J. Home Econ. Jpn. Vol. 48 No. 8 717~722 (1997)

Note

Antigenic Change of Native and Heat-Denatured Ovalbumin Digested with Pepsin, Trypsin or Chymotrypsin

Sumiko ODANI, Hiroko AWATUHARA* and Yukie KATO**

Faculty of Education, Niigata University, Niigata 950-21, Japan
** Faculty of Education, Shinshu University, Nagano 380, Japan*
*** Faculty of Education, Toyama University, Toyama 930, Japan*

Native ovalbumin (NOA) and heat-denatured ovalbumin (HDOA) were digested with pepsin, trypsin or chymotrypsin. The resulting digests were characterized by their degree of hydrolysis, using tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (tricine SDS-PAGE), and by their degree of antigenicity, namely their binding activity to the anti-NOA rabbit IgG antibody, using an enzyme-linked immunosorbent assay (ELISA). When NOA was digested with pepsin, the degree of hydrolysis increased with the time of incubation (0, 10 and 30 min, and 1, 2, 3, 10 and 24 h); that is, large peptides of MW 45,000-18,000 were decreased and small peptides of MW 15,000-2,500 were increased. The antigenicity of the NOA digest after 24 h of incubation with pepsin was reduced to 70.9% of that of intact NOA; however, NOA was barely hydrolyzed with trypsin or chymotrypsin, the antigenicity of the digest remaining almost unchanged. The antigenicity of HDOA, which was obtained by heating a 0.2% NOA solution at pH 7.2 for 10 min at 98℃, decreased to 81.8% of that of NOA. The hydrolysis of HDOA was considerably increased and the antigenicity was decreased (*i.e.*, 8.4%, 82.0% and 21.3% in the 24-h digest with pepsin, trypsin and chymotrypsin, respectively). These results indicate that the antigenic structure of ovalbumin is relatively stable.

(Received January 31, 1997)

Keywords: ovalbumin, heat-denatured, antigenicity, pepsin, trypsin, chymotrypsin.

INTRODUCTION

Hen's egg frequently induces hypersensitivity, particularly in atopic children (Lanageland 1983), and hypersensitivity to egg is the most common food allergy in Japanese infants (Baba 1990). This allergy was initially ascribed to the presence of four predominant proteins that constitute 80% of the total protein content by weight: ovalbumin, ovotransferrin, ovomucoid and lysozyme in hen's egg white (Holen and Elsayed 1990; Djurtoft *et al.* 1991).

In this study, native and heat-denatured ovalbumin samples were each digested with pepsin, trypsin or chymotrypsin, and the resulting digests were characterized by physico-chemical and immunological techniques.

MATERIALS AND METHODS

Materials

Native ovalbumin was purchased from Taiyo Kagaku Co. (Tokyo, Japan). Pepsin (from hog stomach mucosa, 2,500 units/mg), 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 horse radish peroxidase VI (HRP-labeled goat anti-rabbit IgG, 100 purpurogallin units/ml) were each purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Digestion of ovalbumin with pepsin, trypsin or chymotrypsin

Ten milligrams of native ovalbumin was dissolved in 5.0 ml of a 0.2 M acetate-HCl buffer at pH 2.0 for pepsin digestion (Otani and Hosono 1989), and in a 0.1 M NH_4OH/HCOOH buffer at pH 8.5 or pH 8.0 for trypsin or chymotrypsin digestion, respectively (Ametani *et al.* 1987). Protease (0.1 mg) was added to the native ovalbumin solution (5.0 ml), and the mixture

was incubated at 37℃ for 10 or 30 min, and for 1, 2, 3, 10 or 24 h. The enzymes were inactivated by cooling for 5 min in iced water. The digested products were immediately lyophilized and stored at −20℃ before being used. Heat-denatured ovalbumin was prepared by heating 0.2 % native ovalbumin in a 0.01 M phosphate buffer at pH 7.2 with 0.15 M NaCl (PBS) at 98℃ for 10 min, and then cooling for 5 min in iced water. The heat-denatured ovalbumin solution was adjusted to pH 2.0, 8.5 or 8.0 and digested similarly to native ovalbumin.

Antibody

The rabbit anti-native ovalbumin antiserum was prepared by intraperitoneally injecting a female rabbit (New Zealand White), weighing about 2 kg, with 0.25 mg of native ovalbumin in 0.85% NaCl that had been emulsified with an equal volume of Freund's complete adjuvant (Difco Labs., Detroit, U.S.A.). The rabbit was boosted with 0.5 mg of native ovalbumin at 2-week intervals, and 11 weeks after the first immunization, the rabbit was bled. The antiserum was separated, 0.05% sodium azide was added, and the solution was stored at −80℃.

Double radial immunodiffusion

An 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 (tricine SDS-PAGE) analysis of the digested native ovalbumin and heat-denatured ovalbumin was performed according to the method of Schägger and von Jagow (1987) with a slight modification, 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) amino methane (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 native ovalbumin or heat-denatured ovalbumin was dissolved in a mixture of 0.04% SDS, 12% glycerol, 2% β-mercaptoethanol and 0.05% bromophenol blue in 50 mM Tris-HCl 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 standard protein mixtures, cytochrome C and its polymers (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 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.) were used as molecular weight markers.

ELISA

An enzyme-linked immunosorbent assay (ELISA) was performed according to the method of Envall and Perhann (1971) with a slight modification. A polystyrene micro-titer plate (Coster Co., Cambridge, MA, U.S.A.) was used as a solid support. Each well was coated with 100 μl of antigen (20 ng/100 μl of an individual protein in a 0.05 M sodium bicarbonate buffer at pH 9.6) and incubated for 1 h at room temperature. After removing the antigen solution, each well was washed three times with 0.05% Tween-20 in PBS (Tween-PBS). The residual free binding sites were blocked with about 400 μl/well of 2 % casein in Tween-PBS for 1 h at room temperature or overnight at 4 ℃. The plate was then incubated with 100 μl/well of anti-native ovalbumin rabbit IgG antibody, which had been diluted 500 times with 2% casein in Tween-PBS, for 1 h at room temperature after washing three times with Tween-PBS. The IgG antibodies which reacted with the plated-bound antigens were determined by using 100 μl/well of HRP-labeled goat anti-rabbit IgG, which had been diluted 1,000-fold in 2 % casein in Tween-PBS and incubated for 1 h at room temperature. After removing the solution and washing five times with Tween-PBS, 200 μl of a freshly prepared solution of 12 mg of *o*-phenylenediamine in 30 ml of a 0.1 M citrate-0.2 M phosphate buffer at pH 5.5 containing 20 μl of 5% hydrogen peroxide was added to each well. Thirty minutes later, the reaction was stopped by adding 50 μl of 6 N sulfuric acid. The resulting reaction mixture (100 μl) was diluted with 0.9 ml of 1.2 N sulfuric acid and the absorbance was determined at 492 nm. A rabbit serum which was not immunized against native ovalbumin was used as a control. ELISA determinations were carried out in triplicate, and the measurements were averaged.

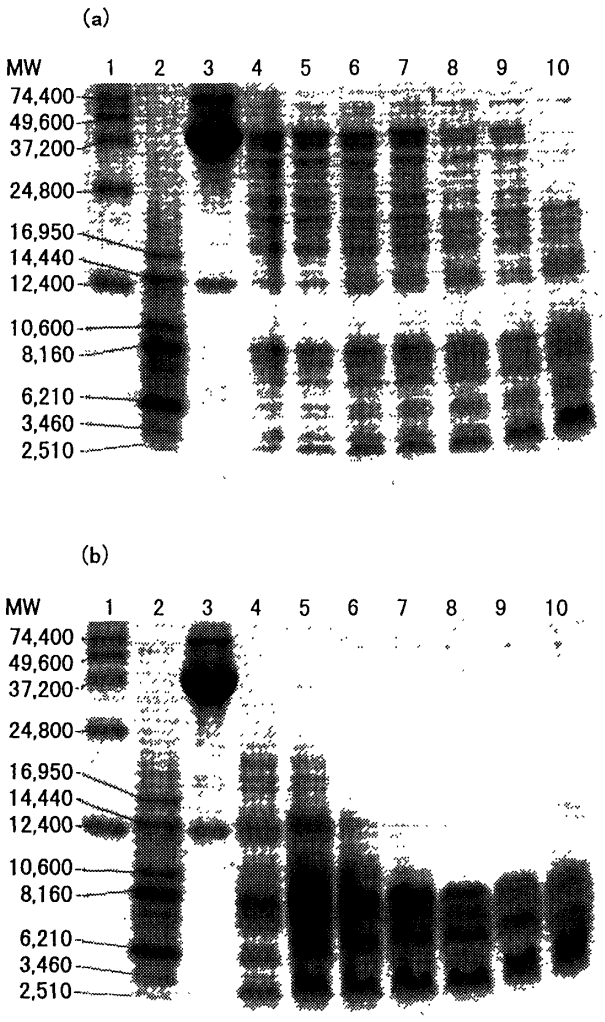

Fig. 1. Tricine SDS-PAGE patterns of the pepsin digests of native ovalbumin and heat-denatured ovalbumin

Native ovalbumin (0.2 %) and heat-denatured ovalbumin (0.2 %, pH 7.2, at 98℃ for 10 min) were digested with pepsin at pH 2.0 (ovalbumin/pepsin: 100/1 w/w) as described in the MATERIALS AND METHODS section. Each digest was dissolved in the SDS sample buffer (10 μg/μl) and heated at 100℃ for 3 min, before 5 μl of the heated sample was loaded on to the gel. (a) native ovalbumin; (b) heat-denatured ovalbumin. Numbers in the left margin refer to the MW of the marker proteins. Marker proteins: lane 1, cytochrome C and its polymers; lane 2, myoglobin fragments and glucagon. Digests: lane 3, 0 min; lane 4, 10 min; lane 5, 30 min; lane 6, 1 h; lane 7, 2 h; lane 8, 3 h; lane 9, 10 h; lane 10, 24 h of incubation.

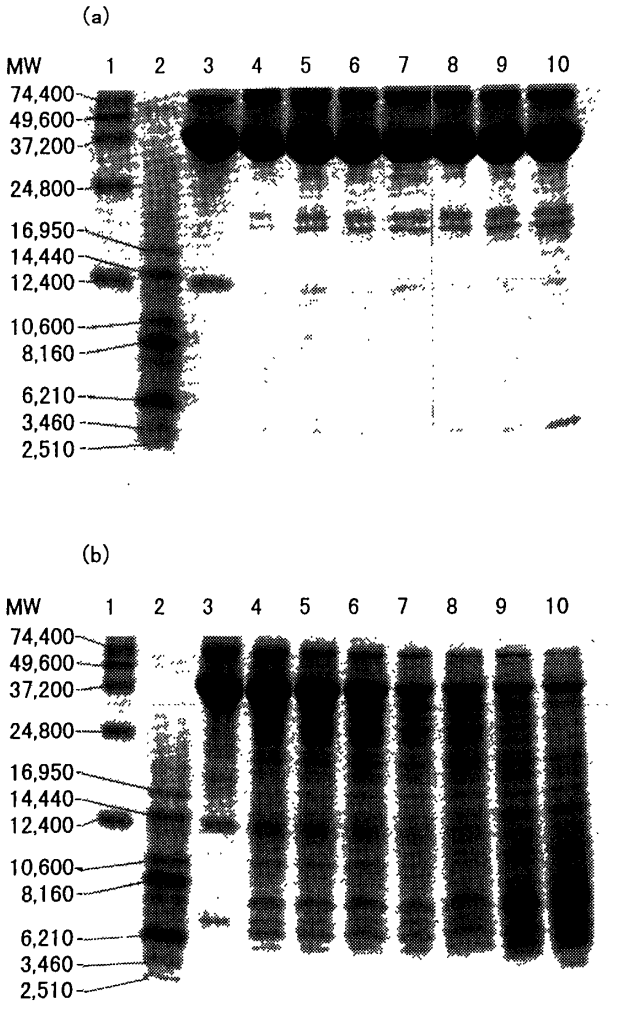

Fig. 2. Tricine SDS-PAGE patterns of the trypsin digests of native ovalbumin and heat-denatured ovalbumin

Native ovalbumin (0.2 %) and heat-denatured ovalbumin (0.2 %, pH 7.2, at 98℃ for 10 min) were digested with trypsin at pH 8.5 (ovalbumin/trypsin: 100/1 w/w) as described in the MATERIALS AND METHODS section. Each digest was loaded on to the gel as described in Fig. 1. (a) native ovalbumin; (b) heat-denatured ovalbumin. Numbers in the left margin refer to the MW of the marker proteins. Numbers of the lanes are the same as those shown in Fig. 1.

RESULTS AND DISCUSSION

Pepsin, trypsin or chymotrypsin digest of native ovalbumin and heat-denatured ovalbumin

The digests of native and heat-denatured ovalbumin after 0, 10 and 30-min, and 1, 2, 3, 10 and 24-h incubations with pepsin, trypsin or chymotrypsin were electrophoresed by tricine SDS-PAGE (Figs. 1-3). The digest of native ovalbumin with pepsin showed an increase in the degree of hydrolysis with incubation time, in which the peptide bands of MW 45,000-18,000 were decreased and peptide bands of MW 15,000-2,500 were increased. Trypsin or chymotrypsin barely

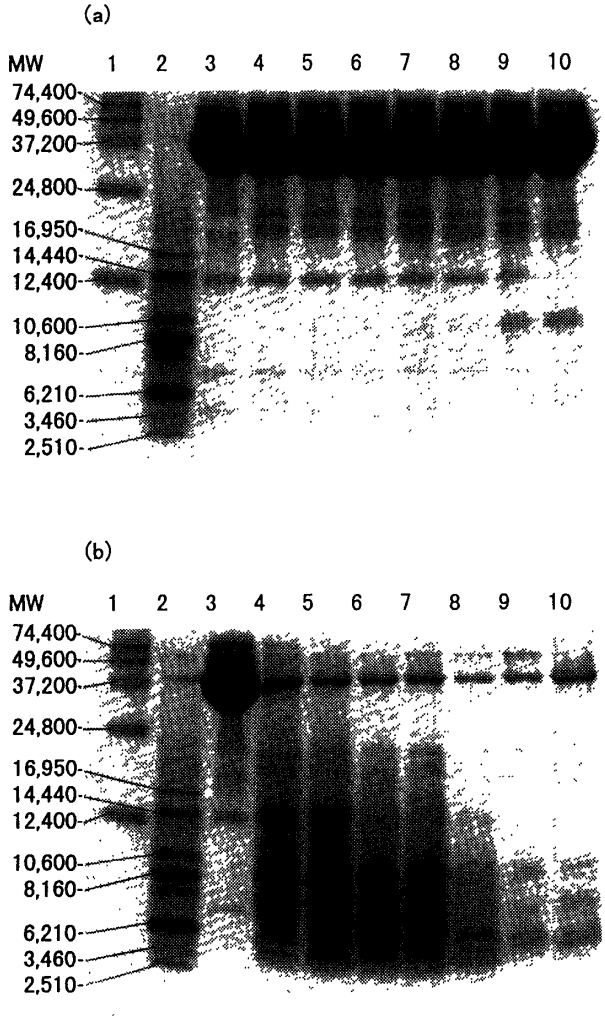

Fig. 3. Tricine SDS-PAGE patterns of the chymotrypsin digests of native ovalbumin and heat-denatured ovalbumin

Native ovalbumin (0.2 %) and heat-denatured ovalbumin (0.2%, pH 7.2, at 98℃ for 10 min) were digested with chymotrypsin at pH 8.0 (ovalbumin/chymotrypsin: 100/1 w/w) as described in the MATERIALS AND METHODS section. Each digest was loaded on to the gel as described in Fig. 1. (a) native ovalbumin; (b) heat-denatured ovalbumin. Numbers in the left margin refer to the MW of the marker proteins. Numbers of the lanes are the same as those shown in Fig. 1.

hydrolyzed native ovalbumin.

The electrophoretic patterns of the hydrolysates showed that almost all heat-denatured ovalbumin was hydrolyzed to small peptides of MW 8,000–2,500 with incubation by pepsin or chymotrypsin, but that intact heat-denatured ovalbumin and high-molecular-weight peptides remained even after a 24-h incubation with trypsin.

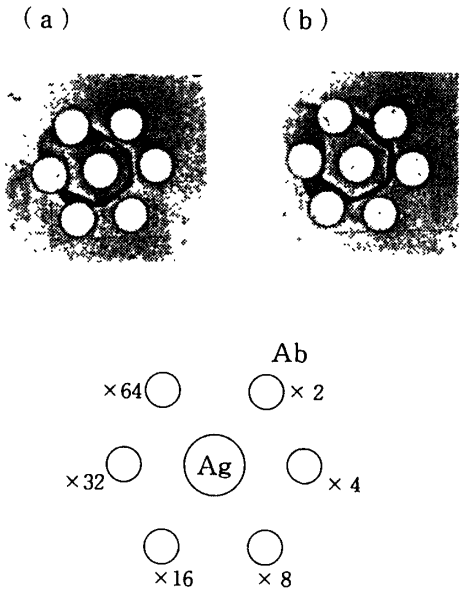

Fig. 4. Double radial immunodiffusion assay of native ovalbumin against anti-native ovalbumin rabbit antiserum

Ouchterlony immunodiffusion was carried out in 1.2 % agarose in PBS containing 0.1% NaN_3. Five microliters of ovalbumin (Ag) and anti-native ovalbumin rabbit antiserum (Ab) was applied to each well. (a) Ag: 100 μg/ml; (b) Ag: 50 μg/ml.

Antigenic reactivity of native ovalbumin, heat-denatured ovalbumin and digests with the anti-native ovalbumin antibody

A rabbit anti-native ovalbumin antiserum was prepared as described in the materials and methods section. The reactivity of the anti-native ovalbumin antibody to the intact molecule of native ovalbumin was tested by the double radial immunodiffusion method. The precipitin line for the antigen-antibody reaction was detected up to a 32-s dilution of the antiserum (Fig. 4).

The antigenicity, namely the binding activity of native ovalbumin, heat-denatured ovalbumin and digests to the anti-native ovalbumin rabbit IgG antibody, was examined by ELISA (Fig. 5). The antigenicity of the pepsin digest of native ovalbumin remained considerably high, with its 24-h incubated peptides being 70.9% of those of native ovalbumin. This result suggests that the peptides digested by incubating with pepsin at pH 2.0 still possessed epitopes. Neither the trypsin nor chymotrypsin treatment of native ovalbumin reduced the antigenicity.

When we heated a 0.2 % native ovalbumin solution of pH 7.2 for 10 min at 98℃, the antigenicity of the

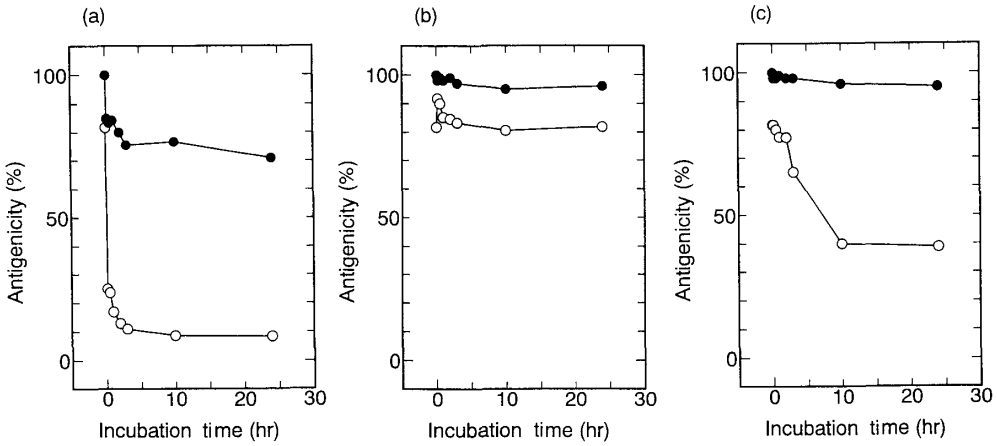

Fig. 5. Antigenic reactivity of native ovalbumin, heat-denatured ovalbumin and digests with the anti-native ovalbumin antibody

Native ovalbumin (0.2%) and heat-denatured ovalbumin (0.2%, pH 7.2, at 98℃ for 10 min) were digested with pepsin at pH 2.0, with trypsin at pH 8.5 or with chymotrypsin at pH 8.0 (ovalbumin/protease: 100/1 w/w) as described in the MATERIALS AND METHODS section. The binding activity of native ovalbumin, heat-denatured ovalbumin and the digests to the anti-native ovalbumin rabbit IgG antibody was examined by ELISA. Each well was coated with 100 μl of the antigen (20 ng/100 μl of an individual protein in a 0.05 M sodium bicarbonate buffer at pH 9.6). (a) pepsin digests; (b) trypsin digests; (c) chymotrypsin digests; ● native ovalbumin; ○ heat-denatured ovalbumin.

heat-denatured ovalbumin was reduced to 81.8 % of that of native ovalbumin. This result indicates that the applied heat treatment had some effects on the antigenicity of native ovalbumin.

Further treatment of heat-denatured ovalbumin with pepsin or chymotrypsin degraded most parts of the epitopes of the molecule. On the other hand, trypsin hydrolysis of heat-denatured ovalbumin resulted in high residual antigenicity. The binding activity between the pepsin, trypsin or chymotrypsin digest of heat-denatured ovalbumin after 24-h of incubation and the anti-native ovalbumin rabbit IgG antibody was reduced to 8.4%, 82.0% and 21.3% of the reactive value for native ovalbumin.

Our results clearly show that incubating heat-denatured ovalbumin with pepsin, trypsin or chymotrypsin led to an increased degree of hydrolysis and decreased antigenicity in comparison with native ovalbumin. However, some antigenicity remained in all the digests.

We investigated in this study the antigenicity of the egg allergen, ovalbumin, by using rabbit IgG. Further analysis of the binding activity of ovalbumin to specific IgE may help to clarify egg allergy.

The authors thank Dr. N. Maeda from the Institute of Animal Experimentation, Faculty of Medicine, Niigata University for his kind help in preparing the antiserum.

REFERENCES

Ametani, A., Kaminogawa, S., Shimizu, M., and Yamauchi, K. (1987) Rapid Screening of Antigenically Reactive Fragments of αs_1-Casein Using HPLC and ELISA, *J. Biochem.*, **102**, 421-425

Baba, M. (1990) Food Allergy and Its Clinical Implications, *Eiyogaku Zasshi*, **48**, 53-62

Djurtoft, R., Pedersen, H. S., Aabin, B., and Barkholt, V. (1991) Studies of Food Allergens: Soybean and Egg Proteins, in *Nutritional and Toxicological Consequences of Food Processing* (ed. by Friedman, M.), Plenum Press, New York, 281-293

Envall, E., and Perhann, P. (1971) Enzyme-linked Immunosorbent Assay (ELISA): Quantitative Assay of Immunoglobulin G, *Immunochemistry*, **8**, 871-874

Holen, E., and Elsayed, S. (1990) Characterization of Four Major Allergens of Hen Egg-White by IEF/SDS-PAGE Combined with Electrophoretic Transfer and IgE-Immunoautoradiography, *Int. Arch. Allergy Appl. Immunol.*, **91**, 136-141

Lanageland, T. (1983) A Clinical and Immunological Study of Allergy to Hen Egg White: I. A Clinical Study of Egg Allergy, *Clin. Allergy*, **13**, 371-382

Otani, H., and Hosono, A. (1989) Immunological Properties of Pepsin, Trypsin and/or Chymotrypsin

Digests of Bovine αs_1-Casein, *Jpn. J. Zootec. Sci.*, **60**, 1143-1150

Ouchterlony, Ö. (1952) Antigen-Antibody Reactions in Gels: ⅣTypes of Reactions in Coordinated Systems of Diffusion, *Acta Pathol. Microbiol. Scand.*, **32**, 231-240

Schägger, H., and von Jagow, G. (1987) Tricine-Sodium Sulfate Polyacrylamide Gel Electrophoresis for the Separation of Protein in the Range from 1 to 100 kDa, *Anal. Biochem.*, **166**, 368-379

未変性および熱変性オボアルブミンのペプシン，トリプシン，キモトリプシン消化による抗原性の変化

小谷スミ子，粟津原宏子*，加藤征江**

（新潟大学教育学部，* 信州大学教育学部，** 富山大学教育学部）

平成9年1月31日受理

ペプシン，トリプシンまたはキモトリプシンで消化した未変性オボアルブミン（NOA）および熱変性オボアルブミン（HDOA）の加水分解度を調べると共に加水分解物の抗原性の変化を調べた．加水分解の程度は低分子ペプチドの分離可能なトリシンSDSポリアクリルアミドゲル電気泳動法で，加熱および消化による抗原性の変化は抗NOAウサギIgG抗体との結合をELISA法で調べることで確かめた．NOAをペプシンで消化すると，消化時間（0，10，30分，1，2，3，10，24時間）と共に分子量45,000～18,000のペプチドが減少し分子量15,000～2,500のペプチドが増加した．また抗原性は24時間後に70.9%に低下した．しかしトリプシンやキモトリプシンで消化しても加水分解の程度と抗原性に大きな変化は見られなかった．0.2% NOA水溶液，pH 7.2を98℃で10分間加熱して得られたHDOAの抗原性はNOAの81.8%に減少した．HDOAはNOAに比べはるかに加水分解されやすく抗原性も低下したが抗原性は残っていた．すなわちペプシン，トリプシンまたはキモトリプシン消化による24時間後の抗原性はそれぞれNOAの8.4%，82.0%，21.3%であった．本結果からオボアルブミンの抗原構造は比較的安定であることが示唆された．

キーワード：オボアルブミン，熱変性，抗原性，ペプシン，トリプシン，キモトリプシン．