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CHARACTERIZATION OF KEX2-ENCODED ENDOPEPTIDASE FROM YEAST SACCHAROMYCES CEREVISIAE

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SUMMARY: Yeast Saccharomyces cerevisiae KEX2 gene previously isolated was characterized as the gene encoding an endopeptidase required for proteolytic processing of precursors of α-factor and killer toxin. In this study, the cloned KEX2 gene was introduced into the kex2 mutant cells and the KEX2 gene product expressed in these cells was partially purified from their membrane fraction. The enzyme preparation exhibits a calcium-dependent endopeptidase activity with a substrate specificity toward the carboxyl side of Lys-Arg, Arg-Arg and Pro-Arg sequences. The enzyme activity was inhibited by serine-protease inhibitors, such as DFP and PMSF, indicating that the KEX2 endopeptidase belongs to a serine-protease family. The optimal pH was determined to be around 5.5. Thus, the KEX2 endopeptidase was found to be a unique calcium-dependent serine-protease distinct from calpain and trypsin.

Many bioactive peptides and secreted proteins are initially synthesized as their own precursors, which are processed to mature forms by limited proteolysis, generally at sites of paired basic residues. However, little is known about the responsible endopeptidases that are involved in precursor processing in vivo (1,2). In yeast Saccharomyces cerevisiae, KEX2 gene was identified as the gene required for proteolytic processing of α-mating factor and killer toxin precursors (3). Introduction of the cloned KEX2 gene into deficient kex2 mutants restored processing activity with hydrolytic specificity toward the carboxyl side of paired basic residues (3,4), indicating that the KEX2 gene product may be an endopeptidase involved in

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

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precursor processing in vivo. The KEX2 endopeptidase was found to exhibit unique enzymatic properties, including membrane-association, calcium-dependency, and substrate specificity toward paired basic residues (3,4). Enzymes with properties similar to those of the KEX2 endopeptidase were partially purified by Wolf's group (5,6) and ours (7). In these studies (3-7), the enzyme was characterized to be a calcium and thiol-dependent neutral protease, resembling the calpains from mammalian tissues (8). However, recent nucleotide sequence analysis of the KEX2 gene showed that the amino acid sequence of the KEX2-encoded protein contains a region extensively homologous to the members of subtilisin-like serine-protease family (9,10). To clarify the molecular mechanisms of the proteolytic action of the KEX2-encoded protein, we studied its enzymatic properties. The cloned KEX2 gene was introduced into the kex2 mutant cells and the KEX2 gene product expressed in these cells was partially purified and characterized. The present study reveals that the KEX2 endopeptidase is a unique Ca^{2+}-dependent serine-protease with optimal pH at around 5.5.

MATERIALS AND METHODS

Strains and plasmid: Saccharomyces cerevisiae K16-57C (MATα leu2 trp1 ura3 kex2-8) was used for the kex2 mutant (10) and X2180-1B used for wild type. The plasmid pYE-KEX2(5.0)b, carrying the 5.0 Kb EcoRI-SalI fragment of KEX2 gene (named KEX2(5.0)), was used for transformation (10).

Medium and growth conditions: Yeast cells were grown at 30°C in SDCA medium (0.7% Bacto-yeast nitrogen base-2% dextrose-2% casamino acids), supplemented as necessary with tryptophan (20 μg/ml).

Preparation of enzyme: Yeast cells (wet weight ca. 1g) were harvested by centrifugation, washed once with 0.9% NaCl and resuspended in 5 ml of 0.1 M sodium phosphate buffer (pH 7.4). They were lysed by incubation with 2.5 mg of Zymolyase-100,000 (Seikagaku Kogyo) at 37°C for 2hr, and homogenized with a Polytron homogeneizer. After centrifugation at 80,000g for 20 min, the pellets were washed four times by resuspension in 5 ml of 10 mM Tris-HCl buffer (pH 7.0), with centrifugation each time at 80,000g for 20 min. The pellets thus obtained were extracted twice with 5 ml of 10 mM Tris-HCl (pH 7.0) containing 0.2 M NaCl and 1% Lubrol (PX type, Nakarai Chemicals). After centrifugation at 80,000g for 20 min, the supernatants were used for measurement of solubilized membrane-associated enzyme activity and protein concentration (11) (Table 1). For further purification, the membrane extracts were desalted with PD-10 column (Pharmacia), equilibrated with 10 mM Tris-HCl (pH 8.0)-0.2% Lubrol, and applied to a column of DEAE-Sepharose CL-6B (1.4 x 44 cm). The column was eluted with a linear gradient from 0 to 0.5 M NaCl in 10 mM Tris-HCl buffer (pH 8.0)-0.2% Lubrol (Fig.1). The active fractions were pooled and used for characterizing their enzymatic properties.

Enzyme assay: Protease activity was measured as follows (7,10): Twenty nanomoles of Boc-Gln-Arg-Arg-MCA were incubated at 37°C with the enzyme in 250 μl of 0.2 M Tris-HCl buffer (pH 7.0) containing 0.1% Lubrol and 1 mM EGTA in the presence or absence of 2 mM $CaCl_2$. In the experiments to characterize the enzymic properties, 0.2 M Tris-HCl buffer was replaced by 0.2 M acetate buffer (pH 5.5). The amounts of AMC released from the substrate were measured by a fluorescence spectrophotometer with excitation at 380 nm and emission at 460 nm. Calcium-dependent endopeptidase activity was evaluated after the addition of an excess amount of $CaCl_2$. One unit of enzyme 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

The 5.0 Kb EcoRI-SalI fragment of the KEX2 gene (named KEX2(5.0)) capable of complementing the kex2 mutation was isolated as previously reported (10). Introduction of the cloned KEX2(5.0) into the kex2 mutants regained both killer activity and Ca^{2+}-dependent endopeptidase activity capable of cleaving a specific fluorogenic substrate (Boc-Gln-Arg-Arg-MCA), as reported (10). The membrane fractions, prepared from the transformants carrying pYE-KEX2(5.0)b (KEX2 cells), were extracted with a buffer containing 1% non-ionic detergent Lubrol and assayed for calcium-dependent endopeptidase activity. For comparison, the relative activities of the membrane extracts from kex2 mutants and wild type cells were also measured. As clealy seen in Table 1, a remarkable increase in a Ca^{2+}-dependent endopeptidase activity was observed in the membrane extracts of KEX2 cells, indicating that the KEX2 gene encodes a Ca^{2+}-dependent endopeptidase. For further analyses, the membrane extracts obtained from the respective cells were chromatographed on a column of DEAE-Sephrose CL-6B (Fig. 1). A major calcium-dependent endopeptidase activity in the membrane extracts of KEX2 cells was observed in the elution position corresponding to that in the membrane extracts of wild type cells (Fig. 1B and 1C). No calcium-dependent endopeptidase activity was detected in the membrane extracts of kex2 mutants (Fig. 1A). These facts indicate that the calcium-dependent endopeptidase activity found in KEX2 cells is the product of the KEX2 gene introduced into the kex2 deficient cells. Furthermore, based on the chromatographic behavior, the KEX2 gene product expressed in KEX2 cells appears to have physicochemical properties similar to those of the enzyme expressed in wild type cells. The active portions, eluted in Fr. 36-40 in Fig. 1B, were pooled and characterized in this study. The enzyme preparation obtained from wild type cells, eluted in Fr. 36-40 in Fig. 1C, exhibited its enzymatic properties very similar to those of the enzyme from KEX2 cells characterized below.

Table 1. Calcium-dependent endopeptidase activity in membrane extracts of yeast cells

Strain	Total activity (units)			Total protein (mg)	Specific activity (units/mg)
	(1) EGTA	(2) EGTA+Ca	(2)-(1)		
K16-57C	5.2	5.4	0.2	23.7	0.01
K16-57C [pYE-KEX2(5.0)b]	3.2	19.8	16.6	9.73	1.71
X2180-1B	8.5	16.9	8.4	14.4	0.58

The membrane extracts were prepared as described in "MATERIALS AND METHODS". Endopeptidase activity was measured by using Boc-Gln-Arg-Arg-MCA as a substrate. Total protein was measured by the method of Lowry et al. (11).

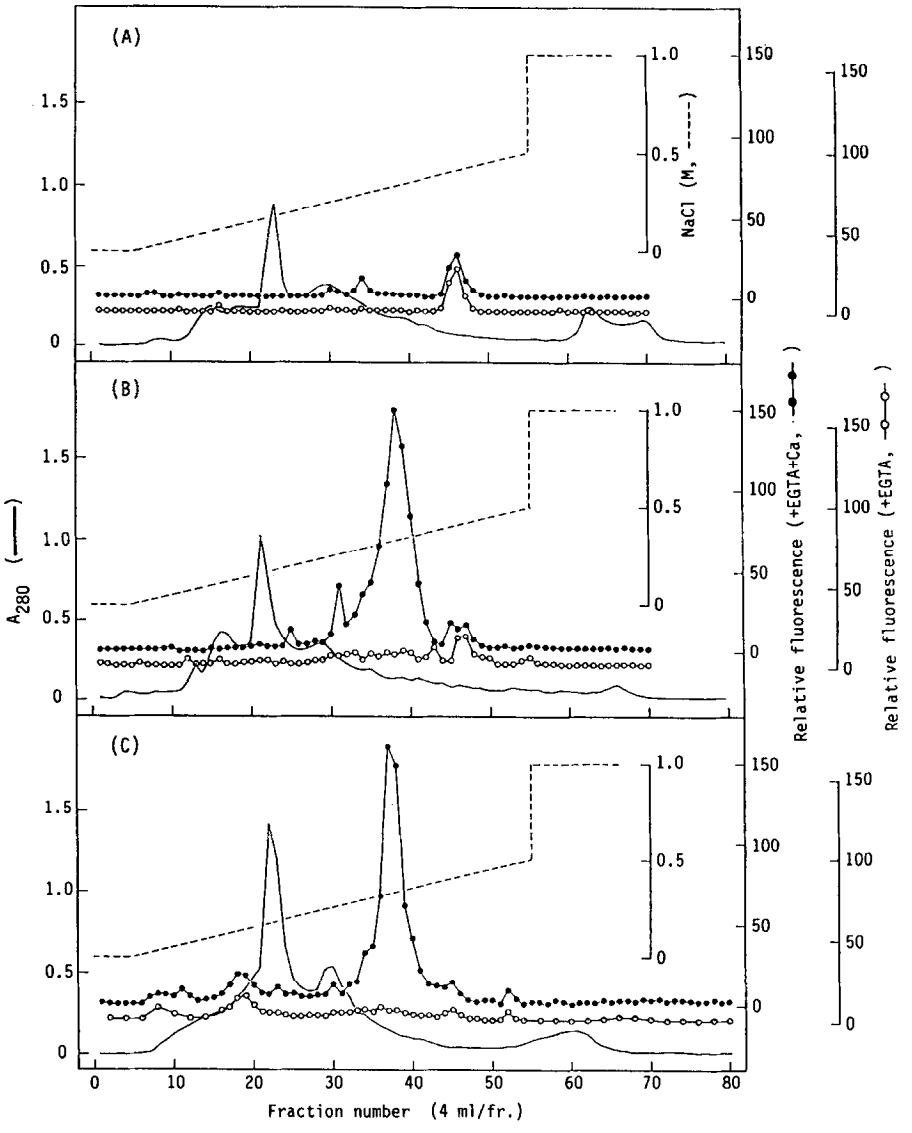

Fig. 1. DEAE-Sepharose CL-6B chromatography of the solubilized membrane-fractions obtained from (A) K16-57C (kex2 mutant), (B) K16-57C[pYE-KEX2(5.0)b] (transformant with KEX2(5.0) gene), and (C) X2180-1B (wild type). Aliquots (150 µl) from each fraction were assayed for endopeptidase activity at 37°C for 6 hr in the presence or absence of an excess amount of $CaCl_2$, as described in "MATERIALS AND METHODS".

To characterize the substrate specificity of the KEX2-encoded protease, various fluorogenic MCA-derivatives were incubated with the enzyme, and the amounts of AMC released from the substrate were measured by a fluorescence spectrophotometer (Table 2). As previously reported (7), MCA-substrates containing Arg-Arg and Lys-Arg sequences were hydrolyzed on the carboxyl side of paired basic residues, although there were some differences in reaction rate. The enzyme also cleaves two substrates containing Pro-Arg sequence on the carboxyl side of Arg residue. On the other hand, typical substrates for

Table 2. Substrate specificity of KEX2 endopeptidase

Substrate	% Activity	Substrate	% Activity
Boc-Gln-Arg-Arg-MCA	100.0	Boc-Glu-Lys-Lys-MCA	1.0
Boc-Leu-Arg-Arg-MCA	116.2	Pro-Phe-Arg-MCA	0.6
Boc-Gly-Arg-Arg-MCA	11.9	Z-Phe-Arg-MCA	0.9
Boc-Leu-Lys-Arg-MCA	92.8	Bz-Arg-MCA	0.7
Boc-Gly-Lys-Arg-MCA	59.2	Arg-MCA	0.9
Boc-Val-Pro-Arg-MCA	38.1	Leu-MCA	1.0
Boc-Ala-Pro-Arg-MCA	17.0		

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 4.5 hr as described in "MATERIALS AND METHODS". AMC released was measured by fluorescence spectrophotometer.

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 KEX2 endopeptidase exhibits the unique substrate specificity toward paired basic residues (Lys-Arg, Arg-Arg) and Pro-Arg sequence. Thus, the enzyme is likely to be involved in proteolytic processing at these signals, found in precursors of α-factor (12), killer toxin (13) and other secretory proteins.

The effects of pH and various reagents on enzyme activity were examined by using Boc-Gln-Arg-Arg-MCA as a substrate. In previous studies, the optimal pH for enzyme activity was reported to be around 7.0 (3-7). However, by using the three different buffer systems (AcONa-AcOH, Tris-HCl, glycine-NaOH), the optimal pH for enzyme activity was determined to be around 5.5 (Fig.2A). The optimal pH of 5.5 is consistent with the internal pH found in secretory vesicles (14), suggesting that the enzyme is actually operative in the vesicles in vivo. Table 3 summarizes the effects of various reagents. As

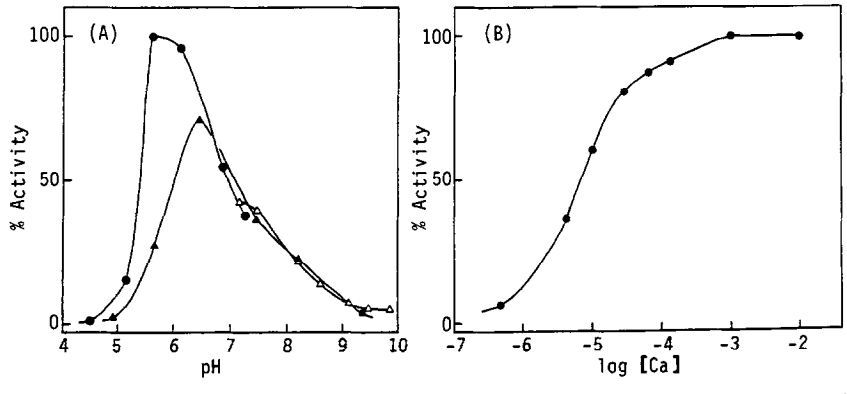

Fig. 2. (A) Effect of pH on enzyme activity. Three buffer systems (sodium-acetate (—●—), Tris-HCl (—▲—), and glycine-NaOH (—△—)) were used at 0.2 M. (B) Effect of calcium ion concentration on enzyme activity. EGTA-$CaCl_2$ buffers were used. Free calcium concentration was calculated by using a dissociation constant of 4.2×10^{-6} M (20).

Table 3. Effects of various reagents on KEX2 endopeptidase activity

Effector			Activity	Effector			Activity
(A) control(+0.5mM $CaCl_2$)			100	$ZnCl_2$	1	mM	6
DFP	1	mM	92	$HgCl_2$	1	mM	1
	3.3	mM	93	STI	0.1	mg/ml	94
	10	mM	5		1	mg/ml	75
PMSF	1	mM	98	Trasyrol	0.1	mg/ml	103
	3.3	mM	3		1	mg/ml	96
	10	mM	1	leupeptin	1	mM	24
pAPMSF	1	mM	82		10	mM	3
	3.3	mM	0	pepstatin A	1	mM	113
	10	mM	0		10	mM	15
TLCK	1	mM	105	bestatin	1	mM	98
	10	mM	37		10	mM	105
ICH_2COONa	1	mM	66	E-64	1	mM	90
	10	mM	0		10	mM	79
ICH_2CONH_2	1	mM	64				
	10	mM	15	(B) control (none)			100
pCMB	1	mM	4	+ 1mM EGTA			9
DTT	1	mM	5	+ 2mM $CaCl_2$			316
β-ME	1	mM	105	+ 2mM $MgCl_2$			72
EDTA	1	mM	4	+ 1mM EGTA + 2mM $CaCl_2$			321
EGTA	1	mM	40	+ 1mM EGTA + 2mM $MgCl_2$			9
	10	mM	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.

previously reported (3-7), the enzyme activity was not inhibited by serine-protease inhibitors, such as DFP, PMSF, and pAPMSF, at 1 mM. At a higher concentration (10 mM) of these reagents, however, the enzyme activity was completely inhibited (9). The enzyme was inhibited by thiol-directed reagents, such as iodoacetate, iodoacetamide, pCMB, and heavy metal ions (Hg^{2+}, Zn^{2+}), but typical inhibitor for thiol-protease, E-64, had no effect on enzyme activity. Thus, the KEX2 endopeptidase may be classified into a serine-protease family. Recent nucleotide sequence analysis of KEX2 gene showed that KEX2 endopeptidase contains a region extensively homologous to subtilisin-like serine-protease family and the amino acid sequence around the active site residues are well conserved (9,10). These findings, taken together, strongly suggest that KEX2 endopeptidase may function as a serine-protease in a manner similar to that of subtilisin. As discussed previously (10), inactivation of KEX2 endopeptidase by thiol-reagents may be due to the blockage of the free cystein residue (Cys-217) located immediately adjacent to a histidine residue in the active site (10).

The enzyme was also inhibited by EDTA and EGTA. The inhibitory effect of EGTA was restored by the addition of an excess amount of $CaCl_2$, but not by the addition of $MgCl_2$ (Table 3(B)). Fig.2B shows the effect of calcium concentration on enzyme activity. The enzyme was half-maximally activated at approximately 10 μM Ca^{2+}. Thus, KEX2 endopeptidase appears to be a unique

calcium-dependent serine-protease, but not related to calpains and pancreatic trypsin.

Paired basic residues are known as the sites of proteolytic processing of various bioactive peptide precursors in a wide range of eukaryotic species from yeast to mammals. Recently, calcium-dependent endopeptidases, which convert proinsulin and proalbumin to their mature products, were characterized from rat insulinoma (15,16) and liver (17,18). Although it is not clear whether these enzymes are classified into a serine-protease family or not, they seem to have some overlapping properties (membrane-association, substrate specificity, calcium-dependency and acidic pH optimum) with KEX2 endopeptidase described in this study. Furthermore, recent report showed that yeast KEX2 gene product expressed in mammalian cells was able to correctly process pro-opiomelanocortin to a set of mature peptides (19). These facts suggest a possibility that endopeptidases with properties similar to those of the KEX2 endopeptidase may be involved in precursor processing in mammalian tissues.

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