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The specificity of bovine spleen cathepsin S

A comparison with rat liver cathepsins L and B

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The peptide-bond-specificity of bovine spleen cathepsin S in the cleavage of the oxidized insulin B-chain and peptide methylcoumarylamide substrates was investigated and the results are compared with those obtained with rat liver cathepsins L and B. Major cleavage sites in the oxidized insulin B-chain generated by cathepsin S are the bonds Glu^{13}–Ala^{14}, Leu^{17}–Val^{18} and Phe^{25}–Tyr^{26}; minor cleavage sites are the bonds Asn^{3}–Gln^{4}, Ser^{9}–His^{10} and Leu^{15}–Tyr^{16}. The bond-specificity of this proteinase is in part similar to the specificities of cathepsin L and cathepsin N. Larger differences are discernible in the reaction with synthetic peptide substrates. Cathepsin S prefers smaller neutral amino acid residues in the subsites S_2 and S_3, whereas cathepsin L efficiently hydrolyses substrates with bulky hydrophobic residues in the P_2 and P_3 positions. The results obtained from inhibitor studies differ somewhat from those based on substrates. Z-Phe-Ala-CH_2F (where Z- represents benzyloxycarbonyl-) is a very potent time-dependent inhibitor for cathepsin S, and inhibits this proteinase 30 times more efficiently than it does cathepsin L and about 300 times better than it does cathepsin B. By contrast, the peptidylmethanes Z-Val-Val-Phe-CH_3 and Z-Phe-Lys(Z)-CH_3 inhibit competitively both cathepsin S and cathepsin L in the micromolar range.

INTRODUCTION

The cysteine proteinase cathepsin S was originally purified from bovine lymph nodes and bovine spleen (Turnšek *et al.*, 1975; Turk *et al.*, 1980, 1983). It was shown that cathepsin S clearly differs from cathepsin L in the hydrolysis of Z-Phe-Arg-NHMec as well as in inhibition by Z-Phe-Phe-CHN_2 (Kirschke *et al.*, 1984, 1986*a*). Moreover, a reactive polyclonal antibody to cathepsin L does not react with cathepsin S (Kirschke *et al.*, 1986*a*).

In the present paper we compare these two proteinases and cathepsin B by further studies of their substrate- and inhibitor-specificities.

EXPERIMENTAL

Enzymes

Cathepsin L (EC 3.4.22.15) and cathepsin B (EC 3.4.22.1) were prepared from the lysosomal fraction of rat liver as described by Kirschke *et al.* (1977) and Barrett & Kirschke (1981). Bovine spleen cathepsin S was isolated by the method of Kirschke *et al.* (1986*a*). All cathepsins used were in electrophoretically homogeneous form and their molarities were determined by active-site titration with E-64 as described by Barrett *et al.* (1982).

Materials

Z-Phe-Ala-CH_2F was kindly provided by Dr. Rasnick (Enzyme Systems Products, Dublin, CA, U.S.A.). Z-Val-Val-Phe-CH_3 and Z-Phe-Lys(Z)-CH_3 were synthesized as described elsewhere (Brömme *et al.*, 1989). Oxidized bovine insulin B-chain, fluorescamine, trifluoroacetic acid, dansyl chloride and dansyl-amino acid standards were purchased from Serva (Heidelberg, Federal Republic of Germany), and Bz-Phe-Val-Arg-NHMec was from Bachem (Bubendorf, Switzerland). Silica sheets with fluorescent indicator and lucifol cellulose sheets were obtained from Eastman–Kodak (Rochester, NY, U.S.A.) and Kavalier (Votice, Czechoslovakia) respectively. Spectrophotometric-grade acetonitrile was purchased from Merck–Schuchardt (Darmstadt, Federal Republic of Germany). All other solvents and chemicals used were of reagent grade and obtained from Laborchemie (Apolda, German Democratic Republic).

Substrates

Z-Phe-Arg-NHMec and HCl,Arg(HCl)-NHMec were synthesized as described elsewhere (Brömme *et al.*, 1989). Boc-Phe-Phe-Arg-NHMec {recryst. from methanol/dioxan/light petroleum; yield 81 %; $[\alpha]_D^{25}$ $-24°$ ($c = 0.5$ in acetic acid); m.p. 169 °C}, Boc-Phe-Leu-Arg-NHMec {recryst. from ethanol/ether; yield 69 %; $[\alpha]_D^{25}$ $-25.9°$ ($c = 0.5$ in dimethylformamide); m.p. 168–172 °C}, Z-Val-Val-Arg-NHMec {recryst. from methanol/ether; yield 57 %; $[\alpha]_D^{25}$ $-26.7°$ ($c = 0.5$ in dimethylformamide); m.p. 252–255 °C} and Z-Ala-Ala-Pro-Arg-NHMec {recryst. from ethanol/light petroleum; yield 39 %; $[\alpha]_D^{25}$ $-22.8°$ ($c = 0.5$ in dimethylformamide); m.p. 156–160 °C} were synthesized by fragment condensation of the *N*-protected di- or tri-peptide with HCl,Arg(HCl)-

Abbreviations used: Boc-, t-butyloxycarbonyl-; Z-, benzyloxycarbonyl-; Bz-, benzoyl-; -CH_3, -methane; -CHN_2, -diazomethane; -CH_2F, -fluoromethane; -NHMec, 7-(4-methyl)coumarylamide; E-64, L-3-carboxy-*trans*-2,3-epoxypropionyl-leucylamido-(4-guanidino)butane. For the discussion of the interactions between proteinase and substrate the nomenclature of Schechter & Berger (1967) was used.

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NHMec by using the mixed-anhydride method. All peptide substrates were obtained in h.p.l.c.-homogeneous form.

Incubation of the oxidized insulin B-chain with cathepsin S

Cathepsin S was activated in 0.5 ml of volatile 50 mM-ammonium carbonate buffer, pH 7.9, containing 6 mM-dithioerythritol and 6 mM-Na_2EDTA at 37 °C for 15 min. The reaction was started by addition of 3 μmol of oxidized insulin B-chain dissolved in 1 ml of 50 mM-ammonium carbonate buffer, pH 7.9, at an enzyme/substrate molar ratio of 1:2600. After digestion times of 30 min and 10 h at 37 °C 750 μl portions were withdrawn and freeze-fried.

Separation and analysis of hydrolysis products

The freeze-dried digests of the oxidized insulin B-chain were precipitated with 400 μl of 1 % (v/v) trifluoroacetic acid and centrifuged at 10000 g for 5 min in a high-speed centrifuge (Janetzki, Engelsdorf, German Democratic Republic). Only after short-time incubation could sufficient undigested insulin B-chain be analysed by dansyl chloride and amino acid analysis. Samples of the clear supernatant were applied in 100 μl portions to a 250-4 Lichrocart 100 RP-18 (5 μm particle size) column (Merck, Darmstadt, Federal Republic of Germany) of a Merck–Hitachi h.p.l.c. apparatus. The cleavage material was eluted from the column with 5% (v/v) acetonitrile in 0.7% (v/v) trifluoroacetic acid for 5 min followed by a linear gradient (5–45%, v/v) of acetonitrile in 0.7% trifluoroacetic acid at a flow rate of 1 ml/min for 45 min. The eluent was monitored at 220 nm and peak fractions were collected. This procedure was repeated four times for each incubation mixture and the correlated fractions were pooled and concentrated. Each peak fraction was characterized as being homogeneous by t.l.c. on cellulose sheets. Non-homogeneous fractions were separated by ascending chromatography in butan-2-ol/formic acid/water (70:7:21, by vol.). The peptides were located by fluorescamine staining and finally eluted with 6 M-HCl (Schiltz *et al.*, 1977). The isolated peptides were hydrolysed with 6 M-HCl in a refluxing condenser for 24 h. The amino acids were determined with a Microtechna 339 amino acid analyser (Prague, Czechoslovakia). The determination of the *N*-terminal amino acid of the purified peptides was done by dansylation followed by t.l.c. on silica sheets with fluorescent indicator (Eulitz *et al.*, 1974).

Enzyme assays with methylcoumarylamide substrates

Initial rates of hydrolysis of the methylcoumarylamide substrates were monitored in 1 cm cuvettes at 25 °C in a Shimadzu UV-300 spectrophotometer with a fluorimetric attachment at an excitation wavelength of 383 nm and with a 450 nm emission filter. The kinetic experiments were carried out with a constant enzyme concentration in 50 mM-sodium acetate buffer, pH 5.5, for cathepsin L, in 50 mM-potassium phosphate buffer, pH 6.5, containing 0.01 % (v/v) Triton X-100 for cathepsin S and in 50 mM-potassium phosphate buffer, pH 6.0, for cathepsin B. The reaction mixtures for cathepsins L and B were activated for 5 min at 25 °C with 2.5 mM-dithioerythritol, 2.5 mM-Na_2EDTA and 0.005 % (v/v) Brij-35 in the assay buffer before starting with substrate, whereas cathepsin S was activated for 15 min with 5 mM-dithioerythritol, 5 mM-Na_2EDTA and 0.01 % Triton X-100 in 50 mM-phosphate buffer, pH 6.5. The kinetic constants $V_{max.}$ and K_m were obtained by non-linear-regression analysis by using the program Enzfitter (Leatherbarrow, 1987).

Inhibition studies

The inhibition assays were performed in a similar manner to the substrate assays. To 1 ml of substrate in assay buffer and different inhibitor concentrations (seven or eight per substrate concentration), taken as 3–15 μl portions from a stock solution of Z-Phe-Ala-CH_2F in acetonitrile, was added 0.5 ml of activated enzyme, and the progress curves were monitored. Since the time-dependent inhibition was studied in the presence of substrate for each inhibitor concentration, two substrate concentrations for an extrapolation to [S] = 0 were used (Z-Phe-Arg-NHMec, 2 and 6 μM for cathepsin L, and 5 and 20 μM for cathepsin B; Z-Val-Val-Arg-NHMec, 10 and 50 μM for cathepsin S). The inhibition parameters k_{+2}, K_i and k_{+2}/K_i were obtained from the plots of pseudo-first-order inhibition rates $k_{app.}$ at [S] = 0 versus [I] by regression to $k_{app.} = k_{+2}[I]/K_i+[I]$ in accordance with Tian & Tsou (1982) by using the program Enzfitter.

The reversible inhibition of the cathepsins by peptidyl methanes was carried out at four substrate concentrations with six inhibitor concentrations in each case. The K_i values were determined by using the Dixon plot ($1/v$ versus [I]).

RESULTS AND DISCUSSION

Action of cathepsin S on the oxidized insulin B-chain

The digestion of the oxidized insulin B-chain by cathepsin S was carried out at pH 7.9. This pH is suited for a specific discrimination between cathepsin S and cathepsin L. The activity of cathepsin L against protein substrates is absolutely zero at this pH value (Kirschke *et al.*, 1989), whereas cathepsin S degrades these substrates with high efficiency even at slightly alkaline pH values. If there should be a possible contamination with the highly effective cathepsin L below its electrophoretic detection limit, it would be inactivated. As shown for cathepsin B (McKay *et al.*, 1983), small contaminations with other proteinases can appreciably alter the pattern of cleavage of the oxidized insulin B-chain under conditions of long periods of incubation.

The analysis of the products of the 30 min incubation led to the identification of eight peptides, including the undigested insulin B-chain. After 10 h seven peptides were identified, but, however, no undegraded insulin B-chain could be found in this case (Table 1). From these data the major cleavage sites were located at bonds Glu^{13}–Ala^{14}, Leu^{17}–Val^{18} and Phe^{25}–Tyr^{26} (Fig. 1). A major cleavage site at bond Leu^{15}–Tyr^{16} is not likely. Although large amounts of the dipeptide Ala^{14}–Leu^{15} were found after both incubation times, the increasing amount of the liberated peptide Ala^{14}–Leu^{17} even after 10 h digestion contradicts a primary cleavage of the Leu^{15}–Tyr^{16} bond. Unfortunately, the dipeptide Tyr^{16}–Leu^{17} could not be detected.

The cleavage sites at bonds Asn^3–Val^4, Ser^9–His^{10} and also Leu^{15}–Tyr^{16} were formed after the liberation of the major cleavage products. This is indicated by the disappearance of the Phe^1–Glu^{13} peptide after prolonged incubation and by the increasing amounts of the smaller peptides Phe^1–Asn^3, Phe^1–Ser^9 and Glu^4–Gln^{13}.

Table 1. Analysis of peptides obtained by digestion of oxidized insulin B-chain with cathepsin S

Hydrolysis was carried out at 37 °C with 3 μmol of oxidized insulin B-chain for 30 min and 10 h at an enzyme/substrate molar ratio of 1:2600. Further information is given in the Experimental section. Cys represents cysteic acid, and the asterisk (*) indicates the residue arbitrarily taken as unity.

Peptide no.	Amino acid analysis (mol/mol)	*N*-Terminal residue	Recovery (nmol)	Peptide
30 min digest	Tyr 0.9 (1), Thr 1.0 (1), Pro 1.1 (1), Lys* 1 (1), Ala 1.0 (1)	Tyr	223	Tyr^{26}–Ala^{30}
	Phe 0.9 (1), Val 1.2 (1), Asp* 1 (1)	Phe	119	Phe^{1}–Asn^{3}
	Ala* 1 (1), Leu 1.0 (1), Tyr 0.4 (0?)	Ala	170	Ala^{14}–Leu^{15}
	Glu 2.1 (2), His 1.4 (2), Leu* 2 (2), Cys 1.0 (1), Gly 1.2 (1), Ser 1.0 (1), Val 1.2 (1)	Glu	60	Gln^{4}–Glu^{13}
	Phe 1.1 (1), Val 2.1 (2), Asp 1.1 (1), Glu 1.9 (2), His 1.8 (2), Leu* 2 (2), Cys 1.1 (1), Gly 1.2 (1), Ser 0.9 (1)	Phe	61	Phe^{1}–Glu^{13}
	Ala 1.2 (1), Leu* 2 (2), Tyr 1.1 (1)	Ala	40	Ala^{14}–Leu^{17}
	Val* 1 (1), Cys 0.9 (1), Gly 2.2 (2), Glu 1.1 (1), Arg 0.8 (1), Phe 1.9 (2)	Val	72	Val^{18}–Phe^{25}
Precipitate	Phe 2.9 (3), Val* 3 (3), Asp 1.4 (1), Glu 3.3 (3), His 2.3 (2), Leu 3.6 (4), Cys 1.8 (2), Gly 3.2 (3), Thr 1.1 (1), Pro 0.6 (1), Lys 1.1 (1), Ser 1.0 (1),Ala 2.2 (2), Tyr 1.3 (2), Arg 0.8 (1)	Phe	115	Phe^{1}–Ala^{30}
10 h digest	Tyr 0.9 (1), Thr 1.0 (1), Pro 1.2 (1), Lys* 1 (1), Ala 1.2 (1)	Tyr	225	Tyr^{26}–Ala^{30}
	Phe 0.9 (1), Val 1.1 (1), Asp* 1 (1)	Phe	288	Phe^{1}–Asn^{3}
	Ala 0.9 (1), Leu* 1 (1), Tyr 0.5 (0?)	Ala	317	Ala^{14}–Leu^{15}
	Glu 2.2 (2), His 1.6 (2), Leu* 2 (2), Cys 1.0 (1), Gly 1.2 (1), Ser 1.1 (1), Val 1.3 (1)	Glu	170	Gln^{4}–Glu^{13}
	Phe 0.8 (1), Val* 1 (1), Asp 0.8 (1), Glu 1.2 (1), His 0.9 (1), Leu 1.2 (1), Cys 0.7 (1), Gly 1.3 (1), Ser 1.0 (1)	Phe	208	Phe^{1}–Ser^{9}
	Ala 1.2 (1), Leu* 2 (2), Tyr 0.7 (1)	Ala	131	Ala^{14}–Leu^{17}
	Val* 1 (1), Cys 0.9 (1), Gly 1.9 (2), Glu 1.1 (1), Arg 0.9 (1), Phe 1.8 (2)	Val	390	Val^{18}–Phe^{25}

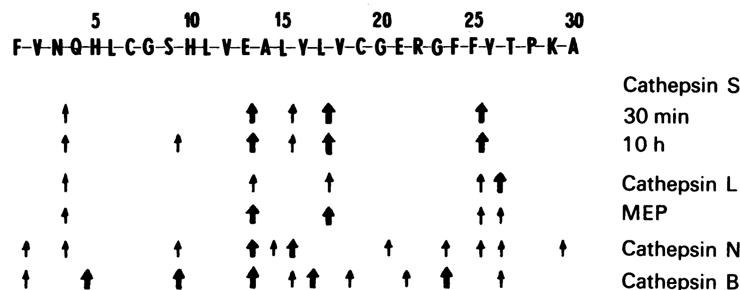

Fig. 1. Action of bovine spleen cathepsin S on the oxidized insulin B-chain

The cleavage sites on the oxidized insulin B-chain are indicated by thick (major sites) and thin (minor sites) arrows. The results are compared with data of Kärgel *et al.* (1980) for cathepsin L, of Gal & Gottesman (1986) for the major excreted protein (MEP), of Evans & Etherington (1979) for cathepsin N and of McKay *et al.* (1983) for cathepsin B.

The bond-specificity of cathepsin S does not reveal a clear preference of specific amino acid sequences as in the case of cathepsin L (Kärgel *et al.*, 1980). Besides bulky amino acid residues such as phenylalanine or leucine in P_2 and P_3, smaller amino acid residues such as glycine, alanine and valine are also accepted in these positions. This specificity correlates in part to cathepsin N (Evans & Etherington, 1979). However, both cathepsin L and cathepsin N display some characteristic differences in their bond-specificities with respect of cathepsin S. The only major cleavage site for cathepsin L is at bond Tyr^{26}–Thr^{27}, whereas cathepsin S splits the B-chain extensively at the neighbouring peptide bond Phe^{25}–Tyr^{26}. Cathepsin S strongly cleaves the Leu^{17}–Val^{18} bond, whereas this bond is not attacked by cathepsin N. On the other hand cathepsin N shows some additional cleavage sites that are not found for cathepsin S (Fig. 1).

Action of the enzymes on peptide methylcoumarylamide substrates

The pH optima for rat liver cathepsin L and bovine spleen cathepsin S for Z-Phe-Arg-NHMec were found to be at pH 6.0 and pH 6.0–6.5 respectively (Fig. 2). Mason *et al.* (1984, 1985) have established a pH optimum of 6.0 for rabbit liver cathepsin L and for human cathepsin L a broader plateau between pH 4.5 and 5.5, which at

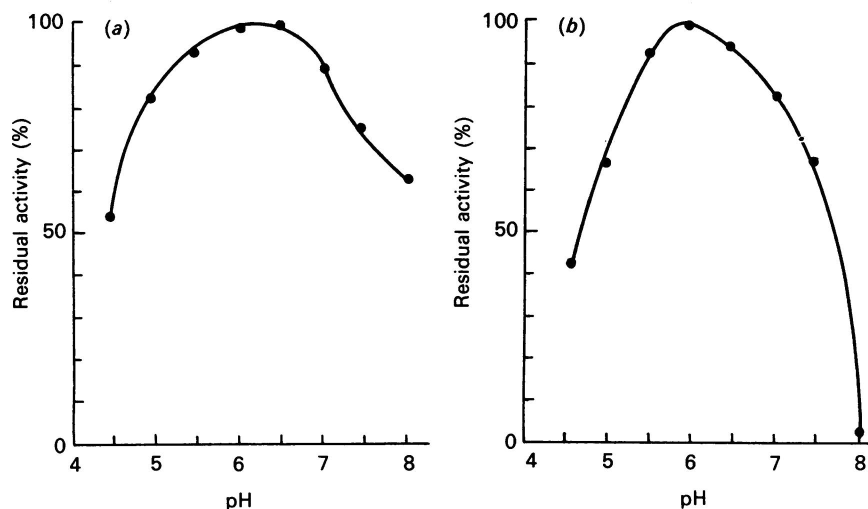

Fig. 2. pH–activity profiles of (*a*) bovine spleen cathepsin S and (*b*) rat liver cathepsin L

The proteinases were activated in 500 μl of thiol reagent in 50 mM buffer at several pH values at 37 °C for 15 min (cathepsin S) and at 25 °C for 5 min (cathepsin L). The residual activity was determined by addition of 1000 μl of substrate (Z-Phe-Arg-NHMec) in the appropriate 50 mM buffer. Buffers used were sodium acetate (pH 4.5–5.5), potassium phosphate (pH 6.0–7.0) and Tris/HCl (pH 7.5–8.0).

prolonged incubation times in buffer without thiol activators drifted to the acid range. For a better comparison with results from the literature we have taken an assay pH of 5.5 for cathepsin L. Although cathepsins S and L differ only slightly in their pH optima, their stabilities at alkaline pH values are very different. The activity of cathepsin L at pH 8.0 after 5 min activation with thiol reagent is zero at 25 °C, whereas cathepsin S still shows a residual activity of 60% after 15 min activation at 37 °C. A similar pH-stability for cathepsin S was also observed in the hydrolysis of azo-casein (Kirschke *et al.*, 1989). Although species variants of cathepsin L show slight differences in their pH-stability (Mason, 1986), the variants of the cathepsins L and B, including preparations from rat and bovine, display comparable rates of substrate hydrolysis and rates of inhibition. Observed rates are distinguished in general by factors between 1- and 3-fold (Kirschke *et al.*, 1984, 1986*b*; Mason, 1986). Therefore a direct comparison of the rat liver cathepsins L and B with cathepsin S from bovine is appropriate.

Peptide methylcoumarylamides are very sensitive substrates of cysteine proteinases (Barrett & Kirschke, 1981). It was shown by Mason *et al.* (1984, 1985) that arginine and lysine are very efficient P_1 residues for the hydrolysis of methylcoumarylamide substrates by cysteine proteinases. We have varied the P_2 and P_3 positions and found that cathepsin S prefers residues with decreasing hydrophobic side chains (Val > Leu > Phe) in the P_2 position. In contrast, cathepsin L shows a preference for rather bulky hydrophobic residues in the P_2 position (Phe ⩾ Leu > Val; Table 2). Both tripeptide substrates (compounds IV and V) with valine in the P_2 position are better substrates for cathepsin S than for cathepsin L, whereas all peptide substrates with phenylalanine or leucine in the P_2 position are favoured substrates for cathepsin L.

However, this notable influence of the occupation of the P_2 position on the specificity constant of the bovine spleen cathepsin S could not be demonstrated for a rabbit spleen proteinase that is also called cathepsin S (Maciewicz & Etherington, 1988). For this enzyme Z-Phe-Arg-NHMec and Bz-Phe-Val-Arg-NHMec are poorer substrates than for cathepsin L. Although a direct comparison between the molar activities presented by Maciewicz & Etherington (1988) and the $k_{cat.}/K_m$ values in the present paper is not possible, the difference in the ability of the enzymes to degrade these two substrates argues against a simple species difference. Furthermore, previously determined molar activities of bovine spleen cathepsin S are one to two orders of magnitude higher (Kirschke *et al.*, 1984) than for the rabbit spleen enzyme. In contrast, cathepsin L from rat, bovine and rabbit, including the values reported by Maciewicz & Etherington (1988), displays comparable values. An additional clear difference in their molecular masses also suggests that bovine spleen cathepsin S and the enzyme from rabbit spleen are different proteins rather than species variations of one enzyme.

In the P_3 position smaller hydrophobic residues such as valine are also favoured for cathepsin S-catalysed hydrolysis of substrates (compound V), whereas cathepsin L again prefers bulky hydrophobic residues in this position (compounds I, II, III), if P_2 is also occupied by a larger residue such as phenylalanine or leucine. This agrees very well with the described substrate-specificity of cathepsin L towards the oxidized insulin B-chain (Kärgel *et al.*, 1980).

Cathepsin B does not reveal a preference for specific residues in the P_2 and P_3 positions of the substrates tested. However, it was noted that all tripeptide substrates display a drastic decrease in $k_{cat.}$ but only a slightly improved affinity compared with the favoured dipeptide Z-Phe-Arg-NHMec.

Proline in the P_2 position (compound VI in Table 2) strongly decreases the specificity constant $k_{cat.}/K_m$ for all three cathepsins. This is in accordance with former results that rat liver cathepsins B and H (Brömme *et al.*, 1987)

Table 2. Specificity of cathepsins S, L and B: kinetic constants for the hydrolysis of peptide methylcoumarylamide substrates

The kinetic constants were determined at pH 6.5 for cathepsin S, at pH 5.5 for cathepsin L and at pH 6.0 for cathepsin B at 25 °C, and represent means ± S.E.M. for two separate determinations per substrate concentration. The concentration of the enzymes for the evaluation of the $k_{cat.}$ values was determined by titration with E-64.

Substrate	Cathepsin S			Cathepsin L			Cathepsin B		
	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)	$k_{cat.}$ (s^{-1})	K_m (μM)	$k_{cat.}/K_m$ ($mM^{-1}\cdot s^{-1}$)
Z-Phe-Arg-NHMec (I)	4.7±0.2	14.7±1.7	320	17.6±1.6	2.8±0.5	6286	364±56	223±46	1632
Boc-Phe-Phe-Arg-NHMec (II)	8.6±0.2	37.5±2.2	229	7.5±0.2	1.4±0.1	5357	22.7±1.4	114±11	199
Boc-Phe-Leu-Arg-NHMec (III)	6.1±0.6	7.3±0.6	836	7.9±0.2	1.5±0.1	5267	46.2±3.5	107±15	432
Bz-Phe-Val-Arg-NHMec (IV)	13.0±0.4	8.1±0.7	1605	1.1±0.03	1.8±0.1	611	17.5±1.4	29±4.5	603
Z-Val-Val-Arg-NHMec (V)	40.5±1.6	17.5±1.7	2314	8.5±0.4	4.8±0.4	1771	14.0±1.7	31.1±7.1	450
Z-Ala-Ala-Pro-Arg-NHMec (VI)	–	–	0.4	–	–	0.5	953±168	19.2±2.4	20

as well as rabbit liver cathepsins B and L (Mason *et al.*, 1984) discriminate against proline in the P_2 position. On the other hand Z-Ala-Ala-Pro-Arg-NHMec (compound VI) is very efficiently hydrolysed by trypsin (result not shown here). The presence of proline in the P_2 and the P_3 position respectively may lead to a good differentiation between similar specificities of serine proteinases and cysteine proteinases, because in contrast with cysteine proteinases several serine proteinases do not accept proline in the P_3 position (Thompson & Blout, 1973; Bauer, 1976; Brömme *et al.*, 1986).

Reversible and irreversible inhibition of the enzymes

Peptidylmethanes are reversible inhibitors of cysteine proteinases (Brömme *et al.*, 1989). According to Smith *et al.* (1988) the formation of a tetrahedral thioketal adduct between the inhibitor and the active-site thiol group of the enzyme probably occurs. Z-Val-Val-Phe-CH_3 proved to be a very effective inhibitor of cathepsin S and cathepsin L and shows no inhibition of cathepsin B up to its maximal solubility of 17 μM in 6.6% (v/v) dimethylformamide (Table 3). The inhibition of the cathepsins S and L in the micromolar range by the P_3–P_2-valylvaline containing peptidylmethane correlates with the high specificity constants determined with the substrate Z-Val-Val-Arg-NHMec (Table 2). In contradiction to the observed specificity on peptidyl substrates for cathepsin S, Z-Phe-Lys(Z)-CH_3 with its bulky hydrophobic residues in the P_1, P_2 and P_3 positions inhibits both cathepsins S and L with nearly the same efficiency in the micromolar range. As expected, the K_i value for this dipeptidylmethane is one order of magnitude greater for cathepsin B (Table 3).

According to Rauber *et al.* (1986) peptidylfluoromethanes are irreversible alkylating inhibitors of serine proteinases and cysteine proteinases. In contrast with the very reactive chloro derivatives, side reactions with other molecules should be diminished through the greatly diminished nucleophilic displaceability of the fluoride. Z-Phe-Ala-CH_2F was proved to be a very potent inhibitor of cathepsin S and shows a competitive type of time-dependent inhibition in the plot of $k_{app.}$ versus [I] (Fig. 3). The inhibitor shows a 30-fold higher second-order rate constant for the inhibition of cathepsin S than for cathepsin L. This is mainly caused by the very low K_i value of 1×10^{-8} M (Table 3). However, this difference in the inhibition rates does not allow the assay of both enzymes in a mixture by total inactivation of one. Cathepsin S still shows a residual activity of 4–7% after 5 min preincubation with 0.1 μM-Z-Phe-Ala-CH_2F (no activity after 10 min), whereas cathepsin L has a residual activity of 80–85% (22–25% after 10 min) against 10 μM-Z-Phe-Arg-NHMec at 25 °C.

The second-order rate constants for the inhibition of cathepsins L and B differ only by a factor of 10-fold. Of course this also is not suited for a differentiation between both enzymes in one mixture. The inactivation of cathepsin B by Z-Phe-Ala-CH_2F has been studied in several laboratories and the determined second-order rate constants display some variation [16000 $M^{-1}\cdot s^{-1}$ at 28 °C for human cathepsin B (Rasnick, 1985); 54000 $M^{-1}\cdot s^{-1}$ at 37 °C for pig liver cathepsin B (Rauber *et al.*, 1986); 21000 $M^{-1}\cdot s^{-1}$ at 25 °C for bovine liver cathepsin B (Smith *et al.*, 1988); 8200 $M^{-1}\cdot s^{-1}$ at 25 °C for rat liver cathepsin B (present work)]. This may be caused by varied temperature conditions in the assays, as

Table 3. Inhibition of cathepsins S, L and B by peptidylmethanes and a peptidylfluoromethane at 25 °C

The inhibition studies were carried out for cathepsin S at pH 6.5, for cathepsin L at pH 5.5 and for cathepsin B at pH 6.0. The values are means ± S.E.M. for double determinations. For further detail see the Experimental section.

	Cathepsin S			Cathepsin L			Cathepsin B		
	k_{+2} (s^{-1})	K_I (μM)	k_{+2}/K_I ($mM^{-1} \cdot s^{-1}$)	k_{+2} (s^{-1})	K_I (μM)	k_{+2}/K_I ($mM^{-1} \cdot s^{-1}$)	k_{+2} (s^{-1})	K_I (μM)	k_{+2}/K_I ($mM^{-1} \cdot s^{-1}$)
Z-Val-Val-Phe-CH_3		1.8±0.3 (competitive)			4.7±0.9 (competitive)			≫ 17*	
Z-Phe-Lys(Z)-CH_3		1.4±0.1 (competitive)			3.0±0.1 (competitive)†			26±1 (competitive)†	
Z-Phe-Ala-CH_2F	0.05±0.002	0.018±0.002	2778	0.05±0.003	0.57±0.07	88	0.07±0.01	8.5±1.8	8.2

* Maximal solubility.
† Data from Brömme *et al.* (1989).

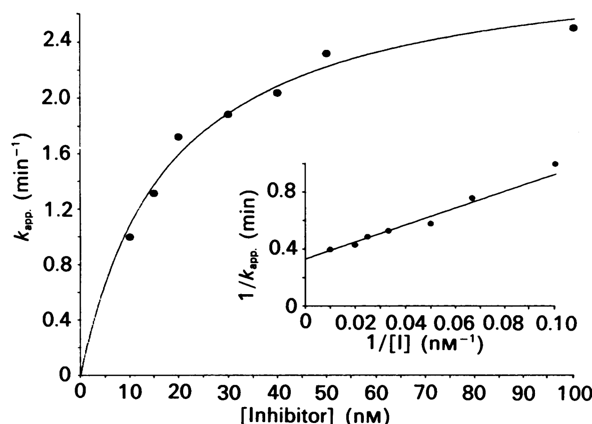

Fig. 3. Fit-to-hyperbola plot for the inhibition of cathepsin S by Z-Phe-Ala-CH_2F

Cathepsin S was inhibited in 50 mM-potassium phosphate buffer, pH 6.5, containing 1.66 mM-dithioerythritol, 1.66 mM-EDTA and 0.01% Triton-X-100 at 25 °C in the presence of substrate (Z-Val-Val-Arg-NHMec). The enzyme concentration was approx. 0.9 nM and the inhibitor concentration range was 10–100 nM.

mentioned by Rauber *et al.* (1986), but also by the use of cathepsin B preparations of different species origin.

CONCLUSIONS

The cathepsins S and L are related cysteine proteinases, but they are characterized by specific differences with regard to their substrate- and inhibitor-specificities. Cathepsin S is distinguished from cathepsin L in the following parameters. (1) Major cleavage sites in the oxidized insulin B-chain differ from those attacked by cathepsin L. (2) The two cathepsins differ in the alkaline range of their pH–activity profiles. Whereas cathepsin L is inactive at pH 8.0, cathepsin S shows a residual activity of 60% against Z-Phe-Arg-NHMec. (3) In the subsites S_2 and S_3 cathepsin S favours rather small non-polar amino acid residues, in contrast with the preference of bulky hydrophobic residues by cathepsin L. (4) Bz-Phe-Val-Arg-NHMec and Z-Val-Val-Arg-NHMec are better substrates for cathepsin S than for cathepsin L. The K_m values of all peptide methylcoumarylamide substrates tested are significantly lower for cathepsin L than for cathepsin S. (5) Cathepsin S is strongly inhibited by Z-Phe-Ala-CH_2F with a rate constant of approx. 2780000 $M^{-1} \cdot s^{-1}$, whereas cathepsin L shows a 30-fold lower second-order rate constant of approx. 90000 $M^{-1} \cdot s^{-1}$.

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