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Determination of Functional Activity of α_1-Protease Inhibitor and α_2-Macroglobulin in Human Plasma Using Elastase

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Summary: The competitive binding of human α_1-antitrypsin and human α_2-macroglobulin to porcine pancreatic elastase was studied. Mixtures of these two protease inhibitors, when titrated against elastase give inhibition curves analogous to those obtained with human plasma. This is however not the case when the individual inhibitors are used. A theoretical treatment enabled us to devise an assay method to determine the amounts of functional activity of α_1-protease inhibitor and α_2-macroglobulin respectively in human plasma.

Introduction

Since the first observations of antiproteolytic activity in blood in 1897 (1) at least nine protease inhibitors have been identified. The two major inhibitors are α_1-antitrypsin (now usually called α_1-protease inhibitor) and α_2-macroglobulin, which represent about 70% of the plasma protease inhibitor activity by weight, and probably all the anti-elastase activity (2). Although both α_1-protease inhibitor and α_2-macroglobulin react with elastase, they do so in different ways. The former completely inhibits human neutrophil elastase using elastin as substrate, whereas α_2-macroglobulin only partly inhibits this system (3).

The relationship between α_1-protease inhibitor deficiency and early onset of emphysema in individuals with genetic deficiency, as well as in smokers is well documented (4, 5). The α_1-protease inhibitor inhibits both the proteolytic and the amidolytic activity (on synthetic substrates) of plasma proteases by binding to them and blocking their active sites (6). The complex formed is very stable and does not dissociate even in the presence of other binders such as α_2-macroglobulin (7).

The α_2-macroglobulin present in human plasma binds to all four classes of endopeptidases (8). On binding, the α_2-macroglobulin molecule undergoes a conformational change which entraps the enzyme. In this way inhibition does not involve interaction with the active site of the protease, and the bound enzyme still possesses activity toward low molecular weight substrates (9) but decreased activity toward native proteins (10). Consequently, when low molecular weight synthetic substrates are used for measuring the protease inhibitory capacity of plasma, the protease bound to α_1-protease inhibitor will be completely inhibited. On the other hand, reaction with α_2-macroglobulin may cause only slight inhibition of protease, or increased activity may even be found. When neutrophil elastase becomes complexed with α_2-macroglobulin, its activity increases 15 to 16 times compared with that of the free enzyme (9).

It has thus become clear that variable concentrations of α_2-macroglobulin in plasma may modulate significantly the inhibitory role of α_1-protease inhibitor in human plasma. In vivo, a balance is necessary in the synthesis and breakdown of elastin. It is possible that this homeostasis can be disturbed either by inactivation of α_1-protease inhibitor by such factors as smoking (11), or by changes in the activity of α_2-macroglobulin. In the latter case it is important to determine

the level of α_2-macroglobulin that can actively bind protease, rather than the immunologically determined level. Several colorimetric methods have been developed to measure α_2-macroglobulin in human blood, based on the ability of α_2-macroglobulin protease complex to hydrolyse a low molecular weight synthetic substrate in the presence of excess protease inhibitor.

Ganrot (12) used trypsin as the protease, benzoyl *D,L*-arginine-*p*-nitroanilide as the substrate and soybean trypsin inhibitor to block excess trypsin. More recently *Cullmann & Dick* (13) described a similar method using carbobenzoxy-Val-Gly-Arg-*p*-nitroanilide as substrate and aprotinin as the inhibitor; and *Rao* et al. (14) used a crude preparation of redwood seed extract *(Adenanthera pavonia)* as the source of inhibitor. None of these methods takes into account the endogenous serum α_1-protease inhibitor in the final calculation of the amount of α_2-macroglobulin.

In this report, we describe the competitive binding of α_1-protease inhibitor and α_2-macroglobulin to porcine pancreatic elastase. The results are used to generate a method for assaying the amounts of functional activity of the two binders in human plasma.

Materials and Methods

Human α_2-macroglobulin (90% pure), human α_1-protease inhibitor and succinyltrialanyl-*p*-nitroanilide were obtained from Hoechst. Porcine pancreatic elastase (EC 3.4.21.11) was obtained from Boehringer, Mannheim.

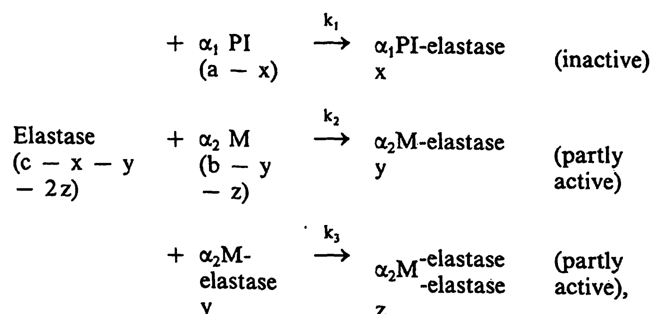
Based on the manufacturer's specifications of 70 U/mg and the rate constant of 73 s^{-1} quoted by *Beith* et al. (15), this elastase is 40% pure.

Fresh human blood (10 ml) was drawn from a non-smoking volunteer into a lithium heparin Venoject tube. The buffer used for all assays was 0.1 mol/l Tris-HCl pH 7.4. All chemicals used were of analytical grade (Merck).

Assumed molecular masses were:
Porcine pancreatic elastase: M_r = 25000;
α_1-protease inhibitor: M_r = 54000;
α_2-macroglobulin: M_r = 725000.

Theoretical model

Consider three simultaneous reactions (α_1PI = α_1-protease inhibitor, and α_2-M = α_2-macroglobulin):



where
a, b, and c are the initial numbers of molecules of α_1PI, α_2M, and elastase, respectively; and
x is the number of molecules of the complex formed with α_1PI, while
y and z represent the numbers of molecules of the binary and ternary complexes respectively between α_2M and elastase at any stage of the reaction.

When a + 2b > c

Here the competition between the two binders depends on the rate constants k_1 and k_2, according to the following equation.

$$\frac{k_1}{k_2} = \frac{\log (a - x)/a}{\log (b - y - z)/b} = \frac{\log (a - x)/a}{\log Xo},$$

where Xo is the mol fraction of α_2-macroglobulin unbound (16, 17). However, the bound fraction of the α_2-macroglobulin will be distributed between binary and ternary complex, and the amounts of each will depend on the amount of enzyme left at any particular time (16), taking into account the amount of α_1-protease inhibitor and the rate of its binding to enzyme. Hence there is no simple relationship. However, since k_2 is greater than k_1 (17, 18), the relative amount of enzyme bound to α_2-macroglobulin will increase as the total amount of added mixture of the 2 binders is increased. Hence we expect to find an increase in enzyme catalytic activity as the volume, V, increases beyond the equivalence point, V_e. This accounts for the increase in catalytic activity after reaching a minimum in figure 1. However, even if the volume added is increased to extremes, the maximum value attainable for the catalytic activity of the complexes is f_2D_o.

D_o = Absorbance produced by the elastase with no plasma added.

f_2 = factor by which the catalytic activity of elastase is changed when it becomes complexed with α_2-macroglobulin in the presence of excess α_1-protease inhibitor.

When a + 2b < c

Since we allow sufficient time for the reaction to proceed to completion, all a and b are bound to enzyme. Also, all the α_2-macroglobulin binds 2 elastase molecules per molecule α_2-macroglobulin (16).

Hence, unreacted enzyme = c − a − 2b
The observed catalytic activity for a volume, V, of mixture = D_v
$D_v = K(c - a - 2b(1 - f_1))t_1$ ... 1,

where f_1 is the factor by which complexing of the enzyme with α_2-macroglobulin decreases (or increases) its catalytic activity.

When plasma is titrated against porcine pancreatic elastase, the amounts of α_1-protease inhibitor and α_2-macroglobulin are in a constant ratio to each other in the plasma.

Thus,
where
a = AV and b = BV,
V = volume of plasma added
A = molar concentration of α_1-protease inhibitor in the plasma
B = molar concentration of α_2-macroglobulin in the plasma
t_1 and t_2 = incubation times
K = a constant such that $D_o = K c t_2$
D_o = absorbance produced by the elastase with no plasma added

therefore

$$D_v = K(c - AV - 2BV(1 - f_1))t_1$$

$$A = \frac{c}{V}\left(1 - \frac{D_v \cdot t_2}{D_o \cdot t_1}\right) - 2B(1 - f_1) \ldots 2$$

This is a linear relationship in V if f_1 is constant. However, it contains 3 unknowns. Hence at least two of these must be determined experimentally.

Methods

Assay of inhibitory capacity of plasma towards porcine pancreatic elastase (fig. 1)

To (3 − x) ml of 0.1 mol/l Tris-HCl buffer, 0.2 ml diluted elastase suspension (270 pmol) was added. Various amounts of plasma (x ml) were added and incubated for 10 min at 37 °C. Fifty µl of succinyl-trialanyl-*p*-nitroanilide (40 g/l) were then added and incubated for 20 min at 37 °C. The reaction was stopped with 0.5 ml 100 g/l citric acid. Absorbance was read at 410 nm.

Titration of α_1-protease inhibitor against elastase (fig. 1)

Various volumes of α_1-protease inhibitor (8.9 mol/l) in buffer were added to 0.2 ml of diluted elastase suspension (267 pmol per assay), and buffer added to a final volume of 3.2 ml. The residual elastase activity was measured as described above for plasma.

Titration of α_2-macroglobulin against elastase (fig. 1)

Various volumes of an α_2-macroglobulin solution (417 nmol/l) were reacted for 5 min at 37 °C with 1.6 nmol of porcine pancreatic elastase in Tris-buffer of pH 7.4. The unreacted elastase was then neutralized with a large excess α_1-protease inhibitor (10 nmol) by reaction for a further 5 minutes at 37 °C. The volume of reaction mixture at this stage was 3.2 ml. Then 50 µl of succinyl-trialanyl-*p*-nitroanilide (40 g/l) was added and incubated for 20 min at 37 °C. The reaction was stopped with 0.5 ml of citric acid solution (100 g/l). Absorbance was read at 410 nm.

Titration of mixtures of α_1-protease inhibitor and α_2-macroglobulin against elastase (fig. 2)

The concentrations of α_1-protease inhibitor and α_2-macroglobulin in the mixtures are shown in the legend to figure 2. The assay was performed as above, using 267 pmol of porcine pancreatic elastase.

Titration of elastase against a mixture of both inhibitors (excess α_1-protease inhibitor added before measuring elastase activity) (fig. 3)

A fixed amount of elastase (267 pmol per assay) was added to varying amounts of a mixture containing 7.5 µmol/l of α_2-macroglobulin and 2.34 µmol/l of α_1-protease inhibitor (0 to 100 µl) in a total volume of 3.2 ml Tris-buffer at pH 7.4. After 5 min reaction at 37 °C the unbound elastase was neutralized by adding 10 µl of α_1-protease inhibitor (57 µmol/l). Then 5 min later 50 µl of enzyme substrate (40 g/l) was added and allowed to react for 20 min at 37 °C. The enzyme reaction was stopped with 0.5 ml of citric acid (100 g/l) and absorbance read at 410 nm.

Determination of f_1

The value of f_1 can be determined by measuring the catalytic activity of a known amount of enzyme alone and after adding an excess of α_2-macroglobulin. The binary and ternary complexes have been shown to be equivalent, i. e. the two binding sites on α_2-macroglobulin are identical (19). Hence the question of which complex is formed when measuring f_1 is irrelevant.

Determination of f_2

Since α_2-macroglobulin bound to elastase cannot be detached by α_1-protease inhibitor, f_2 is determined by first saturating the elastase with α_2-macroglobulin and then adding an excess of α_1-protease inhibitor after binding to α_2-macroglobulin is complete.

Results and Discussion

As shown in table 1 the values obtained for f_1 and f_2 were:

$$f_1 = 0.74 \text{ and } f_2 = 0.70 \text{ at pH } 7.4$$

These values are strongly dependent on the pH. However, since the pH optimum for the complex between α_2-macroglobulin and porcine pancreatic elastase is 7.4, all the assays were performed at this pH. Great care must be taken to adjust the pH exactly in order to obtain the above values for f_1 and f_2.

Tab. 1. The effect of inhibitors on porcine pancreatic elastase activity in the assay (See Methods for determination of f_1 and f_2).

Additions to 53 pmol elastase	$A_{410\,nm}$	Free elastase catalytic activity [%]
None	0.485	100
54 pmol α_2-macroglobulin	0.359	74 = f_1
54 pmol α_2-macroglobulin + 503 pmol α_1-protease inhibitor	0.344	70 = f_2

When normal human plasma was titrated against fixed amounts of elastase, a complex curve was obtained (fig. 1), which has been ascribed by *Meyer* et al. (7) to the two main competing inhibitors in plasma, viz. α_1-protease inhibitor and α_2-macroglobulin.

The complex of α_2-macroglobulin with porcine pancreatic elastase at pH 7.4 is at least 70% as catalytically active as the unbound elastase (tab. 1). The individual effects of each of these inhibitors on elastase, are shown in figure 1. For α_1-protease inhibitor the residual catalytic activity of the elastase was measured after the binding reaction was complete. A linear regression with negative slope was obtained. This was similar to the first half of the curve for plasma and indicated that α_1-protease inhibitor is probably the main factor responsible for the inhibition observed in plasma.

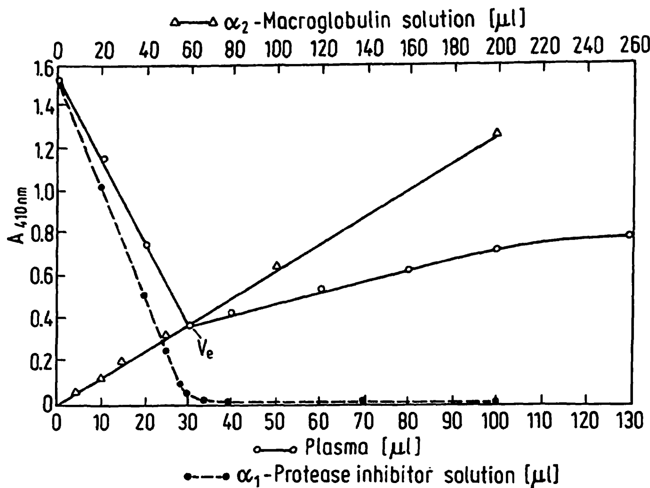


Fig. 1. Titration of enzyme catalytic activity using 267 pmol porcine pancreatic elastase against
a) (o—o) normal plasma and
b) (•—•) α_1-protease inhibitor (8.9 µmol/l).
Also titration of 1.6 nmol porcine pancreatic elastase against α_2-macroglobulin (417 nmol/l) after neutralization of excess elastase with α_1-protease inhibitor (Δ — Δ) is shown (See Methods).

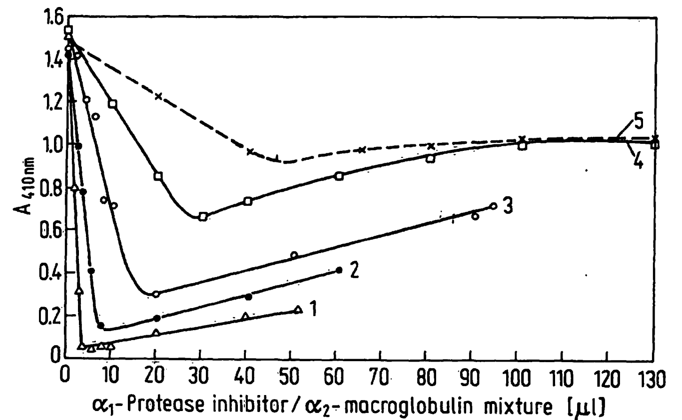


Fig. 2. Titration of different mixtures of α_1-protease inhibitor and α_2-macroglobulin against 267 pmol of elastase. The amounts and ratios of the inhibitors used were as follows:

Curve Number	α_1-protease inhibitor µmol/l	α_2-macroglobulin µmol/l	Molar ratio α_1-protease inhibitor / α_2-macroglobulin
1	71.03	1.41	50.6
2	35.70	1.41	25.3
3	14.30	1.41	10.1
4	4.66	2.31	2.02
5	2.34	2.31	1.01

For the titration of α_2-macroglobulin, we first allowed the binding of elastase to proceed to completion, then neutralized the excess elastase with α_1-protease inhibitor, before adding substrate (see methods section). The catalytic activity therefore represents that of the complex between α_2-macroglobulin and porcine pancreatic elastase in the presence of excess protease inhibitor. This catalytic activity is only 70% of the catalytic activity of the free enzyme (tab. 1). Here we obtained a straight line with positive slope (fig. 1). This confirms that α_2-macroglobulin is probably responsible for the second part of the curve for plasma (7), since this is the only known binder which protects porcine pancreatic elastase from inhibition by α_1-protease inhibitor.

To investigate the phenomenon further we titrated various mixtures of α_1-protease inhibitor and α_2-macroglobulin against fixed amounts of elastase. The results are shown in figure 2. Using a constant amount of elastase (267 pmol) with the various mixtures, the amount of residual catalytic activity was related to the ratio of α_1-protease inhibitor to α_2-macroglobulin, as well as to the absolute amounts of each. As the relative amount of α_1-protease inhibitor was decreased, the equivalence volume (Ve) increased from 4 µl to 44 µl, with a concurrent ten fold increase in the absorbance at Ve. The curve depicted in figure 1 for plasma is similar in shape to those of figure 2, where the second half of the curve is not exactly linear and becomes less linear as the amount of α_1-protease inhibitor is reduced. This is because the inhibition by α_1-protease inhibitor is lower and α_2-macroglobulin becomes more prominent. Hence the competition between the two is more obvious. This is added to the constraint that f_2 is smaller than f_1, and the value of the factor varies as the amount of α_1-protease inhibitor increases (see below). Furthermore, the maximum catalytic activity of the complex in the presence of excess α_1-protease inhibitor cannot exceed 0.7 times the catalytic activity of the uncomplexed enzyme.

Although pure samples of α_1-protease inhibitor and α_2-macroglobulin gave linear regressions when titrated separately, determination of α_2-macroglobulin in a mixture of α_1-protease inhibitor and α_2-macroglobulin produced a non-linear curve (fig. 3). Hence in devising a method for determining α_2-macroglobulin in a mixture containing both inhibitors, this non-linearity must be taken into account. In the suggested assay we use 10 µl of plasma. This corresponds to the initial practically linear portion of the curve.

As a result of the above experiments certain modifications were made of the methods described above, in order to determine α_2-macroglobulin and α_1-protease inhibitor functional activities in plasma.

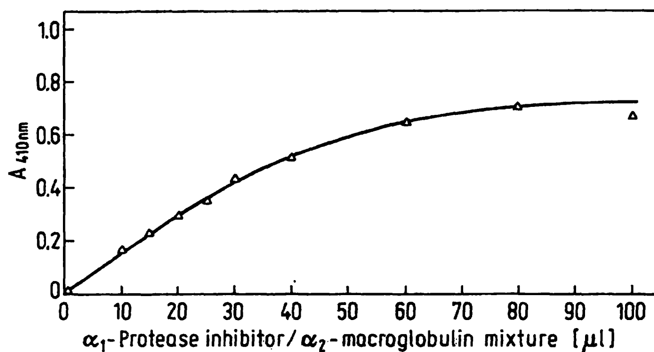


Fig. 3. Titration of a mixture of α_1-protease inhibitor (7.5 µmol/l) and α_2-macroglobulin (2.3 µmol/l) against 267 picomol of elastase. The catalytic activity was measured after neutralization of unbound elastase with 570 pmol of α_1-protease inhibitor (See Methods).

Since the initial region of the curve in figure 3 is practically linear we can make this approximation with only a small error. Over the linear region

$$D_v = 2K \cdot B \cdot V \cdot f_2 \cdot t_3,$$

where

D_v is the absorbance measured
V is the volume of plasma added
t_3 is the incubation time.

Hence, provided we use a sufficiently low volume of plasma we can expect good results for the determination of α_2-macroglobulin.

$D_o = K \cdot c \cdot t_2$ K is pH dependent.
D_o is absorbance with no plasma added.
$D_v = 2\,B \cdot V \cdot f_2\,t_3 \cdot D_o/c \cdot t_2$

$$B = \frac{D_V \cdot c \cdot t_2}{2 \cdot D_o \cdot V \cdot t_3 \cdot f_2}$$

Substituting known values in this equation

$$B = \frac{D_{10}}{D_o} \times 15.2 \text{ [µmol/l]}$$

$$\mathrel{\widehat{=}} 11.2 \times \frac{D_{10}}{D_o} \text{ [g/l]},$$

where D_{10} is value of D_v for V = 10 µl plasma.

Determination of D_o

Using 1.6 nmol (100 µg of 40% pure) of porcine pancreatic elastase per assay the absorbance developed in 3.2 ml of buffer at 37 °C with 50 µl of succinyl-trialanyl-*p*-nitroanilide (2000 µg per assay) is measured. The colour development is only linear for about 3 min under these conditions, hence t_2 is taken as 2 min. The reaction is stopped with 0.5 ml of citric acid solution (100 g/l). D_o is the absorbance at 410 nm developed in two minutes under these conditions. A blank is determined by adding the citric acid solution before the addition of substrate.

Determination of α_2-macroglobulin functional activity in plasma

In order to avoid the necessity of diluting the plasma we suggest an increased amount of porcine pancreatic elastase be used in contrast to that used in figure 1. For most normal plasma 1.6 nmol per assay is satisfactory, giving an equivalence volume of about 40 µl of plasma in 3.2 ml of Tris-HCl buffer (0.1 mol/l) pH 7.4 at 37 °C for 10 min. The excess elastase is then reacted with an excess of α_1-protease inhibitor (4 nmol) for a further 15 min at 37 °C. The residual catalytic activity, D_{10}, for the 10 µl serum is determined by using succinyl-trialanyl-*p*-nitroanilide (2000 µg per assay), and incubating for 15 min (t_3) at 37 °C.

Determination of functional activity of α_1-protease inhibitor in plasma

Except for patients in an acute phase, where the α_1-protease inhibitor could rise to very high levels, we have found that satisfactory results are obtained with 20 µl of plasma and 1.6 nmol of porcine pancreatic elastase (100 µg of the 40% pure product) in 2.3 ml of Tris-buffer (0.1 mol/l at pH 7.4). The two reactants are kept at 37 °C for 5 min. Then 50 µl of a solution of enzyme substrate (40 g/l) is added and colour generation allowed to proceed for a further 5 min. The enzyme reaction is stopped by adding 0.5 ml of citric acid solution (100 g/l). Absorbance is read at 410 nm. A zero time blank is determined by adding the citric acid before adding the substrate in a duplicate assay.

Substituting known values in the equation for A:

$$A = 80 - 32 \times \frac{D_{20}}{D_o} - 0.52\,B \text{ [mol/l]}$$

$$A = 80 - 32 \times \frac{D_{20}}{D_o} - 5.9 \times \frac{D_{10}}{D_o} \text{ [mol/l]}$$

Determination of equivalence point (V_e)

The volume of plasma used for the above assay must not exceed V_e. Hence it is useful to know this value.

At the equivalence point $c = a + 2b$
$= V_e (A + 2B)$

$$V_e = c/(A + 2B)$$

Hence we can calculate the *Total Elastase Inhibiting Capacity* (TEIC) which is defined as the number of ml of plasma required to bind 1 μmol of porcine pancreatic elastase.

$$\text{TEIC} = V_e \times 0.625 \text{ ml in our assay.}$$

As pointed out by *Meyer* et al. (7) the enzymatic determination of α_1-protease inhibitor is more specific and much more rapid than immunological procedures. The ability to determine the functional activities of both α_1-protease inhibitor and α_2-macroglobulin in the same specimen could be of importance in the study of degenerative lung diseases.

A study of patients suffering from emphysema has shown that functional activity levels of α_2-macroglobulin in these patients are significantly higher than those in healthy persons. This data will be published elsewhere.

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