Comparison of the Kinetics of Cathepsins B, L, L-like, and X from the Dorsal Muscle of Mackerel on the Hydrolysis of Methylcoumarylamide Substrates

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Proteolytic kinetics of cathepsins B, L, L-like, and X (a novel cysteine proteinase) from mackerel dorsal muscle were determined using methylcoumarylamide peptides as substrates. From a comparison of K_m and K_{cat}/K_m of these proteinases, cathepsin X showed a lower K_m (0.9 μM) and higher K_{cat}/K_m (1216.7 μM^{-1} min^{-1}) for the hydrolysis of Z-Arg-Arg-MCA. Hydrolysis of Z-Phe-Arg-MCA and Boc-Val-Leu-Lys-MCA by cathepsin L-like had lower K_m (1.5 and 1.3 μM, respectively) and higher K_{cat}/K_m (2404.5 and 437.1 μM^{-1} min^{-1}, respectively). Cathepsins L and L-like did not hydrolyze Boc-Glu-Lys-Lys-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Z-Pyr-Gly-Arg-MCA. Cathepsin B had a higher K_m (157.8 μM) and lower K_{cat}/K_m (94.1 μM^{-1} min^{-1}) for the hydrolysis of Boc-Val-Leu-Lys-MCA. This property could be used to differentiate cathepsin B from cathepsin L or L-like. For the hydrolysis of methylcoumarylamide peptides, the kinetics of cathepsins B, L, L-like, and X were quite different. Peptides with basic amino acids, such as Lys or Arg, at the P_1 site were more susceptible to these proteinases.

INTRODUCTION

Lysosomal cysteine proteinases, cathepsin B (Bonete et al., 1984; Hara et al., 1988; Matsumiya et al., 1989), cathepsin H (Aranishi et al., 1992), and cathepsin L (Yamashita and Konagaya, 1990), from fish muscles have been purified and characterized. Among these proteinases, cathepsins B and L were considered to be related to the muscle softening during spawning migration (Yamashita and Konagaya, 1990) and postmortem aging (Etherington et al., 1990). Proteolytic specificity is a very useful clue in elucidating the limited proteolysis of myofibrillar proteins by these proteinases. The specificity of cathepsin B has been extensively studied using substrate catalysis (Aronson and Barrett, 1978; Towatari and Katanuma, 1983; Koga et al., 1991) and X-ray crystallography (Musil et al., 1991), while the specificity of cathepsin L has been studied using only substrate catalysis (Dufour and Ribadeau-Dumas, 1988; Koga et al., 1990).

In our previous studies, cathepsin B (Jiang et al., 1994a), cathepsin L (Lee et al., 1993), cathepsin L-like (Lee et al., 1993), and a novel cysteine proteinase (designated cathepsin X) (Jiang et al., 1994b) were purified from mackerel dorsal muscle and characterized. This study aims to compare the kinetics of these 4 mackerel cysteine proteinases using 13 methyl-coumarylamide peptides.

MATERIALS AND METHODS

Materials. Mackerel (*Scomber australasicus*), iced for about 4 h before experiment, was obtained from a fisheries market in northern Taiwan. Dithiothreitol (DTT) was ob-

tained from Merck (Darmstadt, Germany). 7-Amino-4-methylcoumarin, bovine serum albumin, cysteine, and 1-(L-*trans*-epoxysuccinyl)leucylamido)-4-guanidinobutane (E-64) were the products of Sigma (St. Louis, MO). Sodium chloroacetate was also obtained from Merck. Dye reagent concentrate for protein concentration assay was obtained from Bio-Rad (Richmond, CA). 4-Methylcoumaryl-7-amide-derived peptides were purchased from Peptide Institute Inc. (Osaka, Japan). Other chemicals were of reagent grade.

Preparation of Mackerel Cysteine Proteinases. Cathepsins B, L, L-like, and X from mackerel muscle were purified according to methods given in previous studies (Jiang et al., 1994a,b; Lee et al., 1993). One unit of enzyme activity was expressed as the amount of enzyme that can hydrolyze methylcoumarylamide substrate (Z-Phe-Arg-MCA for cathepsins B, L, L-like; Z-Arg-Arg-MCA for cathepsin X) and release 1 μmol of aminomethylcoumarin within 1 min of reaction at 35 °C. The concentrations of these proteinases were determined by stoichiometric titration with E-64 (Barrett and Kirschke, 1981).

Hydrolytic Activities against Methylcoumarylamide Substrates. Hydrolytic activities of cathepsins B (at pH 6.5), L (at pH 5.0), L-like (at pH 5.5), and X (at pH 6.0) were fluorometrically measured at 35 °C using 13 methylcoumaryl-amide substrates (L-Arg-MCA, Z-Arg-MCA, Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, Z-Pyr-Gly-Arg-MCA, Suc-Ala-Ala-Ala-MCA, Suc-Ala-Pro-Ala-MCA, Boc-Glu-Lys-Lys-MCA, Boc-Val-Leu-Lys-MCA, Suc-Ala-Ala-Pro-Phe-MCA, Suc-Gly-Pro-MCA, Suc-Gly-Pro-Leu-Gly-Pro-MCA, and Suc-Leu-Leu-Val-Tyr-MCA) according to the method of Barrett and Kirschke (1981). After 10 min of incubation with substrates, the hydrolytic reaction by these proteinases was terminated by adding an equal volume of 0.1 M sodium acetate buffer (pH 4.3) containing 0.1 M sodium monochloroacetate. The intensity of fluorescence of the mixture was measured at an excitation wavelength of 350 nm and an emission wavelength of 460 nm. One unit of enzyme activity was expressed as the amount of enzyme that can hydrolyze methylcoumarylamide substrates and release 1 μmol of aminomethylcoumarin within 1 min of reaction at 35 °C. The catalytic activity was expressed as a percent ratio of the activity of these cathepsins incubated with various substrates to that with maximum activity.

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Determination of K_m and K_{cat}. Catalytic activities of cathepsins B (4.2×10^{-5} μmol), L (4.4×10^{-5} μmol), L-like (4.4×10^{-5} μmol), and X (2.0×10^{-4} μmol) against methylcoumarylamide substrates (calculated from those with relative activity higher than 5%) were measured according to the method of Barrett (1980). In a 4 mL quartz cell, 1.98 mL of various concentrations of substrates (0.5–25 μmol) in either 1.98 mL of 50 mM sodium potassium phosphate buffer (pH 6.5 for cathepsin B; pH 6.0 for cathepsin X) or 50 mM sodium acetate buffer (pH 5.5 for cathepsin L-like proteinase; pH 5.0 for cathepsin L) containing 1 mM EDTA and 2 mM cysteine was separately added. After the addition of 20 μL of proteinase solution, the fluorescent intensity of free aminomethylcoumarin released from the hydrolysis of methylcoumarylamide substrate by these proteinases was measured continuously by a spectrofluorometer (excitation, 350 nm; emission, 460 nm; slit, 3 nm) at 35 °C using a water-circulating holder. The initial velocities (v) were calculated from the reaction curve, i.e. the slop of the initial linear cure. Lineweaver–Burk double-reciprocal plot of substrate concentration (s) versus initial velocity (v) was used to calculate the Michaelis constant (K_m) and maximum velocity (V_m). The catalytic rate constant (K_{cat}) was calculated as

$$V_m = K_{cat}[E]_0$$

where $[E]_0$ is the concentration of enzyme (micromoles).

Determination of Protein Concentration. Protein concentration was determined using the protein–dye binding method with γ-globulin as standard (Bradford, 1976).

RESULTS AND DISCUSSION

Susceptibility of Methylcoumarylamide Substrates to Mackerel Cysteine Proteinases. Cathepsins B, L, L-like, and X hydrolyzed Z-Phe-Arg-MCA, Z-Arg-Arg-MCA, and Boc-Val-Leu-Lys-MCA but did not hydrolyze Suc-Ala-Ala-Ala-MCA, Suc-Gly-Pro-MCA, Suc-Gly-Pro-Leu-Gly-Pro-MCA, Suc-Ala-Ala-Pro-Phe-MCA, Suc-Ala-Pro-Ala-MCA, Z-Arg-MCA, and L-Arg-MCA (Table 1). Cathepsin B seems to have a broader substrate specificity than cathepsin L, since this proteinase could also hydrolyze Boc-Glu-Lys-Lys-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Z-Pyr-Gly-Arg-MCA (Table 1). This result is in accordance with that reported by Towatari and Katanuma (1983), who concluded that cathepsin L had a clearer specificity than cathepsin B. From these results, the methylcoumarylamide peptides with Leu, Lys, Val, Gly, Arg, and Phe in position P_2 were hydrolyzed by cathepsin B; those with Ala, Gly, Leu, Lys, Val, Pro, Arg, and Phe in position P_2 were hydrolyzed by cathepsin X; and those with Leu, Gly, Val, Arg, and Phe in position P_2 were hydrolyzed by cathepsins L and L-like.

In this study, the methylcoumarylamide peptides having basic amino acids such as Lys and Arg at the P_1 site seemed to be good substrates for cathepsin B (Table 1). Rat liver cathepsin B was reported to have basic amino acid residues at the P_1 site and a carboxyl group with pK_a at around 5.5 at the S_1 site (Koga et al., 1991). Although cathepsin B was considered to be an endopeptidase (Aronson and Barrett, 1978; Wada and Tanabe, 1985), it expressed the exopeptidase activity as well, namely dipeptidylcarboxypeptidase (Pohl et al., 1987), and carboxypeptidase activities. Koga et al. (1991) demonstrated that the multiple proteolytic specificity of cathepsin B was pH dependent. The dipeptidylcarboxypeptidase activity of cathepsin B was also studied by X-ray crystallography (Musil et al., 1991). Two histidine residues (His$_{110}$ and His$_{111}$) and a buried subsite (Glu$_{171}$) might be the groups with a pK_a of about 5.5 which were near the active site (Cys$_{29}$). These

Table 1. Substrate Specificity of Cathepsins B, L, L-like, and X from Mackerel

substrate[a] (5 μM)	activity (units/mL)			
	B	L	L-like	X
Suc-Ala-Ala-Ala-MCAa-MCA	0.00	0.00	0.00	2.00
	(0.0)[b]	(0.0)	(0.0)	(0.4)
Suc-Gly-Pro-MCA	0.00	0.00	0.00	2.13
	(0.0)	(0.0)	(0.0)	(0.4)
Boc-Val-Leu-Lys-MCA	36.38	0.19	1.45	5.25
	(29.2)	(6.6)	(9.4)	(1.0)
Boc-Glu-Lys-Lys-MCA	3.31	0.00	0.00	3.00
	(2.7)	(0.0)	(0.0)	(0.6)
Suc-Gly-Pro-Leu-Gly-Pro-MCA	0.00	0.01	0.04	1.75
	(0.0)	(0.4)	(0.3)	(0.3)
Suc-Leu-Leu-Val-Tyr-MCA	10.50	0.01	0.04	0.25
	(8.4)	(0.4)	(0.2)	(0.1)
Z-Pyr-Gly-Arg-MCA	0.56	0.00	0.01	2.00
	(0.5)	(0.0)	(0.1)	(0.4)
Suc-Ala-Ala-Pro-Phe-MCA	0.00	0.00	0.00	1.25
	(0.0)	(0.0)	(0.0)	(0.2)
Suc-Ala-Pro-Ala-MCA	0.00	0.00	0.00	1.50
	(0.0)	(0.0)	(0.0)	(0.3)
Z-Arg-Arg-MCA	34.06	0.18	1.79	523.08
	(27.3)	(6.3)	(11.6)	(100)
Z-Phe-Arg-MCA	124.67	2.88	15.37	14.75
	(100)	(100)	(100)	(2.8)
Z-Arg-MCA	0.31	0.00	0.00	1.50
	(0.3)	(0.0)	(0.0)	(0.3)
L-Arg-MCA	0.00	0.00	0.00	0.63
	(0.0)	(0.0)	(0.0)	(0.1)

[a] Suc, *N*-succinyl; Boc, *N-tert*-butyloxycarbonyl; Z, carbobenzoxy; MCA, 4-methylcoumaryl-7-amide. [b] Values in parentheses are the percentage ratio of the activities on various substrates relative to that on Z-Phe-Arg-MCA (for cathepsins B, L, and L-like) or Z-Arg-Arg-MCA (for cathepsin X).

conformational properties make it express both endo- and exopeptidase activities (Musil et al., 1991).

According to Towatari and Katanuma (1983), cathepsin L cleaved the peptide bonds with at least one hydrophobic amino acid, such as Phe, Leu, Val, and Trp or Tyr, in position P_2. The kind of amino acid in position P_2 was more important than that in position P_1 for the action of cathepsin L (Towatari and Katanuma, 1983). The methylcoumarylamide substrates with Phe, Leu, Val, and Arg in position P_2 were hydrolyzed by cathepsins B, L, and L-like (Table 1).

Kinetic Studies. Lineweaver–Burk plots of cathepsins B, L, L-like, and X for the hydrolysis of Z-Arg-Arg-MCA (Figure 1), Z-Phe-Arg-MCA (Figure 2), and Boc-Val-Leu-Lys-MCA (Figure 3) were used to compare the effects of substrate concentration [s] on the initial velocities (v) and to calculate K_m and Vm. Results (Table 2) indicated that cathepsin X had a lower K_m value (0.9 μM) and a higher K_{cat}/K_m value (1216.7 μM^{-1} min^{-1}) than cathepsins B, L, and L-like for the hydrolysis of Z-Arg-Arg-MCA. In contrast, cathepsins B, L, and L-like proteinases, especially cathepsin L-like proteinase, had high K_{cat}/K_m values (640.5, 760.2, and 2404.5 μM^{-1} min^{-1}, respectively) for the hydrolysis of Z-Phe-Arg-MCA. These kinetics for the hydrolysis of Z-Arg-Arg-MCA and Z-Phe-Arg-MCA can be employed to distinguish cathepsin X from cathepsins B, L, and L-like. Cathepsin L-like has a higher K_{cat}/K_m than cathepsin L for the hydrolysis of Z-Arg-Arg-MCA, Z-Phe-Arg-MCA, and Boc-Val-Leu-Lys-MCA, which made it possible to distinguish it from cathepsin L (Table 2). Synthetic inhibitors have long been considered to be more useful than substrates in discriminating cathepsin B from cathepsin L (Kirschke et al., 1988). Z-Phe-Thr-(4-O-t-Bu)-CHN$_2$ is considered to be an effective inactivator for cathepsin B, while Z-Phe-Phe-CHN$_2$ and Z-Phe-Ala-CHN$_2$ are good inactivators for cathepsin L
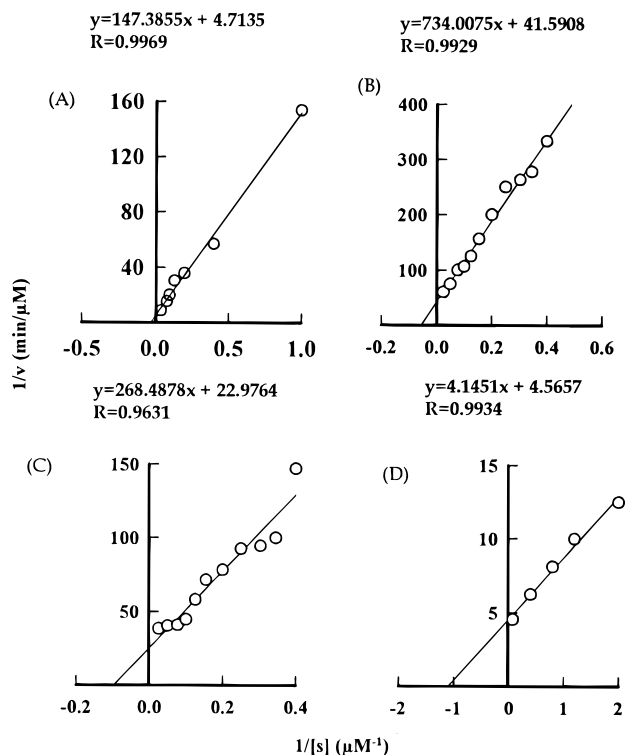
Figure 1. Lineweaver−Burk plots of cathepsins B, L, L-like, and X for the hydrolysis of Z-Arg-Arg-MCA. During incubation at 35 °C, the intensity of fluorescence of various concentrations of Z-Phe-Arg-MCA [s] with cathepsins B (A), L (B), L-like (C), and X (D) was monitored by spectrofluorometer with excitation at 350 nm and emission at 460 nm. The initial velocity (v) was calculated from the reaction curve.
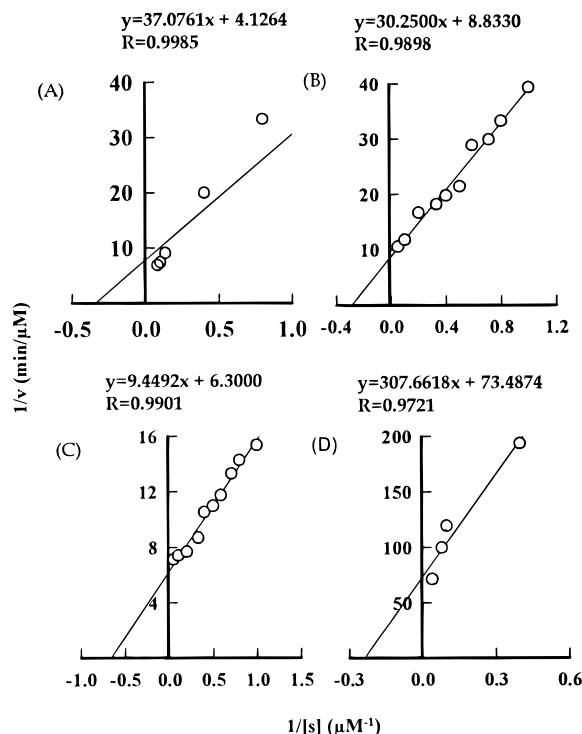

Figure 2. Lineweaver−Burk plots of cathepsins B, L, L-like, and X for the hydrolysis of Z-Phe-Arg-MCA. During incubation at 35 °C, the intensity of fluorescence of various concentrations of Z-Arg-Arg-MCA [s] with cathepsins B (A), L (B), L-like (C), and X (D) was monitored by spectrofluorometer with excitation at 350 nm and emission at 460 nm. The initial velocity (v) was calculated from the reaction curve.

(Barrett et al., 1982; Mason et al., 1985; Dalet-Fumeron et al., 1991). However, as indicated in Table 2, cathepsin B hydrolyzed Boc-Glu-Lys-Lys-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Z-Pyr-Gly-Arg-MCA and had higher K_m (157.8 μM) and lower K_{cat}/K_m (94.1 μM^{-1} min^{-1}) values than cathepsin L and L-like for the hydrolysis of Boc-Val-Leu-Lys-MCA. This property can be used to differentiate cathepsin B from cathepsin L or L-like.

Kinetics parameters of cathepsins B and X for the hydrolysis of Boc-Glu-Lys-Lys-MCA, Z-Pyr-Gly-Arg-MCA, and Suc-Leu-Leu-Val-Tyr-MCA (Table 2) were calculated from Lineweaver−Burk plots (Figures 4 and 5). Although cathepsin B could hydrolyze these three substrates, their K_{cat}/K_m values were low (14.5, 5.9, and 9.5 μM^{-1} min^{-1}, respectively). The K_m and K_{cat}/K_m values of cathepsin X for the hydrolysis of Boc-Glu-Lys-Lys-MCA were 325.9 μM and 0.8 μM^{-1} min^{-1}, respectively (Table 2). These kinetic properties suggested that cathepsins B and X have a wider substrate specificity than cathepsins L and L-like.

The K_m values of cathepsins B and L were 9.0 and 3.42 μM for the hydrolysis of Z-Phe-Arg-MCA, respectively (Table 2). However, the K_m values of cathepsins B and L from human kidney were 252 and 2.2 μM for the hydrolysis of Z-Phe-Arg-MCA, respectively (Baricos et al., 1988). The discrepancy in K_m might be due to the differences in pH and temperature conditions during reaction (Coppes et al., 1992). The K_{cat}/K_m of cathepsin L was higher than that of cathepsin B for the hydrolysis of Z-Phe-Arg-MCA. On the other hand, for the hydrolysis of Z-Arg-Arg-MCA, the K_m of cathepsin B purified in this study (31.3 μM) was much lower than that of cathepsin B from human kidney (184 μM) (Baricos et al., 1988).

Figure 3. Lineweaver−Burk plots of cathepsins B, L, L-like, and X for the hydrolysis of Boc-Val-Leu-Lys-MCA. During incubation at 35 °C, the intensity of fluorescence of various concentrations of Boc-Val-Leu-Lys-MCA [s] with cathepsins B (A), L (B), L-like (C), and X (D) was monitored by spectrofluorometer with excitation at 350 nm and emission at 460 nm. The initial velocity (v) was calculated from the reaction curve.
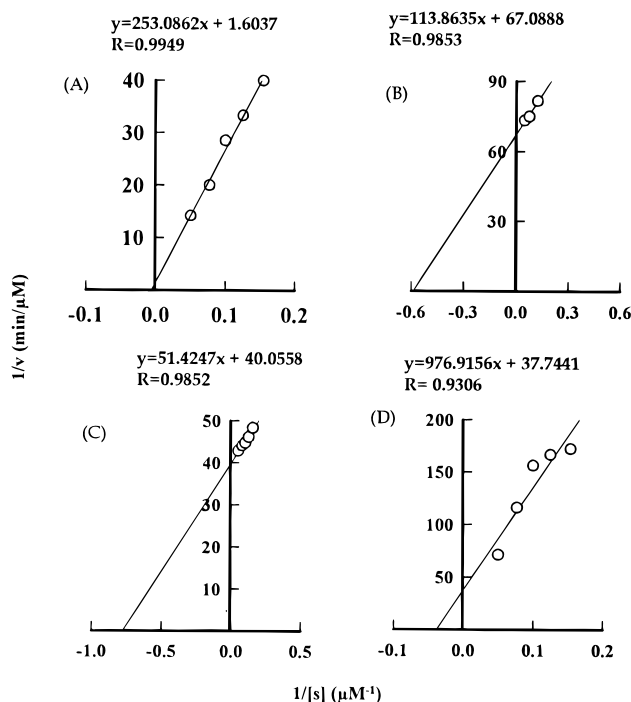
The rabbit liver cathepsins B and L could hydrolyze Z-Phe-Arg-MCA, Bz-Phe-Val-Arg-MCA, and Suc-Ala-

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Table 2. Kinetic Constants for Hydrolysis of Methylcoumarylamide Peptides by Cathepsins B, L, L-like, and X

substrate[a]	K_m (μM)	K_{cat}/K_m (μM^{-1} min^{-1})
Z-Arg-Arg-MCA		
cathepsin B	31.3	161.5
cathepsin L	17.7	30.8
cathepsin L-like	11.7	84.5
cathepsin X	0.9	1216.7
Z-Phe-Arg-MCA		
cathepsin B	9.0	640.5
cathepsin L	3.42	760.2
cathepsin L-like	1.5	2404.5
cathepsin X	4.2	16.2
Boc-Val-Leu-Lys-MCA		
cathepsin B	157.8	94.1
cathepsin L	1.7	199.2
cathepsin L-like	1.3	437.1
cathepsin X	25.9	5.1
Boc-Glu-Lys-Lys-MCA		
cathepsin B	101.3	14.5
cathepsin X	325.9	0.8
Suc-Leu-Leu-Val-Tyr-MCA		
cathepsin B	6.0	9.5
Z-Pyr-Gly-Arg-MCA		
cathepsin B	98.5	5.9

[a] Boc, *N-tert*-butyloxycarbonyl; Z, carbobenzoxy; MCA, 4-methylcoumaryl-7-amide; Suc, *N*-succinyl.
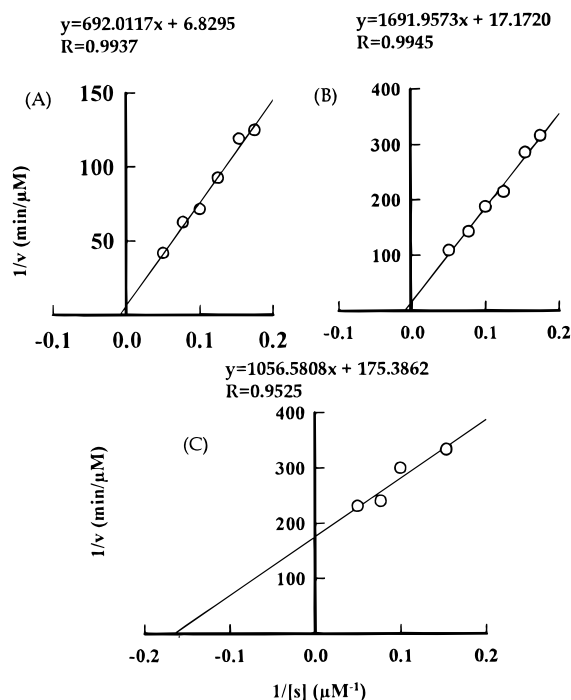

Figure 4. Lineweaver–Burk plots of cathepsin B for the hydrolysis of Boc-Glu-Lys-Lys-MCA (A), Z-Pyr-Gly-Arg-MCA (B), and Suc-Leu-Leu-Val-Tyr-MCA (C). During incubation at 35 °C, the intensity of fluorescence of various concentrations of Boc-Glu-Lys-Lys-MCA, Z-Pyr-Gly-Arg-MCA, and Suc-Leu-Leu-Val-Tyr-MCA (s) with cathepsin B was monitored by spectrofluorometer with excitation at 350 nm and emission at 460 nm. The initial velocity (v) was calculated from the reaction curve.

Phe-Lys-MCA (Mason et al., 1984). For the hydrolysis of Z-Phe-Arg-MCA, the K_m values of rat and rabbit liver cathepsin L were 7.0 and 0.7 μM, while those of rat and rabbit liver cathepsin B were 150.0 and 75.0 μM, respectively (Kirschke et al., 1982; Mason et al., 1984). According to the selective cleavage of peptide bonds by cathepsin L, this proteinase preferred to cleave the peptide bonds with an amino acid residue such as Leu, Phe, Trp, or Tyr in position P_2 and to hydrolyze proteins
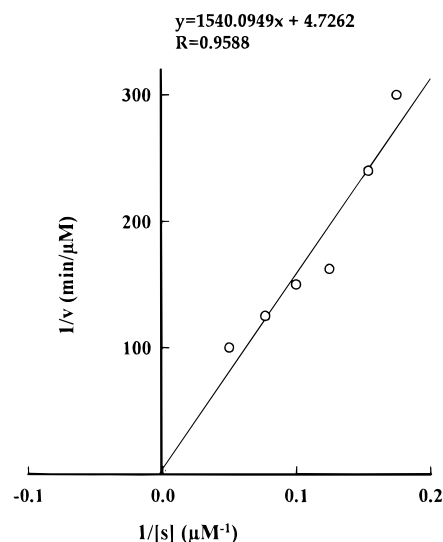

Figure 5. Lineweaver–Burk plots of cathepsin X for the hydrolysis of Boc-Glu-Lys-Lys-MCA. During incubation at 35 °C, the intensity of fluorescence of various concentrations of Boc-Glu-Lys-Lys-MCA [s] with cathepsin X was monitored by spectrofluorometer with excitation at 350 nm and emission at 460 nm. The initial velocity (v) was calculated from the reaction curve.

around hydrophobic amino acid residues which are recognized to bind to subsites S_2 and S_3 (Katanuma et al., 1983).

According to the results of substrate specificity obtained in this study (Table 1), although it is difficult to design a specific synthetic substrate for determining each of these proteinases (cathepsins B, L, L-like, and X) in the crude extracts, the kinetics of these proteinases for the hydrolysis of the methylcoumarylamide peptides (Table 2) could be very useful in differentiating each of these proteinases.

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