Inhibitory Activity of Ovomacroglobulin for Pepsin and Rennin

Akio Kato,* Tomoyuki Kanemitsu, and Kunihiko Kobayashi

Department of Agricultural Chemistry, Yamaguchi University, Yamaguchi 753, Japan

Ovomacroglobulin was shown to have inhibitory activity for pepsin and rennin. The inhibitory activities were greatly enhanced in ovomacroglobulin–pepsin or –rennin at a molar ratio of 1:1. Polyacrylamide gel electrophoresis of ovomacroglobulin–pepsin or –rennin mixtures in the absence of SDS and mercaptoethanol showed the high molecular weight fraction interacting with proteases, while polyacrylamide gel electrophoresis in the presence of SDS and mercaptoethanol showed that the hydrolyzed ovomacroglobulin was cleaved into two peptide fragments whose molecular weights were around 80 000. The surface hydrophobicity of ovomacroglobulin decreased after reaction with rennin. The K_i value of ovomacroglobulin for pepsin was 1.3×10^{-5} M^{-1}.

INTRODUCTION

A number of papers (Barrett and Starkey, 1973; Kitamoto et al., 1982; Nagase et al., 1983, 1986; Molla et al., 1987) have reported the physicochemical properties of and the mechanism of the protease inhibition by ovomacroglobulin. Ovomacroglobulin is a high molecular weight (M_w 720 000) glycoprotein in egg white. The molecule consists of four identical subunits (M_w 180 000) which are linked in two pairs by disulfide bonds. The physicochemical properties and the mechanism of inhibition for proteases have been shown to be analogous to those of α_2-macroglobulin, a plasma protein. It has been proposed that α_2-macroglobulin inhibits the protease activity of a variety of proteases by a trapping mechanism in which the active site of the enzyme remains active (Molla et al., 1987). Ovomacroglobulin also undergoes a similar inhibition mechanism as reported recently (Nagase et al., 1986). The ovomacroglobulin has high enzyme binding activity for a wide variety of proteases, trypsin, chymotrypsin, subtilisin, papain, thermolysin (Nagase et al., 1983, 1986), tadpole collagenase (Barrett and Starkey, 1973), and serratia 56K protease (Molla et al., 1987). However, inhibitory activity of ovomacroglobulin for acidic proteases has never been reported. We found inhibitory activity of ovomacroglobulin for pepsin and rennin. This paper describes the mechanism of the inhibition of ovomacroglobulin for pepsin and rennin.

MATERIALS AND METHODS

Preparation of Ovomacroglobulin. Ovomacroglobulin was prepared by the method of Donovan et al. (1969). Blended egg white was dialyzed for deionized water overnight at 4 °C and centrifuged. The supernatant was brought to 0.3 saturation with ammonium sulfate at pH 6.5. After centrifugation, the supernatant was brought to 0.45 saturation with ammonium sulfate. The precipitate was collected by centrifugation. After dialysis for deionized water, the solution was applied to a column of Sepharose CL-4B. The peak of void volume was collected, concentrated to 25 mL, and applied again to a column of Sepharose CL-4B. The first peak was concentrated and stored at 4 °C.

Assay of Pepsin Activity. Pepsin was from Sigma Chemical Co. Hemoglobin was from Wako Pure Chemical Co. Pepsin activity was determined by measuring trichloroacetic acid soluble peptides or amino acids. A 0.2% ovomacroglobulin solution (125–500 μL) was mixed with 25 μL of a 0.1% pepsin solution. After standing for 5 min, the mixed solution was added to 2.0 mL of a 0.1% hemoglobin solution in 1/10 M citric acid–Na_2HPO_4 buffer, pH 2.2. The reaction was stopped by adding 2 mL of 10% trichloroacetic acid, and the mixture was filtered. The absorbance at 280 nm of the trichloroacetic acid soluble products was determined, and residual activity was calculated by subtraction of the blank value. The activity of pepsin was also measured with κ-casein as substrate. κ-Casein was prepared by the sulfuric acid technique of Zittle and Custer (1963). When κ-casein was used as substrate, the activity was measured in 1/15 M phosphate buffer, pH 5.4, as described below in the assay of rennin activity. The residual activity was estimated by the turbidity after 5 min of reaction. The turbidity reading at 500 nm was measured continuously in a glass cell at 20 °C with a Hitachi 200-10 spectrophotometer.

Assay of Rennin Activity. Rennin was purchased from Sigma. Rennin activity was determined by modifying the method described in a previous paper (Kato et al., 1974). A 0.2% ovomacroglobulin solution (25–100 μL) was mixed with 50 μL of the solution containing 5.0 μg of rennin. After standing for 5 min, the mixed solution was added to 2.5 mL of a 0.05% κ-casein solution in 1/15 M phosphate buffer, pH 5.4.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis was carried out in a slab (50 × 80 × 1 mm) as follows: 15 μL of a 0.1% sample was applied to a 7% gel. Electrophoresis was done for 7 h at 5 mA at 20 °C in a buffer of Tris–glycine, pH 9.5. SDS–PAGE was performed by the method of Laemmli (1970). A 100-μL sample was treated with 15 μL of mercaptoethanol and 100 μL of 0.12 M Tris-HCl buffer, pH 8.6, containing 2% SDS. The 7.5% gel was set in a slab (50 × 80 × 1 mm). Electrophoresis was done for 7 h at 5 mA at 20 °C.

Determination of Surface Hydrophobicity. Surface hydrophobicity was determined by the fluorometric method described previously (Kato and Nakai, 1980). *cis*-Parinaric acid (Wako) was used as a fluorometric probe. A 10-μL portion of ethanolic solution of *cis*-parinaric acid, 3.6×10^{-3} M, was added to 2 mL of protein solution. The mixture was excited at 325 nm, and the relative fluorescence intensity was measured at 420 nm. The initial slope (S_0) was calculated from the fluorescence intensity versus protein concentration.

Determination of the Dissociation Constant of the Pepsin–Ovomacroglobulin Complex. The dissociation constant (K_i) of pepsin–ovomacroglobulin was determined from a Lineweaver–Burk plot in the enzyme/inhibitor molar ratio of 1:1 in various substrate concentrations.

RESULTS

The inhibitory activity of ovomacroglobulin for pepsin is shown in Figure 1. The residual activity of pepsin decreased to 43% in the ovomacroglobulin/pepsin molar ratio of 1:1 when hemoglobin was used as substrate, while it decreased to 0% when κ-casein was used as substrate.

The inhibitory activity of ovomacroglobulin for rennin was investigated by using κ-casein as substrate. κ-Casein is a specific substrate for the primary action of rennin. After cleavage at Phe105–Met106 by rennin, para-κ-casein

0021-8561/91/1439-0041$02.50/0

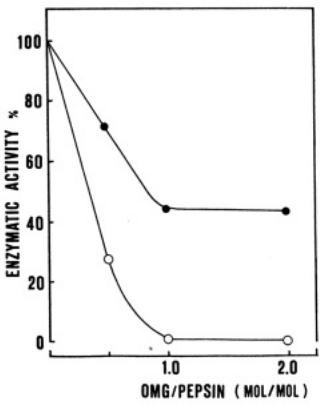

Figure 1. Inhibitory activity of ovomacroglobulin against pepsin. The activity of pepsin was measured with hemoglobin (●) and κ-casein (○) as substrates.

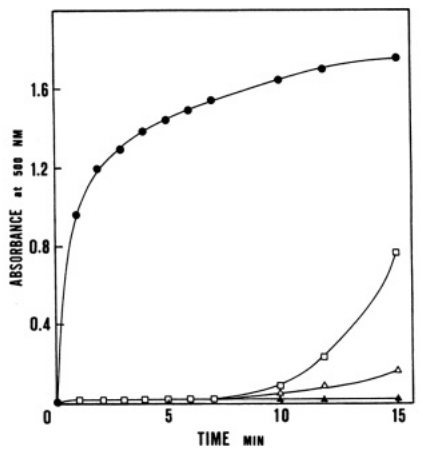

Figure 2. Inhibitory activity of ovomacroglobulin against rennin. Rennin activity was represented by turbidity of κ-casein. (●) Without OMG; (□) OMG/rennin molar ratio of 0.5:1, (△) 1:1, (▲) 2:1.

is associated and the insoluble aggregates are formed. Therefore, rennin activity was followed only by measuring the aggregation of para-κ-casein. Figure 2 shows the effects of ovomacroglobulin on the aggregation of κ-casein by rennin. Increases in the lag phase of aggregation were observed as the concentration of ovomacroglobulin increased. This result indicates the inhibitory activity for rennin by ovomacroglobulin, as well as for pepsin.

To investigate the mechanism of rennin inhibition, ovomacroglobulin was reduced by mercaptoethanol to dissociate it into four subunits. As shown in Figure 3, the inhibitory activity of ovomacroglobulin for rennin was considerably decreased in the presence of mercaptoethanol. The effect of mercaptoethanol on the rennin activity was scarcely observed in the absence of ovomacroglobulin. Thus, the decrease in the rennin inhibition of reduced ovomacroglobulin is due to the dissociation of its structure into subunits. This result suggests that the association form of ovomacroglobulin is important for the inhibition of pepsin and rennin.

The association process of ovomacroglobulin with pepsin or rennin was further examined by using polyacrylamide gel electrophoresis. Figure 4 shows the electrophoretic patterns of untreated, pepsin-treated, and rennin-treated ovomacroglobulins. After reaction with pepsin or rennin for 5 min at 20 °C, polyacrylamide gel electrophoreses were carried out in the absence and presence of SDS and mercaptoethanol. Electrophoresis patterns in the absence of SDS and mercaptoethanol revealed the interaction of ovomacroglobulin with pepsin or rennin. On the other

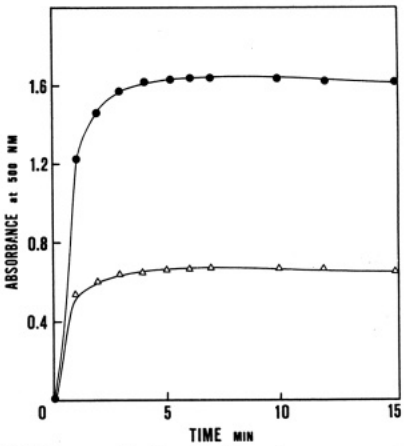

Figure 3. Inhibitory activity of reduced ovomacroglobulin with 2-mercaptoethanol against rennin. Rennin activity was measured in the presence of 0.01 M 2-mercaptoethanol by using κ-casein as substrate. (●) Without OMG; (△) reduced OMG/rennin molar ratio of 1:1.

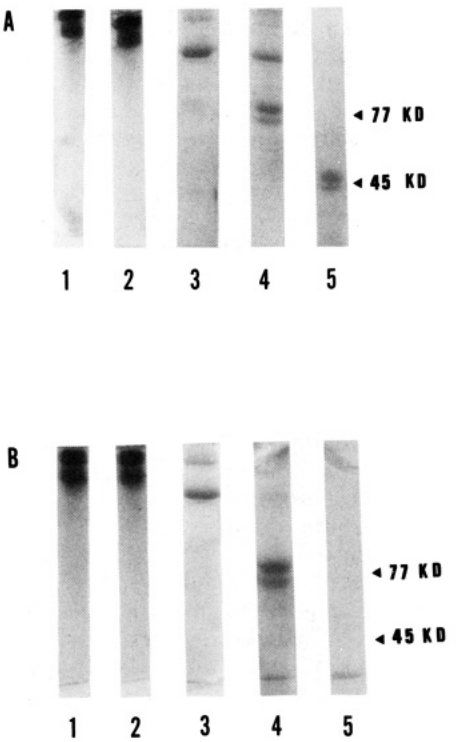

Figure 4. Polyacrylamide gel electrophoresis (lanes 1, 2) and SDS–polyacrylamide gel electrophoresis (lanes 3–5) of various ovomacroglobulin–protease mixtures. (A) OMG–pepsin mixture; (B) OMG–rennin mixture. (Lane 1) Native OMG; (lane 2) OMG–protease mixture (OMG/protease molar ratio of 1:1); (lane 3) reduced OMG; (lane 4) OMG/protease molar ratio of 1:1; (lane 5) protease.

hand, SDS–PAGE patterns showed that ovomacroglobulin was cleaved into two fragments having molecular sizes smaller than that of its subunit and that pepsin and rennin were released. The molecular weight of the smaller fragment was almost the same as that of ovotransferrin (M_w 77 000), while that of another fragment was slightly larger. Although the substrate specificity of rennin is narrow, rennin hydrolyzed the ovomacroglobulin subunit and produced two fragments having molecular weights of about 80 000. A similar observation was obtained in the inhibition mechanism of ovomacroglobulin for other proteases (Kitamoto et al., 1982; Molla et al., 1987). This indicates that the flexible region sensitive to proteases exists in the middle of the ovomacroglobulin subunit.

Table I. Surface Hydrophobicity of Various Ovomacroglobulins

OMG	surface hydrophobicity (S_0)
native	214.5
2-mercaptoethanol treated	355.5
incubated with rennin[a]	162.8

[a] Incubation of OMG with rennin was for 30 min at 20 °C.

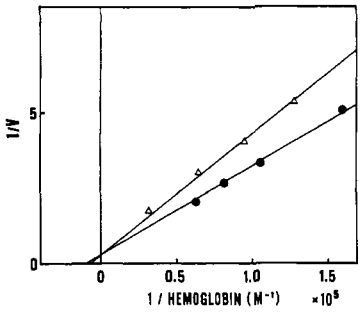

Figure 5. Lineweaver–Burk plot of pepsin. (●) Without OMG; (△) OMG/pepsin molar ratio of 1:1.

Table I shows the surface hydrophobicity of ovomacroglobulin before and after reaction with rennin. A significant decrease in the surface hydrophobicity of ovomacroglobulin was observed after incubation with rennin for 30 min. The mercaptoethanol treatment which results in the dissociation into subunit of ovomacroglobulin greatly increased its surface hydrophobicity, suggesting that the hydrophobic interaction may be involved in the association of subunits.

The pepsin inhibition mechanism was investigated by drawing the Lineweaver–Burk plot as shown in Figure 5. The inhibition mode was competitive for substrate, and the dissociation constant of the ovomacroglobulin–pepsin complex was 1.3×10^{-5} M^{-1}. Since the reaction of rennin is not measurable in a pseudo-first-order manner, the kinetic analysis was not performed.

DISCUSSION

It was reported that α_2-macroglobulin in the sera of pig and horse had inhibitory activity for rennin (Akaeda et al., 1971). Ovomacroglobulin also inhibited rennin and pepsin action, as described in the present paper. The inhibitory activity appeared at a ovomacroglobulin/rennin molar ratio of 1:1. The inhibitory activity was decreased by the reduction of ovomacroglobulin, resulting in the dissociation into its constitutive subunit. This suggests that the association of four subunits of ovomacroglobulin is important for the inhibitory activity for pepsin and rennin. It is interesting that ovomacroglobulin was hydrolyzed into two peptides by rennin, the substrate specificity of which is very limited. This indicates that the exposed flexible region containing potential cleavage sites for most proteases exists in the middle of the ovomacroglobulin subunit. In addition, the surface hydrophobicity of ovomacroglobulin decreased after incubation with rennin. Taking into account the increase in the surface hydrophobicity of dissociated ovomacroglobulin with mercaptoethanol, it seems likely that the hydrophobic regions are involved in the interaction between ovomacroglobulin and rennin in such manner that hydrophobic residues are buried in the complex. From the results of the electrophoresis and the surface hydrophobicity of the ovomacroglobulin–rennin mixture, the cleavage of a peptide bond may trigger a conformational change in ovomacroglobulin which, in turn, traps a pepsin or rennin molecule, as suggested for the inhibitory mechanism of ovomacroglobulin for other proteases.

We reported in a previous paper (Kato et al., 1974) that ovomucin inhibited rennin activity. However, the inhibitory behavior of ovomacroglobulin seems to be different from that of ovomucin. The occurrence of lag time of rennin activity and the recovery of enzymatic action after lag time suggest that the trapped rennin may remain in the active site or may be released from ovomacroglobulin. However, the latter possibility is invalid, because the dissociation constant of the ovomacroglobulin–pepsin complex suggests significantly strong binding.

Rennin is a milk-clotting enzyme that is essential for cheese processing. Therefore, ovomacroglobulin can be used for controlling cheese processing. In addition, the ovomacroglobulin–rennin system can be used for the trapping of unstable enzyme or functional macromolecules.

LITERATURE CITED

Akaeda, H.; Miura, H.; Mikami, M. Studies on the Rennin Inhibitor in Serum. *Nippon Nougeikagaku Kaishi* **1971**, *45*, 449–453.

Barrett, A. J.; Starkey, P. M. The Interaction of α_2-macroglobulin with Proteinases. *Biochem. J.* **1973**, *133*, 709–724.

Donovan, J. W.; Mapes, C. J.; Davis, J. G.; Hamburg, R. D. Dissociation of Chicken Egg-White Macroglobulin into Subunits. *Biochemistry* **1969**, *8*, 4190–4199.

Kato, A.; Hayashi, H.; Yagishita, K. The Inhibitory Properties of Chicken Egg White Ovomucin against Aggregation of κ-Casein by Rennin. *Agric. Biol. Chem.* **1974**, *38*, 1137–1140.

Kato, A.; Nakai, S. Hydrophobicity Determined by a Fluorescence Probe Method and its Correlation with Surface Properties of Proteins. *Biochim. Biophys. Acta* **1980**, *624*, 13–20.

Kitamoto, T.; Nakanishi, M.; Ikai, A. Hen Egg White Ovomacroglobulin Has a Protease Inhibitory Activity. *J. Biochem.* **1982**, *92*, 1679–1682.

Laemmli, U. K. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685.

Molla, A.; Oda, T.; Maeda, H. Different Binding Kinetics of Serratia 56K Protease with Plasma α_2-Macroglobulin and Chicken Egg White Ovomacroglobulin. *J. Biochem.* **1987**, *101*, 199–205.

Nagase, H.; Harris, E. D., Jr.; Woessner, J. F., Jr.; Brew, K. Ovostatin: A Novel Protease Inhibitor from Chicken Egg White. *J. Biol. Chem.* **1983**, *258*, 7481–7489.

Nagase, H.; Harris, E. D., Jr.; Brew, K. Evidence for a Thiol Ester in Duck Ovostatin (Ovomacroglobulin). *J. Biol. Chem.* **1986**, *261*, 1421–1426.

Zittle, C. A.; Custer, J. H. Purification and Some of the Properties of α_s-Casein and κ-Casein. *J. Dairy Sci.* **1963**, *46*, 1183–1188.

Received for review April 3, 1990. Accepted July 16, 1990.

Registry No. Pepsin, 9001-75-6; rennin, 9001-98-3.