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A KINETIC STUDY OF THE INHIBITION OF HUMAN AND BOVINE TRYPSINS AND CHYMOTRYPSINS BY THE INTER-α-INHIBITOR FROM HUMAN PLASMA

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Summary

Human plasma inter-α-inhibitor forms 1 : 1 inactive complexes with human and bovine trypsins (EC 3.4.21.4) and chymotrypsins (EC 3.4.21.1). The association and dissociation rate constants as well as the equilibrium dissociation constants (K_i) of the complexes formed of inter-α-inhibitor and the four proteases have been measured. The most stable complexes are those formed with the bovine enzymes. For instance, $K_i = 2.1 \cdot 10^{-11}$ M for bovine trypsin whereas $K_i = 1.2 \cdot 10^{-8}$ M for human trypsin. Whatever the species, the complexes formed with the chymotrypsins are less stable than those formed with the trypsins.

Introduction

In 1961, Steinbuch and Loeb [1] reported on the isolation of a new plasma protein which they called protein π. A few years later, Heide et al. [2] discovered a new trypsin inhibitor in plasma, the inter-α-inhibitor. These two proteins are identical [3].

The inter-α-inhibitor is responsible for about 5% of the trypsin-inhibiting capacity of plasma, the major inhibitor being α1-antitrypsin [4]. It inhibits bovine trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) [2,4]. However, no data are available in the literature on the kinetic parameters relevant to the inhibition of these two enzymes. These parameters will be reported in the present article.

On the other hand, the physiological functions of the inhibitor are largely unknown. It might protect the plasma proteins against the hydrolytic action of pancreatic proteases which are massively liberated into the blood during acute pancreatitis. To check this hypothesis, we have also performed a detailed kinetic

study of the action of inter-α-inhibitor on human cationic trypsin [5] and chymotrypsin II [6], the major forms of human pancreatic trypsins and chymotrypsins.

Materials and Methods

Bovine trypsin (lot TRL-OGC) and α-chymotrypsin (lot CDS-1CB) came from Worthington Biochemicals. Human cationic trypsin [5] and chymotrypsin II [6] were the generous gifts of Dr. Travis (Athens, Ga. U.S.A.). Active-site titrations of the four proteases were performed with *p*-nitrophenyl-*p'*-guanidinobenzoate HCl [7] (Cyclo Chemicals) and *N*-trans-cinnamoylimidazole [8] (Sigma). These titrations were used to calculate the molarities of active enzyme stated in the text.

Human α_2-macroglobulin was isolated from plasma Fraction III (Centre National de Transfusion Sanguine, Paris) by the method of Steinbuch and Blatrix [9]. The molarities of solutions of this protein were calculated from absorbance readings at 280 nm using $E^{1\%}_{1cm} = 8.1$ [10] and a molecular weight of 725 000 [11].

Inter-α-inhibitor (protein π) which will be referred to as "the inhibitor" in the following text, was the generous gift of Dr M. Steinbuch (Centre National de Transfusion Sanguine, Paris). The molarities of solutions of this protein were calculated from absorbance readings at 280 nm using $E^{1\%}_{1cm} = 7.1$ [2] and a molecular weight of 160 000 [4].

Trypsin activity was measured with benzoyl-DL-arginine-*p*-nitroanilide [12] (Boehringer, Germany), benzoyl-L-arginine-*p*-nitroanilide [13] (Protein Research Foundation, Osaka, Japan) or tosyl-L-arginine-methylester [14] (Sigma). Chymotrypsin activity was measured with succinyl-L-phenylalanine-*p*-nitroanilide [15] (Mann) or acetyl-L-tyrosine-ethylester [16] (Sigma). The substrate hydrolysis was recorded either with a Zeiss PMQ II spectrophotometer (at 410 nm for the chromogenic substrates or at 247 nm for tosyl-L-arginine-methylester) or with a Radiometer pH-stat device for acetyl-L-tyrosine-ethylester. All reactions were carried out at 25° C.

Stoichiometries of the associations

Bovine trypsin (64.3 nM) and inhibitor (varying concentrations) were incubated for 2 min at 25° C in 2.8 ml of 50 mM Tris buffer, pH 8.0, containing 54 mM $CaCl_2$. The enzyme activities were then measured after addition of 0.2 ml of 15.6 mM tosyl-L-arginine-methylester dissolved in water.

Human trypsin (30.5 nM) and inhibitor (varying concentrations) were incubated for 20 min at 25° C in 2.95 ml of the above Tris buffer. The enzyme activities were then measured after addition of 50 µl of 60 mM benzoyl-L-arginine-*p*-nitroanilide dissolved in dimethylformamide.

Bovine chymotrypsin (2.3 µM) and inhibitor (varying concentrations) were incubated for 2 min at 25° C in 2.85 ml of 0.3 M Tris buffer, pH 8. The enzyme activities were then measured after addition of 150 µl of 10 mM succinyl-L-phenylalanine-*p*-nitroanilide dissolved in dimethylformamide. Identical conditions were used for human chymotrypsin except that the enzyme concentration was 1.41 µM and the incubation time 10 min.

Kinetics of the association of bovine trypsin and chymotrypsin with the inhibitor

Bovine trypsin (10.7 nM) and inhibitor (10.7 nM) were incubated at 25°C for varying periods of time in 2.8 ml of 50 mM Tris buffer, pH 8.0, containing 54 mM $CaCl_2$; the enzyme activities were measured with tosyl-L-arginine-methylester as described above.

Bovine chymotrypsin (25 nM) and inhibitor (25 nM) were incubated at 25°C for varying periods of time in 2.925 ml of 1 mM Tris, pH 8.0, containing 100 mM $CaCl_2$ and 25 μg/ml of albumin (which slows down the rate of chymotrypsin degradation). The enzyme activities were measured by adding 75 μl of 300 mM acetyl-L-tyrosine-ethylester dissolved in dimethylsulfoxyde.

Kinetics of the dissociation of the inhibitor/bovine-trypsin and the inhibitor/human-trypsin complexes

Bovine trypsin (32 nM) was first reacted for 10 min at 25°C with an equimolar amount of inhibitor in the Tris buffer. The dissociation was started by addition of a volume of α_2-macroglobulin solution representing 5% of the volume of the above mixture (the final concentration of α_2-macroglobulin was 0.16 μM, 6.4 μM and 32 μM in three separate experiments). This medium was incubated at 25°C and from time to time, a 2.95-ml sample was withdrawn and assayed for trypsin activity, by adding 50 μl of 60 mM benzoyl-L-arginine-*p*-nitroanilide. A control medium containing all the above reagents except the inhibitor, was prepared and assayed in a similar fashion.

Human trypsin (3 μM) was reacted for 2 min at 25°C with an equimolar amount of inhibitor in the Tris buffer. 30 μl of this mixture were then poured into a spectrophotometer cuvette containing 2.97 ml of a medium composed of 50 mM Tris buffer, pH 8.0, 54 mM $CaCl_2$, 3 μM α_2-macroglobulin and 1 mM benzoyl-L-arginine-*p*-nitroanilide and the absorbance was recorded.

Competition between bovine trypsin and bovine chymotrypsin for the binding of the inhibitor

The inhibitor (60 nM) was first reacted at pH 8.0 and 25°C with an excess of bovine chymotrypsin. After 15 min of incubation, when the association was complete, bovine trypsin was added to the mixture to a final concentration of 60 nM (the three partners were dissolved in 50 mM Tris buffer containing 54 mM $CaCl_2$ and 35 μg/ml of bovine serum albumin, which slows down the rate of protease autolysis). The displacement of chymotrypsin by trypsin was monitored by measuring from time to time the remaining trypsin activity in a 2.9-ml sample of the medium, to which 100 μl of 40 mM benzoyl-DL-arginine-*p*-nitroanilide had been added. The new equilibrium was attained after 4—5 h. A medium containing only trypsin and the above buffer was used to measure the total trypsin activity. This activity was stable for the duration of the competition experiments. The procedure was repeated with four different concentrations of bovine chymotrypsin.

Results

1. Stoichiometries of the associations

The stoichiometries of the associations of the proteases with the inhibitor

were determined by measuring the residual activities of mixtures formed of increasing amounts of inhibitor and constant amounts of enzyme. The incubation times indicated in the experimental section have been determined in separate experiments and were found to be necessary for the complete association of enzyme and inhibitor.

For bovine trypsin and bovine chymotrypsin, straight inhibition curves were obtained up to 100% inhibition. The stoichiometries could therefore be determined directly, using these inhibition curves, and were found to be 1 : 1 within the limits of experimental errors.

For human trypsin and human chymotrypsin (Fig. 1), the inhibition curves were concave. The stoichiometries were therefore inferred from Henderson plots [17] in accordance with equation 1 as shown for instance in the insert of Fig. 1:

$$\frac{[I]}{1 - v_i/v_0} = \frac{K_{i(app)}}{n} \cdot \frac{v_0}{v_i} + \frac{[I]}{n} \qquad (1)$$

where [E] and [I] are the total enzyme and inhibitor concentrations, v_0 and v_i are the reaction rates in the absence and presence of inhibitor and n is the number of binding sites on the inhibitor molecule. $K_{i(app)}$ will be defined in the following section. For both human proteases, the stoichiometries were again found to be 1 : 1 (i.e. $n = 1$, see insert in Fig. 1).

2. Binding parameters

The association and dissociation rate constants k_1 and k_{-1}, and the equilibrium dissociation constant K_i corresponding to the following scheme were determined and are reported in Table I:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI$$

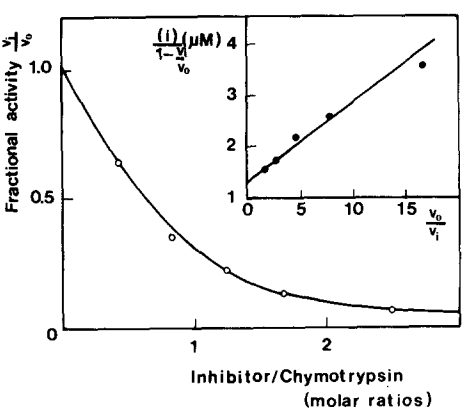


Fig. 1. Determination of the stoichiometry and the dissociation constant K_i of the inhibitor/human-chymotrypsin complex. Final concentrations: chymotrypsin, 1.34 μM; succinyl-L-phenylalanine-*p*-nitroanilide, 0.5 mM; Tris, 0.25 M; dimethylformamide, 5% (v/v), pH 8.0. Inhibitor and enzyme were preincubated for 10 min at 25°C prior to addition of substrate. (○): experimental points, ———: theoretical curve generated using $K_{i(app)}$ = 0.15 μM. Insert: Henderson [17] replot of the data in accordance with eqn. 1.

TABLE I

KINETIC PARAMETERS DESCRIBING THE INTERACTION BETWEEN INTER-α INHIBITOR AND SOME PROTEASES AT pH 8.0 AND 25°C

Proteases	k_1 ($M^{-1} \cdot s^{-1}$)	k_{-1} (s^{-1})	K_i (nM)	$-\Delta G^0$ (kcal · mol^{-1})
Human cationic trypsin	$1.38 \pm 0.33 \cdot 10^5$	$1.7 \pm 0.08 \cdot 10^{-3}$	12.3 ± 2.4	10.8 ± 2.1
Human chymotrypsin II	—	—	128 ± 34	9.4 ± 2.5
Bovine trypsin	$1 \pm 0.09 \cdot 10^6$	$2.3 \pm 0.43 \cdot 10^{-5}$	0.023 ± 0.006	14.5 ± 4
Bovine α-chymotrypsin	$5.5 \pm 0.27 \cdot 10^4$	$5.9 \pm 3.3 \cdot 10^{-5}$	1.08 ± 0.55	12.2 ± 6.2

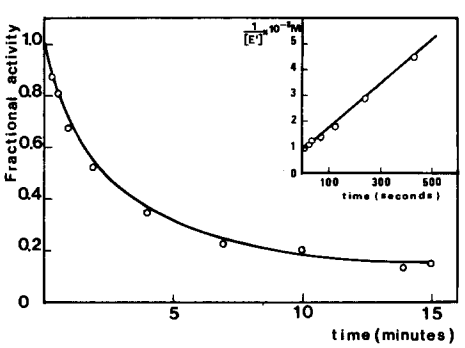
a. Inhibitor/bovine-trypsin interaction. Since the inhibition curve of bovine trypsin was linear up to full inhibition, the dissociation constant K_i could not be evaluated from these data [17,18]. K_i was therefore calculated using the experimentally determined association and dissociation rate constants.

Association kinetics (k_1) were monitored by allowing bovine trypsin and the inhibitor to react for given periods of time before addition of substrate which stopped the association process, and then measuring the remaining enzyme activity (Fig. 2). The rate of association is given by equation 2:

$$\frac{d[EI]}{dt} = k_1[E'][I'] - k_{-1}[EI] \quad (2)$$

where [E'], [I'] and [EI] are the concentrations of enzyme, inhibitor and complex at any time. The second term of eqn. 2 may be neglected since k_{-1} is very low, as will be shown later. Integration of the simplified form of eqn. 2 for [E] = [I] gives:

$$\frac{1}{[E']} = k_1 \cdot t + \frac{1}{[E]} \quad (3)$$



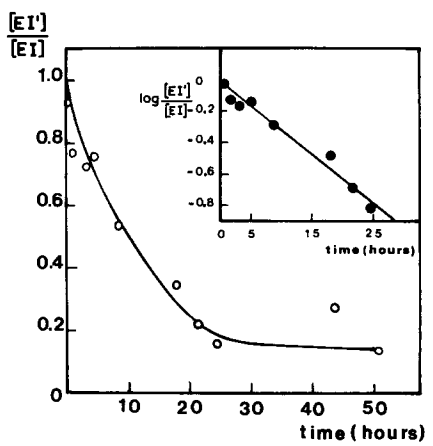


Fig. 2. Determination of the rate constant k_1 for the association of bovine trypsin with inhibitor. Final concentrations: trypsin (= inhibitor), 10 nM; tosyl-L-arginine-methyl-ester, 1.04 mM; Tris, 46.6 mM; $CaCl_2$, 50.4 mM, pH 8.0, 25°C. Insert: second-order replot of the data in accordance with eqn. 3.

Fig. 3. Determination of the rate constant k_{-1} for the dissociation of bovine-trypsin/inhibitor complex at pH 8.0 and 25°C. Final concentrations: complex, 30 nM at time zero, α_2-macroglobulin, 3 μM; benzoyl-L-arginine-*p*-nitroanilide, 1 mM; dimethylformamide, 1.67% (v/v); other conditions as in Fig. 2. Insert: first-order replot of the data in accordance with eqn. 5.

A plot of the data in accordance with eqn. 3 is shown in the insert of Fig. 2. It can be seen that association conforms to second-order kinetics up to 80—90% inhibition. The constant k_1 is equal to $1 \pm 0.09 \cdot 10^6\ M^{-1} \cdot s^{-1}$ (error determined by least square analysis).

Dissociation kinetics (k_{-1}) were monitored by adding α_2-macroglobulin to an equilibrium mixture of bovine trypsin and inhibitor. This globulin forms stable complexes with proteases but does not bind inactive enzymes [19]. Separate experiments showed that the inhibitor was unable to combine with the α_2-macroglobulin/trypsin complex. The α_2-macroglobulin shifts thus the $E + I \rightleftharpoons EI$ equilibrium towards the left. In addition, the α_2-macroglobulin/protease complexes are enzymatically active on synthetic substrates [19]. Dissociation kinetics could thus be monitored by measuring the time-dependent appearance of hydrolytic activity. The activity of a control mixture containing only trypsin and α_2-macroglobulin was taken as the "end-point" of dissociation. This activity was constant during the whole dissociation experiment. Fig. 3 depicts the results of an experiment in which the molar excess of α_2-macroglobulin over the bovine-trypsin/inhibitor complex was 100-fold. Essentially identical results were obtained when the excess was 5- or 20-fold. This indicates that the rate-limiting step of the dissociation process is controlled by k_{-1}. The rate of dissociation is given by eqn. 4:

$$-\frac{d[EI']}{dt} = k_{-1}\,[EI'] \tag{4}$$

which upon integration gives:

$$\log \frac{[EI']}{[EI]} = \frac{k_{-1} \cdot t}{2.303} \tag{5}$$

where [EI] is the concentration of complex at time zero and [EI'] the concentration at any time t. A replot of the data in accordance with eqn. 5 is shown in the insert of Fig. 3. It can be seen that the dissociation process conforms to first-order kinetics up to about 80% dissociation. The value of k_{-1} was found to be $2.3 \pm 0.43 \cdot 10^{-5} \cdot s^{-1}$ (error determined by least square analysis).

b. Inhibitor/human-trypsin interaction. The dissociation constant K_i was determined directly by measuring the fractional activity v_i/v_0 of mixtures formed of constant amounts of human trypsin and increasing amounts of inhibitor as described in the experimental section under the heading Stoichiometries of association. Addition of benzoyl-L-arginine-*p*-nitroaniline resulted in an exponential release of *p*-nitroaniline indicating substrate-induced dissociation of the enzyme/inhibitor complex. The new equilibrium was reached when the rate became constant (after 5—10 min). The data obtained using zero-order rates were plotted in the same manner as shown in Fig. 1 for human chymotrypsin. A rough estimate of $K_{i(app)}$ was obtained from a Henderson [17] plot. A more accurate value of $K_{i(app)}$ and its standard deviation were then calculated by a non-linear regression analysis method fitting the data to eqn. 6 [18]:

$$\frac{v_i}{v_0} = 1 - \frac{[E] + [I] + K_{i(app)} - \sqrt{([E] + [I] + K_{i(app)})^2 - 4[E][I]}}{2[E]} \tag{6}$$

For this purpose, a Fortran IV program was kindly written for us by Dr J.L.

Dimicoli and is available from him upon request *.

Since the substrate dissociates the human-trypsin/inhibitor complex, $K_{i(app)}$ is given by eqn. 7 [20]:

$$K_{i(app)} = K_i\left(1 + \frac{[S]}{K_m}\right) \tag{7}$$

where [S] stands for the total substrate concentration and K_m for the Michaelis constant of the enzyme-substrate complex. To transform $K_{i(app)}$ into K_i, the value of K_m for human trypsin and benzoyl-L-arginine-*p*-nitroanilide was determined in separate experiments and found to be 0.9 mM, a value which is close to that of bovine trypsin [13]. The value of K_i was found to 12.3 ± 2.4 nM.

As mentioned above, the dissociation of the inhibitor/human-trypsin complex is very rapid. The experimental procedure used to measure the constant k_{-1} of the inhibitor/bovine-trypsin complex could therefore not be used. The inhibitor/human-trypsin complex was thus prepared in a small volume and then diluted 100 times in a medium containing substrate and α_2-macroglobulin as described in the experimental section. The appearance of *p*-nitroaniline was followed with respect to time during 35 min. Due to the dissociation of the complex, this appearance was first-order and from these results, k_{-1} could be determined graphically [21] and was found to be $1.7 \pm 0.08 \cdot 10^{-3}$ s^{-1} (error determined by least square analysis).

c. Inhibitor/bovine-chymotrypsin interaction. As for bovine trypsin, the dissociation constant K_i of the bovine-chymotrypsin/inhibitor complex could not be evaluated directly because the inhibition curve was linear. The value of K_i was therefore determined by measuring the competition [21] between bovine trypsin and bovine chymotrypsin for the binding of the inhibitor. The inhibitor was first reacted with an excess of bovine chymotrypsin. Then bovine trypsin (same concentration as the inhibitor) was added in order to dissociate partially the bovine-chymotrypsin/inhibitor complex. After the equilibrium between the three partners was attained, the free trypsin activity was measured and compared to that of the total trypsin activity. The fractional trypsin activities a obtained when x, the molar excess of chymotrypsin over the inhibitor was equal to 10, 20, 40 and 60 were 0.34, 0.52, 0.60 and 0.67 respectively.

The ratio of the two dissociation constants is given by eqn. 8:

$$\frac{K_C}{K_T} = \frac{[C'][TI]}{[T'][CI]} \tag{8}$$

where K_c and K_T are the dissociation constants of the inhibitor/bovine-chymotrypsin and inhibitor/bovine-trypsin complexes and [C'], [T'],]CI] and [TI] are the equilibrium concentrations of bovine chymotrypsin, bovine trypsin and their complexes which are related to the total concentrations [C] and [T] by the following conservation equations:

$$[C] = [C'] + [CI]$$

$$[T] = [T'] + [TI]$$

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Taking into account that $[C]/[T] = x$ and that $[T']/[T] = a$, we get:

$$\frac{1-a}{a^2} = \frac{K_C}{K_T} \cdot \frac{1}{x-a} \qquad (9)$$

Fig. 4 shows a plot in accordance with eqn. 9. The ratio K_C/K_T was found to be 47 ± 11 and K_c = 1.08 ± 0.55 nM. (error determined by least square analysis).

The principle used to determine k_1 was the same as that described for the bovine-trypsin/inhibitor system (see experimental section). The value of k_1 was found to be $5.5 \pm 0.27 \cdot 10^4\ M^{-1}\ s^{-1}$.

d. Inhibitor/human-chymotrypsin interaction. Preliminary experiments showed that the extent of inhibition of human chymotrypsin was dependent upon the substrate concentration. $K_{i(app)}$ was thus determined using the same principle as that described for human trypsin; the data are shown in Fig. 1. To transform $K_{i(app)}$ into K_i, the K_m of the human-chymotrypsin/succinyl-L-phenylalanine-*p*-nitroanilide system was determined separately and found to be 2 mM (i.e. twice the value reported for bovine chymotrypsin [15]). K_i was found to be 128 ± 34 nM.

The dissociation of the enzyme-inhibitor complex was too fast to be measurable. On the other hand, attempts to measure k_1 remained unsuccessful because of the combined effects of the high values of K_i and k_{-1}.

Discussion

From Table I it can be seen that the stabilities ($1/K_i$) of the enzyme-inhibitor complexes vary widely and are governed either by k_1 or k_{-1}. For instance, the differences in the stabilities of the two trypsin-inhibitor complexes are mainly due to differences in the dissociation rate constants whereas the differences in the stabilities of the two bovine protease-inhibitor complexes are essentially due to differences in the association rate constants.

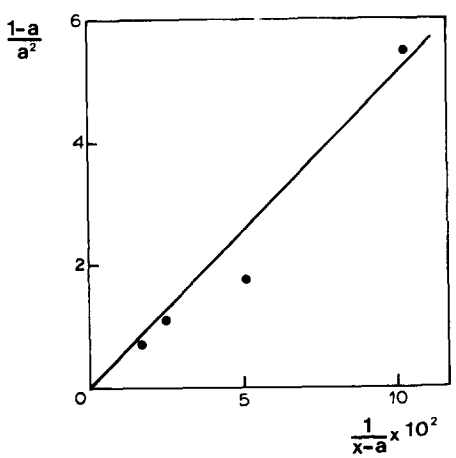

Fig. 4. Determination of the dissociation constant K_i of the bovine-chymotrypsin/inhibitor complex from competition experiments with bovine trypsin at pH 8.0 and 25°C. Final concentrations: trypsin (= inhibitor), 60 nM; chymotrypsin, 0.6, 1.2, 2.4 and 3.6 μM; benzoyl-DL-arginine-*p*-nitroanilide, 1.33 nM; Tris, 46.6 mM $CaCl_2$, 50.4 mM. The data are plotted in accordance with eqn. 9.

Our investigation has led to a very unexpected finding: the complexes formed of human inter-α-inhibitor and human proteases are much less stable than those formed with the bovine proteases. The species differences expressed in terms of differences in $-\Delta G^0$ are 3.7 Kcal · mol^{-1} for the two trypsins and 2.8 Kcal · mol^{-1} for the two chymotrypsins. These differences in free energies of binding may result from differences in the secondary [5,6] and tertiary structures of human and bovine proteases if we assume that the overall binding force which stabilizes an enzyme-inhibitor complex is composed of a number of weak interactions which do not necessarily take place in the vicinity of the enzyme active centers [22].

Another point is worth emphasizing: whatever the species, the complexes formed with chymotrypsin are less stable than those formed with trypsin. The differences in $-\Delta G^0$ are 1.4 Kcal · mol^{-1} and 2.3 Kcal · mol^{-1} for the human and bovine enzymes respectively. On the other hand, the inter-α-inhibitor is inactive on elastase [23]. It is worth noticing that two other potent trypsin inhibitors which are moderately potent on chymotrypsin are also inactive on elastase, namely: the Kunitz soybean trypsin inhibitor [24] and the basic pancreatic inhibitor [25].

In 1969, Feeney et al. [26] reported a comparative study of the inhibition of bovine and human trypsin by a variety of natural protease inhibitors. Only five inhibitors out of sixteen exhibited comparable potencies towards the enzymes from the two species. The other compounds were much less active on human trypsin than they were on bovine trypsin. In addition the compared effects of other inhibitors on bovine and human trypsin have been studied more recently by Travis, Figarella, Feinstein and their colleagues [27—30]. In agreement with the work of Feeney et al. [26], these investigations have led to the conclusion that in most cases human trypsin was more resistant to inhibition than the bovine enzyme. It is shown in this paper that inter-α-inhibitor conforms with this puzzling rule.

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