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On the Determination of Molar Concentration of Plasmin and Plasmin Inhibitors

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The polyvalent protease inhibitor from bovine lung (Trasylol ®) can be used for standardizing purified human autoactivated plasmin in molar units. This enables determination of the molar concentration of plasmin inhibitors in biological material. The rate of dissociation of the complex between the polyvalent protease inhibitor and trypsin and plasmin, respectively, is elucidated.

Casein, fibrin, or synthetic esters of lysine or arginine are often used as substrates for determining plasmin activity.[1] The activity is expressed in arbitrary units. When casein is used as substrate, these units are measurements of the amount of acid soluble degradation products, usually expressed as the increase in optical density at 280 mμ after precipitation with acid. When fibrin is used, the activity units have been defined *inter alia* as the amount of plasmin necessary to dissolve a fibrin clot within a certain time or the amount of plasmin producing lysis of a certain area of a fibrin film per unit of time. When synthetic esters are used, the activity is expressed in units based on the amount of substrate split per unit on time.[2] In all these methods the results are very dependent of the constancy of the prevailing conditions and the units of measurement used are not directly comparable.[3]

Plasmin inhibitors are usually given in terms of plasmin inhibiting activity expressed in the arbitrary units of plasmin activity used. Trautschold, Werle and Fritz [4] suggested as a unit the amount of inhibitor capable of inhibiting an enzyme unit defined as the amount which at 25°C converts one μmole substrate/min. This, however, would imply that the unit would vary with the conditions under which the determination is made, with the substrate used, and with the origin of the enzyme preparation. In the determination of strong inhibitors these difficulties can be obviated by comparing the activity of the inhibitor tested with that of another strong inhibitor of known concentration. The term "strong inhibitor" is to be understood here as an inhibitor that rapidly binds the enzyme in a certain molar ratio forming a complex

with little or no enzyme activity and a low dissociation constant. Trypsin and trypsin inhibitors are standardized in this way against pure crystalline soybean trypsin inhibitor, STI.[5] But STI is far too weak a plasmin inhibitor to be used as standard for plasmin inhibitors. Nissen and Astrup recently suggested the possible use of the polyvalent protease inhibitor from ox-lung (PPI) for determining the plasmin concentration in purified plasmin preparations.[6] Dray, Samama, Vairel and Piette [7] titrated plasmin in plasma against "Kunitz's and Northrop's inhibitor",[8] which is identical with PPI.[9-11] The activity was expressed in inhibitory units. The inhibitor can in turn be standardized against STI with the use of trypsin as an enzyme. If the molar concentration of STI is known, the molar concentration of trypsin, PPI, plasmin, and the plasmin inhibitor can be calculated provided that PPI satisfies the above requirement of a strong inhibitor of trypsin and plasmin. This paper elucidates the justification and the expedience of the use of PPI in the standardization of purified plasmin preparations and thereby of plasmin inhibitors in biological material.

EXPERIMENTAL

Reagents. Crystalline bovine trypsin (Trypure lot No. 6 × 23, Novo A/S, Copenhagen) was dissolved in 0.0025 N HCl. The solution was standardized against STI.

Purified autoactivated human plasmin in 50 % glycerol (Kabi, Stockholm, Grade A, lot No. LmA 5) was used.

Purified protease inhibitor from bovine lung (Trasylol ® 5000 KIE/ml) was obtained from Bayer, Leverkusen, West Germany. Here the inhibitor is referred to as PPI.

Crystalline soy-bean trypsin inhibitor (Worthington, Biochem. Corp., Freehold, N.J.) was dissolved in 0.0025 N HCl. The optical density of the solution at 280 mμ was multiplied by 1.1 to convert it to the concentration of STI expressed in mg/ml.[5] The molecular weight of the STI was taken as 20 000.[12]

Benzyl-DL-arginine-*p*-nitroanilide HCl (BAPNA) was obtained from Fluka AG, Buchs SG, Schweiz. An 0.003 M solution was prepared by heating the substance at about 90°C with distilled water.

Casein according to Hammarsten (E. Merck, Darmstadt, West Germany) was used. A 3 % solution was prepared by heating it with tris buffer at about 90° for 15 min.

All the other reagents were of analytical grade.

Methods. The trypsin activity was determined according to a previously described modification [13] of Erlanger's method [14] with the use of BAPNA as substrate. The activity was given as the change in optical density at 410 mμ. By determining the activity also in the presence of a known amount of STI the trypsin concentration could be expressed in molar units.[5]

Plasmin was determined with casein as a substrate in the way described by Bundy and Mehl for trypsin.[15] The activity was expressed as the increase in optical density at 280 mμ in the supernatant after precipitation with trichloracetic acid to a final concentration of 1.56 %.

PPI was labelled with ^{125}I according to McFarlane.[16] The average labelling was less than 1 atom of iodine per molecule of inhibitor, calculated from the trypsin inhibiting activity of the preparation. The labelling caused only insignificant loss of inhibiting activity. Autoradiography after agarose gel electrophoresis of the labelled preparation showed that ^{125}I was also bound to a contaminant without inhibiting activity and with an electrophoretic mobility corresponding to that of a γ-globulin.

Gel filtration in Sephadex G 200 and G 50 was done at + 4° in the way described previously.[17] The radioactivity of the fraction was measured with a γ-spectrometer. The background activity was about 125 cpm.

RESULTS

Inhibition of trypsin by PPI. The inhibition of trypsin by PPI was studied by mixing trypsin with increasing amounts of PPI. After 10 min incubation of the mixture at 25°C BAPNA was added and the residual trypsin activity was measured. The inhibition was found to vary linearly with the amount of inhibitor added (Fig. 1). The results were largely the same when trypsin was incubated with the inhibitor for only 5 min before addition of the substrate.

In another experiment trypsin was mixed with ^{125}I-labelled PPI in excess (according to calculation from the above experiment) and then fractionated by gel filtration in a column with Sephadex G 50. The pattern of the radioactivity in the fractions showed an initial predominant peak corresponding to the trypsin-PPI complex and two subsequent smaller peaks corresponding to the excess of free inhibitor and ^{125}I-labelled contaminant (Fig. 2). To a minor portion of the fraction with the trypsin-PPI-complex was added unlabelled inhibitor in an amount corresponding to about 10 times the amount bound. After 2 h incubation at 25° the mixture was again fractionated in a Sephadex G 50 column. Now only one peak was obtained at the site of the main fraction after the first gel filtration (Fig. 2).

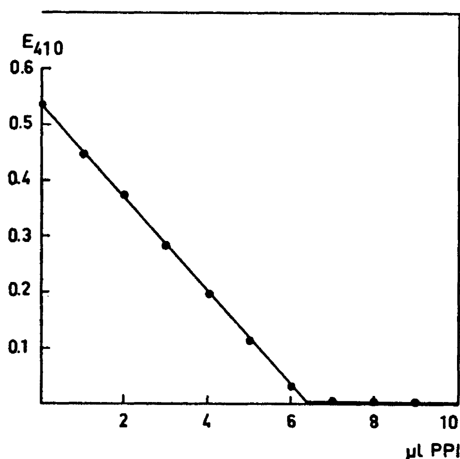

Fig. 1. Inhibition of trypsin with PPI. Varying amounts of PPI were added to 20 µg trypsin in 2 ml tris buffer pH 8.2. Ordinate: Residual trypsin activity expressed as optical density at 410 mµ. Abscissa: Amount in µl of inhibitor added.

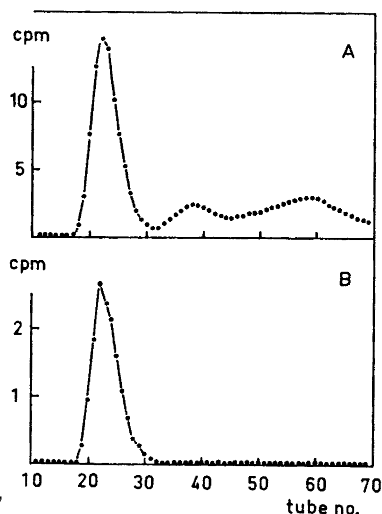

Fig. 2. Distribution of radioactivity in the fractions after gel filtration of a mixture of 200 µg trypsin and ^{125}I-labelled PPI in slight excess (A). The first eluted fraction represents the trypsin-PPI complex. Lower curve (B) shows the distribution of the radioactivity on gel filtration of this complex after incubation for 2 h at 25°C with about tenfold excess of unlabelled PPI. Ordinate: Radioactivity in net cpm × 10^{-3}. Abscissa: Fraction number.

Inhibition of plasmin by PPI. A constant amount of a plasmin solution was mixed with a varying amount of PPI. After 10 min incubation of the mixture the residual plasmin activity was measured with casein as a substrate. The activity decreased linearly with the amount of PPI added down to complete inhibition (Fig. 3). Variation of the incubation period between 5 and 30 min before addition of casein had no effect on the results.

To study the possible dissociation of the plasmin-PPI-complex a mixture of a plasmin solution and ^{125}I-labelled PPI in excess was fractionated by gel filtration (Sephadex G 200). Partial separation of two fractions was obtained, namely plasmin bound PPI and free PPI. Part of the fraction eluted first and containing only very small amounts of free inhibitor was concentrated and purified further by refiltration. To the plasmin-PPI fraction was then added

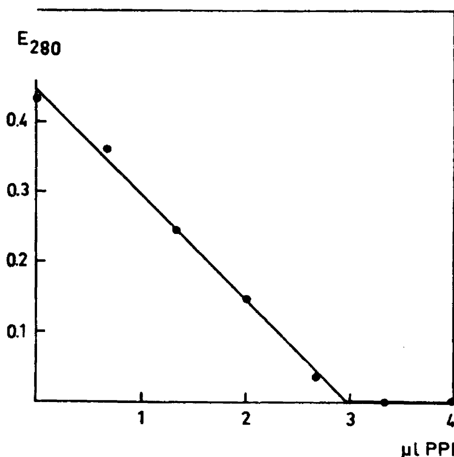

Fig. 3. Inhibition of plasmin with PPI. Various amounts of PPI were added to 50 µl plasmin solution in 1 ml of tris buffer pH 7.6. Ordinate: Residual plasmin activity expressed as optical density at 280 mµ. Abscissa: Amount (in µl) of inhibitor added.

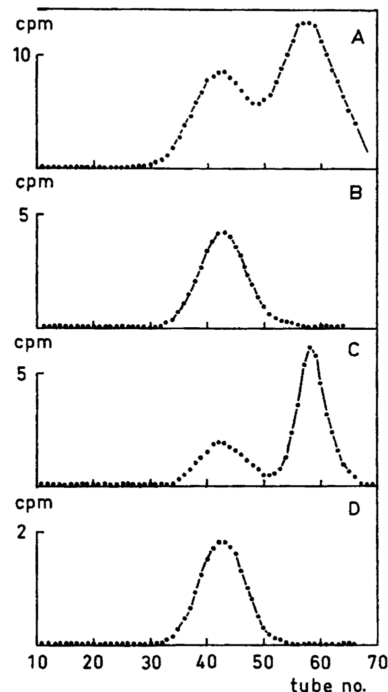

Fig. 4. Distribution of radioactivity in fractions after gel filtration of a mixture of 1 ml plasmin solution and ^{125}I-labelled PPI in excess (A), purified ^{125}I-PPI-plasmin complex (B), purified complex after incubation for 2 h at 37°C with about tenfold excess of unlabelled PPI (C), and purified complex after incubation for 2 h at 37°C in absence of free PPI (D). Ordinate: Radioactivity in net cpm $\times 10^{-3}$. Abscissa: Fraction number.

unlabelled inhibitor in an amount corresponding to 10 times that of the bound amount, the mixture was incubated for 2 h at 37° and then fractionated again by gel filtration. Now about 67 % of the radioactivity was obtained in the fraction corresponding to the free inhibitor and the rest in the complex fraction. In a control experiment the plasmin ^{125}I-labelled PPI complex was incubated in the same way but without free unlabelled inhibitor. After gel filtration radioactivity was found only in the fractions containing the enzyme inhibitor complex.

Calculation of the plasmin concentration in a purified plasmin preparation. As an application of the proposed method for standardizing purified plasmin preparations, the molar plasmin concentration in the plasmin solution used was calculated. The strength was given by the manufacturers as about 10 caseinolytic units/ml according to Sgouris. The activity of 50 μl of a trypsin solution was determined in the presence of a varying amount of STI-solution containing 23.3 nmole/ml. From this the molar concentration of the trypsin solution was calculated as 15.85 nmole/ml. The activity in this trypsin solution was then determined in the presence of a varying amount of the PPI-preparation. 7.9 μl of the PPI-solution was necessary completely to inhibit 50 μl of the trypsin solution. The concentration of the PPI-solution was then calculated as 100 nmole/ml (15.85 × 50/7.9) assuming that PPI is bound to the trypsin equimolarly and in the way described in the introduction for strong inhibitors.

Finally, determinations were made of the activity in 50 μl of plasmin solution in the presence of a varying amount of the standardized PPI-solution (Fig. 3). 2.95 μl of PPI proved necessary to inhibit the plasmin activity completely. With the same assumption as that accepted for the inhibition of trypsin by PPI the concentration of the plasmin solution was calculated as 5.9 nmole/ml (100 × 2.95/50). Attempts to determine the molar plasmin inhibiting activity of α_2-macroglobulin in serum with the thus standardized plasmin preparation will be the subject of a future paper.[18]

DISCUSSION

The direct proportionality found between inhibited activity and amount of inhibitor added suggests that PPI inhibits trypsin and plasmin by being bound to the respective enzymes stoichiometrically. Under the conditions used the binding was so rapid as to be complete within 5 min incubation before addition of the substrate. The fact that no "residual activity" could be demonstrated when inhibitor was used in excess shows that the binding of PPI to trypsin and to plasmin results in total loss of the enzyme activity against the substrate used. The complete inhibition also shows that the binding of the enzyme is either irreversible or "pseudoirreversible" [19] in the sense that the relative affinity of the enzyme for the inhibitor is so much greater than its affinity for the substrate that a true equilibrium is not demonstrable. In such a situation ordinary kinetic analysis cannot decide whether the inhibition is competitive or noncompetitive.[20]

The experiment with incubation of a purified complex of trypsin and ^{125}I-labelled inhibitor with excess of unlabelled inhibitor shows that the trypsin-PPI complex at the pH in question dissociates so slowly that during the incuba-

tion time used (2 h) the PPI must be regarded as an irreversible inhibitor irrespective of the substrate used. This is compatible with the calculation of Trautschold *et al.*[4] that the dissociation constant for the complex is very low (10^{-10}) at neutral or alkaline pH. The plasmin-PPI complex, on the other hand, showed a much higher rate of dissociation, so that the major part of the complex was dissociated in 2 h at 37°C without achievement of complete equilibrium between the bound ^{125}I-labelled and the free unlabelled inhibitor. But in the control experiment without excess of unlabelled inhibitor no free ^{125}I-labelled inhibitor could be demonstrated, which suggests that the dissociation constant was nevertheless so low that the inhibition may in practice be regarded as pseudoirreversible.

In the calculation of the molar concentration of plasmin and plasmin inhibitors it is assumed that the PPI is bound equimolarly to both trypsin and plasmin. Kunitz and Northrop's [8] observations lend strong support to the assumption as far as trypsin is concerned. PPI can probably not bind more than one plasmin molecule at the same time because no form of repetition occurs in the amino acid sequence of PPI,[9,10,11] and because the occurrence of more than one plasmin binding site would presumably result in the binding of more than one trypsin molecule. Assumption of a binding of more than one PPI-molecule per plasmin molecule would have to presuppose the occurrence of more than one proteolytic active group in the plasmin. No support for such an assumption is available. If such binding should occur, it might be assumed that also other strong plasmin inhibitors would be bound to plasmin in the same molar ratio as PPI. This would then not affect the calculated molar concentration of a plasmin inhibitor. The calculated molar concentration of plasmin would, however, then have to be divided by the number of active sites per plasmin molecule in the calculation of the true molar concentration of plasmin.

The advantage of expressing plasmin inhibiting activity as molar concentration of the inhibitor is that the result is largely independent of the method used for determining plasmin activity. PPI has proved to inhibit human, porcine, and bovine plasmin. These preparations, however, showed different degrees of specific activity expressed in caseinolytic units per mole enzyme.[6] It is, however, very likely that any differences with species or differences between plasmin preparations due to different methods used for purification or activation will not affect the molar binding ratio between plasmin and PPI. (On the other hand, the dissociation constant of the complex can very well be affected and should be examined for each type of plasmin). The result obtained in the determination of plasmin inhibitors in molar units is therefore independent also of the type of plasmin preparation used. In the determination of the molar concentration of strong plasmin inhibitors, which can also bind trypsin stoichiometrically, it may be technically advantageous to determine the molar concentration of the inhibitor directly with the use of trypsin as an enzyme.

For weak plasmin inhibitors, which are bound to plasmin much slower (*e.g.* α_1-antitrypsin = "slow plasmin inhibitor")[21-23] or where the complex shows a relatively high dissociation constant, the results of determination of inhibitor activity in arbitrary units are influenced particularly by the condi-

tions under which the determination is made. α_1-Antitrypsin has thus been described as inhibiting autoactivated and streptokinase activated plasmin differently. The biologically relevant plasmin inhibiting activity of such an inhibitor is, of course, dependent on the concentration of the inhibitor and it is generally of less interest to determine the inhibiting activity under artificial conditions against a preparation of more or less denaturated plasmin. In the determination of the "slow plasmin inhibitor" of plasma it therefore seems advantageous to utilize the strong trypsin inhibiting capacity of the inhibitor for the determination and to calculate the concentration of the inhibitor from it in molar units instead of determining the plasmin inhibiting capacity under carefully defined conditions.

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