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A MEMBRANE-BOUND, CALCIUM-DEPENDENT PROTEASE IN YEAST α-CELL CLEAVING ON THE CARBOXYL SIDE OF PAIRED BASIC RESIDUES

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SUMMARY: Paired basic residues are known as a typical site for proteolytic processing of precursors of bioactive peptides. By using a fluorogenic substrate Boc-Gln-Arg-Arg-MCA, a unique endoprotease exhibiting hydrolytic specificity toward the carboxyl side of paired basic residues was partially purified (about 4600-fold) from the membrane fraction of yeast Saccharomyces cerevisiae α-cells. The enzyme is a calcium-dependent thiol protease, with optimal pH at 7.0. It is a glycoprotein, with an apparent molecular weight of about 100,000-120,000. It cleaves fluorogenic substrates and a synthetic model peptide at the carboxyl side of paired basic residues. From its unique substrate specificity, this enzyme may be involved in precursor processing in vivo.

Biologically active peptides are initially synthesized as larger precursor proteins, that are subsequently processed to mature peptides by limited proteolysis. Accumulated data of sequences of precursor proteins indicate that paired basic residues are typical sites for proteolytic processing. However, little is known about the responsible proteases that are physiologically involved in precursor processing (1-3). Yeast Saccharomyces cerevisiae α-cells synthesize and secrete α-mating factor, a peptide of 13 amino acids, which is processed from a larger precursor by cleavage at paired basic residues (4). In a previous study we used these yeast cells as a simple model system for the study of prohormone processing and reported the identification, in the soluble fraction of yeast α-cell, of a serine-protease which specifically cleaves the peptide bonds between consecutive basic residues (5). In this paper, we report the partial purification and characterization, in the membrane fraction of yeast α-cells, of a unique

Abbreviations: MCA,4-methylcoumaryl-7-amide; AMC,7-amino-4-methylcoumarin; Boc-,t-butyloxycarbonyl-; PMSF,phenylmethylsulfonyl fluoride; DFP,diisopropyl fluorophosphate; TPCK,p-tosyl-L-phenylalanine chloromethyl ketone; TLCK,p-tosyl-L-lysine chloromethyl ketone; pCMB,p-chloromercuribenzoate; NEM,N-ethylmaleimide; DTT,dithiothreitol; β-ME,2-mercaptoethanol; EGTA,ethylene glycol bis(β-aminoethyl ether)-tetraacetic acid; o-PT,o-phenanthroline; pAPMSF,p-amidinophenylmethylsulfonyl fluoride; pABA,p-aminobenzamidine; STI, soybean trypsin inhibitor, Con A,concanavalin A.

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calcium-dependent thiol protease that specifically cleaves on the carboxyl side of paired basic residues.

MATERIALS AND METHODS

Materials: A substrate peptide was synthesized by solid-phase procedures. MCA-substrates were synthesized as previously reported (6). Con A-Sepharose, Arg-Sepharose, benzamidine-Sepharose and FPLC columns were purchased from Pharmacia. Inhibitors of microbial origin were purchased from Peptide Institute, Minoh, Osaka. Other reagents were purchased from Nakarai Chemicals. Preparation of enzyme: Haploid strain S. cerevisiae X-2180 1B (α-mating type cell) was supplied by Drs. T. Tanaka & T. Kodama, Suntory Ltd. Cells were grown aerobically at 28°C in YM-medium, containing 0.3% yeast extract, 0.3% malt extract, 0.5% polypeptone and 1% glucose. Cells (wet weight 200 g) were harvested by centrifugation, washed once with 0.9% NaCl and resuspended in 0.1 M sodium phosphate buffer (pH 7.0). They were broken by mechanical rupture with a Dyno-Mill and then homogenized with a Polytron homogenizer. After centrifugation at 1,000g for 10 min, the supernatant was centrifuged at 80,000g for 30 min. The pellet fraction was washed four times with 10 mM Tris-HCl buffer (pH 7.0), with centrifugation each time at 80,000g for 30 min. The washed membrane thus obtained was extracted three times with 220 ml of 10 mM Tris-HCl buffer (pH 7.0)-0.1 M NaCl-1% Lubrol (PX type, Nakarai Chemicals). The extract was diluted with the same volume of 10 mM Tris-HCl buffer (pH 8.0) and directly applied to a column of DEAE-cellulose (DE-52) (4.0 x 30 cm). The column was eluted by 10 mM Tris-HCl buffer (pH 8.0)-0.2% Lubrol with a linear gradient from 0.05 to 0.45 M NaCl (Fig.1A). The active fractions (Fr. 127-141) were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.0)-0.2% Lubrol-1 mM $CaCl_2$. The dialyzate was applied to a column (2 x 10 cm) of benzamidine-Sepharose (Fig. 1B). The column was washed with the same buffer, and then the enzyme activity was eluted with the same buffer containing 0.5 M NaCl. The active fractions (Fr. 48-50) were pooled and directly applied to a column (2 x 13 cm) of Con A-Sepharose (Fig. 1C), equilibrated with 20 mM Tris-HCl buffer (pH 7.0)-0.2% Lubrol-0.5 M NaCl. After washing the column with the equilibration buffer, the enzyme activity was eluted with the same buffer containing 0.5 M α-methyl-D-mannoside. The active fractions (Fr. 26-46) were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 7.0)-0.2% Lubrol-1 mM $CaCl_2$. The dialyzate was applied to a column (2 x 6.5 cm) of Arg-Sepharose (Fig. 1D). The enzyme activity was eluted with the same buffer containing 0.5 M NaCl. The active fractions (Fr. 28-29) were pooled and further purified by FPLC. A portion (1/10) of the enzyme active fraction was desalted with a PD-10 column and then applied to a column (0.5 x 5 cm) of Mono Q (Fig. 1E). Elution was carried out with a linear gradient (40 min) of NaCl concentration from 0 to 0.5 M in 10 mM Tris-HCl (pH 8.0)-0.2% Lubrol. The active fractions (Fr. 21-22) were pooled and further purified by gel-filtration on a column (1.6 x 50 cm) of Superose 12, equilibrated with 10 mM Tris-HCl buffer (pH 7.0)-0.2% Lubrol-0.05 M NaCl (Fig. 1F). The active fractions (Fr. 30-37) were pooled and their enzymic properties were characterized in this study.

Enzyme assays: Assay for proteolytic activity used in this study was as follows: twenty nanomoles of Boc-Gln-Arg-Arg-MCA was incubated with an enzyme preparation, in a final volume of 250 µl containing 0.2 M Tris-HCl buffer (pH 7.0), 0.1% Lubrol and 1 mM $CaCl_2$. The reaction mixture was kept at 37°C for 1-24 hr. Then 3.0 ml of distilled water was added, and the amounts of AMC released from the substrate were measured by a fluorescence spectrophotometer (Hitachi 204) with excitation at 380 nm and emission at 460 nm. One unit of activity is tentatively defined as the amount of enzyme that can release 10 nanomoles of AMC from the substrate under the above assay conditions in 1 hr.

RESULTS AND DISCUSSION

Proteolytic activity specifically cleaving the carboxyl side of paired basic residues was monitored by using the fluorogenic peptide derivative Boc-Gln-Arg-Arg-MCA as a substrate. The enzyme was 4600-fold purified from the

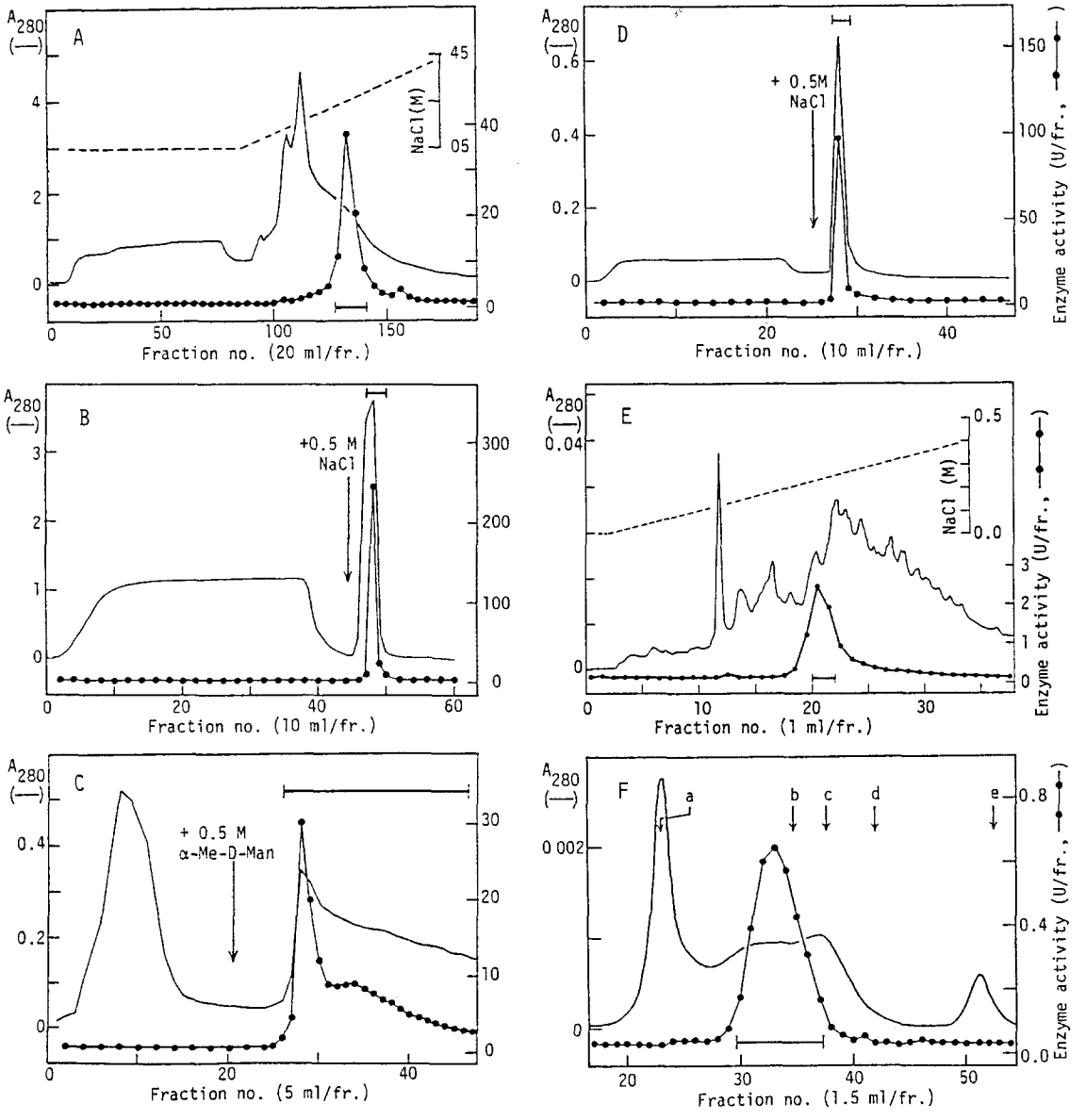

Fig. 1. Purification of the enzyme from membrane fraction of yeast α-cell by using a column of DE-52 (A), benzamidine-Sepharose (B), Con A-Sepharose (C), Arg-Sepharose (D), Mono Q (E) and Superose 12 (F). The elution positions of standard proteins in (F) are indicated by arrows: a, thyroglobulin (680 K); b, bovine serum albumin (68 K); c, ovalbumin (43 K); d, soybean trypsin inhibitor (21.5 K); e, cytochrome c (14 K).

membrane fraction of yeast α-cell (wet weight 200 g) in the manner summarized in Fig. 1 and Table 1. The membrane proteins, extracted with a buffer containing the non-ionic detergent Lubrol, were chromatographed on a column of DEAE-cellulose (Fig. 1A). The active fraction, marked with a bar, was further purified by successive chromatographies on benzamidine-Sepharose (Fig. 1B),

Table 1. Purification of a membrane-bound calcium-dependent protease from yeast α-cell

Step	Total protein[a] (mg)	Total activity (units)	Specific activity (U/mg)	Yield (%)	Purification fold
1. membrane extract	5060	-----[b]	0.064	(100.0)	(1.0)
2. DE-52	416.6	323.7	0.78	100.0	12.2
3. Benzamidine-Seph.	38.02	283.7	7.46	87.7	116.6
4. Con A-Sepharose	19.88	161.1	8.10	49.8	126.6
5. Arg-Sepharose	7.655	106.1	13.86	32.8	216.6
6. Mono Q	0.400	41.77	104.43	12.9	1631.7
7. Superose 12	0.108	31.89	295.28	9.9	4614.8

a) Protein concentration was determined spectrophotometrically using $A^{0.1\%}_{280nm} = 1.0$. b) Total activity was not determined in this fraction.

Con A-Sepharose (Fig. 1C), and Arg-Sepharose (Fig. 1D). The enzyme activity was adsorbed on a Con A-Sepharose column and eluted with a buffer containing 0.5 M α-methyl-D-mannoside, indicating that the enzyme is a glycoprotein. Further purification of the enzyme was carried out by FPLC using a column of Mono Q (Fig. 1E) and Superose 12 (Fig. 1F). From the elution position of enzyme activity on a Superose 12 column, the molecular weight of the enzyme was estimated as 100,000-120,000. The enzymic properties of the active portion, eluted in Fr. 30-37, were characterized in this study.

To characterize the substrate specificity of the enzyme, various fluorogenic MCA-derivatives were incubated with the enzyme, and AMC released from the substrate was measured by a fluorescence spectrophotometer (Table 2). The reaction products obtained from Boc-Gln-Arg-Arg-MCA with the enzyme were analyzed on reverse-phase HPLC (Fig. 2A). On the basis of amino acid analyses and comparison of retention time on HPLC with authentic specimens, three peaks were identified as Boc-Gln-Arg-Arg (peak 1), AMC (peak 2) and the starting Boc-Gln-Arg-Arg-MCA (peak 3), indicating that the enzyme exclusively hydrolyzes on the carboxyl side of Arg-Arg sequence in the substrate. Similarly, HPLC analysis confirmed that the enzyme specifically cleaved on the carboxyl side of Lys-Arg pair in Boc-Leu-Lys-Arg-MCA (Fig. 2B). Three other MCA-substrates containing Arg-Arg and Lys-Arg sequences were hydrolyzed on the

Table 2. Substrate specificity of the enzyme

Substrate	% Activity	Substrate	% Activity
Boc-Gln-Arg-Arg-MCA	100.0	Boc-Glu-Lys-Lys-MCA	0.8
Boc-Leu-Arg-Arg-MCA	97.8	Pro-Phe-Arg-MCA	0.5
Boc-Gly-Arg-Arg-MCA	14.3	Z-Phe-Arg-MCA	0.3
Boc-Leu-Lys-Arg-MCA	118.1	Bz-Arg-MCA	1.1
Boc-Gly-Lys-Arg-MCA	74.9	Arg-MCA	1.1
Boc-Val-Pro-Arg-MCA	51.4	Leu-MCA	0.5
Boc-Ala-Pro-Arg-MCA	25.3		

Protease activity for Boc-Gln-Arg-Arg-MCA was taken as 100%. Each substrate (20 nmole) was incubated with the enzyme at 37°C for 16 hr as described in "MATERIALS AND METHODS". AMC released was measured by fluorescence spectrophotometer.

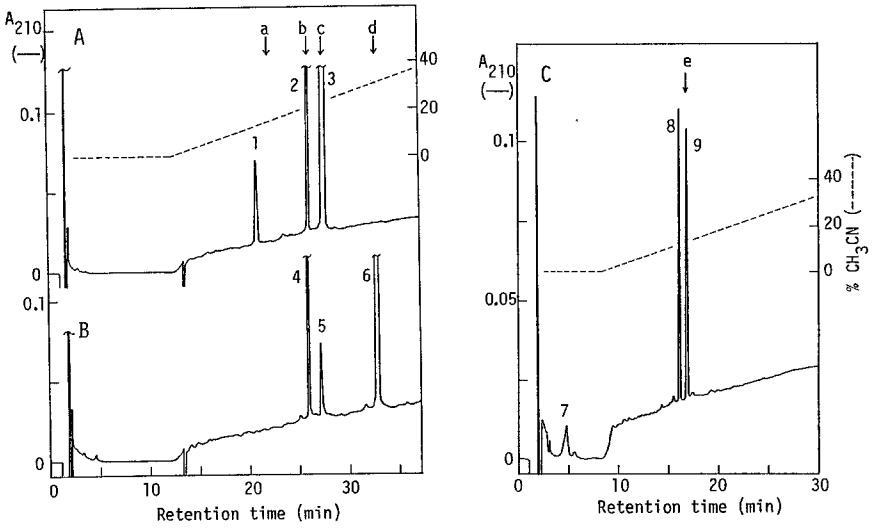

Fig. 2. HPLC patterns of reaction products from Boc-Gln-Arg-Arg-MCA (A), Boc-Leu-Lys-Arg-MCA (B) and Pro-Met-Tyr-Lys-Arg-Glu-Ala-Glu-Ala-NH_2 (C). Two MCA-substrates (20 nmoles) and one peptide substrate (4 nmoles) were incubated with the enzyme for 20 hr at 37°C and subjected to HPLC on a column of TSK LS-410 ODS-SIL (0.4 x 25 cm, Toyosoda). Elution was carried out with a linear gradient (40 min) of acetonitrile concentration from 0 to 60% in 0.1% trifluoroacetic acid at a flow rate of 2 ml/min. Based on amino acid composition and comparison of retention time with authentic specimen, the respective peaks were identified (yields of the peptides are designated in parentheses). A: 1, Boc-Gln-Arg-Arg (3.2 nmol); 2, AMC; 3, Boc-Gln-Arg-Arg-MCA (9.0 nmol); B: 4, AMC; 5, Boc-Leu-Lys-Arg (2.9 nmol); 6, Boc-Leu-Lys-Arg-MCA (8.5 nmol); C: 7, Glu-Ala-Glu-Ala-NH_2 (1.4 nmol); 8, Pro-Met-Tyr-Lys-Arg (1.6 nmol); 9, Pro-Met-Tyr-Lys-Arg-Glu-Ala-Glu-Ala-NH_2 (1.2 nmol). The elution positions of authentic specimens are indicated by arrows: a, Arg-MCA; b, AMC; c, Boc-Gln-Arg-Arg-MCA; d, Boc-Leu-Lys-Arg-MCA; e, Pro-Met-Tyr-Lys-Arg-Glu-Ala-Glu-Ala-NH_2.

carboxyl side of paired basic residues, although there were some differences in reaction rate. Furthermore, we found that the enzyme also converts Pro-Met-Tyr-Lys-Arg-Glu-Ala-Glu-Ala-NH_2, whose sequence corresponds to the site of cleavage for pro-α-factor processing, to Pro-Met-Tyr-Lys-Arg and Glu-Ala-Glu-Ala-NH_2 by specific cleavage after Lys-Arg sequence (Fig. 2C). In addition to the substrates containing paired basic residues, the enzyme also cleaves two substrates containing Pro-Arg sequence (Boc-Val-Pro-Arg-MCA and Boc-Ala-Pro-Arg-MCA) on the carboxyl side of Arg residue. The site of cleavage was confirmed by HPLC analyses as above (data not shown). On the other hand, typical substrates for trypsin and kallikreins (Bz-Arg-MCA, Pro-Phe-Arg-MCA and Z-Phe-Arg-MCA), two aminopeptidase substrates (Arg-MCA and Leu-MCA) and a substrate containing Lys-Lys sequence (Boc-Glu-Lys-Lys-MCA) were not affected by the enzyme. These findings indicate that the enzyme is distinct from pancreatic trypsin and other related proteases. It preferentially cleaves on the carboxyl side of Arg residue of paired basic residues (Lys-Arg, Arg-Arg) and Pro-Arg sequence of the substrates examined. The unique substrate specificity exhibited by the enzyme suggests that it may be involved in

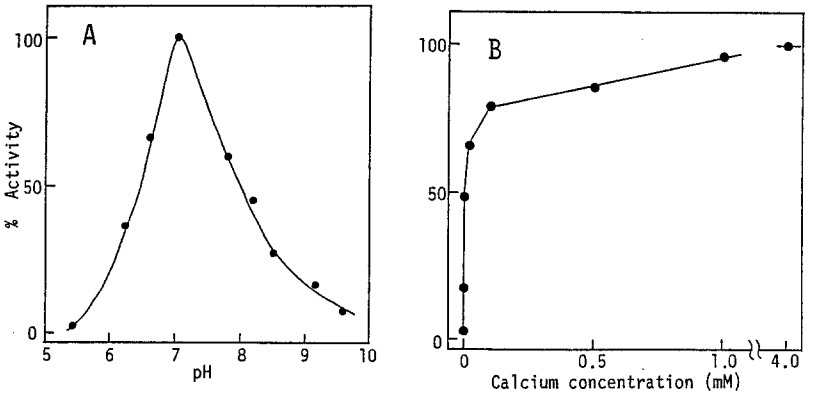

Fig. 3. (A) Effect of pH on enzyme activity. (B) Effect of calcium concentration on enzyme activity. EGTA-$CaCl_2$ buffers were used. Free calcium concentration was calculated by using a dissociation constant of 4.2×10^{-6} M (14).

precursor processing in vivo. The enzyme may possibly be involved in proteolytic processing at Pro-Arg signal (7,8) as well as paired basic signal.

The effects of pH and various reagents on enzyme activity were examined using Boc-Gln-Arg-Arg-MCA as a substrate. The optimal pH for enzyme activity was around 7.0 (Fig. 3A). Table 3 summarizes the effects of various reagents. Serine-protease inhibitors, such as PMSF and DFP, and general trypsin inhibitors, such as TLCK, STI, Trasyrol and pAPMSF, had no effect on enzyme activity. The enzyme was inhibited by thiol-directed reagents, such as iodoacetate, iodoacetamide, pCMB and heavy metal ions (Hg^{2+}, Zn^{2+}, Cu^{2+}), indicating that a thiol group is required for enzyme activity. The enzyme was also inhibited by EDTA and EGTA. The inhibitory effect of EGTA was restored by

Table 3. Effects of various reagents on the enzyme activity

	Effector		Activity		Effector		Activity
(A)	control(+0.5mM $CaCl_2$)		100		STI	100 µg/ml	97
	PMSF	1 mM	107		Trasyrol	100 µg/ml	96
	DFP	1 mM	106		leupeptin	100 µg/ml	88
	TPCK	1 mM	105			1.7 mg/ml	79
	TLCK	1 mM	106		antipain	100 µg/ml	72
	ICH_2COONa	1 mM	16			1.7 mg/ml	61
	ICH_2CONH_2	1 mM	15		chymostatin	100 µg/ml	100
	pCMB	1 mM	0		pepstatin A	100 µg/ml	101
	NEM	1 mM	117		elastatinal	100 µg/ml	111
	DTT	1 mM	2		phosphoramidon	50 µg/ml	119
	β-ME	1 mM	96		bestatin	100 µg/ml	104
	L-cystein	1 mM	95		amastatin	100 µg/ml	105
	EDTA	1 mM	2		E-64	100 µg/ml	103
	EGTA	1 mM	4				
	o-PT	1 mM	88	(B)	control (none)		100
	pAPMSF	1 mM	98		1mM EGTA		4
	pABA	1 mM	100		2mM $CaCl_2$		179
	$ZnCl_2$	1 mM	4		2mM $MgCl_2$		92
	$HgCl_2$	1 mM	0		1mM EGTA + 2mM $CaCl_2$		175
	$CuSO_4$	1 mM	9		1mM EGTA + 2mM $MgCl_2$		5

Protease activity was measured as described in "MATERIALS AND METHODS". The enzyme was preincubated with the reagent at 30°C for 30 min in the presence of 0.5 mM $CaCl_2$ (A) or in the absence of $CaCl_2$ (B). In the reactivation experiments in (B), the enzyme was inactivated with 1 mM EGTA at 30°C for 30 min, and then incubated with $CaCl_2$ or $MgCl_2$ as above.

the addition of an excess amount of $CaCl_2$, but not by the addition of $MgCl_2$ (Table 3(B)). Fig. 3B shows the effect of calcium concentration on enzyme activity. The enzyme is half-maximally activated at approximately 3-5 μM Ca^{2+}. The low Ca^{2+} requirement for activation of the enzyme suggests that a local concentration of Ca^{2+} may regulate the action of the enzyme in yeast cells. Enzyme activity was inhibited by 1 mM DTT, but not by 1 mM β-ME or L-cystein. Among the microbial inhibitors, leupeptin and antipain partially inhibited enzyme activity, but others had no effect.

The enzyme identified in this study appears to be a unique calcium-dependent thiol protease distinct from pancreatic trypsin and other putative prohormone processing proteases reported (1-3). The enzyme in this study has some overlapping properties with calcium-dependent neutral proteases (calpains) found in various mammalian and avian tissues (9,10), but it is distinct from calpains because calpains are known to be located in the cytosol. Julius et al. reported that yeast kex2 mutants are defective in the activity of proteolytic processing of the precursors of α-factor and killer toxin but reintroduction of the normal KEX2 gene to these mutants restores this activity (11). They observed a protease activity cleaving on the carboxyl side of paired basic residues in the membrane preparations of the complementated mutants carrying the KEX2 gene on a multicopy plasmid (11,12). Achstetter et al. found a protease activity (proteinase yscF) with specificity toward paired basic residues in the membrane preparations of yeast mutants deficient in major vacuolar proteases(13). Although these two enzyme activities found in membrane preparations of their mutants have properties (substrate specificity, membrane-association and calcium-dependency) similar to those of the enzyme reported here, identity of these enzymes is not clear at present when any enzyme has not been isolated to homogeneity. In order to clarify the reaction mechanism of the enzyme and its function, regulation and localization in yeast cells, further purification is now going on in our laboratory.

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REFERENCES

1. Docherty, K. & Steiner, D.F. (1982) Ann. Rev. Physiol. 44, 625-638.
2. Douglass,J., Civelli,O. & Herbert,E.(1984) Ann. Rev. Biochem. 53, 665-715.
3. Loh,Y.P., Brownstein,M.J. & Gainer,H.(1984)Ann. Rev. Neurosci. 7, 189-222.
4. Kurjan,J. & Herskowitz,I. (1982) Cell 30, 933-943.
5. Mizuno,K. & Matsuo,H. (1984) Nature 309, 558-560.
6. Morita,T., Kato,H., Iwanaga,S., Takada,K., Kimura,T. & Sakakibara,S.(1977) J. Biochem. 82, 1495-1498.

7. Bostian,K.A., Elliott,Q., Bussey,H., Burn,V., Smith,A. & Tipper,D.J. (1984) Cell 36, 741-751.
8. Kangawa,K. et al. (1984) Nature 312, 152-155.
9. Imahori,K. (1982) in Calcium and cell function, vol.3, ed. Cheung,W.Y., (Academic Press, Inc., New York), pp. 473-485.
10. Murachi,T. (1983) in Calcium and cell function, vol.4, ed. Cheung,W.Y., (Academic Press, Inc., New York), pp. 377-410.
11. Julius,D., Brake,A., Blair,L., Kunisawa,R. & Thorner,J. (1984) Cell 37, 1075-1089.
12. Fuller,R., Brake,A. & Thorner,J.(1986) in Microbiology 1986, ed. Levine,L. (American Society for Microbiology, Washington,DC), pp. 273-278.
13. Achstetter, T. & Wolf, D.H. (1985) EMBO J. 4, 173-177.
14. Allen,D.G., Blinks,J.R. & Prendergast,F.G. (1976) Science 195, 996-998.