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Inhibitory Specificity against Various Trypsins and Stability of Ovomucoid from Japanese Quail Egg White

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Summary The inhibitory specificity and stability of ovomucoid from Japanese quail egg white (OMJPQ) were examined to understand its nutritional significance. OMJPQ showed strong inhibitory activities toward trypsins from various origins including human, and the trypsin inhibitions occurred at molar ratios of enzyme to inhibitor between 1/1 and 2/1. On the other hand, an equimolar mixture of the second and third domains of OMJPQ inhibited bovine trypsin more strongly than the corresponding native OMJPQ did. This distinction was partly explained by the presence of steric hindrance on the formation of a 2:1 trypsin-OMJPQ complex. OMJPQ retained about 100% of its original activity over a pH range from 1 to 12 after a 24-h incubation at 37°C. The inhibitor was most thermostable between pH 2 and 5, where more than 70% of its original activity was maintained after a 1-h incubation at 100°C and about 25% of the activity even after a 30-min incubation at 121°C. OMJPQ was also considerably resistant to pepsin attack. Pepsin digestion of the protein resulted in only about 40% loss of the original trypsin-inhibitory activity even after a 24-h digestion. Furthermore, the addition of bovine serum albumin to the digestion mixture brought about rapid elevation in the trypsin-inhibitory activity during an initial 30-min digestion. SDS-PAGE and immunoblot suggested that this was due to the liberation of active inhibitory domains from the native molecule by inter-domain proteolysis.

Key words ovomucoid, Japanese quail, egg white, proteinase inhibitor, trypsin, stability, inhibitory specificity, pepsin

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Abbreviations: OMJPQ, ovomucoid from Japanese quail egg white; BAPA, α-*N*-benzoyl-DL-arginine-*p*-nitroanilide; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PVDF, polyvinylidenefluoride.

Avian eggs containing a variety of proteins are very important foodstuffs for human beings. Ovomucoid is one of the major components of avian egg whites and is responsible for most of the inhibitory activity of egg white against serine proteinases. The classical study of Lineweaver and Murray (*1*) on chicken ovomucoid is well-known. Kato et al. (*2,3*) reported the structure and function of ovomucoids from various avian species. We have also reported an improved method for purification of OMJPQ (*4*). Like chicken ovomucoid, OMJPQ consists of three tandem homologous domains (*2*). However, OMJPQ is different from the chicken in that it can inhibit human trypsin (*5*). Furthermore, compared to chicken ovomucoid, food chemical and nutritional studies on OMJPQ are very rare.

We are interested in the inhibitory specificity and stability of OMJPQ, since these properties are related to the nutritional significance of the protein. In this paper, we describe the inhibitory activity toward trypsins from various origins, the pH and heat stabilities, and the changes in trypsin inhibitory activity and molecular structure of OMJPQ during pepsin digestion.

MATERIALS AND METHODS

Materials. Pure OMJPQ was prepared as reported previously (*4*). Bovine trypsin (type III), porcine trypsin (type IX), porcine pepsin (crystallized and lyophilized), bovine serum albumin (BSA) (fraction V, powder), ovalbumin (Grade III), and enterokinase (partially purified) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Horseradish peroxidase-labeled goat anti-rabbit IgG, *Staphylococcus aureus* V8 protease (Spase V8) and PVDF membrane were from Bethyl Laboratory, Inc. (Montgomery, AL, U.S.A.), Pierce Chemical (Rockford, IL, U.S.A), and Atto Corp. (Tokyo), respectively. Pancreas acetone powders of rat, rabbit, ovine, guinea pig, cat, dog, and equine were also purchased from Sigma Chemical Co. Human pancreatic juice was obtained from Shiga Medical University and stored at $-80°C$. BAPA was obtained from Peptide Institute, Inc.(Osaka). The active-site titrant, *p*-nitrophenyl-*p*′-guanidinobenzoate HCl (NPGB) was purchased from Nutritional Biochemicals (Cleveland, OH, U.S.A.). CNBr-activated Sepharose 4B and DEAE-Sepharose CL-6B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals used were of special grade.

Preparation of second and third domains. Purified OMJPQ was digested by Spase V8 at 37°C in 0.1 M Tris-HCl buffer, pH 8.3, containing 0.2 M NaCl according to the method of Kato et al. (*3*). Released second and third domains were then isolated by gel permeation chromatography and affinity chromatography.

Preparation of purified trypsins from various origins. Pancreas acetone powders of rat, rabbit, ovine, guinea pig, cat, dog, and equine were homogenized with 0.01 N HCl containing 20 mM $CaCl_2$ using a homoblender (Matsushita Electric Industrial Co. Ltd.). Each homogenate was centrifuged at $10{,}000 \times g$ for 60 min at

4°C. To the clear supernatant was added solid ammonium sulfate to give 20% saturation. The mixture was centrifuged and the supernatant was next brought to 80% ammonium sulfate saturation. After centrifugation, the precipitate obtained was dialyzed against 2 mM HCl. A clear supernatant of dialyzate was then lyophilized to obtain a crude preparation. The crude trypsins were stored at −20°C until use. The crude human trypsin was also prepared by the same procedure from human pancreatic juice in place of pancreas homogenate. For preparation of purified trypsins, the crude preparations were treated with enterokinase and chromatographed on an immobilized rice bran trypsin inhibitor column (*6*). Each purified enzyme was found to be homogeneous by disc-PAGE at pH 4.0 (data not shown).

Preparation of antiserum against OMJPQ. OMJPQ was emlsified with an equal volume of Freund's complete adjuvant and injected into a male rabbit (3 kg body weight) intracutaneously three times at 2-week intervals at a dose of 2 mg/rabbit. The rabbit was bled a week after the last injection to collect serum. Double immunodiffusion was carried out in 1% agarose gel according to the method of Ouchterlony (*7*) and showed that the antiserum was specific for OMJPQ. The IgG fraction was then prepared from the serum by salting-out and the subsequent chromatography on DEAE-Sepharose CL-6B and polyclonal antibody against OMJPQ was purified from the IgG by affinity chromatography on antigen-Sepharose 4B.

Measurement of inhibitory activity. Trypsin-inhibitory activity was estimated from the residual trypsin activity in the presence of the inhibitor. The inhibitor in 2.2 ml of 0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM $CaCl_2$ was incubated with 0.1 ml of trypsin solution for 5 min at 37°C and then 0.1 ml of BAPA solution (10 mg/ml in dimethylsulfoxide) (*8*) was added to the mixture. After incubation for 10 min at 37°C, the reaction was stopped by the addition of 1.0 ml of 10% acetic acid and the absorbance of the mixture was measured at 410 nm.

SDA-PAGE and immunoblot analysis. SDS-PAGE was done in 15% slab gels by the method of Laemmli (*9*). After electrophoresis, proteins in a slab gel were transferred electrophoretically to a sheet of PVDF membrane as described by Towbin et al. (*10*). Proteins with antigenicity were then stained by an enzyme immunoassay technique (*11*) with the purified rabbit anti-OMJPQ, horseradish peroxidase-labeled goat anti-rabbit IgG, 4-chloro-1-naphthol, and hydrogen peroxide.

Estimation of pH and heat stabilities. An aqueous solution (0.01 ml) of OMJPQ (0.872 mg/ml) was mixed with 0.2 ml of each of the following buffers: 0.05 M NaCl-0.5 M HCl, pH 1; 0.05 M Na-citrate-0.05 M HCl, pH 2 and 3; 0.05 M Na-acetate-0.05 M acetic acid, pH 4 and 5; 0.05 M NaH_2PO_4-0.05 M Na_2HPO_4, pH 6, 7, and 8; 0.05 M Na_2CO_3-0.05 M $NaHCO_3$, pH 9 and 10; and 0.05 M NaOH-0.05 M Na_2HPO_4, pH 11 and 12. Each of the mixtures was incubated for 24 h at 37°C for 1 h at 60, 80, or 100°C, and for 30 min at 121°C. After incubation, each mixture was rapidly cooled in ice-cold water and the remaining trypsin-inhibitory

activity was determined.

Pepsin digestion of OMJPQ. OMJPQ (3.6 mg) was digested by pepsin in 2 ml of 0.1 M NaCl-0.1 N HCl, pH 1.8 for 24 h at 37°C in a substrate-enzyme ratio of 10:1 or 100:1 (w/w). Pepsin digestion of OMJPQ in the presence of ovalbumin (18 mg) or BSA (18 mg) was also performed in a OMJPQ-pepsin ratio of 10:1 (w/w) under the same condition as above. After appropriate incubations, aliquots (0.1 ml) were removed from the digestion mixture and neutralized by the addition of 0.2 ml of 0.1 M Na-phosphate buffer, pH 7.0, to terminate the digestion. The neutralized sample solutions were then used for SDS-PAGE and the trypsin-inhibitory assay.

RESULTS AND DISCUSSION

Inhibition of the trypsins from 10 animal species by OMJPQ

Inhibitory activities of OMJPQ were determined against the trypsins from 10 animal species as target enzymes. Among the enzymes used, bovine and porcine trypsins were from commercial preparations and the other trypsins were purified by affinity chromatography in our laboratory. The concentration of each trypsin was determined by active-site titration with NPGB according to the method of Chase and Shaw (*12*) and the concentration of OMJPQ by amino acid analysis.

Figure 1 shows the inhibitory activities of OMJPQ against the BAPA-hydrolytic activities of the trypsins. All the enzymes tested were strongly inhibited by the inhibitor and enzyme-inhibitor molar ratios at 100% inhibition obtained by extrapolation were between 1/1 and 2/1 in all cases. The results mean that OMJPQ interacts with each of the trypsins to form 1:1 and 2:1 (M/M) trypsin-OMJPQ complexes. This also agrees with the fact that OMJPQ consists of 3 domains among which the second and third domains are responsible for the trypsin-inhibitory activity (*2*). The reason why OMJPQ did not inhibit trypsin at strict enzyme-inhibitor molar ratio of 2/1 is thought to be very complicated. Probably, it is related to trypsin-binding affinity of the second or third domain of OMJPQ, enzyme and inhibitor concentrations in a reaction mixture, steric hindrance on an enzyme-inhibitor interaction, and so on.

As mentioned above, OMJPQ is a double-headed trypsin inhibitor consisting of an inactive first domain and active second and third domains. Therefore, the inhibitory activity of an equimolar mixture of the second and third domains was compared with that of native OMJPQ using bovine trypsin as a target enzyme. The result is shown in Fig. 2. Obviously, the mixture of the domains inhibited bovine trypsin more strongly than the corresponding OMJPQ did. That is to say, the dissociation at a 2:1 equivalence point ([inhibitor]/[trypsin]=0.5 in Fig. 2) was larger in the OMJPQ than in the domain mixture. This result suggests the presence of steric hindrance on the formation of a 2:1 (M/M) trypsin-OMJPQ complex.

OMJPQ is the only ovomucoid known to inhibit human trypsin (*5*). Our present results also confirmed that OMJPQ showed a strong inhibition toward

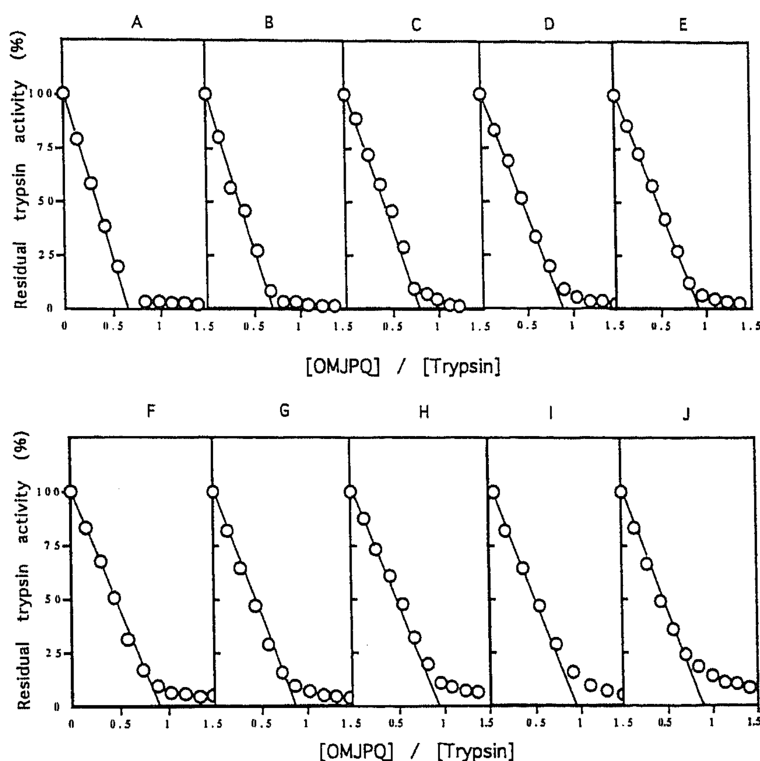

Fig. 1. The inhibitory activies of OMJPQ against the trypsins from 10 animal species. A fixed amount of each trypsin was mixed with increasing amounts of the inhibitor, and the residual enzyme activity was determined by the method described in MATERIALS AND METHODS. The enzyme concentration in the reaction mixture was A (cat): 5.76×10^{-8} M; B (dog): 5.91×10^{-8} M; C (rat): 2.47×10^{-7} M; D (bovine): 2.17×10^{-7} M; E (guinea pig): 1.18×10^{-7} M; F (ovine): 2.15×10^{-7} M; G (equine): 2.12×10^{-7} M; H (rabbit): 5.91×10^{-8} M; I (porcine): 1.62×10^{-7} M; J (human): 1.55×10^{-7} M.

human trypsin. However, the inhibition degree for human trypsin was slightly lower than those for the other trypsins except for porcine trypsin, because the dissociation at a 1 : 1 equivalence point was relatively large. Figarella et al. (*13*) reported that chicken ovomucoid showed a weak inhibition toward human trypsin 2 (anionic type) and no inhibition toward human trypsin 1 (cationic type) which represents the major part of the potential trypsin activity of human pancreatic juice. In our study, human trypsin prepared was judged as the cationic type by disc-PAGE at pH 4.0. Compared to chicken ovomucoid, therefore, OMJPQ seems to have more significant inhibition toward human trypsin *in vivo.*

pH and heat stabilities of OMJPQ

Figure 3 shows the pH and heat stabilities of OMJPQ. The inhibitor was stable at 37°C, pH 1 to 12, where full inhibitor activity was maintained after a 24-h incubation. Even at 60 or 80°C, almost full activity was still maintained in an acidic pH region after a 1-h incubation. At 100 or 121°C the inhibitory activity

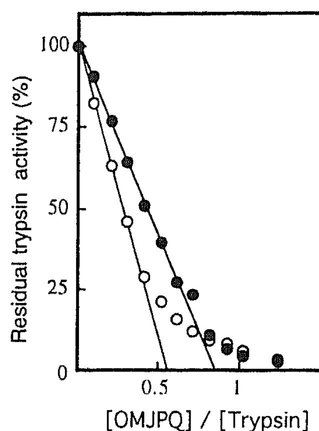

Fig. 2. The inhibitory activities of an equimolar mixture of the second and third domains and the corresponding intact OMJPQ against bovine trypsin. Ten microliters of bovine trypsin solution (3.4×10^{-5} M) was mixed with increasing volumes of the domain mixture (second domain: 4.0×10^{-6} M, third domain: 4.0×10^{-6} M) or intact OMJPQ solution (4.0×10^{-6} M), and the residual enzyme activity was determined. The concentration of the domain mixture was expressed as that of the corresponding OMJPQ solution. ○, the mixture of the second and third domain; ●, intact OMJPQ.

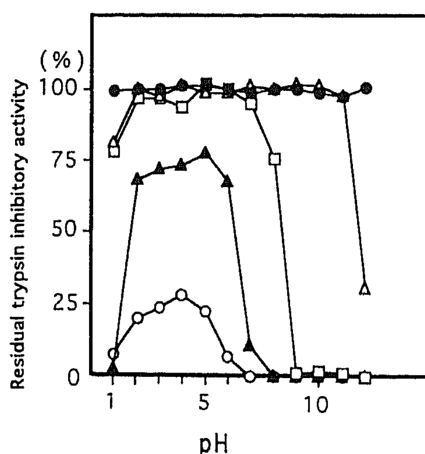

Fig. 3. pH and heat stabilities of OMJPQ. The inhibitor solutions were incubated at various pH's for 24 h at 37°C (●), for 1 h at 60°C (△), 80°C (□), and 100°C (▲), and for 30 min at 121°C (○). The remaining trypsin inhibitory activity of each incubation mixture was determined at pH 8.0.

decreased even in the acidic pH region. However, the inhibitor retained about 70% of its original activity at pH 2 to 6 for a 1-h incubation at 100°C, and about 25% in the region of pH 2 to 5 for 30 min at 121°C. These findings clearly indicate that OMJPQ is as thermostable as chicken ovomucoid (*1*). This property of OMJPQ may cause a problem in relation to human nutrition, since OMJPQ may not be completely inactivated by cooking.

Pepsin digestion of OMJPQ

OMJPQ was digested by pepsin at 37°C and pH 1.8 in a OMJPQ-pepsin ratio of 10:1 or 100:1 (w/w). The digestion was also carried out in the presence of ovalbumin or BSA to examine the effect of a dietary protein on the digestion. Figure 4 shows the change in trypsin-inhibitory activity of OMJPQ during the pepsin digestions. The activity is given as a percentage of the original inhibitor activity. Two typical patterns of the activity change were observed. One is that OMJPQ was rapidly inactivated to lose about 30% of the original activity during the initial 30-min incubation, though the inhibitor still retained about 60% of the original activity even after a 24-h digestion. The other is that the inhibitory activity rapidly increased to about 150% of the original activity during the initial 30-min incubation and then slowly decreased to near the original activity after a 24-h incubation. The former pattern is the case of the pepsin digestions at a OMJPQ-enzyme ratio of 10:1 (w/w) with and without ovalbumin. On the other hand, the latter is the case of the pepsin digestions at a OMJPQ-enzyme ratio of 100:1 (w/w) and at the ratio of 10:1(w/w) in the presence of BSA. These results indicate that OMJPQ is considerably resistant to pepsin digestion, and under certain conditions it can be activated.

To examine the change in the molecular structure of OMJPQ during pepsin digestion, SDS-PAGE and immunoblot of the pepsin digests were carried out and the results are shown in Fig. 5. SDS-PAGE and immunoblot patterns of the digests at OMJPQ:pepsin=10:1 (w/w) without ovalbumin or BSA clearly indicated that OMJPQ was rapidly degraded by pepsin within the initial 30-min incubation to produce small molecular mass products of 6.5, 8.5, and 9.5 kDa (Fig. 5A). These immunoreactive degradation products are probably the domains as constituents of

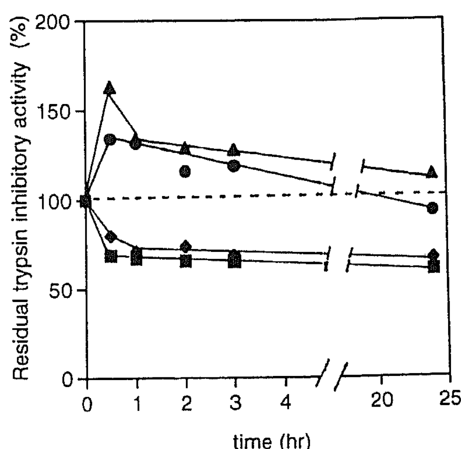

Fig. 4. Effect of pepsin digestion on the trypsin-inhibitory activity of OMJPQ. OMJPQ was digested by pepsin at 37°C and pH 1.8. The ratio of substrate to enzyme was 100:1 (w/w) (▲) or 10:1 (w/w) (■). Pepsin digestion was also carried out at substrate:enzyme ratio of 10:1 in the presence of ovalbumin (◆) or BSA (●).

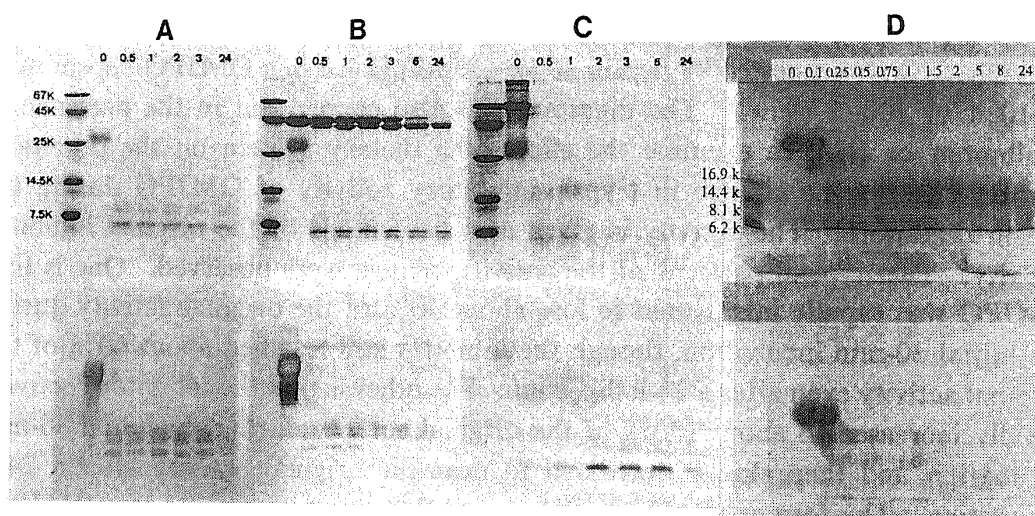

Fig. 5. SDS-PAGE and immunoblot of the pepsin digests of OMJPQ. The upper half of the pictures indicates SDS-PAGE patterns and the lower half shows immunoblot patterns. A: OMJPQ : pepsin = 10 : 1 (w/w) without other proteins; B: OMJPQ : pepsin = 10 : 1 (w/w) with ovalbumin; C: OMJPQ : pepsin = 10 : 1 (w/w) with BSA; D: OMJPQ : pepsin = 100 : 1 (w/w) without other proteins.

OMJPQ. The amounts of the products were shown to gradually decrease during further incubation. Similar behavior of OMJPQ was also shown in immunoblot patterns of the pepsin digests in the presence of ovalbumin (Fig. 5B). In this case, ovalbumin was scarcely digested by pepsin. On the other hand, SDS-PAGE and immunoblot of the digests at OMJPQ : pepsin = 100 : 1 (w/w) without ovalbumin and BSA showed a 16-kDa degradation product of OMJPQ corresponding to the first domain connecting with the second or the second domain with the third in addition to the above-mentioned three products at an initial stage of digestion (Fig. 5D). This indicates that the releasing rate of the three domains from intact OMJPQ molecule decreased because of the lower enzyme concentration. A similar result was also obtained in immunoblot patterns of the digests in the presence of BSA. In contrast with ovalbumin, BSA was highly susceptible to pepsin. Therefore, the effect of BSA is most likely attributed to competition with OMJPQ for pepsin. Finally, it is suggested that pepsin rapidly hydrolyzes the inter-domain peptide bonds of OMJPQ to release domains and then slowly cleaves intra-domain peptide bonds to reduce the inhibitory activity. The elevation in the inhibitory activity observed at an early stage of the pepsin digestion of OMJPQ in the presence of BSA is probably due to the liberation of active inhibitory domains from intact OMJPQ molecule by inter-domain proteolysis, since the inhibitory activity of the mixture of the inhibitory domains is higher than that of the corresponding OMJPQ (Fig. 2). Therefore, some dietary proteins susceptible to pepsin, such as BSA, are expected to protect OMJPQ against pepsin attack in the stomach.

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