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Antigenic Peptides of Ovalbumin Digested with Trypsin or Chymotrypsin

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Ovalbumin (OVA) is a major allergen of hen's egg white and often induces hypersensitivity in atopic children. Further knowledge of the antigenic and allergenic determinants of OVA will allow better treatment to be provided for this disease. We investigated the antigenic determinants of OVA by immunoblotting and a microsequence analysis. OVA was digested with trypsin or chymotrypsin, and the peptides were separated by tricine SDS-polyacrylamide gel electrophoresis. The antigenicity was tested by binding an anti-OVA rabbit IgG antibody with the peptides. Binding studies revealed that almost all bands of the tryptic peptides with MW of more than 5,520 and almost all bands of the chymotryptic peptides with MW of more than 7,280 were positive. It is proposed that a part of the antigenic determinant existed in the OVA sequence of 253-261 (or 264).

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INTRODUCTION

Ovalbumin is a major allergen of hen's egg white and often induces hypersensitivity in atopic children (Langeland 1983). Knowledge of the antigenic and allergenic determinants of ovalbumin may lead to an increased understanding of egg allergy and may contribute to better diagnosis and treatment of the disease: *e.g.*, by blocking the allergic reaction with haptenic peptides (Honma *et al.* 1996) or by inducing protective antibodies by other haptenic peptides (Kahler *et al.* 1992).

The antigenic and allergenic determinants of ovalbumin have been investigated by Elsayed *et al.* (1986), and a subsequent study that has shown that the N-terminal decapeptide (Elsayed *et al.* 1988) encompassed an Ig-binding haptenic epitope. Peptide 323-339 has been shown to be a T-cell epitope in mice and the antigenicity was distributed over the whole region of 11-70 (Elsayed and Stavseng 1994).

This study aimed to characterize the antigenic determinants of ovalbumin by immunoblotting and microsequence analysis methods. For this purpose, ovalbumin was digested with trypsin or chymotrypsin, and the antigenicity was measured as the binding activity of the peptide with the anti-ovalbumin rabbit IgG antibody.

MATERIALS AND METHODS

Materials

Chicken ovalbumin was purchased from Taiyo Kagaku Co. (Tokyo, Japan). L-(1-Tosylamide-2-phenyl)ethyl chloromethyl ketone-treated trypsin (TPCK-trypsin, from bovine pancreas type XIII, 11,700 units/mg), N-*p*-Tosyl-L-lysine chloromethyl ketone-treated chymotrypsin (TLCK-chymotrypsin, from bovine pancreas, 50 units/mg) and goat anti-rabbit IgG conjugated with Sigma horseradish peroxidase VI (HRP-labeled goat anti-rabbit IgG, 100 purpurogallin units/mg) were each purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Digestion of ovalbumin with trypsin or chymotrypsin

Digestion was performed according to the method of Ametani *et al.* (1987). A solution of 2 mg of OVA in 1.0 ml of a 0.1 M NH_4OH/HCOOH buffer at pH 8.5 or 8.0 was treated with 0.02 mg of TPCK-trypsin or TLCK-chymotrypsin, and the mixture was incubated for 24 h at 37 ℃. The enzymes were inactivated by cooling for 5 min in iced water, the digested products being immediately lyophilized and stored at −20℃.

Preparation of the rabbit anti-ovalbumin antibody

A female rabbit (New Zealand White), weighing about 2 kg, was intraperitoneally injected with 0.25 mg of OVA in 0.85% NaCl that had been emulsified with

an equal volume of Freund's complete adjuvant (Difco Labs., Detroit, MI, U.S.A.). The rabbit was boosted with 0.5 mg of OVA at 2-week intervals, and 11 weeks after the first immunization, the rabbit was bled. The antiserum was separated and 0.05% sodium azide was added, after which the solution was stored at −80℃.

Double radial immunodiffusion

Ouchterlony immunodiffusion (Ouchterlony 1952) was carried out in 1.2% agarose in PBS containing 0.1 % NaN_3.

Tricine SDS-polyacrylamide gel electrophoresis

A tricine SDS-polyacrylamide gel electrophoresis for separating the trypsin or chymotrypsin digest of ovalbumin was performed according to the modified method of Schägger and Jagow (1987), using a discontinuous gel comprising a 4% stacking gel, 10% spacer gel and 16.5% separating gel. The anodic buffer was 0.2 M tris(hydroxymethyl)aminomethane (Tris)-HCl at pH 8.9, while the cathodic buffer was 0.1 % SDS in a 0.1 M Tris-0.1 M N-[tris (hydroxymethyl) methyl] glycine (Tricine) buffer at pH 8.2. The protease digest of ovalbumin was dissolved in a mixture of 0.04% SDS, 12% glycerol, 2% β-mercaptoethanol and 0.05 % bromophenol blue in a 50 mM Tris-HCl buffer at pH 6.8, and the solution was incubated for 3 min at 100℃. A constant current of 10 mA was supplied for about 1 h, allowing each sample to completely enter the stacking gel. The current was then raised to 20 mA and maintained at this level for about 4 h of electrophoresis. The gel sheet was stained with 0.2 % Coomassie brilliant blue R250 in a mixture of 45 % methanol and 7 % acetic acid, and destained with a mixture of 10% isopropanol and 7% acetic acid.

Two kinds of standard protein mixtures were used as molecular weight markers. One of them was cytochrome C and its polymers (the hexamer of MW 74,400, tetramer of MW 49,600, trimer of MW 37,200, dimer of MW 24,800, and monomer of MW 12,400; Oriental Yeast Co., Tokyo, Japan), and the other was myoglobin fragments and glucagon (myoglobin 1-153 of MW 16,950, myoglobin 1-131 of MW 14,440, myoglobin 56-153 of MW 10,600, myoglobin 56-131 of MW 8,160, myoglobin 1-55 of MW 6,210, glucagon of MW 3,480, and myoglobin 132-153 of MW 2,510; Sigma Chemical Co., St. Louis, MO, U.S.A.).

Immunoblotting

Immunoblotting was performed according to the modified method of Ohmi *et al.* (1995). The separated peptides in the gel were electroblotted on to a nitrocellulose membrane (0.45 μm in pore size, Nippon Bio-Rad Laboratories, Tokyo, Japan) in a 0.025 M Tris-0.192 M glycine buffer at pH 8.7 containing 20 % methanol at 2 mA/cm^2 for 60 min with a semi-dry blotting system (Bio Craft Co., Tokyo, Japan). The western blotted membrane was then cut in two. One sheet of the membrane, for detecting peptides, was stained with 0.02% Amido black 10 B in 45% methanol-7% acetic acid, and destained with 10% methanol-7% acetic acid. The other sheet of the membrane, for detecting antigenicity, was blocked with 2% casein in a 0.01 M phosphate buffer containing 0.15 M NaCl at pH 7.2 (PBS) for 1 h at room temperature or overnight at 4℃. After washing three times with 0.05 % Tween-20 in PBS (Tween-PBS), the membrane was then incubated with the anti-ovalbumin rabbit IgG antibody or rabbit serum, which had been diluted 500 times with 2% casein in Tween-PBS, for 1 h at room temperature. The IgG antibodies which reacted with the membrane-bound antigens were determined by using HRP-labeled goat anti-rabbit IgG, which had been diluted 250 times with 2% casein in Tween-PBS, for 1 h at room temperature after washing three times with Tween-PBS. After subsequently washing five times more with Tween-PBS, the bound antibodies were detected by incubating a freshly prepared solution of 3 mg of 4-chloro-1-naphtol and 2 μl of 30% hydrogen peroxide in 1.0 ml of methanol and 4.0 ml of PBS. Rabbit sera which had not been immunized against ovalbumin were used as a control.

N-terminal sequence analysis

The N-terminal amino acid sequence of the peptide on a PVDF membrane was analyzed by a 476A protein sequencer (Applied Biosystems, CA, U.S.A.). Western-blotting of the peptides was performed according to the methods of Matsudaira (1987) and Hirano (1993). The gel was electroblotted on to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P^{SQ}, 0.1 μm pore size, Millipore Co., Bedford, MA, U.S.A.) at 1 mA/cm^2 for 90 min with a semi-dry blotting system. Before electroblotting, the gel, PVDF membrane and one of three kinds of filter paper (cathodic side) were dipped into a mixture of 25 mM Tris, 40 mM ε-amino-*n*-caproic acid, 20 % methanol and 0.05 % SDS. Another filter paper (next to the former paper) was dipped into a mixture of 25 mM Tris, 20% methanol and 0.05% SDS, and a third filter paper (anodic side) was dipped into a mixture of 0.3 M Tris, 20% methanol and 0.05% SDS. The western blotted PVDF membrane was stained with 0.02% Coomassie Brilliant Blue R250 in a 45 % methanol-7% acetic acid mixture, and

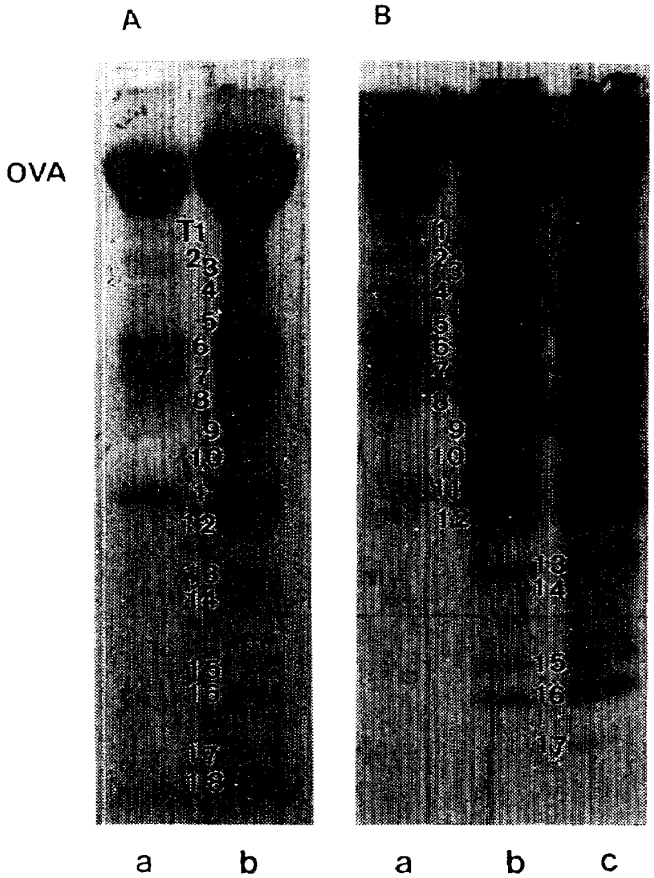

Fig. 1. Tricine SDS-polyacrylamide gel electrophoresis and immunoblotting of the tryptic peptides of ovalbumin

Ovalbumin was digested with trypsin at pH 8.5. Tricine SDS-polyacrylamide gel electrophoresis for separating the trypsin digest of ovalbumin was performed according to the method of Schägger and Jagow (1987), using a discontinuous gel comprising a 4% stacking gel, 10% spacer gel and 16.5% separating gel. Immunoblotting was performed according to the method of Ohmi *et al.* (1995). Binding of the anti-ovalbumin IgG antibody on western-blotted peptides on an NC membrane was examined. A: Amido black 10B-stained peptides; a, 10 μg; b, 50 μg. B: Immunoblotted peptides; a, 0.2 μg; b, 1 μg; c, 5 μg.

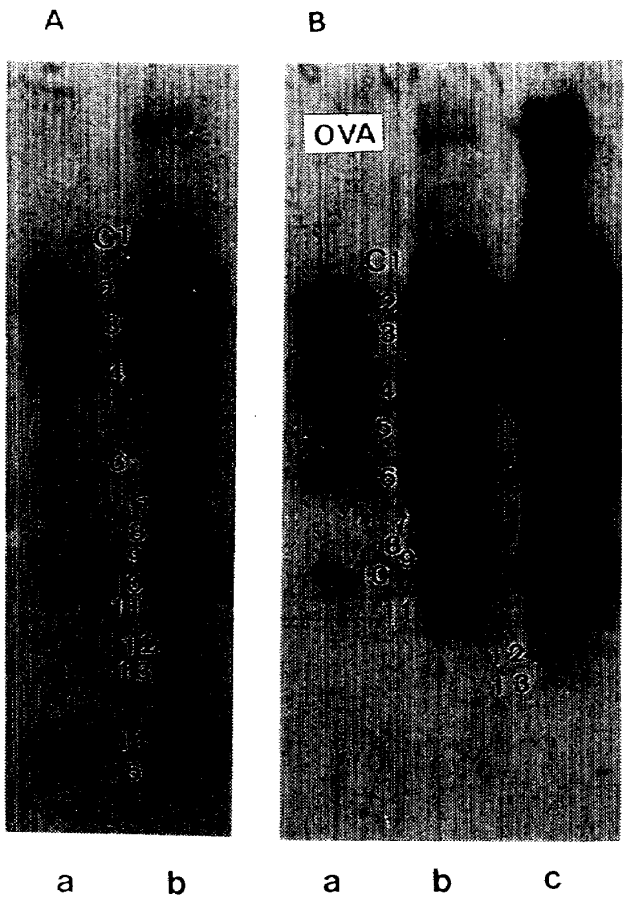

Fig. 2. Tricine SDS-polyacrylamide gel electrophoresis and immunoblotting of the chymotryptic peptides of ovalbumin

Ovalbumin was digested with chymotrypsin at pH 8.0. The analytical conditions are same as these in the legend to Fig. 1. A: Amido black 10B-stained peptides; a, 10 μg; b, 50 μg. B: Immunoblotted peptides; a, 0.2 μg; b, 1 μg; c, 5 μg.

destained with 60% methanol.

RESULTS AND DISCUSSION

Trypsin or chymotrypsin digestion of ovalbumin

Ovalbumin was exhaustively digested with trypsin or chymotrypsin. The mixtures of the peptides were analyzed by tricine SDS-polyacrylamide gel electrophoresis. Figures 1-A and 2-A show the western-blotted peptides after staining the nitrocellulose membrane with Amido black 10B. Trypsin or chymotrypsin itself was not visible under this condition. Trypsin digested ovalbumin into 18 peptides with MW between 31,500 (T1) and 4,400 (T18), the main peptides being T6, T7 and T11. Chymotrypsin digested ovalbumin into 15 peptides with MW between 25,500 (C1) and 4,000 (C15), the main peptides being C2, C3, C4, C6, C10 and C14.

Antigenicity from immunoblotting with the nitrocellulose membrane

A rabbit anti-ovalbumin antiserum was prepared as described in the MATERIALS AND METHODS section. The reactivity of the anti-ovalbumin antibody to an intact molecule of ovalbumin was tested by the double radial immunodiffusion method. The precipitin line for the antigen and antibody reaction was detected up to a thirty-second dilution of the antiserum (Odani *et al.* 1997). The antigenicity of the peptide, *i.e.*, the binding activity of the anti-ovalbumin

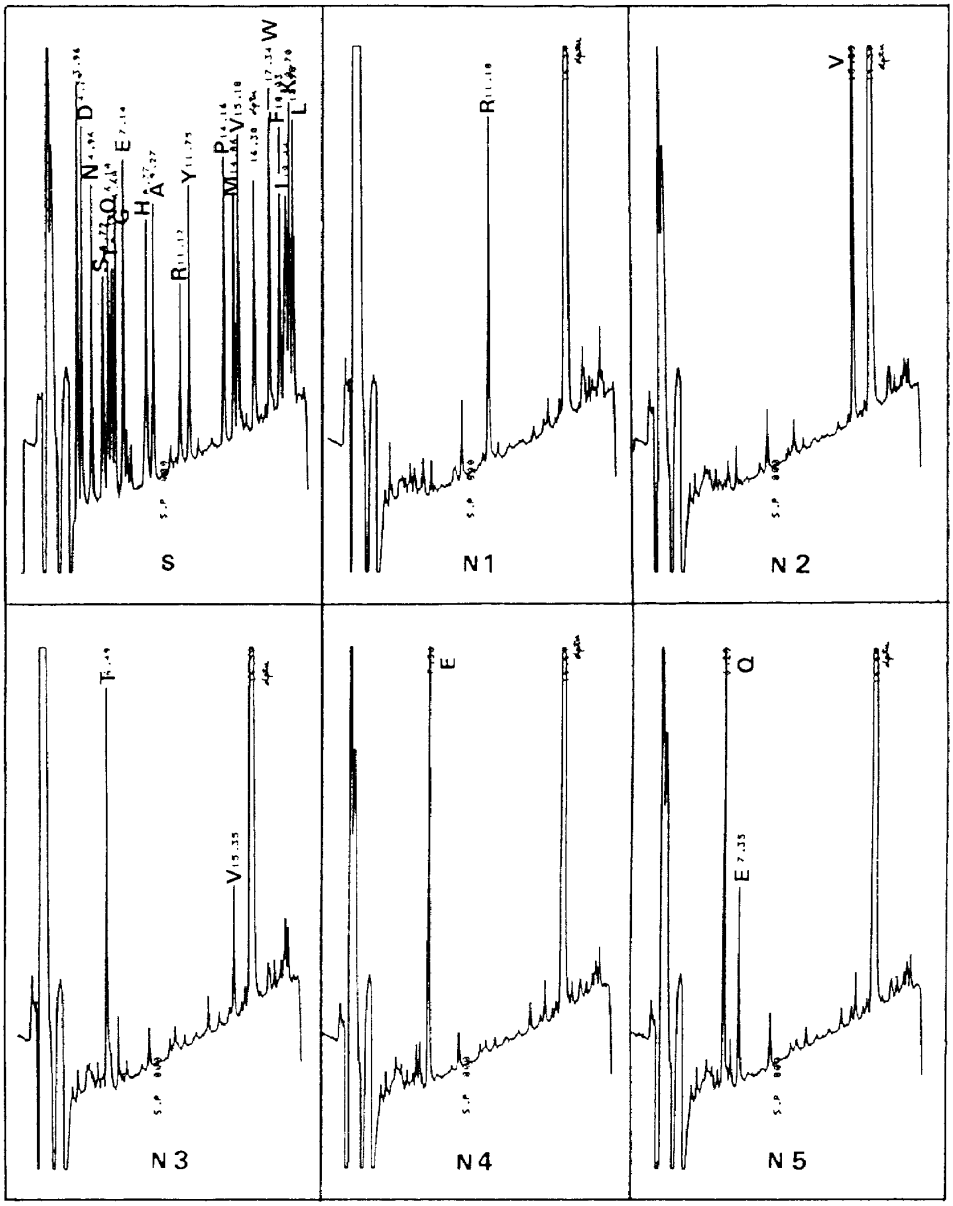

Fig. 3. HPLC chromatograms from the sequence analysis of antigenic peptide C13

Antigenic peptide C13, which was reactive to the anti-ovalbumin IgG antibody, was submitted to a 476A protein sequencer (Applied Biosystems, CA, U.S.A.), after having been transferred to a PVDF membrane and Coomassie-stained. S, a standard mixture of PTH-amino acids; N1-N5, N-terminal PTH-amino acid at each step.

rabbit IgG antibody with the western-blotted peptide on the nitrocellulose membrane, is shown in Figs. 1-B and 2-B. A binding test of the blotted peptide with non-immunized rabbit serum was carried out for detecting non-specific binding, but not a single peptide was bound to the serum (data are not shown). The tryptic peptides with MW of more than 5,520 (T17) and the chymotryptic peptides with MW of more than 7,280 (C13) showed antigenicity. Only T18 (MW 4,400), C14 (MW 6,070) and C15 (MW 4,000) failed to react with the anti-ovalbumin rabbit IgG antibody.

N-terminal analysis of the peptide

Some of the peptide bands which were reactive and non-reactive to anti-ovalbumin IgG antibody were submitted to a microsequence analysis after their transfer on to a PVDF membrane and Coomassie staining. Only five amino acids from the N-terminal end of the peptide were analyzed, since the primary structure of ovalbumin is already known: *i.e.*, oval-

Table 1. N-terminal sequences of the tryptic peptides of ovalbumin

Peptide	MW*	Antigenicity	Sequence (MW**)
T 6	22,000	+++	V_{200} TEQE-P_{385} (22,425)
T 7	19,500	+++	AcG_1-R_{158} (19,887)
T 8	18,100	+++	V_{200} TEQE-R_{339} (17,548)
T 10	16,300	++	V_{200} TEQE-K_{322} (15,792)
T 13	10,700	++	AcG_1-R_{104} (11,575)
T 15	8,750	++	I_{278} KVVL-R_{339} (8,697)
T 16	7,000	++	E_{143} LINS-K_{206} (7,255)
T 17 a	5,520	+	N_{159} VLQP-K_{206} (5,413)
T 17 b			I_{229} LELP-R_{276} (5,410) or K_{277} (5,539)
T 18 a	4,400	−	V_{56} VRFD-K_{92} (4,045)
T 18 b			L_{105} YAEE-R_{142} (4,458)

Tryptic peptides were western-blotted on to PVDF membranes and Coomassie-stained. Five amino acids from the N-terminal end of the peptide spots were analyzed by a protein sequencer. *MW by a tricine SDS-polyacrylamide gel electrophoresis analysis. **MW by the sequence analysis; antigenicity: +++, strong; ++ , medium; + , weak; − , not detectable.

Table 2. N-terminal sequences of the chymotryptic peptides of ovalbumin

Peptide	MW*	Antigenicity	Sequence (MW**)
C 2	21,800	+++	R_{199} VTEQ-P_{385} (22,687)
C 3	20,100	+++	AcG_1-F_{180} (20,157)
C 4	17,700	+++	V_{243} LLPD-P_{385} (17,700)
C 6	14,100	++	V_{243} LLPD-F_{358} (14,657)
C 9	11,100	+	S_{98} FSLA-F_{198} (11,486)
C 10	9,400	++	A_{107} EERY-F_{188} (9,298)
C 13	7,280	+	R_{199} VTEQ-F_{261} (7,092) or L_{264} (7,463)
C 14	6,070	−	R_{199} VTEQ-L_{252} (6,019)

Chymotryptic peptides were western-blotted on to PVDF membranes and Coomassie-stained. Five amino acids from the N-terminal end of the peptide spots were analyzed by a protein sequencer. *MW by the tricine SDS-polyacrylamide gel electrophoresis analysis. **MW by the sequence analysis; antigenicity: +++, strong; ++, medium; +, weak; −, not detectable.

bumin consists of 385 amino acid residues, the molecular weight of the polypeptide chain is 42,699, the N-terminus is acetylated, the carbohydrate moiety is located at N_{292}, and two phosphorylated serines are in residues 68 and 344 (Nisbet *et al.* 1981). The molecular weight of the carbohydrate moiety was estimated to be about 2,000: *i.e.*, the molecular weight of ovalbumin (45,000) as determined by physical methods (Warner 1954) minus the sum of the molecular weights of the polypeptide chain, an acetyl group and two phosphoryl groups.

HPLC chromatograms showed that the antigenic C13 peptide band was pure, because only one peak of phenylthiohydantoin (PTH)-amino acid was detected at each step (Fig. 3). The primary structure of the peptide was deduced from the N-terminal sequence, the molecular weight by the tricine SDS-polyacrylamide gel electrophoresis analysis, and the C-terminal amino acid that was specifically cleaved with trypsin or chymotrypsin. Eleven tryptic peptides and eight chymotryptic peptides were determined. The results are listed in Tables 1 and 2, and in Fig. 4.

The results of the tricine SDS-polyacrylamide gel electrophoresis, the immunoblotting experiment and

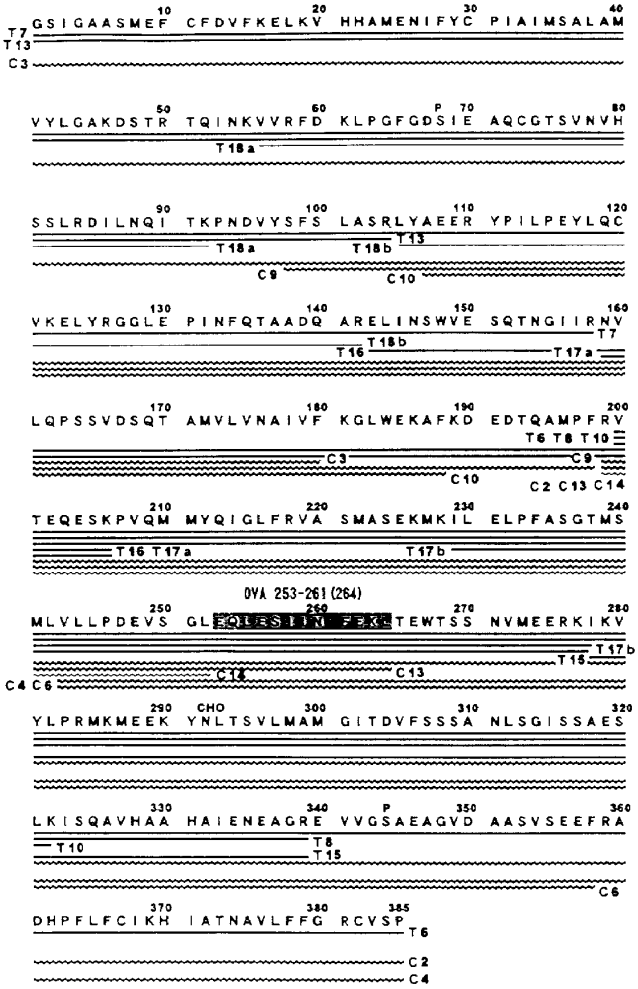

Fig. 4. Location of the antigenic peptides of ovalbumin digested with trypsin or chymotrypsin

The bold line shows the antigenically positive tryptic peptide, and the light line shows the antigenically negative tryptic peptide. The bold curved line shows the antigenically positive chymotryptic peptide, and the light curved line shows the antigenically negative chymotryptic peptide. In the primary structure of ovalbumin (Nisbet *et al.* 1981), "S-P" indicates a phophoserine residue, and "N-CHO" indicates an asparagin residue with a polysaccharide chain.

the sequence analysis revealed some of the antigenic structures of ovalbumin. The antigenicity of T6 was higher than that of T7, the antigenicity of T12 was higher than that of T11, and the antigenicity of T13 was higher than that of T14. Although the amount of T17 peptides was little, they were bound with the anti-ovalbumin rabbit IgG antibody. On the contrary, although the amount of T18 peptides was more, they did not bind with the antibody. The antigenicity of C2 seemed to be higher than that of C3 (Fig. 2-B), even though the amount of C2 was less than that of C3 (Fig. 2-A). The antigenicity of C4 was the highest of all, while the antignicity of C10 was weak. The antigenicity of T11 (MW 14,000), which had first been assumed to be one of the main tryptic peptides, was weaker than that of T6 or T7. However, the T11 peptide was confirmed to be chicken lysozyme (MW 14,300), an impurity in the ovalbumin preparation, judging from its N-terminal sequence (KVFGR) which was the same as the N-terminal sequence of chicken lysozyme.

The antigenically active sequence of C2 and C4 was the same, for both peptides contained antigenically negative C14; however, the antigenicity of C4 was much higher than that of C2, suggesting that the C4 band would have been a mixture of some antigenically active peptides. The antigenicity of T6 (OVA 200-385) was higher than that of T7 (OVA 1-158), while the antigenicity of C2 (OVA 119-385) was higher than that of C3 (OVA 1-180) or of C10 (OVA 107-188). These results suggest that the C-terminal region of ovalbumin might have acted as a stronger antigen than the N-terminal region of ovalbumin.

Antigenically positive peptide C13 contained antigenically negative peptide C14; therefore, the C-terminal sequence of C13 stretching from residues 253 to 261 (or 264) might have acted as an antigenic determinant. This sequence was also involved in the peptides of T6, T8, T10, T17b, C2, C4 and C6. A comparison of the antigenic structure with information on the crystal structure of ovalbumin (Stein *et al.* 1991) reveals that this antigenic sequence is located on a flexible α-helix hG (OVA 254-260) which is located at the surface of the molecule. It has been shown that the α -helix hG was contained in the immunodominant peptides of OVA 257-264 (Wick and Pfeifer 1996), OVA 258-276 (Heeg *et al.* 1991; Falk et al. 1991) and OVA 258-281 (Shimojo et al. 1994) which bind to the major histocompatibility complex (MHC) class I and are recognized by T cells. This information implies that the C-terminal sequence of C13 would have contributed to the immunogenicity of ovalbumin.

In this study, we suggest that a part of the antigenic determinant would exist in OVA 253-261 (or 264) after digesting ovalbumin with the combination of trypsin and chymotrypsin. Binding studies with synthetic peptides, which are deduced to be antigenic structures, would allow further identification of the antigenic binding sites of ovalbumin. Furthermore, if we use the IgE antibody from an allergic patent's sera

to hen's egg white, more beneficial information about the egg allergy would be provided. We hope that this information could provide insight to researchers on allergy-related studies, especially on egg allergy.

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トリプシン，キモトリプシン消化により得られるオボアルブミンの抗原性ペプチド

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オボアルブミン（OVA）をトリプシンまたはキモトリプシンで消化して得られたペプチドの抗原性と一次構造を調べた．消化ペプチドをトリシン-SDS-ポリアクリルアミドゲル電気泳動法で分離し，イムノブロット法で抗原性を検出したところ，トリプシン消化で分子量5,520以上，キモトリプシン消化で分子量7,280以上のバンドはすべて抗OVAウサギIgG抗体と反応した．抗原性を示すペプチドをゲルからPVDF膜にブロットしたのち，気相シークエンサーでN末端アミノ酸配列分析を行い一次構造を推定した．これらのアミノ酸配列のうち，抗原性を示す領域および抗原性を示さない領域から抗原決定基と考えられる部位は，OVA 253～261（または264）に含まれることが示唆された．

キーワード：オボアルブミン，アレルギー，抗原，トリプシン，キモトリプシン，ペプチド．