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Proteolytic activity and cleavage specificity of cathepsin E at the physiological pH as examined towards the B chain of oxidized insulin

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Proteolytic activity and cleavage specificity of cathepsin E were investigated in a wide range of pHs from 3.0 to 10.5 using the B chain of oxidized insulin as substrate. Contrary to the previous notion that cathepsin E is virtually inactive above pH 6, significant proteolytic activity was observed at pH 7.4 and above. Further, cleavage specificity appeared to change significantly with pH and rather specific cleavage occurred at pH 7.4 and above as compared to pH 5.5 and 3.0. These results suggest that cathepsin E may function in vivo at the physiological pH with a rather restricted specificity.

Cathepsin E; Gastric mucosal aspartic proteinase; Proteolytic activity; Cleavage specificity; B chain of oxidized insulin

1. INTRODUCTION

Human gastric cathepsin E (previously called slow moving protease [1], cathepsin D-like proteinase [2], etc.) is an aspartic proteinase present in human gastric mucosa. In the previous studies [2,3], we purified this enzyme to apparent homogeneity and investigated its several properties. Similar studies were also performed by Samloff et al. [4]. Further, the complete amino acid sequence of human procathepsin E has been deduced recently by Azuma et al. [5] from the analysis of its cDNA clones. On the other hand, we have reported the amino acid sequences of the NH_2-terminal regions of human gastric cathepsin E as well as procathepsin E [6,7] and the autocatalytic processing of procathepsin E into cathepsin E at pH 3.5 [7]. Cathepsin E has been localizaed not only to stomach mucosa, but also to erythrocyte membranes and several lymphoid-associated tissues and cells including thymus, spleen, macrophages, and polymorphonuclear lymphocytes [8,9]. However, its physiological function and the process of activation remain obscure. Due to its intracellular localization in lymphoid-associated tissues and cells, it has been suggested to have a role in immune function [4,10]. It has also been suggested to be involved in the processing of big endothelin-1 [11].

The optimum pH of cathepsin E towards hemoglobin is 3–3.5, so its proteolytic activity and substrate specificity were investigated previously only at acidic pHs [3,12–14]. As far as cathepsin E can be active only at acidic pHs, its physiological roles in vivo would be highly restricted. Therefore, in order to shed light on the physiological roles of cathepsin E, it is thought to be very important to investigate its potential proteolytic activity and substrate specificity at around neutral (physiological) pHs. Furthermore, it was not always clear whether the enzyme preparations used in the previous studies were procathepsin E or cathepsin E. In fact, the enzyme preparations we obtained previously [2,3] were proved to be procathepsin E [6,7]. Therefore, it is also thought to be important to use the activated form but not the proform of cathepsin E to elucidate its characteristics, especially at the physiological pH where the autocatalytic activation of procathepsin E would be very slow or would not take place. In the present study, the proteolytic activity and cleavage specificity of cathepsin E were investigated in a wide range of pH from 3.0 to 10.5 using the B chain of oxidized insulin as substrate. The results demonstrated for the first time that the enzyme possesses significant proteolytic activity at the physiological pH with an apparently rather restricted cleavage specificity.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Nph, nitrophenylalanine; cya, cysteic acid.

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2. MATERIALS AND METHODS

2.1. *Materials*

Specimens of human stomachs were generously supplied by Dr. M. Ukai. The B chain of oxidized bovine insulin was purchased from Sigma. Procathepsin E was purified from the human gastric mucosa essentially as described previously [2,3] and the major component was used. Procathepsin E was activated to cathepsin E completely by dialyzing it against 0.05 M sodium acetate buffer, pH 4.0, containing 0.2 M NaCl at 4°C for 3 h and the activation mixture was applied to a pepstatin-Sepharose column (1.0 × 1.0 cm) equilibrated with the

same buffer. After washing the column with the same buffer (60 ml), the protein was eluted with 0.05 M Tris-HCl buffer, pH 8.0, containing 1 M NaCl, and further purified by Mono-Q chromatography (data not shown). The enzyme fraction was desalted by passage through a Sephadex G-25 column and used in the present study. The purity of the cathepsin E sample thus prepared was confirmed by SDS-PAGE and the NH_2-terminal amino acid sequencing.

2.2. *Digestion of peptides and analysis of the digests*

The B chain of oxidized insulin (100 nmol) was digested at 37°C with cathepsin E (0.2 nmol, 8 μg) in 300 μl of each of the following buffers: 0.5 M sodium formate buffer, pH 3.0, 0.5 M sodium acetate buffer, pH 5.5, 0.5 M Tris-HCl buffers, pH 7.4 and 8.5, and 0.25 M sodium borate buffers, pH 9.5 and 10.5. Aliquots of 100 μl were removed at appropriate time intervals and submitted to high performance liquid chromatography (HPLC) using a Hitachi 655A-11 system on a column (0.46 × 25 cm) of TSKgel ODS-120T (Tosoh Co.). The peptides were eluted with a gradient of acetonitrile (0–50% in 60 min) in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min. The effluent was monitored by measuring the absorbance at 215 nm and the peptide peak fractions were pooled and lyophilized. An aliquot of each peptide fraction dissolved in water was submitted to amino acid analysis using an Applied Biosystems automated derivatizer-analyzer (420A/130A). For comparison, a synthetic chromogenic peptide substrate [15], Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu (7.2 nmol), was also digested by cathepsin E (0.014 nmol, 0.56 μg) at pH 7.4 for 2 h under similar conditions and the digest was analyzed in the same way.

3. RESULTS

Fig. 1 shows the HPLC patterns of the 3-h digest of the B chain of oxidized insulin at pH 3.0, 5.5 and 7.4 as typical examples. The B chain was cleaved rapidly and extensively at pH 3.0 (Fig. 1a), but was also hydrolyzed to a considerable extent at pH 5.5 (Fig. 1b) and 7.4 (Fig. 1c). As can be seen from the figure, the HPLC patterns were significantly different among the three pH values. Particularly the HPLC pattern of the digest at pH 7.4 appeared to be much simpler than those of the digest at pH 3.0 and 5.5. Upon longer incubation, hydrolysis proceeded further, but the cleavage pattern at each pH value (data not shown) appeared to be roughly similar to that obtained with the 3-h digest. The HPLC analyses of the digests at pH 8.5, 9.5 and 10.5 showed that hydrolysis could also occur at these pH values although the rate of hydrolysis became much slower as the pH was raised (data not shown). The HPLC patterns of the digests at these higher pH values appeared to be qualitatively similar to that of the digest at pH 7.4.

The cleavage sites and the extents of hydrolysis estimated from amino acid analysis are shown in Fig. 2 for the digests at pH 3.0, 5.5 and 7.4. At pH 3.0 and 5.5, several peptide bonds, especially Phe–X, Tyr–X, and Leu–X bonds, were simultaneously cleaved to marked extents, whereas at pH 7.4, the Glu^{13}–Ala^{14} was cleaved fairly selectively. The extents of cleavage of this bond was estimated to be 22% at pH 3.0, 19% at pH 5.5, 22% at pH 7.4, 19% at pH 8.5, 16% at pH 9.5, and 7% at pH 10.5. In addition, the Glu^{21}–Arg^{22} bond was cleaved to significant extents: 6% at pH 7.4, 5% at pH 8.5, 4% at pH 9.5, and 3% at pH 10.5, whereas this bond was not hydrolysed at pH 3.0 and 5.5. Judging from the HPLC patterns of the digests at pH 8.5, 9.5 and 10.5 (data not shown), the cleavage sites appear to be similar to those observed at pH 7.4 although the extents of hydrolysis decreased as the pH was raised. By summing up the

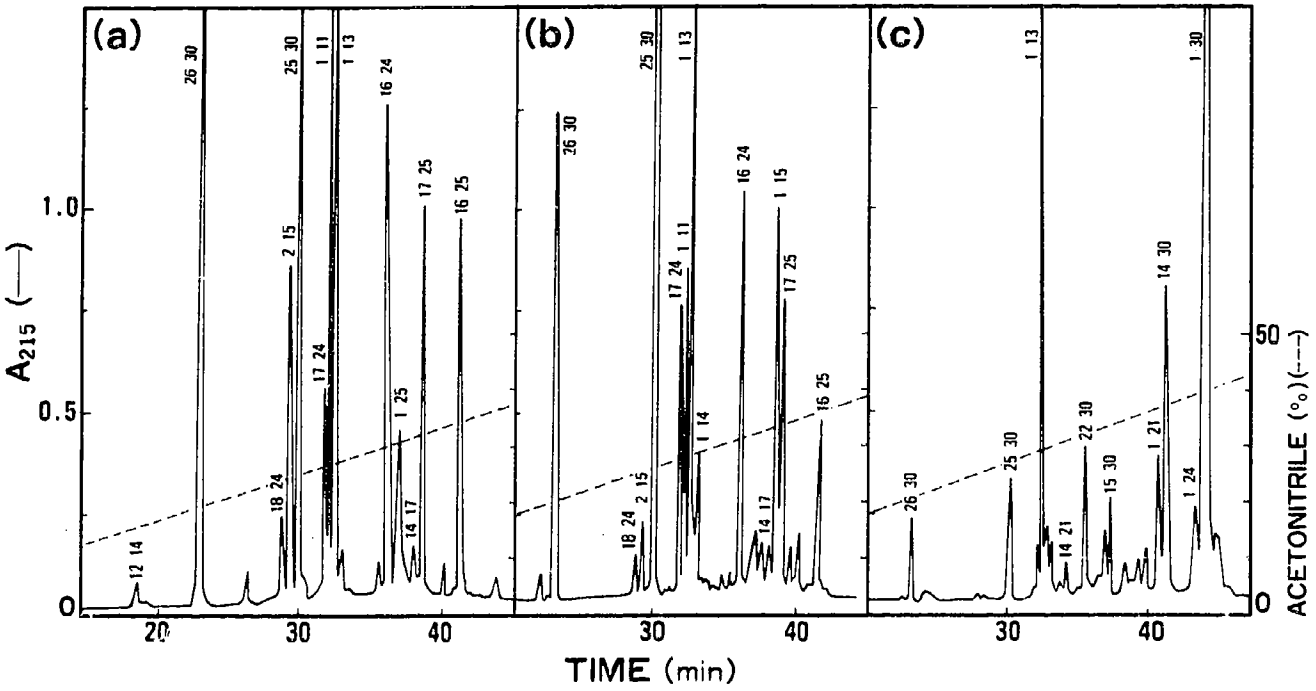

Fig. 1. HPLC patterns of 3-h cathepsin E digests of the B chain of oxidized insulin at pH 3.0 (a), 5.5 (b), and 7.4 (c) on a TSK gel ODS-120T column. The identity of each peak is shown with the residue numbers in the B chain of oxidized insulin. Peptides obtained in yields of 1% or less are not shown.

estimated extents of hydrolysis of individual peptide bonds, the total extents of hydrolysis could be calculated. These values relative to that at pH 3.0 (taken as 100%) were 77% at pH 5.5, 17% at pH 7.4, 13% at pH 8.5, 12% at pH 9.5, and 6% at pH 10.5. In addition, the enzyme was also shown to hydrolyze about 26% of the chromogenic substrate, Lys-Pro-Ile-Glu-Phe-Nph-Arg-Leu, at the Phe–Nph bond at pH 7.4 under the conditions used (data not shown).

The proteolytic activity of cathepsin E towards the B chain of oxidized insulin was completely inhibited by 0.1 mM pepstatin at pH 3.0 and 5.5, whereas 0.1 mM pepstatin had no inhibitory effect when used at pH 7.4. Further, when cathepsin E, which had been inhibited by incubation with 0.1 mM pepstatin at pH 3.0 or 5.5, was brought to pH 7.4, the enzyme restored full activity and essentially the same HPLC pattern as that obtained without pepstatin was obtained. Neither ATP (6 mM) nor any of the following protease inhibitors: 0.1 mM diisopropylfluorophosphate, 1 mM phenylmethanesulfonyl fluoride, 10 mM *o*-phenanthroline, 0.05 mM diprotin A, 0.1 mM leupeptin, 0.1 mM antipain, 0.1 mM E-64, and 0.1 mM amastatin, affected the hydrolysis at pH 7.4.

4. DISCUSSION

Gastric cathepsin E is an aspartic proteinase optimally active at pH around 3 and has been thought to be devoid of proteolytic activity at neutral pH values. Indeed, it was reported to have rather low activity at pH 5 and practically no activity at pH above 6 towards hemoglobin as substrate [2,16]. In the present study, however, human gastric cathepsin E was found to possess rather high activity at pH 5.5 and still significant activity at pH 7.4 (and above) towards the B chain of oxidized insulin. Reduced and carboxymethylated ribonuclease A and several other peptides were also shown to be hydrolyzed to significant extents by cathepsin E at pH 7.4 analyzed in the same manner (Athauda, S.B.P. et al., to be published). Further, the activity at pH 7.4 towards the B chain of oxidized insulin was not inhibited by any of the serine, cysteine, and metalloproteinase inhibitors tested, indicating that this activity is due to cathepsin E itself and not to a contaminating proteinase(s). Thus, gastric cathepsin E was shown for the first time to be significantly active towards certain polypeptide substrates at neutral to slightly alkaline pH values. This finding suggests that cathepsin E may function in vivo in a wider physiological pH range than was hitherto considered.

Previously, Thomas et al. [17] reported that cathepsins E from human gastric mucosa and red blood cells were virtually inactive at pH 5.8 towards synthetic chromogenic peptide substrates and [^{3}H]casein, but that the activity was expressed at pH values up to about 6.6 in the presence of ATP (6.25 mM), due to stabilization of the enzyme by ATP. In the present study, however, our cathepsin E preparation hydrolyzed one of the same synthetic peptide substrates rather rapidly at pH 7.4 at

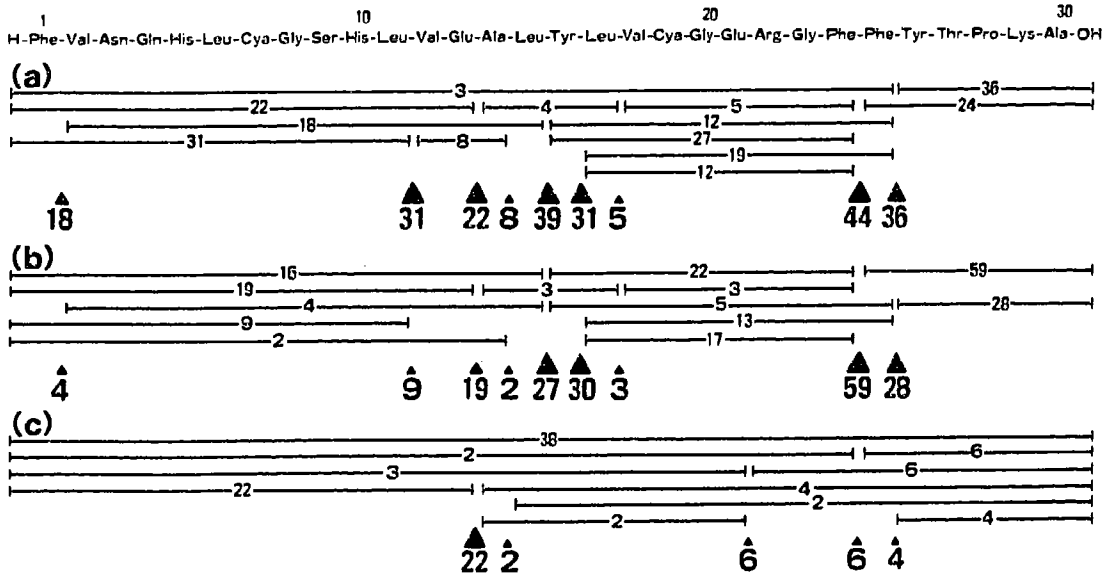

Fig. 2. Yields of peptides produced from the B chain of oxidized insulin by cathepsin E and the sites of cleavage at pH 3.0 (a), 5.5 (b), and 7.4 (c). Bars indicate peptide fragments produced, and the number in each bar shows the percent yield of the peptide. Arrowheads indicate the cleavage sites, and the number under each arrowhead shows the estimated extent of cleavage in percent of the peptide bond. Large, medium, and small arrowheads indicate major, secondary, and minor cleavage sites, respectively.

the Phe–Nph bond in the absence of ATP. Further, ATP (6.25 mM) had no effect on the rate of hydrolysis of the B chain of oxidized insulin at pH 7.4. The reason for this discrepancy is not certain at present. It is now known that human gastric cathepsin E purified under the conditions at pH above 7 is actually procathepsin E, which can be autocatalytically activated to cathepsin E under acidic conditions [6,7]. Therefore, the enzyme preparation which displayed some activity at pH 6.6 only in the presence of ATP might possibly be procathepsin E rather than cathepsin E.

The rather selective cleavage of the Glu^{13}–Ala^{14} bond in the B chain of oxidized insulin at pH 7.4 suggests that cathepsin E may be involved in limited proteolysis of certain proteins and/or peptides under neutral (physiological) conditions. The reason for the change in cleavage specificity with pH is not very clear, but the difference in ionization state in the enzyme active site region and/or the substrate molecule would be primarily responsible for this change. The enzyme appears to prefer the ionized form to be unionized form of glutamic acid residue in the Glu–X bonds to be cleaved. Interestingly, the enzyme was not inhibited by pepstatin at pH 7.4, although it was strongly inhibited by pepstatin at pH 3.0 and 5.5. This indicates that the enzyme has little affinity to pepstatin at pH 7.4. Therefore, the enzyme is no more a pepstatin-sensitive proteinase at neutral pH.

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