Abstract Variations in bovine enteropeptidase (EP) activity were shown to result from autolysis caused by the loss of calcium ions; the cleavage sites were determined. The native enzyme preferred its natural substrate, trypsinogen (Kᵩ₀ = 2.4 µM), to the peptide and fusion protein substrates (Kᵩ₀ = 200 and 125 µM, respectively). On the other hand, the truncated enzyme composed of the C-terminal fragment 466-800 of EP heavy chain and intact light chain did not distinguish these substrates. The results suggest that the N-terminal fragment 118-465 of the enteropeptidase heavy chain contains a secondary substrate-binding site that interacts directly with trypsinogen.

Key words: Enteropeptidase (enterokinase); Autolysis; Trypsinogen activation; Fusion protein; Enzyme activity

1. Introduction

Enteropeptidase (enterokinase; EC 3.4.21.9) is a highly specific processing protease of the duodenal mucosa that initiates a cascade of reactions activating digestion enzymes [1]. The first reaction of this cascade is the conversion of trypsinogen into trypsin via EP-catalyzed cleavage after the (Asp)₄-Lys sequence. In naturally occurring proteins, this sequence has been found only in N-terminal activation peptides of various trypsinogens [2]. The bovine EP molecule consists of disulfide-linked heavy (135-120 kDa) and light (47 kDa) chains; the latter is a trypsin-like serine protease (Fig. 1). It was found that ca. 40% of the apparent molecular mass of EP is provided by glycosylation [3-5]. The enzyme is synthesized as a single-chain precursor of 1035 amino acid residues and then cleaved after Arg⁸⁰⁰ to produce the disulfide-linked heterodimer [5]. Earlier, we developed a preparative method for purifying EP [6] and its inhibitor (DI) from the duodenum [7]. A highly purified form of the enzyme was found to be stabilized by calcium ions and did not contain any other proteolytic enzyme contaminations [6]. The activity of these preparations was an order of magnitude higher than of those obtained by the previously described method of Liepins and Light [3]. N-terminal sequencing (15 amino acid residues) of the enzyme heavy chain was performed for the first time; Ser¹¹⁸ of the precursor was shown to be the N-terminal residue in the heavy chain of the active bovine EP [8] (Fig. 1). Removal of the 1-117 fragment from the proEP molecule during processing was earlier found only for the porcine enzyme [9].

A variety of fusion proteins containing the (Asp)₄-Lys sequence were hydrolyzed by the obtained EP preparations [8,10,11]. Kinetic data indicated that the peptide and artificial protein substrates bound through the linker (Asp)₄-Lys sequence to the light (catalytic) chain; a secondary binding site may participate in a more efficient hydrolysis of the physiological substrate trypsinogen [8,10].

Here, we studied the properties of the truncated enzyme (EP autolysis product), which allowed the suggestion that the secondary binding site lies within the N-terminal fragment 118-465 of the EP heavy chain.

2. Materials and methods

2.1 Materials

All reagents used were purchased from Sigma (United States), Serva (Germany), Bio-Rad (United States), and Merck (Germany), if not stated otherwise, and were of analytical grade. Bovine trypsin was from the Plant of Medical Preparations (St. Petersburg, Russia). Basic bovine protease inhibitor (BPI) was kindly provided by Dr. N.I. Larionova (Moscow State University, Russia). Bovine EP was isolated and purified as described by Mikhailova and Rumsh [6]; EP inhibitor from the duodenum (DI) was isolated as described by Mikhailova et al. [7].

2.2 Protein determination

Protein was determined by the Bradford method [12] with a Bio-Rad Protein Assay Kit, Standard 1 (bovine IgG) or spectrophotometrically by the A₂₈₀-A₂₃₅⁺ difference [13].

2.3 Activity assay

EP activity was determined from the activation of trypsinogen according to Liepins and Light [3] with some modifications. The amount of EP that produced 1.0 nmol of trypsin from trypsinogen in the activation mixture within 1 min was taken as one unit of activity. The reaction mixture contained EP (<0.03 units/ml), 0.1 M sodium acetate (pH 5.0), 50 mM CaCl₂ (buffer A) and 50 µl of trypsinogen solution (1.0 mg/ml in 1 mM HCl) in a total volume of 0.5 ml and was incubated for 30 min at 37°C. The reaction was terminated with 50 µl of 1 M HCl. The tryp tic activity in 75-µl aliquots was measured spectrophotometrically at 405 nm at 25°C with 0.5 mg/ml BAPNA [14] in 0.1 M Tris-HCl (pH 8.0) containing 50 mM CaCl₂ and 10% dimethyl sulfoxide in a total volume of 1.5 ml. Trypsinogen autoactivation was taken into account in the calculations (the amount of trypsin resulting from trypsinogen autoactivation did not exceed 5-10% of the amount of EP-derived trypsin). The calibration curve was plotted using known trypsin concentrations. The trypsin molar

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concentration in solutions was evaluated by active-site titration with Gdn-Bz-ONp [15]. Trypsinogen preparations (Sigma) were shown to contain 75% of potentially active proenzyme. Kinetic parameters for trypsinogen activation were determined at pH 5.0 and 8.0 at 37°C [8]. The concentrations of EP solutions were determined by active-site titration with Gdn-Bz-OMum according to Liepnieks and Light [3] using trypsin titrated with Gdn-Bz-ONp as a standard. The activity of pure EP (100%) in experiments was 1840 units/mg.

2.4. Substrate hydrolysis

Kinetic parameters for the GD$_4$K-NA cleavage were determined as described previously for the human EP [16]. The release of $\beta$-naphthylamine was followed with a Hitachi MPF-4 spectrofluorimeter with excitation at 330 nm and emission at 410 nm [10].

The expression and purification of the fusion protein PrA-P26 were described by Kutusov et al. [17]. The kinetic parameters of the PrA-P26 cleavage were determined as described previously [10]. The initial rate of substrate hydrolysis was estimated as a decrease in the area of the PrA-P26 peak in the course of its incubation with EP by SE-HPLC on an Ultrapac TSK G-2000 SW column (0.75 cm x 60 cm; LKB) at a flow rate of 0.5 ml/min; 25 mM sodium phosphate (pH 6.6) containing 0.2 M NaCl (buffer B) was used as an eluent.

2.5. EP autolysis in the presence of EDTA

The effects of EDTA on EP activity and structure were studied using the enzyme preparations purified in the absence of CaCl$_2$ or with lyophilized EP (Boehringer Mannheim, Germany).

Method A: 5 ml of the freshly dissolved Boehringer EP (0.3 mg/ml in H$_2$O) was incubated with 1 mM EDTA in the absence or presence of inhibitors (2 $\mu$M BPI, 2 $\mu$M DI, 3 $\mu$M OVO, or 5 $\mu$M STI) in 100 ml of 0.1 M Tris-HCl (pH 8.0) at 20°C. The resulting enzyme concentration was 70 nM. The control samples were incubated without EDTA either in the presence or absence of inhibitors. 2-$\mu$l aliquots were withdrawn at different time intervals, diluted 1:100 with buffer A, and the EP activity toward trypsinogen was measured. The reaction mixture samples (30–45 $\mu$l) were simultaneously analyzed by SE-HPLC on a Toyo Soda TSK G 4000 SW column (0.75 cm x 60 cm) in buffer B at a flow rate of 0.5 ml/min.

Method B: EP solutions (30–60 $\mu$g in 0.1–0.6 ml of 3–10 mM Tris-HCl, pH 8.0) were incubated with 1–2 mM EDTA at 20–37°C for 16–24 h. The active enzyme concentration was 0.65–1.7 $\mu$M. 2-$\mu$l aliquots were withdrawn at different time intervals, diluted 1000–1500-fold with buffer A, and the EP activity towards trypsinogen was measured. Incubation was performed up to the complete loss of the enzyme activity. The protein solution was concentrated (>10-fold) in a Speed-Vac, diluted with the sample loading buffer for SDS-PAGE (3% SDS and 2% $\beta$-mercaptoethanol), and incubated for denaturation at 55–60°C for 5–6 h. SDS-PAGE was carried out in 7% gel according to Laemmli [18]. The protein bands were transferred onto Immobilon membrane (Millipore, USA) by electroblotting, N-terminal amino acid sequencing of the autolysis products was performed by automated Edman’s degradation with an Applied Biosystem 470 A gas-phase sequencer.

3. Results and discussion

The known procedures of EP purification, including the method of Liepnieks and Light [3], did not involve Ca$^{2+}$. We found that, when the enzyme preparations had been purified without CaCl$_2$, their long-term storage (for several months) or incubation with EDTA (for several hours) resulted in the appearance of the second EP form, the retention time of which in SE-HPLC was higher as compared to the native enzyme (Fig. 2). The proteolytic activity of this modified EP towards trypsinogen comprised only several percent of the initial enzyme activity; however, this form completely retained its activity towards fusion protein substrates and GD$_4$K-NA (Table 1). The number of active sites in the modified EP did not change as determined by titration with Gdn-Bz-OMum. The increase in the retention time upon SE-HPLC testified that an apparent decrease (approx. 2-fold) in the molecular mass accompanied enzyme modification. The acceleration of the modification process in the presence of EDTA (but not phenanthroline) suggested that the loss of calcium ions by the protein was the reason for this phenomenon. This suggestion was confirmed by isolation and purification of EP in the presence of CaCl$_2$ from the very first step. In this case, only the high-molecular-weight EP form was obtained that could not be converted into the truncated enzyme even upon long-term incubation with EDTA. In the presence of BPI or DI, the enzyme activity towards trypsinogen was completely retained upon addition of EDTA; no transition into the modified form was observed (Fig. 2). In the presence of STI or OVO, this transition became somewhat slower (Fig. 2); it should be noted that STI and OVO are not EP inhibitors, but STI has been

![Fig. 1. Schematic structure of bovine enteropeptidase and its truncated form.](image-url)
shown to inhibit the isolated light chain [4]). Therefore, we may conclude that the EP modified form is an enzyme autolysis product. Apparently, the process was intramolecular: the autolysis rate of EP was independent of the enzyme concentration. As a rule, removal of calcium ions from Ca\(^{2+}\)-containing proteins makes their structure more unfolded, decreases their stability in the presence of various denaturing agents and promotes autolysis [19]. Trypsin, which binds one Ca\(^{2+}\) ion per molecule [20], is an example of such enzymes.

After SDS-PAGE under reducing conditions (Fig. 3) and electroblotting of the heavy chain fragments on Immobilon, N-terminal amino acid sequences of the autolysis products were determined. The 70–80 kDa protein band was shown to be composed of the heavy chain C-terminal fragment beginning with Thr\(^{422}\) (TIFQ) and the heavy chain N-terminal fragment beginning with Ser\(^{115}\) (SIIV), i.e. with the N-terminal residue of the active EP heavy chain [8]. Hence, in the presence of Ca\(^{2+}\) the EP heavy chain undergoes autolysis at the peptide bond formed by the Lys\(^{465}\) carboxyl group. Note that in the proEP amino acid sequence [5] the residue at position 464 is glutamic acid, and the heavy chain does not contain the EP-specific sequence (Asp)\(^4\)Lys [5]. However, the central fragment of the heavy chain (residues 359–465) contains not only Lys\(^{465}\), but also Lys\(^{460}\), Arg\(^{384}\) and Arg\(^{422}\) preceded by Glu residues [5] (Fig. 1). Sequencing of the broad protein band of 56–66 kDa (Fig. 3) showed that these autolysis products began with Ser\(^{115}\), probably representing the N-terminal fragments derived by the heavy chain cleavage by the above-mentioned basic residues. Indeed, in some experiments we found another C-terminal fragment with the N-terminal sequence TQGS resulting from the heavy chain fragment autolysis at Arg\(^{384}\). Heavy chain fragments beginning with Val\(^{425}\) and Thr\(^{466}\) were previously found in EP commercial preparations [21]. Their presence was ascribed to trypsin-like contaminations [21], but we consider them to be EP autolysis products. Fragment 466–800 of the EP heavy chain is obviously the terminal product of enzyme autolysis (Fig. 1). The specificity of autolysis and the structural aspects will be discussed in our future publications. In the presence of various different denaturing agents, EP may undergo autolysis in the Ca\(^{2+}\)-independent manner. Thus, we ascribed the appearance of the 115 kDa minor protein band (Fig. 3) to the extra cleavage of the EP heavy chain at Lys\(^{254}\) under the SDS treatment of the sample.

By using fusion proteins with the (Asp)\(^4\)Lys linker sequence, we significantly increased the number of substrates suitable for experimental studies of this highly specific enzyme and determined for the first time the catalytic parameters of the fusion protein hydrolysis with EP [10]. To diminish the probability of trypsinogen autoactivation at pH 8.0 the EP activity is usually determined from the rate of trypsinogen activation at pH 5.0–6.0 rather than at the pH optimum of 7.0–8.0 [3]. We estimated for the first time the kinetic parameters of trypsinogen activation not only at pH 5.0, but also at the optimal pH for more correct comparison of hydrolysis parameters of various substrates. In the case of EP purified in the presence of Ca\(^{2+}\) the kinetic parameters were determined at pH 8.0 for a series of substrates containing the (Asp)\(^4\)Lys sequence: the low-molecular substrate GD\(_{1}\)K-NA (K\(_M\) = 200 \(\mu\)M, k\(_{cat}\) = 1000 min\(^{-1}\)), fusion protein PrA-P26 (K\(_M\) = 125 \(\mu\)M, k\(_{cat}\) = 157 min\(^{-1}\)) and the natural substrate

![Graph](image_url)

**Table 1**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Native enzyme (residues 118–1035)</th>
<th>Truncated enzyme (residues 466–1035)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsinogen, pH 5.0</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Trypsinogen, pH 8.0</td>
<td>0.7</td>
<td>50.0</td>
</tr>
<tr>
<td>PrA-P26</td>
<td>73.6</td>
<td>73.6</td>
</tr>
<tr>
<td>GD(_{1})K-NA</td>
<td>18.3</td>
<td>18.3</td>
</tr>
</tbody>
</table>

The rates of trypsinogen activation by the native and truncated enzymes were determined experimentally as described in Section 2.3. Data for other substrates were calculated with the determined K\(_M\) and k\(_{cat}\) values (see text).
trypsinogen ($K_M = 2.4$ and $1.8 \, \mu M$, $k_{cat} = 700$ and $435 \, \text{min}^{-1}$ at pH 8.0 and 5.0, respectively) [8,10]. We believe that the discrepancies between our and literature data on the $K_M$ and $k_{cat}$ values for trypsinogen activation at acidic pH ($K_M = 17 \, \mu M$, $k_{cat} = 380 \, \text{min}^{-1}$ [3]) are due to the admixture of the truncated (low-active) EP form in the enzyme preparations obtained without Ca$^{2+}$. Recently, the kinetic constants for GD$_4$K-NA cleavage and trypsinogen activation were determined for the native and recombinant bovine EP [22]. The reported values agree well with our data. In the case of native EP, the $K_M$ values for the fusion protein and the peptide substrate were similar and appeared to be by two orders of magnitude higher than those for trypsinogen. Moreover, the $K_M$ values for the artificial substrates were of the same order of magnitude as the $K_i$ values (100–140 $\mu M$) for the inhibition of human and porcine EPs by the (Asp)$_4$Lys peptide as determined using trypsinogen and GD$_4$K-NA as substrates [23,24]. These results suggest that the peptide and fusion protein substrates bind to the enzyme through the linker sequence (Asp)$_4$Lys. More efficient hydrolysis of trypsinogen, the natural substrate, provides evidence for a significant contribution of other sites of the substrate and the enzyme to the productive binding [8,10].

The EP light chain was always less active towards trypsinogen as compared to the C-terminal fragment (residues 466–800) of the heavy chain linked by the Cys$^{788}-$Cys$^{912}$ disulfide bond to the light chain (Fig. 1). The activity of the truncated enzyme towards trypsinogen and low-molecular substrates was similar to that of the isolated light chain. The rate of trypsinogen activation by this modified enzyme decreased by two orders of magnitude as compared to the native enzyme and appeared to be similar to the hydrolysis rates of peptides and fusion proteins with the EP-specific sequence (Asp)$_4$Lys (Table 1). These results may help in elucidating the role in the EP heavy chain in trypsinogen recognition: the secondary substrate-binding site that interacts directly with trypsinogen [22] may be located in the N-terminal fragment 118–465 (or, with respect to the data of Lu et al. [22], fragment 198–465) of the heavy chain. The heavy chain central fragment (residues 360–465) was shown to be the most susceptible to Ca$^{2+}$-dependent autolysis. The EP heavy chain has an unusual mosaic structure. It contains an assortment of structural motifs found in other protein families; the function of these motifs in EP is unknown [5]. The fragment 358–520 is homologous to the membrane-bound metalloprotease meprin (the so-called MAM domain) [5]. Related domains in other proteins bind ligands or mediate protein-protein interaction [5]. In the EP heavy chain, the MAM domain along with the sites of Ca$^{2+}$-dependent autolysis contains a substantial amount of negatively charged amino acid residues, especially between Lys$^{886}$ and Arg$^{889}$, providing the potential binding site for calcium ions. Clarification of the MAM domain role in trypsinogen recognition and the features of its interaction with EP active site in the presence and absence of Ca$^{2+}$ may be of great importance for understanding the EP specificity.

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References