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Species variants of cathepsin L and their immunological identification

Robert W. MASON
Department of Biochemistry, Strangeways Research Laboratory, Worts Causeway, Cambridge CB1 4RN, U.K.

Cathepsin L variants purified from sheep and ox liver are shown to have similar catalytic properties to those from rat, rabbit and man with regard to activity against the substrate benzyloxycarbonyl-Phe-Arg-7-(4-methyl)coumarylamide and inhibition by benzyloxycarbonyl-Phe-Phe-diazomethane, thus identifying cathepsin L in these species for the first time. All five variants of cathepsin L are shown to be immunologically related by their interaction with antibodies raised to the human enzyme. Sheep liver was found to yield more enzyme than any other species, suggesting that this tissue is a good source of cathepsin L. Cathepsin S, a closely related enzyme, could not be detected in livers of any of these species.

INTRODUCTION

Cathepsin L is the most active of the lysosomal proteinases, as judged by its activity towards azocasein (Barrett & Kirschke, 1981), collagen (Kirschke *et al.*, 1982) and elastin (Mason *et al.*, 1986*a*). The enzyme may play an important role in bone resorption and emphysema (Delaisse *et al.*, 1984; Mason *et al.*, 1986*a*). To pursue the potential role of cathepsin L in human diseases, a good source of the enzyme is required. Cathepsin L is difficult to isolate because it forms tight complexes with cystatins in tissue homogenates (Mason *et al.*, 1985; Barrett *et al.*, 1986), but small quantities have been purified from livers of rat, rabbit and man and shown to have similar catalytic properties (Kirschke *et al.*, 1977; Mason *et al.*, 1984, 1985). Cathepsin L has not previously been purified from the larger domestic mammals, which represent an abundant source of tissue from which the enzyme might be isolated.

An enzyme which was originally called 'cathepsin S' has been purified from ox spleen (Turk *et al.*, 1978). It has similar proteolytic properties to the species variants of cathepsin L, and this led to its name being altered to 'cathepsin L' (Turk *et al.*, 1983). However, it has different kinetics for hydrolysis of the synthetic substrate Z-Phe-Arg-NHMec and inhibition by Z-Phe-Phe-CHN_2 and it is proposed that its original name, 'cathepsin S', should be retained (Kirschke *et al.*, 1984). It has not been clear, however, whether cathepsin S is the ox species variant of cathepsin L or a different enzyme, because there has been no independent evidence for the existence of ox cathepsin L.

The purpose of the present investigation was to identify a suitable source of cathepsin L for use as a model of the human enzyme. Attempts were therefore made to purify cathepsin L from the livers of ox and sheep and to compare them both enzymologically and immunologically with cathepsin L from rat, rabbit and man. The ox liver enzyme was also compared with ox spleen cathepsin S in order to determine whether or not these are the same enzymes.

MATERIALS AND METHODS

Materials

Aminomethylcoumarin substrates were from Cambridge Research Biochemicals, Harston, Cambridge, U.K. Z-Phe-Phe-CHN_2 was a gift from Dr. E. N. Shaw, Friedrich-Miescher-Institut, Basel, Switzerland. E-64 was purchased from Sigma Chemical Co., Poole, Dorset, U.K. Nitrocellulose paper was from Bio-Rad, Richmond, CA, U.S.A. Peroxidase-conjugated antibodies were bought from Dakopatts, Glostrup, Denmark, and normal sheep serum from Imperial Laboratories, Salisbury, Wiltshire, U.K. 4-Chloro-1-naphthol was from Aldrich Chemical Co., Gillingham, Dorset, U.K.

Enzyme assays and purification

Activity against Z-Phe-Arg-NHMec and inhibition by E-64 and Z-Phe-Phe-CHN_2 were measured as previously described (Mason *et al.*, 1985). Hydrolysis of azocasein was measured as described by Barrett & Kirschke (1981). Human cathepsin L was purified as described by Mason *et al.* (1985). Rat cathepsin L was a gift from Dr. H. Kirschke. Cathepsin L was isolated from livers of rabbit, sheep and ox essentially as described for the purification of human cathepsin L (Mason *et al.*, 1985). Two minor alterations were made to this protocol. The initial extracts were autolysed by incubating overnight at pH 4.2 and 20 °C. Enzyme fractions were applied to the final Mono S column, which had been equilibrated in 50 mM-sodium acetate, pH 4.5, and the enzyme was eluted with a linear gradient of NaCl (2 × 25 ml, 0–1 M) dissolved in this buffer.

Immunological procedures

Anti-(rabbit cathepsin L) antibodies were raised in a goat as described previously (Etherington *et al.*, 1984). Polyclonal antibody to pure human cathepsin L was raised in a rabbit by intramuscularly injecting a total of 50 μg of enzyme emulsified in Freund's complete adjuvant at two sites. At 2 weeks and 4 weeks later,

Abbreviations used: E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane; Ac-Ep-459, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-acetamido)butane; Z, benzyloxycarbonyl; NHMec, 7-(4-methyl)coumarylamide; -CHN_2, diazomethane.

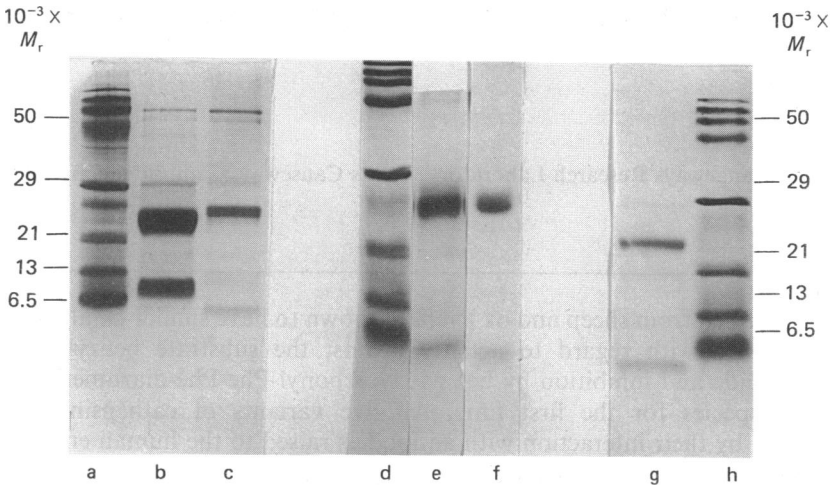

Fig. 1. SDS/polyacrylamide-gel electrophoresis of five species variants of cathepsin L

Enzyme samples were subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions as described previously (Mason *et al.*, 1985). Samples are: a, d and h, M_r standards; b, rabbit cathepsin L; c, ox cathepsin L; e, human cathepsin L; f, sheep cathepsin L; and g, rat cathepsin L.

Table 1. Kinetic constants for hydrolysis of Z-Phe-Arg-NHMec by five species variants of cathepsin L

Enzyme concentrations were determined by titration with E-64 and K_m and $k_{cat.}$ values determined at pH 5.5 and 30 °C. Experimental details were described previously (Mason *et al.*, 1985). Values for human, rat and rabbit cathepsin L were obtained from Mason *et al.* (1985). Results for human, rabbit, ox and sheep cathepsin L were obtained from at least two separate preparations of enzyme and are shown with standard deviations. Only one preparation of rat cathepsin L was available.

Enzyme source	K_m (μM)	$k_{cat.}$ (s^{-1})
Human	2.4±0.12	17±0.51
Rabbit	1.8±0.11	20±1.4
Rat	4.5±0.45	26±2.2
Ox	2.2±0.15	10±0.45
Sheep	1.8±0.14	30±1.8

booster injections of 50 μg of enzyme in Freund's incomplete adjuvant were given. After a further 3 weeks the rabbit was killed and serum collected.

Purified enzymes were allowed to react with the irreversible active-site-directed inhibitor [^{3}H]Ac-Ep459 before SDS/polyacrylamide-gel electrophoresis and radioactivity was determined in gel slices as described previously (Mason *et al.*, 1985). Western blot analysis was based on the method of Towbin *et al.* (1979), using 10% normal sheep serum in 50 mM-Tris/HCl buffer, pH 7.7, as blocking buffer. The horseradish peroxidase bound to the second antibody was stained with a solution containing 10 mg of 4-chloro-1-naphthol, 3.3 ml of methanol, 16.7 ml of Tris/HCl buffer, pH 7.7, and 5 μl of H_2O_2 (30%, v/v).

To determine the specificity of the antibody and to see if autolysis resulted in proteolytic cleavage of the enzyme, crude non-autolysed fractions of human liver were prepared, and the enzyme was detected after electroblotting. Liver was homogenized in 10 vol. of 50 mM-sodium acetate buffer, pH 5.0, containing 1 mM-EDTA and 0.1% Brij. Insoluble material was removed by centrifugation and filtration through Millipore filters (0.2 μm pore size). Protein (100 mg) was applied to a Mono S column (HR 10/10) equilibrated in the same buffer. Unbound protein was washed off with 5 column volumes of this buffer (20 ml) and bound protein was eluted in two steps (10 ml each) of 200 mM-NaCl, followed by 600 mM-NaCl dissolved in this same buffer. Samples (50 μg) were electrophoresed on SDS/polyacrylamide gels. Electroblotting was performed by using a Bio-Rad transblot cell, with 20% methanol in Tris/glycine, pH 8.3, as transfer buffer. Protein was transferred to nitrocellulose overnight (100 V, 20 mA) as described by the manufacturers. The nitrocellulose paper was then allowed to react with rabbit anti-(human cathepsin L), peroxidase-conjugated, second antibody and stained as described above.

RESULTS AND DISCUSSION

The procedure employed for the purification of cathepsin L from sheep and ox livers was similar to the method used to purify cathepsin L from human liver (Mason *et al.*, 1985). The yields of purified cathepsin L from the livers of ox and sheep were 100 μg and 1.2 mg per 500 g of wet tissue respectively. This shows that, whereas ox liver is a poor source of cathepsin L, sheep liver provides more purified enzyme than human or rabbit livers and as much as rat liver (Kirschke *et al.*, 1977; Mason *et al.*, 1984, 1985). Furthermore, the purification procedure for the sheep enzyme does not require the isolation of a lysosomal fraction, making the method much simpler than that used to purify rat liver cathepsin L.

The concentrations of NaCl required to elute ox cathepsin L from the CM-Sephadex C-50 column (pH 5.5) and Mono S, run first at pH 5.5 and then at pH 4.5, were 400, 390 and 420 mM respectively, and for sheep cathepsin L, 420, 400 and 440 mM respectively. Such tight binding for cathepsin L to cation-exchange columns has also been noted for the human and rat

Table 2. Rate constants for inactivation of cathepsin L by E-64 and Z-Phe-Phe-CHN_2

Second-order rate constants for inactivation were determined at pH 5.5 and 30 °C as described previously (Mason *et al.*, 1985). Values for human and rat enzymes were obtained from Mason *et al.* (1985) and Barrett *et al.* (1982) respectively. Values for human, rabbit, ox and sheep cathepsin L were mean values of at least four separate determinations with standard deviations. Values for rat cathepsin L were mean values of three separate determinations, but standard deviations were not determined.

Species	Second-order rate constants ($M^{-1} \cdot s^{-1}$)	
	E-64	Z-Phe-Phe-CHN_2
Human	47800 ± 10700	136000 ± 15000
Rat	96000	160000
Rabbit	60000 ± 10000	240000 ± 40000
Ox	36000 ± 6800	130000 ± 18000
Sheep	39000 ± 4800	2000000 ± 37400

enzymes (Kirschke *et al.*, 1977; Mason *et al.*, 1985). As recognized previously (Mason *et al.*, 1984), rabbit cathepsin L required lower concentrations of NaCl for elution (260, 260 and 310 mM respectively). No activity corresponding to a distinct form of cathepsin S with high azocaseinolytic activity but low activity against Z-Phe-Arg-NHMec could be found in any fractions. All species variants of cathepsin L were found to be two-chain forms, with a heavy chain of M_r 22000–25000 and a light chain of M_r 5000–7000 (Fig. 1). Furthermore, the active-site-directed inhibitor [^{3}H]Ac-Ep459 labelled the heavy chain of all variants. This inhibitor binds to the active-site cysteine residue, which is located towards the *N*-terminus of the mature cysteine proteinase (Barrett *et al.*, 1984). For human cathepsin L, *N*-terminal sequence analysis has shown that the active-site cysteine residue is at position 25 of the heavy chain and that the light chain is derived from the *C*-terminal region of the mature protein (Mason *et al.*, 1986*b*). It therefore appears likely that all variants of cathepsin L are cleaved at a site towards the *C*-terminus of the enzyme to give two-chain forms of the enzymes.

Kinetic constants for the hydrolysis of Z-Phe-Arg-NHMec were determined for sheep and ox cathepsin L and compared with values obtained previously for rat, rabbit and man (Mason *et al.*, 1985) (Table 1). All five species have K_m values in the range 1–5 μM and $k_{cat.}$ values in the range 8–30 s^{-1} (Table 1). Rates of inactivation by the active-site-directed irreversible inhibitors E-64 and Z-Phe-Phe-CHN_2 were also determined (Table 2). Again, all five species variants of cathepsin L had similar inhibition characteristics. The species variants of cathepsin L differed in their pH stability. The ox and human enzymes were unstable above pH 5.5, whereas the sheep and rabbit enzymes were stable up to pH 6.0 and 6.5 respectively (Fig. 2).

The antibody to human cathepsin L recognized a single band of M_r 25000 in crude fractions from the CM-Sephadex C-50 column, indicating that the antibody is specific for the heavy chain of cathepsin L (Fig. 3). The cleaved enzymes probably exist as such *in vivo*, as Western blot analysis of the non-autolysed fractions of

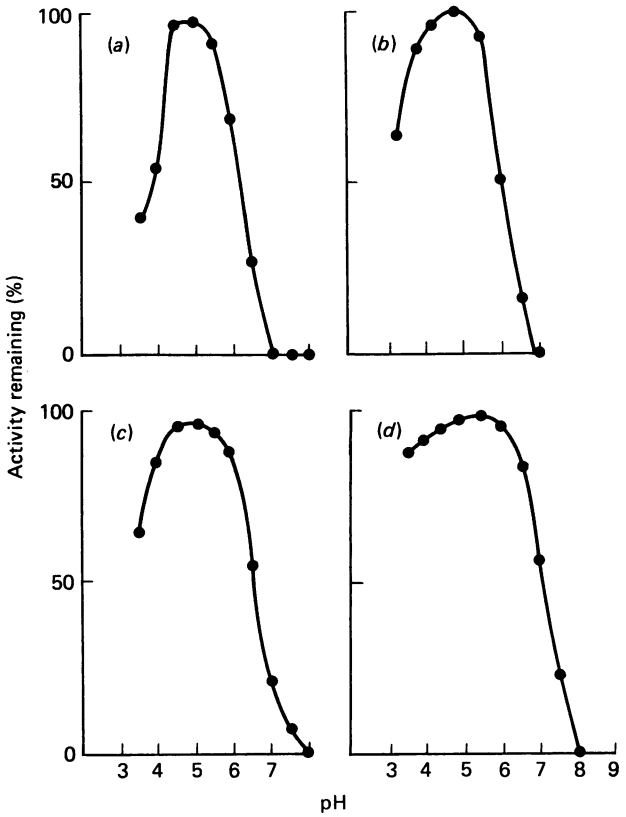

Fig. 2. pH stability of species variants of cathepsin L

Enzymes were incubated with 100 mM-buffer at various pH values for 1 h at 30 °C and residual activity assayed with Z-Phe-Arg-NHMec. Buffers used were sodium formate (pH 3.5–4.5), sodium acetate (pH 4.5–5.5) and sodium phosphate (pH 5.5–8.0), all with a cation concentration of 200 mM. (*a*) human cathepsin L; (*b*), ox cathepsin L; (*c*), sheep cathepsin L; and (*d*), rabbit cathepsin L.

human liver revealed only one band with an M_r of 25000, corresponding to the heavy chain of the enzyme purified after autolysis. Western blot analysis shows that all species variants of cathepsin L react with antibody to the human enzyme, suggesting that all variants contained common antigenic sites. The antibody does not recognize the other lysosomal cysteine proteinase, cathepsins B and H. An antibody raised to rabbit liver cathepsin L also recognizes other species variants (results not shown).

Cathepsin S is a cysteine proteinase that is closely related to cathepsin L. However, cathepsin S differs in catalytic properties in being only slowly inhibited by Z-Phe-Phe-CHN_2 and having low activity against Z-Phe-Arg-NHMec (Kirschke *et al.*, 1984). The present study has shown that ox liver contains an enzyme that has properties characteristic of cathepsin L and unlike those of cathepsin S. A distinct enzyme with the characteristics of cathepsin S was not found in liver from any species, however, suggesting that cathepsin S is not expressed in significant quantities in liver and may be confined to lymphoid tissues.

The characteristics of mature cathepsin L, isolated from five different species, can now be clearly defined as follows: (1) it consists of two chains with approx. M_r 25000 and 5000, with the active-site cysteine residue located in the heavy chain; (2) it hydrolyses Z-Phe-

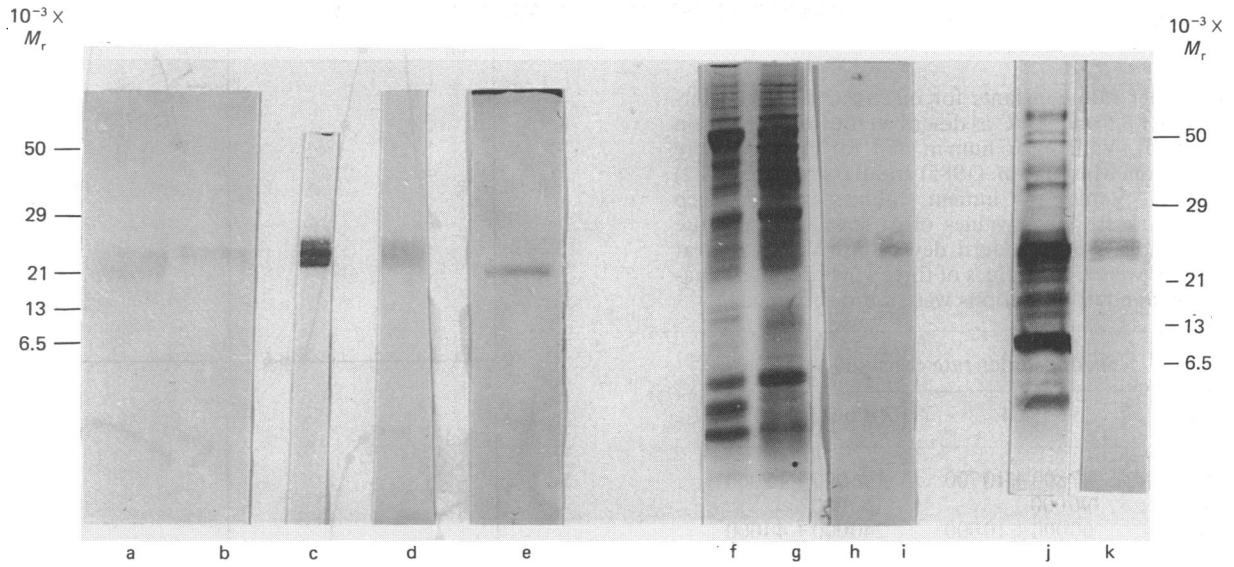

Fig. 3. Western blots of species variants of cathepsin L

Western blots of cathepsin L from rabbit, ox, human, sheep and rat, taken from the SDS/polyacrylamide gels shown in Fig. 1, were allowed to react with anti-(human cathepsin L) antiserum as described in the Materials and methods section (tracks a, b, c, d and e respectively). Protein samples (50 μg) from the non-autolysed pools of human liver eluted from the Mono S column by 200 mM-NaCl and 600 mM-NaCl were electrophoresed on SDS/polyacrylamide gels (tracks f and g respectively). Parallel tracks were electroblotted on to nitrocellulose sheets and allowed to react with anti-(human cathepsin L) (tracks h and i respectively). Tracks j and k show the gel and Western blot of a fraction of cathepsin L from the CM-Sephadex C-50 column.

Arg-NHMec with a K_m of approx. 2 μM and $k_{cat.}$ of approx. 20 s^{-1}; (3) it is inactivated irreversibly by Z-Phe-Phe-CHN_2 with a rate constant of approx. 150000 $M^{-1} \cdot s^{-1}$; (4) it is inactivated irreversibly by E-64 with a rate constant of approx. 60000 $M^{-1} \cdot s^{-1}$; (5) it is unstable at neutral pH.

The demonstration that sheep liver contains cathepsin L with characteristics essentially identical with those of human cathepsin L suggests that this tissue can be used as a most convenient source of a model for the human enzyme.

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