The Enhancing Effect of Gelatin on Aprotinin Activity

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Abstract—Following preincubation of aprotinin with gelatin, the inhibitory effect on the trypsin activity was enhanced under the same condition as in the case of enhanced antigen-antibody reaction of aprotinin in gel in the presence of gelatin. In the immunochemical titration of aprotinin, gelatin treatment decreased the value of the equivalence point of aprotinin. Immunoprecipitation of aprotinin preincubated with or without gelatin adjusted to equal activity per unit volume resulted in overlapping equivalence points. These data suggest that the increase in aprotinin activity in the presence of gelatin is due to increase in the active form of aprotinin.

Aprotinin is used clinically to treat pancreatitis and shock due to acute peritonitis, as this compound has inhibitory effects on trypsin, chymotrypsin, plasmin and kallikrein (1). In the enzyme immunoassay of aprotinin, the recovery of this protease inhibitor from serum was high, and there was an enhancing effect of serum on the antigen-antibody precipitation of aprotinin in the gel. The influence of serum on the assay was eliminated by using a gelatin-containing buffer as a sample diluent in the first step of the antigen-antibody reaction, and the antigen-antibody reaction of aprotinin was enhanced in the presence of gelatin. The enhancing effect of serum on the antigen-antibody precipitation of aprotinin in the gel was not observed in the presence of gelatin (2). However, the effect of gelatin on aprotinin activity remained to be investigated. In the present study, we looked at the effect of gelatin on the inhibitory activity of aprotinin on trypsin, under the same condition as in the case of enhanced antigen-antibody reaction of aprotinin in gel.

Materials and Methods

Materials: A commercial sample of Trasylol, 10,000 kallikrein inhibitor units (KIU) per ml (Bayer A.G., W. Germany), served as aprotinin. The activity of one KIU corresponds to 0.14 μg of crystalline aprotinin. Gelatin and complete Freund’s adjuvant were obtained from Difco Laboratories (U.S.A.), trypsin from Miles Laboratories (U.S.A.), tosyl-L-arginine methyl ester hydrochloride (TAME) from Protein Research Foundation (Japan), and agarose and amido black 10 B from Nakarai Chemicals, Ltd. (Japan). DEAE-cellulose was obtained from Whatman Ltd. (England).

Solutions: Solution A: Solution A was 0.9% (w/v) NaCl containing 0.01% (w/v) NaN3.

Solution B: Solution B contained 0.05% (w/v) gelatin in solution A.

Antibody and IgG fraction of antibody: Antibody to aprotinin was produced in rabbits by injecting an emulsion of Trasylol and adjuvant (3). The IgG fraction of antiaprotinin serum was obtained by fractionation with Na2SO4 (4) followed by passage through a DEAE-cellulose column (5).

Double diffusion test: The immuno-diffusion plate was prepared using 1% (w/v) agarose in solution A with or without gelatin. Aprotinin was serially diluted 2-fold with solution A with or without gelatin, and it was reacted with antiaprotinin IgG solution (4 mg/ml) at 4°C for 72 hr. After washout of the unreacted protein with 0.9% (w/v) NaCl solution, the precipitin line was stained with a solution of amido black 10 B.

Effect of gelatin on aprotinin activity:
Various amounts of aprotinin in solution A with or without gelatin were prepared. The solution was incubated at 4°C for 72 hr or at 37°C for 24 hr, and the aprotinin activity in the solution was measured.

Inhibition study: Ninety-six KIU/ml of aprotinin in solution A was reacted with an equal volume of antiaprotinin IgG fraction (0 to 4 mg/ml) in solution A. The mixture was incubated at 37°C for 4 hr and then left to stand at 4°C overnight. After centrifugation at 30,000×g for 20 min, aprotinin activity in the supernatant was measured.

Immunoechemical titration of aprotinin: The immunoprecipitation procedure was according to the method of Joh et al. (6), as adapted from that described by Feigelson and Greengard (7). With this method, increasing amounts of aprotinin are added to a constant amount of antibody, i.e., that amount which will completely precipitate or inactivate the smallest amount of aprotinin used. This procedure results in disappearance of aprotinin activity in the supernatant. As more aprotinin is added, it saturates the available antibody and increasing amounts of aprotinin remain in the supernatant. The concentration of aprotinin that is titrated by the antibody is termed the equivalence point, and is graphically identified as the point on the abscissa at which aprotinin activity is first detected in the supernatant. In immuno-titration experiments, various amounts of aprotinin in solution A and in solution B were prepared. The solutions were incubated at 4°C for 72 hr and were then added respectively to equal volumes of antiaprotinin IgG fraction (1 mg/ml) in solution A and in solution B. The mixture was incubated at 37°C for 4 hr and left overnight at 4°C. After centrifugation at 30,000×g for 20 min, aprotinin activity in the supernatant was measured.

Measurement of aprotinin activity: The inhibitory effect of aprotinin on trypsin activity was measured with TAME as a substrate, using the method of Simlot and Feeney (8). In this case, the assay sample was incubated with trypsin (5 μg/ml) at 37°C for 5 min, after which the substrate (TAME) was added.

Results

Double diffusion test: Figure 1 shows the effect of gelatin on the double diffusion test of aprotinin. Comparing the antigen-antibody precipitation in gel prepared with solution A (Fig. 1, I) and that with gelatin-containing solution (Fig. 1, II and III), the enhancing effect of gelatin on the antigen-antibody

![Fig. 1. Effect of gelatin on the double diffusion test of aprotinin. (I) The immunoplate was prepared using 1% (w/v) agarose in solution A. Aprotinin (17.5 μg/ml) was diluted with solution A. Serial 2-fold dilutions of aprotinin were put in wells No. 1-5, and antiaprotinin IgG solution (4 mg/ml) was placed in the central well. (II) The immunoplate was prepared using 1% agarose in solution A with 0.05% (w/v) gelatin. Aprotinin (17.5 μg/ml) was diluted with solution A with 0.05% gelatin. Other conditions were the same as in (I). (III) The immunoplate was prepared using 1% agarose in solution A with 0.4% (w/v) gelatin. Aprotinin (17.5 μg/ml) was diluted with solution A with 0.4% gelatin. Other conditions were the same as in (I).]
reaction of aprotinin was apparent. With the 2-fold diluted solution of 17.5 μg/ml of aprotinin, a precipitate was clearly observed in (II) and (III), whereas in (I), a precipitin line was not observed. However, aprotinin in solution A with 0.05% gelatin or with 0.4%

Fig. 2. Effect of gelatin on aprotinin activity. Aprotinin activity in an aliquot of aprotinin preincubated with (G(+)) or without gelatin (G(-)) at 4°C for 72 hr or at 37°C for 24 hr was measured. Each value shows the mean of six assays and the standard error. Student's t-test was used for statistical analysis. There are significant differences between the group of no gelatin (G(-)) and gelatin-containing groups (0.05% and 0.4%) (**P<0.01).
gelatin produced a precipitin line in the gel with gelatin at the same dilutions (Fig. 1, II and III). The enhancing effect of gelatin on the antigen-antibody precipitation of aprotinin in gel was observed even at 0.00625% of gelatin dissolved in solution A.

**Effect of gelatin on aprotinin activity:** As shown in Fig. 2, approximately 1.25-fold enhanced inhibitory effect of aprotinin on the esterolytic activity of trypsin was observed after preincubation with gelatin, compared to that of aprotinin without gelatin; and the extent of the enhancing effect of gelatin on the aprotinin activity remained unchanged, regardless of differences in the concentration of gelatin (Fig. 2, G(+) 0.05% and G(+) 0.4%). The least effective concentration of gelatin dissolved in solution A on the aprotinin activity was 0.00625%. The concentration of gelatin used had no inhibitory activity on the esterolytic activity of trypsin. The change of pH in solution A was not observed with the addition of 0.05% of gelatin. No significant difference in aprotinin activity was observed between the samples preincubated with gelatin at 4°C for 72 hr and that at 37°C for 24 hr.

**Inhibition study of aprotinin:** As shown in Fig. 3, antiaprotinin IgG inhibited aprotinin activity, and the inhibition of this activity was increased with increments in the amount of antiaprotinin IgG added to the reaction. Two mg/ml of antiaprotinin IgG all but completely inhibited the 48 KIU/ml of aprotinin activity. IgG from control rabbit serum (0 to 2 mg/ml) did not inhibit aprotinin activity.

**Immunochemical titration of aprotinin:** In the first experiment (Fig. 4, I), increasing amounts of aprotinin preincubated with or without gelatin, were added to a constant amount of antibody. The equivalence point of aprotinin preincubated with gelatin (G(+)) was 0.094 μg/ml, whereas that of aprotinin preincubated without gelatin (G(−)) was 0.118 μg/ml. Its ratio was 1:1.25. In the second experiment (Fig. 4, II), aprotinin preincubated with gelatin was adjusted to an equal activity per unit volume as that of aprotinin preincubated without gelatin, and this was added to a constant amount of antibody. The equivalence point of aprotinin preincubated with gelatin overlapped that of aprotinin preincubated without gelatin.

![Fig. 3. Inhibition study of aprotinin.](image-url)
Fig. 4. Immunochemical titration of aprotinin. (I) Various amounts of aprotinin in solution A and in solution B were prepared. The solutions were incubated at 4°C for 72 hr, and then they were added respectively to equal volumes of antiaprotinin IgG fraction (1 mg/ml) in solution A and in solution B. (II) The activity of aprotinin in solution B preincubated at 4°C for 72 hr was adjusted with solution B (dilution of 1:1.25 with solution B) so that aprotinin activity per unit volume was equal to that of a unit volume of aprotinin in solution A preincubated at 4°C for 72 hr, after which an equal volume of antiaprotinin IgG fraction in solution B (1 mg/ml) was added. After the mixture was centrifuged, the inhibitory effect of aprotinin on trypsin activity was measured in the supernatant. The trypsin activity without aprotinin was taken as 100%, and trypsin inhibition % shown on the ordinate was calculated from the trypsin activity with aprotinin in the supernatant. In (I), note that gelatin treatment shifted the equivalence point of aprotinin to the left. In (II), note that the curves of both groups (G(+) and G(-)) overlap and that the equivalence points are identical.

Discussion

In the sandwich enzyme immunoassay of aprotinin (2), gelatin was found to enhance the antigen-antibody reaction of aprotinin in the medium of 10 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl, 1 mM MgCl₂, 0.01% (w/v) NaN₃ and 0.1% (w/v) bovine serum albumin. However, the effect of gelatin on aprotinin activity could not be studied under the above described condition, as the medium contained bovine serum albumin, and the buffering capacity of sodium phosphate in the medium interfered with changes in the absorbance at 395 nm of the indicator, m-nitrophenol. In the present work, the enhanced antigen-antibody reaction of aprotinin in gel was observed in the presence of gelatin dissolved in solution A (Fig. 1), and the effect of gelatin on aprotinin activity under the same condition as in the case of the enhanced antigen-antibody reaction of aprotinin could be investigated.

The preincubation time of aprotinin, with or without gelatin, was chosen to be 72 hr at 4°C or 24 hr at 37°C, since in the double diffusion test, the reaction was developed for 72 hr at 4°C. Under these conditions, enhanced aprotinin activity was observed after preincubation with gelatin (Fig. 2).

Regarding the enhancing effect of gelatin on aprotinin activity, at least two possibilities have to be considered: a) conformational change in the aprotinin molecule which would lead to an increase in aprotinin activity may occur in the presence of gelatin and b) aprotinin might exist in two different states in solution A. Only one state has aprotinin activity ("active form of aprotinin").
In the presence of gelatin, the ratio of the two states might be altered to increase the "active form of aprotinin". The immunochemical titration technique is used to demonstrate whether enhanced enzyme activity results from accumulation of more specific enzyme protein or from activation of preexisting enzyme molecules (6, 7). Using this technique, we found that each unit volume of aprotinin preincubated with gelatin contains approximately 1.25 times higher amount of the more "active form of aprotinin" than that of aprotinin preincubated without gelatin (Fig. 4, I). The 1.25-fold increase in the "active form of aprotinin" corresponds exactly to the incremental rise of aprotinin activity induced by gelatin in Fig. 2. Immuno-precipitation of aprotinin preincubated with or without gelatin adjusted to equal activity per unit volume resulted in overlapping equivalence points (Fig. 4, II). These results, therefore, demonstrate that the increase in aprotinin activity in the presence of gelatin is due to increase in the "active form of aprotinin".

The existence of aprotinin in dimer-monomer equilibrium in neutral solution has been reported (9). With regard to the molecular form of aprotinin which reacts with trypsin, the following supports the idea that aprotinin reacts in the monomeric state, and not in the dimeric state. The molecular weight of the complex of aprotinin and trypsin determined by gel filtration on Sephadex (10) and with sedimentation analysis (11) is 26,000 and 30,500±1,000, respectively. According to the reports that the reaction of aprotinin with trypsin is stoichiometric, i.e., the molar ratio is 1:1 (12), and in the physiological pH range, a 100% inhibition of 0.56 to 0.7 µg of trypsin is achieved by 1.2 KIU of aprotinin (13), the molecular weight of aprotinin in the complex with trypsin is approximately 6,000 to 7,000. Since the activity of one KIU corresponds to 0.14 to 0.15 µg of aprotinin (13). These investigators also suggested that the "active form of aprotinin" comes from the monomeric state.

The pH of the solution has an effect on the dimer-monomer equilibrium of aprotinin (9). However, the change of pH in solution A was not observed with the addition of the effective concentration of gelatin which enhanced the aprotinin activity. The exact mechanism by which gelatin increases the "active form of aprotinin" is the subject for a future study.

Regarding the enhancing effect of gelatin on antigen-antibody precipitation of aprotinin in gel, it was suggested in the previous paper that in the presence of gelatin, the ratio of two states, one which readily binds the antibody and another which does not, might be altered to increase the form which favors the binding of antibody (2). The present finding suggests that the "active form of aprotinin" favors the binding of the antibody used in this study.

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References
8 Simlot, M.M. and Feeney, R.E.: Relative reactivi-


