Species variations amongst lysosomal cysteine proteinases

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Properties of cathepsin L from rat liver lysosomes were compared with those of a similar enzyme, cathepsin S from beef spleen. Major characteristics of cathepsin L are the high activity against Z-Phe-Arg-methylcoumarylamide and sensitivity to the fast reacting irreversible inhibitor Z-Phe-Phe-diazomethane. In contrast, cathepsin S hydrolyzes Z-Phe-Arg-methylcoumarylamide only slowly and Z-Phe-Phe-diazomethane cannot be regarded as a potent inhibitor of this enzyme. The differences in the substrate specificity of cathepsin L from rat liver and cathepsin S from beef spleen are discussed in comparison with the substrate specificity of cathepsin B from rat and human liver and beef spleen.

Substrate specificity *Enzyme differentiation* *Cysteine proteinase* *Cathepsin B* *Cathepsin L* *Cathepsin S*

1. INTRODUCTION

Our purpose is to consider whether a cysteine proteinase that has been isolated from bovine spleen should be regarded as a variant of cathepsin L, or a new enzyme. This enzyme was initially called beef spleen cathepsin S [1], because of its similarity to beef lymph node cathepsin S [2], and then cathepsin L [3].

The criteria for identification of the lysosomal cysteine proteinases are by no means settled. For cathepsin B, the availability of the specific substrates Z-Arg-Arg-NNap and Z-Arg-Arg-NMec is important [4], but as yet we have no specific substrate for cathepsin L. Nevertheless, studies of rat [5,6], rabbit [7] and human [8] cathepsin L have shown that important characteristics are high proteolytic activity, high activity against Z-Phe-Arg-NMec at low substrate concentrations, and rapid irreversible inactivation by Z-Phe-Phe-CHN_2. With these properties in mind, we have re-examined the beef spleen enzyme, which we describe as cathepsin S.

Abbreviations: Bz-, benzoyl; -CHN_2, diazomethane; E-64, L-3-carboxy-*trans*-2,3-epoxypropyl-L-leucylamido(4-guanidino)butane; -NMec, 7-(4-methyl)coumarylamide; -NNap, 2-naphthylamide; -NNapOMe, 2-(4-methoxy)naphthylamide; Z-, benzyloxycarbonyl

2. MATERIALS AND METHODS

E-64 was kindly provided by Dr A.J. Barrett, Strangeways Research Laboratory, Cambridge. Azocasein was prepared from casein, vitamin-free (Serva, Heidelberg), by Dr J. Langner, Physiol.-chem. Institut, Halle (Saale), GDR. (Characterization of the azocasein preparation is given below.) Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2 were kindly given by Dr E. Shaw, Brookhaven National Laboratory, Upton, NY. Bz-Arg-NNap and Bz-Arg-NH_2 were obtained from Serva. Z-Arg-Arg-NNapOMe and Z-Arg-Arg-NMec were from Bachem, Torrance, CA, and Z-Phe-Arg-NMec and Bz-Phe-Val-Arg-NMec were obtained from Cambridge Research Biochemicals, Cambridge.

2.1. *Enzyme preparation*

Cathepsin B from human liver was kindly provided by Dr A.J. Barrett, Strangeways Research Laboratory. Cathepsins B and L were purified from rat liver lysosomes as in [5,9] and stored in

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0.1 M acetate buffer (pH 5.0) containing 0.5 mM $HgCl_2$ and 1 mM Na_2EDTA. Cathepsins B and S from beef spleen were purified as in [10].

2.2. *Enzyme assays*

The final concentrations of substrates were: peptide-NMec, 10 μM; peptide-NNap, 1 mM; Bz-Arg-NH_2, 10 mM; azocasein, 1%.

(After termination of the incubation the azocasein concentration was 0.5% in 5% trichloroacetic acid and ΔE^{1cm}_{366nm} = 20 after total hydrolysis and E^{1cm}_{366nm} = 0.05–0.1 for the blank have been determined using this azocasein preparation.) The reaction mixtures were buffered at pH 6.0 with 50 mM phosphate buffer, at pH 5.5 and 5.0 with 0.1 M acetate buffer. The enzymes were generally activated 5 min at 40°C with 2.5 mM dithiothreitol and 2.5 mM Na_2EDTA prior to incubation with the substrates. The incubation time was 10 min for the peptides as substrates and 30 min for azocasein.

The assays with all substrates were generally as in [4].

2.3. *Determination of enzyme concentration*

The enzyme concentrations were determined by active-site titration with E-64 as detailed in [4]. In these assays Z-Phe-Arg-NMec was used as substrate to determine the concentrations of cathepsin L, cathepsin S and cathepsin B.

3. RESULTS

Table 1A shows the activities against several substrates of preparations of rat cathepsin L and beef cathepsin S that had been standardized by active site titration. The data can be compared with

Table 1
Activities of cathepsins L, S and B

(A) Cathepsins L and S

Substrate	pH	Cathepsin L rat liver	Cathepsin S beef spleen
Azocasein	6.0	259	343
Azocasein-urea	5.0	710	967
Z-Phe-Arg-NMec	5.5	1229	71
Bz-Phe-Val-Arg-NMec	5.5	67	120
Z-Arg-Arg-NMec	5.5	2.4	0.3
Bz-Arg-NH_2	5.5	94	9

(B) Cathepsins B from 3 species

Substrate	pH	Cathepsin B from		
		Rat liver	Human liver	Beef spleen
Azocasein	6.0	29	35	35
Azocasein-urea	5.0	9	10	8
Z-Phe-Arg-NMec	6.0	400	554	700
Bz-Phe-Val-Arg-NMec	6.0	294	412	479
Z-Arg-Arg-NMec	6.0	367	414	552
Z-Arg-Arg-NNapOMe	6.0	2856	8960	9352
Bz-Arg-NNap	6.0	288	389	468

1 unit corresponds to 1 mg azocasein and 1 μmol peptide, respectively, degraded per min at 40°C. The concentration of the enzymes was determined by titration with E-64. Substrate concentration in the assays was 10 μM for the NMec substrates, 1 mM for the NNap substrates, 10 mM for Bz-Arg-NH_2 and 1% of azocasein. Concentration of urea was 3 M in the assay mixtures. Values are expressed as units/μmol

those for cathepsin B from 3 different species in table 1B. Cathepsin L and cathepsin S showed approximately similar levels of proteolytic activity against azocasein that were much higher than those of cathepsin B. It has been shown that cathepsin L is much more stable to 3 M urea at pH 5.0 in the presence of azocasein than is cathepsin B [11]. Since the urea sensitizes the azocasein to proteolysis, it makes the assays more sensitive to cathepsin L, but less sensitive to cathepsin B, since it is destroyed. It can be seen that cathepsin S resembled cathepsin L and differed from cathepsin B in this type of assay. We detected almost no activity of cathepsin L or cathepsin S against Bz-Arg-NNap (<0.1 unit/μmol) and Arg-NNap was not hydrolysed at all (not shown).

Z-Arg-Arg-NMec is a highly selective substrate for cathepsin B, but we have repeatedly detected traces of activity (less than 1%) in cathepsin L preparations; this is reflected in the data of table 1. The activity is not due to contaminating cathepsin B, since it is very sensitive to inhibition by Z-Phe-Phe-CHN_2 (not shown). Cathepsin S did not show any activity significant within the limits of experimental error against this substrate. Bz-Arg-NH_2 is a low-M_r substrate that is hydrolysed by rat cathepsin L [5]. Z-Phe-Arg-NMec has been detected as a very sensitive substrate of the enzyme [6]. However, these are only poor substrates of beef cathepsin S (table 1). The molar activity of cathepsin L with Bz-Arg-NH_2 is about 10-fold and with Z-Phe-Arg-NMec about 17-fold higher than

Table 2

Relative activities of cathepsins L, S and B

Substrate	Cathepsin				
	L_{rat}	S_{beef}	B^{rat}	B^{beef}	B_{human}
Z-Phe-Arg-NMec	100.0	100.0	100.0	100.0	100.0
Z-Arg-Arg-NMec	0.2	0.4	91.8	79.0	75.0
Bz-Phe-Val-Arg-NMec	5.5	169.0	73.5	68.4	74.4
Azocasein	21.1	483.0	7.3	5.0	6.3
Azocasein-urea	57.8	1362.0	2.2	1.1	1.9

For experimental details see table 1 and section 2.2. Values are expressed as %

Table 3

Residual activity of cathepsins L and S after reaction with irreversible inhibitors

Inhibitors (10^{-7} M)	Time of preincubation (min)	% residual activity	
		Cathepsin L, rat liver (1.7×10^{-7} M)	Cathepsin S, beef spleen (1.33×10^{-7} M)
Z-Phe-Phe-CHN_2	5	36	98
	10	15	90
	20	14	82
Z-Phe-Ala-CHN_2	5	49	85
	10	21	72
	20	16	68

The activated enzymes were incubated with the inhibitors; the concentrations refer to the preincubation mixture. After 5, 10 and 20 min at room temperature the same volume of 2% azocasein in 6 M urea was added and the assay was incubated 30 min at 40°C to determine the residual activity of the enzyme

those of cathepsin S. The data are only valid for substrate concentrations of 10 mM for Bz-Arg-NH_2 (K_m of 3 mM has been determined for rat liver cathepsin L [5]) and 10 μM for Z-Phe-Arg-NMec (K_m of 7 μM has been determined for cathepsin L from rat [9]).

Bz-Phe-Val-Arg-NMec was found to be the best of the synthetic substrates tested for beef cathepsin S, whereas Z-Phe-Arg-NMec is much better for cathepsin L (table 1).

A comparison of the substrate specificity of cathepsins B from 3 species (table 1) showed no major differences. The molar activities of cathepsin B from beef, rat and human are of the same order of magnitude with each substrate. The same is true of 10 additional peptides we tested with cathepsin B from these species (not shown). The biggest difference in the activities has been found with Z-Arg-Arg-NNapOMe; the enzymes from beef and human have a 3-fold higher value than the rat cathepsin B. These results show that there are almost no species differences in the catalytical properties of rat, beef and human cathepsin B (table 2).

Just as they differ in substrate specificity, cathepsins L and S differ in their sensitivity to inhibitors. Peptide diazomethyl ketones exhibit a high degree of specificity for individual cysteine proteinases [12]. Thus, Z-Phe-Phe-CHN_2 has been found to react very fast and stoichiometrically with rat cathepsin L but only slowly with cathepsin B [6]. Z-Phe-Ala-CHN_2 is a potent inhibitor of cathepsins L and B from rat. As shown in table 3, these inhibitors cannot be regarded as potent inhibitors of cathepsin S from beef spleen.

4. DISCUSSION

A comparison of the molar activities of rat cathepsin L and beef cathepsin S showed only small differences with azocasein as substrate, but major differences with low-M_r substrates. In contrast, cathepsins B from different species showed a distinctive pattern of relative activities with several substrates. It has been shown by HPLC analysis that rat, beef and human cathepsins B produce closely similar mixtures of peptides in the digestion of insulin B chain [13].

Cathepsin L from rat liver corresponds more to the enzyme from rabbit liver [7] than to cathepsin S from beef spleen with regard to the substrate specificity with low-M_r substrates (table 2). On the other hand, the same split position between Met and Arg in the hexapeptide Leu-Trp-Met-Arg-Phe-Val has been detected for cathepsin L from rat liver [14] and for cathepsin S from beef spleen [3].

Similarly to cathepsin S, cathepsin N from beef spleen was also reported to have a lower activity towards Z-Phe-Arg-NMec than found for the rabbit liver isoenzymes of cathepsin L [15]. However, it has not been established whether cathepsin N from beef spleen degrades azocasein [16].

Cathepsins L and S differ in some physical properties as well. Cathepsin S consists of a single chain of M_r 24000 [1], whereas cathepsin L from rat has an M_r of 29000 and two chains can be separated after reduction by SDS-gel electrophoresis (M_r ~24000 and ~5000) [17]. We found that the enzymatic activity remained in the heavy chain of rat cathepsin L after removal of the light chain [17]. However, we could not establish differences in the substrate specificity of the two forms of cathepsin L (unpublished).

Rat cathepsin L has isoelectric points in the range 5.5–6.1 [5] which are similar to those of rabbit cathepsin L (pI = 5.0–5.9) [7], but different to the pI of 6.2–6.8 of beef spleen cathepsin S [1].

5. CONCLUSIONS

We detected differences in the substrate specificity and sensitivity towards inhibitors of cathepsin L from rat liver and cathepsin S from beef spleen. The pI and M_r values of the enzymes differ [1,5]. The properties of high activity on Z-Phe-Arg-NMec and rapid inactivation by Z-Phe-Phe-CHN_2 seem to be shared by cathepsin L from several other species, but are not seen with the beef spleen enzyme. For this reason, it may be more appropriate to retain the name 'cathepsin S' for the beef spleen enzyme.

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