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Purification of the complex of cathepsin L and the MHC class II-associated invariant chain fragment from human kidney

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The complex of cathepsin L and the fragment of the MHC class II-associated invariant chain was purified from human kidney. M_r of the complex, as determined by gel filtration, is about 40,000. Both components were identified by amino acid and sequence analyses. The bound invariant chain fragment is almost identical to the additional segment found in p41, but not in the p31 form of the invariant chain. The complex has significantly enhanced stability at neutral and slightly alkaline pH, and reduced proteolytic activity against the synthetic substrate Z-Phe-Arg-MCA compared to free cathepsin L. The complex exhibits no enzymatic activity against the protein substrate azocasein. For the first time, the invariant chain was found in a complex with a protein, which was not an MHC molecule.

Cathepsin L; Cysteine proteinase; MHC class II molecule; Invariant chain; p41

1. INTRODUCTION

Cathepsin L (EC 3.4.22.15) is known to be the most powerful lysosomal cysteine proteinase. It belongs to the papain superfamily and plays an important role in the degradation of intracellular proteins [1]. It degrades a variety of protein substrates, such as collagen, elastin, insulin and glucagon, with much higher specific activity than the other lysosomal proteinases [2,3]. Cathepsin L is routinely assayed with azocasein in the presence of urea, and with the synthetic substrate Z-Phe-Arg-MCA [2]. Its activity and stability are highest in slightly acidic media [4]. At neutral and alkaline pH, it is rapidly inactivated [4–6]. The most potent physiological regulators of cathepsin L are inhibitors from the cystatin superfamily [7].

Cathepsin L has been purified from many organisms, such as bovine [8,9], chicken [10], rat [11], rabbit [12] and human [4,6,13]. The species variants share similar properties. In human, it is expressed at the highest level in liver, lungs and kidney; its level in other tissues is lower [14].

Lysosomal proteinases participate in many biological processes. One of them is antigen recognition by the MHC class II-restricted T lymphocytes, which requires two, still poorly understood, proteolytic events: proteolytic degradation of the MHC class II-associated invariant chain (Ii) and processing of protein antigens [15]. Although there have been attempts to clarify the identity and the precise location of the responsible enzyme(s), not much is yet known about it [16–21].

Here we report for the first time purification of the complex of cathepsin L and the fragment of the MHC class II-associated invariant chain. The amino-terminal sequence of the bound fragment was determined, and its stabilizing and inhibitory effect on cathepsin L is shown.

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Abbreviations: MHC, major histocompatibility complex; Ii, invariant chain; Z-, benzyloxycarbonyl-; -MCA, -4-methyl-7-coumarylamide; DEAE-, diethylaminoethyl-; Cm-, carboxymethyl-; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular weight; IEF, isoelectric focusing; EDTA, ethylenediaminetetraacetic acid.

2. MATERIALS AND METHODS

2.1. *Materials*

Cm-Sephadex C-50, Sephacryl S-200, DEAE-Sephacel, Mono Q anion-exchange column and PhastGel Separation Media were purchased from Pharmacia (Sweden). Z-Phe-Arg-MCA and Protein Molecular Weight Standards used for calibration of the gel filtration column were from Serva (Germany). ChromSpher C8 column was from ChromPack (Netherlands). Sequencing reagents were obtained from Applied Biosystems (USA). All other chemicals used were of analytical grade.

2.2. *Purification procedure*

The cathepsin L–Ii complex was purified from human kidneys using a method modified from that previously described for the purification of cathepsin L [6]. Frozen kidneys were thawed, cut into pieces and homogenized in 2 vol. of 0.1 M sodium acetate buffer, pH 5.0, containing 0.3 M NaCl, 1 mM EDTA and 0.2% Triton X-100. Insoluble material was removed by centrifugation (10,800 × *g*, 20 min), and the supernatant was adjusted to pH 4.2 and incubated at 37°C for 4 h. After the removal of precipitated proteins by centrifugation, the supernatant was precipitated with solid ammonium sulfate up to 75% saturation at 0°C. The precipitate was resuspended in 20 mM sodium acetate buffer, pH 5.2, containing 1 mM EDTA, and insoluble material was removed by centrifugation. The supernatant was applied to

a Cm-Sephadex C-50 column (4 × 37.5 cm), equilibrated with the same buffer. The column was washed with three bed volumes of the buffer, and bound proteins were eluted with a linear gradient of NaCl (0–1.0 M) in the same buffer. Fractions containing the complex (at 0.6 M NaCl) were pooled, dialysed against 0.1 M sodium acetate buffer, containing 0.3 M NaCl and 1 mM EDTA, pH 5.0, and concentrated. The concentrate was applied to a Sephacryl S-200 column (3 × 140 cm), equilibrated with the same buffer. Collected fractions were assayed using Z-Phe-Arg-MCA as a substrate, as described previously [22]. Active fractions in the 25–45 kDa range were pooled, dialysed against 20 mM piperazine buffer, containing 1 mM EDTA, pH 5.5. The sample was applied to a DEAE-Sephacel column (1.7 × 25 cm), equilibrated with the same buffer. The column was washed, and bound proteins were eluted with a 0–0.3 M NaCl linear gradient in the starting buffer. Fractions, active on Z-Phe-Arg-MCA at pH 7.5 (cathepsin L–Ii complex), were eluted at 0.12 M NaCl, while fractions, active on the same substrate at pH 5.5 (free cathepsin L), were eluted at 0.24 M NaCl. Fractions, containing the complex, were combined, concentrated and dialysed against 20 mM bis-tris buffer, containing 1 mM EDTA, pH 6.2, and then chromatographed on a Mono Q column of a Pharmacia FPLC system, equilibrated with the same buffer. A linear gradient of NaCl (0–0.1 M) in the starting buffer was used for the elution of bound proteins. The complex was eluted at 0.05 M NaCl.

2.3. *HPLC*

The complex, eluted from the Mono Q column, was analysed by HPLC (Milton Roy LCD, UK) on a ChromSpher C8 column (3 × 100 mm), equilibrated with 0.1% trifluoroacetic acid. Bound proteins were eluted with a linear gradient of acetonitrile (0–70%) in the starting solution. Absorbance was monitored continuously at 215 nm.

2.4. *Enzyme assays*

The complex was assayed with Z-Phe-Arg-MCA at pH 7.5 as described for cathepsin S [22]. Assays with the same substrate at pH 5.5 were also performed [2].

2.5. *SDS-PAGE and isoelectric focusing*

Electrophoretic separations were performed using PhastSystem (Pharmacia, Sweden), following the instructions of the manufacturer. Either 8–25% gradient polyacrylamide gels (0.45 × 43 × 50 mm) with molecular weight markers ranging from M_r 14,400 to 94,000 (Pharmacia) or 20% homogeneous polyacryramide gels (0.45 × 43 × 50 mm) together with low molecular weight markers (M_r from 2,500 to 16,950) (LKB) were used for SDS-PAGE, while PhastGel IEF 3–9 slabs (0.35 × 43 × 50 mm) were used for isoelectric focusing.

2.6. *M_r determination by gel filtration*

A column of Sephacryl S-200 (8 × 1,050 mm) was equilibrated with 0.1 M sodium acetate buffer, pH 5.0, containing 0.3 M NaCl and 1 mM EDTA, and calibrated with Dextran blue, rabbit muscle aldolase, egg albumin, chymotrypsinogen and cytochrome *c*. The purified complex was applied to the column in a volume of 700 μl, and protein concentrations in eluted fractions (1 ml) were measured at 280 nm.

2.7. *Continuous measurement of proteolytic activity of the complex*

The complex was preactivated at 37°C for 5 min in 5 mM DTE in 0.3 M sodium acetate buffer, pH 5.7, containing 1 mM EDTA. Buffers of various pH values, ranging from pH 3.0 to 8.0, were prepared by mixing 0.1 M citric acid and 0.2 M Na_2HPO_4. They also contained 1 M NaCl and 1 mM EDTA. The activity of the complex against Z-Phe-Arg-MCA was measured at room temperature, at pH values in 0.5 unit intervals. The reaction was started by the addition of 0.250 ml of the preactivated complex (2 nM final concentration) to 2.750 ml of Z-Phe-Arg-MCA (5 μM final concentration) in the appropriate buffer. The product formation was continuously followed by measuring fluorescence at excitation and emission wavelengths of 370 and 460 nm, respectively. Progress curves were recorded using a Perkin Elmer LS-3 spectrofluorimeter connected to an IBM-XT microcomputer. Flusys software [23] was used for computer control of the spectrofluorimeter.

2.8. *Amino acid and sequence analyses*

Amino acid composition was analysed by Applied Biosystems (USA) 421 Amino Acid Analyser, with pre-column derivatization with phenylisothiocyanate.

Automated sequence analyses were performed on Applied Biosystems liquid phase sequenator model 475A, connected on-line to a 120A phenylthiohydantoin-amino acid analyzer from the same manufacturer.

3. RESULTS AND DISCUSSION

The cathepsin L–Ii complex, as well as free cathepsin L, were detected in human kidney. The two forms of the enzyme were separated on DEAE-Sephacel at pH 5.5. The elution profile is shown in Fig. 1. The complex was recognized by high activity against Z-Phe-Arg-MCA at pH 7.5, while cathepsin L had no activity under the assay conditions. On the other hand, free cathepsin L exhibited much higher activity than its counterpart in the complex at pH 5.5. Moreover, the complex was not active against the protein substrate azocasein, using the standard assay for cathepsin L [2]. These were the first indications for the potential stabilizing and inhibitory role of Ii.

The fragment of the invariant chain remained associated with cathepsin L during the whole purification

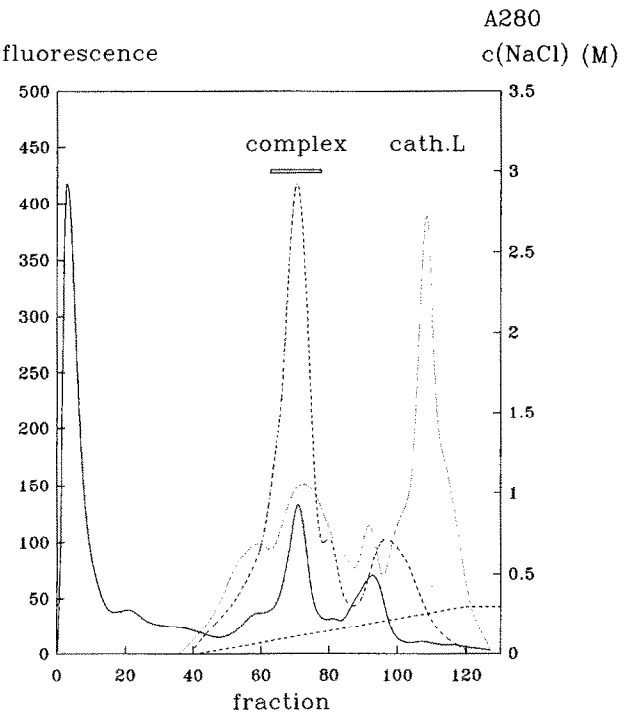


Fig. 1. Ion-exchange chromatography of the pooled fractions from the Sephacryl S-200 column on DEAE-Sephacel. Experimental conditions are described in Section 2. The solid line represents absorbance at 280 nm. The dotted and the dashed lines represent activity against Z-Phe-Arg-MCA at pH 5.5 and 7.5, respectively. The horizontal bar indicates the pooled fractions. Fifty times less diluted samples were taken from fractions for the assay at pH 7.5 than for the assay at pH 5.5.

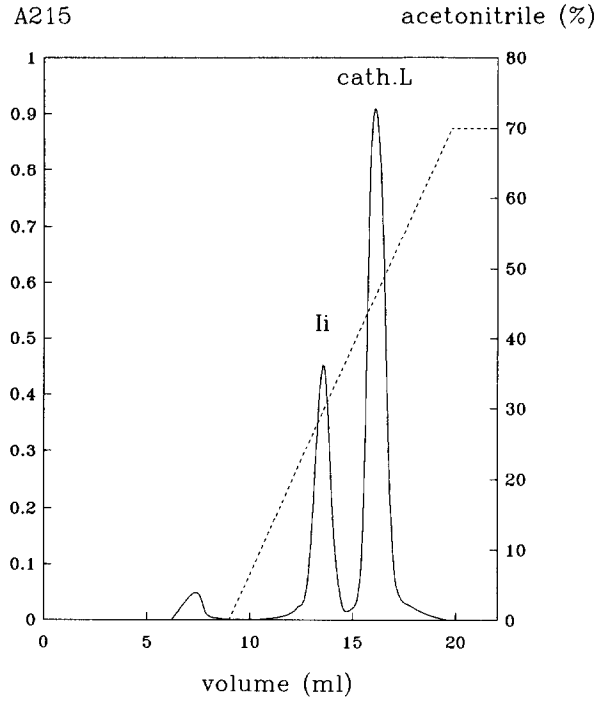


Fig. 2. Dissociation of the complex by reversed-phase HPLC. Experimental details are described in Section 2.

procedure. It dissociated under the denaturing conditions on the ChromSpher C8 column. The elution pattern revealed the presence of two components (Fig. 2). The first peak represented a fragment of the p41 form of the MHC class II-associated invariant chain, as determined by sequence analysis. 26 amino acid residues on the amino-terminal end were determined, and they are identical to the amino-terminal part (starting with the second amino acid residue) of the additional fragment of p41, which is derived from the alternatively spliced exon [15], and which is not found in the p31 form of the invariant chain (Fig. 3). The amino acid composition of the fragment bound to cathepsin L (not shown) is nearly identical to the amino acid composition of the extra fragment, unique to p41, suggesting that carboxyl-terminal parts probably do not differ very much either. The second peak was recognized as cathepsin L by its amino-terminal sequence.

The complex was eluted as a single peak with M_r of ~ 40,000 on the analytical Sephacryl S-200 column (not shown). This peak was applied to SDS-PAGE, where, under non-reducing conditions, it migrated predominantly as two bands, one with M_r 32,000 and the other with M_r below 14,000, indicating complex dissociation (not shown). The band with M_r 32,000 corresponds to cathepsin L, consistent with the published data [13], and the other band corresponds to the Ii fragment. Under reducing conditions, three major bands can be seen, one with M_r ~ 26,000, corresponding to the heavy chain of cathepsin L, and two below M_r 14,000. In order to determine the molecular weight of these two bands, electrophoresis was also performed on a 20% homogeneous polyacrylamide gel in the presence of low molecular weight markers (not shown). One band of protein appeared at M_r 9,000 (Ii fragment), the other appeared at M_r 6,000 (light chain of cathepsin L).

On the isoelectric focusing gel, a single band with pI value 5.3 can be seen (Fig. 4), demonstrating the homogeneity of the complex.

In order to study the pH-dependence of the interaction of the invariant chain with cathepsin L, continuous measurement of the proteolytic activity of the complex against Z-Phe-Arg-MCA at various pH was performed. We tried to dissociate the complex by combining the effect of high substrate concentration, dilution and pH changes. Experimental data obtained at pH 3.5, 6.5 and 8.0 are shown in Fig. 5. The proteolytic activity of the complex at pH 3.0 (not shown) and 3.5 (Fig. 5a) resulted in an exponential release of the fluorescent product. The progress curve could be fitted by nonlinear regression analysis to the following integrated equation [24]:

$$[P] = v_s t + (v_i - v_s)(1 - e^{-kt})/k \quad \text{Eq. (1)}$$

where [P], v_i, v_s and k represent the product concentra-

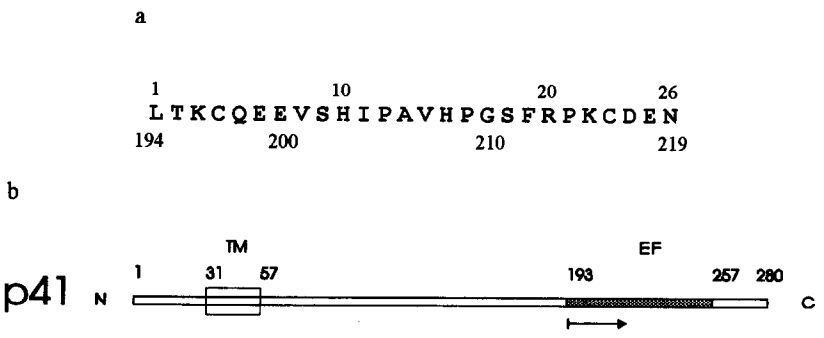


Fig. 3. (a) Amino-terminal sequence of the Ii fragment bound to cathepsin L. The upper numbering starts with the first amino acid of the bound fragment. The lower numbering is according to p41. (b) Schematic representation of p41 form of Ii [26]. TM stands for transmembrane region, and EF for extra fragment, unique to p41. The horizontal arrow indicates the position of the determined amino-terminal part of the fragment bound to cathepsin L.

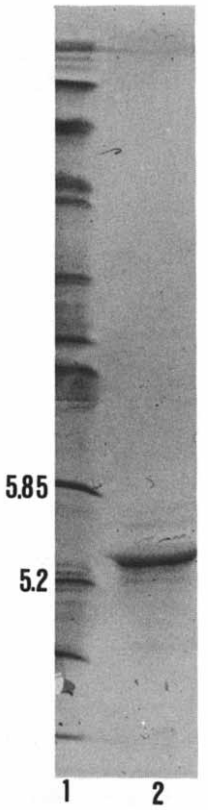


Fig. 4. Isoelectric focusing of the complex. Lanes: 1, standards; 2, complex.

tion, the initial velocity, the steady-state velocity and the apparent first-order rate constant respectively. This equation describes the following situation:

$$\begin{array}{l} E + S \rightleftharpoons ES \longrightarrow E + P \\ + \\ L \\ \Updownarrow \\ EL \end{array}$$

E stands for enzyme (cathepsin L), L for ligand (Ii fragment), S for substrate and P for product. At other pH values (from 4.0 to 7.5), the rate of product formation was constant (Fig. 5b). At pH 8.0, practically no proteolytic activity against the substrate could be detected (Fig. 5c). After 7 min of incubation at pH 8.0, the reaction mixture was acidified with acetic acid to pH 3.5. An exponential increase of fluorescence was observed, indicating that active cathepsin L had been released from the complex. These results demonstrate that the fragment of the invariant chain is bound to cathepsin L in a way, that it inhibits and stabilizes the enzyme. At pH 3.0 and 3.5 the complex dissociates, active enzyme molecules are released and the product formation rate (the slope of the progress curve) increases. Human kidney cathepsin L is known to be very unstable above pH 5.5 [4], has a very short half life at pH 8.0 [6], and

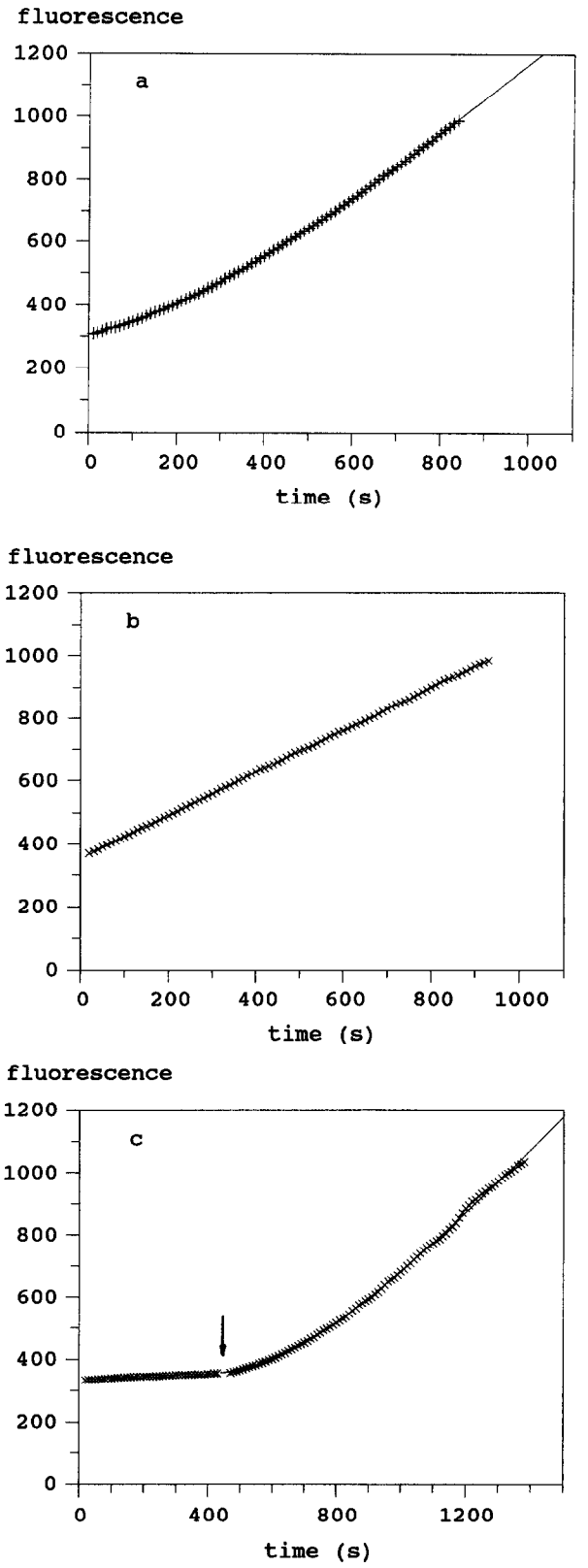


Fig. 5. Progress curves for the proteolytic action of the complex at various pH. Experimental conditions are described under section 2. Experimental points are marked with (+). The solid lines represent theoretical curves calculated using Equation 1. (a) pH 3.5, (b) pH 6.5, (c) initial pH was 8.0. The arrow indicates the time when the reaction mixture was acidified to pH 3.5.

would be therefore completely denatured in 7 min without the possibility of restoring its activity simply by acidification. This is evidence that cathepsin L is strongly stabilized by the invariant chain. The enzyme assay, by which the complex was traced during the purification procedure, was based on this assumption. The assay started with one hour of preincubation at pH 7.5 and 37°C. This allowed us to determine the activity of the complex in the presence of free cathepsin L, which is very unstable at neutral and alkaline pH [4,25]. In the complex with Ii, cathepsin L has been shown to be stabilized, thus enabling measurement of its activity even in such unfavourable conditions. Using the same procedure, the activities of two other cysteine proteinases, namely cathepsins J and S, could be detected. However, cathepsin J was separated from other cysteine proteinases by gel filtration, whereas the activity of cathepsin S in human kidney was too low to interfere with our measurements.

In conclusion, cathepsin L was found in a complex with the invariant chain fragment, which has stabilizing and inhibitory effects on it. This might be of physiological importance in the in vivo regulation of cathepsin L activity. However, additional studies will be needed to clarify the site and the mechanism of complex formation, and its precise role within the cell.

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