

# Cathepsin L is capable of truncating cystatin C of 11 N-terminal amino acids

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**Abstract** Cystatin C with the 11 N-terminal amino acids truncated shows a much lower affinity for cysteine proteinases than the intact inhibitor. Such truncation of cystatin C is recorded after action of glycyl endopeptidase and cathepsin L. Incubation of cystatin C with papain, cathepsin B or cathepsin H led to no changes in the cystatin C molecule. Isoelectric focusing of the cathepsin L and cystatin C mixture showed the formation of two new bands. One of them appeared whether E-64 or PMSF was added or not, evidently representing a cystatin C/cathepsin L complex. The other band is the truncated cystatin C molecule. N-terminal sequencing after separation by HPLC showed that cystatin C is cleaved by cathepsin L at the Gly11–Gly12 bond. The action of cathepsin L on cystatin C may be explained by the cleavage of the scissile bond in an inappropriate complex.

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**Key words:** Cystatin C; Cathepsin L; Limited proteolysis

## 1. Introduction

Human cystatin C is the most studied extracellular low molecular weight inhibitor of cysteine proteinases. It consists of 120 amino acid residues with two disulfide bridges and belongs to family 2 of the cystatin superfamily [1–4]. It is widespread in human tissues, and is found mainly in secretions and extracellular fluids such as cerebrospinal fluid, seminal plasma, milk, synovial fluid, urine, saliva and blood plasma [5,6]. It is thought that cystatin C is the physiologically most important inhibitor, regulating activities of endogenous cysteine proteinases such as cathepsins B, H, L and S in the extracellular environment. On the basis of the crystal structures of chicken cystatin and the stefin B/papain complex, it has been shown that three contiguous regions on the folded human cystatin C polypeptide chain are involved in the interaction with the proteinase active site cleft [7,8]. Two evolutionarily conserved hairpin loop segments correspond to the Gln55–Ile–Val–Ala–Gly59 and Pro105–Trp106 regions in the C-terminal part of the molecule. The third enzyme binding region of human cystatin C is the N-terminal part with the conserved Gly11 residue. Truncation of the inhibitor by 10 or 11 N-terminal residues results in major loss of affinity for the target enzyme [9–12]. These findings indicate that the main significance of the conserved Gly11 residue of cystatin C is

to allow adaptation of the N-terminal segment in a conformation optimal for interaction with the active site region of cysteine proteinase [9,13]. The first 10 amino acid residues are missing in the cystatin C variant isolated from amyloid deposits of patients with hereditary cystatin C amyloid angiopathy [15,16]. Differently truncated forms of cystatin C, together with the full-length form, were found in urine of patients with nephrological disorders [14]. Leukocyte elastase was shown to cleave a single bond between Val10 and Gly11 at the N-terminal end of cystatin C in vitro [9]. Since both proteins are extracellular it was suggested that such a cleavage could be of physiological importance by controlling the extent of inhibition of cysteine proteinases, especially cathepsin B, at the site of inflammation [9]. It is believed that these truncations are the result of proteolytic cleavage of full-length cystatin C, since the N-terminal part of the inhibitor molecule is exposed and has no ordered structure in solution [17].

In the present work we have investigated whether cysteine proteinases are involved in the formation of N-terminally truncated cystatin C variants detected by isoelectric focusing (IEF) in samples of normal and ‘pathological’ urine [14], in particular those truncated by 10 or 11 residues with significantly lower inhibitory activity.

## 2. Materials and methods

### 2.1. Materials

Bz-DL-Arg-NA, Arg-NA, E-64, PMSF and 2 times crystallized papain (EC 3.4.22.2) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Z-Phe-Arg-MCA and Arg-MCA were from Bachem (Bubendorf, Switzerland). Coomassie brilliant blue G-250 was from Bio-Rad (USA). E-64 was from Peptide Research Foundation (Osaka, Japan). Superdex 75 HR10/30, 8–25% polyacrylamide gradient gels, low molecular weight calibration kit, polyacrylamide gels with Pharmalyte pH 3–9 and broad pI kit were supplied by Amersham Pharmacia Biotech (Uppsala, Sweden). The Chromospher C8 column was from Chrompack (Frankfurt, Germany). Glycyl endopeptidase (EC 3.4.22.25) was a generous gift from Dr. Alan J. Barrett, Strangeways Research Laboratory, Cambridge, UK.

### 2.2. Purification of cysteine proteinases

Human cathepsin B (EC 3.4.22.1), cathepsin H (EC 3.4.22.16) and cathepsin L (EC 3.4.22.15) were purified from human kidney by the method involving gel filtration, covalent chromatography on thiol-Sepharose and cation exchange chromatography [18]. Papain from Sigma was additionally purified by FPLC on a Mono S column using 0.02 M sodium acetate buffer, pH 5.0, containing 1 mM EDTA. Papain was eluted with a linear gradient of NaCl (0–0.5 M). Concentrations of cathepsins and papain were determined taking  $A_{280}$  (1%) for cathepsin B to be 18, for cathepsins H and L 14 [19] and for papain 25 [20]. Active concentrations of cathepsins B, L and papain were determined by titration with E-64 [21], while the active concentration of cathepsin H was obtained by titration with stefin A [22].

### 2.3. Purification of cysteine proteinase inhibitors

The full-length form of human cystatin C was isolated from urine

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**Abbreviations:** Bz, benzoyl; E-64, 1-3-carboxy-2,3-trans-epoxypropionyl-leucylamido(4-guanidino)butane; MCA, 4-methyl-7-coumaryl amide; IEF, isoelectric focusing; NA, 2-naphthylamide; PMSF, phenylmethylsulfonyl fluoride; Z, benzyloxycarbonyl

of patients with various nephrological disorders by the modified method of Brzin et al. [2,14] using gel filtration, affinity chromatography on carboxymethylated papain-Sepharose and ion exchange chromatography. Recombinant human cystatin C, which differed from the wild-type protein by three additional amino acid residues (Gly-Ser-Met) at the N-terminal end, was prepared as described previously [23]. In most experiments human recombinant cystatin C was used, since it was available in sufficient amounts and exhibited essentially identical physicochemical properties to the native protein, including  $K_i$  values for tested cysteine proteinases [23,24].

Stefin A was purified from human leukocytes [25]. Concentrations of inhibitors were determined spectrophotometrically, taking  $A_{280}$  (1%) for cystatin C and stefin A to be 8.5 and 7.4, respectively, as calculated from the amino acid composition. The percent of fully active cystatin C was obtained by titration with titrated papain [23].

All concentration stated below refer to the active forms of enzymes or inhibitors.

#### 2.4. Treatment of cystatin C and stefin A with proteinases

Recombinant cystatin C and stefin A were incubated separately at a final concentration of 0.5 mg/ml with glycyI endopeptidase, papain, cathepsin B, cathepsin H and cathepsin L at different molar ratios at 37°C. Buffers used were the same as for enzyme assays [23,26]. After different times samples were taken and the reaction stopped by adding 5 mM E-64. The effect of enzyme action was monitored by PAGE in the presence of SDS and by IEF. SDS-PAGE was carried out in an 8–25% polyacrylamide gradient gel on the PhastSystem apparatus as recommended by the manufacturer. Gels were stained with 0.2% Coomassie brilliant blue G-250. A low molecular weight calibration kit was used. IEF was carried out on polyacrylamide gels with Pharmalyte carrier ampholines in the pH range of 3–9, using the PhastSystem apparatus, as recommended by the manufacturer. A mixture of standard proteins was run parallel to the samples. Gels were stained with Coomassie brilliant blue G-250.

#### 2.5. Preparation and purification of N-terminally truncated cystatin C

Recombinant cystatin C was incubated with glycyI endopeptidase in 0.1 M phosphate buffer, pH 6.5, containing 2 mM dithiothreitol and 1.5 mM EDTA, for 4 h at 37°C in a molar ratio of enzyme to inhibitor of 1:100. The truncated cystatin C was then purified by gel chromatography on a Superdex 75 column on FPLC system, using 0.1 M ammonium bicarbonate buffer, pH 8.0.

Cystatin C truncated by cathepsin L was made by incubating recombinant cystatin C at a final concentration of 0.5 mg/ml with cathepsin L in an enzyme/inhibitor ratio of 1:8, in 0.34 M sodium acetate buffer, pH 5.5, containing 2 mM dithiothreitol and 1.5 mM EDTA for 4 h at 37°C. The cleavage products were purified by HPLC on a C8 Chromosphere column, using an acetonitrile gradient (0–80%) in 0.1% trifluoroacetic acid. Eluted proteins were N-terminally sequenced using an Applied Biosystems 475 liquid sequencer connected on-line to a 120 A phenylthiohydantoin amino acid analyzer from the same manufacturer.

#### 2.6. Determination of inhibition constants

Inhibition kinetics of cathepsins B, H and L were studied under pseudo-first-order conditions [27] in continuous kinetic assays, and followed using a Perkin Elmer LS-3 spectrofluorimeter, connected to an IBM personal computer, running Flusys software [28]. Truncated cystatin C in various concentrations was mixed with substrate solution in the fluorimetric cuvette. The reaction was initiated by the addition of the enzyme in a negligible volume. Cathepsin B was assayed in 0.1 M sodium phosphate buffer, pH 6.0, cathepsin H in 0.1 M sodium phosphate buffer, pH 7.0, and cathepsin L in 0.34 M sodium acetate buffer, pH 5.5. All buffers contained 1.5 mM EDTA and 2 mM dithiothreitol. The substrates used were Z-Phe-Arg-MCA for cathepsins B and L and Arg-MCA for cathepsin H, all at 5  $\mu$ M final concentration. Apparent  $K_i$  values were calculated by linear regression analysis of the initial and steady-state rates of substrate hydrolysis obtained at different inhibitor concentrations according to Morrison [29]. The  $K_i$  values were corrected for substrate competition with the use of  $K_m$  values for these enzymes [23].

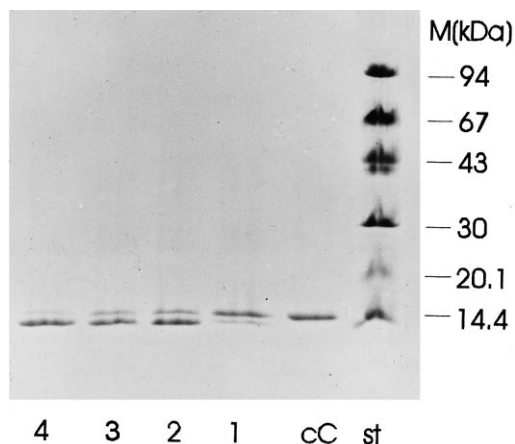


Fig. 1. Cleavage of recombinant human cystatin C by cathepsin L and glycyI endopeptidase detected by SDS-PAGE. Lanes 1–3 indicate cystatin C after incubation with cathepsin L at a molar ratio of 8:1 for 2, 4 and 6 h, respectively, at 37°C. Lane 4: cystatin C after incubation with glycyI endopeptidase in molar ratio 100:1 for 30 min at 37°C. Incubation buffers were as indicated in Section 2. Standard proteins are indicated with ST and cystatin C with cC.

### 3. Results

The purity of cathepsin L used in these experiments was checked by SDS-PAGE, IEF and N-terminal analysis. The preparation of the enzyme contained about 50% of active enzyme. The proportions of inhibitory active recombinant and native cystatin C used in these experiments were about 71% and 55%, respectively, as determined by titration with papain.

Incubation of recombinant human cystatin C with cathepsin L in an enzyme/inhibitor molar ratio of 1:8 at 37°C resulted in gradual conversion of full-length cystatin C into the truncated form, as revealed by SDS-PAGE (Fig. 1). Overnight incubation of recombinant cystatin C with cathepsin L eventually resulted in complete disappearance of the cystatin C band (not shown). A control experiment with cystatin C alone in the same buffer at 37°C revealed no breakdown of the inhibitor, even after 24 h incubation. The ability of cathepsin L to cleave the native full-length human cystatin C with pI 9.2 isolated from urine was also tested on SDS-PAGE. The result was similar to that with recombinant inhibitor (not shown). Incubating recombinant cystatin C with glycyI endopeptidase in an enzyme/inhibitor molar ratio of 1:100 for 30 min resulted in similar truncation of cystatin C (Fig. 1).

Isoelectric focusing of the mixture of recombinant human cystatin C and cathepsin L in molar ratio 8:1 assayed after 3 h of incubation showed a band with pI 9.2 and formation of a new major band with pI 8.1 and a weak band with pI 8.25 (Fig. 2). Formation of these two bands was not prevented by addition of PMSF to the incubation mixture at a final concentration of 50 mM. On the other hand, addition of E-64 to the enzyme at a final concentration of 1 mM completely abolished the appearance of these new bands. In addition, a band with pI 5.9 appeared in all incubation mixtures, including those containing PMSF and E-64, probably due to the formation of cystatin C/cathepsin L complex since the isoelectric point of cathepsin L is 5.4. When cystatin C was incubated

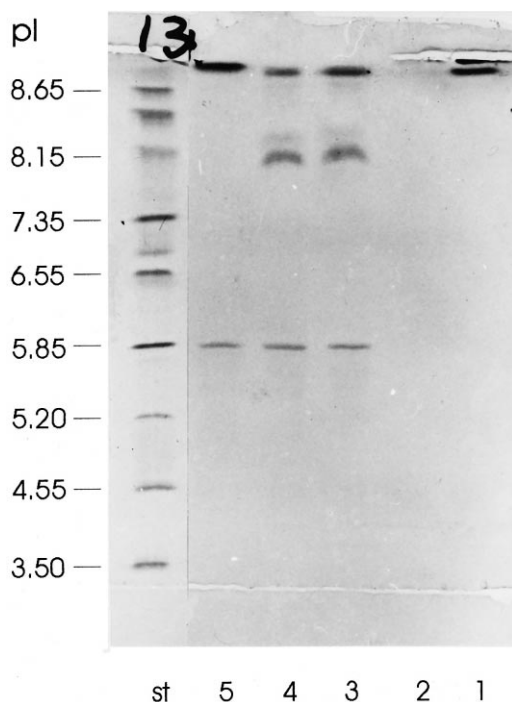


Fig. 2. Cleavage of recombinant human cystatin C by cathepsin L detected by isoelectric focusing. Cystatin C was incubated for 2 h at 37°C with cathepsin L at a ratio of 8:1 in the incubation buffer as indicated in Section 2 (lane 3). Lanes 4 and 5: PMSF (10 mM) and E-64 (1 mM), respectively, were added to the enzyme before the incubation with the inhibitor. Lanes 1 and 2: cystatin C and cathepsin L were applied separately at the same concentrations and after incubation as above.

with glycyl endopeptidase in an enzyme/inhibitor molar ratio of 1:100 for a short period (about 10 min) the bands with  $pI$  8.1 and 8.25 appeared. Prolonged incubation resulted in formation of a single band with  $pI$  8.1. No band presumably corresponding to cystatin C and glycyl endopeptidase complex could be detected in isoelectric focusing gel.

SDS-PAGE of incubation mixtures of cystatin C with papain in molar ratio 5:1, with cathepsin B in molar ratio 4:1 or cathepsin H in molar ratio 4:1, all in appropriate incubation buffers for 5 h at 37°C, revealed only intact cystatin C, with no truncation being observed (Fig. 3).

Experiments to examine the effect of different enzymes on stefin A were performed by mixing stefin A with cathepsin B, cathepsin L or papain, all in a molar ratios 4:1, and with glycyl endopeptidase in molar ratio 50:1, in appropriate incubation buffers for 5 h at 37°C. In all cases SDS-PAGE of the incubation mixtures revealed no change in stefin A polypeptide chain (not shown).

To elucidate the nature of the cleavage of cystatin C with cathepsin L, 0.5 mg of recombinant cystatin C was mixed with cathepsin L in a ratio of 8:1 for 4 h at 37°C. The reaction was terminated by addition of E-64 to 1 mM final concentration. The incubation mixture was resolved into two peaks on C8 Chrompack column on the HPLC system under acetonitrile gradient (Fig. 4). Both peaks were N-terminally sequenced. The first peak contained the N-terminally truncated cystatin C with sequence starting with Gly12 (Gly-Pro-Met-Asp-Ala-), according to human cystatin C numbering, and a minor contamination with cathepsin L. The second peak corresponded

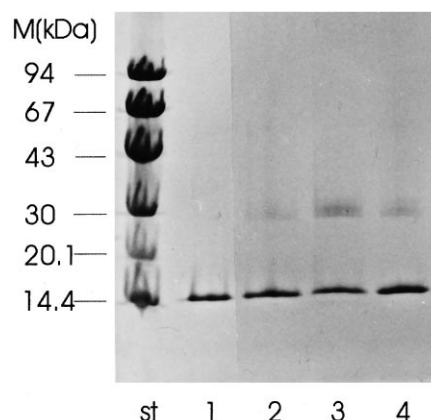


Fig. 3. SDS-PAGE of the incubation mixtures of recombinant human cystatin C with papain, cathepsin B and cathepsin H. Cystatin C was incubated for 5 h with papain (lane 2), cathepsin B (lane 3), or cathepsin H (lane 4), all in ratios 4:1 in incubation buffers as described in Section 2. In lane 1 cystatin C was applied. Standard proteins are indicated with ST.

to the intact recombinant human cystatin C with N-terminal sequence starting with Gly(-3) (Gly-Ser-Met-Ser-Ser-Pro-).

Larger amounts of truncated human cystatin C, lacking the first 11 N-terminal amino acids, were obtained by incubating 2.5 mg of recombinant human cystatin C with glycyl endopeptidase in a ratio of 1:100 for 4 h. SDS-PAGE of the incubation mixture revealed total conversion of the recombinant cystatin C into the truncated form. After purification on Superdex 75 the truncated inhibitor was used for inhibition studies. By continuous rate assays equilibrium dissociation constants ( $K_i$ ) for complexes of the truncated inhibitor with cathepsins H and L were determined, while the value for cathepsin B was estimated to be more than 500 nM. The results agree well with the determinations of Hall et al. [10]. The N-terminal truncation of human cystatin C thus strongly affected the affinity of the inhibitor for all the enzymes tested.  $K_i$  values for cathepsin B and cathepsin L increased by at least three orders of magnitude and for cathepsin H by about two orders of magnitude.

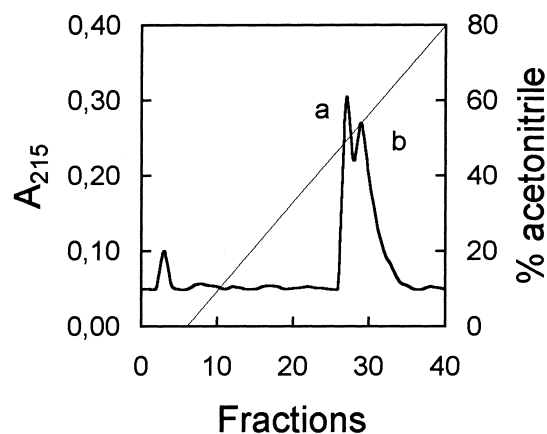


Fig. 4. Separation of the incubation mixture of the recombinant human cystatin C with cathepsin L on C8 Chromspher column of the HPLC system. A 0–60% gradient of acetonitrile was applied. As determined by N-terminal sequencing, peak (a) corresponds to truncated cystatin C, starting with Gly12, and peak (b) intact recombinant human cystatin C.

#### 4. Discussion

The majority of our experiments were performed using a recombinant cystatin C extended by three amino acids at its N-terminus, which was comparable to the wild-type cystatin C with respect to all the physicochemical properties tested. The expression product showed a molar inhibitory activity of 71%. The additional three amino acid residues are not expected to influence the binding of the inhibitor to the proteinase, as the first 10 amino acid residues in cystatins have been shown to be unstructured and highly mobile [17], and pointing away from the proteinase surface [7]. Control experiments with full-length cystatin C of human origin were also performed. Its homogeneity was checked by the usual electrophoretic methods and by N-terminal analysis. It displayed a molar inhibitory activity of 55%, a value commonly observed for preparations of this inhibitor from human sources [30]. Our data, which document initial complex formation, followed by truncation of cystatin C by cathepsin L, with which it is mixed in excess, cannot be adequately explained by the assumption of a common mode of interaction with target proteinases for all cystatins [7]. This presumes that, in contrast to standard substrates, the inhibitor's N-terminal trunk will be sufficiently removed from the proteinase catalytic residues in the complex and thus not be cleaved.

Four possible causes for the Gly11-Gly12 bond to become substrate-like for proteolytic cleavage with cathepsin L can be envisaged: (a) a minute amount of glycyl endopeptidase, originating as contamination of the papain bonded to Sepharose used for the isolation of cystatin C, (b) the presence of an atypical cysteine proteinase in the purified cathepsin L preparation, (c) conformational change in cystatin C that renders the Gly11-Gly12 bond susceptible to hydrolysis, and (d) hydrolysis of the sensitive bond of cystatin C in complex with cathepsin L by a free cathepsin L molecule.

Cleavage of the bond by contaminating glycyl endopeptidase in the cystatin C preparation was ruled out by control experiments with cystatin C alone, which revealed no breakdown of the inhibitor.

Although cathepsin L was shown to be homogeneous by N-terminal analysis, SDS-PAGE and IEF, its activity was about 50%, which may reflect the presence of atypical, partly inactivated (abnormal/denatured) molecules, possibly partly degraded by autolysis or by reduction of disulfide bonds. One could envisage a different mode of interaction of the N-terminus of cystatin C with atypical cathepsin L, as a result of differences in the active site region of the latter. On the other hand, the presence of a minute quantity of a cysteine proteinase with glycyl endopeptidase specificity co-purified with cathepsin L cannot be completely ruled out, although no protease with similar properties has been reported from human origin. Reactive glycyl bonds in free cystatin C molecules could be cleaved, while those engaged in complex with cathepsin L would be protected within the complex. Our observation that all cystatin C is entirely degraded therefore does not support the above possibility. It should be added that a preparation of bovine cathepsin L behaved identically to human enzyme (our observation) and that human cathepsins B and H and papain showed no cleavage.

Given the evidence presented here the most probable cause for the Gly11-Gly12 hydrolysis could be conformational distortion in the cystatin C molecule within the cystatin C/ca-

thepsin L complex, which brings the Gly11-Gly12 bond into a substrate-like position. A similar phenomenon was observed in complexes of papain and cathepsin L with mutants of chicken cystatin characterized by distorted contacts of one of the hairpin loops [31]. The Gly9-Ala10 bond in chicken cystatin, analogous to the Gly11-Gly12 bond in cystatin C, was cleaved in a substrate-like manner, resulting in a truncated inhibitor of much lower activity and dissociation of the complex.

The calculated value of 71% for molar inhibitor concentration may represent two populations of cystatin C molecules, those that are fully active, and those that carry small local conformational changes and have a lower binding affinity for cathepsin L, and with the Gly11-Gly12 bond suitably located to be cleaved within the complex. As papain, cathepsin B and cathepsin H are inactive under the same conditions, it can be concluded that the mode of interaction of the N-terminal region with target proteinases depends also on the enzyme and reflects structural particularities in the active site region of the latter.

On the other hand, the truncation of cystatin C could be explained as the attack of the second cathepsin L molecule on the cathepsin L/cystatin C complex. The phenomenon of the action of a second enzyme molecule on an enzyme/inhibitor complex has been observed by Berti and Storer [32] studying proteolytic susceptibility of cystatin C depending on acidic pH-dependent local conformational changes, where the second papain molecule acts on a papain/inhibitor complex by cleavage of the exposed regions in the inhibitor molecule. In our case N-terminal cleavage of cystatin C by free cathepsin L in solution is not likely for several reasons. The amount of free cathepsin L in solution would be negligible, judging from the very low inhibition constant of  $10^{-12}$  M, and it would be further reduced by the initial excess of cystatin C. The Gly11-Gly12 bond under the experimental conditions would be buried in the complex, therefore shielded from cleavage by the free enzyme if present. However, the stability of the native inhibitor towards unfolding would be reduced in the lysosomal pH range of 4.5–5.0, therefore, according to this mechanism, enabling rapid degradation of cystatin C after it had been sequestered free or complexed into the lysosome, in order to prevent intra-lysosomal accumulation of non-degradable proteins.

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