Activation of recombinant proenteropeptidase by duodenase

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Abstract Duodenase, a serine proteinase from bovine Brunner's (duodenal) glands that was predicted to be a natural activator of enteropeptidase zymogen, cleaves and activates recombinant single-chain bovine proenteropeptidase ($k_{cat}/K_m = 2700$ M^{-1} s^{-1}). The measured rate of proenteropeptidase cleavage by duodenase was about 70-fold lower compared with the rate of trypsin-mediated cleavage of the zymogen. The role of duodenase is supposed to be the primary activator of proenteropeptidase maintaining a certain level of active enteropeptidase in the duodenum. A new scheme of proteolytic activation cascade of digestive proteases is discussed.

Key words: Duodenase; Proenteropeptidase activation; Serine protease; Duodenal mucosa

1. Introduction

The duodenum is the central part of the digestive system. Normal function of the duodenum is important for health. Digestion in the duodenum depends on various enzymatic processes in which numerous enzymes of pancreatic (trypsin, chymotrypsins, carboxypeptidases, kallikrein and elastase) and intestinal origin take part. Pancreatic enzymes are synthesized as inactive forms and activated in the duodenum by a proteolytic cascade [1,2]. After hydrolysis by pancreatic proteinases dietary proteins are metabolized by peptidases of the mucosal epithelial cells into amino acids, which are absorbed through the epithelium [3].

A new proteolytic enzyme, duodenase, was discovered by us in the bovine duodenal mucosa [4,5]. The primary structure of the enzyme is related to that of chymases from mast cells, granzymes from cytotoxic T-lymphocytes and cathepsin G from leukocytes [6]. Nevertheless, duodenase has no relation to haemopoietic cells. It is the first protease localized in secretory granules of epitheliocytes in the Brunner's (duodenal) glands presented in the duodenal mucosa [7]. The enzyme as constituent of the gland secrete enters duodenal slit and this suggests duodenase functions extracellularly. Duodenase has unique amino acid residues within its substrate binding pocket [6], determining unusual specificity of the enzyme. The substrate specificity of duodenase is dual: trypsin-like and chymotrypsin-like. However, it hydrolyzes protein substrates yielding large and stable fragments [5]. This fact allows us to suggest that duodenase belongs to the group of processing enzymes. It was supposed that duodenase is the primary activator of proenteropeptidase [7].

Enteropeptidase is a intrinsic membrane protein of enterocytes of the proximal small intestine. The enzyme plays the key role in the digestive cascade, cleaving the N-terminal activation peptide from trypsinogen to produce trypsin, thereby leading to subsequent activation of other pancreatic zymogens by trypsin [2]. Enteropeptidase is synthesized as single chain precursor which apparently undergoes proteolytic processing. The questions concerning the mechanism of enteropeptidase activation and the identification of the enzyme or enzymes responsible for proenteropeptidase processing has been a focus of many investigations [8–12].

The exclusive localization of duodenase in the duodenum, its high content in this tissue and its potential role in initiating of the activation cascade of digestive hydrolases (activation of proenteropeptidase) permits to speculate the importance of duodenase in the function of the duodenum.

To prove the role of this enzyme, we studied activation of the recombinant single-chain bovine enteropeptidase precursor by duodenase.

2. Materials and methods

2.1. Materials

Recombinant proenteropeptidase and duodenase were prepared as described previously [5,12]. Native bovine enteropeptidase was kindly provided by Dr. A.G. Mikhaylova (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia). The chromogenic substrate glutamyl-glycyl-arginine 4-nitroanilide (Glu-Gly-Arg-pNA) was kindly provided by Prof. V.A. Tkachuk (Cardiology Centre, Moscow, Russia). The enteropeptidase substrate glycyl-tetra-aspartyl-lysine β-naphthylamide (Gly-$(Asp)_4$-Lys-NA) was purchased from Bachem, Germany. Bovine trypsin was from Miles Lab. Research Products, Republic of South Africa. Chicken egg white ovomucoid was from Serva, Germany. Soybean trypsin inhibitor and bovine trypsinogen were purchased from Sigma, USA.

2.2. Determination of the concentration and purity of the enzyme preparations

Concentration of active duodenase was determined using titration with soybean trypsin inhibitor as described earlier [7]; trypsin solution was titrated with *p*-nitrophenyl-*p*'-guanidinobenzoate [13]. Enzymatic purity of duodenase was confirmed by SDS–PAGE using substrate gel with co-polymerized azocasein [14]. Purity of duodenase and trypsin preparations used in the study was confirmed additionally by comparative study of their proteolytic action on melittin and proinsulin [15].

2.3. Successive activation of zymogens

The usual method for assaying enteropeptidase by activation of trypsinogen and subsequent measuring of the generated trypsin [16] was modified in our study. Recombinant proenteropeptidase (15 nM) was incubated with duodenase (13 nM) in 10 mM Tris–HCl, pH 8.0 at 37°C. Samples (5 μl) were withdrawn after certain time periods and

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Abbreviations: -pNA, 4-nitroanilide; -NA, β-naphthylamide

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added to 245 μl of a reaction mixture containing trypsinogen (0.4 μM), ovomucoid (4 μM), and 0.05 M $CaCl_2$ in 0.1 M Na–acetate buffer (pH 5.0). The control sample contained proenteropeptidase, trypsinogen, and ovomucoid in the same buffer. The reaction mixture was incubated for 30 min at 37°C, and then 25 μl of 1 M HCl were added to achieve a final pH of 2.0 in order to inactivate enteropeptidase and dissociate the trypsin–ovomucoid complex. The activity of the formed trypsin was determined spectrophotometrically at 405 nm from hydrolysis of Glu-Gly-Arg-pNA at 37°C. Trypsin-containing sample (5 μl) was added to the solution of the substrate (0.8 mM; 500 μl) in 0.1 M Tris–HCl (pH 8.0), containing 0.05 M $CaCl_2$. The hydrolysis rate was measured from the initial slope of the charted curve.

2.4. Direct monitoring of enteropeptidase activity

Proenteropeptidase (38 nM) and duodenase (13 nM) were incubated at 37°C in 30 mM Tris–HCl, pH 8.0. At certain times, samples were withdrawn, and the reaction was stopped by adding soybean trypsin inhibitor to a final duodenase/inhibitor ratio of 1/10. Enteropeptidase activity was determined using the enteropeptidase substrate Gly-$(Asp)_4$-Lys-NA with a Hitachi 650-60 (Japan) fluorescence spectrophotometer at 37°C. The reaction mixture (300 μl) contained 0.1 M Tris–HCl (pH 8.0), 0.05 M $CaCl_2$, and 0.1 mM substrate. The reaction was initiated by adding samples containing the activated enteropeptidase. The concentration of liberated β-naphthylamine was determined from the fluorescence increment ($\lambda_{ex} = 330$ nm and $\lambda_{em} = 410$ nm).

The concentration of recombinant proenteropeptidase was assumed to be equal to the concentration of active enteropeptidase released after total activation of proenteropeptidase by trypsin [12].

The k_{cat}/K_m value was determined at the concentrations of proenteropeptidase and duodenase of 70 nM and 5 nM, respectively, using the equation $v = (k_{cat}/K_m)[E]_0[S]$, when $[S] \ll K_m$ [17]. All steps were performed as described above. The concentration of duodenase-activated enteropeptidase was calculated using the rate of liberation of β-naphthylamine and kinetic parameters of Gly-$(Asp)_4$-Lys-NA hydrolysis by native enteropeptidase at pH 8.0 [18].

2.5. Activation of proenteropeptidase: analysis by SDS–PAGE

The reaction mixture (8.5 μl) contained recombinant proenteropeptidase (66 nM) and duodenase (6 nM) in 0.03 Tris–HCl, pH 8.0. Duodenase was absent in the control mixture. The mixtures were incubated at 37°C for 3 h; then reducing sample loading buffer containing 2-mercaptoethanol was added. SDS–PAGE was performed in 10% gels according to Laemmli [19]. Silver staining was performed as described by Damerval et al. [20].

3. Results

3.1. Successive activation of zymogens

A mini-cascade of zymogen activation was studied including activation of proenteropeptidase by duodenase and subsequent activation of trypsinogen by enteropeptidase. The experimental design (see Section 2) included preincubation of proenteropeptidase with duodenase at pH 8.0 and 37°C. At certain times, samples containing activated enteropeptidase were withdrawn and added to trypsinogen solution (pH 5.0, to minimize autoactivation). The released trypsin was monitored by hydrolysis of the substrate Glu-Gly-Arg-pNA. Under these conditions, proenteropeptidase activation by duodenase was observed. However, the trypsin formed during the reaction also activated an additional amount of proenteropeptidase, complicating the analysis of proenteropeptidase cleavage by duodenase.

To avoid this effect, ovomucoid was included in the trypsinogen-activation reaction. Before measuring the trypsin activity, the trypsin–inhibitor complex was dissociated by decreasing the pH to 2. A high substrate concentration (0.8 mM Glu-Gly-Arg-pNA) was chosen to prevent reassociation of this complex in the reaction mixture at pH 8.0. In the presence of ovomucoid, the interference from proenteropeptidase activation by trypsin was decreased but not eliminated. The increase of the trypsin activity in the analyzed samples was proportional to the time of incubation of duodenase with proenteropeptidase, that confirmed activation of proenteropeptidase by duodenase (Fig. 1A). However, in this assay design we were unable to determine the rate of proenteropeptidase activation by duodenase with sufficient accuracy.

3.2. Determining the k_{cat}/K_m value of proenteropeptidase activation by duodenase

To exclude the confounding effect of trypsin-mediated cleavage of proenteropeptidase, the activation of proenteropeptidase by duodenase was studied using a sensitive fluorescent enteropeptidase substrate Gly-$(Asp)_4$-Lys-NA. The time course of proenteropeptidase (70 nM) activation by 5 nM duodenase (Fig. 1B) indicated that generation of active enteropeptidase was linear over 4 h, and about 20% of the zymogen was converted into the active enzyme. When the duodenase/proenteropeptidase molar ratio was increased to 1:3 (duodenase concentration was 13 nM and proenteropeptidase concentration was 38 nM), about 90% of the precursors were activated within 3 h. No autocatalytic cleavage or activation was observed in the absence of duodenase.

The concentrations of proenteropeptidase tested (up to 70 nM) were not sufficient to allow determination of k_{cat} and K_m for cleavage of the zymogen by duodenase. Because the concentration of proenteropeptidase in our experiments was much smaller than the predicted K_m value for the duodenase cleavage (> 1 μM), it was possible to estimate only the k_{cat}/K_m value using the equation $v = (k_{cat}/K_m)[E]_0[S]$ (when $[S] \ll K_m$) [17]. The calculated k_{cat}/K_m value for duodenase activation of proenteropeptidase was 2700 M^{-1} s^{-1}.

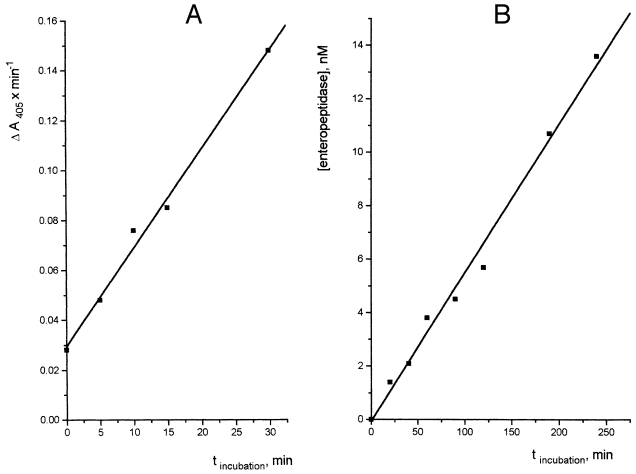

Fig. 1. Time course of proenteropeptidase activation by duodenase. A: Proenteropeptidase (15 nM) was incubated with duodenase (13 nM) in 10 mM Tris–HCl, pH 8.0 at 37°C. At the indicated times, samples (5 μl) were assayed for enteropeptidase activity by incubation with trypsinogen and subsequent measurement of trypsin as described in Section 2. B: Proenteropeptidase (70 nM) was incubated with duodenase (5 nM) in 10 mM Tris–HCl, pH 8.0 at 37°C. At the indicated times, samples (10 μl) were withdrawn, and the reaction was stopped by adding soybean trypsin inhibitor (duodenase/inhibitor ratio was 1:10). Enteropeptidase activity was determined using Gly-$(Asp)_4$-Lys-NA as described in Section 2.

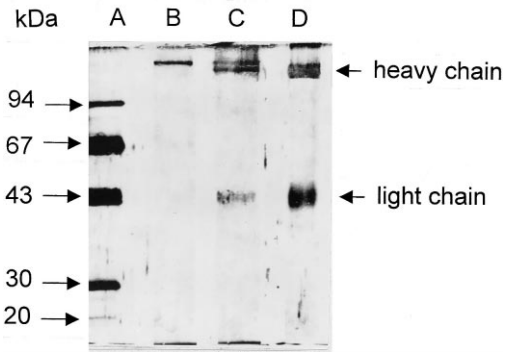

Fig. 2. Cleavage of recombinant proenteropeptidase by duodenase (SDS–PAGE). Samples (see Section 2) were loaded onto a 10% gel and electrophoresed according to Laemmli [19] under reducing conditions (2% 2-mercaptoethanol). Proteins were visualized using the silver-staining method [20]. Lane A, molecular weight standards (Pharmacia, Sweden); lane B, control of proenteropeptidase (0.5 pmol) incubated without duodenase for 3 h; lane C, proenteropeptidase (0.5 pmol) incubated with duodenase for 3 h; lane D, native bovine enteropeptidase (2 pmol).

3.3. SDS–PAGE analysis of proenteropeptidase activation by duodenase

The reaction mixture containing proenteropeptidase (66 nM) was incubated with duodenase (6 nM) for 3 h (pH 8.0; 37°C) and analyzed by SDS–PAGE in comparison with control sample of proenteropeptidase and native enteropeptidase from the bovine duodenal mucosa (Fig. 2). Upon gel electrophoresis under reducing conditions proenteropeptidase migrated as a single protein band of about 150 kDa (Fig. 2, lane B). Incubation of proenteropeptidase with duodenase resulted in proenteropeptidase cleavage to produce the fragments of apparent masses of 130–140 and 43 kDa (Fig. 2, lane C). The apparent molecular masses of the obtained fragments of recombinant enteropeptidase agree to those of native enteropeptidase heavy and light chains, respectively (Fig. 2, lane D). The protein bands of the native enteropeptidase are diffuse due to its multiple glycoforms.

4. Discussion

Enteropeptidase is a membrane-bound serine proteinase synthesized by enterocytes of the duodenal mucosa [21]. The biological role of this enzyme is conversion of trypsinogen into trypsin, which is a key step in the activation of other digestive hydrolases [1]. Bovine enteropeptidase is synthesized as a single-chain precursor of 1035 amino acid residues [22]. The precursor requires proteolytic activation. Consequently, enteropeptidase may not be the 'first' protease of the digestive hydrolase cascade. Active enteropeptidase is a disulfide-linked heterodimer consisting of a 120–135 kDa heavy chain and of a 42–47 kDa light chain [23]. The activation cleavage site between the heavy and light chains has the sequence Val-Ser-Pro-Lys↓Ile, which might be recognized by trypsin or other trypsin-like proteinases. An increase in the enteropeptidase activity in the presence of trypsin was described by V.V. Savich in his dissertation 'Separating of intestinal juice' in 1904. As recently was demonstrated, trypsin efficiently activated recombinant proenteropeptidase [12]. The existence of insoluble enteropeptidase precursor, which is present in the upper part of the intestine and able to be activated by trypsin, was predicted in 1967 by G.K. Shlygin [24]. The author discussed the pathway of digestive proteinase activation, including reciprocal activation of enteropeptidase precursor and trypsinogen. The mechanism of the primary initiation of the closed trypsin-enteropeptidase cycle was also discussed. The assumption of alternative, trypsin-independent activation of the enteropeptidase zymogen was based on the results of experiments of Shlygin [24]. In the investigation, pancreatic ducts of a dog were blocked, what led to significant decrease in the enteropeptidase activity in the duodenum. However, the absolute concentration of the active enteropeptidase remained still high even 3 months after blocking of the pancreatic secretion. Hence, some activator of enteropeptidase precursor of non-pancreatic origin was believed to be present in the intestine.

Duodenase, a serine proteinase from the bovine duodenal mucosa, was earlier predicted to be a natural activator of enteropeptidase precursor [7]. This suggestion was made taking into account the preference of duodenase for substrates that contain amino acid sequences resembling the activation cleavage site of proenteropeptidases of different origin. Moreover, duodenase in duodenal gland secretions may enter the duodenal lumen and contact enterocytes containing membrane-bound proenteropeptidase. Considering the structural and functional peculiarities of the small intestine and the close relationship between the mucus layer, villi and duodenal glands [25], duodenase meets proenteropeptidase earlier than proenteropeptidase meets trypsin. Therefore, duodenase may be a significant primary activator of proenteropeptidase.

To determine whether duodenase could initiate proenteropeptidase activation, recombinant full-length bovine proenteropeptidase [12] was used as a substrate for duodenase. Proenteropeptidase was found to have no catalytic activity toward effective enteropeptidase substrate Gly-$(Asp)_4$-Lys-NA. When proenteropeptidase was incubated with trypsinogen alone, about 50% of trypsinogen was activated within 30 min. This can occur if traces of trypsin activity in the trypsinogen preparation activate small amounts of proenteropeptidase. The active enteropeptidase, in turn, efficiently activates trypsinogen generating a new portion of active trypsin, what leads to an avalanche-like activation of trypsinogen. In the presence of ovomucoid, which is a potent trypsin inhibitor, the activation of proenteropeptidase during incubation with trypsinogen significantly decreased. This allowed us to investigate the activating properties of duodenase using a coupled 'mini'-cascade of reactions. It included proenteropeptidase cleavage by duodenase as the first step, and trypsinogen cleavage by enteropeptidase as the second step. The formed trypsin was monitored using the substrate Glu-Gly-Arg-pNA. Under these conditions, we observed activation of trypsinogen that was proportional to the time of duodenase incubation with proenteropeptidase (Fig. 1A). As reported previously [7], duodenase did not activate trypsinogen.

Duodenase-catalyzed activation of proenteropeptidase was also observed when the enteropeptidase synthetic substrate Gly-$(Asp)_4$-Lys-NA was substituted for the natural substrate trypsinogen. This allowed us to be sure that the possible effect of the presence of trypsin on the proenteropeptidase activation would be excluded (the enzymatic purity of duodenase was proved as described in Section 2). Proenteropeptidase was unable to cleave the enteropeptidase synthetic substrate even after prolonged incubation with it, but was readily converted to the active enzyme by incubation with duodenase. Omission or inactivation of duodenase prevented any conversion of the

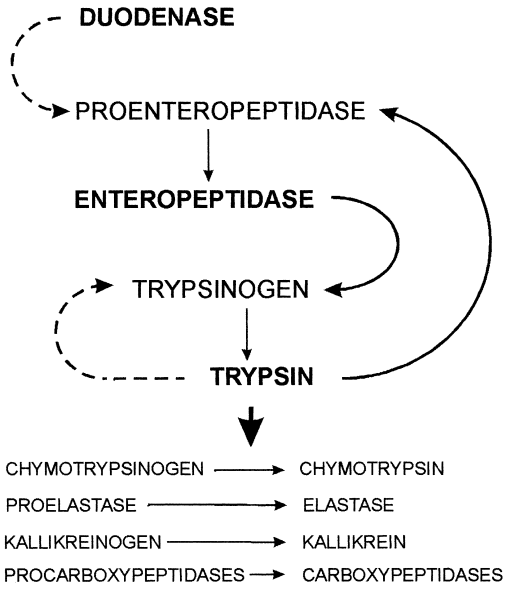

Fig. 3. Role of duodenase in the pancreatic zymogen activation cascade. Dashed arrows: 'slow' activation; solid arrows: 'rapid' activation.

precursor to active enteropeptidase. The activated enteropeptidase had properties very similar to those of native enteropeptidase, that suggested proenteropeptidase had been correctly processed by duodenase. Since the rate of proenteropeptidase processing by duodenase (0.05 nM min^{-1}) was about 70-fold lower than the earlier determined rate of trypsin-mediated activation of the zymogen under similar conditions [12], the prolonged time of incubation with duodenase was about 3 h. It was necessary to obtain readily detectable protein bands corresponding to the proenteropeptidase cleavage products by SDS–PAGE. When the incubation time was over 4 h, the control sample of proenteropeptidase underwent some degradation, making correct interpretation of the SDS–PAGE data difficult. However, the size of the bands generated upon duodenase cleavage of proenteropeptidase (Fig. 2, lane C) corresponded to those of the heavy and light chains of native enteropeptidase (Fig. 2, lane D). This cleavage would occur at the sequence Val-Ser-Pro-Lys↓Ile, that confirms the reported substrate specificity of duodenase [7]. Although we were not able to sequence the generated protein bands because the amount of the protein sample was too small, we believe that the duodenase-mediated processing of proenteropeptidase was correctly performed, since the generated product possessed the same activity and properties as the native enteropeptidase.

The rate of proenteropeptidase processing by duodenase was estimated, and a k_{cat}/K_m value of 2700 M^{-1} s^{-1} was calculated using Gly-$(Asp)_4$-Lys-NA as enteropeptidase substrate. This k_{cat}/K_m value is similar to the value of 2600 M^{-1} s^{-1} for duodenase cleavage of the substrate Suc-Ser-Pro-Lys-pNA, corresponding to the bovine proenteropeptidase activation site [7]. For comparison, duodenase cleaves peptide analogous to the P_9-P_5' sequence (Schechter and Berger nomenclature [26]) of the bovine proenteropeptidase activation site (LVTQEVSPKIVGGS) with the k_{cat}/K_m value of 16 000 M^{-1} s^{-1} [7].

Activities of duodenase, trypsin and enteropeptidase toward proenteropeptidase, trypsinogen and synthetic substrates derived from the zymogens (Suc-Ser-Pro-Lys-pNA (proenteropeptidase) and Gly-$(Asp)_4$-Lys-NA (trypsinogen)) are given in Table 1. From these data it is clear that trypsin is a potent activator of proenteropeptidase that cleaves the zymogen and related synthetic substrate with similar high k_{cat}/K_m values. Duodenase demonstrates a 100-fold lower efficiency toward the same substrates. As was showed earlier [7], duodenase compared to trypsin is generally a 'slow' enzyme. At the same time it possesses high selectivity toward substrates and prefers compounds with the proenteropeptidase activation sequence.

Duodenase exhibits no activity towards trypsinogen and very slowly cleaves the synthetic substrate Gly-$(Asp)_4$-Lys-NA, containing the activation site sequence of trypsinogen (Table 1). Trypsin appears to be a inefficient activator of trypsinogen cleaving it with an extremely low k_{cat}/K_m value. Enteropeptidase, being a natural activator of trypsinogen converts it into active enzyme with very high catalytic efficiency (k_{cat}/K_m value) and hydrolyzes the corresponding synthetic substrate about 50-fold less efficiently than the natural substrate.

Although the biological function of duodenase is still unknown, these results suggest that duodenase may facilitate the reciprocal activation of proenteropeptidase and trypsinogen. Duodenase is about 100-fold less effective as activator of proenteropeptidase in vitro than trypsin [12]. However, taking into account the relatively high content of duodenase in the duodenal mucosa (0.3% of total protein) [4], it is possible that the processing activity of duodenase is sufficient to maintain a certain basal level of active enteropeptidase in the duodenum. When the next portion of pancreatic zymogens enters the

Table 1
Comparison of activities of proteolytic cascade activation enzymes towards their natural and synthetic substrates

Substrate	Enzyme	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})	Ref.
Recombinant proenteropeptidase	duodenase	–	–	2 700	present report
	trypsin	–	–	270 000	calculated from [12]
Suc-Ser-Pro-Lys-pNA	duodenase	1.00	2.6	2 600	[7]
	trypsin	0.06	15.0	250 000	[7]
Trypsinogen	duodenase	0	0		[7]
	trypsin	0.4	2.5×10^{-3}	6	[33]
	enteropeptidase	1.2×10^{-3}	6.9	5 750 000	[12]
		2.4×10^{-3}	11.7	4 900 000	[18]
Gly-$(Asp)_4$-Lys-NA	duodenase	0.68	0.016	24	[7]
	trypsin			no data	
	enteropeptidase	0.22	24.0	109 000	[12]
		0.20	16.7	83 000	[18]

duodenal lumen, the duodenase-activated proenteropeptidase may activate trypsinogen in turn, and the resultant active trypsin can activate additional proenteropeptidase. This process would lead to rapid activation of the remaining trypsinogen and, consequently, other pancreatic hydrolases (Fig. 3). On the other hand, some properties of duodenase similar to the ones of mast cell chymases [27] suggest that duodenase could be involved in processes like those regulated by mast cell proteinases.

Like the related mast cell chymases and leukocyte proteases [28,29], duodenase is expressed initially as a preproenzyme comprised of a 17 residue signal peptide, a two residue prosequence (Gly-Lys) at the N-terminus and the mature protein (E. Smirnova, unpublished). The dipeptide prosequence has been shown to be sufficient to render the proforms of catalytically inactive chymase [30,31] and granzyme B [32]. Hence, produodenase appears to require proteolytic activation. An enzyme responsible for the activation of duodenase may be an exopeptidase of the enterocyte brush-border membrane. Further studies are necessary to characterize this activation process.

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