Immunochemical Characterization of Ovomucoid from Japanese Quail Egg White Using Monoclonal Antibodies

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Summary The ovomucoid of Japanese quail egg white is known as a proteinase inhibitor or protein component responsible for egg allergy. In order to characterize the antigenic properties of ovomucoid in relation to its molecular structure, we have prepared two monoclonal antibodies, E9 (IgG1) and E11 (IgG1), recognizing distinct epitopes from each other. These monoclonal antibodies bound to the SDS/mercaptoethanol-treated ovomucoid, but not to the reductively carboxymethylated and pyridyl-ethylated ovomucoid. By immunoblotting analysis of the peptic digests of ovomucoid, it was shown that E9 bound to the fragment consisting of two domains, I and II, of the ovomucoid, and that E11 reacted with the fragment containing domain III. These results indicate that the antigenic activity depends on the conformational structure of domains tightly folded by disulfide linkages. Ovomucoids from hen and duck were also recognized by both the antibodies, although having less affinity compared to the one from Japanese quail. These antibodies proved to be effective in establishing an enzyme-linked immunosorbent assay system for quantitative analysis of quail ovomucoid.

Key Words ovomucoid, quail egg white, monoclonal antibody, epitope, antigenicity

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Avian eggs are very important foodstuffs for human beings, but contain some anti-nutritional protein components. One of the major components of egg white, comprising about 10% of the total egg white proteins (1), is ovomucoid, which serves not only as a proteinase inhibitor but also as a food allergen. Avian ovomucoid consists of three homologous domains (DI, II and III) and binds a few proteinase molecules (multi-headed type inhibitor) (2). Hen ovomucoid does not inhibit human trypsin, whereas ovomucoid from Japanese quail (Coturnix coturnix japonica) egg white (OMJPQ) inhibits human enzyme (3). This phenomenon is very important for human nutrition. Ovomucoid has also been numbered among the typical allergens in egg white (4, 5). There are several reports describing the heat stability of the antigenicity of hen ovomucoid and the effects of proteolytic digestion on the inhibitory activities against the proteinase of hen and quail ovomucoids (6–9). We demonstrated that the antigenic activity of OMJPQ could be found in the small intestine even when boiled egg was ingested (9). This suggests that peptide fragments from ovomucoid may share in antigenicity. From studies on the reactivities of egg white with sera of patients allergic to egg, Hoffman (5) reported that ovalbumin retained strong allergenicity in cooked egg white, and that ovomucoid had weak but not ignorable allergenicity in cooked egg. Furthermore, there is a report on the T-cell response to hen egg ovomucoid in relation to antigenicity (10). A study on the molecular structure of ovomucoids is, therefore, very important to understand egg antigenicity or allergenicity. On the other hand, reliable methods of detection or determination of ovomucoids in processed foods should be rapidly established in view of safely using processed foods by patients allergic to egg white. We attempted to prepare monoclonal antibodies (mAbs) against OMJPQ as a probe for analysis and detection of ovomucoid in processed foods containing egg white.

In the present paper, we describe the characterization of mAbs raised against OMJPQ and the antigenic properties of OMJPQ analyzed using mAbs.

MATERIALS AND METHODS

Materials. Commercial eggs of Japanese quail (C. c. japonica) were obtained from a local store. The materials used in the present study were obtained from the sources indicated in parentheses: Bovine trypsin (type III) and pepsin (2× crystallized) (Sigma, MO, USA), α-N-benzoyl-DL-arginine-p-nitroanilide HCl (Bz-DL-Arg-pNA) (Peptide Institute, Osaka, Japan), mouse myeloma cell line, P3×63 Ag8U1 (P3U1) (Shino Test Institute, Sagamihara, Japan), BALB/c mice (Nihon Clea, Tokyo, Japan), complete and incomplete Freund’s adjuvant (DIFCO, Detroit, USA), fetal bovine serum, sterilized L-glutamine and streptomycin-penicillin mixture (Life Technologies, NY, USA), RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan), a mouse monoclonal antibody isotyping kit (Amersham International PLC, Amersham, UK), aminopterin (Sigma), polyethylene glycol 4000 (Merck, Darmstadt, Germany), protein A (Bio-Rad, CA, USA), nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), mouse IgG (mg/mL) (MLB, Nagoya, J Nutr Sci Vitaminol
Immunocchemical Characterization of Ovomucoid

Preparation of quail, hen and duck ovomucoids. The purification of OMJPQ was done as described previously (9). Briefly, crude OMJPQ was obtained in a 75% alcohol precipitate and dissolved in 50 mM NH₄HCO₃, put on a Sephacryl S-200 column (2.5 × 90 cm) equilibrated with the same buffer, and chromatographed. The fractions containing protein with trypsin inhibitory activity were collected and lyophilized. The lyophilized preparation was dissolved in 50 mM sodium acetate/HCl buffer (pH 3.5) and put on a column (2.5 × 45 cm) of SP-Sephadex C-25 equilibrated with the same buffer. The inhibitor fractions eluted from the column were collected, desalted, and lyophilized. Ovomucoids of hen and duck were prepared from their egg whites in the same manner as mentioned above. The reductively carboxymethylated OMJPQ (RCM-OMJPQ) and pyridylethylated OMJPQ were prepared by the methods of Stein et al (11) and Hermodson et al (12), respectively. RCM-OMJPQ and pyridylethylated OMJPQ were finally purified by HPLC with a column of ODS-80Ts (4.6 mm × 15 cm, Tohso, Tokyo). The OMJPQ preparations obtained gave a single protein band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of mAbs. Female 8-wk-old BALB/c mice were intraperitoneally injected with a mixture of intact OMJPQ (50 μg/mouse) and complete Freund’s adjuvant. At intervals of 3 wk, booster shots were carried out two times with a mixture of OMJPQ (50 μg/mouse) and incomplete Freund’s adjuvant. Finally, the mice were intraperitoneally injected with OMJPQ (50 μg/mouse) in 10 mM sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl (PBS). The fusion between spleen cells obtained from the immunized mice and myeloma cells (P3U1) was essentially according to the method of Tsuji et al (13). The hybridoma cells thus obtained were intraperitoneally injected into female BALB/c mice which had been primed with pristane within 1 wk. After 10 to 14 d, ascitic fluids that formed were collected. The mAbs against OMJPQ in the ascites were fractionated with (NH₄)₂SO₄. An IgG mAb was further purified with a protein A column. Amounts of the mAbs were determined by the method of Lowry et al (14) using nonimmune mouse IgG as a standard. Mice were cared for in line with the standards in the Guideline for the Care and Use of Laboratory Animals of the University of Tokushima School of Medicine and Mukogawa Women’s University.

Enzyme-linked immunosorbent assay (ELISA). Each 100 μL of the intact-OMJPQ (100 μg/mL) was infused into wells of a 96-well microplate. After incubation at 4°C overnight, the wells were loaded with 100 μL of 1% bovine serum albumin (BSA) dissolved in PBS. The wells were washed four times with PBS containing 0.05% Tween 20 (PBS/Tween) and incubated with culture supernatants or either of the mAbs. Next, the wells were washed with PBS/Tween and mixed with peroxidase-conjugated sheep anti-mouse IgG (mg/mL), and diluted...
1:1,000 with PBS/Tween containing 0.1% BSA. The immunocomplexes on the wells were incubated for 15 min at room temperature with o-phenylenediamine (0.4 mg/mL) and 0.01% H₂O₂ as the substrates in 0.1 M citrate/0.1 M phosphate buffer (pH 5.0) and absorbance of the reaction mixtures in the wells at 490 nm were taken on a microplate reader, Model 450 (Bio-Rad).

**Binding of mAbs to RCM- and pyridylethylated OMJPQ immobilized on the wells of a microplate.** One-hundred microliters of the RCM and pyridylethylated OMJPQ (100 µg/mL) in PBS was infused into the wells of a 96-well microplate and incubated at 4°C overnight. After blocking with 1% BSA in PBS and washing with PBS/Tween, the protein on the well was mixed with varying concentrations of mAb (E9 or E11). The immunocomplexes were assayed as described in ELISA.

**Sandwich ELISA.** Peroxidase-conjugated E11 used in a sandwich ELISA system was prepared according to the method of Nakane and Kawaoi (15). One-hundred microliters of E9 (100 µg/mL) was infused into the wells of a 96-well microplate and incubated overnight at 4°C, followed by the addition of 1% BSA in PBS at room temperature for 1 h and then washing four times with PBS/Tween. The E9 adsorbed on the wells was allowed to react with varying amounts of OMJPQ for 2 h and then with a fixed concentration (10 µg/mL) of peroxidase-conjugated E11. The immunocomplexes were assayed as described in ELISA.

**Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis (SDS-PAGE).** SDS-PAGE was done in 15% slab gels by the method of Laemmli (16). The proteins separated on the gels were stained with 0.1% Coomassie Brilliant Blue R250.

**Immunoblot analysis.** After electrophoresis, proteins in a slab gel were electrophoretically transferred on a nitrocellulose membrane. The membrane was incubated with 1% BSA in 20 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl and 0.05% Tween 20 (buffer A), and then subjected to react with a mAb against the OMJPQ. As the control, nonimmune IgG was used instead of mAbs (E9 and E11). The bound mAb was treated with peroxidase-conjugated sheep anti-mouse IgG (diluted 1:2,000 with buffer A containing 0.1% BSA). The immunocomplexes on the membranes were detected by incubation for 1 h at room temperature with 4-chloro-l-naphthol (0.3 mg/mL) and 0.05% H₂O₂ as the substrates in 50 mM Tris-HCl buffer (pH 7.3). Immunoblotting of the peptic digest of OMJPQ was done in the same manner.

**N-terminal sequences of the peptic digest of OMJPQ.** The digestive products were separated by SDS-PAGE, and proteins on a slab gel were electrophoretically transferred to a sheet of PVDF membrane as described by Towbin et al (17). The PVDF membrane was then stained with 0.1% Coomassie Brilliant Blue R250 dissolved in methanol/acetic acid/water (5:1:4, by vol.). The amino acid sequences were determined by an Applied Biosystems model 477A/120A protein sequencer and PTH-analyzer according to the instructions for users.
RESULTS AND DISCUSSION

Preparation of mAbs

BALB/c mice intraperitoneally immunized with intact OMJPQ produced specific antisera with high antibody titer. The spleen cells of an immunized mouse were fused with P3U1 myeloma cells. Finally, two hybridoma cell lines producing mAbs against OMJPQ have been established. The two mAbs thus obtained were named E9 and E11. The class and subclass of the mAbs were examined using a mouse monoclonal antibody isotyping kit. The class and light chain of both mAbs proved to be IgG1 and κ, respectively.

Properties of mAbs

As shown in Fig. 1B and C, both mAbs E9 and E11 were bound to the SDS/mercaptoethanol-treated OMJPQ, but not to the RCM-OMJPQ and pyridylethylated OMJPQ. This fact was also confirmed by ELISA as shown in Fig. 2, suggesting that the epitopes of two mAbs are not sequential. In this assay system, the apparent dissociation constants (Kd) of immunocomplexes between E9 or E11 and OMJPQ were estimated at about $10^{-7}$ M or $10^{-11}$ M. As shown in Fig. 1A, the SDS/mercaptoethanol-treated OMJPQ in lane 1 was smaller in molecular weight than the RCM-OMJPQ in lane 2. It is well known that avian ovomucoids consist...
of three domains, one of which locates in the one-third region from the NH₂-terminal (2). This domain contains three intradomain disulfide bridges and is totally inactive as a trypsin inhibitor. The other two-thirds region contains 2nd and 3rd domains with 6 intramolecular disulfide bonds, which have proteinase inhibitory activity. These domains are tightly folded by multiple disulfide bonds so that the ovomucoid molecule is considered to behave as a low-molecular protein in SDS-PAGE beyond expectation. As a result of reduction and carboxymethylation of cystine residues, OMJPQ really gave a higher molecular weight by SDS-PAGE (Fig. 1A). The irreversibly reductive cleavage of disulfide bonds leads to the unfolding of a higher dimensional structure. For this reason, the conformation of epitopes collapsed and became insensitive to immuno-reaction. This result suggests that the allergenicity of ovomucoid may be reduced by destruction of the higher dimensional structure. Ovomucoid is comprised of domain structures rich in disulfide bonds; thus, heat treatment alone is not sufficient to denature or unfold their higher dimensional structures. Furthermore, it was shown by immunoblotting that the two mAbs could recognize both hen and duck ovomucoids like OMJPQ (Fig. 3). Hen and duck ovomucoids consist of three tandem domains homologous to OMJPQ (18) and resemble ovomucoids in amino acid sequences as well. The ELISA assay, however, revealed that the binding activity of these ovomucoids to the mAb (E9 or E11) was very weak compared with that of OMJPQ (Fig. 4). In order to examine the epitope regions of OMJPQ against mAbs (E9 and E11), intact OMJPQ was digested with pepsin (OM: pepsin = 1 mg: 10 μg) in sodium citrate HCl buffer (pH 3.0) for 1 h. The peptic digest was separated by SDS-PAGE and immunoblotted with each mAb (Fig. 5). E9 could recognize 22.0, 17.8, and 17.0 kDa peptides in addition to OMJPQ, while E11 could recognize 22.0, 17.8, and 10.5 kDa peptides but not the 17.0 kDa peptide (Table 1). These results show that the two mAbs
Fig. 3. Immunoblots of SDS/mercaptoethanol-treated ovomucoids of quail (lane 1), hen (lane 2) and duck (lane 3) with mAbs (E9 and E11). A and B show the ovomucoid immunoblotted with E9 and E11, respectively. M indicates the standard proteins.

Fig. 4. Binding of E11 to the ovomucoid (OM) of quail (○), hen (●) and duck (△) immobilized on the wells of a microplate.

recognize distinct epitopes on OMJPQ, suggesting that these mAbs are applicable to sandwich ELISA, which requires more than two different antibodies. In the present study, we tried to determine OMJPQ by sandwich ELISA using E9 and E11. Consequently OMJPQ could be assayed in the range of 0.5–100 ng (Fig. 6). The peptic digests separated on SDS-PAGE were subjected to the sequencing of N-terminal amino acids. The N-terminal amino acid sequences of the peptides are shown in Table 1. Although each peptide fragment with molecular weight of 22.0, 17.8, or 17.0 kDa showed broad bands on SDS-PAGE, they gave the same sequences for the N-terminal side. It is known that peptide fragments possessing carbohydrate
Fig. 5. Immunoblotting patterns of SDS/mercaptoethanol-treated OMJPQ and peptic hydrolyzates of OMJPQ. A: proteins stained with Coomassie Brilliant Blue R250. B and C: proteins immunoblotted with mAbs E9 and E11, respectively. Lanes 1, 4 and 6 show SDS/mercaptoethanol-treated OMJPQ. Lanes 2, 3, 5 and 7 show peptic hydrolyzates of OMJPQ.

Table 1. N-Terminal sequences of the pepsin-digested products and reactivity against mAbs.

<table>
<thead>
<tr>
<th>Peptides (kDa)</th>
<th>Sequences</th>
<th>E9</th>
<th>E11</th>
</tr>
</thead>
<tbody>
<tr>
<td>22.0</td>
<td>LRLI-GTDG---</td>
<td>+*</td>
<td>+</td>
</tr>
<tr>
<td>17.8</td>
<td>LRLI-GTDG---</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>17.0</td>
<td>LRLI-GTDG---</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10.5</td>
<td>VSVD-SEYP---</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*a* + and − indicate that the peptides could be recognized or not, respectively.

chains give a broad migration band on SDS-PAGE (19). Accordingly, the molecular size of each fragment may depend on the difference in sequence of the C-terminal side. The fragment with the molecular weight of 10.5 kDa was different from those of the 22.0, 17.8, and 17.0 kDa fragments in the N-terminal amino acid sequence. Two 22.0 and 17.8 kDa fragments were bound to both mAbs, while the 17.0 kDa fragment was bound only to E9 and the 10.5 kDa fragment bound only to E11. Based on these results, possible regions of the mAb-binding OMJPQ site are illustrated in Fig. 7. It has become apparent that two mAbs can recognize distinct domain structures (20) on the OMJPQ molecule; namely E9 can bind domain I, II, and E11 can bind domain III. The role of the carbohydrate moieties of OMJPQ in the reaction between OMJPQ and mAbs requires further discussion. In any case, these mAbs can be practically used not only to detect and quantify allergenically positive ovomucoid or its related peptide fragments in processed foods, but also to analyze the allergenicity of ovomucoids in more detail.

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Fig. 6. Dose response curves for intact OMJPQ in sandwich ELISA. Experimental details are described in the text.

Fig. 7. Peptic fragments of OMJPQ recognized by mAb E9 (●) and mAb E11 (◆).
REFERENCES


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