Serum (○) and Normal Human Serum (●) as Measured by ELISA. ELISA plates were coated with Red-PEP-I. The reactivity of Red-PEP-I against patient and normal human serum was expressed as peroxidase activity. Each point represents the mean of two measurements.

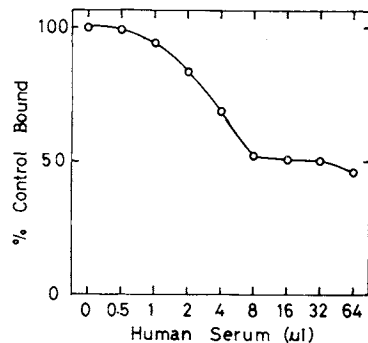


FIG. 5. ELISA Competitive Inhibition Analysis for the Allergic Reactivity of Red-PEP-I Using the Patient Serum as a Competitor.

Each point represents the mean of three measurements.

The immunoreactivity of Red-PEP-I against mouse anti-ovomucoid antibody was examined by the Ouchterlony method,¹⁵⁾ ELISA, and its competitive inhibition analysis. Though no immunoprecipitation line was detected by the Ouchterlony method (data not shown), the ELISA clearly demonstrated that Red-PEP-I was reactive to the antibody specific to ovomucoid. Figure 3 shows the ELISA profile of Red-PEP-I against both the mouse antiserum and the normal serum. The Red-PEP-I was positive against the antiserum diluted 10⁶ times or more, but completely negative to the normal serum, indicating that the Red-PEP-I was immunologically reactive.

The immunoreactivity of the Red-PEP-I to

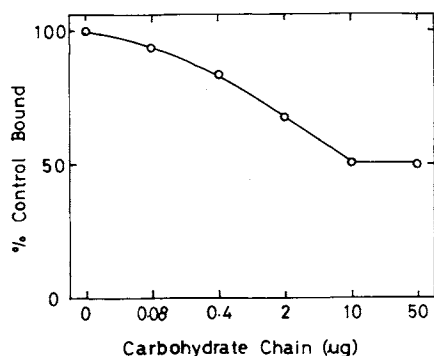


FIG. 6. ELISA Competitive Inhibition Analysis for the Immunoreactive Specificity of Red-PEP-I Using Ovomucoid Carbohydrate Chains as a Competitor.

Each point represents the mean of three measurements.

IgE antibody of serum from allergic patients was detected directly by the ELISA method and was negative to normal human IgE antibody (as shown in Fig. 4). Figure 5 shows that about fifty percent of Red-PEP-I immunoreactivity against anti-ovomucoid mouse antibody was inhibited by the serum of patients specific to ovomucoid, using the ELISA competitive inhibition analysis.

The ovomucoid carbohydrate chains was obtained from a hydrolysate of ovomucoid treated with a large amount of Pronase. It was detected by an amino acid analysis that the carbohydrate chains contain almost no other amino acid residues except aspartic acid residues (from asparagine). In the case of the ovomucoid carbohydrate chains as a competitor, again 50% of the immunoreactivity of Red-PEP-I against the mouse anti-ovomucoid antibody was inhibited by the ELISA competitive inhibition analysis (Fig. 6).

DISCUSSION

It was not easy to isolate some glycopeptides from the PEP-I fraction and to locate it in the ovomucoid amino acid sequence directly from its amino acid composition, because the peptides in the PEP-I fraction consisted of some smaller fragments which were cross-linked with each other by cystine disulfide bonds. Hence, only the glycopeptides could be sepa-

rated after the reductive cleavage of disulfide bonds.

Based on the homology of amino acid composition of the carbohydrate containing peptide Red-PEP-I, it could be said that Red-PEP-I was derived from the sequence Gly⁵¹-Tyr⁷³ on ovomucoid containing two cysteine residues.

It has already been reported that the reductive cleavage of ovomucoid disulfide strongly reduced its immunological and biological activity, probably because drastic changes of polypeptide conformation were induced. Nevertheless, the Red-PEP-I retained its immunoreactivity to mouse antiserum against ovomucoid (Fig. 3). It seems reasonable that the glycopeptide originally has no ordered conformation. Hence, the antigenic determinants of the Red-PEP-I might depend rather on its amino acid and carbohydrate sequence than on its conformation. Because the carbohydrate chains have antigenic activity (Fig. 6), it would be reasonable that the antigenic determinants of ovomucoid remained immunoreactive even after the drastic proteolysis with pepsin and heating at 100°C for a long time. Recently, we have also shown that ovomucoid carbohydrate chains linked to bovine serum albumin reacted with mouse anti-ovomucoid antiserum (data not shown).

In this study, the immunoreactivity of Red-PEP-I was detected against both the IgE antibody and IgG antibody of serum from allergic patients as shown in Fig. 4. More evidence was provided that the glycopeptide is immunoreactive to the patients serum of egg allergy by the ELISA competitive inhibition assay as shown in Fig. 5. It has been reported that the IgG and IgE antibodies of serum from patients allergic to pollen protein recognized the same epitope or a very near region.¹⁶⁾ Similarly, Red-PEP-I reactivity with the IgE antibody of patients' serum might be partly inhibited by the IgG antibodies of patients' serum. We had already shown that the carbohydrate chain attached to the third domain was important in the immunoreactivity of the carbohydrate-containing third domain to the patients' IgE

antibody.⁹⁾ Recently, we found that the immunoreactivity of ovomucoid against the patients' IgE antibody was decreased by chemical deglycosylation (manuscript in preparation). All these results clearly suggest that carbohydrate moieties of ovomucoid are important in immunoreactivity against the human IgE antibody. Furthermore, carbohydrate moieties of glycoproteins have a resistance to enzymatic hydrolysis and heat-denaturing. These properties of carbohydrate moieties seem to be important for ovomucoid as a dietary allergen.

We don't insist that the carbohydrate moiety of this glycopeptide is the only constituent having immunoreactive properties. Although enough evidence cannot be given, its peptide moiety must contribute to the immunoreactivity of this glycopeptide. This work only shows that the carbohydrate moiety might contribute to the immunoreactivity of the ovomucoid molecule. Further studies would have been done about the contribution of the carbohydrate moieties to the immunoreactivity of whole ovomucoid molecules.

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