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The catalytic domain of cathepsin C (dipeptidyl-peptidase I) alone is a fully functional endoprotease

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Abstract

Cathepsin C is a tetrameric lysosomal protease that acts as a dipeptidyl-peptidase due to the presence of the exclusion domain that is unique among papain-like cysteine proteases. Here we describe a recombinant form of cathepsin C lacking its exclusion domain (CatCΔEx) produced in bacterial expression system (*E. coli*). CatCΔEx is a monomer with endoprotease activity and affinity for hydrophobic residues such as Phe, Leu or Pro, but not Val, in the P2 position. As opposed to cathepsin C, it does not require chloride ions for its activity. Despite lower turnover rates of hydrolysis of synthetic substrates, CatCΔEx has elastolytic and gelatinolytic activity comparable to other cysteine cathepsins.

Key words

proteolysis, oligomeric proteins, exclusion domain, elastolysis, gelatinolysis

Abbreviations

AMC-, 7-amido-4-methylcoumarin; Boc-, t-butoxy-carbonyl; Bz-, benzoyl; DTT, 1,4-dithiothreitol; E-64, trans-Epoxy succinyl-L-leucylamido(4-guanidino)butane; EDTA, Ethylenediaminetetraacetic acid; IPTG, isopropyl β-d-1-thiogalactopyranoside; Suc-, Succinyl; Z-, benzyloxycarbonyl;

Introduction

Human cathepsin C (also known as dipeptidyl-peptidase I; EC 3.4.14.1) is a lysosomal protease from the family of papain-like cysteine proteases, also called cysteine cathepsins.¹ Like other members of the family it is responsible for non-specific protein degradation in the mildly acidic and reducing milieu of the lysosome. However, experiments with knock-out mice and genetic studies have also identified specific roles for cathepsin C in cells of the immune system that become apparent when the enzyme's activity is inappropriate.² It has been shown that cathepsin C activates effector serine proteases in immune cells and its excessive activity has been connected with different inflammatory diseases such as chronic obstructive pulmonary disease and cystic fibrosis.^{3,4} Insufficient activity, on the other hand, leads to autoimmune diseases such as Papillon-Lefèvre and Haim-Munk syndrome, characterized by palmoplantar keratoderma and severe periodontitis.⁵⁻⁷ On account of its involvement in various diseases, cathepsin C has become a promising target for drug development.⁸

The unique feature of cathepsin C is an additional part termed the exclusion domain whose fold is similar to metalloprotease inhibitors.⁹ The crystal structure of DPPI has shown that the active form of the enzyme is a homotetramer (Figure 1A)⁹ and each subunit consists of the exclusion domain non-covalently bound to the catalytic domain. The exclusion domain sterically blocks access to subsites beyond S2 of the active site,¹⁰ making cathepsin C a dipeptidyl-peptidase (Figure 1B). In addition, the side chain of residue Asp1 interacts with the N-terminus of the substrate and thereby contributes to substrate binding.

Figure 1: Three dimensional structure of human cathepsin C. A) Active cathepsin C tetramer (PDB accession code 1K3B). Catalytic domains are shown in surface representations and coloured alternatingly grey and blue, respectively. The exclusion domain is shown as yellow ribbons. **B)** Closer view of the active site of a single subunit. Active site residues Cys234 and His381 are coloured yellow and blue, respectively, and subsites S1 and S2 are denoted. The essential chloride ion is shown as a green sphere. The exclusion domain (yellow ribbons) blocks subsites beyond S2. The side chain of Asp1 (shown as sticks) interacts with the N-terminus of the substrate. Images were created using UCSF Chimera software.⁴²

Like other cysteine cathepsins, cathepsin C is synthesized as a preproenzyme. The exclusion domain has been proposed to function as an intramolecular chaperone and assist in the folding of the enzyme.¹¹ It is also important in tetramer formation, along with proper N-glycosylation.¹² In the lysosome, cathepsin C is activated by other lysosomal proteases, the pro-domain is removed and the catalytic domain is split into heavy and light chains, leaving the exclusion domain connected to the heavy chain only by non-covalent bonds.^{9-11,13} This leaves room for possibility that a single catalytic domain can dissociate from the tetrameric complex. Studies of the related papain-like exoprotease cathepsin B have shown that the enzyme can switch between exo- and endoproteolytic activities in response to changes in pH.¹⁴ It is also known that cathepsin H lacking its mini chain, a propeptide remnant responsible for aminopeptidase activity, acts as an endoprotease.¹⁵ This indicates that if the catalytic domain of cathepsin C were to “escape” the tetramer it could also act as an endoproteolytic enzyme. It has, in fact, been shown that canine and rabbit cathepsin C can cleave substrates typical for cysteine cathepsin endoproteases under certain circumstances.^{16,17} However, the molecular basis for these observations has remained uninvestigated.

In this work, we report the production of recombinant procathepsin C lacking the exclusion domain (proCatCΔEx) in soluble form in a previously established bacterial expression system.¹⁸ We activate the proenzyme by pepsin treatment, demonstrate its endoproteolytic activity and characterize its interactions with different synthetic and macromolecular substrates.

Materials and methods

Materials

Primers, restriction enzymes and T4 DNA ligase, were from ThermoFischer Scientific (USA). All cysteine proteases (human cathepsins B, K, L and S) were produced in-house by the protocol described by Novinec et al.¹⁸ The Ni-NTA Superflow column was from Qiagen (Germany). Column Superdex 75 10/300 was from GE Healthcare, (UK). The fluorogenic substrates, irreversible inhibitor E-64 and pepstatin A were from Bachem (Switzerland). Soluble calf skin collagen was from Worthington Biochemical Company (USA); Soluble ETNA elastin was from Elastin Products Company (USA).

Molecular cloning

The expression plasmid pET-32/28-His₆-proCatCΔEx was constructed by amplifying the cDNA sequence coding for proCatCΔEx (human cathepsin C residues 145 to 463) by polymerase chain reaction using the forward primer 5'- CGG GCT AGC GCC TCT GAG AAT GTG TAT GTC -3' reverse primer 5'-CCG CTC GAG CTA CAA TTT AGG AAT TGG TGT GG -3' (restriction sites *NheI* and *XhoI*, respectively, are underlined) and inserting the PCR product into the pET-32/28 vector, assembled as described previously [18], using T4 DNA ligase and restriction sites *NheI* and *XhoI*. Correct PCR amplification was verified by sequencing using a commercial sequencing service.

Expression, purification and activation of proCatCΔEx

CatCΔEx was produced according to the protocol published for expressing other cysteine cathepsins in soluble form in *E.coli*.¹⁸ In brief, the expression strain Rosetta-gami 2 (DE3) pLysS (Novagen) was transformed with the pET-32/28-proCCΔEx plasmid. Cells were cultivated at 37 °C in LB medium with ampicillin, chloramphenicol and tetracycline until A₆₀₀ 0.6 - 0.8. Expression of CatCΔEx was then induced with 0.4 mM IPTG and cells grown for 20 hours at 18 °C. Cells were harvested by centrifugation, resuspended in binding buffer (20 mM sodium phosphate buffer pH 7.4 with 500 mM sodium chloride and 20 mM imidazole) and lysed by sonication. The cleared cell lysate was applied to a pre-equilibrated Ni-NTA Superflow affinity column, the latter washed with 10 column volumes of binding buffer and bound proteins eluted in a linear gradient of imidazole from 20 to 250 mM. Fractions containing proCatCΔEx were dialysed against 20 mM sodium phosphate buffer pH 7.4 with 300 mM NaCl, concentrated to a protein concentration between 0.5-1.5 mg/ml and stored at -80 °C. Activation of CatCΔEx was carried out by addition of 5% of sample volume of 3 M sodium acetate buffer pH 3.8, pepsin (final concentration 50 µg/ml), DTT (final concentration 5 mM) and 0.05% Triton X-100. The activation mixture was then incubated at 37 °C. The activation was monitored using the synthetic substrate Z-Phe-Arg-AMC. When peak activity was achieved, pepsin was inhibited with the addition of pepstatin A in a 1:1

molar ratio. The active concentration of CatCΔEx was determined by active site titration with the irreversible inhibitor E-64.

Size-exclusion chromatography

Size-exclusion chromatography was optionally for additional purification of proCatCΔEx prior to activation and to determine the oligomeric composition of purified proCatCΔEx. Samples were applied to a Superdex 75 10/300 column (GE Healthcare, USA) equilibrated in 50 mM sodium acetate buffer pH 5.5 with 300 mM sodium chloride and 1 mM EDTA. The elution volumes of calibrating proteins were taken from the manufacturer's product manual.

Kinetic measurements

All kinetic measurements were carried out in a Perkin Elmer LS 50 B fluorescence spectrometer. Continuous assays were performed with constant stirring at 25 °C in 50 mM sodium acetate buffer pH 5.5 with 100 mM sodium chloride (unless otherwise indicated), 1 mM EDTA and 2.5 mM DTT. Hydrolysis of all fluorogenic substrates was monitored at λ_{ex} 370 nm and λ_{em} 455 nm. All experimental data were processed and analyzed with GraphPad Prism 5.0 software (USA).

Discontinuous assays were performed by incubating the enzyme (final concentration 5 nM) with 100 μ M substrate in the same reaction buffer used for continuous assays. Samples were incubated at 25 °C for 3 hours and then their fluorescence measured. All experiments were performed in triplicates. Specific enzyme activity (expressed as μ mol product produced per mg enzyme per minute) was then calculated for each substrate.

The dependence of enzyme activity on the concentration of sodium chloride was measured in buffers with increasing sodium chloride concentrations, obtained by mixing two 50 mM sodium acetate buffers pH 5.5 containing 0 and 1 M sodium chloride, respectively, in varying proportions. Each buffers also contained 2.5 mM DTT and 1 mM EDTA.

To determine the pH profile, activity was measured at a low substrate concentration (0.5 μ M Z-Phe-Arg-AMC) in a range of buffers with varying pH values.¹⁹ Citrate buffer was used for pH 3.0 to 4.0, acetate buffer for pH 4.5 to 5.8, phosphate buffer for pH 6.0 to 8.0 and tris/HCl buffer for pH 8.5 to 9.0. All buffers also contained 100 mM NaCl, 2.5 mM DTT and 1 mM EDTA. Experimental data were analysed with the simplified form of the four protonation state model,²⁰ using the equation:

$$\left(\frac{k_{cat}}{K_m}\right)_{obs} = \frac{\left(\frac{k_{cat}}{K_m}\right)_{lim}}{\frac{[H^+]}{K_1} + 1 + \frac{K_2}{[H^+]}} \quad (1)$$

where $(k_{cat}/K_m)_{lim}$ is the limiting k_{cat}/K_m value and K_1 and K_2 correspond to the pKa values of the ionisable groups.

Thermal stability experiments were carried out by incubating the enzyme (final concentration 1 μ M) in buffers with different pH values at 37 °C. Acetate buffer was used for pH 4.5 and phosphate buffer for pH 6 and 7.4. All buffers also contained 100 mM NaCl, 2.5 mM DTT and 1 mM EDTA. Samples were withdrawn in regular time intervals and their activity measured using 5 μ M Z-Phe-Arg-AMC. Data were analysed by GraphPad Prism 5.0 using the equation for one phase exponential decay.

Azocasein degradation assay

The caseinolytic activity of CatCΔEx was measured by incubating each enzyme (final concentration 0.1 μM) with a 3 mg/ml azocasein solution in 50 mM sodium acetate buffer pH 5.5 with 100 mM NaCl and 1 mM EDTA. All reactions mixtures were incubated at 37 °C for to 2 hours and the proteins then precipitated with 5% (w/v) trichloroacetic acid. Samples were cleared by centrifugation and cleared supernatants mixed with 4 volumes of 0.5 M NaOH. Absorption of the samples was then measured at 440 nm.

Elastin degradation assay

Soluble ETNA-elastin was dissolved in 50 mM sodium acetate buffer pH 5.5 with 100 mM sodium chloride and 1 mM EDTA to a concentration of 5 mg/ml. 200 μL aliquots were incubated with each enzyme (final concentration 0.1 μM) for 3 hours at 37 °C. Proteins were then precipitated with 5% (w/v) trichloroacetic acid. After centrifugation, clear supernatants were mixed with 2.8 ml 0.2 M sodium borate buffer pH 8.5 and 1 ml of fluorescamine solution (0.15 mg/ml in acetone). The fluorescence of the samples was then measured at λ_{ex} 390 nm and λ_{em} 480 nm. A standard curve was constructed using a range of concentrations of Ala.

Gelatin degradation assay

Soluble calf skin collagen was diluted in reaction buffer (50 mM sodium acetate buffer pH 5.5 with 100 mM NaCl and 1 mM EDTA) to a concentration of 1 mg/ml and then heat-denatured by incubation at 45 °C for 20 min. Denatured collagen samples were then incubated with each enzyme at final concentration 0.1 μM. Reactions were stopped by the addition of an excess amount of E-64 and protein bands were visualised by SDS-PAGE on a 7% polyacrylamide gel.

Collagen degradation assay

Soluble calf skin collagen was diluted in reaction buffer (50 mM sodium acetate buffer pH 5.5 with 100 mM NaCl and 1 mM EDTA) to a concentration 0.4 mg/ml. Samples were then incubated with each enzyme at final concentration 1 μM for 4, 8 and 24 hours, respectively, at 28 °C. Reactions were stopped by the addition of an excess amount of E-64 and protein bands were visualised by SDS-PAGE on a 7% polyacrylamide gel.

Results

Production and activation of recombinant CatCΔEx

For the production of recombinant CatCΔEx, the expression plasmid pET-32/28-His₆-proCatCΔEx was constructed by amplifying the proCatCΔEx sequence (comprising residues 145 to 463 of full-length human cathepsin C) by PCR and inserting it in to the pET-32/28 expression vector in frame with an N-terminal His₆ tag using *Nhe*I and *Xho*I restriction sites. The recombinant protein was expressed in soluble form in the cytoplasm of *E. coli* strain Rosetta-gami 2 (DE3) pLysS using the same protocol used for the preparation of other soluble cysteine cathepsins.¹⁸ SDS-PAGE analysis of cell lysates before and after induction of expression showed a weak band at approximately 40 kDa which corresponds to the calculated molecular mass of CatCΔEx which is 38,442 Da (Figure 2A). Soluble recombinant proCatCΔEx was purified from the cell lysate by immobilized nickel ion-affinity chromatography and the yields of each step in the procedure are presented in Table 1. Eluted fractions were further purified by size exclusion chromatography, which at the same time provided information about the oligomeric composition of proCatCΔEx. The protein was eluted at an elution volume corresponding to a molecular mass of about 40 kDa (Figure 2B). Since proCatCΔEx has a calculated molecular mass of approx. 38 kDa, these data show that proCatCΔEx is a monomer. The protein was electrophoretically pure (Figure 2C) and the final purification yield was about 2 mg of proCatCΔEx per litre of bacterial culture.

Table 1: Yields of individual steps in the production of recombinant proCatCΔEx per liter bacterial culture.

Step	Protein (mg)	Total yield (in%)	Purity (in%)
<i>E. coli</i> culture	10 ^a	100 ^a	
Purified proenzyme	2 ^b	20 ^b	95 ^c
Active enzyme	0.2 ^d	2 ^d	>95 ^c

^a Determined by SDS–PAGE using different concentrations of BSA as standard.

^b Determined from A280 of the purified protein.

^c Determined by SDS–PAGE. 95% purity indicates that the protein is electrophoretically pure.

^d Determined by active site titration with the irreversible inhibitor E-64.

ProCatCΔEx did not activate autocatalytically, nor could it be activated by cathepsin L like full-length cathepsin C.²¹ However, it could be converted to its enzymatically active form by treatment with pepsin, similar to several other cysteine cathepsin endoproteases.²² Processing by pepsin was accompanied by significant precipitation. After incubation, the activation mixture was cleared by centrifugation and analysed on SDS PAGE (Figure 2C). The gel showed the presence of a single band with a molecular mass of about 27 kDa, which corresponds well to the theoretical molecular mass of 25,919 Da calculated for the catalytic domain of cathepsin C and indicates that the catalytic domain is not split into heavy and light chains by pepsin treatment and hence represents activated CatCΔEx.

Active CatCΔEx did not cleave the commonly used cathepsin C substrate H-Gly-Phe-AMC. Instead, enzyme activity was observed with fluorogenic substrates that are commonly used to measure the activity of cysteine cathepsin endoproteases. The activation was thus followed using the substrate Z-Phe-Arg-AMC (Figure 2D). The

enzyme reached maximum activity after 4 hours activation. We followed the reaction for an additional 4 hours and observed a slow and steady decline of enzyme activity. In subsequent activation experiments the activation was therefore always stopped by the addition of a 1:1 molar ratio of pepstatin A when peak activity was reached. In comparison, cathepsin L treatment also resulted in an initial increase of activity towards Z-Phe-Arg-AMC followed by a rapid decline (results not shown). Moreover, since the specificity of CatCΔEx obtained by pepsin treatment appeared to be similar to other cysteine cathepsin endopeptidases, activation with cathepsin L or S, which are both known to activate full-length cathepsin C,²¹ was not further investigated as it would result in contamination of the CatCΔEx sample by other proteases with similar specificity.

The active enzyme concentration of CatCΔEx could be determined by active site titration with the irreversible inhibitor E-64. As opposed to high expression yields, the activation efficiency was relatively low (about 10%), thus the final yield of active CatCΔEx was about 0.2 mg per litre of bacterial culture. Nevertheless, we were able to obtain sufficient amounts of active enzyme to perform all the desired experiments.

Figure 2: Expression, activation and oligomeric composition of recombinant proCatCΔEx. **A)** SDS-PAGE analysis of the expression of recombinant proCatCΔEx. *Escherichia coli* cell lysates were analysed prior to induction (-IPTG) and after overnight expression at 18°C (+IPTG) on a 12.5% polyacrylamide gel. Proteins were stained with Coomassie brilliant blue R-250. **B)** Size exclusion chromatography of proCatCΔEx, purified from the bacterial cell lysate by immobilised metal ion affinity chromatography, on a Superdex 75 column. The peak corresponding to proCatCΔEx is marked by the arrow. The positions of calibrating standards were taken from the manufacturer manual. **C)** SDS-PAGE analysis of purified proCatCΔEx and activated enzyme (CatCΔEx) after pepsin treatment on a 12.5% polyacrylamide gel. Proteins were stained with Coomassie brilliant blue R-250. **D)** Time course of CatCΔEx activation followed by activity measurements using the substrate Z-Phe-Arg-AMC.

Substrate specificity of CatCΔEx

To establish a basic substrate specificity profile of CatCΔEx we have tested its activity on several commonly used synthetic substrates and determined the corresponding kinetic parameters K_m and k_{cat} . These experiments were performed under conditions optimal for wild type cathepsin C, i.e. in the presence 100 mM sodium chloride and at pH 5.5. The results are collected in Table 2. The enzyme showed selectivity for specific residues in the P2 position of the substrate, which is typical for cysteine cathepsin proteases, with preference for residues with large hydrophobic side chains. Highest activity was observed with substrates Z-Phe-Arg-AMC and Z-Leu-Arg-AMC as well as Boc-Val-Leu-Lys-AMC indicating that position P3 does not play an important role in substrate recognition. Interestingly, the enzyme showed appreciable activity on the substrate Z-Gly-Pro-Arg-AMC, it thus accepts Pro in position P2 which is an uncommon preference in papain like cysteine proteases. In contrast, very little activity was observed with substrates containing small hydrophobic residues (Val, Ala or Gly) or Arg in position P2, as well as Bz-Arg-AMC. The specific activity of CatCΔEx on these substrates could be determined only after prolonged incubation for 3 hours and is 2 to 3 orders of magnitude lower than for optimal substrates.

Table 2: Kinetic parameters for the hydrolysis of synthetic substrates by CatCΔEx. All experiments were performed in 50 mM sodium acetate pH 5.5 with 100 mM sodium chloride, 1 mM EDTA and 2.5 mM DTT at 25 °C. The parameters were determined using GraphPad Prism 5.0 software.

	K_m (μM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (× 10 ⁴ M ⁻¹ s ⁻¹)	Specific activity x10 ⁻³ (μmol/mg)
Z-Phe-Arg-AMC	6.5 ± 1.0	0.23 ± 0.05	3.5 ± 0.9	500 ± 110 ¹
Z-Leu-Arg-AMC	2.3 ± 0.3	0.18 ± 0.03	7.8 ± 1.7	400 ± 70 ¹
Boc-Val-Leu-Lys-AMC	2.8 ± 0.7	0.16 ± 0.01	5.7 ± 1.5	360 ± 20 ¹
Z-Gly-Pro-Arg-AMC	28 ± 2	0.13 ± 0.01	0.46 ± 0.05	230 ± 20 ¹
Z-Val-Val-Arg-AMC	n.d.	n.d.	n.d.	2.34 ± 0.06
Z-Phe-Val-Arg-AMC	n.d.	n.d.	n.d.	3.00 ± 0.08
Suc-Ala-Ala-Phe-AMC	n.d.	n.d.	n.d.	0.17 ± 0.06
H-Gly-Phe-AMC	n.d.	n.d.	n.d.	0.2 ± 0.1
Z-Arg-Arg-AMC	n.d.	n.d.	n.d.	0.17 ± 0.07
Bz-Arg-AMC	n.d.	n.d.	n.d.	8 ± 1

¹ specific activity was calculated from values of K_m and k_{cat} using the Michaelis-Menten equation.

Activity profile of CatCΔEx

Cathepsin C requires the presence of halide ions, most efficiently chloride, for enzymatic activity.^{23,24} Therefore, we have initially examined the activity of CatCΔEx in the presence of increasing concentrations of sodium chloride to establish whether the same is true for CatCΔEx. The substrate Z-Leu-Arg-AMC was chosen because its highest k_{cat}/K_m value. The lowest concentration of chloride tested was 0.15 mM, due to the presence of sodium chloride in the enzyme preparation. This concentration is significantly below the 4 mM equilibrium dissociation constant determined for chloride ions and wild type cathepsin C at this pH.²⁴ The results in Figure 3A show that sodium chloride does have a potentiating effect on the enzyme and optimal activity was observed between 50 and 200 mM sodium chloride. However, enzyme activity in the presence of the lowest chloride concentration was about 55% of optimal activity. The kinetic parameters K_m and k_{cat} determined for hydrolysis of Z-Leu-Arg-AMC at the lowest chloride concentration were $6.3 \pm 1.0 \mu\text{M}$ and $0.21 \pm 0.01 \text{ s}^{-1}$, respectively. Thus, removal of chloride ions causes only a minor (2 to 3-fold) increase in the K_m value, but no drastic changes in the turnover rate. In contrast, wild type cathepsin C had about 20-fold higher activity in 50 mM sodium chloride than in 0.2 mM.²⁴ These results indicate that CatCΔEx does not require chloride ions for its activity. This unique characteristic of cathepsin C thus appears to be directly linked to its dipeptidyl-peptidase activity.

In the continuation we established the pH profile for the hydrolysis of Z-Phe-Arg-AMC. This substrate was chosen due to its higher K_m value as opposed to Z-Leu-Arg-AMC, allowing us to perform experiments at a substrate concentration significantly below the K_m value ($[S] \ll K_m$). Experiments were performed in presence of 100 mM sodium chloride across a broad range of pH values (3.0 to 9.0). We found, that the enzyme is optimally active between pH 5 and 7 with peak activity at pH 6.0 (Figure 3B). Finally, we tested the thermal stability of CatCΔEx at 37 °C at three different pH values, i.e. under optimal conditions (pH 6.0), under acidic, lysosome-like conditions (pH 4.5) and at physiological plasma pH of 7.4 (Figure 3C). At pH 4.5 and 6.0 we did not observe any significant changes in enzyme activity over 4-hour incubation periods. At pH 7.4 the enzyme was less stable and total loss of activity was observed after 200 min of incubation. The calculated half-life of the enzyme under these conditions was $52 \pm 2 \text{ min}$.

Figure 3: Activity profile of CatCΔEx. **A)** Salt dependence of CatCΔEx activity on the substrate Z-Leu-Arg-AMC. **B)** pH profile for the hydrolysis of Z-Phe-Arg-AMC by CatCΔEx. The substrate concentration was $0.5 \mu\text{M}$ ($[S] \ll K_m$) and k_{cat}/K_m values were calculated directly from the slopes of the progress curves. The fit was obtained with Equation 1. **C)** Thermal stability of CatCΔEx at 37 °C at pH 4.5, 6.0

and 7.4, respectively. Residual activity was measured with the substrate Z-Phe-Arg-AMC. The fits were obtained with the one phase exponential decay equation in GraphPad Prism 5.0.

Degradation of macromolecular substrates

For further insight into the proteolytic activity of CatCΔEx we investigated its activity on macromolecular substrates and compared it with other human cysteine cathepsins. The substrates included azocasein as a non-specific protein substrate that is successfully cleaved by most endoproteases, as well as elastin and type I collagen, structural proteins that are often targets of cysteine cathepsins under pathological conditions.²⁵ CatCΔEx cleaved azocasein, elastin and denatured collagen (gelatin), but not native type I collagen (Figure 4). The caseinolytic activity of CatCΔEx was on average 5 to 10-fold lower than that of other cysteine cathepsins (Figure 4A), thus reflecting its lower turnover rates. Nonetheless, CatCΔEx was only a few-fold weaker than cathepsin B. Interestingly, cathepsin V was the strongest caseinolytic protease by far, with 20-fold higher activity than CatCΔEx and 2- to 4-fold higher activity than other cysteine cathepsins.

Elastin degradation assays were performed at two different pH values, i.e. at pH 5.5 which is optimal for the stability of most proteases and physiological plasma pH 7.4. At pH 5.5 the activity of CatCΔEx was on average 3 to 4-fold lower than other proteases. At pH 7.4 on the other hand, CatCΔEx was more active than cathepsins B, L and V, likely due to a comparatively smaller loss of enzyme activity. Only cathepsins K and S that are known to be relatively stable under these conditions^{26,27} had higher observed activity (Figure 4B).

Similar to previous assays, CatCΔEx degraded gelatin, albeit with lower efficiency than cathepsins K and L (Figure 4C). Nevertheless after the prolonged incubation (40 minutes) CatCΔEx was able to completely degrade gelatin into small fragments not observable on the SDS-PAGE (not shown). Native type I collagen, on the other hand, is a tough substrate for proteases that is successfully degraded by cathepsin K and in weaker manner by cathepsin L.^{19,28} Samples of native type I collagen incubated with CatCΔEx showed no detectable collagenolytic activity of the protease (Figure 4D).

Figure 4: CatCΔEx activity on macromolecular substrates. A) Caseinolytic activity of CatCΔEx and selected cysteine cathepsins. Data represent the A_{440} of released peptides and are normalised to CatCΔEx. **B)** Elastolytic activity of CatCΔEx and selected cysteine cathepsins, presented as the quantity of elastin peptides released per enzyme molecule per second. Experiments were performed at pH 5.5 (top) and pH 7.4 (bottom). All experiments in panels A and B were performed in triplicates. Error bars represent the s.e.m. of the experiments. **C)** SDS-PAGE analysis of gelatin degradation by CatCΔEx and cathepsins K and L. Lanes marked 0 represent untreated gelatin. **D)** SDS-PAGE analysis of collagen degradation by CatCΔEx and cathepsins K and L. Lanes marked 0 represent untreated gelatin. Samples in panels C and D were run on 7% polyacrylamide gel and stained with Coomassie brilliant blue R-250.

Discussion

In this work we successfully prepared a recombinant form of (pro)cathepsin C without its exclusion domain ((pro)CatCΔEx) in *E. coli*. The wild-type protein is unique in the papain-like family for being a tetramer in its active state¹³ which forms via a dimeric zymogen²¹. In contrast, both proCatCΔEx and CatCΔEx were monomers like other cysteine cathepsins, demonstrating the crucial role of the exclusion domain in oligomerization. The high expression yield of soluble proCatCΔEx also shows that neither the exclusion domain nor N-glycosylation are necessary for proper folding of the remaining part of cathepsin C *in vitro*.^{11,12}

While there is no evidence of such a form of cathepsin C *in vivo*, its existence is not entirely impossible, given that an alternative spliced variant, consisting only of the first two exons has been identified.²⁹ Moreover, alternative forms produced by exon skipping were reported for other cysteine cathepsins, e.g. cathepsin B.³⁰ A more likely scenario is, however, that under certain stimuli a catalytic domain can be released from the tetramer. Cathepsin C is primarily a lysosomal enzyme but it can also be found in other cellular compartments. Neutrophils, for example, contain active cathepsin C in the Golgi apparatus, cytoplasmic granules and also secrete active enzyme into the extracellular space.³¹ Similarly, active cathepsin C is secreted from tumor cells, either by lysosome exocytosis³² or directly³³. Lysosomal cathepsin C can also enter the cytosol due to lysosomal membrane permeabilisation associated with apoptosis, aging and neurological disorders.³⁴ Endoproteolytically active cathepsin C could potentially be produced in any compartment, where cathepsin C is present.

The specificity and affinity of CatCΔEx for small synthetic substrates are similar to other cysteine cathepsin proteases,³⁵ with the marked preference for Leu and Phe but not Val in the position P2. Interestingly, the enzyme also accepts Pro in the position P2. This preference has been observed in cathepsin K and associated with the ability to cleave triple helical collagen.³⁶ However, CatCΔEx does not cleave intact collagen (Figure 4D), whereas cathepsin L, which lacks proline specificity does.²⁸ Therefore, the relation between collagenolytic activity and proline specificity is not straight forward. CatCΔEx has significantly lower k_{cat} values for the hydrolysis of synthetic substrates than other cysteine cathepsins³⁵ and consequently k_{cat}/K_m values are 1 or 2 orders of magnitude lower in comparison to the dipeptidyl-peptidase activity of cathepsin C.³⁷ Nevertheless, k_{cat} values in this order of magnitude are not unusual for proteolytic enzymes and similar values have recently been determined for the papain-like protease responsible for cell death in the plant *N.benthamiana*.³⁸

Importantly, CatCΔEx shows significant activity towards protein substrates including extracellular matrix components (gelatin, elastin) that are targets for proteolytic degradation by cysteine cathepsins in normal physiological processes such as wound healing and also in various diseases.²⁵ Interestingly, cathepsin C has also been shown to degrade gelatin in solution before.¹⁷ Considering our results obtained with CatCΔEx we can assume that the measured activity was likely due to disruption of the tetrameric structure resulting in endoproteolytic activity of a liberated catalytic domain. The prospect of extracellular activity of CatCΔEx is reinforced by its significantly higher stability at physiological plasma pH (Figure 3D) in comparison to other ubiquitously expressed lysosomal proteases, such as cathepsins B and L.^{39,40} Its comparably higher stability closes the gap between the elastolytic activities of CatCΔEx and other cysteine cathepsins at pH 7.4 as opposed to pH 5.5, where all enzymes are optimally stable (Figure 4B). Apart from the cleavage of structural proteins, CatCΔEx could also

be involved in the shedding of proteins from the cell surface and thus affect cell signalling.⁴¹

Cathepsin C is a ubiquitously expressed enzyme with important roles in the immune system and a possible target for the treatment of the immune disorders such as autoimmune diseases.⁸ However, little is known about its biological function outside of the immune system. The results presented herein provide significant information on the biochemical properties of cathepsin C and present a good basis for further studies of CatCΔEx characteristics and potential roles in biological processes.

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Author contributions

MR performed experiments and wrote the manuscript. BL secured funding. MN designed the experiments. All authors interpreted data and discussed the results.

Conflict of interest

The authors declare no conflicts of interest.

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