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Effect of Calcium Ions on Enteropeptidase Catalysis

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Abstract—The effects of calcium ions on hydrolysis of low molecular weight substrates catalyzed by different forms of enteropeptidase were studied. A method for determining activity of truncated enteropeptidase preparations lacking a secondary trypsinogen binding site and displaying low activity towards trypsinogen was developed using N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester (Z-Lys-S-Bzl). The kinetic constants for hydrolysis of this substrate at pH 8.0 and 25°C were determined for natural enteropeptidase (K_m 59.6 μM, k_{cat} 6660 min^{-1}, k_{cat}/K_m 111 $\mu M^{-1}\cdot min^{-1}$), as well as for enteropeptidase preparation with deleted 118-783 fragment of the heavy chain (K_m 176.9 μM, k_{cat} 6694 min^{-1}, k_{cat}/K_m 37.84 $\mu M^{-1}\cdot min^{-1}$) and trypsin (K_m 56.0 μM, k_{cat} 8280 min^{-1}, k_{cat}/K_m 147.86 $\mu M^{-1}\cdot min^{-1}$). It was shown that the enzymes with trypsin-like primary active site display similar hydrolysis efficiency towards Z-Lys-S-Bzl. Calcium ions cause 3-fold activation of hydrolysis of the substrates of general type GD_4K-X by the natural full-length enteropeptidase. In contrast, the hydrolysis of substrates with one or two Asp/Glu residues at P2-P3 positions is slightly inhibited by Ca^{2+}. In the case of enteropeptidase light chain as well as the enzyme containing the truncated heavy chain (466-800 fragment), the activating effect of calcium ions was not detected for all the studied substrates. The results of hydrolysis experiments with synthetic enteropeptidase substrates GD_4K-$F(NO_2)G$, G_5DK-$F(NO_2)G$ (where $F(NO_2)$ is *p*-nitrophenyl-L-phenylalanine residue), and GD_4K-Nfa (where Nfa is β-naphthylamide) demonstrate the possibility of regulation of undesired side hydrolysis using natural full-length enteropeptidase for processing chimeric proteins by means of calcium ions.

Key words: enteropeptidase, calcium ion, trypsinogen, activation, autolysis, trypsin, peptide substrates, fusion proteins

The influence of Ca^{2+} on the efficiency of hydrolysis of different substrates catalyzed by enteropeptidase remains unclear; contradictory results have been obtained by different authors for different substrates [1]. For instance, trypsinogen activation must be performed in the presence of Ca^{2+} to avoid trypsin autolysis; an earlier study revealed that low calcium concentrations (1-2 mM) increase enteropeptidase efficiency towards this (natural) substrate [2, 3], while high concentrations (>1 mM), in contrast, lead to the inhibition of activation [4]. Baratty and Maroux [2, 5] showed that the secondary substrate binding site of enteropeptidase contains a cluster of positively charged amino acid residues interacting with negatively charged aspartic acid residues at positions P2-P5 of trypsinogen activation peptide; high ionic strength inhibits trypsinogen activation due to a steep increase in K_m values. In theory, the neutralization by Ca^{2+} should also prevent binding of residues at positions P2-P5 with enzyme, and therefore calcium must possess an inhibiting effect. However, using the synthetic substrate glycyl-tetra-L-aspartyl-L-lysine β-naphthylamide (GD_4K-Nfa), which also contains four residues of aspartic acid at positions P2-P5, Grant and Hermon-Taylor [1] at the same time found activating effect of calcium ions: hydrolysis of GD_4K-Nfa by human enteropeptidase at pH 8.4 in the presence of 10 mM Ca^{2+} was three times faster than at $[Ca^{2+}]$ of 0.1 mM. This effect was mainly caused by decrease in K_m value [1].

In this context, the match of activating effect of calcium ion on substrate hydrolysis with a cluster of negatively charged amino acid residues at positions P2-P5 of enteropeptidase with the similar effect discovered by Abita et al. [6] and Delaage et al. [7] in the case of trypsin is unexpected. These classic studies demonstrate that unfavorable influence of four aspartic acid residues at positions P2-P5 of the activation peptide of trypsinogen on trypsin hydrolysis is apparently caused by negative electrostatic influence on binding of Arg/Lys at position P1 with the S1 site, and it is necessary for the prevention of undesired trypsinogen autoactivation. Abita et al. [6] revealed that in the presence of at least two Asp residues at P2-P3 substrate positions binding of Ca^{2+} with these residues reduces K_m value 3-4-fold without affecting k_{cat}. Trypsinogen contains two calcium-binding sites [8]: the

Abbreviations: GD_4K-Nfa) glycyl-tetra-L-aspartyl-L-lysine β-naphthylamide; Z-Lys-S-Bzl) α-N-benzyloxycarbonyl-L-lysine thiobenzyl ester; DTDP) 4,4′-dithiodipyridine; $F(NO_2)$) *p*-nitrophenyl-L-phenylalanine residue; TFA) trifluoroacetic acid.

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first, high affinity site ($pK_{Ca^{2+}}$ 4.5 [9]), matches an analogous site in trypsin (calcium-binding loop Glu70-Glu80) and provides stability of this molecule against autolysis; the second site, with lower affinity ($pK_{Ca^{2+}}$ 2.2 [9]), is located in the region of four Asp residues of the activation peptide and regulates autoactivation. At the same time, the regulation of autolysis and autoactivation by Ca^{2+} has opposite character: binding of this ion (4 mM) by the first site prevents the hydrolysis of the Arg117–Val118 bond (autolysis) and vice versa, the saturation of the second site with calcium ions (50 mM) promotes the hydrolysis of the Lys15–Ile16 bond (autoactivation). Hence, the promoting effect of 50 mM Ca^{2+} on the hydrolysis of substrates containing sequence -$(Asp)_n$-Lys- (n = 2-4) detected by Abita et al. [6] corresponds to the saturation of the second calcium binding site of trypsinogen, which is supposed to consist of two aspartic acid residues (Asp13 and 14) adjacent to the hydrolyzed Lys15–Ile16 bond of trypsinogen. This is quite logically explainable from the perspective of electrostatic interactions.

The fact that trypsinogen containing -$(Asp)_4$-Lys- sequence in its activation peptide is a bad substrate for trypsin has important physiological sense: trypsin activates all other food digestion enzymes from their precursor except for itself. In this case, the role of an activator is taken by a specialized enzyme, enteropeptidase. This is the only way to prevent activation of proenzymes in the pancreas and therefore the destruction of the organism's own tissues. By using this fine mechanism (activation cascade of food digestion enzymes), mammals solve the task of food protein cleavage without destroying their own proteins.

In contrast with trypsin, the biological role of enteropeptidase is concluded in highly efficient activation of trypsinogen. Typical substrates of enteropeptidase contain four negatively charged Asp/Glu residues in P2-P5 positions, and one of the prevailing factors in the unique specificity of enteropeptidase is considered to be the electrostatic interaction between these residues and Lys889 in the secondary binding site of enteropeptidase light chain [10].

In this connection, from our point of view the fact that the promotion of GD_4K-Nfa hydrolysis by enteropeptidase during neutralization of negatively charged aspartic acid residues by calcium ions [1], which even quantitatively matches the analogous three-fold activation of trypsin hydrolysis of the substrates of this type, is unexplained [6]. In addition, as we discovered earlier, the presence or the absence of the whole enteropeptidase heavy chain or its fragments significantly affects the hydrolysis of its natural substrate, trypsinogen. The difference in activity between natural and truncated enteropeptidase towards trypsinogen reaches the order of two, while the hydrolysis efficiency of artificial substrates containing -$(Asp)_4$-Lys- sequence (either low molecular weight such as GD_4K-Nfa or high molecular weight recombinant proteins such as $PrAD_4K$-P26) by all forms of this enzyme is virtually the same [11-13]. However, later we discovered that the activity of enteropeptidase preparations with full-length or truncated heavy chain towards the artificial substrates is after all not exactly the same, although the difference is much less profound than in the case of trypsinogen. It also appeared that the data depend on the presence of calcium ions in the incubation mixture.

In the literature, we could also see a certain difference in efficiency of GD_4K-Nfa hydrolysis by the full-length bovine enteropeptidase and by its light chain (in the presence of 10 mM $CaCl_2$, pH 8.4) [14]. The interesting point is that hydrolysis constant values for this substrate at pH 8.4 by the full-length (natural and recombinant) bovine enteropeptidase in the presence of Ca^{2+} (k_{cat}/K_m = 6573 and 6600 $\mu M^{-1}\cdot min^{-1}$, respectively) virtually match the corresponding ones for human enteropeptidase [1], and also are very similar to GD_4K-Nfa hydrolysis constants at pH 8.0, which were obtained by us [13] (Table 1). At the same time, the efficiency of GD_4K-Nfa hydrolysis by the light chain of the enzyme in the presence of 10 mM Ca^{2+} is three times lower (due to higher K_m value), and all constants virtually match the analogous ones for the case of full-length human enzyme and low content of Ca^{2+} (0.1 mM) [1] (Table 1).

Now having two efficient synthetic substrates of enteropeptidase at our disposal, GD_4K-$F(NO_2)$G and G_5DK-$F(NO_2)$G, where $F(NO_2)$ is a *p*-nitrophenylalanine residue, as well as highly purified preparation of natural bovine enteropeptidase [11], preparation of a truncated enteropeptidase, containing C-terminal region of the heavy chain (466-800), and a recombinant form of the light chain of this enzyme, containing only the last 17 residues of the heavy chain (784-800), we have performed a systematic study of the effect of calcium ion on the hydrolysis of all the abovementioned substrates by these enteropeptidase forms.

Studies involving the use of truncated forms of enteropeptidase, which lacked the secondary trypsinogen-binding site and had low activity (compared to natural enzyme) towards trypsinogen, required a search for efficient low molecular weight substrates. Esters and amides of N-protected amino acids (arginine or lysine) are extremely slowly hydrolyzed by this highly specific enzyme. However, it was proved that a well known highly active substrate for trypsin-like enzymes, N-benzyloxycarbonyl-L-lysine thiobenzyl ester (Z-Lys-S-Bzl) [16], is also efficiently hydrolyzed by enteropeptidase with rates compared to the rate of hydrolysis of this compound by trypsin [17]. The use of GD_4K-Nfa substrate requires spectrofluorimetric techniques for studies of enzyme activity. Meanwhile, in the case of Z-Lys-S-Bzl in combination with one or another reagent for the detection of SH-groups, for instance, with 4,4′-dithiodipyridine (DTDP), the substrate hydrolysis could be monitored spectrophotometrically.

Table 1. Efficiency of substrate hydrolysis by natural enteropeptidase (EP 118-1035), autolyzed enteropeptidase (EP 466-1035), and enteropeptidase light chain (EP 784-1035) in the presence of 50 mM Ca^{2+} (I) and its absence (II) (pH 8.0, 37°C)

Substrate	EP 118-1035						EP 466-1035		EP 784-1035			
	I			II			I	II	I			II
	k_{cat}, min^{-1}	K_m, mM	k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$	k_{cat}, min^{-1}	K_m, mM	k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$	k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$	k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$	k_{cat}, min^{-1}	K_m, mM	k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$	k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$
G**D$_4$K**-Nfa			5.570			1.830	1.660	1.704			1.530	2.040
						1.660						
	1000	0.200	5.000 [13]						1494	0.600	2.490***	
	1698	0.280	6.064*	1290	0.525	2.457**						
G**D$_4$K**-			5.640			1.425	2.290	1.590			1.130	2.270
-F(NO_2)G	1070	0.160	6.700 [15]								1.280	1.585
G$_5$**DK**-			1.870			1.980	1.230	1.380			1.410	1.600
-F(NO_2)G	1040	0.437	2.380 [15]									2.090
LTA**EEK**-A			4.930×10^{-3}	4.20	29.40	7.00×10^{-3} [15]						
						6.48×10^{-3}						
LTA**EEK**-AAV			0.293			0.400 [15]						
			0.332			0.452						

* 10 mM Ca^{2+}, pH 8.4, human enteropeptidase [1].
** 0.1 mM Ca^{2+}, pH 8.4, human enteropeptidase [1].
*** 10 mM Ca^{2+}, pH 8.4 [14].

MATERIALS AND METHODS

Materials. In this work we used N-benzyloxycarbonyl-L-lysine thiobenzyl ester hydrochloride (Z-Lys-S-Bzl) and 4,4′-dithiodipyridine (DTDP) from Sigma (USA), glycyl-tetra-L-aspartyl-L-lysine β-naphthylamide (GD_4K-Nfa) from ICN Biomedical Inc. (USA), crystallized trypsin from Medical Drug Factory of St. Petersburg meat packing plant (Russia). Other reagents were purchased from Merck and Serva (Germany), Sigma and Bio-Rad (USA), Reanal (Hungary), Kriokhim (Russia); HPLC was performed on a Beckman System Gold instrument (USA). Spectrophotometric measurements were carried out using a Gilford 2400-2 spectrophotometer (USA).

Peptide substrates GD_4K-F(NO_2)G, G_5DK-F(NO_2)G, LTAEEKA, and LTAEEKAAV were synthesized according to the earlier described techniques [15].

Determination of enteropeptidase activity. Activity of enteropeptidase was determined by monitoring the increase in absorbance at 324 nm occurring upon hydrolysis of Z-Lys-S-Bzl (66.7 μM) in the presence of 0.2 mM DTDP in a thermostatted spectrophotometer cuvette; 0.1 M Tris-HCl buffer, pH 8.0, 25°C. The determined value $\Delta\varepsilon_{324}$ was 16,067 $M^{-1} \cdot cm^{-1}$. The concentration of the active sites of EP 466-1035 and EP 785-1035 preparations, and also for the control of known concentration of full-length enzyme (EP 118-1035), was determined by titration with known concentrations of bovine basic pancreatic trypsin inhibitor (BPTI), when corresponding amounts of the inhibitor in the range from [I] = 0 to [I] = [E] were introduced into the enzyme–substrate incubation mixture.

Enteropeptidase (EP 118-1035) was extracted from bovine duodenal mucosa and purified according to the technique developed previously [11]. Activity of the natural enzyme preparations was determined by trypsinogen activation [11].

To obtain an apo-form, the EP 118-1035 sample (10^{-7} M) was incubated in 10 mM HEPES-KOH buffer, pH 6.5, containing 2 mM EGTA, for 24 h at 4°C. The chelator was removed via repeated dialysis (1 : 5,000,000) in a Centricon-100 cell by the same buffer without EGTA. Apo-form of EP 466-1035 was obtained via autol-

ysis of the full-length apo-enteropeptidase at pH adjusted to 8.0. After the autolysis was finished (60 min, 37°C), enzyme 466-1035, which retained 100% activity towards Z-Lys-S-Bzl and 2-3% activity towards trypsinogen, was dialyzed as described above. According to electrophoretic data, both apoenzymes were homogeneous.

Proenzyme EP 784-1035 was kindly provided by Prof. Sadler from the Howard Hughes Medical Institute, Saint Louis, USA. Active EP 784-1035 was obtained via autoactivation of this preparation upon its storage in 0.1 M Tris-HCl buffer, pH 8.0, containing 50 mM $CaCl_2$, at 4°C.

Determination of hydrolysis constants for Z-Lys-S-Bzl by enteropeptidase, enteropeptidase light chain (EP 785-1035), and trypsin. A corresponding amount of enzyme was added to the spectrophotometer cuvette containing $(2\text{-}13.33)\cdot10^{-5}$ M Z-Lys-S-Bzl and 0.2 mM DTDP in 0.1 M Tris-HCl, pH 8.0, 25°C until the final concentration of 0.2 nM (enteropeptidase), 0.61 nM (light chain EP 785-1035), and 0.65 nM (trypsin). The initial rate of the substrate hydrolysis (six-eight concentrations for each enzyme, at least three series) was determined directly at 324 nm.

Determination of kinetic parameters (k_{cat} and K_m) of substrate hydrolysis was based on the calculations according to Eisenthal and Cornish-Bowden [18]. Error did not exceed 10-20%.

Study of the effect of Ca^{2+} on the efficiency of enteropeptidase hydrolysis. Hydrolysis of all the substrates was monitored by HPLC analysis of the aliquots of incubation mixtures. The incubation was performed in 0.1 M Tris-HCl buffer, pH 8.0, or 0.1 M Tris-HCl buffer, pH 8.0, containing 50 mM $CaCl_2$, at 37°C. Low substrate concentrations were used for determination of k_{cat}/K_m values for enteropeptidase hydrolysis ([S] << K_m): $3.64\cdot10^{-5}$ M in the case of GD_4K-Nfa, GD_4K-F(NO_2)G, and G_5DK-F(NO_2)G; $5\cdot10^{-5}$ M in the case of LTAEEK-A, and also $3\cdot10^{-5}$ and $4\cdot10^{-6}$ M in the case of LTAEEK-AAV. Enzyme concentrations were 1.0-1.4 nM (natural enteropeptidase EP 118-1035), 1.0-1.2 nM (autolyzed enteropeptidase EP 466-1035), and 1-4 nM (enteropeptidase light chain EP 785-1035) for the hydrolysis of GD_4K-Nfa, GD_4K-F(NO_2)G, and G_5DK-F(NO_2)G. For the hydrolysis of LTAEEK-A and LTAEEK-AAV in the absence and presence of 50 mM Ca^{2+}, we used only full-length enteropeptidase EP 118-1035 at the concentrations of 14.5 and 5.8 nM, accordingly. All possible combinations of incubation mixture (substrate–enzyme–buffer) with or without Ca^{2+} were tested in different combinations at least three times. At certain periods of time (corresponding to less than 20% of substrate conversion), 6-8 samples (2-5 μl) were collected from the incubation mixture, diluted 10-25 times with 10% trifluoroacetic acid (TFA) and stored at –70°C. Content of the aliquots was analyzed using HPLC in 0.1% TFA on a Luna C18 (2 × 250 mm) column from Phenomenex (USA), 0-60% acetonitrile gradient; elution rate 0.3 ml/min. The initial reaction rate was calculated according to the ratio of peak area between the substrate and one of the products; a correction for difference in molecular absorbance at 222 nm was made after the hydrolysis had been completed. The determined k_{cat}/K_m (±10%) values are presented in Table 1.

RESULTS

Using an example of two enteropeptidase substrates containing four aspartic acid residues at P2-P5 positions (GD_4K-Nfa and GD_4K-F(NO_2)G), our results demonstrate that calcium ions at concentrations exceeding 10 mM have indeed an activating effect on hydrolysis (approximately 3-fold) (Table 1). Also, the data for the first of these substrates completely match the results obtained by Grant and Hermon-Taylor for human enteropeptidase [1]. However, this was only found for the natural, full-length bovine enteropeptidase (EP 118-1035). In the case of two other truncated forms of bovine enteropeptidase, which contain only C-terminal region of the heavy chain (EP 466-1035) or only the last 17 amino acid residues of the heavy chain (EP 784-1035), the activating effect is not observed and the data on the efficiency of hydrolysis of these substrates (considering experimental error of 10-20%) are virtually the same. It should be noted that in our case the values of k_{cat}/K_m constants, which determine the efficiency of hydrolysis of these substrates by truncated forms of enteropeptidase (Table 1), are quite similar to each other, as well as to the values obtained by Lu et al. for hydrolysis of one of these substrates (GD_4K-Nfa) by recombinant enteropeptidase light chain [14].

Despite the replacement of three aspartic acid residues in P3-P5 positions with glycine residues [15], the third investigated peptide, G_5DK-F(NO_2)G, which unexpectedly appeared as a very efficient enteropeptidase substrate, was hydrolyzed by all three enteropeptidase forms (including the natural full-length enzyme) with virtually the same efficiency both in the absence and in the presence of calcium ions (Table 1). In this case, it is worth mentioning that all the results regarding the hydrolysis of substrates with -D_4K- truncated enteropeptidase forms and also in all cases of hydrolysis of the substrate with one aspartic acid residue (-G_4DK-) indicate insignificant inhibition by calcium ions. This effect is not too profound (10-30%), but it was observed in practically all experiments (except for GD_4K-F(NO_2)G hydrolysis by autolyzed enteropeptidase EP 466-1035 in the presence and in the absence of Ca^{2+}), which confirms that it is not an experiment error.

The data required a further expansion of experimental substrate base. The fact is that the substrate with shortened linker G_5DK-F(NO_2)G at P2-P5 positions used by us contained only one aspartic acid residue. But according to Abita et al. [6, 7] at least two Asp residues are required to develop Ca^{2+} effect (in this case, 3-fold acti-

vation); for instance, for substrate with one such residue the effect was not observed. Hence, we studied calcium ion effect on the hydrolysis of two other and less efficient enteropeptidase substrates, LTAEEK-A and LTAEEK-AAV [15], containing two negatively charged amino acid residues. However, the phenomena observed in this case (24-30% inhibition of these substrates hydrolysis by 50 mM $CaCl_2$) were virtually the same as for the case of the substrate with one negatively charged G_5DK-F(NO_2)G residue (Table 1).

Earlier we, as well as other researchers, did not pay much attention to the absence or presence of Ca^{2+} in the reaction mixtures, except for the case of trypsinogen activation, when it is necessary for the prevention of autolysis of trypsinogen itself as well as formed trypsin. As we revealed earlier, calcium ions are also required for the prevention of autolysis of enteropeptidase heavy chain, but the purification of this enzyme in the presence of $CaCl_2$ at all stages results in relatively stable preparations, whose autolysis requires an extensive incubation with EDTA or EGTA chelators [11]. Therefore, in those cases when the addition of $CaCl_2$ into substrate–enteropeptidase incubation mixture was undesired, we performed the hydrolysis without Ca^{2+}.

For instance, for the determination of hydrolysis constants of the recombinant protein $PrAD_4K$-P26, where an aliquot content was analyzed using highly efficient gel filtration on an Ultropac TSK G-2000 SW column in 25 mM Na-phosphate buffer [13], calcium ions were naturally absent in the incubation mixture; this fact can explain lower efficiency (3-4-fold) of this substrate hydrolysis compared to synthetic low molecular weight substrates GD_4K-Nfa and GD_4K-F(NO_2)G, for whom the hydrolysis constants were initially determined (spectrofluorimetrically and spectrophotometrically) only in the presence of 50 mM $CaCl_2$ [13, 15]. Though, in the absence of calcium ions the hydrolysis efficiency of all three natural substrates of enteropeptidase investigated by us and containing -GD_4K- sequence was practically the same.

It should also be noted that during the study of hydrolysis of the substrates with shortened linker using HPLC technique, the incubation mixture usually did not contain $CaCl_2$ [15, 19-21], except for chromophoric substrates GD_4K-F(NO_2)G and G_5DK-F(NO_2)G. In the latter case, when hydrolysis constants were determined both by spectrophotometric and HPLC techniques, the incubation with enzyme was performed in the presence of Ca^{2+}. In this work, we revealed that the absence or presence of Ca^{2+} has insignificant influence on the efficiency of hydrolysis of the substrates with 1-2 negatively charged amino acid residues (20-30% inhibition in the case of Ca^{2+} presence).

Hydrolysis of Z-Lys-S-Bzl by enzymes with a primary trypsin site (Table 2) is usually monitored spectrophotometrically in 0.1 M Tris-HCl buffer, pH 8.0, and without Ca^{2+} not only in the case of enteropeptidase [17], but even trypsin [16]. We discovered that the addition of calcium ions does not influence the hydrolysis efficiency for this substrate (which does not contain aspartic acid residues) by either the natural enzyme or by its truncated forms, and also by trypsin (data not shown).

However, attention should be drawn to the difference in efficiency between Z-Lys-S-Bzl hydrolysis by full-length enteropeptidase EP 118-1035 and light chain EP 784-1035. Regardless whether Ca^{2+} is present in the substrate–enzyme mixture or not, the efficiency of full-length enteropeptidase is still 3 times higher than the efficiency of light chain due to lower K_m value (Table 2). Hence, for the general substrate -D_nK-, where n = 0, [Ca^{2+}] = 0-50 mM and n = 4, [Ca^{2+}] = 50 mM, we discovered equal 3-fold difference in efficiency of EP 118-1035 and EP 784-1035 (but not EP 466-1035; the efficiency of Z-Lys-S-Bzl hydrolysis by autolyzed enteropeptidase is not different from the efficiency of the full-length enzyme). At the same time, in the case of n = 1-2 and all three enteropeptidase forms (EP 118-1035, EP 466-1035, and EP 784-1035), as well as n = 4 and two truncated enzyme variants (EP 466-1035 and EP 784-1035) we observed the same kind of [Ca^{2+}] influence: 20-30% inhibition by 50 mM $CaCl_2$.

Based on the results we can make the following conclusion: directed hydrolysis of the recombinant proteins by full-length natural enteropeptidase should be performed in the presence of $CaCl_2$. First, because in this case the hydrolysis efficiency is increased threefold (and is threefold more active than the truncated recombinant enzyme). Second, the earlier investigated [15, 19-21] ability of enteropeptidase to fairly efficiently hydrolyze the peptide bond after Lys/Arg residues, which are preceded by 1-3 Asp/Glu residues, is an obstacle during the processing of recombinant proteins containing built-in enteropeptidase linker -D_4K-. We should take into account the possibility of degradation of both protein carrier and target product if they contain -(Asp/Glu)$_n$-Lys(Arg)-, where n = 1-3 [22-25]. Experimental results, where we used peptide substrates GD_4K-F(NO_2)G,

Table 2. Kinetic parameters of Z-Lys-S-Bzl hydrolysis by several trypsin-like enzymes

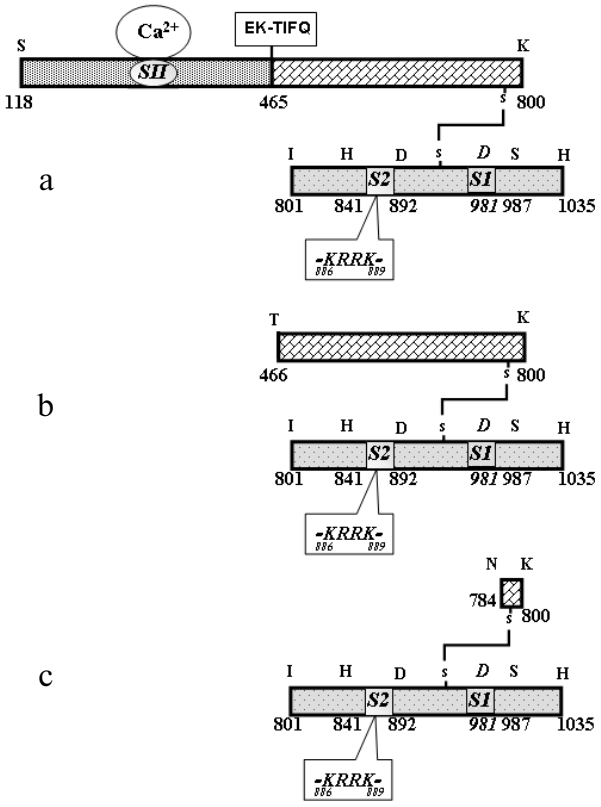
Enzyme	$K_m \times 10^5$, M	k_{cat}, min^1	k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$
Enteropeptidase EP 118-1035	5.96	6660	111.00
Light chain of enteropeptidase EP 784-1035	17.69	6694	37.84
Trypsin	5.60	8280	147.90

G_5DK-F(NO_2)G, and GD_4K-Nfa, allow (in the case of natural two-chain enteropeptidase) to control this undesired hydrolysis. Calcium ions possess the ability for threefold activation of hydrolysis of GD_4K- bond, but they cause 20-30% inhibition of undesired hydrolysis of -(D/E)$_n$-K(R)- bonds, where n = 1-3.

Vice versa, the addition of Ca^{2+} is not necessary when the recombinant enteropeptidase light chain is used for this purpose (however, according to the numerous literature data all techniques as a rule require the presence of $CaCl_2$). In reality, our data reveal even a small inhibition of the hydrolysis.

DISCUSSION

As a result of the systematic study of enzymatic and structural features of enteropeptidase, we have concluded that the unique properties of this highly specific enzyme are associated with the existence of the three substrate binding sites (figure). The light (or catalytic) enteropeptidase chain contains two of them. This is a primary site (S1) with Asp981 residue determining the primary trypsin specificity of the light chain, and, furthermore, the secondary binding site Lys889 (S2), which coordinates four aspartic acid residues at the P2-P5 substrate positions [10], thus providing high specificity of the enzyme. To achieve high enteropeptidase activity towards its natural substrate (trypsinogen) and retain at the same time high specificity of hydrolysis, the participation of one more secondary substrate binding site (SII) located in the region between 118 and 465 of the heavy chain is required [11-13]. In this way, there is a strict hierarchy of the secondary substrate binding sites, i.e., one provides specificity whereas the other provides efficiency of hydrolysis. Only in the case of its physiological substrate, trypsinogen, enteropeptidase realizes all hydrolysis capacity embedded in its molecule: the k_{cat}/K_m value for trypsinogen activation is $3 \cdot 10^8$ $M^{-1} \cdot min^{-1}$ [13, 14], which corresponds to typical efficiency of hydrolysis of polypeptide substrates by serine proteinases.



Schematics of: a) full-length bovine enteropeptidase (EP 118-1035); b) autolyzed bovine enteropeptidase (EP 466-1035); c) light chain of recombinant bovine enteropeptidase containing 17 C-terminal amino acid residues from the heavy chain (EP 784-1035)

The assumption stated above regarding the fact that the heavy chain is only involved in hydrolysis of the natural substrate of enteropeptidase (trypsinogen) should be slightly corrected as a result of the present study of calcium ion influence on hydrolysis of the model peptide substrates GD_4K-Nfa and GD_4K-F(NO_2)G by enteropeptidase. It was found that Ca^{2+} at the concentration of 50 mM, which corresponds to the saturation of the calcium-binding site located in the tetra-aspartyl sequence of the trypsinogen activation peptide, increases the efficiency of hydrolysis of these two substrates; however, this occurs only in the case of full-length enteropeptidase retaining the N-terminal region of the heavy chain 118-465. So, GD_4K-X + Ca^{2+} substrate, where X = Nfa or -F(NO_2)G, technically acts as trypsinogen, but with significantly lower effect, 3 versus 10^2.

The uniqueness of calcium ion as enteropeptidase activator was supported by Rinderknecht and Friedman [26], who observed that salts of rare-earth elements (La, Pr, Nd, Tb, Ho, Lu) are effective inhibitors of trypsinogen activation catalyzed by enteropeptidase. Lanthanide ions are effectively bound to the tetra-aspartyl sequence in trypsinogen activation peptide and inhibit enzyme catalysis: $I_{50} = 3.5 \cdot 10^{-6}$ M. Inhibitory effect of ionic strength also affecting the efficiency of trypsinogen activation by enteropeptidase is considerably less: $I_{50} \geqslant$ 50 mM for NaCl [26]. It should also be noted that the concentration of lanthanide salts is also much lower than is needed for the detection of the three-fold activating effect of Ca^{2+} (50 mM). Upon trypsinogen activation by trypsin, lanthanide ions act as activators in a same way as Ca^{2+}, but at significantly lower concentrations (1 : 100) [27]. Another unusual attribute of the action of rare-earth ions on enteropeptidase is the noncompetitive inhibition mechanism [26]. In all these cases of activation of enteropeptidase hydrolysis by calcium ions [1] as well as trypsin hydrolysis by calcium ions [6, 7] or lanthanides [27], the increase in the rate of enzymatic reaction occurs mainly due to decrease in the K_m value. However, the

addition of lanthanide salts has practically no influence on the K_m value for the reaction of trypsinogen activation by enteropeptidase; inhibition is due to decrease in the V_{max} value [26].

It should be also mentioned that the activating effect of calcium ions is displayed only for the substrates with four aspartic acid residues, like in trypsinogen. In the presence of 1-2 Asp/Glu residues at P2-P3 positions the corresponding peptides are hydrolyzed by the full-length enteropeptidase in the presence of 50 mM Ca^{2+} resulting in an opposite effect (weak inhibition), which is the same as in the case of hydrolysis of substrates with four Asp residues by enteropeptidase preparations lacking N-terminal region 118-465. It should be noted that Abita et al. [6] and Delaage et al. [7] observed Ca^{2+} activating effect on trypsin hydrolysis by calcium ions already in the presence of at least two Asp residues. However that may be, coordination of calcium ion by the four residues of aspartic acid in positions P2-P5 of the substrate results in certain interaction with some region in the N-terminus of enteropeptidase (118-465), and such interaction increases the efficiency of hydrolysis.

Upon investigation of Ca^{2+}-dependent autolysis of enteropeptidase heavy chain, it became obvious that the heavy chain contains a calcium-binding site, which is one of the most important structure–functional determinants of enteropeptidase [21]. It is tempting to assume that the interaction between the natural enteropeptidase substrate, trypsinogen, and putative secondary site located within the 118-465 fragment of the heavy chain involves the cooperative coordination of Ca^{2+} by aspartic acid residues at the positions P2-P5 of trypsinogen and calcium-binding site of enteropeptidase heavy chain. Such calcium binding also occurs in non-natural substrates containing four Asp residues at P2-P5.

One should also point out the fact that three-dimensional structure, based on which the main contribution of Lys889 residue of enteropeptidase light chain in ionic interactions with the four negatively charged substrate residues was determined, was obtained by crystallization of a light chain with corresponding inhibitor [10] (and of course in the absence of Ca^{2+}, but in this case it does not matter). It can also be assumed that the threefold increase in activity upon cooperative coordination of Ca^{2+} by the substrate and enzyme heavy chain is a result of a such conformational rearrangement of the secondary substrate-binding site S2 of the light chain that not only Lys889 (among Lys886-Arg887-Arg888-Lys889) is involved in the substrate binding, but all four residues. And is such coordination of Ca^{2+} by the natural substrate (trypsinogen) in cooperation with calcium-binding site of N-terminal region 118-465 of enteropeptidase heavy chain a more efficient factor increasing enzyme activity by two orders of magnitude? The answer for this question can be obtained only as a result of a planned series of experiments with mutant trypsinogen forms.

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