Inhibition of human pancreatic proteinases by mucus proteinase inhibitor, eglin c and aprotinin

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The kinetic investigation of the inhibition of human pancreatic trypsin 1, trypsin 2 and chymotrypsin A by mucus proteinase inhibitor, eglin c and aprotinin reveals that (i) the first protein is a potent inhibitor of chymotrypsin A ($k_{ass.} = 1.4 \times 10^6$ $M^{-1} \cdot s^{-1}$, $K_i = 71$ pM) but forms loose complexes with trypsin 1 ($K_i = 0.5$ μM) and trypsin 2 ($K_i = 18$ nM), (ii) eglin c does not inhibit the two trypsins but forms a tight complex with chymotrypsin A ($k_{ass.} = 3.3 \times 10^6$ $M^{-1} \cdot s^{-1}$, $K_i < 0.1$ nM) and (iii) aprotinin is a potent inhibitor of trypsin 1 ($k_{ass.} = 1 \times 10^5$ $M^{-1} \cdot s^{-1}$, $K_i < 0.2$ nM) and trypsin 2 ($k_{ass.} = 2.4 \times 10^5$ $M^{-1} \cdot s^{-1}$, $K_i < 1$ nM) but forms a loose complex with chymotrypsin A ($K_i = 0.17$ μM). These data, together with those published previously on human pancreatic elastase, suggest that a cocktail of aprotinin + eglin c might be a better intensive-care drug for acute pancreatitis than aprotinin alone, because it will efficiently inhibit all four human pancreatic proteinases. On the other hand, human gastric juice inactivates mucus proteinase inhibitor by pepsin-mediated cleavage. This indicates that the fraction of mucus proteinase inhibitor that reaches the stomach following aerosol delivery to cystic fibrosis patients does not reach the duodenum in an active form and, therefore, does not aggravate the pancreatic insufficiency of these patients.

INTRODUCTION

The pancreas synthesizes and stores large quantities of the zymogen forms of proteolytic enzymes. Upon stimulation, the zymogens are released in the duodenum where they are activated to accomplish their digestive task. Human pancreas has been shown to synthesize a number of proteinases including trypsin 1 and 2, chymotrypsin A ([1,2] and refs. therein) and elastase [3]. There are at least two human pathological conditions involving pancreatic proteinases. One of them, cystic fibrosis, is accompanied by a hyposecretion of proteolytic zymogens [4] with subsequent insufficient food digestion [5]. The other disease, acute pancreatitis, is due to sudden intrapancreatic zymogen activation which results in rapid glandular self-destruction, diffusion of proteinases in the peritoneum and blood and, frequently, fatal shock [6].

Aprotinin, a 6.5 kDa trypsin and kallikrein inhibitor isolated from bovine organs [7], has been used for more than three decades as an intensive-care drug for acute pancreatitis [8]. This inhibitor forms very tight complexes with human pancreatic trypsin 1 and trypsin 2 [9,10]. However, it does not inhibit human pancreatic chymotrypsin A [9] and does not bind human pancreatic elastase [11]. The fact that aprotinin is unable to inhibit the bulk of pancreatic proteinases might explain why its therapeutic efficacy has sometimes been questioned [12].

One aim of the present investigation was to search for proteinase inhibitors that would have a broader specificity than aprotinin while still having a low molecular mass to be as diffusible as this inhibitor. Mucus proteinase inhibitor (MPI) and eglin c appeared to be potential candidates for such a function. The former is an 11.7 kDa basic protein present in human mucus secretions and now produced by genetic engineering [13]. Its conformation is stabilized by eight disulphide bonds and it is formed of a single chain of 107 amino acid residues whose sequence is arranged in two domains [14,15]. MPI is a potent inhibitor of neutrophil elastase and cathepsin G [16,17] and of bovine pancreatic trypsin and chymotrypsin [18]. Its action on human pancreatic trypsins and chymotrypsin has never been tested. Eglin c is an 8.1 kDa protein first isolated from the leech and now produced by recombinant technology [19]. This inhibitor is composed of a single chain of 70 amino acid residues. It is extremely stable despite the lack of disulphide bridges [20]. Like MPI, it potently inhibits neutrophil elastase and cathepsin G and bovine pancreatic chymotrypsin, but forms a loose complex with bovine pancreatic trypsin [21–23]. It is not known whether it inhibits human pancreatic trypsins and chymotrypsin. This paper describes the kinetics of inhibition of these three enzymes by recombinant eglin c and MPI.

The main clinical feature of cystic fibrosis is the destruction of lung connective tissue due to the proteinases released in large quantities from the neutrophils recruited in the airways. MPI is thought to be a potential drug for preventing lung destruction since it is a potent elastase and cathepsin G inhibitor [16,17]. In preliminary clinical trials, the inhibitor was administered via an aerosol at a dose of 100 mg twice daily for 1 week after which the elastase activity was measured in bronchoalveolar lavage fluid. With this dose, the elastase activity was significantly depressed, suggesting that MPI may indeed be of therapeutic usefulness in cystic fibrosis [24]. With half the dose, however, no elastase inhibition was observed [25]. The use of such large quantities of MPI, a significant part of which reaches the stomach [26], and hence the duodenum, raises the question as to whether MPI may inhibit the patients' pancreatic proteinases and therefore aggravate their well-known pancreatic insufficiency [4,5]. To answer

Abbreviations used: MPI, mucus proteinase inhibitor (secretory leucoprotease inhibitor); BAPA, benzoyl-L-arginine-*p*-nitroanilide; Suc, succinyl; MeOSuc, methoxysuccinyl; pNA, *p*-nitroanilide.

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this question we have studied the stability of MPI in human gastric juice.

MATERIALS AND METHODS

The zymogens of human pancreatic trypsin 1, trypsin 2 and chymotrypsin A were purified from pancreatic juice and activated as described [1,2]. Human pancreatic elastase isolated as described previously [3] was a gift from Dr. M. Rabaud, INSERM U306, Université de Bordeaux II, France. Human neutrophil elastase was isolated as described previously [27]. Stock solutions of trypsin 1 and chymotrypsin A were prepared in 1 mM HCl containing 20 mM $CaCl_2$, while stock solutions of trypsin 2 and elastase were prepared in 20 mM $CaCl_2$ and 50 mM sodium acetate, pH 5.0, respectively. These solutions were stored frozen until used. Once a sample was thawed it was used within 1 day. Human gastric juice, collected from a healthy volunteer under pentagastrin stimulation [28], was given to us by Dr. M. Armand, INSERM U130, Marseille, France. Its pH was 4.1 and its pepsin activity was 1007 Anson units/ml, which is within the range of activity commonly found for normal gastric juice [29]. Pepstatin was from Sigma and RNase from Pharmacia. Recombinant MPI and eglin c were obtained from Ciba-Geigy, Basel, Switzerland, through the courtesy of Dr. H. P. Schnebli. Aprotinin was from Biosys, Compiègne, France. Stock solutions of these inhibitors were made in the pH 7.4 buffer described below and frozen until used. Benzoyl-L-arginine-*p*-nitroanilide (BAPA) and Suc-Ala_2-Pro-Phe-*p*-nitroanilide were from Bachem, Bubendorf, Switzerland. MeOSuc-Ala-Arg-pNA (where MeOSuc is methoxysuccinyl and pNA is *p*-nitroanilide) was synthesized for us by Enzyme Systems Products, Dublin, CA, U.S.A. Stock solutions of these substrates were prepared in dimethylformamide and stored at 4 °C. All experiments were done at 25 °C in a solution containing 50 mM Hepes, 100 mM NaCl and 10 μg/ml BSA (Sigma), pH 7.4. This solution will be referred to as the pH 7.4 buffer. The enzymic reactions were started by adding 10 μl of stock solution of substrate to 990 μl of buffered enzyme $\pm$ inhibitor solution. The reaction rates were measured by recording the absorbance at 410 nm and 25 °C.

Determination of $k_{ass.}$

MPI, eglin c and aprotinin are all reversible competitive inhibitors [7,26,27] whose reaction with proteinases is described by Scheme 1.

$$E + I \underset{k_{diss.}}{\overset{k_{ass.}}{\rightleftharpoons}} EI$$

Scheme 1

The association rate constant $k_{ass.}$ was determined by reacting E and I for variable periods of time before addition of substrate which slows down the association process enough to allow measurement of residual enzyme activity [30]. If the dissociation reaction is slow with respect to the interval of time needed to follow association, the rate of association $-d[E]/dt$ is given by [30]:

$$-\frac{d[E]}{dt} = k_{ass.}[E][I] \quad (1)$$

The rate of association was measured either under second-order conditions ($[E]_0 = [I]_0$) or pseudo-first-order conditions ($[I]_0 \geqslant 10\,[E]_0$). In the former case the data were fitted to eqn. (2) by non-linear regression analysis using the ENZFITTER Software (Biosoft, Cambridge, UK):

$$[E] = \frac{[E]_0}{1 + [E]_0 \cdot k_{ass.} \cdot t} \quad (2)$$

where [E] is the concentration of free enzyme at any time t and $[E]_0$ is the concentration at $t = 0$. $[E]_0$ and [E] are proportional to the rate of substrate hydrolysis at $t = 0$ and at any time t, respectively. When pseudo-first-order conditions were used, the data were fitted to the following exponential equation:

$$[E] = [E]_0 \cdot e^{-k_{ass.}[I]_0 t} \quad (3)$$

and $k_{ass.}$ was calculated from the pseudo-first-order rate constant $k_{ass.}[I]_0$. The concentration conditions used to determine $k_{ass.}$ are reported in Table 1.

Table 1 Concentration conditions used to determine the association rate constants $k_{ass.}$

Enzyme (E) and inhibitor (I) were allowed to react for variable periods of time before addition of a small volume of a stock solution of substrate (S). The progress of substrate hydrolysis was then recorded to measure the reaction velocity. All experiments were done at 25 °C in the pH 7.4 buffer.

	Inhibitor concentration		
Enzyme	MPI	Eglin c	Aprotinin
Trypsin 1			
$[E]_0$	0.12 μM	0.12 μM	0.12 μM
$[I]_0$	1.2 μM	84 μM	0.12 μM
$[S]_0$	1 mM[a]	1 mM[a]	1 mM[a]
Trypsin 2			
$[E]_0$	0.16 μM	0.16 μM	0.16 μM
$[I]_0$	0.16 μM	21 μM	0.16 μM
$[S]_0$	50 μM[b]	50 μM[b]	150 μM[b]
Chymotrypsin A			
$[E]_0$	15 nM	10 nM	33 nM
$[I]_0$	15 nM	10 nM	660 nM
$[S]_0$	0.2 mM[c]	0.2 mM[c]	0.2 mM[c]

[a] BAPA; [b] MeOSuc-Ala-Arg-pNA; [c] Suc-Ala_2-Pro-Phe-pNA.

Determination of $k_{diss.}$

The $k_{diss.}$ of the MPI–trypsin 1 complex was measured in the following way. The complex, prepared by reacting 1 μM trypsin 1 with 1 μM MPI for 30 min, was diluted 100-fold into 2.2 mM BAPA and the release of *p*-nitroaniline was recorded for 20 min. The slopes of the tangents drawn along the progress curve were used to calculate the concentration of free trypsin during the dissociation process. This concentration increased with time until equilibrium between E, S and I and their complexes was attained, i.e. when about 60 % dissociation of EI had occurred. The rate constant $k_{diss.}$ was calculated using an equation that takes into account both the dissociation of EI and its re-association (see eqn. 7 of ref. [27]). The $k_{diss.}$ of the MPI–trypsin 2 complex was measured in a similar way except that 180 nM EI was diluted 13-fold into 4.3 mM BAPA and the progress curve was recorded for 1 h.

The $k_{diss.}$ of the MPI–chymotrypsin A complex was measured using human neutrophil elastase as a dissociating agent. The MPI–chymotrypsin complex, prepared by reacting 0.33 μM MPI with 0.33 μM chymotrypsin for 30 min, was diluted 10-fold into 0.36 μM elastase and samples from this mixture were withdrawn

from time to time and mixed with 0.2 mM Suc-Ala$_2$-Pro-Phe-pNA to measure the chymotrypsin released during the 6 h dissociation reaction. Two control mixtures, one containing 33 nM chymotrypsin alone and the other containing 33 nM chymotrypsin + 0.36 μM elastase, were incubated and assayed in an identical way. The loss of chymotrypsin activity was less than 10 % after 6 h of incubation of these two mixtures. If elastase efficiently scavenges MPI, the release of chymotrypsin will be exponential (Scheme 1) and $k_{diss.}$ may be calculated using non-linear regression analysis by fitting the data to eqn. (4):

$$[E] = [E]_0(1 - e^{-k_{diss.}t}) \quad (4)$$

where [E] and $[E]_0$ are the chymotrypsin concentrations at any time t and at the end of the dissociation respectively.

Determination of K_i

The K_i of the MPI–trypsin 1 complex was determined by reacting constant concentrations of trypsin 1 (0.12 μM) with variable concentrations of MPI for 20 min and measuring the residual trypsin activity with 1 mM BAPA. The progress curves of substrate hydrolysis were concave, diagnosing dissociation of EI during the assay [31]. Accordingly, the substrate solution was mixed as rapidly as possible with the medium and initial rates of substrate hydrolysis were calculated from the slopes of the tangents drawn to the progress curves at $t = 0$. These rates were therefore assumed to measure the concentration of free E present in the equilibrium mixture of E, I and EI before perturbation of this equilibrium by the substrate. A curvilinear titration curve was obtained. The data were therefore fitted by non-linear regression analysis to the quadratic equation described previously [31]:

$$a = 1 - \frac{([E]_0 + [I]_0 + K_i) - \{([E]_0 + [I]_0 + K_i)^2 - 4[E]_0[I]_0\}^{\frac{1}{2}}}{2[E]_0} \quad (5)$$

where a is the fractional enzyme activity, i.e. the ratio of the enzyme velocity in the presence of inhibitor to the velocity in its absence.

The K_i of the aprotinin–chymotrypsin A complex was determined in a similar way with $[E]_0 = 33$ nM, an incubation time of 40 min and 0.2 mM Suc-Ala$_2$-Pro-Phe-pNA as the substrate. Here the progress curves of substrate hydrolysis were linear. In addition, their slopes increased with the substrate concentration, as shown in separate experiments. This indicates that the substrate partially dissociates the enzyme–inhibitor complex and that the new equilibrium is attained within the time required to mix the reagents [31]. A curvilinear titration curve was again obtained. The data were analysed using a variant of eqn. (5) in which $K_{i(app)}$ replaces K_i. This apparent constant is given by $K_{i(app)} = K_i(1 + [S]_0/K_m)$. The K_m for the chymotrypsin A-catalysed hydrolysis of Suc-Ala$_2$-Pro-Phe-pNA, measured in a classical way, was found to be 88 μM.

Four enzyme–inhibitor systems yielded linear inhibition curves, namely: chymotrypsin A–MPI, chymotrypsin A–eglin c, trypsin 1–aprotinin and trypsin 2–aprotinin. The enzyme concentrations used for these titrations were 10 nM chymotrypsin A, 20 nM trypsin 1 and 100 nM trypsin 2 and the enzyme–inhibitor incubation times were 40 min, 6 h and 30 min respectively. The substrates and their concentrations were the same as those given in Table 1.

Inactivation of MPI by gastric juice

MPI (1 μM) was incubated at 37 °C with gastric juice and 10 μl aliquots of the mixture were withdrawn after selected intervals of time and diluted into 990 μl of a buffer (500 mM Hepes, 100 mM NaCl, pH 7.4) containing 10 nM human chymotrypsin A. After 30 min at 25 °C, 10 μl of 20 mM Suc-Ala$_2$-Pro-Phe-pNA was added to measure the residual chymotrypsin activity. This experiment was repeated using gastric juice supplemented with 1 mM pepstatin. Experiments using human trypsin 1 and 2 as indicator enzymes were done in a similar way except that 2 μM MPI was incubated with gastric juice, 150 μl aliquots were withdrawn, the trypsin concentration was 300 nM and the substrate was either 1 mM BAPA (trypsin 1) or 50 μM MeOSuc-Ala-Arg-pNA (trypsin 2).

The electrophoretic characterization of gastric juice-inactivated MPI was done as follows. A sample (10 ml) of gastric juice containing 1 μM MPI was incubated at 37 °C for 6 h, after which 6 ml of the medium was loaded on to a Pharmacia Mono S HR 5/5 column equilibrated with 20 mM Bicine buffer, pH 8.7, and eluted with a linear salt gradient (0–0.5 M NaCl). The MPI-containing fractions were pooled, desalted on a Pharmacia Sephadex PD-10 column and lyophilized. The protein was dissolved in a minimum of water and denatured and electrophoresed as described previously [32].

RESULTS AND DISCUSSION

Proteinase inhibition by native MPI, eglin c and aprotinin

The association rate constant $k_{ass.}$ could be measured precisely for all enzyme–inhibitor systems that yielded inhibitory complexes except for the chymotrypsin A–aprotinin complex whose association was found to be both slow and loose. Figure 1 illustrates a typical $k_{ass.}$ measurement: trypsin 1 and aprotinin reacted under second-order conditions yielding an inhibition curve that conformed to second-order kinetics. The $k_{ass.}$ of this system was found to be $(1.00 \pm 0.02) \times 10^5$ $M^{-1} \cdot s^{-1}$ as calculated by non-linear regression analysis.

The dissociation rate constant $k_{diss.}$ could only be measured for the three MPI–proteinase systems. The complexes of MPI with the two human trypsins were sufficiently loose to be dissociable by dilution into substrate (results not shown; see the

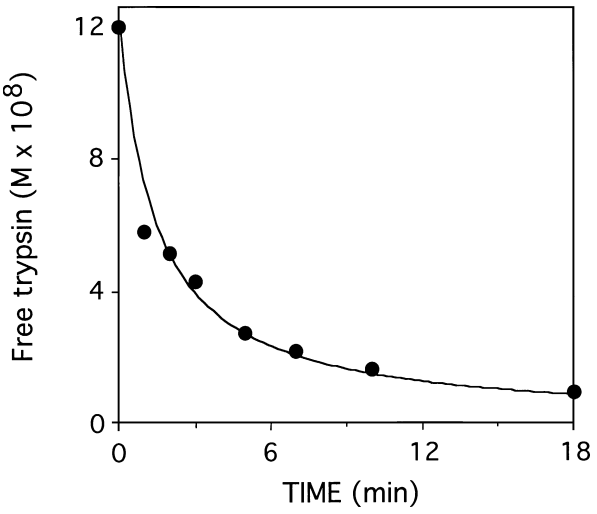


Figure 1 Kinetics of association of human pancreatic trypsin 1 and aprotinin at pH 7.4 and 25 °C

Trypsin (0.12 μM) and aprotinin (0.12 μM) were reacted for variable periods of time before addition of 1 mM BAPA which measures the residual enzyme activity, i.e. the concentration of free trypsin. (●) Experimental points, (—) theoretical curve generated using eqn. (2) and the best estimate of $k_{ass.}$ calculated by non-linear regression analysis.

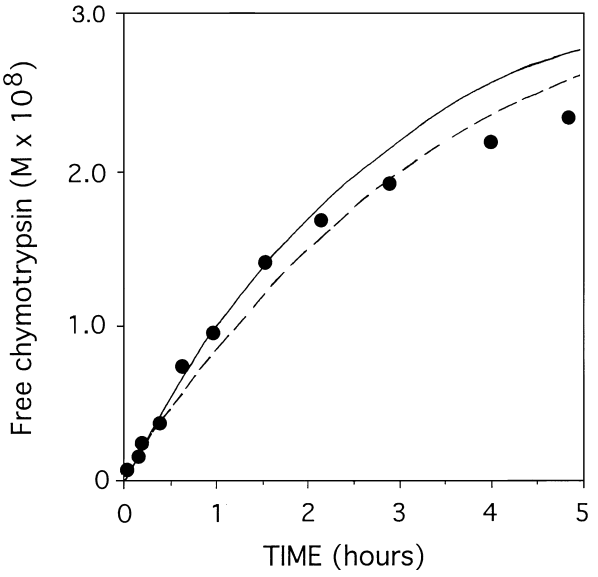
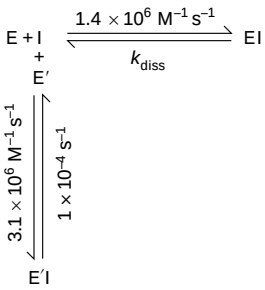


Figure 2 Kinetics of dissociation of the MPI–human chymotrypsin A complex by neutrophil elastase at pH 7.4 and 25 °C

The enzyme–inhibitor complex was diluted 10-fold into an elastase solution and the time-dependent appearance of chymotrypsin activity was assessed with 0.2 mM Suc-Ala_2-Pro-Phe-pNA. The final concentrations in the dissociation medium were: complex, 33 nM; elastase, 360 nM. (●) Experimental points, (solid and broken lines) theoretical curves generated using eqn. (4) and the best estimates of $k_{diss.}$ calculated by non-linear regression analysis on the first eight data points (solid line) and on the whole set of data points (broken line).

Materials and methods section). In contrast, the chymotrypsin A–MPI complex did not significantly dissociate under these conditions. We therefore used neutrophil elastase as a dissociating agent. This enzyme forms a very tight reversible complex with MPI whose $k_{ass.}$ and $k_{diss.}$ values have been measured in the same conditions as those used in the present work [33]. The following scheme helps discussing the possible use of elastase to measure the $k_{diss.}$ of the chymotrypsin–MPI complex:



Scheme 2

where E, E′ and I stand for chymotrypsin A, neutrophil elastase and MPI respectively. Two conditions must be fulfilled to get dissociation of EI by E′: (i) $k_{diss.}$ should not be much smaller than 10^{-4} s^{-1}, and (ii) E′ should favourably compete with E for the binding of I. The latter condition is theoretically fulfilled since (i) the $k_{ass.}$ values for the binding of I with either E or E′ are very close, and (ii) $[E']_0 = 10\,[EI]_0$ (see the Materials and methods section) which means that $[E'] > [E]$ at the start of the dissociation process. Therefore, an exponential release of E should be observed as long as [E] is small enough not to compete with E′ for the binding of I.

Table 2 Inhibition of human pancreatic proteinases by MPI, eglin c and aprotinin at pH 7.4 and 25 °C

The errors on the kinetic parameters are less than 15% (experimental constants) or less than 30% (calculated constants).

		Inhibitor		
Enzyme	Constants	MPI	Eglin c	Aprotinin
Trypsin 1	$k_{ass.}$ ($M^{-1}\cdot s^{-1}$)	1.6×10^{4}	–	1.0×10^{5}
	$k_{diss.}$ (s^{-1})	9.0×10^{-3}	–	$<2\times10^{-5}$
	K_i (M)	5.6×10^{-7} [a,b]	$>2\times10^{-3}$	$<2\times10^{-10}$
Trypsin 2	$k_{ass.}$ ($M^{-1}\cdot s^{-1}$)	1.6×10^{5}	–	2.4×10^{5}
	$k_{diss.}$ (s^{-1})	2.9×10^{-3}	–	$<2\times10^{-4}$
	K_i (M)	1.8×10^{-8} [a]	$>2\times10^{-3}$	$<10^{-9}$
Chymotrypsin A	$k_{ass.}$ ($M^{-1}\cdot s^{-1}$)	1.4×10^{6}	3.3×10^{6}	$\sim10^{4}$
	$k_{diss.}$ (s^{-1})	9.9×10^{-5}	$<3\times10^{-4}$	$\sim2\times10^{-3}$ [a]
	K_i (M)	7.1×10^{-11} [a]	$<10^{-10}$	1.7×10^{-7}
Elastase	$k_{ass.}$ ($M^{-1}\cdot s^{-1}$)	–	7.3×10^{5} [d]	–
	$k_{diss.}$ (s^{-1})	–	2.7×10^{-4} [d]	–
	K_i (M)	6.7×10^{-8} [c]	3.7×10^{-10} [d]	$>2\times10^{-3}$

[a] Calculated from $K_i = k_{diss.}/k_{ass.}$.
[b] This value is of the same order ofmagnitude as that determineddirectly, i.e. 2.6×10^{-7} M.
[c] From Laurent et al. [36].
[d] From Faller et al. [37].

Figure 2 shows the time-dependent appearance of chymotrypsin activity following reaction of elastase with the chymotrypsin–MPI complex. Analysis of the whole set of data by non-linear regression based on eqn. (4) gave $k_{diss.} = (9\pm0.4)\times10^{-5}$ s^{-1}. The theoretical curve corresponding to this constant (broken line in Figure 2) does not, however, fit the experimental points well as can be seen by inspecting the Figure. A plot of the residuals versus time using the ENZFITTER software confirmed that eqn. (4) did not appropriately describe the experimental data. It was hypothesized that the poor fit was due to the fact that, after a certain extent of dissociation, the back reaction ($E + I \rightarrow EI$) becomes significant, slows down the overall rate of E release and is thus responsible for the deviation of the data from first-order kinetics. Accordingly, we performed non-linear regression analyses using fewer and fewer data points until the fit was satisfactory. Figure 2 shows that the exponential calculated using the first eight data points fits these points well, indicating that up to about 50% EI dissociation the release of E is first-order. The $k_{diss.}$ calculated in this way was found to be $(9.9\pm0.2)\times10^{-5}$ s^{-1}. Attempts to use higher elastase concentrations in order to get an exponential release of chymotrypsin A for more than one half-life were unsuccessful because of the progressive instability of chymotrypsin.

Most of the other inhibitory complexes were so tight that dissociation by a proteinase or by inhibitors such as α_2-macroglobulin [34] or α_1-proteinase inhibitor [35] were unsuccessful. The systems whose $k_{diss.}$ could not be measured also exhibited linear inhibition curves when the enzymes were titrated with increasing amounts of inhibitor. The K_i values of these complexes could therefore not be evaluated using the data of the titration curves. Theory predicts that a reversible inhibitor yields a straight inhibition curve if $K_i \leqslant 0.01[E]_0$ [31]. This relationship was used to calculate the upper limits of K_i. In turn, the upper limits of $k_{diss.}$ were estimated from $k_{diss.} = k_{ass.}\cdot K_i$. Three enzyme–inhibitor systems did not form inhibitory complexes even when

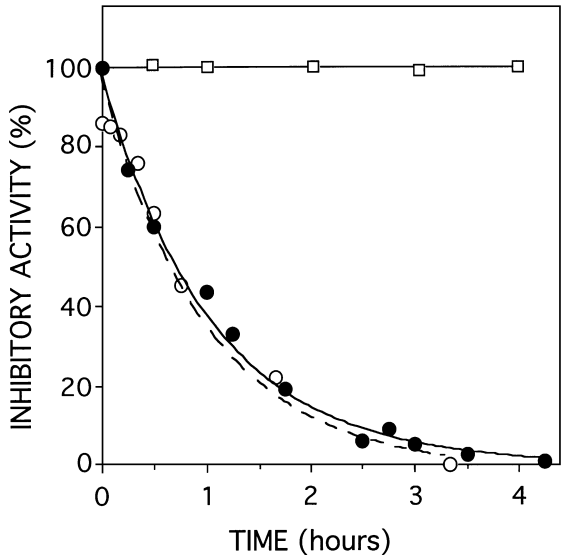


Figure 3 Kinetics of inactivation of MPI by gastric juice at pH 4.1 and 37 °C

MPI was incubated with gastric juice and samples were withdrawn after selected intervals of time and tested for their ability to inhibit human pancreatic chymotrypsin A (●) and trypsin 2 (○). The effect of gastric juice supplemented with 1 mM pepstatin on the activity of MPI (□) was tested using chymotrypsin A as an indicator enzyme.

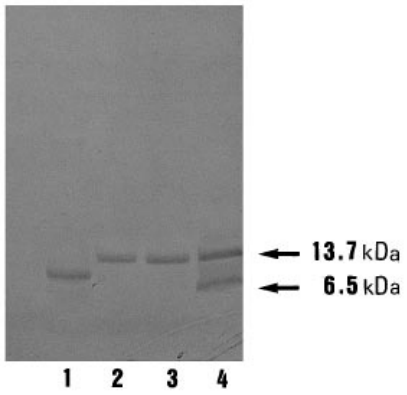


Figure 4 SDS/PAGE of reduced samples of MPI

Lane 1, MPI incubated for 6 h with human gastric juice and subsequently isolated by cation-exchange chromatography. Lanes 2 and 3, native and acid-treated MPI, respectively. Lane 4, molecular-mass markers, aprotinin (6.5 kDa) and RNase (13.7 kDa).

tested with a 0.1 mM concentration of inhibitor. This indicates that their K_i values are greater than 2 mM.

Table 2 compiles the kinetic data found for the various enzyme–inhibitor systems. The results obtained previously for the inhibition of human pancreatic elastase by MPI [36] and eglin c [37] have also been included. We confirm earlier studies showing that aprotinin has a narrow specificity and a high affinity for human trypsins 1 and 2 [9–11]. We also show for the first time that this protein is a fast-acting inhibitor of the two trypsins. This may account for its therapeutic usefulness in acute pancreatitis [8]. The specificity of MPI and eglin c is as narrow as that of aprotinin. While the latter is essentially a trypsin inhibitor, the former are basically chymotrypsin inhibitors. The three inhibitors exhibit a much broader specificity for bovine pancreatic trypsin and chymotrypsin [7,18,21–23].

Inactivation of MPI by human gastric juice

MPI was mixed with human gastric juice and samples of the medium were withdrawn at given intervals of time and tested for trypsin 2 and chymotrypsin A inhibitory activity at pH 7.4. Figure 3 shows that both inhibitory activities decay exponentially with about identical rate constants [$(1.9 \pm 0.2) \times 10^{-4}$ s^{-1} for trypsin 2 and $(2.6 \pm 0.1) \times 10^{-4}$ s^{-1} for chymotrypsin A]. Trypsin 1 gave similar results (results not shown). Control experiments showed that MPI was very stable at acidic pH, in agreement with earlier reports [16,38]. In addition, the inhibitor was not inactivated when gastric juice was supplemented with pepstatin (see Figure 3), indicating that the inactivation is due to proteolytic cleavage by pepsin or pepsin-like enzymes present in gastric juice [39].

To check this hypothesis, we chromatographically isolated MPI from its reaction medium with gastric juice and subjected it to electrophoresis. MPI was incubated with gastric juice for 6 h to ensure its full inactivation (6 h = 6 $t_{\frac{1}{2}}$). Control chromatographic runs showed that (i) most gastric juice proteins eluted before the start of the salt gradient while a small proportion of protein desorbed at 150 mM NaCl, and (ii) native MPI was eluted at 280 mM NaCl. With these controls, it was easy to identify inactive MPI following chromatography of the MPI + gastric juice mixture. The inactive inhibitor eluted at 260 mM NaCl, i.e. slightly ahead of the native protein. Experiments with chymotrypsin showed that the isolated material was indeed inactive. Figure 4 shows that inactive MPI migrates as an approx. 8600 Da protein on PAGE whereas acid-treated native MPI migrates about as expected from its molecular mass (11 700 Da). This experiment thus confirms that proteolysis is responsible for the inactivation of MPI by gastric juice. Cysteine proteinase inhibitors have been shown to be inactivated by aspartic proteinases [40–42]. To the best of our knowledge, however, this is the first report of an inactivation of a serine protease inhibitor by aspartic proteinase(s).

Conclusion

The therapeutic efficacy of aprotinin as an intensive-care drug for acute pancreatitis has sometimes been questioned [12]. This is possibly due to the fact that this compound inhibits only the two human pancreatic trypsins. Our data suggest that a cocktail of aprotinin + eglin c would be much more beneficial for this disease, since eglin c rapidly forms tight complexes with human pancreatic chymotrypsin A and elastase and thus efficiently complements the action of aprotinin.

Large quantities of aerosol-delivered MPI are required to inhibit neutrophil elastase in the airways of cystic fibrosis patients [25]. A significant part of this inhibitor reaches the stomach [26]. Our data show that the human trypsin and chymotrypsin inhibitory activity of MPI are destroyed by human gastric juice with a half-life of 1 h. It is therefore likely that the inhibitor present in the stomach reaches the duodenum in an inactive form. Hence, treatment of cystic fibrosis patients with aerosolized MPI does not aggravate their pancreatic insufficiency.

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