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Effect of α_2M on earthworm fibrinolytic enzyme III-1 from *Lumbricus rubellus*

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Abstract

Though it is known that human α_2-macroglobulin (α_2M) inhibits most proteases, the effect of α_2M has not been investigated on earthworm fibrinolytic enzymes (EFEs) from *Lumbricus rubellus*, which could be transported from intestine epithelium into blood as an intact molecule (Fan et al., Biochim. Biophys. Acta 1526 (2001) 286). The activity of earthworm fibrinolytic III-1 (EFE-III-1) decreased to 65% when incubated with α_2M, while it decreased to 30% in plasma under the same conditions. The first order rate of the inactivation of EFE-III-1 with α_2M was similar to that of fast phase with plasma, indicating that α_2M may be the inhibitor initially binding to the enzyme in blood. SDS-PAGE showed that incubation of EFE-III-1 with α_2M a released fragment ($\sim$90 kDa), followed by formation of a high molecular weight complex ($\sim$700 kDa). There was a linear relationship between the apparent inhibition rate constant (k_1) and [α_2M], by double reciprocal plot. It was suggested, as described by Tsou (Acta Biochem. Biophys. Sinica 5 (1965) 398) and Tian (Biochem. J. 21 (1982) 1028), that the mechanism of α_2M/EFE-III-1 interaction could be coincided with a complexing irreversible inhibition. Experiments in both the inactivation and the intrinsic fluorescence showed that α_2M bound to the enzyme mole by mole equivalently. The intrinsic fluorescence of α_2M was enhanced with an observable blue shift in emission maxima, suggesting that α_2M was one of the important inhibitors to EFEs when it absorbed into blood.

Keywords: Earthworm fibrinolytic enzymes; α_2-Macroglobulin; Plasma; Inhibition

1. Introduction

It was reported that α_2-macroglobulin (α_2M) was present at high concentration (approximately 2.0 μM) in plasma and inhibited almost all four classes; cysteine-, serine-, aspartate- and metallo-proteases [3]. The tetrameric molecule of α_2M ($\sim$720 kDa) was formed by the noncovalent association of two disulfide-bonded dimers ($\sim$360 kDa) in which the monomers ($\sim$180 kDa) constituted in an antiparallel manner [4]. When the tetrameric α_2M reacted with a protease [5,6], which underwent a major conformational change so that the protease was irreversibly 'trapped' [7–11]. The protease-induced transition was characterized by cleavage of a reactive β-cysteinyl-γ-glutamyl thiol ester present on each of the four α_2M subunits, and by the appearance of receptor recognition sites that led to rapid clearance of α_2M-protease complexes from the circulation [12].

Earthworm has been used as a material for preparation of Chinese traditional drug to treat clotting diseases in eastern countries for over 2000 years. In *Compendium of Materia Medica* edited by a Chinese traditional medical doctor Shizhen Li (1518–1593), earthworm that was dried and made into powder, was described as a medicine 'Earth-dragon' to treat clotting diseases. In 1878, Frédéricq found some components that were secreted from the alimentary tract of earthworm, could dissolve fibrin [13]. Some years ago, Mihara et al. isolated a group of fibrinolytic enzymes from earthworm (*Lumbricus rubellus*) extract [14], and then obtained

Abbreviations: EFEs, earthworm fibrinolytic enzymes; α_2M, α_2-macroglobulin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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three partial fractions through purification. These three fractions were further subdivided into six homogeneous isozymes, which were regarded as novel fibrinolytic enzymes at that time [15,16]. Two of the six earthworm fibrinolytic enzymes (EFEs), earthworm fibrinolytic III-1 (EFE-III-1) and EFE-III-2, were alkaline trypsin-like serine proteases, and EFE-II was an elastase-like serine protease [17]. These three isomers showed strong fibrinolytic activities, broad optimal pH (pH 9–11) and high resistance to thermal or guanidine–HCl denaturation [18,19]. In 90th last century, the enzymes (EFE-III-1 as a main component) were successfully made into an oral fibrinolytic medicine both in China [20] and South Korea [21]. However, EFE-III-2 lost about 95% of its initial activity in 20 min when incubated with rat plasma at 37 °C [22]. It was demonstrated that EFE-III-1 could be absorbed into blood through intestinal epithelium as an intact form, remaining approximately 5% of the initial activity [23]. It indicated that some inhibitors in plasma possibly inactivated EFE-III-1 when it was absorbed. To understand interactions between EFE-III-1 and inhibitors, the kinetics of the inactivation, for example α_2M as an important inhibitor in blood, should be investigated. In this paper, conformational changes of α_2M were recorded by monitoring the intrinsic fluorescence when EFE-III-1 was inactivated, indicating the interaction between α_2M and EFE-III-1 underwent a complexing irreversible process.

2. Materials and methods

2.1. Materials

We isolated EFEs (six isomers) of *L. rubellus*, and then purified EFE-III-1 from the isomers as described before [19]. The EFE-III-1 showed a single band (~30 kDa) in SDS-PAGE with a specific activity of 48 U mg^{-1} (1 U was defined as the activity required to convert 1 μmol substrate per min per mg of enzyme) [5]. It was lyophilized and stored at 4 °C and the activity did not change over 6 months. The chromophoric substrate (Chromozym TH) for the assay was from Boehringer (Mannheim, Germany). The human α_2M, with a molecular mass of ~720 kDa came from Sigma (St. Louis, USA), indicating that this macroglobulin is an active form. Molecular weight markers were from Amersham Phamacia Biotech (UK). Fresh human plasma anticoagulated (3.8% sodium citrate) was obtained from healthy volunteers. The other reagents were all analytical grades without further purification. Absorbance and light scattering were measured on a spectrophotometer (PE-λ12) and a fluorescence spectrophotometer (Hitachi-F4500), respectively. Centricon-30 (Amicon Inc., USA) was manipulated for centrifugal ultrafiltration.

2.2. Association between α_2M and EFE-III-1

The enzyme (1.0 μM) was incubated with α_2M at the equimolar ratio. All incubations involving α_2M and the EFE were conducted at 37 °C. Aliquots were taken at desired time intervals and placed into the wells of 5% polyacrylamide gel electrophoresis (PAGE) without denaturants, for instance sodium dodecyl sulfate (SDS), and 12% SDS-PAGE. The gel was visualized with Coomassie brilliant blue.

2.3. Assay of EFE-III-1 with α_2M

The enzyme (1.0 μM) was incubated with α_2M at different concentrations (0.1, 0.2, 0.5, 1.0, 2.0 and 10 μM) in HEPES buffer (25 mM HEPES, pH 7.4, containing 150 mM NaCl) at 37 °C. Aliquots were taken and assayed at desired incubation times. Chromozym TH (Tos–Gly–Pro–Arg–4-NA) was used as a substrate [24], which was cleaved, releasing an oligopeptide and a chromophoric product (4-nitraniline, $\varepsilon_{405\ nm} = 9.75\ mM^{-1}\ cm^{-1}$).

2.4. Assay of EFE-III-1 in human plasma

The plasma used for assay was centrifuged (3000 rpm, RT, 10 min) and diluted to 50% (v/v) with HEPES buffer (25 mM HEPES, pH 7.4, containing 150 mM NaCl). EFE-III-1 (0.5 μM) was incubated with the plasma at 37 °C. The aliquots were taken at desired time intervals and assayed as described above. We studied the interaction of EFE-III-1 with plasma rather than with serum because the concentration of protease inhibitors, as described by Tian and Tsou is significantly lower in serum [2].

2.5. Inhibiting kinetics of EFE-III-1 with α_2M

α_2M and EFE-III-1 of different molar ratios (α_2M/EFE-III-1: 0.1, 0.2, 0.5, 1.0 and 2.0) were incubated in HEPES buffer. We recorded the time course of the inactivation at each molar ratio. The apparent rate constants at different concentrations of α_2M were determined from the slopes in the plot of $\log([RA_t]-[RA_\infty])/(1-[RA_\infty])$ against time (t) according to Eq. (1) [25], as follows:

$$\log([RA_t]-[RA_\infty])/(1-[RA_\infty]) = -0.43k_1t, \quad (1)$$

where $[RA_\infty]$ was the remaining activity of EFE-III-1 at the time approaching infinity, $[RA_t]$ was the activity at any time (t), and k_1 was the apparent rate constant detected at different concentrations of α_2M [I]. The second order rate constant (k_2), as described by Kitz and

Wilson 1962, could be calculated by the double reciprocal plot of $1/k_1$ via $1/[I]$, as shown with Eq. (2) [26]:

$$k_1 = k_2[I]/(k_s + [I]). \quad (2)$$

2.6. Measurements of the intrinsic fluorescence

Previously, to study the kinetics of the reaction of nerve growth factors with α_2M, the intrinsic fluorescence of the α_2M/enzyme-incubated mixture was conveniently monitored with an excitation wavelength at 280 nm, as described by Wolf et al. [27]. In fact, changes in the intrinsic fluorescence were related to conformational changes of protein. Thus, we recorded the time courses of the fluorescence changes when α_2M (0.1 μM) interacted with EFE-III-1 at different concentrations (0.05, 0.1, 0.2, 0.4 and 1.0 μM) in HEPES buffer (pH 7.4). The molar ratio of a formed complex (EFE-III-1/α_2M) was determined by this measurement. When the equimolar concentration of EFE-III-1 and α_2M was reacted, the apparent second order rate constant (k_2') could be approximated by Eq. (3) [26]:

$$1/(1 - \Delta F_t/\Delta F_{max}) = E_0 k_2' t + 1, \quad (3)$$

where ΔF_t was the fluorescence change at any time (t), ΔF_{max} was the increase in the intrinsic fluorescence monitored when the reaction was complete, and E_0 was the initial concentration of the enzyme.

3. Results

3.1. Association of α_2M with EFE-III-1

EFE-III-1, as described previously, could be transported through the intestine epithelium into blood as an intact form [23], however, it was inactivated significantly when the enzyme was absorbed in serum. Since there was a high concentration of α_2M in blood, which inhibited almost all proteases of four classes, we have studied the effect of α_2M on the activity of EFE-III-1. According to Barrett and Starkey [9], a protease first cleaves the peptide bond in the bait region of α_2M, giving rise to a ~90 kDa subunit (or called fragment). Similarly, as shown in Fig. 1A, α_2M (tetramer ~720 kDa) shifted faster than α_2M alone as control, becoming clearer after 30 min, when the globulin was incubated with EFE-III-1. It suggested this macroglobulin was partially digested. As we know, digested fragments (< 200 kDa) would run off the gel (5%). Consequently, electrophoresis in 12% gel resulted in a band (~90 kDa), whose density increased with time (Fig. 1B).

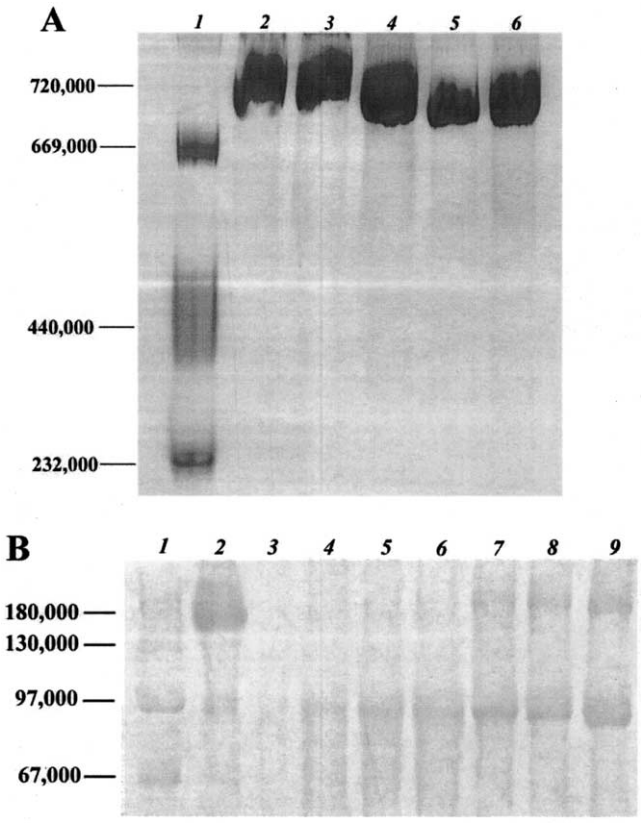

Fig. 1. Digestion of α_2M and formation of complexes with EFE-III-1. EFE-III-1 (1.0 μM) was incubated with α_2M (1.0 μM) in 25 mM HEPES (pH 7.4) at 37 °C. (A) The aliquots were taken and loaded to 5% nondenaturing PAGE. Lane 1, high molecular weight markers; lane 2, α_2M alone as control; line 3 through 6 show the samples incubated with EFE-III-1 for 15, 30, 60 and 120 min, respectively. (B) Conditions were referred to a, except 12% SDS-PAGE was used. Lanes 1, molecular weight markers; lane 2, α_2M alone as control; lanes 3 through 9 represent samples incubated for 5, 10, 20, 30, 60, 90 and 120 min, respectively.

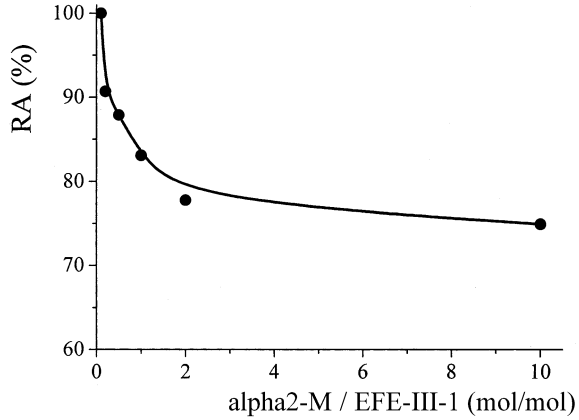

Fig. 2. Inactivation of EFE-III-1 incubated with α_2M at different molar ratios. The EFE (1.0 μM) was assayed in the presence of α_2M at different molar ratios (α_2M/EFE-III-1, 0.1, 0.2, 0.5, 1.0, 2.0 and 10) in 25 mM HEPES buffer containing 150 mM NaCl (pH 7.4) at 37 °C. RA represents relative remaining activity.

3.2. Inactivation of EFE-III-1 by α_2M

The EFE lost about 35% activity (compared with the native one) at an equivalent molar ratio of α_2M/EFE-III-1. No further inactivation could be detected at any higher ratios (Fig. 2). It implied that the complex contained 1 mol of α_2M tetramer and 1 mol of EFE-III-1.

On the basis of previous reports, α_2M bound to some proteins in an irreversible way, such as hemorrhagic metalloproteinases [6] and growth factors [26,28]. In general, for an irreversible inhibition, inactivation depended upon time, whereas a reversible inhibition usually underwent only an initial decrease in activity [29]. Thus, the inactivation kinetics of EFE-III-1 in the presence of α_2M indicated an irreversible inhibition mechanism, because the inhibiting process, as shown in Fig. 3, depended on time in plotting the relative activity against time. The inactivation underwent a monophasic procedure, whose rate constant was $\sim 0.26\ s^{-1}$, implying that α_2M was at least one important inhibitor contributing to the inactivation of EFE-III-1 in plasma.

When EFE-III-1 was incubated in human plasma at 37 °C (Fig. 4), it lost about 70% of the activity. The inactivation kinetics in plasma, however different from the inhibition by α_2M alone, was a biphasic procedure: a fast and a slow phase, whose rate constant (fast phase) was $\sim 0.27\ s^{-1}$. It has been known that plasma contains antiprotease components, not only α_2M [30,31], but also anti-thrombin-III [32], α_2-antiplasmin [1] and other inhibitors. They might act as inhibitors onto the EFE in blood. Anyway, the apparent inhibition rate constant of EFE-III-1 by equimolar α_2M was similar to that (fast phase) by plasma, suggesting that α_2M as an inhibitor bound to the protease initially, except for the other inhibitors.

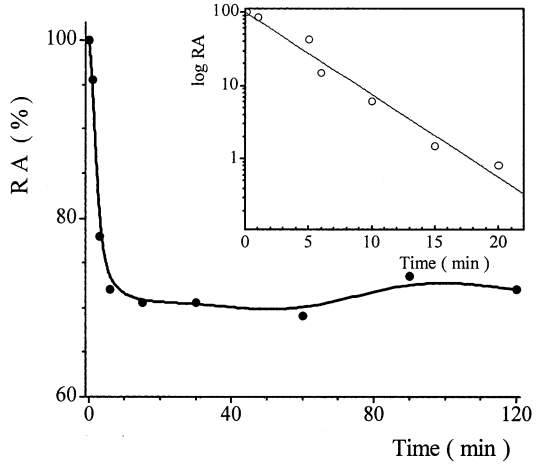

Fig. 3. Kinetics in inactivation of EFE-III-1 incubated with α_2M. Conditions were referred to Fig. 2, except time course was monitored. The enzyme was equimolarly added to α_2M, followed by the measurement of activities at different time intervals (0, 5, 10, 20, 40, 60, 90 and 120 min). The inset was the same data plotted in semilogarithm. RA represents relative remaining activity.

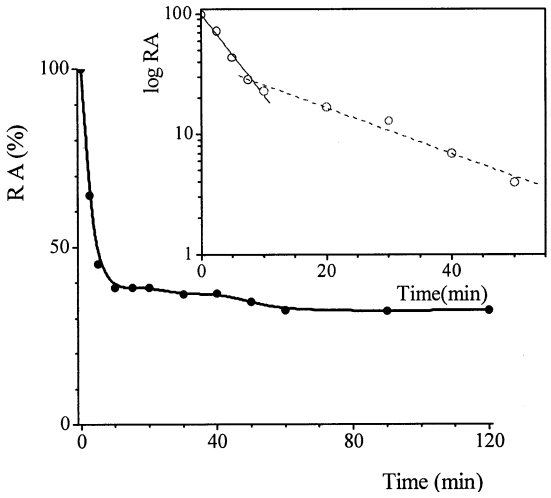

Fig. 4. Inactivation of EFE-III-1 incubated with plasma. Conditions were as for Fig. 3, except EFE-III-1 was incubated with plasma (50% dilution, v/v). The inset was the same data plotted in semilogarithm: the fast-phase (solid line), slow-phase (dash line) and remaining activity (RA). The rate constant was determined from the slope of the inset.

As described by Tsou [1] and Tian [2,33], the apparent inhibition rate constants (k_1) under different concentrations of inhibitor could be approximated by using Eq. (1). At different molar ratios (α_2M/EFE-III-1), k_1 was calculated with the slopes in plot of $\log([RA_t]-[RA_\infty])/(1-[RA_\infty])$ against time (Table 1). A complexing irreversible inhibition could be distinguished from a noncomplexing one by a double reciprocal plot of $1/k_1$ against $1/[I]$ according to Tsou [1]. For a simple irreversible inhibition, k_1 was independent of $[I]$, whereas k_1 of the complexing irreversible one was a function of $[I]$. In the present study (Fig. 5), a straight line suggesting that inhibition of EFE-III-1 by α_2M could be identified to a complexing irreversible type.

Table 1
Apparent inhibition rate constants of EFE-III-1 inhibited by α_2M at different molar ratios

Molar ratios (α_2M/EFE-III-1)	Apparent inhibition rate constant k_1 (s^{-1})
2.0	0.59
1.0	0.26
0.5	0.19
0.2	0.054
0.1	0.022

EFE-III-1 (1.0 μM) and α_2M at different molar ratios (α_2M/EFE-III-1: 0.1, 0.2, 0.5, 1.0 and 2.0) were incubated in HEPES buffer. Apparent inhibition rate constants (k_1) were determined from the slopes in the graph of $\log([RA_t]-[RA_\infty])/(1-[RA_\infty])$ against time.

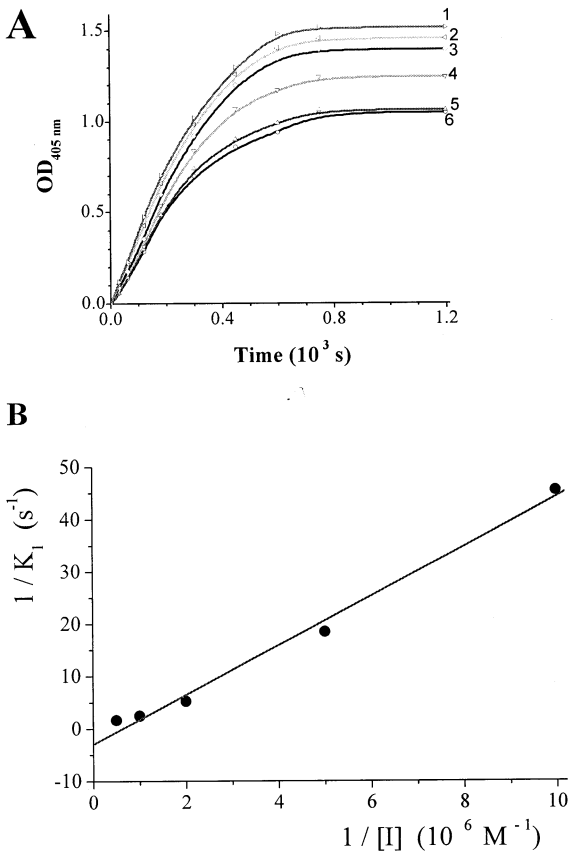

Fig. 5. Assay of EFE-III-1 in the presence of α_2M at different concentrations. Conditions were the same as Fig. 2. (A) A plot of RA against time. (B) Double reciprocal plot with apparent rate constants (k_1) and concentrations of α_2M ([I]). RA represents the relative remaining activity at any time (t).

3.3. Changes in the intrinsic fluorescence when α_2M binding to EFE-III-1

The electrophoresis, mentioned above, has already revealed that there formed an α_2M/EFE-III-1 complex. We should confirm it by detecting changes in the intrinsic fluorescence of the two proteins. When interacting with a protease, α_2M underwent a major conformational change [3–6], which could be measured by the intrinsic fluorescence around 335 nm. As shown in Fig. 6, the intensity of the intrinsic fluorescence of α_2M (0.1 μM) increased when incubated with the EFE. That EFE-III-1 was bound by α_2M (tetramer) at the equimolar ratio caused a 20% enhancement in the emission intensity. No further increase in the intensity could be detected at any higher molar ratios (EFE-III-1/α_2M). This suggested that 1 mol of α_2M and 1 mol of EFE-III-1 constituted a binary complex. The enhancement was accompanied by a 3-nm blue shift in emission maximum, which was also a characteristic for α_2M to associate with a protease and form a complex, as described before [26].

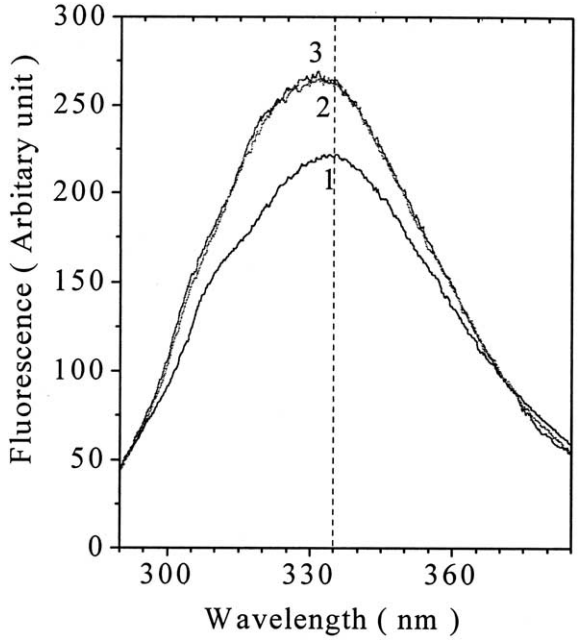

Fig. 6. The intrinsic fluorescence of α_2M/EFE-III-1 complexes. EFE-III-1 was incubated with α_2M (0.1 μM) in HEPES buffer at 37 °C for 10 min, followed by measurement of the intrinsic fluorescence at 335 nm (Ex 280 nm). Spectra shown were native α_2M (0.1 μM, curve 1), which associated with EFE-III-1 (0.1 μM, curve 2) and (0.2 μM, curve

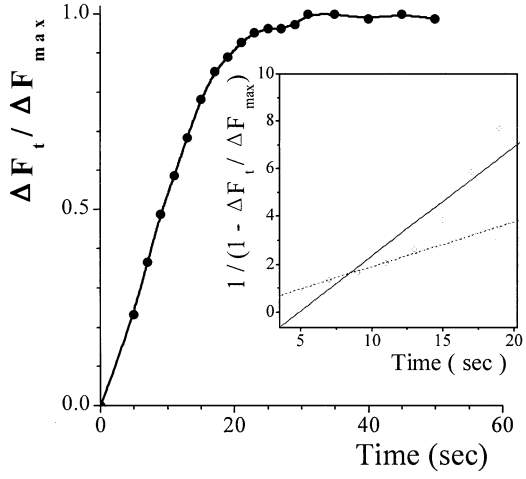

Fig. 7. Kinetics in the intrinsic fluorescence of α_2M as incubated with EFE-III-1. The intrinsic fluorescence was measured when α_2M (0.1 μM) was equimolarly added to EFE-III-1 at 37 °C. ΔF_t is the fluorescence change at any time (t) and ΔF_{max} is the fluorescence change when the reaction reached the completion. Inset was the same data plotted in $1/(1-\Delta F_t/\Delta F_{max})$ against time.

In order to study the rate constant of α_2M conformational changes when it associated with EFE-III-1, the time course of the increase in the intrinsic fluorescence was recorded (Fig. 7). That the intrinsic fluorescence increased and reached the maximum in 1 min showed a rapid conformation change when the inhibitor bound to the enzyme. The graph of $1/(1-\Delta F_t/\Delta F_{max})$ against time

Table 2
The second order rate constants (k_2) of EFE-III-1 inhibited by α_2M

Assay by Chromozym TH k_2 ($\times 10^5$ M^{-1} s^{-1})	Intrinsic fluorescence measurement k_2' ($\times 10^5$ M^{-1} s^{-1})
4.7	4.3

The second order rate constant (k_2) of assay under the same conditions as Table 1 was obtained from the graph (Fig. 5) of $1/k_1$ against $1/[I]$. The intrinsic fluorescence was measured when α_2M (0.1 μM) was equimolarly added to EFE-III-1, at 37 °C. The second order rate constant (k_2') was obtained from the slope (Fig. 7, inset) of $1/(1-\Delta F_t/\Delta F_{max})$ against time.

(t) was nonlinear, indicating that the interaction between the inhibitor and the enzyme was a second order process. Forced linear regression during the first 10 s yielded k_2' at 2.0×10^5 M^{-1} s^{-1}. The analysis of the increase in the fluorescence through 20 s yielded k_2' at 4.3×10^5 M^{-1} s^{-1}. These values were in good agreement with the constant ($k_2 = 4.7 \times 10^5$ M^{-1} s^{-1}) determined by Chromozym TH hydrolysis (Table 2). The similarity between the second order rate constants of the inactivation of EFE-III-1 and those of the conformational changes of α_2M supported the 'trap model' [9–11]. It suggested that a series of events occurred in a rapid succession: a bait-region cleavage of α_2M followed by a conformational change, and the inhibition of EFE-III-1 as a result.

4. Discussion

The association rate of a protease with α_2M to form a complex is related to the nature and the size of the substrate [34,35]. The active site of the trapped protease is generally inhibited with respect to large substrates. The trapped protease, however, is still accessible to either small substrate or small inhibitor [36]. Except for α_2M, several inhibitors such as soybean trypsin inhibitor (SBTI), aprotinin and diisopropyl fluorophosphates (DFP) [16] have been used to study the inhibiting mechanism of EFEs. Furthermore, α_2M, which is at a high concentration (2–4 μM) in blood, is widely employed as a strong inhibitor for almost all kinds of proteases [1,26]. In our previous experiments, EFE-III-1 is demonstrated to transport from intestine epithelium to blood [23], and its activity decreases to 5%, suggesting a strong inhibition occurred after the absorption. Thus, it is necessary to investigate how α_2M acts as an important inhibitor to the EFE in plasma.

Feldman et al. [8], Barrett and Starkey [9] applied the 'trap model' to explain the mechanism of the protease inhibition by α_2M. It was found that the protease first cleaved the peptide bond in 'bait region' of α_2M, resulting a 90 kDa fragment which is not released in physiological conditions: the cleaveage leads to the

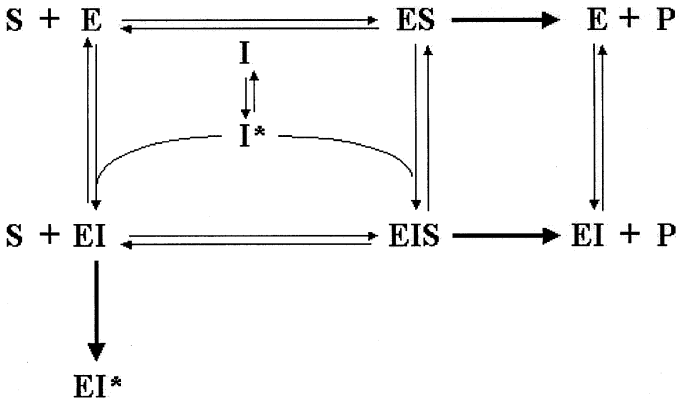

Fig. 8. The mechanism of the interaction between EFE-III-1 and α_2M. (E)nzyme: EFE-III-1; (I)nhibitor: α_2M; (I*)nhibitor: α_2M subunit; (S)ubstrate: Chromozym TH; (P)roduct: 4-nitroaniline.

opening of the reactive thioester that covalently bounds the proteases. In this degradation procedure, α_2M tetramer undergoes a conformational change, which can be conveniently detected by monitoring the increase in the intrinsic fluorescence. As mentioned above, an increase in the intrinsic fluorescence accompanied with a blue shift around 335 nm, when α_2M is incubated with EFE-III-1, is a characteristic involved in the α_2M/protease interaction [27]. Here, we suppose, the 'trap model' can also explain the mechanism of α_2M to inhibit EFE-III-1. The enzyme first cleaves α_2M in 'bait region', and then initiates a series of events in which the macroglobulin rearranges and traps the protease.

Furthermore, our experiments show that EFE-III-1 can be markedly inhibited by α_2M through an irreversible complexing procedure. This view is based on the observations, as mentioned above: (i) formation of a band (enzyme/inhibitor complex) with a molecular mass less than 720 kDa in the gel without SDS; (ii) a distinguish inactivation in the presence of α_2M; (iii) the apparent rate constant depending on α_2M's concentration and finally; (iv) the second order rate constant of inactivation is close to that of changes in the intrinsic fluorescence of the complex. It is suggested that α_2M inhibits EFE-III-1 by the following mechanism (Fig. 8).

The fragment ($\sim$90 kDa) from α_2M might have undergone a major conformational change before the appearance of EFE-III-1/α_2M complex ($\sim$700 kDa). Then the inhibitor associates with the enzyme to form a reversible complex (EI), before the irreversible modification that rearranged the complex into an irreversible form (EI*). Although EFE-III-1 contains eight Cys residues [16], the previous study with DTNB shows that all of these residues are involved in formation of disulfide bonds in the native enzyme [19]. Thus, when the cleaved α_2M traps EFE-III-1, there could not be a chance to form disulfide bonds between the globulin subunits and the protease.

5. Conclusion

Investigation of the effect of α_2M on the EFE, one of the six isozymes (EFE-III-1), shows that the activity of EFE-III-1 decreased to 65% when α_2M was added. The first order rate of the inactivation of EFE-III-1 with α_2M was similar to that of fast phase with plasma, indicating that α_2M may be the inhibitor initially binding to the enzyme in blood. The polyacrylamide gel electrophoresis without any denaturants reveals the formation of a complex of EFE-III-1 and α_2M ($\sim$700 kDa). Simultaneously, SDS-PAGE shows that it produces a fragment ($\sim$90 kDa) at the 'bait site', during the 'trap' procedure. Experiments in both the inactivation and the intrinsic fluorescence showed that α_2M bound to the enzyme mole by mole equivalently. The relationship between the apparent inhibition rate constant (k_1) and [α_2M] is a linear by double reciprocal plot. It appears that the mechanism of α_2M/EFE-III-1 interaction is coincided with a complexing irreversible inhibition.

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References

[1] Tsou CL. Acta Biochem Biophys Sinica 1965;5:398.
[2] Tian WX, Tsou CL. Biochem J 1982;21:1028.
[3] Jensen PE, Sottrup-Jensen L. J Biol Chem 1986;261:15863.
[4] Harpel PC. J Exp Med 1973;138:508.
[5] Christensen U, Sottrup-Jensen L. Biochemistry 1984;23:6619.
[6] Baramova EN, Shannon JD, Bjarnason JB, Gonias SL, Fox JW. Biochemisty 1990;29:1069.
[7] Waller EK, Schleuning WD, Reich E. Biochem J 1983;215:123.
[8] Feldman SR, Gonias SL, Pizzo SV. Proc Natl Acad Sci USA 1985;82:5700.
[9] Barret AJ, Starkey PM. Biochem J 1973;133:709.
[10] Salvesen GS, Barrett AJ. Biochem J 1980;187:695.
[11] Werb Z, Burleigh MC, Barrett AJ, Starkey PM. Biochem J 1974;139:359.
[12] Olhsson K. Acta Physiol Scand 1971;81:269.
[13] Frédéricq L. Arch Zool Expér 1878;7:391.
[14] Mihara H, Sumi H, Akazawa K, Yoneta T, Mizumoto H. Thromb Haemost 1983;50:258.
[15] Mihara H, Sumi H, Mizumoto H, Yoneta T, Ikeda R, Maruyama M. Oral administration of earthworm powder as a possible thrombolytic therapy. In: Tanaka K, editor. Recent advances in thrombosis and fibrinolysis. New York, USA: Academic Press, 1990:287.
[16] Mihara H, Sumi H, Yoneta T, Mizumoto H, Ikeda R, Seiki M, Maruyama M. Jpn J Physiol 1991;41:461.
[17] Sumi N, Nakajima N, Michara HA. Comp Biochem Physiol 1993;106:763.
[18] Nakajima N, Mihara H, Sumi H. Biosci Biotech Biochem 1993;57:1726.
[19] Zhou J, Wu C, He RQ. J Biochem Mol Bio Biophys 1998;5:1.
[20] Hou QM. Prog Biochem Biophys 1994;21:85.
[21] Park Y, Kim J, Min B, Seo J, Jeong J. Biotechnol Lett 1998;20:169.
[22] Nakajima N, Ishihara K, Sugimoto M, Sumi H, Mikuni K, Hamada H. Biosci Biotech Biochem 1996;60:293.
[23] Fan Q, Wu C, Li L, Fan R, Meng JM, Wu C, He RQ. Biochim Biophys Acta 2001;1526:286.
[24] Zhou J, Fan Q, Wu C, He RQ. Protein Peptide Lett 1997;4:409.
[25] Arnesen H, Fagerhol MK. Scand J Clin Lab Invest 1972;29:259.
[26] Chu CT, Rubenstein DS, Enghlid JJ, Pizzo SV. Biochemistry 1991;30:1551.
[27] Wolf BB, Vasudevan J, Gonias SL. Biochemistry 1993;32:1875.
[28] Engel PC. Enzyme kinetics, 2nd ed.. USA: Chapman & Hall, in Association with Methuen, Inc, 1981:26.
[29] Ogston D, Bennett B, Herbert RJ, Douglas AS. Clin Sci 1973;44:73.
[30] Sottrup-Jensen L, Petersen TE, Magnusson S. FEBS Lett 1981;128:127.
[31] Christensen U, Clemmensen I. Biochem J 1978;175:635.
[32] Moroi M, Aoki KN. J Biol Chem 1976;251:5956.
[33] Tian WX. J Graduate School USTC 1990;7:94.
[34] Barrett AJ. Methods Enzymol 1981;80:737.
[35] Sottrup-Jensen L. In: Putnam FW, editor. The plasma proteins, vol. 5. Orlando, USA: Academia press, 1987:192.
[36] Zhou J, Jiang K, He RQ, Han YM. Acta Biophys Sinica 1997;13:531.