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Recombinant cathepsin E has no proteolytic activity at neutral pH

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Abstract

Cathepsin E (CatE) is a major intracellular aspartic protease reported to be involved in cellular protein degradation and several pathological processes. Distinct cleavage specificities of CatE at neutral and acidic pH have been reported previously in studies using CatE purified from human gastric mucosa. Here, in contrast, we have analyzed the proteolytic activity of recombinant CatE at acidic and neutral pH using two separate approaches, RP-HPLC and FRET-based proteinase assays. Our data clearly indicate that recombinant CatE does not possess any proteolytic activity at all at neutral pH and was unable to cleave the peptides glucagon, neurotensin, and dynorphin A that were previously reported to be cleaved by CatE at neutral pH. Even in the presence of ATP, which is known to stabilize CatE, no proteolytic activity was observed. These discrepant results might be due to some contaminating factor present in the enzyme preparations used in previous studies or may reflect differences between recombinant CatE and the native enzyme.

Keywords: Recombinant cathepsin E; Cleavage specificity; Proteolytic activity; FRET substrate

Cathepsin E (CatE, EC 3.4.23.34) is a non-lysosomal aspartic protease with a limited distribution in certain cell types, including gastric epithelial cells [1], macrophages [2], lymphocytes [2], microglia [3], and dendritic cells [4]. It is localized to different cellular compartments. In a number of cells it appears to be present in vesicular structures associated with the endoplasmic reticulum and Golgi apparatus [3,5,6]. It is also reported to be localized to endosomal structures [3] and the plasma membrane [7]. It is the active form of CatE that shows endosomal localization [8]. CatE plays an important role in the MHC class II antigen processing pathway in B cells [9,10], microglia [11], and dendritic cells [4]. CatE is also expressed in pancreatic ductal adenocarcinoma [12], and its presence in pancreatic juice is reported to be a diagnostic marker for this cancer [13]. Increased levels of CatE in neurons and glial cells of aged rats are suggested to be related to neuronal degeneration and reactivation of glial cells during the normal aging process of the brain [14]. CatE-deficient mice develop atopic dermatitis-like skin lesions [15] and are more susceptible to bacterial infection associated with decreased expression of multiple cell surface Toll-like receptors [16]. According to a most recent study [17], CatE deficiency induces a novel form of lysosomal storage disorder characterized by the accumulation of lysosomal membrane sialoglycoproteins and the elevation of lysosomal pH in macrophages. CatE is normally most active at acidic pH, which corresponds to active endosomal form, preferring substrates with bulky hydrophobic amino acids at the P1 and P1′ positions [18]. However, according to some reports, CatE also retains activity at neutral pH [19,20]. In one of these [19], proteolytic activity and cleavage specificity towards the B chain of oxidized insulin was examined. It was reported that the cleavage specificity changed significantly, with more specific cleavage at pH 7.4 and above, as compared to pH 5.5 and 3.0. At acidic pH, several peptide bonds, especially Phe-X, Tyr-X, and Leu-X were cleaved, whereas at pH 7.4 the Glu^{13}-Ala^{14} bond

Abbreviation: CatE, Cathepsin E.

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was selectively cleaved. In a more recent study [20], preferential cleavage of Arg-X and Glu-X bonds at pH 7.4 was reported, with the Arg-Arg bond the preferred cleavage site.

This reported unique specificity of CatE at pH 7.4, together with the fact that it is localized in different cellular compartments, suggested the possibility that it could be involved in processing or degradation of certain proteins and peptides at or near neutral pH *in vivo*. However, in all the studies reporting proteolytic activity of CatE at pH 7.4, the enzyme was isolated either from human gastric mucosa [19–21] or human red blood cells [21]. In the present study, we determined the proteolytic activity of recombinant CatE in order to avoid any possible contamination of the isolated enzyme with other factors. Peptide substrates such as glucagon, neurotensin, and dynorphin A were used because these were previously reported to be hydrolysed by CatE at neutral pH [20]. The experiments were carried out with or without ATP, which is reported to stabilize CatE [21]. Our data clearly indicate that at neutral pH, recombinant human CatE shows no cleavage activity and is not stabilized by ATP.

Materials and methods

Materials. Cathepsin E was purchased from R&D systems (Wiesbaden, Germany) and stored as 0.1 mg/ml stock solution in 25 mM MES, 0.15 M NaCl, pH 6.5, and 50 % glycerol, at −20 °C. ATP was purchased from Sigma (Taufkirchen, Germany) prepared and used according to the instructions of the supplier.

Solid-phase peptide synthesis. The peptides HSQGTFTSDYSKYLDSRRAQDFVQWLMNT (glucagon), ELYENKPRRPYIL (neurotensin), and YGGFLRRIRPKLKWDNQ (dynorphin A) were synthesized using standard Fmoc/*t*-Bu chemistry [22], performed on the multiple peptide synthesizer Syro II (MultiSynTech, Witten, Germany) on a 0.025-mmol scale using a 6-fold molar excess of Fmoc amino acids (MultiSynTech, Witten, Germany) on TCP-resin (PepChem, Reutlingen, Germany). All other reagents and solvents for peptide synthesis were purchased from Merck KGaA (Darmstadt, Germany). In situ activation was performed using TBTU (6 eq) and HOBt (1 eq) followed by the addition of *N*-methylmorpholine (12 eq) in *N*,*N*-dimethylformamide. After completion of the automated synthesis, the resin-bound peptides were Fmoc deprotected using 20% (v/v) piperidine in *N*,*N*-dimethylformamide twice for 15 min and washed subsequently with *N*,*N*-dimethylformamide, isopropyl alcohol, and diethyl ether. To release the peptides from the resin and to remove the side chain protecting groups, the following solution was used: 92% (v/v) trifluoroacetic acid containing 3% (v/v) thioanisol, 3% (w/v) phenol, and 2% (v/v) ethanedithiol. The peptides were precipitated and washed twice in diethyl ether, dried, and dissolved in 80% (v/v) *tert*-butanol in water followed by lyophilization. Crude peptides were purified using preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and their identity was confirmed using MALDI-MS. Peptide purities were determined via analytical RP-HPLC and proved to be greater than 95%.

A FRET-based peptide substrate derived from glucagon sequence; Mca-KYLDSRRAQDFVQWL-K(Dnp)-NH_2 was also synthesized. Peptide: KYLDSRRAQDFVQWL-K(Dnp)-NH_2 was synthesized as described above. After deprotecting Fmoc using piperidine, on-resin labelling of the peptides with Mca ((7-methoxycoumarin-4-yl)acetyl) was performed. The fluorophore Mca was coupled at a 3-fold excess directly to the α-amino group of the side chain-protected resin-bound peptide in DMF using the TBTU/HOBt activation method for 3 h in the dark [23]. Subsequently, the peptide was released from the resin and processed as described above.

Digestion of peptides and analysis of digestion products using RP-HPLC. Hydrolysis of each peptide substrate by cathepsin E and analysis of the resulting peptides were performed as follows. Neurotensin (5 μg), glucagon (5 μg), and dynorphin A (5 μg) were digested in the absence or presence of ATP (6.25 mM), at 37 °C for 18 h with cathepsin E (10 or 50 ng) in 50 mM sodium phosphate buffer, pH 7.4 or 50 mM sodium acetate buffer, pH 4.0. The digestion experiments were performed simultaneously at pH 4.0 and 7.4, CatE was taken from the same stock aliquot and activated at pH 4.0 prior to digestion at different pH values as recommended by the suppliers (R&D systems). The reaction was terminated by addition of 25 μl stop solution (95% (v/v) ACN, 1% (v/v) trifluoroacetic acid in water.

Peptide fragments were separated via analytical RP-HPLC using a C18 column (150 × 2 mm, Reprosil 100, Dr. Maisch GmbH, Tuebingen, Germany) with the following solvent system: (A) 0.055% (v/v) trifluoroacetic acid in H_2O, and (B) 0.05% (v/v) trifluoroacetic acid in ACN/H_2O (4:1, v/v). The column was eluted with a 20–80% gradient of solvent B for 40 min. UV detection was carried out at 214 nm (UV detector SPD-10AV, Shimadzu, Duisburg, Germany). Manually collected fractions were subsequently analyzed by MALDI-MS.

MALDI-MS. 0.5 μl of each RP-HPLC fraction was mixed with 0.5 μl DHB-matrix [10 mg/ml (w/v) 2,5-dihydroxybenzoic acid in 60% [22] ethanol containing 0.1% (v/v) TFA] and applied on a gold target for MALDI-MS using a MALDI time-of-flight system (Reflex IV, serial no. 26159.00007, Bruker Daltonics, Bremen, Germany). Signals were generated by accumulating 120–210 laser shots. Raw data were analyzed using the software Flex Analysis 2.4 (Bruker Daltonics).

FRET-based assay for the determination of cathepsin E cleavage activity. Catalytic activity of CatE was determined fluorometrically by hydrolysis of the substrate Mca-KYLDSRRAQDFVQWL-K(Dnp)-NH_2. CatE (10 ng) was added to 80 μl digestion buffer (50 mM sodium acetate buffer, pH 4.0 or 50 mM sodium phosphate buffer, pH 7.4) and the reaction was started by addition of 1 nmol substrate (Stock solution 1 mM in DMSO, 1 μl of substrate solution was dissolved in 10 μl of the buffer separately and then added to the reaction mixture). Progress of fluorescent product formation was recorded using a fluorescence reader (Tecan Spectra Fluor, Crailsheim, Germany) on kinetic mode at 37 °C ($\lambda_{ex} = 340$, $\lambda_{em} = 405$). As a positive control for the Arg cleavage site trypsin 0.1 μg was added to 80 μl digestion buffer (50 mM NH_4HCO_3, pH 8.0) and the reaction was started by addition of 1 μl substrate solution as describe above.

Results and discussion

To investigate the proteolytic potential of recombinant CatE at neutral pH, we tested the peptide substrates glucagon, neurotensin, and dynorphin A, previously reported to be cleaved at pH 7.4 [20]. Fig. 1 shows the expected cleavage sites in these peptides at neutral pH. Glucagon was digested *in vitro* with CatE and the peptide fragments thus generated were separated by RP-HPLC (Fig. 2B–E) and identified by MALDI-MS (Table 1). Fig. 2A shows the chromatogram of the undigested peptide substrate (glucagon) as a negative control. Fig. 2B depicts the results of digestion of the glucagon with CatE at pH 4.0 in the presence of ATP at a concentration (6.25 mM) approximating that within the cells, and reported to stabilize CatE [21]. As expected, at acidic pH glucagon was cleaved between Phe-Val residues (Fig. 2B and Table 1) as identified by MALDI-MS. Digestion of glucagon with CatE at pH 4.0 without ATP (Fig. 2C) yielded a similar RP-HPLC-profile as in the presence of ATP. In contrast, when digested at pH

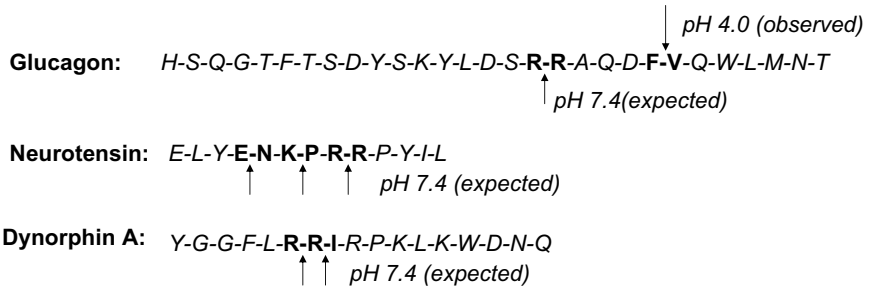

Fig. 1. Different peptides used in the study and potential cathepsin E cleavage sites. All the potential CatE cleavage sites at pH 7.4 and the observed cleavage site at pH 4.0 (glucagon) are printed in bold.

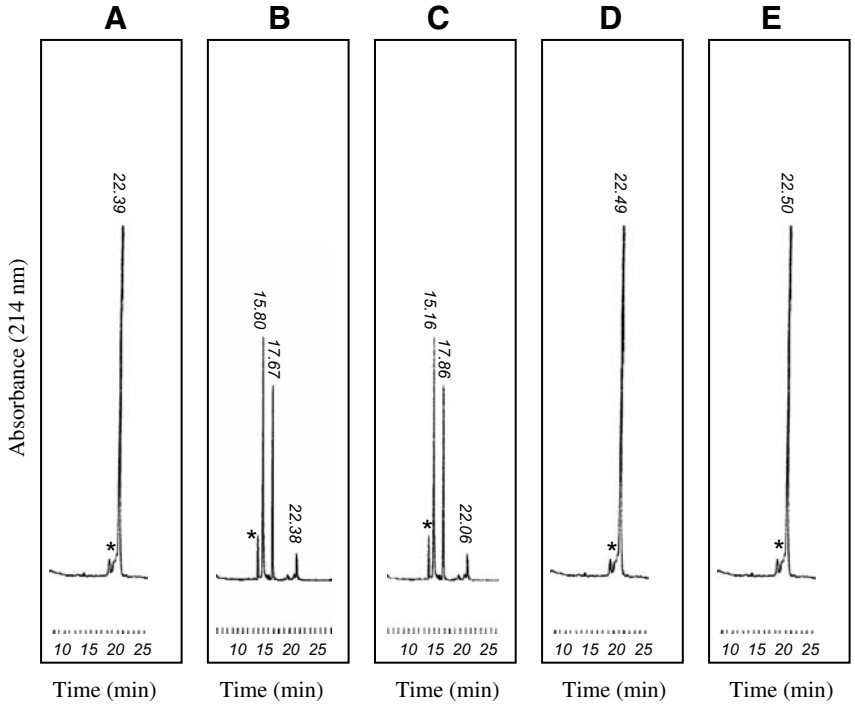

Fig. 2. RP-HPLC profiles depicting digestion of synthetic glucagon peptide with CatE. (A) Undigested peptide. (B) Peptide digested with CatE (10 ng) at pH 4 (0.05 M sodium acetate buffer) in the presence of ATP (6.25 mM). (C) Peptide digested with CatE (10 ng) at pH 4.0 without ATP. (D) Peptide digested with CatE (50 ng) at pH 7.4 (0.05 M sodium phosphate buffer) in the presence of ATP (6.25 mM). (E) Peptide digested with CatE (50 ng) at pH 7.4 without ATP. Digestion of glucagon with CatE at pH 4.0 with or without ATP resulted in one cleavage reaction giving two peaks. Whereas, digestion at pH 7.4 with or without ATP showed no cleavage activity even when five times larger amount of CatE was applied. Peaks were collected and peptides were analyzed using MALDI-MS (see Table 1). *Corresponds to peptides with oxidized methionine.

7.4 no proteolytic activity was observed with or without ATP (Fig. 2D–E and Table 1). Neurotensin, previously reported to be cleaved at Arg-Arg, Lys-Pro, and Glu-Asn [20] by human CatE at pH 7.4 was also examined (Fig. 3). Again no cleavage activity was observed at pH 7.4 with or without ATP (Fig. 3B–C and Table 1). Finally, digestion of dynorphin A with CatE at pH 7.4 also showed no proteolytic activity with or without ATP (Table 1).

In order to confirm the above mentioned results another approach, i.e., FRET-based proteinase assay was used for determining proteolytic activity at neutral pH. According to the sequence of glucagon, a FRET-based peptide substrate for CatE, i.e., Mca-KYLDSRRAQDFVQWL-K(Dnp)-NH_2 [where Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is dinitrophenyl] was synthesized. It is an intramolecularly quenched fluorogenic peptide derivative in which the fluorescent signal of the fluorophore Mca is quenched by the chromophoric residue Dnp. After cleavage of the peptide, the quenching efficiency is decreased, resulting in an increase in fluorescence. Using this substrate, cleavage activity of CatE at pH 7.4 and 4.0 was determined. As shown in Fig. 4, no increase in fluorescence

Table 1
Identified peptides after digestion of neurotensin, glucagon, and dynorphin A by CatE, as determined by MALDI-MS (digestion experiments performed in the presence of 6.25 mM ATP)

Substrate	pH	Peptides identified by MALDI-MS	$[M+H]^+$		Retention time
			Theoretical	Observed	
Glucagon HSQGTFTSDYSKYLDSRRAQDFVQWLMNT	4	HSQGTFTSDYSKYLDSRRAQDF	2609.77	2609.39	15.8
		VQWLMNT	891.06	891.06	17.67
		Glucagon (uncleaved)	3482.82	3481.95	22.38
	7.4	No cleavage activity was observed. Only undigested glucagon recovered	3482.82	3481.86	22.49
Neurotensin ELYENKPRRPYIL	7.4	No cleavage activity was observed. Only undigested neurotensin recovered	1690.98	1690.88	15.55
Dynorphin A YGGFLRRIRPKLKWDNQ	7.4	No cleavage activity was observed. Only undigested dynorphin A recovered	2147.53	2147.53	17.60

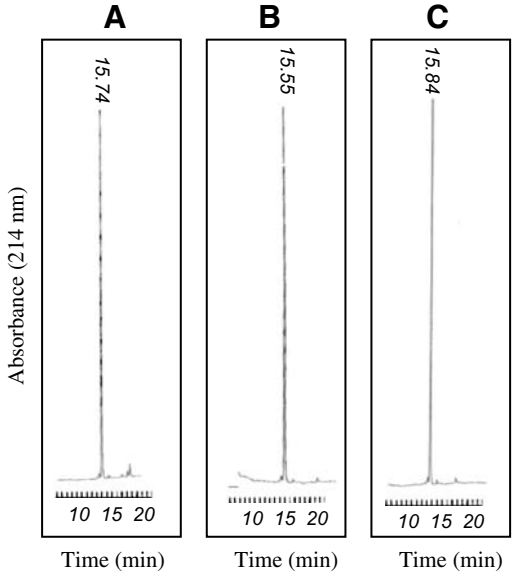

Fig. 3. RP-HPLC profiles depicting digestion of synthetic neurotensin peptide with CatE. (A) Undigested peptide. (B) Peptide digested with CatE (50 ng) at pH 7.4 (0.05 M sodium phosphate buffer) in the presence of ATP (6.25 mM). (C) Peptide digested with CatE (50 ng) at pH 7.4 without ATP.

was observed at pH 7.4 whereas at pH 4.0 there was a rapid increase. To have a positive control for the Arg cleavage site trypsin was included in the experiment as it cleaves C-terminal to Arg residues. As also shown in Fig. 4, trypsin cleaved the substrate efficiently at pH 8.0.

Our results fail to reveal any proteolytic activity of recombinant CatE at neutral pH even in the presence of ATP. Thus, the purified enzyme used in previous studies [18,19] might have been contaminated with some other factor. Alternatively, these discrepant results may reflect conformational differences between recombinant CatE and the native enzyme. These differences may also arise from post-translational modification of the native enzyme *in vivo*.

Acknowledgments

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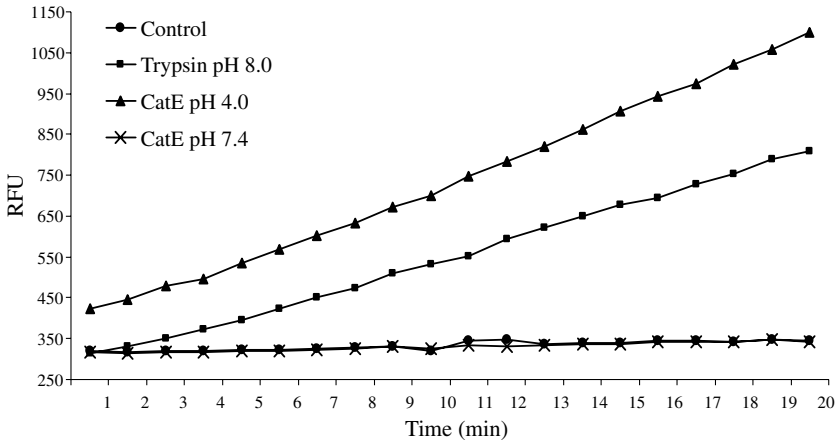

Fig. 4. FRET-based assay for the determination of CatE cleavage activity at pH 4.0 and 7.4. Hydrolysis of the fluorogenic peptide substrate based on glucagon sequence (Mca-KYLDSRRAQDFVQWL-K(Dnp)-NH_2) (10 μM) by 10 ng of CatE in 50 mM sodium acetate buffer (pH 4) at 37 °C or 0.05 M sodium phosphate buffer (pH 7.4) at 37 °C or 0.1 μg of trypsin in 50 mM NH_4HCO_3 (pH 8.0). Control is the sample containing only the substrate and no enzyme.

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