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Kinetic Characterization of Lysine-Specific Metalloendopeptidases from *Grifola frondosa* and *Pleurotus ostreatus* Fruiting Bodies[1]

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Two zinc-metalloendopeptidases, GFMEP (accession number P81054) and POMEP (accession number P81055), from the fruiting bodies of two edible mushrooms, *Grifola frondosa* and *Pleurotus ostreatus*, respectively, specifically hydrolyze peptidyl-lysine bonds (-X-Lys-) in polypeptides. To understand detailed substrate specificities and kinetic characters of these enzymes, we have synthesized various intramolecularly quenched fluorescent peptide substrates and determined their kinetic constants with these substrates. Each synthesized fluorogenic peptide has a fluorescent residue, tryptophan, at its carboxyl terminus and a quenching group, dinitrophenyl (Dnp), at its amino terminus. Quenching of the Trp fluorescence in an intact substrate is relieved on hydrolysis of the -X-Lys- bond, giving rise to a continuous increase in fluorescence. The octapeptide substrate, Dnp-Ser-Thr-Ala-Thr-Lys-Leu-Ser-Trp, was an efficient substrate for both enzymes, the k_{cat}/K_m values being 9.8×10^6 and 7.0×10^5 $M^{-1}\cdot s^{-1}$ for GF- and POMEP, respectively. Peptides with aspartic acid adjacent to the Lys residue were found to be poor substrates for both enzymes. Neither the shortest peptide, Dnp-Thr-Lys-Trp, nor peptides with substitution of L-Arg, L-ornithine, or D-Lys for Lys were hydrolyzed by either enzyme. These results confirmed the strict specificities of GF- and POMEP toward the peptide bond, -X-Lys-. Substitution of Co^{2+} for Zn^{2+} enhanced the activity, while the K_m values were comparable. All peptides not hydrolyzed by either enzyme had inhibitory effects on GFMEP activity. The active site structure is discussed in relation to these observations.

Key words: active site structure, intramolecularly quenched fluorescent substrate, mushroom, peptidyl-Lys specific metalloendopeptidase, substrate specificity.

We have purified and characterized metalloendopeptidases [EC 3.4.24.20], GFMEP (accession number P81054) and POMEP (accession number P81055), from popular edible mushrooms, *Grifola frondosa* (*2*) and *Pleurotus ostreatus* (*3*), respectively. These enzymes consist of single polypeptide chains with apparent molecular masses of 20 kDa and contain 1 atom of zinc per molecule, which is indispensable for their proteolytic activity. Both MEPs exhibit strict specificity for peptidyl-Lys bonds in proteins and peptides. While POMEP exhibits a pH optimum of 5.6, GFMEP is most active at pH 9.5 and exhibits more than 50% of the maximal activity within the pH range of 6-10.5.

A primary structural study (*4*) revealed that GF- and POMEP are composed of 167 and 168 amino acid residues, respectively, and share 61.3% sequence identity between them. Although both MEPs contain putative zinc binding sites common to zinc-metalloproteinases, -His-Glu-X-X-His-, they do not show any significant resemblance to thermolysin nor astacin. They may belong to a new metalloproteinase subfamily with some fungal metalloproteinases categorized as deuterolysins (21-26% sequence identity with either MEP), although their substrate specificities are quite different.

While GF- and POMEP exhibit unique specificities toward -X-Lys- bonds, kinetic characterization with peptide substrates has not been carried out yet. Most, if not all, commercially available chromogenic or fluorogenic peptide substrates were designed for enzymes cleaving on the carboxyl side of specific residues. However, they are not suitable for proteinases splitting on the amino side of

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Abbreviations: P1, P2, P3, *etc.*, and P1′, P2′, P3′, *etc.* designate substrate/inhibitor residues amino-terminal ("non-primed side") and carboxyl-terminal ("primed side") to the scissile bond, respectively, and S1, S2, S3, *etc.*, and S1′, S2′, S3′, *etc.* represent the corresponding subsites of the proteinase (*1*); AECys, *S*-aminoethyl cysteine; Boc, *tert*-butoxycarbonyl; DMF, *N,N*-dimethylformamide; Dnp, 2,4-dinitrophenyl; Fmoc, 9-fluorenylmethoxycarbonyl; GFMEP, *Grifola frondosa* metalloendopeptidase; MEP, metalloendopeptidase; POMEP, *Pleurotus ostreatus* metalloendopeptidase; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; RCM, reduced and *S*-carboxymethylated; RPE, reduced and *S*-pyridylethylated; RP-HPLC, reversed-phase high performance liquid chromatography; TFA, trifluoroacetic acid.

specific residues, such as thermolysin, and GF- and POMEP.

For a convenient assay system for proteinases including ones specific to peptidyl-amino acid bonds, several intramolecularly quenched fluorescent substrates have been developed (*5*). Stack and Gray described a convenient, continuous recording fluorescence assay for rabbit collagenase and gelatinase based on the hydrolysis of Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 (*6*). The key feature of this peptide is the presence of both a fluorescent Trp residue at the P2′ position and a quenching 2,4-dinitrophenyl (Dnp) group at the amino terminus. Hydrolysis of the Gly-Leu bond relieves the quenching of the Trp fluorescence in an intact substrate, and the resulting fluorescence increase serves as the basis for the assay.

In this study, we examine the catalytic properties of GF- and POMEP, using the intramolecularly quenched fluorescent substrates we have designed and synthesized. The data obtained in this work confirmed the strict specificities of GF- and POMEP toward peptidyl-Lys bonds, and provided some structural information around the active site.

EXPERIMENTAL PROCEDURES

Materials—Fruiting bodies of the popular edible mushrooms, *G. frondosa* and *P. ostreatus*, were purchased from producers in Niigata and Gunma Prefectures, respectively, and stored at −40°C. The sources of other materials and chemicals used in this work were as follows: 1-hydroxybenzotriazole, PyBOP, Fmoc-L-amino acids (SM8-Pack mix), Fmoc-D-Lys (Boc)-OH, and *p*-benzyloxybenzyl alcohol resin pre-coupled with Fmoc-Trp residue (Fmoc-Trp-resin) from Shimadzu (Kyoto); anisole, thioanisole, 1,2-ethanedithiol, 2-methylindole, *N*-methylmorpholine, and piperidine from Nacalai Tesque (Kyoto); 2,4-dinitrofluorobenzene and *N*-ethylmorpholine from Kanto Chemicals (Tokyo); thiophenol from Tokyo Kasei (Tokyo); Fmoc-L-Orn (Boc)-OH from Calbiochem-Novabiochem (La Jolla, CA); ethyl methyl sulfide from Fluka Chemie (Buchs, Switzerland); methanesulfonic acid (4 N) from Pierce (Rockford, IL); soybean trypsin inhibitor from Sigma (St. Louis, MO); and neurotensin from the Peptide Institute (Minoh). Other chemicals used were of the highest grade available.

Purification of GFMEP and POMEP—GFMEP was isolated from *G. frondosa* fruiting bodies as described previously (*2*). POMEP was purified by the method of Dohmae *et al.* (*3*). Cobalt-substituted POMEP (Co^{2+}-POMEP) was prepared as follows: POMEP (0.37 mg) was treated with 20 mM EDTA and then the mixture was dialyzed against 20 mM Tris-HCl buffer, pH 7.2 (buffer A). Zn^{2+}-depleted POMEP was dialyzed against buffer A containing 2 mM $CoCl_2$ overnight, followed by exhaustive dialysis against buffer A to remove excess Co^{2+}. Enzyme concentrations were determined by amino acid analysis using a Waters Pico-Tag system (*7*).

Chemical Synthesis of Intramolecularly Quenched Fluorescent Peptides—All peptides were synthesized by the solid-phase method with N_α-Fmoc-amino acids (*8*) using a Shimadzu PSSM-8 peptide synthesizer according to the standard protocol described in the manual. The amino termini of resin-bound peptides were dinitrophenylated according to Netzel-Arnett *et al.* (*9*) with some modification: the peptides on the resin were reacted with a mixture of 2,4-dinitrofluorobenzene (10 equivalents) and *N*-ethylmorpholine (15 equivalents) in 500 μl dry DMF at room temperature for 2 h. After consecutive washes with DMF, methanol, and *t*-butyl methyl ether, the peptides were freed from the resin, and from side chain protecting groups by treatment with a cleavage cocktail (total, 500 μl) of 94% (v/v) TFA, 3% (v/v) anisole, and 3% (v/v) 1,2-ethanedithiol containing 5 mg 2-methylindole at room temperature for 2 h. For Arg-containing peptides, a mixture of 82% (v/v) TFA, 5% (v/v) water, 5% (v/v) thioanisole, 3% (v/v) 1,2-ethanedithiol, 3% (v/v) thiophenol, and 2% (v/v) ethyl methyl sulfide containing 5 mg 2-methylindole was used at room temperature for 8 h (*8*). Prior to dinitrophenylation, a small aliquot of each resin-bound peptide was saved for confirmation of the sequence, being cleaved and deprotected in a similar manner.

Purification of Peptides and Peptide Analyses—All peptides were purified by RP-HPLC on a Waters Pico-Tag column (4.6×150 mm), with a Gilson HPLC system and a Hewlett Packard HP 1040M diode-array detector. Peptides were eluted using solvents A, 0.09% (v/v) aqueous TFA, and B, 80% (v/v) acetonitrile containing 0.075% (v/v) TFA, and monitored as to the absorbance at 215, 254, 275, and 290 nm. The peptides, in 30% (v/v) acetonitrile containing 0.09% (v/v) TFA, were applied to the column, which was equilibrated with 40% B and eluted with a linear gradient of 40-42-80% B in 0-15-20 min at the flow rate of 0.5 ml/min. For amino acid compositions, peptides were hydrolyzed in 4 N methanesulfonic acid at 110°C for 20 h and the resulting hydrolyzates were subjected to amino acid analyses with a Hitachi 835 amino acid analyzer. The molecular masses of the peptides were determined by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry with a Reflex mass spectrometer (Bruker, Bremen, Germany) using α-cyano-4-hydroxycinnamic acid as the matrix. Amino acid sequences were confirmed with peptides without dinitrophenylation and digestion products with a Model 477A protein sequencer connected on-line to a Model 120A PTH-analyzer (Perkin Elmer, Foster City, CA) (*10*).

Kinetic Measurements—Assays were performed at 37°C in 50 mM Na-borate buffer, pH 9.5, for GFMEP, and in 50 mM Na-borate buffer, pH 8.5, and Na-acetate buffer, pH 5.0, for POMEP. Co^{2+}-POMEP was assayed in 50 mM Na-borate buffer, pH 8.5. The fluorescence changes were recorded at 37°C, with excitation at 280 nm and emission at 360 nm, with a Hitachi spectrofluorometer F-4010. In general, 2 ml of assay buffer containing 2-28 μM substrates was pre-incubated in a thermostated cell (10×10×45 mm) for 5 min, and then the reaction was initiated by the addition of 5 μl enzyme solution to give a final concentration of 0.7-35.7 nM. Individual kinetic parameters (k_{cat} and K_m) were calculated from the initial rate of hydrolysis by means of double-reciprocal plots.

Inhibitory Effects of Uncleaved Peptides on GFMEP Activity—An uncleaved peptide (12 μM) was mixed with SUB14 as the substrate (12 μM) in 50 mM Na-borate buffer, pH 9.5, and then pre-incubated at 37°C for 10 min. The reaction was started by the addition of GFMEP (final concentration, 2.86 nM) and the initial rate was recorded. The K_i value was determined by means of double-reciprocal plots of the initial hydrolysis rates observed with 4-28

μM SUB14 in the presence of 10 μM inhibitor.

RESULTS

Design of the Synthetic Peptide Substrates—To design the amino acid sequences of substrates, the sequences around GFMEP cleavage sites in some proteins and peptides were compared. Alignment of the sequences around the cleaved or uncleaved -X-Lys-bonds in various substrates is shown in Table I. GFMEP specifically hydrolyzed -X-Lys-, even if the amino acid residue at the P1 or P2′ position was acidic, basic, hydrophobic, or Pro. GFMEP cleaved all -X-Lys- bonds in horse heart cytochrome *c* except for Glu4-Lys5, which is the most amino-terminal among the expected cleavage sites. The sequence including the putative substrate site is -Asp2-Val-Glu-Lys-Gly6-, in which the amino acids at the P1 and P3 positions are both acidic (*2*), although another site with a similar sequence, -Thr19-Val-Glu-Lys-Gly23-, in the same protein was cleaved. A similar pattern was recognized for the uncleaved putative substrate sites in GFMEP and soybean trypsin inhibitor. Considering these observations together with the peptide solubility, we first designed SUB2, Dnp-Ala-Thr-Lys-Leu-Ser-Trp, as the standard substrate and synthesized the 15 different peptide substrates shown in Table II. The integrity of the synthetic peptides was confirmed by composition, sequence, and mass spectrometric analyses (data not shown).

Characterization of Intramolecularly Quenched Fluorescent Substrates—Peptides containing a fluorescent group (Trp) on one side of the scissile bond and a quenching group (Dnp-) on the other side are potential fluorogenic substrates. In an intact peptide, the indole fluorescence is quenched by the dinitrophenyl group, and the cleavage can be monitored continuously as the relief from the quenching by recording the increase in fluorescence during conversion of the substrate to products. Initial hydrolysis rates were calculated from the progress curves.

The fluorescence emission spectrum of each substrate was recorded before and after total enzymatic hydrolysis to calculate the intramolecular quenching efficiency. For hexapeptide substrates (SUB2-7), quenching efficiencies close to 90% were observed, which were independent of their concentration up to 20 μM (data not shown). For the longer peptides, quenching was less effective (about 80% for SUB14 and SUB15) due to the increased distance between the fluorophore and the quenching group, but still sufficient for routine assays. Figure 1 shows the fluorescence intensities of equimolar mixtures of Dnp-Ala-Pro and Lys-Leu-Ser-Trp (cleavage products of SUB7) in a buffer

TABLE I. **Cleavage patterns of some proteins and peptides with GFMEP.**[a]

Substrate	Cleaved						Uncleaved					
	P3	P2	P1	P1′	P2′	P3′	P3	P2	P1	P1′	P2′	P3′
RPE-GFMEP[b]	A	A	A	K	S	L	D	P	D	K	A	V
	G	G	T	K	D	Y						
	A	F	W	K	A	P						
Cytochrome *c*[c] (horse)	E	K	G	K	K	I	D	V	E	K	G	K
	K	G	K	K	I	F						
	F	V	Q	K	C	A						
	T	V	E	K	G	G						
	G	K	H	K	T	G						
	F	G	R	K	T	G						
	D	A	N	K	N	K						
	N	K	N	K	G	I						
	I	T	W	K	E	E						
	E	N	P	K	K	Y						
	N	P	K	K	Y	I						
	P	G	T	K	M	I						
	A	G	I	K	K	K						
	G	I	K	K	K	T						
	I	K	K	K	T	E						
	A	Y	L	K	K	A						
	Y	L	K	K	A	T						
RCM-Soybean trypsin inhibitor	L	S	L	K	F	D	E	L	D	K	G	I
	P	A	V	K	I	G	E	D	D	K	B	G
	G	E	N	K	D	A						
	N	N	Y	K	L	V						
	V	V	S	K	N	K						
	Q	F	Q	K	L	D						
Neurotensin	Y	E	N	K	P	R						

[a]Amino acids are given in one-letter codes. B: *S*-carboxymethylcysteine. RCM-soybean trypsin inhibitor was digested at 37°C in 0.1 M NH_4HCO_3 buffer, pH 9.5, containing 2 M urea for 18 h at an enzyme to substrate ratio of 1 : 50 (mol/mol). Neurotensin was digested at 37°C in 0.1 M Tris-HCl buffer, pH 9, for 6 h. The peptides generated were separated by RP-HPLC, and then subjected to amino acid sequence, composition, and/or mass spectrometric analyses. [b]Nonaka, T. *et al.* (*4*). [c]Nonaka, T. *et al.* (*2*).

TABLE II. **Kinetic constants for the hydrolysis of intramolecularly quenched fluorescent substrates by GFMEP.**[a]

Substrate	P4 P3 P2 P1 P1′ P2′ P3′ P4′ P5′ P6′	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)
SUB1	Dnp - T - K - W		Not cleaved	
SUB2	Dnp - A - T - K - L - S - W	12.1	3.0	2.5×10^5
SUB3	Dnp - A - D - K - L - S - W	11.6	1.3	1.1×10^5
SUB4	Dnp - A - T - K - D - S - W	24.9	0.25	1.0×10^4
SUB5	Dnp - A - R - K - L - S - W	15.2	54.7	3.6×10^6
SUB6	Dnp - A - T - K - R - S - W	17.7	3.7	2.1×10^5
SUB7	Dnp - A - P - K - L - S - W	11.1	5.5	5.0×10^5
SUB8	Dnp - A - T - K - P - S - W		Not cleaved	
SUB9	Dnp - A - T - K*-L - S - W[b]		Not cleaved	
SUB10	Dnp - A - T - R - L - S - W		Not cleaved	
SUB11	Dnp - A - T - O+-L - S - W[c]		Not cleaved	
SUB12	Dnp - D - A - T - K - L - S - W	8.8	5.1	5.8×10^5
SUB13	Dnp - D - A - D - K - L - S - W	15.5	0.12	7.7×10^3
SUB14	Dnp - S - T - A - T - K - L - S - W	11.4	111.1	9.7×10^6
SUB15	Dnp - A - T - K - L - S - T - S - W	13.3	17.6	1.3×10^6

[a]Assays were carried out in 50 mM Na-borate buffer, pH 9.5. K_m and k_{cat} values were determined from double-reciprocal plots. [b]K*: D-lysine. [c]O+: L-ornithine.

solution at various concentrations. The intermolecular quenching was evident at more than 40 μM. Therefore, this assay system can be effectively utilized with less than 20 μM of the product.

Substrate Specificities of the Enzymes—Substrates were designed so that both MEPs cleaved at one site only. Only one Dnp-product was released in each cleaved case, as verified by RP-HPLC (data not shown). Amino acid sequence analyses of the reaction products demonstrated that cleavage occurred on the amino-terminal side of Lys residues (data not shown), and that neither peptide bond Ala-Arg in SUB5 nor Lys-Arg in SUB6 was cleaved by either enzyme. No cleavage was observed for the shortest substrate, SUB1, or peptides with substitution of D-Lys (SUB9), L-Arg (SUB10), or L-ornithine (SUB11) with either enzyme. These results provide proof of the strict specificities of GF- and POMEP toward peptide bonds, -X-Lys-.

Kinetics of GFMEP—The kinetic parameters for the hydrolysis of peptide substrates were determined from double-reciprocal plots. Since substrate inhibition was observed at higher concentrations (>30 μM) of each substrate, substrate concentrations of lower than 30 μM were used in routine assays. As shown in Table II, the K_m values of GFMEP remained at 10–20 μM regardless of the substrate sequence except for SUB4. Substrates containing an Arg residue adjacent to the P1′ Lys residue (SUB5 and SUB6), and the longest peptides (SUB14 and SUB15) were found to be better substrates than the standard substrate, SUB2. GFMEP hydrolyzed SUB14 with the highest efficiency, k_{cat}/K_m. On the other hand, the enzyme cleaved Asp-containing SUB3 and SUB4 with much lower k_{cat}/K_m values. The highest K_m value was obtained with SUB4. Of all the substrates hydrolyzed, SUB13, which has two Asp residues at the P3 and P1 positions, was found to be the poorest substrate. Although the peptide with a Pro residue at P1, SUB7, turned out to be a rather good substrate, the peptide with a Pro residue at P2′ (SUB8) was found refractory to the enzyme.

Kinetics of POMEP at pH 8.5 and pH 5.0—Figure 2 summarizes the kinetic characters of POMEP. At pH 8.5, POMEP generally shows the kinetic character of GFMEP. It failed to cleave SUB8 and SUB13 to measurable extents, and SUB4 was the poorest substrate with the highest K_m value (42 μM). Since POMEP was reported to be most active under weakly acidic conditions with azocasein as the substrate (*3*), we examined the kinetic constants of POMEP at pH 5.0 with the fluorogenic peptide substrates. With almost all the cleaved substrates, POMEP was found to be rather active at pH 8.5 than at pH 5.0. The catalytic efficiencies were higher at pH 8.5 by one order of magnitude with remarkable decreases in K_m values. In contrast, the efficiency for Asp-containing SUB3 or SUB4 was comparable, although at pH 5.0, the k_{cat} value for SUB3 doubled and the K_m value for SUB4 decreased significantly. SUB13 was susceptible to POMEP at pH 5.0, but was resistant to it at pH 8.5.

Kinetics of Co^{2+}-Substituted POMEP (Co^{2+}-POMEP)—Both MEPs are readily inhibited by EDTA or *o*-phenanthroline, and reactivated on the addition of several divalent metal ions. Manganese and cobalt are known as the most effective metal ions for GF- and POMEP, respectively (*2, 3*). To examine the effect of divalent metal ion substitution on the proteolytic activity, we prepared Co^{2+}-POMEP and determined its kinetic constants at pH 8.5. As compared with the kinetic constants of the native POMEP (Fig. 2), the K_m values remained at about the same level, whereas the

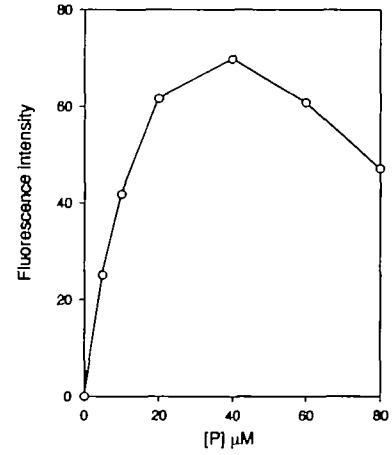

Fig. 1. **Effect of intermolecular quenching of an equimolar mixture of Dnp-Ala-Pro and Lys-Leu-Ser-Trp.** Fluorescence intensities were plotted against the concentration of the complete SUB7 hydrolysate with GFMEP. Assays were carried out in 50 mM Na-borate buffer, pH 9.5, at 37°C.

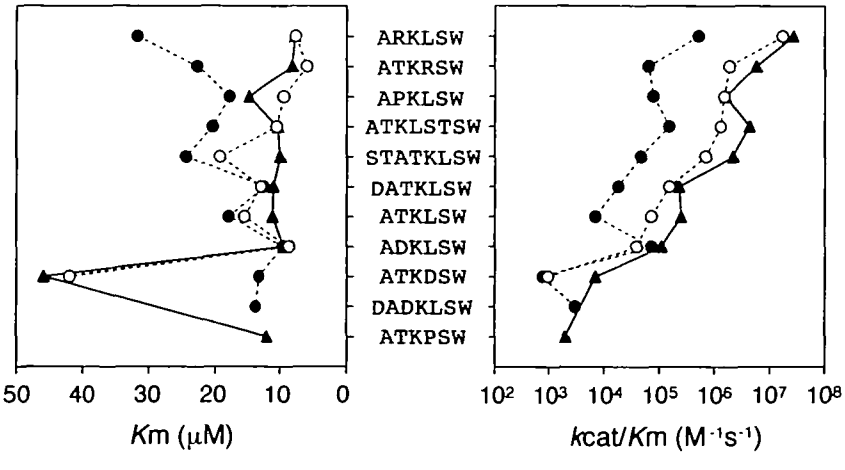

Fig. 2. **Comparison of kinetic parameters of POMEP.** Kinetic parameters (K_m and k_{cat}/K_m) were calculated from the initial rate of hydrolysis by means of double-reciprocal plots. For clarity, substrates are arranged in the order of efficiency (k_{cat}/K_m) with POMEP at pH 8.5 rather than the substrate number. ○, POMEP at pH 8.5; ●, POMEP at pH 5.0; ▲, Co^{2+}-POMEP at pH 8.5.

k_{cat} values were higher than those of the native enzyme. SUB8 with a Pro residue at P2′ was cleaved by Co^{2+}-POMEP, which was resistant to native Zn^{2+}-POMEP. These results indicate that the replacement of Zn^{2+} with Co^{2+} enhances the amidolytic activity but not the affinity for substrates.

Inhibition of GFMEP by Uncleaved Substrates—GFMEP (2.86 nM) was assayed with 12 μM SUB14 as a substrate in the presence of 12 μM SUB1, SUB8, SUB9, SUB10, or SUB11, which are not susceptible to either MEP. These uncleaved peptides were found to inhibit GFMEP activity by 26.5-37.8%. The D-lysine containing peptide, SUB9, which exhibited the strongest inhibitory activity toward GFMEP, was selected to study the mode of inhibition and found to inhibit the GFMEP activity competitively (data not shown) with a K_i value of 39.9 μM.

DISCUSSION

The unique specificities of GF- and POMEP toward peptidyl-Lys bonds were revealed by analyses of digests of horse heart cytochrome *c* (*2*) and oxidized bovine insulin B-chain (*3*), respectively. Both enzymes have been assayed with azocasein as a substrate, because convenient substrates suitable for both MEPs have not been available owing to their specificities for peptidyl-Lys bonds. Hence, kinetic characterization of these enzymes has not been performed. To study the kinetic behavior of the two MEPs, intramolecularly quenched fluorescent substrates (*5, 6, 9*) were synthesized. In order to obtain a quenching efficiency of more than 50%, the donor (Trp) and acceptor (Dnp) must be located within about 30 Å of each other (*11*). Comparison of the cleavage patterns of several proteins and peptides with GFMEP (Table I) proved to be valuable for peptide substrate design. A similar spectrum was observed when the reported cleavage patterns of *Armillaria mellea* protease (*12, 13*) were examined, although the specificity of this enzyme was not as strict as those of the two MEPs. Taking solubility into account, we first designed SUB2, Dnp-ATKLSW, as a standard substrate, and a total of 15 peptide substrates with various characters were synthesized. As the first trial, P2, P3′, and P4′ were fixed as Ala, Ser, and Trp (except in SUB15), respectively.

Since NH_4HCO_3 or Tris-HCl buffer, pH 9.5, had been used frequently for the digestion of proteins with GFMEP, we initially used NH_4HCO_3 buffer (50 mM) for the assay in this study. However, the k_{cat} value of GFMEP for SUB2 (0.235 s^{-1}) was about 10 times lower than that of trypsin. To examine the effect of the assay buffer on the GFMEP activity, we tried several other buffer solutions. In 50 mM Na-borate buffer, pH 9.5, the enzyme hydrolyzed SUB2 maximally, with a 10-fold increased k_{cat} value, although it was equally active toward azocasein even in NH_4HCO_3 buffer (data not shown). Considering the specificity of the enzyme, ammonium ions or the primary amino group of Tris may act as a weak competitive inhibitor for the GFMEP activity with peptide substrates. Furthermore, POMEP activity was assayed with SUB2 under various buffer conditions. Surprisingly, POMEP was found to be most active in 50 mM Na-borate buffer, pH 8.5, rather than pH 5.6, which was reported to be optimal for azocasein (*3*). Thus, we used the borate buffer, pH 9.5 for GFMEP and pH 8.5 for POMEP, as the standard assay buffer.

Efficient enzymatic hydrolysis of peptide bonds requires precise positioning of the scissile bonds as to the catalytic mechanism and slight disorientation results in lower efficiency (k_{cat}). From the data for only 15 peptide substrates, it is too early to speculate the active center structure, but some substrate preferences became clear. Apparently, the introduction of a positive charge was favoured at the P1 position (SUB5) and, to a lesser extent, at the P2′ position (SUB6) as shown in Table II and Fig. 2. On the contrary, a negative charge at the P2′ position (SUB4) and, to a lesser extent, at the P1 position (SUB3) lowered the efficiency. The k_{cat}/K_m values obtained were very low with SUB3, SUB4, and SUB13 at both pH 9.5 and 8.5. Negative charge(s) on Asp residue(s) adjacent to the Lys residue in a peptide substrate may prevent proper alignment of the substrate polypeptide chain in the active center. Only SUB4 showed significantly higher K_m values, especially with POMEP, suggesting the presence of a negative charge around the S2′ pocket. Although a negative charge at the P3 position alone (SUB12) was rather favourable, an additional negative charge at the P1 position made SUB13 one of the poorest substrates. SUB13 was designed based on uncleaved sequences including the amino terminus of horse heart cytochrome *c*, Asp2-Val-Glu-Lys5-.

While Pro at P1 (SUB7) was rather preferred (Table II and Fig. 2), at P2′ (SUB8) it was found to have a strongly negative effect. In the assay with SUB8, a fluorescence intensity increase was barely detectable and kinetic constants could not be calculated for either MEP. This critical effect of Pro at P2′ may indicate the importance of the hydrogen bond between the P2′ carbonyl and MEP for fixation of the substrate amide bond in the active site. The small difference in the P2′ NCO angle between imino and amino acids may be enough to dislocate the scissile bond from the active center. It is not clear yet why the shortest peptide (SUB1) is resistant to both MEPs. These enzymes may have much longer substrate binding sites, like metzincins (*14*), since extended peptides (SUB14 and 15) were hydrolyzed efficiently. The enzymes may not be able to transmit enough strain to short substrates with limited binding interactions.

GF- and POMEP are strictly specific for Lys residues in peptides and proteins. Neither Arg (SUB5, 6, and 10), Orn (SUB11), nor D-Lys (SUB9) was cleaved. Trypsin and *Achromobacter* protease I (API, also known as lysylendopeptidase) from *Achromobacter lyticus* (*15*) are serine proteinases with specificities toward Arg and Lys, and Lys, respectively. The active site pocket in these serine proteinases contains an acidic amino acid residue which is indispensable for the recognition of Arg and/or Lys residues in substrates through the charge interaction (*16*). Therefore, it is quite likely that both MEPs also contain essential acidic amino acid residues in their S1′ pockets which interact with the ε-NH_2 groups of Lys residues in substrates. There are four acidic residues common to Lys-specific GF- and POMEP but not conserved in other deuterolysins (*4*). One of them may be responsible for the substrate specificity. Recently, an extracellular caseinolytic protease from *Aeromonas hydrophila* (*17*) was reported to have a sequence homologous to those of GF- and POMEP (44.6 and 44% sequence identity, respectively). Although its substrate specificity has not been reported yet, the enzyme is likely to be specific for basic residues, since the

four acidic residues are also conserved.

As a weakly acidic pH optimum was reported for POMEP with azocasein as the substrate (*3*), POMEP was assayed at pH 5.0 with these peptide substrates. Contrary to our expectation, POMEP was clearly shown to be more active at pH 8.5 than at pH 5.0 with these peptide substrates (Fig. 2). Among these substrates, only those with Asp at the P1 position (SUB3 and SUB13) exhibit better k_{cat}/K_m values at pH 5.0. Since casein is an acidic protein with a pI value of 4.6, the negative charges of the substrate protein might have led to the wrong non-general optimum pH.

Both MEPs contain essential zinc atoms and are inhibited by chelating agents such as EDTA and *o*-phenanthroline. The zinc-depleted apoenzyme is easily reactivated by the addition of several divalent metal ions (*2, 3*). Co^{2+}-substituted POMEP was found to be more active and hydrolyzed SUB8, which was resistant to both enzymes. All peptides not susceptible to either MEP (SUB1, SUB8, SUB9, SUB10, and SUB11) were shown to inhibit GFMEP activity, most likely in a competitive manner, as seen for SUB9 with a K_i value of 39.9 μM. Interestingly, the most effective inhibitory peptide contained D-Lys not Arg or Orn. The strict sidechain specificity of the enzyme was also reflected in the spectrum of inhibitory peptides.

GF- and POMEP are very useful and interesting metalloproteinases owing to their strict specificities for peptidyl-Lys and peptidyl-AECys bonds, high heat stability, and resistance to denaturing agents. To determine the three-dimensional structures of GF- and POMEP by X-ray crystallography, crystallization has been attempted and crystal structural analysis is in progress. This kinetic and structural information will be useful for elucidation of the reaction mechanism for the specific hydrolysis of peptidyl-Lys bonds by GF- and POMEP.

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