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Substrate Specificities and Kinetic Properties of Proteinase A from the Yeast *Saccharomyces cerevisiae* and the Development of a Novel Substrate

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The substrate specificities and kinetic properties of proteinase A, an intracellular aspartic proteinase from the yeast *Saccharomyces cerevisiae*, were determined using a series of synthetic chromogenic peptides with the general structure P5-P4-P3-P2-Phe-(NO_2)Phe-P2′-P3′ [P5, P4, P3, P2, P2′, P3′ are various amino acids; (NO_2)Phe is *p*-nitro-L-phenylalanine]. The nature of the residues occupying the NH_2-terminal region of the substrate had a strong influence on the kinetic constants. Among those tested, Ala-Pro-Ala-Lys-Phe-(NO_2)-Phe-Arg-Leu had the best kinetic constants (K_m = 0.012 mM, k_{cat} = 14.4 s^{-1}, k_{cat}/K_m = 1,200 $M^{-1} \cdot s^{-1}$). Compared with such aspartic proteinases as pepsin, cathepsin D, and renin, the substrate specificity of proteinase A was unique. Based on these results, a novel fluorescent substrate, MOCAc-Ala-Pro-Ala-Lys-Phe-Phe-Arg-Leu-Lys(Dnp)-NH_2, was developed for the sensitive measurement of proteinase A.

Key words: aspartic proteinase, fluorescent substrate, kinetic property proteinase A, *Saccharomyces cerevisiae*, substrate specificity.

Proteinase A (PrA) [EC 3.4.23.6], an aspartic proteinase, is located in the lysosome-like vacuoles of *Saccharomyces cerevisiae*, together with several other hydrolases including two carboxypeptidases, Y and S, a serine type of proteinase B, two aminopeptidases, I and Co, several phosphatases, and a ribonuclease (*1*). PrA has been purified to homogeneity and its properties have been characterized in detail (*2-4*). The amino acid sequence of PrA shows similarity to those of porcine pepsin (40.4%) (*5, 6*) and lysosomal porcine cathepsin D (44.1%) (*7*) and E (45.0%) (*8*), but less homology to human renin (37.9%) (*9*).

PrA is thought to be involved in intracellular proteolysis, especially under sporulation conditions (*10, 11*). PrA is also known to be sorted *via* multiple pathways into the vacuole (*12*) and to be involved in the activation of other vacuolar proteases, such as proteinase B and carboxypeptidase Y, by processing of the pro-peptides (*13*). Therefore, it is important to investigate the substrate specificity of PrA in clarifying the functional vacuolar proteolysis system.

Yeast cell vacuoles share some biochemical and functional features with lysosomes in animal cells (*14*). The substrate specificity of cathepsin D, a lysosomal aspartic proteinase, is well studied (*15*), and a sensitive substrate for cathepsin D has been developed. The peptide hydrolyzing activities of the secretion types of other animal aspartic proteinases have also been investigated (*16, 17*). On the other hand, the substrate specificities of PrA have not yet been explored in detail. Although several proteins, including unfolded myoglobin, insulin A and B-chains, and a ribonuclease, and various synthetic substrates have been used for PrA measurement (*18-20*), their sensitivities are not ideal. A recent study using a denatured protein substrate (*21*) showed that PrA preferentially cleaves bonds between hydrophobic residues, a similar characteristic to lysosomal cathepsin D and pepsin.

Here, we systematically screened a set of substrates in order to obtain more information about the specificity of PrA within various subsites. Based on the results, we have developed a sensitive fluorescent substrate for use in a novel PrA assay.

MATERIALS AND METHODS

Peptide Substrates and Enzymes—PrA from *S. cerevisiae* (Lot No. 102H8510), porcine kidney renin (Lot No. 31H9600), porcine pepsin (Lot No, 39F8196), aminopeptidase M, pepstatin, and renin inhibitor (Na-Cbz-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Ne-Boc-Lys-methylester) were purchased from Sigma. PrA, renin and pepsin were further purified using Mono Q and Mono P columns on a FPLC

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Abbreviations: PrA, Proteinase A; MOCAc, (7-methoxycoumarin-4-yl)acetyl; AMC, 4-Methyl-coumaryl-7-amide; Dnp, 2,4-dinitrophenyl; Suc, succinyl; DMSO, dimethylsulfoxide; Boc, *t*-butyloxycarbonyl.

system. The proteinases were dissolved in water at 1 mM and stored at −80°C until use. The resulting preparations were homogenous as judged by SDS-PAGE and HPLC analysis. The amount of protein was determined by amino acid analysis and the method of Lowry *et al.* (*22*).

The chromogenic peptide substrates used were synthesized and characterized by the methods of Dunn *et al.* (*23-25*). All have the general structure P5-P4-P3-P2-Phe-(NO_2)Phe-P2′-P3′ [where P*n* represents a variety of amino acids and (NO_2)Phe is *p*-nitro-L-phenylalanine]. The parent peptide was Lys-Pro-Ala-Lys-Phe-(NO_2)Phe-Arg-Leu. All gave single peaks on HPLC analysis and were readily soluble in water. They were stored at −80°C as 10 mM aqueous stock solutions.

The fluorescent peptide substrates used, Suc-A-P-A-K-F-F-R-L-MCA (substrate A), Suc-R-P-F-H-L-L-V-Y-MCA (substrate B), Suc-L-F-A-L-E-V-A-Y-D-MCA (substrate C), and MOCAc-A-P-A-K-F-F-R-L-K(Dnp)-NH_2, were synthesized at the Peptide Institute, Osaka. All preparations were homogenous as determined by HPLC. They were dissolved in DMSO at 1 mM and stored at −80°C until use.

PrA Assays Using Chromogenic Peptides and the Determination of Kinetic Parameters—Stock chromogenic substrate solutions were diluted with 0.1 M McIlvaine buffer (0.2 M disodium hydrogen phosphate plus 0.1 M citric acid, pH 4.5). Chromogenic octapeptides (1, 5, 10, 25, 50, 75, 100, 150, and 200 nmol) were incubated with purified PrA in a total volume of 0.5 ml in 0.1 M McIlvaine buffer (pH 4.5) for 15 min at 30°C. Substrate cleavage between Phe and (NO_2)Phe residues yields a decrease in absorbance at 300 nm. The initial linear rate of this change was measured as the initial velocity (v). K_m and V_{max} values were derived from direct fitting by nonlinear regression analysis from at least eight different initial substrate concentrations. k_{cat} values were obtained from the equation: $V_{max} = k_{cat}[E]$, where [E] is the enzyme concentration determined by amino acid analysis. The estimated error of the derived K_m and k_{cat} values is less than 5%.

Enzyme Assays Using MCA-Substrates and the Determination of Kinetic Parameters—The buffers used were as follows: 0.1 M McIlvaine buffer (pH 4.5) for PrA, 0.1 M ammonium formate buffer (pH3 .0) for pepsin, and 0.1 M sodium phosphate buffer (pH 6.8) for renin. Stock MCA-substrate solutions were diluted in buffers for rate determinations. Enzyme reactions were performed by the method of Yokosawa *et al.* (*19*). Reaction mixtures contained 250 μl of 0.2 M buffer, 2 μl of fluorescent substrate (0.5-1,000 nmol: optimal concentrations determined from the kinetic analyses), and 1 μg of enzyme in a total volume of 500 μl. The reaction mixtures were incubated at 30°C for 30, 60, 120, 180, 360, 480, 600, 720, or 900 s, and the reactions were terminated by adding 200 μl of 0.5 M Tris/HCl (pH 9.0) and 1 nmol of enzyme inhibitor (pepstatin for pepsin and PrA, or renin inhibitor for renin). After addition of 5 μl of aminopeptidase M solution (0.5 mg/ml aqueous solution), the mixtures were incubated for 60 min at 37°C to release AMC from the peptides. The fluorescence of the released AMC was measured at an excitation wavelength of 370 nm and an emission wavelength of 460 nm on a Shimadzu spectrofluorophotometer, Model RF-1500. The initial rate of AMC release was measured to obtain the initial velocity (v). AMC fluorescence was linear over the substrate concentration range used for K_m determination. K_m and V_{max} values were estimated from the intercepts and slopes of double-reciprocal plots of rates *versus* substrate concentrations. K_{cat} values were obtained from the equation: $V_{max} = k_{cat}[E]$, where [E] is the enzyme concentration.

A Novel Assay Procedure for the Measurement of PrA Using MOCAc-Substrate—Reaction mixtures containing 250 μl of 0.2 M McIlvaine buffer (pH 4.5), 2 μl of 1 mM substrate solution [MOCAc-A-P-A-K-F-F-R-L-K(Dnp)-NH_2 in DMSO], and appropriate amounts of sample solutions were prepared in a total volume of 500 μl. The reaction mixture was incubated at 30°C for 20 min and the reaction was terminated by adding 5 μl of 0.1 M NaOH. The PrA activity was estimated by measuring the fluorescence of the cleaved MOCAc-A-P-A-K-F peptide at 393 nm upon excitation at 328 nm.

RESULTS

Substrate Specificity of PrA for Synthetic Chromogenic Substrates—A series of experiments was designed to provide insight into subsite interactions at each position of the substrates (Table I). The kinetic constants of the substrate Lys-Pro-Ala-Lys-Phe-(NO_2)Phe-Arg-Leu will be discussed as the control parent peptide. Substitutions at substrate positions P5-P2 and P2′-P3′ included Ala, Ser, Leu, Asp, and Arg, to evaluate the effects of size, hydrogen bonding potential, and charge. As the parent peptide has Lys residues at P5 and P2, and a Pro residue at P4, six substitutions were evaluated at these positions.

(i) Site-specific cleavage of chromogenic peptide substrate by PrA: In all peptides examined, the bond cleaved by PrA was between Phe and (NO_2)Phe when a peptide (200 nmol) was incubated with 10 μg of purified PrA in a total volume of 0.5 ml of 0.1 M McIlvaine buffer (pH 4.5) for 15 min at 37°C. The products generated were examined by HPLC and amino acid analysis (data not shown).

(ii) The effects of P2′ and P3′ positions: No amino acid alteration at the P3′ position of the substrates improved the rate of hydrolysis: the cleavage efficiency was much lower than that of the parent peptide when estimated from the k_{cat}/K_m values. Substitution of Ala, Asp, Leu, or Ser at P2′ was also not favorable. Thus, the substrate with Arg and Leu in the P2′ and P3′ positions was the most favorable for PrA hydrolysis.

(iii) The effects of P2 and P3 positions: Replacement of the Lys at the P2 position with Ala, Leu, or Ser resulted in improvement in the kinetic constants: the k_{cat}/K_m value increased about 4-6 times over that of the parent peptide. In contrast, substitution of Asp or Arg at the P2 position decreased the k_{cat}/K_m value. Ala was the most favorable residue for the P2 position. In the case of the P3 position, no amino acid changes resulted in a considerable change in the kinetic constants. Replacement of Ala with Leu or Ser resulted in a slight increase in the k_{cat}/K_m value to 2-3 times that of the parent peptide. On the other hand, replacement of Ala by Asp or Arg decreased the k_{cat}/K_m values. Ser was the most favorable residue for the P3 position.

These data are in general agreement with results reported earlier (*23*).

(iv) The effects of P4 and P5 positions: Replacement of

TABLE I. **Kinetic parameters for the hydrolysis of chromogenic peptide substrates by proteinase A from *Saccharomyces cerevisiae*.**

Peptide number	Substrate P5	P4	P3	P2	P1	P1′	P2′	P3′	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
1	Lys	Pro	Ala	Lys	Phe	XPhe	Arg	Leu	0.030	4.0	133
2								Ala	0.095	6.7	71
3								Asp	0.024	1.7	71
4								Arg	0.126	2.7	21
5								Ser	0.250	4.7	19
6							Ala		0.042	1.5	36
7							Asp		0.068	2.0	29
8							Leu		0.017	1.7	100
9							Ser		0.073	0.9	12
10				Ala					0.020	15.9	795
11				Asp					0.017	1.7	100
12				Leu					0.017	8.4	494
13				Arg					0.097	9.4	97
14				Ser					0.015	6.7	447
15			Asp						0.028	1.9	68
16			Leu						0.038	10.6	279
17			Arg						0.093	3.3	35
18			Ser						0.028	10.4	371
19		Ser							0.037	2.4	65
20		Asp							0.033	5.8	176
21		Arg							0.094	6.7	71
22		Ala							0.037	3.5	95
23		Leu							0.031	6.8	219
24	Ser								0.015	6.6	440
25	Asp								0.026	18.6	715
26	Arg								0.020	14.7	735
27	Ala								0.012	14.4	1,200
28	Leu								0.014	13.8	986
29	Gly	Ala	Phe	XPhe	Arg	Leu			0.019	19.7	1,040
30	Ala	Leu	Ser	Ala	Phe	XPhe	Arg	Leu	0.010	10.9	1,090

XPhe: 4-(NO_2)-phenylalanine.

TABLE II. **Specificity constants of proteinase A and other aspartic proteinases.**

Peptide number	Substrate P5	P4	P3	P2	P1	P1′	P2′	P3′	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1}$) Proteinase A	Pepsin	Cathepsin D	Cathepsin E	Rhizopuspepsin
1	Lys	Pro	Ala	Lys	Phe	XPhe	Arg	Leu	0.14	0.8	<0.02	0.07	1.13
2								Ala	0.07	0.13		0.04	0.81
3								Asp	0.07	0.09		0.02	0.72
4								Arg	0.02	0.15		0.03	0.38
5								Ser	0.02	0.08		0.02	0.61
6						Ala			0.04	0.60		0.03	1.15
7						Asp			0.03	0.06		<0.01	0.09
8						Leu			0.10	1.0		0.08	0.73
9						Ser			0.01	0.50		0.03	0.55
10				Ala					0.80	0.8	0.2	0.7	2.39
11				Asp					0.10	0.5	0.3	0.10	0.83
12				Leu					0.49	0.7	1.0	1.6	2.31
13				Arg					0.10	0.15	<0.02	0.14	2.17
14				Ser					0.46	0.5	0.1	0.7	1.03
15			Asp						0.07	0.2	<0.02	0.09	0.7
16			Leu						0.28	0.6	<0.02	0.15	1.4
17			Arg						0.04	<0.02	<0.02	0.14	1.4
18			Ser						0.38	0.2	<0.02	0.07	0.6
19		Ser							0.06	0.09		0.008	0.42
20		Asp							0.18	0.04		0.01	0.73
21		Arg							0.07	0.04		0.004	0.06
22		Ala							0.10	0.30		0.01	0.75
23		Leu							0.22	0.06		0.009	1.57
24	Ser								0.45	0.20		0.01	1.21
25	Asp								0.71	0.50		0.01	0.76
26	Arg								0.75	0.40		0.01	1.74
27	Ala								1.17	0.30		0.02	1.14
28	Leu								0.97	0.70		0.02	0.82

Pro at the P4 position with Leu resulted in a slight increase in the k_{cat}/K_m value to about 2 times that of the parent peptide. Introduction of Ser, Arg, or Ala, however, resulted in a decrease in the k_{cat}/K_m value; and introduction of Asp produced no significant change. On the other hand, replacement of the Lys at P5 position with any of five different amino acid residues resulted in considerable improvement in the kinetic constants: the K_m values for peptides with Ser, Ala, or Leu at P5 decreased to one half that of the control substrate, while the k_{cat}/K_m values increased to about 4-9 times that of the control. Ala was the most favorable residue at P5: the k_{cat}/K_m value increased to approximately 9 times that of the control.

Kinetic Constants for the Optimized Amino Acid Substrate—Based on the results described above, we synthesized a unique peptide combining the amino acid residues with the best kinetic constants at each position from P5 to P3′, that is, Ala-Leu-Ser-Ala-Phe-(NO_2)Phe-Arg-Leu. The kinetic constants of this peptide for PrA were $K_m = 0.010$ mM, $k_{cat} = 10.9\ s^{-1}$, and $k_{cat}/K_m = 1{,}090\ mM^{-1} \cdot s^{-1}$ (Table I, peptide No. 30). We also synthesized a pentapeptide, Gly-Ala-Phe-(NO_2)Phe-Arg-Leu (peptide No. 29), reported to be the most sensitive substrate for pepsin (*5, 6, 21*). The kinetic constants of this pentapeptide for PrA were $K_m = 0.019$ mM, $k_{cat} = 19.7\ s^{-1}$, and $k_{cat}/K_m = 1{,}040\ mM^{-1} \cdot s^{-1}$. Among of the 30 synthetic peptides in Table I, Ala-Pro-Ala-Lys-Phe-(NO_2)Phe-Arg-Leu (peptide No. 27) and Ala-Leu-Ser-Ala-Phe-(NO_2)Phe-Arg-Leu (peptide No. 30) showed the highest specificity constants, k_{cat}/K_m.

Comparison of Specific Constants of PrA with Those of Other Aspartic Proteinases—Specificity constants of PrA were compared with those of other aspartic proteinases such as pepsin (*26, 27*), cathepsin D (*27*), cathepsin E (*28*), and rhizopuspepsin (*27, 29*) (Table II). Pepsin readily cleaves the substrates containing P3′ Leu (No. 1), P2′ Leu (No. 8), P2 Ala (No. 10), or Leu (No. 12), P3 Leu (No. 16), P4 Ala (No. 22), and P5 Leu (No. 28). Cathepsin D, a lysosomal aspartic proteinase, cleaves these peptides more selectively. That is, only one of the peptides having Leu at P2 is readily cleaved. Cathepsin E also shows similarly strict substrate specificity. On the contrary, rhizopuspepsin, isolated from *Rhizopus chinensis* exhibits a very broad substrate specificity, having high specificity constants for a wide range of peptides containing variations from the P5 to P3′ positions. Compared with these enzymes, PrA showed similar substrate specificity to that of pepsin.

Comparison of the Substrate Specificities of Proteinase A, Pepsin, and Renin Using Three Fluorescent Substrates—We constructed a fluorescent substrate based on the sequence of peptide No. 27 (Table I) with the fluorescent compound MCA added to the COOH-terminal, that is, Suc-A-P-A-K-F*-F-R-L-MCA (substrate A, the asterisk indicates the position of proteolysis). We also synthesized two other fluorescent substrates: Suc-R-P-F-H-L-L*-V-Y-MCA (substrate B), reported as a sensitive substrate for renin and PrA (*19, 20*); and Suc-L-F-A-L-E-V*-A-Y-D-MCA (substrate C), previously reported as a substrate for PrA (*30*). PrA cleaved between F and F in substrate A, releasing F-R-L-MCA; between L and V in substrate B, releasing V-Y-MCA; and between V and A in substrate C, releasing A-Y-D-MCA. The positions cleaved by pepsin and renin were not determined.

The kinetic constants obtained for PrA using these three fluorescent substrates are summarized in Table III. The K_m value of PrA for substrate A was 0.006 mM, which was about one-seventh of the K_m value for substrate B, and one-third of that for substrate C. The k_{cat}/K_m value of PrA for substrate A was $3{,}040\ mM^{-1} \cdot s^{-1}$, which was about 15 times higher than the values for substrate B and C. Thus, Suc-A-P-A-K-F-F-R-L-MCA (substrate A) showed the best kinetic constants for PrA and its sensitivity is much superior to the previously used substrates B (*19, 20*) and C (*30*).

The kinetic constants of renin for three fluorescent substrates were compared (Table III). It is considered that renin might hydrolyze the L-L bond of substrate B and the L-MCA bond of substrate A. The k_{cat}/K_m value for substrate B was $4{,}030\ mM^{-1} \cdot s^{-1}$, about 1,700 times higher than that for substrate A. Renin did not hydrolyze substrate C effectively; the K_m value was above 1 mM.

The kinetic constants for pepsin using the three fluores-

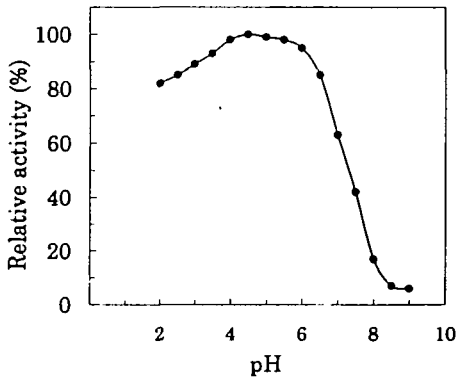

Fig. 1. **The pH dependence of hydrolysis of the substrate MOCAc-A-P-A-K-F-F-R-L-K(Dnp)-NH$_2$ by proteinase A.** Reaction mixtures containing 250 μl of 0.2 M solutions of various buffers (pH 2.0-9.0), 2 μl of 1 mM substrate solutions, and appropriate amounts of PrA solutions were prepared in a total volume of 500 μl. Each reaction mixture was incubated at 30°C for 20 min and the reaction was terminated by adding 5 μl of 0.1 M NaOH. The PrA activity was estimated by measuring the fluorescence emission of the cleaved MOCAc-A-P-A-K-F peptide at 393 nm upon excitation at 328 nm. Relative activity is expressed as a percentage of the maximum value.

TABLE III. **Kinetic parameters of proteinase A, renin and Pepsin for substrates A, B, and C.**

Proteinase	Substrate A			Substrate B			Substrate C		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Proteinase A	6.07	18.5	3,040	39.3	8.25	210	18.1	3.73	206
Renin	370	0.9	2.4	2.53	10.2	4,030	1,000	—	—
Pepsin	5.81	20.7	3,560	8.34	9.11	1,090	7.6	2.00	263

Substrate A: Suc-Ala-Pro-Ala-Lys-Phe-Phe-Arg-Leu-MCA. Substrate B: Suc-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-MCA. Substrate C: Suc-Leu-Phe-Ala-Leu-Glu-Val-Ala-Tyr-Asp-MCA. —: No meaningful data obtained.

cent substrates were also compared (Table III). Pepsin hydrolyzed all fluorescent substrates effectively. The k_{cat}/K_m value for substrate A was 3,560 $mM^{-1} \cdot s^{-1}$, 3 times higher than for substrate B (1,090 $mM^{-1} \cdot s^{-1}$) and 14 times higher than for substrate C (263 $mM^{-1} \cdot s^{-1}$).

Development of a Novel Method for PrA Measurement Using a Fluorescent Peptide, MOCAc-Ala-Pro-Ala-Lys-Phe-Phe-Arg-Leu-Lys(Dnp)-NH_2, as a Substrate—We developed a novel method for PrA measurement using MOCAc-A-P-A-K-F-F-R-L-K(Dnp)-NH_2 as a substrate to increase sensitivity and simplify the PrA activity assay. This peptide is designed based on peptide No. 27 by adding MOCAc at the amino terminus, and adding additional residue, (ε-Dnp)Lys at the carboxyl terminus. The highly fluorescent group, MOCAc, in the substrate is efficiently quenched by the Dnp before proteolysis by PrA. When PrA cleaves the Phe-Phe bond, the fluorescence emission at 393 nm upon excitation at 328 nm increases several hundred fold. Reaction mixtures containing 250 μl of 0.1 M McIlvaine buffer (pH 4.5), 2 μl of 1 mM substrate solutions, and appropriate amounts of PrA solutions were prepared in a total volume of 500 μl. The reactions were carried out as described in "MATERIALS AND METHODS." By varying the substrate concentration, we were able to determine kinetic parameters for PrA, pepsin, and renin as shown in Table IV. The catalytic efficiency of PrA, represented by k_{cat}/K_m, is in the same range as that observed for peptides No. 27 and 30. The data for comparison of the substrate specificities of PrA, pepsin, and renin using this MOCAc-substrate were consistent with those for MCA-substrate shown in Table III. In addition to being a sensitive substrate, the MOCAc-substrate allows use of a simpler assay procedure than the method using MCA as a fluorescent compound.

TABLE IV. **Kinetic parameters of proteinase A, renin and Pepsin for MOCAc-A-P-A-K-F-F-R-L-K(Dnp)-NH_2.**

Proteinase	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Proteinase A	6.18	18.1	2,930
Renin	560	0.8	1.4
Pepsin	6.17	21.1	3,420

The pH dependence of hydrolysis of the substrate MOCAc-A-P-A-K-F-F-R-L-K(Dnp)-NH_2 by PrA was examined, with optimal hydrolysis seen at approximately pH 4.5–5.0 (Fig. 1). The peptide-hydrolyzing activity was stable at acidic pH, but low at alkaline pH.

DISCUSSION

PrA has an extended active-site cleft (*4*) that can accommodate at least eight amino acids in the S5-S3′ subsites. PrA has two aspartic acid residues, Asp^{32}-Thr-Gly and Asp^{215}-Thr-Gly, which are involved in catalytic action. A recent study showed that mutation of Asp^{215} in PrA blocks the maturation of other vacuolar proteases (*4*). Thus, PrA plays an important role in proteolysis in yeast cells.

PrA is also known to hydrolyze peptides at sites between adjacent hydrophobic residues (*21*): Phe, Leu, and Glu are favored at substrate position P1, and Phe, Ile, Leu, and Ala at P1′. This proteolytic characteristic of PrA is similar to those of cathepsin D and pepsin, although the amino acid sequence of the active site of PrA differs from those of pepsin and cathepsin D (*6*). It is also reported that PrA has more limited hydrolysis spectrum than pepsin in the cleavage of myoglobin (*21, 31*).

In this paper we demonstrated that our octapeptide, A-P-A-K-F-(NO_2)F-R-L (peptide No. 27), and A-L-S-A-F-(NO_2)F-R-L (peptide No. 30) were well accommodated in the active site of PrA and that PrA hydrolyzed the Phe-(NO_2)Phe bond effectively.

We also found that the substrate specificity of PrA resembled that of pepsin among the aspartic proteinases reported so far (Table II). Both PrA and pepsin showed high activity when Lys at the P2 position was replaced with Ala, Leu, or Ser. However, additional differences in substrate specificity were observed. Comparison of the effects of substitution at each of the six positions evaluated that the largest change in k_{cat}/K_m was caused by change at P5 in the

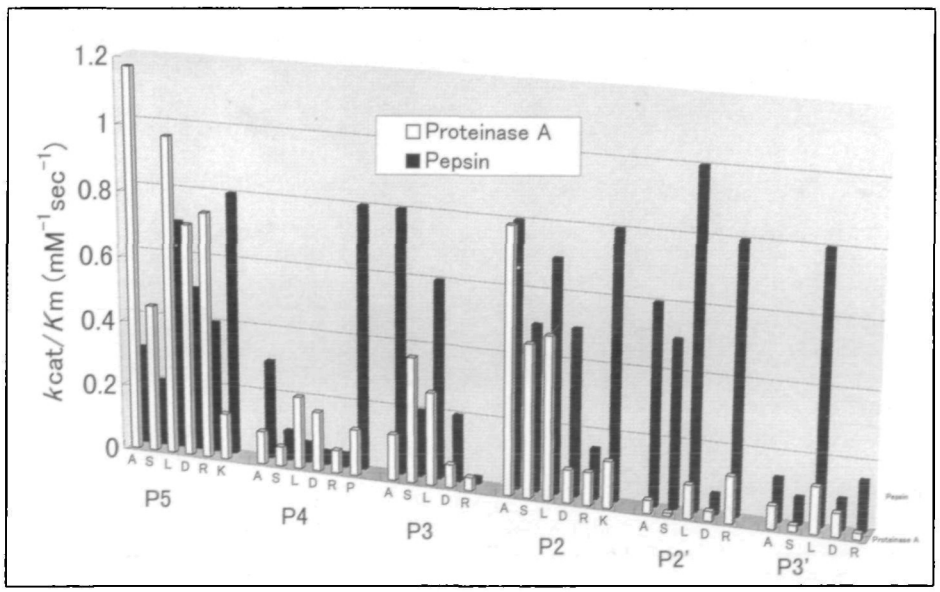

Fig. 2. **Specific constants for hydrolysis of a series of synthetic peptides by PrA and pepsin.** The white row and black row show the data of PrA and pepsin, respectively.

case of PrA, and by changes at P4 and P3 in the case of pepsin, while changes at P2, P2′, and P3′ produced similar effects (Fig. 2).

Recently, the three-dimensional structure of PrA was elucidated (*32*), that was found two notable structural features. First, no negatively charged amino acid residues were found in the active site cleft of PrA. In contrast, pepsin has at least four negatively charged amino acid residues (Glu13, Asp242, Glu244, and Glu287). Second, the S5 subsite of PrA was composed of such hydrophobic amino acid residues as Leu10, Ala12, and Val278. These structural differences re between PrA and pepsin may cause differences in substrate specificity, especially in the P5 preference, as described above.

PrA may also be compared with human cathepsin D, as both enzymes operate in vacuolar (or lysosome) compartments. Cathepsin D shows much stricter specificity, especially with respect to the P2 position. The Lys residue present in the parent sequence and in all but five of the peptides shown in Table II is not acceptable to cathepsin D. No cleavage is observed unless the P2 Lys is replaced by Ala, Asp, Ser, or, preferably, Leu. In this respect PrA and cathepsin D show significant differences. Based on the crystal structure of PrA complexed to renin inhibitor, the S2 subsite is more accessible to the yeast enzyme than to cathepsin D. Furthermore, the substitution of Thr^{216} residue in PrA for the Met found in cathepsin D (287 in pepsin numbering) is also responsible for the acceptance of a Lys residue in P2 of a substrate. Our observation that peptide No. 27 is also an excellent substrate for PrA correlates with the lack of charged residues within the active site cleft. In this respect, PrA and cathepsin D show similar characteristics.

The modified peptide substrate, Ala-Leu-Ser-Ala-Phe-(NO_2)Phe-Arg-Leu, prepared by combining the amino acids with the best kinetic constants at each position, showed almost the same kinetic constants as Ala-Pro-Ala-Lys-Phe-(NO_2)Phe-Arg-Leu. Substrate specificities might be determined through the combined interactions of all amino acid residues in the substrate, and a substrate that combines the amino acid residues with the best kinetic constants at each position would, therefore, not necessarily yield the highest kinetic constants. Nevertheless, peptide No. 30 has a sequence that fits extremely well into the structure of PrA previously determined (*32*).

Investigation of the substrate specificity of PrA might be useful for clarifying the functional vacuolar system. It has been proposed that the precursors of vacuolar proteins are activated in a cascade by which pro-PrA is activated to PrA, PrA activates pro-PrB to PrB, and PrB activates pro-CPY to mature CPY (*33, 34*). Pro-CPY can also be processed directly by PrA (*35*). PrA is considered to be involved particularly in the final maturation stage of pro-PrB (*36*). Pro-PrB, a protein of MW 42,000, is further processed by PrA in vacuole at or near amino acid 293 (PrB numbering) to yield the 293-amino acid PrB with a mature C-terminus. Although the amino acid sequence of flanking region of positions 293(Pro) and 294(Glu) of PrB is not necessarily favorable to proteolysis by PrA, the sequence of the C-terminus of pro-PrB includes several positions which might be digested by PrA: for example, T-P-N-V-L^{300}-I^{301}-Y-N (PrB numbering). Leu-Ile is favorable for positions P1 and P1′ as a substrate for PrA, and the flanking region of these residues is also considered to be suitable for proteolysis by PrA. Further processing of the C-terminus, the mature form might be caused by carboxypeptidases.

Finally, this paper also describes a novel method for the measurement of PrA activity using a fluorescent nonapeptide, MOCAc-Ala-Pro-Ala-Lys-Phe-Phe-Arg-Leu-Lys-(Dnp)-NH_2, that was designed based on our initial findings regarding substrate preference. This method provides a powerful tool for measuring trace amounts of PrA activity.

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