N-terminal amino acid sequences of the heavy and light chains of chicken liver cathepsin L

Kenji Wada and Tadashi Tanabe

Laboratory of Cell Biology, National Cardiovascular Center Research Institute, Fujishiro-dai, Suita, Osaka 565, Japan

Received 17 September 1986

Avian cathepsin L (EC 3.4.22.15) was first purified from chicken liver. The enzyme was composed of two polypeptides with M_r values of 28 000 (heavy chain) and 5000 (light chain). The two polypeptide chains of the enzyme were separated by gel filtration after cleavage of disulfide bonds. The N-terminal amino acid sequences of the heavy and light chains were APRSVDWREKGYVTPVKDQGQCGSCWAFSTTGALEGQ and GKKYWIVKNSWGEKWGDKGYIYMAKDRKNHCGIATAASYP, respectively. These sequences show high homologies with those of the N-terminal and C-terminal portions of papain, respectively. The residues of the surrounding area of cysteine-25 in the heavy chain were quite similar to those in the region around the active-site cysteine of papain.

(Chicken liver) *Cathepsin L* *Cysteine proteinase* *Amino acid sequence*

1. INTRODUCTION

Cathepsin L, a lysosomal cysteine proteinase plays an important role in the turnover of intracellular proteins [1,2]. It has a much higher specific activity in the degradation of a variety of physiological protein substrates than the other lysosomal proteinases [2,3]. Recently, cathepsin L has been purified from mammalian tissues and the properties have been studied in detail [1–3], however, the purification and properties of avian enzymes have not been reported. Recent studies on the primary structure of two lysosomal cysteine proteinases, cathepsin B and H indicated that their amino acid sequences are strikingly homologous both to each other and to a plant cysteine proteinase papain in spite of their different specificities [4]. In view of the elucidation of the molecular reaction mechanisms and evolution of cysteine proteinases, it is of interest to investigate the primary structure of cathepsin L. In the present paper we describe the purification, characterization and N-terminal amino acid sequence of chicken liver cathepsin L.

Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Z-Phe-Arg-MCA, benzyloxycarbonyl-L-phenylalanyl-L-arginine-4-methyl-7-coumarylamide

2. MATERIALS AND METHODS

2.1. *Purification of cathepsin L*

All procedures were carried out at 4°C. The buffer used for the purification contained 50 mM sodium acetate buffer, pH 5.0, and 1 mM EDTA (buffer A). Chicken livers (800 g) stored at −80°C were thawed, minced and homogenized with an Ultra Turrax homogenizer at top speed for 2 min in 2 vols of a solution containing 0.5 M NaCl, 1 mM EDTA, 15 mM HCl, 0.1 mM phenylmethylsulfonyl fluoride and 5 μg/ml pepstatin. The homogenate was acidified to pH 4.0 by 4 M HCl and stirred for 2 h. After removal of insoluble materials by centrifugation, the supernatant adjusted to pH 5.0 with 2 M NaOH was mixed with 300 ml of a 50% (v/v) suspension of phosphocellulose in buffer A containing 0.5 M NaCl. The mixture was stirred for 1 h and filtered

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50 © 1986 Federation of European Biochemical Societies

on a Buchner funnel by aspiration. The phosphocellulose was washed with 500 ml of the same buffer. Cathepsin L was then eluted with 500 ml of buffer A containing 1.5 M NaCl. The eluate was diluted with the same volume of buffer A and subjected to precipitation by 30–75% saturated ammonium sulfate. The precipitate was dissolved in buffer A and dialyzed against the same buffer. The dialysate was precipitated by 35–70% acetone (v/v). After centrifugation, the pellets were dissolved in 50 ml of buffer A containing 0.5 M NaCl and applied to a column (2 × 17 cm) of phosphocellulose. The column was eluted with 300 ml of a linear gradient of NaCl, 0.5–1.5 M in buffer A. The pooled fractions containing the enzyme activity were concentrated by precipitation with 80% saturated ammonium sulfate. The concentrated enzyme solution (3 ml) was applied to a column (2 × 95 cm) of Sephadex G-75 which had been equilibrated against buffer A containing 0.5 M NaCl. The column was eluted with the same buffer. The pooled active fractions were adjusted to 25% saturation with solid ammonium sulfate and then applied to a column (1.4 × 1.5 cm) of phenyl-Sepharose. After the column was washed with 30 ml of buffer A containing ammonium sulfate at 25% saturation, the enzyme was eluted with 50% (v/v) ethylene glycol in buffer A. The active fraction (4 ml) was dialyzed against 0.05 M potassium phosphate buffer, pH 6.0, containing 1 mM EDTA and 0.2 M NaCl and stored at −80°C. Under these conditions the enzyme was stable for a month.

2.2. *Determinations*

Cathepsin L activity was measured using Z-Phe-Arg-MCA as described in [3]. One unit of enzyme activity is defined as 1 μmol of aminomethyl coumarin formed per min. Protein was determined by the method of Lowry et al. [5]. M_r of the native enzyme was determined by gel filtration on a Sephadex G-75 column (2 × 95 cm) equilibrated with 50 mM sodium acetate buffer (pH 5.0) containing 0.5 M NaCl. M_r values of polypeptides were determined by SDS-PAGE (15% gel) [6].

2.3. *Amino acid composition and amino acid sequence analysis*

Approx. 15 μg of the purified enzyme was subjected to 6 M HCl hydrolysis in vacuo at 110°C for 20 h. Amino acid analysis was performed by reversed-phase high-performance liquid chromatography of phenylthiocarbamoyl derivatives [7] using the Waters Pico-Tag system. Tryptophan was determined by amino acid analysis after hydrolysis in 4 M methanesulfonic acid [8]. Half-cystine was determined as cysteic acid after performic acid oxidation [9]. Reduction and carboxymethylation were carried out by the method of Hirs [10]. Amino acid sequence was determined by automated Edman degradation with an Applied Biosystems gas-phase sequencer 470A using a program adapted from [11].

3. RESULTS

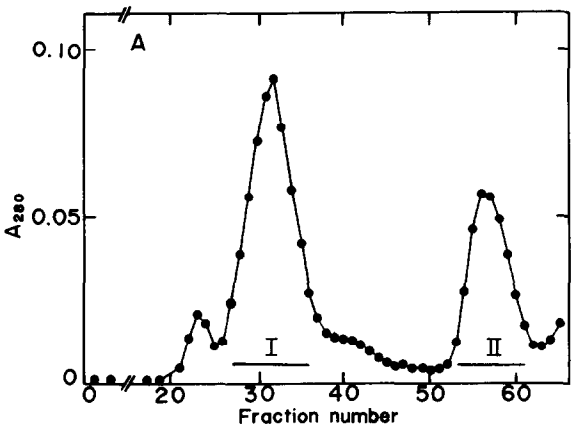
One of the recent advances in the study of lysosomal cysteine proteinases was determination of the primary structure of cathepsin B and H, whereas protein chemical properties of cathepsin L have not been studied because of its relatively low cellular content [3]. In order to study the protein structure of cathepsin L, an efficient purification

Table 1

Amino acid composition of chicken liver cathepsin L

Amino acid	Residues[a]
Asp	27.1
Glu	26.9
Ser	17.3
Gly	28.8
His	5.1
Arg	8.4
Thr	8.0
Ala	19.2
Pro	8.9
Tyr	14.0
Val	16.6
Met	2.6
1/2Cys[b]	7.0
Ile	6.8
Leu	10.1
Phe	7.7
Trp[c]	4.5
Lys	14.7

[a] Based on an M_r of 24000
[b] Determined as cysteic acid
[c] Determined after 20 h hydrolysis in 4 M methanesulfonic acid



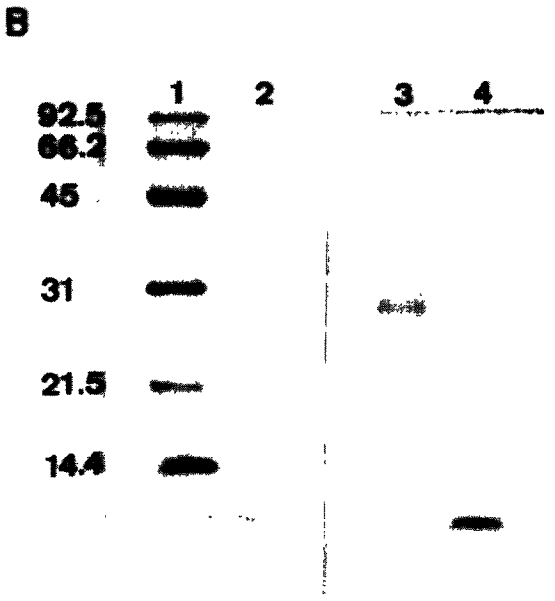


Fig.1. Separation of the heavy and light chains of chicken liver cathepsin L. (A) Sephadex G-75 column chromatography. Reduced and carboxymethylated cathepsin L (0.5 mg) was applied to a Sephadex G-75 column (1.5 × 62 cm) equilibrated with 0.1 M ammonium acetate (pH 9.0) and eluted with the same buffer. The fractions of 1.5 ml were collected and monitored at 280 nm for protein. Two peaks as indicated by horizontal bars were separately pooled and lyophilized. (B) SDS-PAGE of the heavy and light chains. Lanes: 1, M_r marker proteins; 2, the native enzyme (2 μg); 3, peak I (2 μg); 4, peak II (2 μg). M_r ($\times 10^3$) of standards is shown to the left of the gel.

method, which is applicable to a large-scale preparation of the enzyme, was developed by eliminating cellular fractionation steps. Based on the activity on a synthetic substrate, Z-Phe-Arg-MCA, the final enzyme preparation was purified 1300-fold over the crude extract. The overall yield of the enzyme was 0.7 mg with a specific activity of 31 units/mg protein from 800 g of chicken liver. The enzyme was stable between pH 5.0 and 6.0, but fairly unstable above pH 6.6. The hydrolyzing activity of the enzyme on Z-Phe-Arg-MCA was maximal between pH 5.3 and 6.0, and decreased markedly above pH 6.6 and below pH 4.5. Kinetic analysis was performed with Z-Phe-Arg-MCA at pH 6.0 and 30°C. The values of K_m and k_{cat} were 8 μM and 25 s^{-1}, respectively. The molecular mass of the native enzyme was determined by gel filtration through a Sephadex G-75 column. Its M_r was calculated to be approx. 24000. The amino acid composition of the enzyme is shown in table 1. When the enzyme preparation was examined by SDS-PAGE, it displayed two polypeptides with M_r values of 28000 and 5000 (fig.1). Separation of the reduced and carboxymethylated polypeptides of cathepsin L (0.5 mg) on a column of Sephadex G-75 is shown in fig.1A. Two major peaks I and II were obtained and analyzed by SDS-PAGE in fig.1B. Peak I (0.4 mg protein) contained the heavy chain, and peak II (0.1 mg protein) was the light chain. The separated chains were then subjected to automated sequence analyses (fig.2). The analyses were done with 0.93 nmol of heavy chain and 0.86 nmol of the light chain. Initial yields of the analyses were approx. 78% for the heavy chain and 83% for the light chain. Average repetitive yields were calculated to be about 86% for both analyses.

4. DISCUSSION

In regard to kinetic properties, pH-stability and M_r of the native enzyme, avian cathepsin L isolated from chicken liver was found to be very similar to mammalian enzymes [1–3]. Chicken liver cathepsin L as well as the human enzyme was obtained in a two-chain form composed of a heavy chain and a light chain [12]. Differently from cathepsin B and H purified as a mixture of a single-chain form and a two-chain form [4], no single-chain forms of chicken liver cathepsin L with M_r values larger

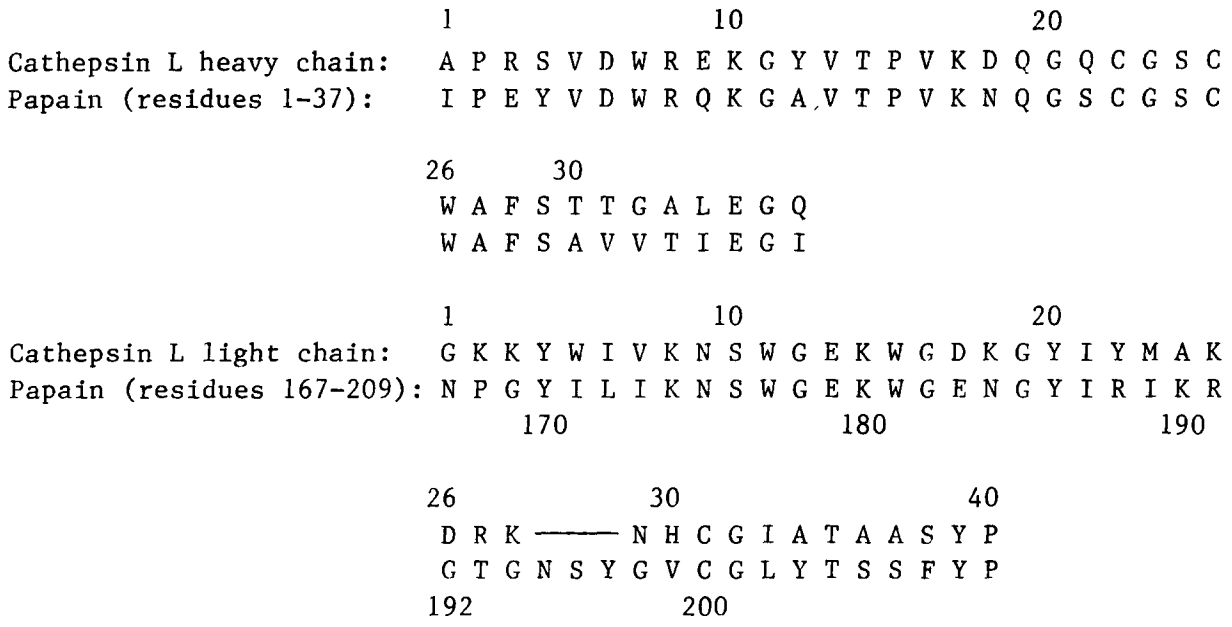


Fig.2. N-terminal sequence of cathepsin L. Analysis was performed by automated Edman degradation as described in section 2. The sequence of papain [13] is also shown for comparison. Gaps are indicated by long solid lines.

than 28000 have been found in our preparations. Molar ratios of the two polypeptides in the cathepsin L preparations were approx. 1 : 1 as analyzed by N-terminal amino acid determination.

The N-terminal sequences of the heavy and light chains of chicken liver cathepsin L are compared to the amino acid sequence of papain (fig.2). The N-terminal 37 residues of the heavy chain show homology with the N-terminal sequence of papain (65% identity). Cysteine-25 of papain is the active site of the enzyme [13] and very high sequence identity is found in the regions surrounding the cysteine-25. This fact is in agreement with the finding that the large chain of human cathepsin L contained the active site [12]. When the N-terminal 40 residues of light chain are compared to the primary structure of papain, the sequence of the light chain shows homology with that of the C-terminal portion (residues 167–209) of papain. A quite similar result was reported with a two-chain form of cathepsin H [4]. Since a striking homology has been shown among the primary structures of cathepsin B and H, and papain [4], it is reasonable to assume from our present study that cathepsin L resembles other cysteine proteinases in primary structure, suggesting a common evolutionary origin.

ACKNOWLEDGEMENTS

We thank Mr Hiroyuki Fukuda of Nihon Waters Ltd for the amino acid analyses. This investigation was supported in part by a research grant from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

[1] Barrett, A.J. (1977) in: Proteinases in Mammalian Cells and Tissues (Barrett, A.J.) pp.181–208, North-Holland, Amsterdam.
[2] Kirschke, H., Kargel, H.-J., Riemann, S. and Bohley, P. (1980) in: Proteinases and their Inhibitors (Turk, V. and Vitale, L.J. eds) pp.93–101, Mladinska knjiga-Pergamon Press, Ljubljana-Oxford.
[3] Barrett, A.J. and Kirschke, H. (1981) Methods Enzymol. 80, 535–561.
[4] Takio, K., Towatari, T., Katunuma, N., Teller, D.C. and Titani, K. (1983) Proc. Natl. Acad. Sci. USA 80, 3666–3670.

[5] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[6] Laemmli, U.K. (1970) Nature 227, 680–685.
[7] Bidlingmeyer, B.A., Cohen, S.A. and Tarvin, T.L. (1984) J. Chromatogr. 336, 93–104.
[8] Liu, T.-Y. and Moor, S. (1972) in: Chemistry and Biology of Peptides (Meienhofer, J. ed.) pp.629–653, Ann Arbor Science Publishers, Ann Arbor.
[9] Hirs, C.H.M. (1967) Methods Enzymol. 11, 59–62.
[10] Hirs, C.H.M. (1967) Methods Enzymol. 11, 199–203.
[11] Hunkapiller, M.W., Hewick, R.M., Dryer, W.J. and Hood, L.E. (1983) Methods Enzymol. 91, 399–413.
[12] Mason, R.W., Green, G.D.J. and Barrett, A.J. (1985) Biochem. J. 226, 233–241.
[13] Drenth, J., Jansonius, J.N., Koekoek, R. and Walthers, B.G. (1971) Adv. Protein Chem. 25, 79–115.