Endo- and Aminopeptidase Activities of Rat Cathepsin H

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Employing soluble denatured protein substrates and their derivatives, the proteolytic activity of rat cathepsin H was investigated. The enzyme showed aminopeptidase activity which sequentially released amino acid from the N-terminal of the substrate. The aminopeptidase activity did not act on N_α-acetylated peptides and showed moderate ionic-strength dependence when methionyl-methylcoumarylamide was employed as a substrate. These results indicate that the activity essentially requires an N-terminal free amino group of the substrate and recognizes it electrostatically to some extent. On the other hand, the enzyme was also indicated to exhibit endopeptidase activity by employing appropriate N_α-acetylated peptide substrates. In contrast to the aminopeptidase activity, the endopeptidase activity showed rather strict specificity, preferring hydrophobic residues at P2 and P3 sites. Because of the broad specificity and high efficiency of the aminopeptidase activity, it was difficult to directly observe endopeptidase activity in the digestion of large peptide substrates with a free α-amino terminal. Thus, this is the first experimental evidence that indicates endopeptidase activity by assigning internal peptide bonds cleaved by this activity. From this data, we proposed a model of the binding site of this enzyme.

Keywords cathepsin H; cysteine protease; papain superfamily; specificity

Introduction

Cathepsin H is a member of the lysosomal cysteine proteases which are involved in intracellular protein degradation.[2)] The nature of this enzyme has been well characterized.[2,3)] Kirschke *et al.* reported that rat cathepsin H was an endoaminopeptidase,[4,5)] which could behave as an aminopeptidase as well as endopeptidase. Although there have been some studies on the specificity of the aminopeptidase activity employing small peptide derivatives,[6-8)] the endopeptidase activity remains unclear as does its specificity towards protein or large peptide substrates, which are much closer to its true substrates *in vivo*.

Therefore, we studied the proteolytic activity of rat cathepsin H employing reduced and *S*-(3-trimethylammonio)-propylated (TAP-) lysozyme,[9)] TAP-ribonuclease A, reduced and *S*-aminoethylated (AE-) insulin B-chain and their derivatives as substrates.

On the other hand, cathepsin H belongs to the cysteine protease family named the papain superfamily which includes plant cysteine proteases, papain and actinidin, and mammalian lysosomal cysteine proteases, cathepsins B and L. In spite of their structural similarities,[10,11)] they have a great diversity of proteolytic activity.[2,3)] Recently, we investgated the proteolytic activities of papain, cathepsin L[12)] and cathepsin B[13)] and found a close relationship between their endopeptidase activities and amino acid replacements at two residues corresponding with Vall33 and 157 in papain, which are supposed to be involved in the k_{cat} of its endopeptidase activity as well as K_m.[14)] The amino acid replacements in the cathepsins are as follows: cathepsin L, Ala and Leu[11)]; cathepsin B, Ala and Gly[15,16)]; cathepsin H, Ala and Val.[15)] Depending upon the hydrophobicity and bulkiness of these residues, the endopeptidase activities of cathepsins L[12)] and B[13)] seem to be conserved to different extents. Since the amino acid replacements in cathepsin H are the same as those of actinidin, which is reported to have certain endopeptidase activity,[17)] it is interesting to investigate whether cathepsin H has distinct endopeptidase activity.

From this point of view, we compared the proteolytic activity of rat cathepsin H with that of proteases belonging to the papain-superfamily and further discussed amino acid replacements around the substrate-binding sites responsible for their different specificities.

Experimental

Materials Hen egg white lysozyme and bovine pancreas ribonuclease A were purchased from Seikagaku Kogyo Co., Ltd. (Tokyo, Japan) and Sigma, respectively. TAP-[9)] or AE-proteins[18)] and N_α-acetylated peptide derivatives[19)] were prepared according to the literature. Tryptic digestion of TAP-lysozyme and AE-insulin B-chain was carried out as described in the literature.[20)] Tryptides derived from TAP-lysozyme (LT1+2, Lys–Val–Phe–Gly–Arg; LT5, His–Gly–Leu–Asp–Asn–Tyr–Arg; LT11, Asn–Leu–TAPCys–Asn–Ile–Pro–TAPCys–Ser–Ala–Leu–Leu–Ser–Ser–Asp–Ile–Thr–Ala–Ser–Val–Asn–TAPCys–Ala–Lys; LT17+18, Gly–TAPCys–Arg–Leu) and from AE-insulin B-chain (IT2, Gly–Ser–His–Leu–Val–Glu–Ala–Leu–Tyr–Leu–Val–AECys) and their N-acetylated derivatives were isolated by reversed-phase high-performance liquid chromatography (HPLC). All peptides prepared here were highly soluble in water.

Aminomethylcoumarin substrates, arginine 4-methyl-coumaryl-7-amide (Arg-MCA) and methionine 4-methyl-coumaryl-7-amide (Met-MCA), were purchased from the Peptide Research Foundation, Osaka, Japan. Sephadex G-75, diethylaminoethyl (DEAE)-Sephacel, Mono S (FPLC) and molecular weight standard markers for sodium dodecyl suefate (SDS)-polyacrylamide gel electrophoresis (PAGE) were obtained from Pharmacia Fine Chemicals. Acrylamide, *N,N'*-methylenebisacrylamide, SDS and Triton X-100 were purchased from Katayama Chemical Co. All other chemicals used were reagent grade materials from various commercial sources.

Enzyme Purification and Assay Cathepsin H from rat liver lysosomal fraction was isolated to homogeneity essentially by the method of Kirschke *et al.*[4)] The electrophoretic pattern of the purified cathepsin H showed a homogenous single protein band. The result of gas-phase amino acid sequencing of N-terminal ten residues was as follows; Tyr–Pro–Ser(Gln)–Ser–Met–Asp(Ser)–Trp(Ala)–Arg(Thr)–Lys–Lys–. The amino acid sequence agreed with that of rat cathepsin H except for some minor amino acids in the parentheses. Since the purification was performed according to the conventional method, we regarded the minor component as its isozyme. Therefore, the preparation was employed for the following experiments without any further purification.

Enzyme assays were performed with Arg-MCA at pH 6.8 according to the method described previously.[3)] Released 7-amino-4-methylcoumarin (AMC) was determined by measuring fluorescence with a Hitachi F-2000 fluorescence spectrophotometer (excitation at 380 nm, emission at 460 nm).

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TABLE I. Reaction Condition for Digestion with Cathepsin H

Substrate[a)]		Cathepsin H (unit)[b)]	Volume (ml)	Time[c)] (h)	References
TAP-Lysozyme					
(Run 1)	5 mg	21	2.5 (pH 6.8)[d)]	T.C.[e)]	Fig. 1A
(Run 2)	2 mg	15	1.0 (pH 6.8)	4	Fig. 2A
TAP-Ribonuclease A					
	3 mg	23	1.5 (pH 6.8)	24	Fig. 2C
AE-Insulin B-chain					
(Run 1)	200 nmol	15	2.0 (pH 6.5)	T.C.	Fig. 1B
(Run 2)	100 nmol	8	1.0 (pH 6.5)	26	Fig. 3
N_α-Acetylated tryptide derived from TAP-lysozyme					
AC-LT11	87 nmol	5	1.0 (pH 6.5)	24	Table V
AC-LT1+2	20 nmol	5	1.0 (pH 6.5)	24	No degradation[f)]
AC-LT5	20 nmol	5	1.0 (pH 6.5)	24	No degradation
AC-LT17+18	20 nmol	5	1.0 (pH 6.5)	24	No degradation
N_α-Acetylated tryptide derived from AE-insulin B-chain					
AC-IT2	40 nmol	8	1.0 (ph 6.0)	26	Table V

a) Abbreviations are given in the text. *b)* Cathepsin H activity against Arg-MCA was assayed as described in the literature.[3)] 1 unit = 1 nmol aminomethylcoumarin/min. *c)* Digestion was terminated by adding appropriate amount of iodoacetic acid solution (final concentration, 0.3—3.2 mM). *d)* In 0.1 M phosphate buffer (pH 6.0—6.8) containing 1 mM EDTA and 3.8 mM 2-mercaptoethanol. *e)* Time-course experiment. *f)* Since no hydrolysis was observed, the results of reversed-phase HPLC separation are not shown.

One unit of the enzyme activity was defined as that quantity releasing one nmol of AMC per minute at pH 6.8 and 40 °C.

Analytical Methods Separation of the peptides with HPLC was performed on a reversed-phase column (4 × 300 mm) of TSK-gel ODS-120T (5 μm, Tosoh, Japan) with a Hitachi HPLC system. The column of reversed-phase HPLC was eluted with a gradient of 40 ml of 1% acetonitrile and 40 ml of 40% acetonitrile, both containing 0.1% concentrated HCl at a flow rate of 0.8 ml/min. The elution of peptides was monitored by the absorbance at 210 nm.

Catheptic products were determined with a Hitachi 835 amino acid analyzer after the following treatments: i) amino acid composition; samples were previously hydrolyzed in 6 N HCl under a vacuum at 110 °C for 20 h, lyophilized and dissolved in 0.02 N HCl; ii) free amino acids; samples were diluted with 0.02 N HCl.

Determination of the N-terminal amino acid sequences of the peptides was performed with an Applied Biosystems Model 470A 120A gas-phase protein sequencer.

Catheptic Digestion Proteins and peptide substrates were incubated at 40°C with rat cathepsin H in an appropriate buffer, pH 3—7, containing 3.8 mM 2-mercaptoethanol and 1 mM ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA) for an appropriate time period. Details are specified in Table I.

Double-Digestion of Protein Substrates with Cathepsin H and L-1-(*p*-Tosylamido)-2-phenylethyl-chloromethyl Ketone-Treated (TPCK) Trypsin As described under the introductory section, aminopeptidase activity of cathepsin H gives a complicated mixture of peptide fragments with heterogeneity at their N-terminal. In order to facilitate detection of cleavages at internal peptide bonds by the endopeptidase activity, hydrolysates of TAP-proteins were further digested with TPCK-trypsin (double-digestion). Catheptic digestion was performed at pH 6.8 and 40 °C for an appropriate time and terminated with iodoacetic acid (final concentration; 0.4 mM). TPCK-trypsin (1/50, w/w) was added to the catheptic digest (pH 6.8) and incubated at 40 °C for 3 h. The tryptic digestion was terminated by lowering the pH with phosphorous acid. The double-digests were analyzed by reversed-phase HPLC peptide mapping. By comparison with the tryptic peptide maps derived from intact substrates, the new peaks which appeared and disappeared in the double-digestion peptide map were assigned by analyzing their amino acid compositions.

Ionic-Strength Dependence of the Aminopeptidase Activity Ionic-strength dependence of the aminopeptidase activity against Met-MCA was measured at pH 6 and 8 (0.01 M phosphate buffer containing 1 mM EDTA, 4 mM cysteine and an appropriate concentration of NaCl). Assays were carried out similarly to the routine method for enzyme-activity against Arg-MCA (described above).

Results

Hydrolysis of Soluble Denatured Protein Substrates The proteolytic action of cathepsin H toward TAP-lysozyme, TAP-ribonuclease A and AE-insulin B-chain was investigated.

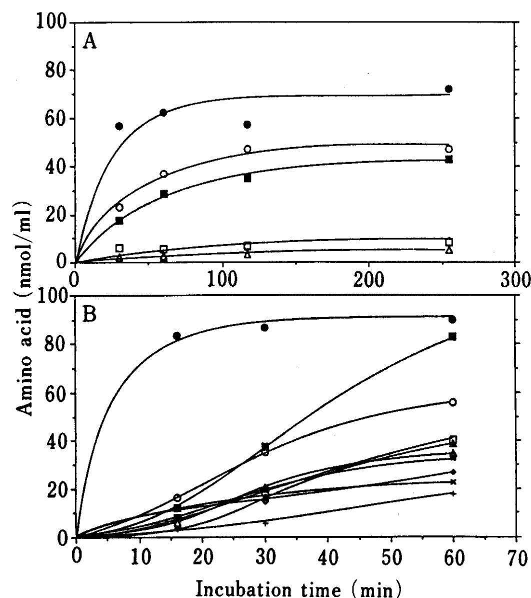

Fig. 1. The Time-Course of the Release of Amino Acids in the Catheptic Digestion of TAP-Lysozyme (A) and AE-Insulin B-Chain (B)

The catheptic digest was subjected to amino acid analysis. Because Gln and Asn showed retention times close to those of Thr and Ser, respectively, they could not be determined separately in the amino acid analyzing system employed here. Gln and Asn were determined together with Thr and Ser, respectively (B). A, amino acids released from TAP-lysozyme (N-terminal, Lys–Val–Phe–Gly–Arg–···). ●, Lys; ○, Val; ■, Phe; □, Ala; △, Trp. B, amino acids released from AE-insulin B-chain (N-terminal, Phe–Val–Asn–Gln–His–···). ●, Phe; ■, Leu; ○, Val; □, His; ▲, Gln and Thr; △, Tyr; ⊞, Ala; ◇, Lys; ×, Asn and Ser; +, Gly. The yields of Lys at 260 min in A and of Phe at 60 min in B were 51% and 90%, respectively.

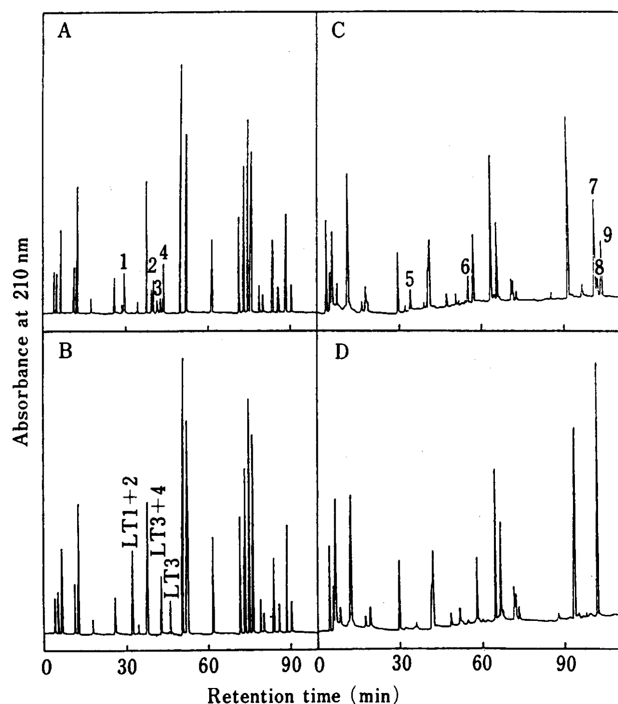

Fig. 2. Double-Digestion of TAP-Lysozyme (A) and TAP-Ribonuclease A (C)

Catheptic digests of TAP-lysozyme and TAP-ribonuclease A were doubly-digested with TPCK-trypsin. Details are given in the text. Doubly-digested samples were analyzed with reversed-phase HPLC employing the linear gradient system described under METHODS. The peaks were collected and analyzed for their amino acid composition and/or amino acid sequence. The analyzed data was shown in Table III. A, Double-digest of TAP-lysozyme; B, tryptic peptides derived from TAP-lysozyme (control); C, Double-digest of TAP-ribonuclease A; D, tryptic peptides from TAP-ribonuclease A (control).

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TABLE II. Proteolytic Activities of Cathepsin H

Substrate	Cleaved sequence[a]						Residue number	References
	P3	P2	P1	P1′	P2′	P3′		
A. Hydrolyzed by aminopeptidase activity								
TAP-Lysozyme			Lys	Val	Phe	Gly	1—4	Figs. 1A, 2A and 3A
			Val	Phe	Gly	Arg	2—5	Table III
			Phe	Gly	Arg	TAPCys[b]	3—6	
			Gly	Arg	TAPCys	Glu	4—7	
TAP-Ribonuclease A			Lys	Glu	Thr	Ala	1—4	Fig. 3B
			Glu	Thr	Ala	Ala	2—5	
			Thr	Ala	Ala	Ala	3—6	
			Ala	Ala	Ala	Lys	4—7	
AE-Insulin B-chain			Phe	Val	Asn	Gln	1—4	Fig. 1B
			Val	Asn	Gln	His	2—5	
B. Hydrolyzed by endopeptidase activity								
TAP-Lysozyme	Asn	Leu	TAPCys	Asn	Ile	Pro	74—79	Fig. 2A
	Ile	Pro	TAPCys	Ser	Ala	Leu	78—83	Tables III and V
	Asp	Val	Gln	Ala	Trp	Ile	119—124	
TAP-Ribonuclease A	Tyr	Pro	Asn	TAPCys	Ala	Tyr	92—97	Fig. 2C
	Ile	Val	Ala	TAPCys	Glu	Gly	107—112	Table III
AE-Insulin B-chain	Leu	Val	Glu	Ala	Leu	Tyr	11—16	Tables IV and V
	Leu	Tyr	Leu	Val	AECys[c]	Gly	15—20	
	Leu	Val	AECys	Gly	Glu	Arg	17—22	
C. Not hydrolyzed								
AC-LT1+2			AcLys[d]	Val	Phe	Gly	1—4	No degradation[g]
AC-LT5			AcHis[e]	Gly	Leu	Asp	15—18	No degradation
AC-LT17+18			AcGly[f]	TAPCys	Arg	Leu	126—129	No degradation

a) Peptide bond between P1 and P1′ was hydrolyzed by cathepsin H. *b*) *S*-Aminoethylated cysteine. *c*) *S*-(3-Trimethylammonio)propylated cysteine. *d*) N_α,N_ε-Diacetylated lysine. *e*) N_α-Acetylated histidine. *f*) N_α-Acetylated glycine. *g*) Since no hydrolysis was observed, the results of RP-HPLC separation are not shown.

TABLE III. Amino Acid Composition[a] of Peptides Derived from the Double-Digestion[b] of TAP-Lysozyme and TAP-Ribonuclease A

Amino acid	Peak[c]								
	1	2	3	4	5[e]	6[e]	7	8	9
Asp					1.15 (1)		2.14 (2)	1.67 (2)	1.85 (2)
Thr					1.03 (1)				
Ser					1.97 (1)		1.01 (1)	0.88 (1)	0.96 (1)
Glu		1.11 (1)	1.13 (1)		1.34 (1)		0.81 (1)		1.09 (1)
Gly		1.00 (1)			1.52 (1)		1.00 (1)		1.00 (1)
Ala		2.91 (3)	3.06 (3)			1.24 (1)	1.68 (2)	1.00 (1)	1.18 (1)
Val						1.03 (1)	3.60 (4)	2.60 (3)	2.62 (3)
Met		0.63 (1)	—[d] (1)						
Ile						1.05 (1)	0.67 (1)		
Leu		0.99 (1)	1.08 (1)						
Tyr					0.82 (1)		0.85 (1)	0.77 (1)	0.68 (1)
Phe	1.00 (1)						1.13 (1)	0.86 (1)	0.93 (1)
TAPCys		0.94 (1)	0.50 (1)				0.51 (1)		
Lys		0.98 (1)	1.14 (1)		1.00 (1)				
His						1.00 (1)	1.50 (2)	0.78 (1)	0.95 (1)
Trp				1.00 (1)					
Arg		1.98 (2)	1.15 (1)						
Pro					0.98 (1)		2.05 (2)	1.50 (2)	1.79 (2)
Residue number[f]	— (38)	4—14[g] (15)	6—14[g] (9)	— (29)	86—94[h] (13)	105—109[h] (31)	105—124[h] (26)	113—124[h] (20)	111—124[h] (25)

a) Values are residues per molecule. Numbers in parentheses are the values expected for assignment. *b*) Catheptic peptides mixtures derived from TAP-proteins were hydrolyzed by TPCK-trypsin. Details are given in the text. *c*) See Fig. 2, A; peak 1—5, B; peak 6—10. *d*) Methionine was detected in its oxidized form. *e*) Amino acid sequencing was carried out (Data not shown). *f*) Numbers in parentheses are the yields which are expressed as percentages of recovery on amino acid analysis. *g*) Residue number in TAP-lysozyme. *h*) Residue number in TAP-ribonuclease A.

In the digestion of TAP-lysozyme with cathepsin H, free amino acids, Lys, Phe and Val were sequentially released (Fig. 1A) and the N-terminal tryptic peptides, LT1+2, LT3, and LT3+4 disappeared in the tryptic peptide map (Fig. 2A). Thus, TAP-lysozyme was degraded by the aminopeptidase activity. The sequential release of N-terminal amino acids was also observed in the digestion of AE-insulin B-chain (Fig. 1B). These results clearly indicate that cathepsin H degrades these substrates mainly via aminopeptidase activity.

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If cathepsin H behaves solely as an aminopeptidase, the yields of amino acids sequentially released from the N-terminal should gradually decrease. But some amino acids could not be determined as products derived solely by the aminopeptidase activity (Fig. 3). In addition, SDS-PAGE of the catheptic hydrolysate of TAP-lysozyme showed some protein bands whose molecular weights were smaller than those of the predictable catheptic products resulting solely from the aminopeptidase activity (data not shown). Moreover, tryptic peptide mapping of the catheptic peptide mixture derived from TAP-ribonuclease A indicated that cathepsin H cleaved the internal peptide bonds at the C-terminal side of Asn-94 and Ala-109 (Fig. 2C). These results suggest that cathepsin H also has endopeptidase activity.

In the case of the AE-insulin B-chain, we successfully found some catheptic peptides resulting from the cleavage of the internal peptide bonds (Table IV). Thus, cathepsin H showed distinct endopeptidase activity as well as aminopeptidase activity. The results are summarized in Table II.

Hydrolysis of N_α-Acetylated Protein and Peptide Derivatives In order to clarify the role of the N-terminal α-amino group at the P1 site in the aminopeptidase activity, Nα-acetylated peptide derivatives were employed as substrates.

At first, acetylated TAP-lysozyme tryptides, AC-LT1+2, AC-LT5, and AC-LT17+18 were incubated with cathepsin H. They were not degraded at all although all intact TAP-lysozyme tryptides could be degraded mainly by the aminopeptidase activity (data not shown). Thus, it is indicated that the enzyme cannot cleave off the N_α-acetylated amino acid moiety by its aminopeptidase activity.

Next, we analyzed the endopeptidase activity against N_α-acetylated peptides. AC-LT11 and AC-IT2 (tryptic peptide of AE-insulin B-chain, Gly-8-AECys-19) were degraded by the endopeptidase activity and the resulting peptides, with newly appearing intact N-termini, were further degraded by the aminopeptidase activity (Table V). The catheptic peptides with acetylated α-amino group were assigned and their cleaved bonds were determined (Table II). The results show that the endopeptidase activity prefers hydrophobic residues at P2 and P3 sites.

Ionic-Strength Dependence of the Aminopeptidase Activity The aminopeptidase activity has been indicated to require a free α-amino group on the N-terminal of the peptide (Table II). An electrostatic interaction between

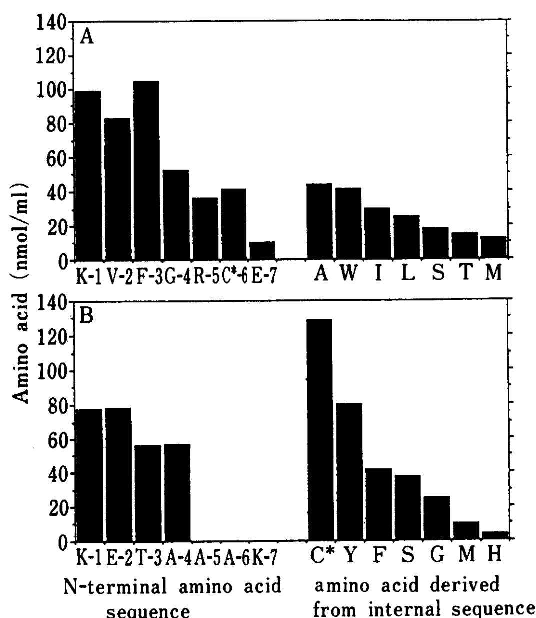

Fig. 3. Aminopeptidase Activity toward TAP-Proteins

The amounts of released amino acid were plotted along N-terminal amino acid sequences of the substrates. Amino acids were expressed as one letter codes (C*, TAP-Cys). A, 4 h digestion of TAP-lysozyme; B, 24 h digestion of TAP-ribonuclease A. The yields of Lys in A and B were 70% and 56%, respectively.

TABLE IV. Amino Acid Composition[a)] of Catheptic Peptides Derived from AE-Insulin B-Chain

Amino acid	Peptide 1	2	3	4	5	6
Thr	2.00 (2)					2.42 (2)
Ser					0.62 (1)	
Glu				1.00 (1)	1.13 (1)	1.38 (1)
Gly					1.00 (1)	2.00 (2)
Val				1.12 (1)	1.18 (1)	
Leu		2.00 (2)		0.90 (1)	1.04 (1)	
Tyr		0.54 (1)				0.37 (1)
Phe			1.00 (1)			2.31 (2)
Lys	1.18 (1)					1.28 (1)
His					0.94 (1)	
Arg						1.21 (1)
Pro	1.11 (1)					0.83 (1)
Residue number[b)]	27—30 (25)	15—17 (72)	— (128)[c)]	11—13 (8)	8—13 (9)	20—30 (25)

a) Values are residues per molecule. Numbers in parentheses are the values expected for assignment. b) Numbers in parentheses are the yields which are expressed as percentages of the recovery on amino acid analysis. c) The yield was more than a hundred percent since the AE-insulin B-chain had three phenylalanyl residues per molecule.

TABLE V. Amino Acid Composition[a)] of Catheptic Peptides Derived from N_α-Acetylated Peptides, AC-LT11 and AC-IT2

Amino acid	Peptide[b)] 1	2	3	4	5	6
Asp		1.00 (1)	2.11 (2)	2.22 (2)	3.84 (4)	
Thr			1.13 (1)	1.00 (1)	0.91 (1)	
Ser			2.46 (2)	3.90 (4)	3.52 (4)	0.79 (1)
Glu						0.86 (1)
Gly						1.00 (1)
Ala			2.13 (2)	3.31 (3)	3.00 (3)	
Val			1.20 (1)	1.18 (1)	1.01 (1)	0.94 (1)
Met						
Ile	1.00 (1)		1.06 (1)	2.13 (2)	1.91 (2)	
Leu		1.05 (1)		2.24 (2)	2.67 (3)	0.80 (1)
Tyr						
Phe						
TAPCys	0.53 (1)	1.01 (1)	0.50 (1)	1.50 (2)	3.35 (3)	
Lys			1.06 (1)	1.24 (1)	1.15 (1)	
His						0.77 (1)
Trp						
Arg						
Pro	0.94 (1)			1.14 (1)	1.00 (1)	
Residue number[e)]	78—80[c)] (25)	74—76[c)] (68)	86—96[c)] (26)	78—96[c)] (24)	74—96[c)] (43)	8—13[d)] (30)

a) Values are residues per molecule. Numbers in parentheses are the values expected for assignment. b) Peptides 1—5 and peptide 6 were derived from AC-LT11 and AC-IT2, respectively. c) Residue number in TAP-lysozyme. d) Residue number in AE-insulin B-chain. e) Numbers in parentheses are the yields which are expressed as percentages of the recovery on amino acid analysis.

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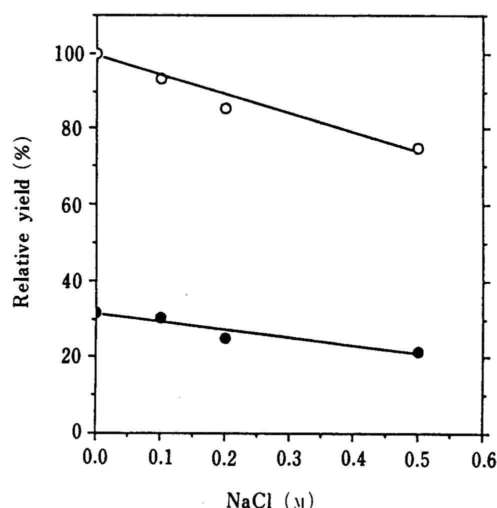

Fig. 4. Ionic-Strength Dependence of the Aminopeptidase Activity

The aminopeptidase activity was measured at various concentrations of sodium chloride. The assay was performed according to the method of Barret *et al.*[3] employing Met-AMC in place of Arg-AMC. ○, pH 6: ●, pH 8.

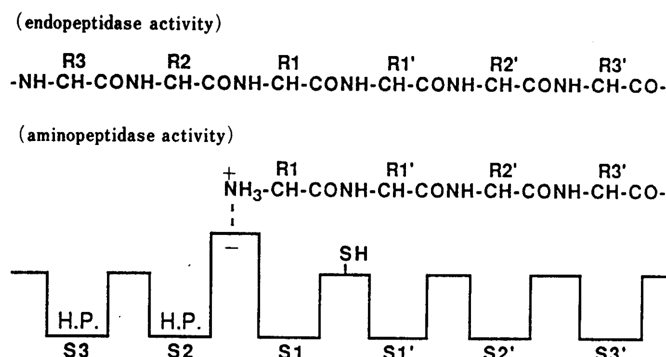

Fig. 5. Hypothetical Model of the Active Site of Cathepsin H

The model of the binding site of the enzyme is supposed to have six subsites around the catalytic thiol group (SH). The binding site S1 interacts with the amino group at the P1 site electrostatically (broken lines) and the steric barrier between the S1 and S2 sites. The S2 and S3 sites tends to prefer hydrophobic amino acids (expressed as H.P.). The aminopeptidase and endopeptidase activity is governed by the S1-P1 interaction and the S2-P2 and S3-P3 interactions, respectively. Details are given in the text.

the α-amino group and the S1 site was evaluated by investigating the ionic-strength dependence of the activity against Met-AMC at pH 6 and 8 (Fig. 4). At both pH's, the release of AMC was depressed in the manner of ionic-strength dependence, and the activity at pH 8 was about 30% of that at pH 6 regardless of the concentration of sodium chloride. Assuming that the pK_a of Met-MCA is 7.6, the ratio of the activity is consistent with that of the N_α-protonated fraction of the substrate. This data suggests that the activity essentially requires the substrate to have a protonated α-amino group, and the enzyme recognizes it electrostatically.

Discussion

The results represented in this paper clearly indicate that rat cathepsin H has distinct endopeptidase activity as well as aminopeptidase activity toward soluble denatured protein and its derivatives.

Takahashi *et al.*[7] reported that porcine spleen cathepsin H hydrolyzed oligopeptide solely by aminopeptidase activity. Since the enzyme could also cleave Bz-Arg-2-NNaph at the C-terminal side of Arg, the enzyme was expected to have similar activity to that of rat liver cathepsin H. Following are two possible reasons why no endopeptidase activity was observed: i) inappropriate substrates which might have no peptide bonds scissile to the endopeptidase activity were used, ii) the hydrolyzing conditions at a low temperature (21 °C) and acidic pH (pH 2.8) would have been far from the suitable condition.

Rat cathepsin H showed aminopeptidase activity with broad specificity (Table II). Its essential requirement is an α-amino group of the substrate because the peptides with N_α-acetylated amino group couldn't be cleaved by the aminopeptidase activity (Table II). This requirement is supposed to be performed by two factors.

One is an electrostatic interaction. The activity showed certain dependence on the fraction of the protonated α-amino group and the ionic-strength (Fig. 4). This suggests that cathepsin H has a negatively charged residue around the S1 site, which recognizes the protonated α-amino group electrostatically. However, this suggestion does not agree with the structure predicted from its amino acid sequence because it has no negatively charged residue near the active site.[7] The existence of negatively charged residue around the S1 site is also deduced from the fact that rat cathepsin B, the other member of the papain superfamily, is suggested to have a negatively charged residue which electrostatically interacts with positively charged residue at the P1 site.[13] However, as indicated by the moderate ionic-strength dependence (Fig. 4), the electrostatic interaction does not seem to be so strong that it completely governs the activity.

The other factor is a steric hindrance at the N-terminal side of the S1 site. The steric hindrance is not so severe that it can be overcome by the hydrophobic interaction at the S2 site in the binding mode of the endopeptidase activity (see below). The N-acetylated amino acid residue seems to be too large for the S1 site in the binding mode of the aminopeptidase activity and the acetyl group seems to be too small for the S2 site in that of the endopeptidase activity. Therefore, the N-acetylated peptides could not be degraded by this enzyme. Thus, the aminopeptidase activity shows broad specificity concerning amino acid residues because the only essential requirement is a free amino group.

In contrast to the broad specificity of the aminopeptidase activity, the endopeptidase activity showed rather strict specificity. This activity favors peptide bonds which have hydrophobic residues at the P2 site (Table II). The preference of the S2 site corresponds with that of human cathepsin H whose K_m toward Bz-Arg-2-NNap is greater than that toward Arg-2-NNap.[6] This preference of the endopeptidase activity is a common feature among papain-superfamily proteases, such as papain,[12] and cathepsins B[13] and L.[12] As described in the introductory section, the residues corresponding with Val-133 and -157, which are main components of the P2 site of papain, are supposed to be involved in the specificity. In rat cathepsin L, the hydrophobicity and size of the residues are conserved by complimentary amino acid replacements, that is Val133Ala and Val157Leu.[11] With the complimentary amino acid replacements, this protease is supposed to have distinct endopeptidase activity whose specificity is similar

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to that of papain.[12] On the other hand, the amino acid replacements in rat cathepsin B, that is Val133Ala and Val157Gly,[15,16] reduce hydrophobicity and the net size of the corresponding residues. Because of these replacements, the endopeptidase activity of rat cathepsin B seems to be weakened, but it maintains a similar tendency in the preference of hydrophobic residues at the S2 site.[13] Considering this data and the amino acid replacements in rat cathepsin H, Val133Ala and conserved Val157, it is reasonable that rat cathepsin H has some endopeptidase activity with similar specificity to those of the endopeptidases belonging to papain-superfamily.

From the above discussion, we conclude that although cathepsin H plays a role as an aminopeptidase in intracellular protein degradation, it can be also behave as an endopeptidase if the substrate-binding energy generated by the subsites is sufficient to overcome the lack of electrostatic interaction and the steric hindrance around the S1 site, both of which govern the aminopeptidase activity (Fig. 5).

Thus, our discussion is based on an assumption that the enzyme is pure enough for its characterization. Although the preparation was performed by the conventional method,[4] we could not deny completely that the dual proteolytic action, that is the endo- and aminopeptidase activities, might result from some contaminant and/or its isozyme detected by the N-terminal amino acid sequence analysis (see Experimental).

Recently, other members of papain-superfamily, human cathepsin L[21] and papain,[22] have been expressed in *E. coli* and in a baculovirus-insect cell system, respectively. Now the papain-superfamily is becoming a target protein for investigating the relationship between the structure and function of a protease from the viewpoint of protein engineering. The date in this paper will give some direction to understanding the structural basis for the functional diversity of the papain superfamily by means of the advanced techniques of protein engineering.

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References and Notes

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