SANDWICH ENZYME IMMUNOASSAY OF APROTININ

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Enzyme immunoassay, using the rabbit antiaprotinin Fab'- β -D-galactosidase complex and the rabbit antiaprotinin IgG-coupled polystyrene ball as a solid phase was used to measure sera levels of aprotinin. The least measurable amount of the aprotinin in the enzyme immunoassay was about 0.002—0.005 kallikrein inhibitor unit/ml.

In the enzyme immunoassay of aprotinin in serum, the influence of serum on the assay was eliminated by using a buffer with gelatin as a sample diluent in the first step of the antigen and antibody reaction.

Aprotinin level in the serum, as estimated by enzyme immunoassay correlated highly with levels obtained by inhibition assay.

Keywords—sandwich enzyme immunoassay; inhibition assay; aprotinin; gelatin; aprotinin in serum

INTRODUCTION

Aprotinin is used clinically to treat patients with acute pancreatitis and shock due to acute peritonitis. This compound has inhibitory effects on trypsin, chymotrypsin, plasmin and kallikrein.¹⁾ Levels of aprotinin in plasma or tissue are usually measured by enzymatic determination²⁾ or by an isotope technique.^{3,4)} In the evaluation of various methods, I have found the sandwich enzyme immunoassay to be a highly sensitive method capable of determining 10^{-3} kallikrein inhibitor unit/ml levels of aprotinin.

MATERIALS AND METHODS

Materials—A commercial sample of Trasylol, 10000 kallikrein inhibitor units (KIU) per ml (Bayer A.G., W.Germany), served as aprotinin. DEAE-cellulose was obtained from Whatman Ltd. (England), β -D-galactosidase from Boehringer Mannheim GmbH (W.Germany), *N,N'*-*o*-phenylenedimaleimide from Aldrich Chemical Company, Inc. (U.S.A.), bovine serum albumin from Armour Pharmaceutical Company (U.S.A.). 4-Methylumbelliferyl- β -D-galactoside, 4-methylumbelliferone, agarose and amino black 10 B were purchased from Nakarai Chemicals,

Ltd. (Japan). Adjuvant and gelatin were products of Difco Laboratories (U.S.A.). Polystyrene balls were purchased from Medical and Biological Laboratories Ltd. (Japan). Tosyl-L-arginine methyl ester hydrochloride (TAME) was obtained from the Protein Research Foundation (Japan), and trypsin from Miles Laboratories (U.S.A.).

Buffer—Buffer A: Buffer A was 0.01 M sodium phosphate (pH 7.0) containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.01% (w/v) NaN_3 and 0.1% (w/v) bovine serum albumin.

Buffer B: Buffer B contained 0.4% (w/v) gelatin in buffer A.

Buffer C: Buffer in which the bovine serum albumin is omitted from buffer A.

Buffer D: Buffer in which the bovine serum albumin is omitted from buffer B.

Antibody and Antiaprotinin Fab'- β -D-galactosidase Complex—Antibody to aprotinin was produced in a rabbit by injecting an emulsion of Trasylol and adjuvant.⁵⁾ The IgG fraction of antiaprotinin serum or normal rabbit serum was obtained by fractionation with Na_2SO_4 ⁶⁾ followed by passage through a DEAE-cellulose column.⁷⁾ Antiaprotinin Fab' fragment was pre-

pared by the method of Nisonoff and Rivers.⁸⁾ β -D-Galactosidase from *Escherichia coli* was coupled to antiaprotinin Fab' fragment by use of *N,N'*-*o*-phenylenedimaleimide.⁹⁾

IgG-coated Polystyrene Ball — Polystyrene balls (d = 6 mm) were coated with antiaprotinin IgG by physical adsorption at 4°C for 24 h,¹⁰⁾ and were stored in buffer A at 4°C until use.

Sandwich Enzyme Immunoassay — Each of the IgG-coated polystyrene balls was incubated with various amounts of aprotinin in a final volume of 0.75 ml of buffer A or buffer B with shaking at 37°C for 4 h, and then allowed to stand at 4°C overnight. Each ball was washed three times with 1 ml of buffer A, and was incubated with 800 units of antiaprotinin Fab'- β -D-galactosidase complex, in a volume of 0.5 ml of buffer A, at 37°C for 6 h. Each ball was then washed three times with 1 ml of buffer A, and was transferred to another test tube. The activity of β -D-galactosidase bound to the ball was assayed.

Assay of β -D-Galactosidase Activity — Each ball was preincubated in 0.3 ml of buffer A at 37°C for 6 h. Each ball was then washed three times with 1 ml of buffer A, and was transferred to another test tube. The activity of β -D-galactosidase bound to the ball was assayed.

reaction was stopped by adding 2.5 ml of 0.1 M glycine-NaOH buffer, pH 10.3. The fluorescence intensity of the 4-methylumbelliferone released was measured against a freshly prepared standard of 1×10^{-6} M 4-methylumbelliferone. The wavelengths used were 360 nm for excitation and 450 nm for emission analysis. One unit of β -D-galactosidase activity was defined as that which hydrolyzes 1 picomole of 4-methylumbelliferyl- β -D-galactoside per min, under the condition described.

Serum Level of Aprotinin in Rat — Aprotinin (60000 KIU/kg) was administered intravenously in a single dose to 27 male Wistar rats weighing about 250 g. After the injection, three rats were decapitated every 20 min, and sera prepared separately. Control sera were obtained from rats without aprotinin treatment. Sera from each rat were assayed using two methods, i.e. sandwich

enzyme immunoassay and inhibition assay. In the enzyme immunoassay, serum samples were diluted with buffer B, and concentration of aprotinin in the sample was adjusted to be in the range of 0.002–0.1 KIU/ml.

Inhibition Assay — Aprotinin was extracted from the sera with perchloric acid, according to the method of Fritz *et al.*,²⁾ and the inhibitory effect of aprotinin on the esterolytic activity of trypsin, using TAME as substrate, was measured by the method of Simlot and Feeney.¹¹⁾

RESULTS

Standard Curve for the Sandwich Enzyme Immunoassay

Fig. 1 shows a typical standard curve for the sandwich enzyme immunoassay of aprotinin. β -D-Galactosidase activity bound to the antiaprotinin IgG coated ball increased with increase in the amount of aprotinin, and the increment was greater with buffer B (G(+)) than with buffer A (G(-)), whereas the activity bound to the nonspecific IgG coated ball did not increase. The least detectable concentrations of aprotinin (concentration yielding double the increase in the activity of galactosidase bound to the ball than that in the absence of antigen) were estimated to be 0.002 KIU/ml in buffer B and 0.005 KIU/ml in buffer A, respectively. These values correspond to 0.0015 KIU/assay tube and 0.00375 KIU/assay tube, respectively. When the balls, coated with various concentration of antiaprotinin IgG solutions ranging from 0.5 mg/ml to 4 mg/ml, were used, the least measurable concentration of aprotinin was the same as that of one mg/ml.

Recovery of Aprotinin from the Serum

As shown in Fig. 2, the values obtained in the assay without gelatin were approximately three times higher than when aprotinin was added, and the influence of serum was eliminated by using the gelatin-containing buffer in the assay. The recovery percent tended to decrease with increasing concentration of gelatin in the buffer, and the optimal concentration of gelatin in the assay was 0.4% (w/v). However, no significant differences were observed among the assays with 0.3%, 0.4%

and 0.5% gelatin-containing buffer.

Precision Analysis

Precision of the sandwich enzyme immunoassay was analyzed on the serum sample which was used in recovery study. The coefficients of within- and between-assay variations in the serum obtained with buffer B were 9.0% ($n=6$, mean=40.0 KIU/ml) and 9.5% ($n=4$, mean=40.1 KIU/ml), respectively.

FIG. 1. Typical Standard Curve for the Sandwich Enzyme Immunoassay of Aprotinin

The rabbit antiaprotinin IgG coated polystyrene balls (A, B) or nonspecific IgG coated polystyrene balls (C, D), prepared with one mg/ml of IgG fractions, were incubated with various concentrations of aprotinin in buffer B (G(+)) or in buffer A (G(-)), and then incubated with 800 units of rabbit antiaprotinin Fab- β -D-galactosidase complex. Each point shows the mean value of duplicate assays.

Effect of Serum and/or Gelatin on Double Diffusion Test of Aprotinin

In the upper panel of Fig. 3, aprotinin in serum (A) and aprotinin (B) were serially 3-fold diluted with buffer A. Comparing the (A) and (B), the enhancing effect of serum on antigen-antibody precipitation was apparent. Rat serum *per se*

FIG. 2. Recovery of Aprotinin from Rat Serum

The rat serum, containing 40 KIU/ml of aprotinin, was diluted with buffer A (gelatin concentration; 0%) or with gelatin-containing buffer A, and recovery of aprotinin was calculated from the corresponding standard curve. Each value shows the mean of six assays and its standard error. Student's *t*-test was used for statistical analysis. Significant difference is observed between the group of 0% and gelatin-containing groups (0.3%, 0.4%, 0.5%) ($p < 0.01$).

showed no antigen-antibody reaction in the gel. However, in gel with gelatin (in the lower panel of Fig. 3) aprotinin in serum (A) and aprotinin (B)

produced a precipitin line at the same dilutions. Fig. 3 also shows the enhancing effect of gelatin on antigen-antibody precipitation in gel. With 3-

FIG. 3. Effect of Serum and/or Gelatin on Double Diffusion Test of Aprotinin

Bovine serum albumin was omitted from buffer A or buffer B in the preparation of immunoplate, for the precipitin line was stained with a solution of amino black dye.

Upper panel: Immunoplate was prepared by one % (w/v) agarose in buffer C. Antigen [aprotinin (250 KIU/ml) in rat serum (A) or aprotinin (250 KIU/ml) in saline (B)] was diluted with buffer A. Serial 3 fold dilutions of antigen were put in wells No.1—5, and antiaprotinin IgG solution (7 mg/ml) was placed in the central well.

Lower panel: Immunoplate was prepared by one % agarose in buffer D. Antigen [aprotinin (250 KIU/ml) in rat serum (A) or aprotinin (250 KIU/ml) in saline (B)] was diluted with buffer B. Other conditions were as in the upper panel.

fold diluted solution of 250 KIU/ml of aprotinin, a precipitate was clearly observed in the lower panel in (B), whereas in the upper panel in (B) precipitin line was not observed.

Serum Level of Aprotinin in Rat

Aprotinin was administered intravenously to rats, and levels of this compound in the sera were measured by both sandwich enzyme immunoassay and inhibition assay. In the enzyme immunoassay, serum samples were diluted with buffer B to remove the interference by serum. Fig. 4 shows the serum level of aprotinin measured by the sandwich enzyme immunoassay. The first-

FIG. 4. Serum Level of Aprotinin in Rats measured by Sandwich Enzyme Immunoassay

Each point shows the mean value of aprotinin in sera from three rats. Other details are given in the METHODS' section.

order curve was observed at 20 to 140 min after injection. Using the hypothetical serum concentration at zero-time, volume of distribution (V_d) was calculated to be approximately 0.55 l/kg of the body weight, and the biological half-time ($t_{1/2}$) of aprotinin was about 40 min. The same values of V_d and $t_{1/2}$ were also obtained with the inhibition assay, and here, aprotinin was extracted from the serum with perchloric acid. In this process, recovery percent of aprotinin from the serum was 47.6 ± 1.1 (mean $\pm$ standard error of 9 experiments). The values of aprotinin determined by inhibition assay were corrected for the loss in the extraction process by multiplying $1/0.476$, and the base line trypsin inhibitor activity extracted from control rat serum obtained from the rat without aprotinin treatment, was taken as zero value. As shown in Fig. 5, aprotinin levels in the

FIG. 5. Correlation between Aprotinin Concentration in Rat Serum measured by Enzyme Immunoassay (EIA) and by Inhibition Assay

The values of aprotinin shown in Fig. 4 and the values obtained by inhibition assay, in which measured values were corrected for the loss of aprotinin in the extraction process by multiplying $1/0.476$, were plotted.

$$Y = 0.97X + 0.21 \quad (r = 0.995)$$

serum, as estimated by sandwich enzyme immunoassay correlated highly with the levels obtained by inhibition assay.

DISCUSSION

The commercial sample of Trasylol which was used as aprotinin contained at least two components with molecular weights of about 6500 (component 1) and about 900 (component 2).⁵⁾ Based on activity and molecular weight, component 1 was obviously aprotinin whereas component 2 was an impurity in the commercial product, and component 1 gave an antigen-antibody reaction, whereas component 2 did not.⁵⁾

In the enzyme immunoassay of aprotinin, the least measurable concentration of aprotinin was estimated to be as little as 0.002–0.005 KIU/ml which corresponds to 0.28–0.75 ng of aprotinin/ml since the activity of one KIU is equivalent to 0.14–0.15 μ g of aprotinin.¹²⁾ This value was not changed through the present study using the 800 units of complex and the balls coated with one mg/ml of antiaprotinin IgG solution. The amount of bound IgG could not be determined by measuring the change in the concentration of IgG solution before and after coupling, since the change was too small to be determined precisely. The plateau value of bound galactosidase was obtained at or close to 0.3 KIU/ml of aprotinin, using the 800 units of complex (Fig. 1), and the plateau value increased with increase in the amount of complex. This suggests that the amount of aprotinin coupled to the antiaprotinin IgG coated ball was sufficient to bind more than 800 units of complex.

Gelatin, a hydrophobic protein, has been used in complement assays as a stabilizing agent to decrease the spontaneous lysis of sheep erythrocytes.¹³⁾ In the present sandwich enzyme immunoassay of aprotinin, samples diluted with buffer B, showed the lower blank value and an enhanced antigen-antibody reaction, as compared to the samples diluted with buffer A (Fig. 1). The sensitivity of the assay with buffer B was increased by these factors compared with that with buffer A. With regard to the enhancing

effect of gelatin on the antigen-antibody reaction, at least two possibilities have to be considered, a) the antiaprotinin IgG fractions, used in this study, might be heterogeneous on the specificity for aprotinin, and the enhanced antigen-antibody reaction might be caused by newly appearing antigenic determinants in aprotinin, in the presence of gelatin. b) Aprotinin might exist in two different states such as monomer and dimer in buffer A. One readily binds the antibody whereas the other does not. In the presence of gelatin, the ratio of two states might be altered to increase the form which favors the binding of antibody. The first hypothesis is untenable, since using the fixed amount of antiaprotinin Fab'- β -D-galactosidase complex which was prepared with antiaprotinin IgG fractions, the plateau value of bound galactosidase activity was the same with buffer A as with buffer B (Fig. 1). On the other hand, the second hypothesis would explain the enhancing effect of gelatin or serum on antigen-antibody precipitation, in the double diffusion test in gel (Fig. 3). In the ultracentrifuge study, it has been reported that aprotinin shows a monomer-dimer equilibrium, with the dimer predominating in neutral solution.¹⁴⁾ The exact molecular form or state of aprotinin which favors the binding of antibody is the subject for a future study. The enhancing effect of serum on antigen-antibody precipitation was not observed in the presence of gelatin (Fig. 3, lower panel). This suggests that the equilibrium of the two states of aprotinin established in the gelatin-containing buffer is not affected by the presence of serum. These results parallel the results of recovery percent of aprotinin in serum using the buffer with or without gelatin (Fig. 2). Thus, in the enzyme immunoassay of aprotinin in serum, samples were diluted with gelatin-containing buffer.

There have been numerous pharmacokinetic studies of aprotinin, after administration.^{3,15–17)} In the present study, the objective was to compare the values of aprotinin in serum measured by enzyme immunoassay with values measured by inhibition assay. To perform both of the assays, at least 1 ml of serum sample was needed each time

From this reason three rats were used at each sampling. After the injection of aprotinin, the first order curve was observed at 20 to 140 min. From this curve, the biological half-time of aprotinin in the serum was about 40 min, and the volume of distribution was about 0.55 l/kg of the body weight. The same results were obtained using the inhibition assay. Reports on the half-life of aprotinin in serum include 59 min in rats,⁴⁾ 70 min in rats,¹⁶⁾ 150 min in humans¹⁵⁾ and 0.7 h and 7 h in humans.¹⁷⁾ However, the half-life estimated was highly dependent on the time of measurement, since the serum concentration curves of aprotinin cannot be interpreted on the basis of a simple pharmacokinetic model. values estimated immediately after injection resulted in a very short half-life of a few minutes, while values obtained after one hour resulted in a relatively long half-life. It was also reported that the volume of distribution in humans calculated from the elimination curve of aprotinin in serum was 0.63 l/kg of the body weight.¹⁵⁾ Aprotinin levels in serum measured by enzyme immunoassay correlated highly with the levels obtained by inhibition assay. This suggests that the amounts of aprotinin measured by enzyme immunoassay reflect those of the biologically active aprotinin which has an inhibitory effect on an enzyme such as trypsin. Degradation of aprotinin in serum might not occur at least during the first three hours after the administration, since it was confirmed in a preliminary study, that there was no difference between the amount of aprotinin measured either by enzyme immunoassay or by inhibition assay before incubation and that of aprotinin measured after incubation of the mixture which contained aprotinin and rat serum, for 4 h at 37°C.

The disadvantage of enzyme immunoassay of aprotinin was that at least two days were required to obtain the results. However, the assay was almost as sensitive as or even more sensitive than radioimmunoassay,^{4,18)} and the sample volume needed for the assay was exceedingly small compared with the volume needed for the inhibition assay.

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