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Interaction between Duodenase and α_1-Proteinase Inhibitor

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Abstract—The interaction between duodenase, a newly recognized serine proteinase belonging to the small group of Janus-faced proteinases, and α_1-proteinase inhibitor (α_1-PI) from human serum was investigated. The stoichiometry of the inhibition was 1.2 mol/mol. The presence of a stable enzyme–inhibitor complex was shown by SDS-PAGE. The mechanism of interaction between duodenase and α_1-PI was shown to be of the suicide type. The equilibrium and inhibition constants are 13 ± 3 nM and $(1.9 \pm 0.3) \cdot 10^5$ $M^{-1} \cdot sec^{-1}$, respectively. Based on the association rate constant of the enzyme–inhibitor complex and localization of duodenase and α_1-PI in identical compartments, α_1-PI is suggested to be a duodenase inhibitor *in vivo*.

Key words: duodenase, α_1-proteinase inhibitor

The duodenum is the central organ of the digestive system. Zymogen (catalytically inactive proenzyme) forms of digestive proteinases are secreted into the duodenum from the pancreas and are activated via proteolytic cleavage of a specific peptide bond near the N-terminus of the molecule. Activated proteinases (trypsin, chymotrypsin, etc.) hydrolyze proteins to peptides; peptidases then degrade the latter to amino acids, which can be absorbed by the intestinal mucosa. Elucidation of the mechanism of activation of proteinases secreted by the pancreas will increase understanding of the processes of digestion of proteins.

Duodenase, a newly described serine proteinase from bovine intestine having molecular mass 30 kD, is localized in secretory granules of epithelial cells of Brunner's glands of the duodenal mucosa [1]. Duodenase has been shown to participate in activation of proenteropeptidase, a single-stranded precursor of enteropeptidase [2]. Enteropeptidase, an internal membrane protein of enterocytes of the upper part of the small intestine, plays a leading role in activation of trypsin by N-terminal processing; this results in sequential activation of other pancreatic zymogens by trypsin [3]. Thus, activating proenteropeptidase, duodenase can play the main role in initiation of a sequence of proteolytic cleavage reactions resulting in activation of pancreatic enzymes.

The substrate specificity of duodenase is unusual, combining the properties of trypsin- and chymotrypsin-like proteinases. With respect to synthetic substrates, the trypsin-like duodenase activity manifests itself more clearly than the chymotrypsin-like activity. However, interacting with protein proteinase inhibitors as protein substrate analogs, duodenase exhibits its chymotrypsin-like properties. Thus, duodenase binds to the antichymotrypsin site (Leu-Ser) of Bowman–Birk type inhibitor from soybean (BBI), whereas the antitrypsin site (Lys-Ser) of the inhibitor remains vacant [4].

The goal of this work was to study the interaction between duodenase and α_1-proteinase inhibitor (α_1-PI) from human serum [5]. α_1-PI, a protein with molecular mass 54 kD [5], is an inhibitor of the serpin group and participates in regulation of a sequence of proteolytic processes such as blood coagulation, complement activation, fibrinolysis, and inflammation [6]. Study of the interaction between duodenase and serpins may clarify the normal regulation of duodenase and that in inflammation or in intestinal tissue damage.

MATERIALS AND METHODS

The following reagents were used in this study: bovine trypsin and α-chymotrypsin and N,N,N′,N′-

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tetramethylethylenediamine (TEMED) from Merck (Germany); α_1-PI from Miles (Canada); Tris-HCl, acrylamide, and bis-acrylamide from ICN (USA); ethyl ester of N-benzoyl-L-arginine (Bz-Arg-EE), ethyl ester of N-benzoyl-L-tyrosine (Bz-Tyr-EE), Suc-Ala-Ala-Pro-Phe-pNA, and Tos-Gly-Pro-Lys-pNA from Sigma (USA); TSK-Gel Toyopearl HW-50 from ToyoSoda MFG (Japan); ammonium persulfate and Coomassie Brilliant Blue R-250 from Reanal (Hungary). Duodenase preparation was kindly given by Dr. E. Sokolova of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry. Other reagents were of extra pure, chemically pure, and analytically pure grade.

All spectrophotometric measurements were performed using a Shimadzu UV-265 FW spectrophotometer (Japan).

The active-site concentration in trypsin determined according to Chase and Shaw [7] was 61%.

Purification and determination of activity of α_1-PI. A commercial preparation was purified by gel filtration on a Toyopearl HW-50 column (eluent, dilute HCl, pH 3.0). The main protein peak was pooled and lyophilized. The active inhibitor content determined by titration with trypsin was 75%.

Determination of duodenase activity. Protein concentration in the duodenase preparation was assayed according to Lowry [8]. The active-site concentration in duodenase was assayed by titration with BBI of known activity [4] and also spectrophotometrically by the rate of *p*-nitroaniline release (molar extinction $\varepsilon_{410} = 8{,}800\ M^{-1}\cdot cm^{-1}$) during hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA at 25°C, using k_{cat} and K_m values 0.15 sec^{-1} and 0.56 mM, respectively.

Isolation and determination of activity of BBI. Classical Bowman–Birk inhibitor (BBI) was isolated from soybean by a method developed by us earlier [9]. The concentration of the active inhibitor in the BBI preparation assayed by its suppression of the activity of trypsin and α-chymotrypsin was 100%.

Determination of k_{cat} and K_m. Kinetic parameters of hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA and Tos-Gly-Pro-Lys-pNA were determined from the dependence of the rate of their hydrolysis on the substrate concentration. The constants were determined from the equations of Lineweaver–Burk or Idie [10]; for Tos-Gly-Pro-Lys-pNA, k_{cat} and K_m were 13 sec^{-1} and 0.37 mM, respectively.

Dependence of remaining enzymatic activity of duodenase on time of incubation with α_1-PI. The remaining activities of duodenase during incubation with α_1-PI at enzyme/inhibitor concentration ratios 0.5 : 1, 1 : 1, and 2 : 1 were assayed by suppression of the duodenase activity. All kinetic measurements were performed in 0.05 M Tris-HCl buffer, pH 8.0, at 25°C in a thermostatted cuvette; Suc-Ala-Ala-Pro-Phe-pNA was used as the substrate. The initial amidase activity was assayed as follows: 0.277 ml of buffer, 0.01 ml of 0.33 μM duodenase aqueous solution, and 0.013 ml of 10 mM substrate solution were placed in the cuvette; the optical density was monitored spectrophotometrically at 410 nm. To assay the remaining enzymatic activity, $(0.277 - x)$ ml of the buffer, 0.01 ml of duodenase solution, and x (0.01, 0.02, or 0.04) ml of α_1-PI solution in the working buffer were placed in the cuvette. The mixture was incubated for 2-240 min, then 0.013 ml of the substrate solution was added and the reaction rate monitored. The remaining enzymatic activity versus the incubation time was plotted at various inhibitor concentrations.

Determination of the stoichiometry of interaction between duodenase and α_1-PI. The stoichiometry of interaction between duodenase and α_1-PI was estimated by suppression of the amidase activity of duodenase by the inhibitor after preincubation of the enzyme with the inhibitor for 20 or 40 min. The remaining enzymatic activity versus the inhibitor concentration was plotted, the equivalence point was determined by linear extrapolation, and then the stoichiometry of the process was calculated.

Determination of inhibition constant (K_i). K_i was determined according to Duranton [11] by estimation of the remaining enzymatic activity at various inhibitor concentrations and constant enzyme concentration; the reaction was initiated by addition of the enzyme to the substrate–inhibitor mixture.

Determination of the association rate constant (k_{as}). The working buffer (0.38 ml), 30 nM duodenase solution (0.5 ml), and α_1-PI solution in the working buffer (0.02 ml) were placed in a 1-ml cuvette. The mixture was incubated for 5-40 min, and then 10 mM Tos-Gly-Pro-Lys-pNA solution in dimethylsulfoxide (0.05 ml) was added. The optical density was monitored spectrophotometrically at 410 nm. The remaining duodenase activity was plotted versus the inhibitor concentration. This dependence was approximated using Eq. (1):

$$v/v_o = 1/(1 + [E]_o \cdot k_{as} \cdot t), \quad (1)$$

where v_o and v are the initial reaction rate and the reaction rate at the time moment t, respectively.

SDS-PAGE analysis. SDS-PAGE of α_1-PI and the α_1-PI–duodenase complex was performed using a Mini Protean II (Bio-Rad, USA) in 8 and 12% gel, pH 8.3 [12], with 5.9 μg of protein per lane. Electrophoresis was performed for 40 min at 20°C with voltage 200 mV. The gels were stained for 15 min with solution containing 0.15% Coomassie Brilliant Blue R-250, 30% methanol, and 10% acetic acid and washed for one day with solution containing 10% methanol and 10% acetic acid.

Samples for electrophoresis were prepared as follows: a mixture of 15 μl of duodenase preparation (3 μM,

1.5 μg) and 4 μl of α_1-PI solution (21 μM, 4.4 μg) was incubated for 0.5-120 min; then 2 μl of 2 mM phenylmethylsulfonyl fluoride and 6 μl of fivefold buffer for the sample were added. Before applying on the plate, samples were kept on a water bath (60°C) for 10 min and centrifuged.

RESULTS AND DISCUSSION

The mechanism of action of serpins and the structure of their complexes with proteinases are still not well known. Proteinase inhibitors of the serpin group (*ser*ine *p*roteinase *in*hibitors) interact with serine proteinases via a suicide mechanism, which suggests a branching of reaction with irreversible formation of a covalent complex with a certain proteinase and release of a free active enzyme and inactive inhibitor (Fig. 1) [13]. Thus, along with formation of stable complexes with proteinases, serpins can be their substrates. Irreversibility and branching of the inhibition process with formation of a covalent complex makes serpins different from all other groups of serine proteinase inhibitors such as Kunitz, Casal, and Bowman–Birk inhibitors, and proteinase inhibitors of the potato family, which form tight but dissociating noncovalent complexes with proteinases.

We began studying the interaction between duodenase and α_1-PI by investigating how the inhibition proceeds with time. The dependence of the remaining activity of duodenase versus incubation time with α_1-PI is presented in Fig. 2. As shown, the minimal duodenase activity is observed on incubation of the enzyme with the inhibitor for 40 min at all enzyme/inhibitor ratios. Then recovery of the enzymatic activity to approximately 50% is observed.

It is known from the literature that recovery of the enzymatic activity is not typical of every serpin–proteinase pair. Thus, some target proteinases of corresponding serpins bind to them irreversibly, and recovery of the enzymatic activity is not observed with time. The granulocyte fraction of polymorphonuclear human leukocytes contains equal quantities of two serine proteinases, HLE and cathepsin G, which are considered to be the most destructive enzymes of the human body [14]. In blood serum, there is a certain physiological inhibitor corresponding to each of these enzymes: α_1-PI with HLE [14] and α_1-antichymotrypsin (ACT) with cathepsin G, respectively [15]. In the normal state, these enzymes are complexed with the corresponding serpins, and the active enzymes are not released [11]. Binding to ACT, chymotrypsin forms an initially covalent complex with conformational changes, which then transforms into another complex; the presence of the latter was shown by SDS-PAGE [13]. This process is accompanied by release of the active enzyme. Another extreme case is the interaction between serpins and metalloproteinases; for the latter, these inhibitors are substrates, and loss of enzymatic activity is not observed at all [16, 17].

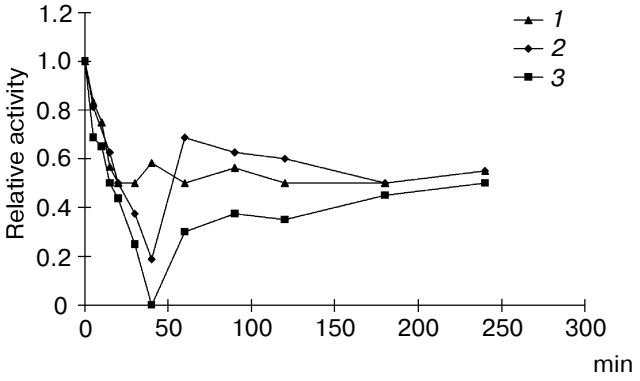

Fig. 2. Remaining activity of duodenase versus incubation time with α_1-PI. Concentrations: $4.3 \cdot 10^{-4}$ M Suc-Ala-Ala-Pro-Phe-pNA; [E] = 11 nM; 0.05 M Tris-HCl, pH 8.0, 25°C. $[I]_o$: 5.5 (*1*), 11 (*2*), and 22 nM (*3*).

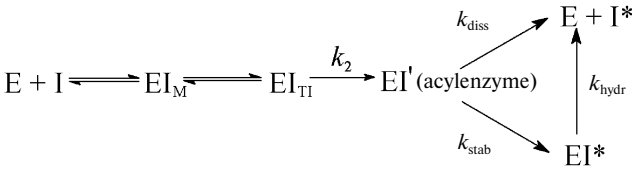

Fig. 1. The suicide mechanism of proteinase inhibition by serpins. EI_M, Michaelis enzyme–inhibitor complex; EI_{TI}, tetrahedral intermediate; EI′, acylenzyme; EI*, covalent complex in which the inhibitor is in a degraded state (a reactive site loop is inserted into the β-layer of A); I*, degraded inhibitor.

Incomplete inhibition of duodenase by α_1-PI at their ratio 1 : 1 indicates that the inhibitor acts via a suicide mechanism (Fig. 1).

The reaction products are divided between the degraded inhibitor I* and a covalent complex of proteinase with degraded inhibitor in the ratio regulated by the stoichiometry of inhibition (SI) = $1 + k_{diss}/k_{stab}$ [11]; SI is an important characteristic of this process. Thus, in the case of serpins, the stoichiometry of their interaction with proteinases is determined by the dissociation and acylenzyme stabilization rate constants rather than the active site content of the enzyme. Stoichiometry is characteristic of the interaction of each particular serpin–proteinase pair, since it does not depend on the enzyme concentration [18].

The results of studying stoichiometry of duodenase–α_1-PI reaction are presented in Fig. 3. The stoichiometry of the process was determined for two incubation time intervals: at the minimal enzymatic activity

(incubation of the enzyme with inhibitor for 40 min) and for 20-min incubation. To show independence of stoichiometry from the enzyme concentration, two concentrations of duodenase, 11 and 110 nM, were used. The reaction stoichiometry was 1.2 ± 0.1 for 40-min incubation and at various duodenase concentrations and 2.3 ± 0.2 for 20-min incubation. Thus, it was demonstrated that for the α_1-PI–duodenase pair the stoichiometry also does not depend on the enzyme concentration, but it does depend on the incubation time. The fact that stoichiometries differ approximately twofold confirms the dependence of the remaining enzymatic activity on the incubation time (Fig. 2). Stoichiometry can also determined from this dependence. At the minimal enzymatic activity, for duodenase/α_1-PI ratio 1 : 1, 80% inhibition is observed; hence, 100% inhibition will be observed for duodenase/α_1-PI ratio 1 : 1.2. The stoichiometry value more than 1 confirms the suicide mechanism of interaction between α_1-PI and duodenase.

Analysis of the interaction between duodenase and α_1-PI by SDS-PAGE. To clearly demonstrate the formation of a stable enzyme–inhibitor complex, SDS-PAGE under non-dissociating conditions in 8, 12, and 18% gels was performed. For electrophoresis, the enzyme concentration should be not less than 1 μM; this is two orders of magnitude more than the concentrations we use in kinetic experiments. Thus, in this case the inhibitory complex is formed after several seconds and the method does not allow us to demonstrate the kinetics of accumulation of the EI* complex. As shown in Fig. 4, a stable complex is formed even after a short incubation time. A minor band of a complex (approximately 60 kD), intermediate between a band of degraded inhibitor and a main band of the enzyme–inhibitor complex, is also observed. As a structure typical of the interaction between various chymases and α_1-PI, this complex is mentioned in the literature but its nature is not discussed [18].

Determination of K_i. K_i was measured by a method based on competition of substrate and inhibitor for binding to the enzyme (the initial substrate–inhibitor complex is formed as fast as the enzyme–substrate complex). Significant inhibition of the enzyme was observed at inhibitor concentrations close to the enzyme concentrations (Fig. 5). The apparent equilibrium inhibition constant ($K_{i(app)}$) is 21 ± 4.5 nM; the true inhibition constant is 13 ± 3 nM.

Determination of the association rate constant of duodenase with α_1-PI. To quantitatively characterize the inhibition of duodenase by α_1-PI, a simplified reaction scheme was used:

$$E + I \rightleftarrows EI_M \rightarrow EI^*.$$

If the inhibitor concentration is significantly lower than the apparent equilibrium inhibition constant

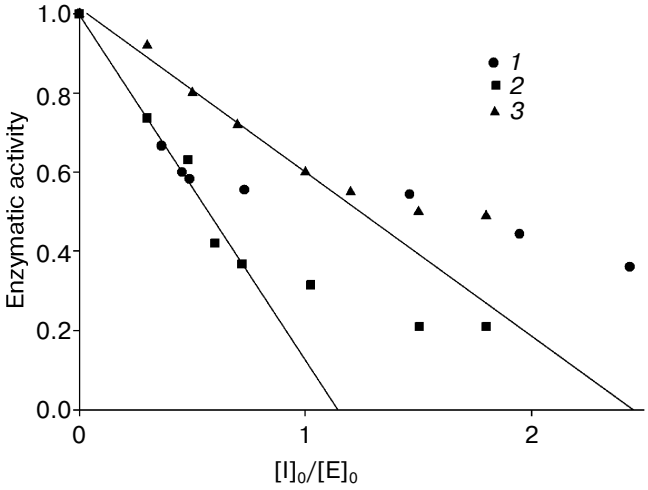

Fig. 3. Determination of stoichiometry of interaction between α_1-PI and duodenase. Concentrations: 0.43 mM Suc-Ala-Ala-Pro-Phe-pNA, 0.05 M Tris-HCl buffer, pH 8.0, 25°C. *1*) [E] = 11 nM, incubation time 40 min; *2*) [E] = 110 nM, incubation time 40 min; *3*) [E] = 11 nM, incubation time 20 min.

Fig. 4. SDS-PAGE in 8% gel. Concentrations: $[E]_o$ = 1 μM, $[I]_o$ = 3 μM. [EI]*, a covalent enzyme–inhibitor complex; [I], native α_1-PI; [I]*, degraded α_1-PI. The free enzyme band moves together with the dye front. Lane *1*, initial inhibitor preparation. Incubation times: 30 sec (*2*); 2 (*3*); 10 (*4*); 20 (*5*); 40 (*6*); 90 (*7*), and 120 min (*8*).

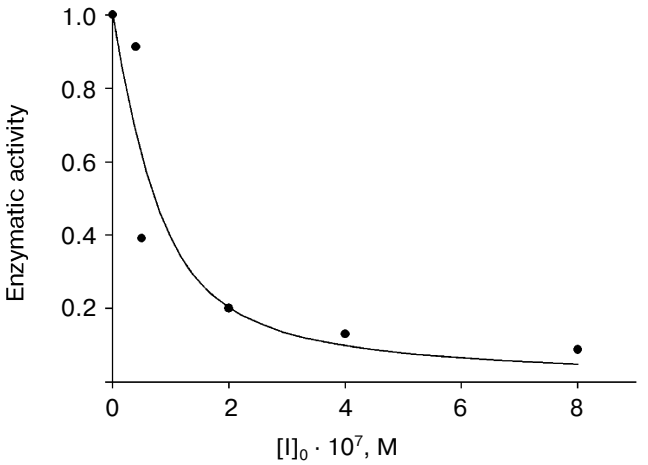

Fig. 5. Determination of K_i for the interaction between duodenase and α_1-PI. Concentrations: 0.43 mM Suc-Ala-Ala-Pro-Phe-pNA, $[E]_o$ = 11 nM, 0.05 M Tris-HCl buffer, pH 8.0, 25°C. The curve was calculated using Duranton's equation [11].

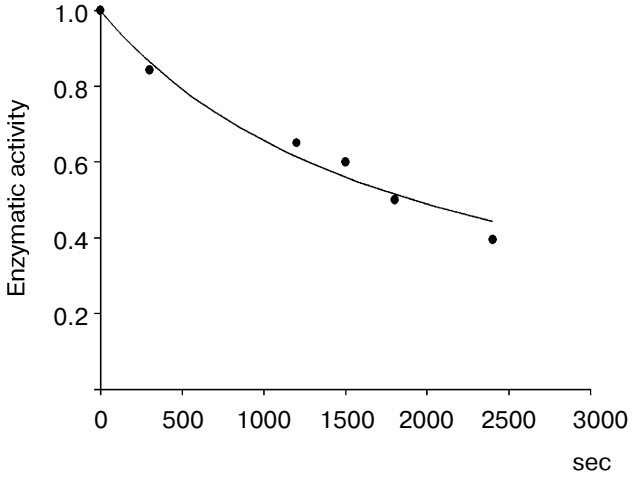

Fig. 6. Determination of k_{as} for the interaction between duodenase and α_1-PI. Concentrations: 1.04 mM Tos-Gly-Pro-Lys-pNA, $[E]_o = [I]_o = 2$ nM, 0.05 M Tris-HCl buffer, pH 8.0, 25°C.

($K_{i(app)}$), the inhibition proceeds as a bimolecular reaction with the second-order rate constant k_{as} (Fig. 6). The value of k_{as} calculated from Eq. (1) is $(1.9 \pm 0.3)\cdot 10^5$ $M^{-1}\cdot sec^{-1}$.

The value of k_{as} allows calculation of the time necessary for essentially complete development of the proteinase inhibition reaction, $t_{99\%}$, using Eq. (2) by Bieth [14].

$$t_{99\%} = 5/k_{as}\cdot[I]_o. \quad (2)$$

Thus, for inhibitor concentration 11 nM, $t_{99\%} = 38$ min is obtained (using $k_{as} = 2.2\cdot 10^5$ $M^{-1}\cdot sec^{-1}$). This value is confirmed by the data obtained during study of dependence of the remaining duodenase activity versus its incubation time with α_1-PI (Fig. 2). Along with this, using Eq. (2), it can be shown kinetically that under the conditions of electrophoresis at the duodenase concentration of 1 μM, the enzyme–inhibitor complex is formed in approximately 1 sec.

Association rate constants of α_1-PI with serine proteinases

Enzyme	k_{as}, $M^{-1}\cdot sec^{-1}$		
	sheep α_1-PI	horse α_1-PI	human α_1-PI
Bovine duodenase			$1.9 \cdot 10^5$
SMCP-1	$1.1 \cdot 10^3$ [22]	$7.5 \cdot 10^2$ [22]	
Human cathepsin G			$6.8 \cdot 10^4$ [11]
Bovine α-chymotrypsin	$1.3 \cdot 10^6$ [21]	$1.7 \cdot 10^6$ [22]	$8.0 \cdot 10^5$ [24]

Duodenase is a homolog of human granulocyte cathepsin G [19, 20] and sheep mast cell proteinase SMCP-1 [21]. All the three enzymes are members of a small group of so-called Janus-faced proteinases with unusual dual trypsin- and chymotrypsin-like substrate specificity [7]. Cathepsin G interacts with α_1-PI with stoichiometry 1.15 [11], which is close to the corresponding value for duodenase. Human mast cell chymase interacts with α_1-PI with stoichiometry 4.5 [18]; this indicates that α_1-PI is a better substrate for this enzyme.

Let us compare proteinases with dual specificity in the degree of suppression of their activity by α_1-PI. The true inhibition constant of cathepsin G by this inhibitor is 125 nM [11]; this is one order of magnitude larger than for duodenase. Pemberton et al. [21] studied the interaction of SMCP-1 with sheep and horse α_1-PI: horse α_1-PI suppresses trypsin- and chymotrypsin-like activity (that is, activity estimated against trypsin and chymotrypsin substrates) by 90% [22]; sheep α_1-PI is virtually identical to human α_1-PI and is also a highly efficient inhibitor of granulocyte elastase and trypsin [23]. The inactivation rate of a certain proteinase by the inhibitor is a criterion of the functional activity of the protein inhibitor. The association rate constants of the mentioned proteinases and chymotrypsin with human, sheep, and horse α_1-PI are summarized in the table. Human α_1-PI binds to bovine duodenase almost three times more efficiently than human granulocyte cathepsin G and by almost two orders of magnitude more efficiently than sheep α_1-PI binds to SMCP-1.

Now let us go back to the earlier mentioned dual specificity of duodenase and SMCP-1 and consider how α_1-PI inhibits duodenase and SMCP-1 compared to bovine α-chymotrypsin. Human α_1-PI binds to duodenase four times less efficiently and sheep α_1-PI inhibits SMCP-1 by three orders of magnitude less efficiently compared to bovine α-chymotrypsin. Thus, in spite of high structural identity (more than 80%) and similar substrate specificity, duodenase and SMCP-1 are significantly different with respect to α_1-PI.

α_1-PI is mainly synthesized in liver cells and, due to its relatively low molecular mass, is distributed among blood vessels and extra-vessel space [25]. However, there are evidences for its presence in epithelial cells of the small intestine [26], human pancreatic juice [27], and in Brunner's glands of the duodenal mucosa [28]. Thus, duodenase and α_1-PI are localized in the same compartments of intestinal mucosa and consequently, α_1-PI may be considered as a potential endogenous inhibitor of this enzyme. It should be noted that α_1-PI concentration in

pancreatic juice increases threefold in chronic pancreatitis [26]. In addition, with inflammation or tissue damage the intestinal mucosa may be exposed to blood; as a result, α_1-PI can regulate duodenase activity.

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