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Some characteristics of hormone (pheromone) processing enzymes in yeast

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The *KEX2* gene-encoded, membrane-bound Ca^{2+}-dependent thiol endoproteinase, proteinase yscF, responsible for processing of the precursor protein of the sex pheromone α-factor of the yeast *Saccharomyces cerevisiae* was solubilized from the membraneous fraction and partially purified. Gel filtration revealed an apparent M_r of the native protein of around 150000. Ca^{2+} concentration for half-maximal activity was in the micromolar range and concentration of the substrate Cbz-Tyr-Lys-Arg-4-nitroanilide for half-maximal velocity was 0.05 mM. The enzyme is able to cleave basic amino acids from the carboxy-terminus of peptides and probably involved in final maturation of the α-factor peptides generated by proteinase yscF is membrane-associated, active at neutral pH and responds strongly to the serine proteinase inhibitor phenylmethylsulfonyl fluoride as well as to -SH group blocking agents.

Sex pheromone; Precursor; Hormone processing; Proteinase yscF; Carboxypeptidase yscα; *(Saccharomyces cerevisiae)*

1. INTRODUCTION

The α-factor of the yeast *Saccharomyces cerevisiae* is one of two oligopeptide hormones (pheromones) triggering sexual conjugation of the haploid cell types *a* and α of this organism [1,2]. The extracellularly secreted tridecapeptide pheromone is synthesized intracellularly as a precursor protein, which undergoes proteolytic maturation [3,4]. The cloned α-factor precursor gene (*MFα1*) uncovered the possible maturation sites in the precursor protein [5]. Four repeats of the α-factor are flanked by peptide spacers each starting with the amino acid sequence Lys-Arg followed by Glu-Ala or Asp-Ala sequences [5]. Sequences of two basic amino acids have been found in mammalian hormone precursor molecules and are thought to represent the initial processing sites [6]. Using a chromogenic peptide substrate which harbors the carboxy-terminal tyrosine residue of the α-factor molecule and the consecutive Lys-Arg sequence of the spacer peptide (Cbz-Tyr-Lys-Arg-4-nitroanilide), we identified a membrane-associated proteinase splitting the peptide substrate after the arginine residue. We called the enzyme proteinase yscF [7]. Using the artificial substrate Boc-Gln-Arg-Arg-7-amido-4-methylcoumarin the same enzyme was identified [8]. Several facts are in excellent agreement with the notion that this enzyme is the initial α-factor precursor processing catalyst. (i) The enzyme is missing in a sterile mutant (kex2) which accumulates inactive α-factor precursor [7,8]. (ii) Introduction of the cloned gene coding for the proteinase into the mutant restores mating and enzyme activity [8]. (iii) The enzyme cleaves only after two basic amino acid residues [7,8]. (iv) The proteinase is able to mature human proalbumin [9]. Proteinase yscF is a Ca^{2+}-dependent thiol proteinase with a pH optimum for cleavage of Cbz-Tyr-Lys-Arg-4-nitroanilide of 7.2 [7].

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Abbreviations: Boc, butyloxycarbonyl; Cbz, benzyloxycarbonyl; HPLC, high-performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; pHMB, *p*-hydroxymercuribenzoate

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Endoproteolytic processing of the α-factor precursor by proteinase yscF leaves α-factor molecules which need further maturation at the amino- and at the carboxy-terminus. The amino-terminal processing enzyme was identified as a membrane-bound dipeptidyl aminopeptidase (dipeptidyl aminopeptidase A or dipeptidyl aminopeptidase yscIV) removing the Glu-Ala and Asp-Ala residues [10]. A membrane-associated carboxypeptidase possibly involved in removal of the carboxy-terminally remaining Lys-Arg residues after endoproteolytic cleavage of the α-factor precursor was found and called carboxypeptidase yscα [7]. Here we report on partial purification and further characterization of proteinase yscF and on further identification of the properties of carboxypeptidase yscα.

2. MATERIALS AND METHODS

For all studies strain BYS232-31-42 (*α prb1-1 prc1-1 cps1-3 his7 lys2 leu2*) defective in the unspecific vacuolar peptidases proteinase yscB, carboxypeptidase yscY and carboxypeptidase yscS was used [7,11]. Membranes of this strain were prepared as outlined in [7]. For proteinase yscF purification membranes were prepared from 25 g cells suspended in 20 mM Tris-Cl buffer, containing 0.5 mM $CaCl_2$, pH 7.2. Purified membranes were suspended in the same buffer yielding a volume of about 7.6 ml. Purification was done with 2-ml portions. Proteinase yscF was solubilized by adding per ml membrane suspension: 6.4 ml of 100 mM Tris-Cl buffer, pH 7.2; 60 μl of 1 M $CaCl_2$ and 0.35 ml Zwittergent 3-14 (10% in 100 mM Tris-Cl buffer, pH 7.2). Solubilization was brought about by shaking the mixture for 60 min at 30°C and subsequent centrifugation at 160000 $\times$ *g* for 60 min at 4°C. The supernatant contained the solubilized enzyme. The solubilized enzyme was dialyzed for 16 h at 4°C against 10 mM Tris-Cl buffer containing 10 mM $CaCl_2$, pH 7.2. The enzyme was applied onto a DEAE-Sepharose CL-6B ion-exchange column (2.25 ml gel bed per mg protein), equilibrated with 10 mM Tris-Cl buffer containing 10 mM $CaCl_2$, pH 7.2. The enzyme was eluted with a linear gradient of 0–250 mM NaCl in the same buffer. Active fractions were pooled and concentrated by centrifugation in Centriflo CF 25 cones (Amicon). Alternatively, the DEAE-Sepharose CL-6B column chromatography could be replaced by HPLC (LKB) using a TSK-DEAE 5 PW ion-exchange column (0.175 ml gel bed per mg protein). Elution of the enzyme was done with a linear gradient of 0–250 mM NaCl in 10 mM Tris-Cl buffer containing 10 mM $CaCl_2$, pH 7.2. Gel filtration was performed using Sephadex G-150 chromatography (column size 95 $\times$ 0.5 cm; 75 ml gel bed). The buffer was 20 mM Tris-Cl, 10 mM $CaCl_2$, 200 mM NaCl, pH 7.2. Standard proteins used for M_r determination were hen egg ovalbumin (M_r 43000), bovine serum albumin (M_r 67000), bovine liver catalase (M_r 232000) and horse spleen ferritin (M_r 440000).

Proteinase yscF was tested with Cbz-Tyr-Lys-Arg-4-nitroanilide as described in [7]. EGTA inhibition and Ca^{2+} reactivation of proteinase yscF in its membrane environment was done by dialyzing the enzyme for 20 h at 4°C against 1 mM EGTA in 0.1 M Tris-Cl buffer, pH 7.2, and thereafter for 4 h at 4°C in the same buffer without EGTA.

Carboxypeptidase yscα was tested as outlined in [7] by measuring the increase in fluorescence brought about by carboxyterminal liberation of amino acids from Cbz-Tyr-Lys-Arg and Cbz-Tyr-Lys and subsequent reaction with *o*-phthaldialdehyde. Final test volume was 0.4 ml. Aliquots of 20 μl were removed, diluted into 0.4 ml of 10 mM potassium phosphate buffer, pH 7.3, 0.2 ml of the fluorescence solution [7] was added. The pH optimum of carboxypeptidase yscα was determined between pH 4.8 and 8.0 with 50 mM potassium phosphate buffer adjusted to the respective pH values. The actual pH was measured in each reaction mixture. Inhibition studies on carboxypeptidase yscα were done by incubating membranes and inhibitor at pH 6.5 or 7.5 for 60 min at 30°C. Peptide substrates were from Bachem (Bubendorf, Switzerland). All other chemicals were from Calbiochem (Frankfurt), Sigma (Taufkirchen), Serva (Heidelberg) and Roth (Karlsruhe) and were of the highest purity available.

3. RESULTS AND DISCUSSION

Table 1 summarizes a typical purification scheme leading to an about 80–130-fold enriched preparation of proteinase yscF. Proteinase yscF activity increases more than 2-fold upon solubiliza-

Table 1

Purification of proteinase yscF

Purification step	Volume (ml)	Activity (mU)	Protein (mg)	Spec. act. (mU/mg)	Purification (-fold)	Yield (%)
(1) Crude extract	7	57	217	0.26	1	100
(2) Membranes	2	57	43	1.32	5	100
(3) Supernatant of centrifuged, solubilized membranes	13.8	129	19	6.8	26	226
(4) DEAE-Sepharose CL-6B chromatography	12.3	13	0.8	16.2	62	23
(5) Centriflo CF 25 concentration	1.45	9.1	0.43	21.2	81	16
(4a)HPLC TSK-DEAE 5 PW column	2.5	15	0.45	33.6	129	26

Purification of proteinase yscF and assays were performed as outlined in section 2. As the crude extract contains additional soluble Cbz-Tyr-Lys-Arg-4-nitroanilide splitting activity which overlaps with the membrane-bound proteinase yscF, only the membrane-associated Cbz-Tyr-Lys-Arg-4-nitroanilide splitting activity was taken to calculate the purification of proteinase yscF

tion with Zwittergent 3-14, a phenomenon that had been also observed upon addition of 0.1% Triton X-100 to the membrane preparation [7]. From the detergents tested for solubilization of proteinase yscF Zwittergent 3-14 proved to be the most potent. While proteinase yscF was highly stable in its membraneous environment at −20°C, the solubilized enzyme gradually lost its activity (about 50% within 5 days). The stability of the solubilized enzyme could be significantly increased upon addition of bovine serum albumin (2.5 mg/ml or more). Determination of the M_r of native proteinase yscF by gel filtration on Sephadex G-150 resulted in a value of approximately 150 000 (mean of 4 independent determinations). Depending on the preparation additional species of varying amounts of higher M_r (> 400 000) were visible after gel filtration. We consider these to be aggregation products of proteinase yscF which form under the detergent-free conditions used during these experiments.

We had shown that proteinase yscF is strongly inhibited by mercurials, Zn^{2+} and EGTA. The EGTA inhibition could be reversed by Ca^{2+} but not by Mg^{2+}, indicating that proteinase yscF is a Ca^{2+}-dependent proteinase [7]. Micromolar concentrations of Ca^{2+} are needed to regenerate 50% of the maximum activity, which can be reactived after dialysis for about 20 h at 4°C against EGTA followed by dialysis against buffer. 0.5–1 mM Ca^{2+} was needed to regenerate the maximum proteinase yscF activity possible within 30 min at 30°C after the same EGTA treatment. The high Ca^{2+} concentration needed for full reactivation of proteinase yscF may be due to a low speed of the reactivation process because of partial denaturation of the enzyme during the time of dialysis. The Michaelis constant (substrate concentration at half-maximal velocity) of proteinase yscF was determined for its substrate Cbz-Tyr-Lys-Arg-4-nitroanilide. A value of 0.05 mM was determined.

While a variety of characteristics are known for proteinase yscF [7,8]; this paper) little is known as yet about carboxypeptidase yscα, the enzyme possibly involved in further processing of the proteinase yscF generated α-factor molecules from the high M_r precursor [7]. As found for proteinase yscF, the enzyme is membrane-associated [7]. Carboxypeptidase yscα exhibits a broad pH optimum for cleavage of Cbz-Tyr-Lys-Arg, which is between pH 6 and 7.5. The activity drops dramatically to near zero when a pH value of 8 is reached. The response of carboxypeptidase yscα activity to a variety of agents known to influence proteolytic activity is shown in table 2. Carboxypeptidase yscα is strongly inhibited by PMSF and is extremely sensitive to $HgCl_2$ action. Concentrations of 10^{-7} M inactivate the enzyme nearly completely. The same inactivation pattern is found for cleavage of both

Table 2

Effect of inhibitors on carboxypeptidase yscα activity

Inhibitor	Final concentration	Activity (% of control)
None	–	100
PMSF	1 mM	<1
EDTA	0.5 mM	95
EDTA	0.5 mM	87
o-Phenanthroline	1 mM	47
$ZnCl_2$	1 mM	79
$CaCl_2$	1 mM	90
Dithiothreitol	1 mM	46
Chymostatin	5 μg/ml	72
Chymostatin	50 μg/ml	46
Antipain	5 μg/ml	69
Antipain	50 μg/ml	50
Pepstatin	5 μg/ml	73
Pepstatin	50 μg/ml	54
Leupeptin	5 μg/ml	43
Leupeptin	50 μg/ml	27
E64a	5 μg/ml	58
E64a	50 μg/ml	35
$HgCl_2$	10^{-7} M	9
$HgCl_2$	10^{-6} M	8
$HgCl_2$	2.5×10^{-5} M	<1
pHMB	10^{-6} M	16
pHMB	10^{-5} M	12
pHMB	10^{-4} M	<1

Incubations and tests were done as outlined in section 2. Stock solutions of PMSF, chymostatin, antipain, pepstatin, leupeptin and E64a were in dimethyl sulfoxide. All other inhibitors were dissolved in distilled water. Specific activity of carboxypeptidase yscα without addition was 0.69 mU/mg and was set at 100%

peptide substrates, Cbz-Tyr-Lys-Arg and Cbz-Tyr-Lys and at two different pH values of 6.5 and 7.5. This inhibition pattern suggests that carboxypeptidase yscα is a serine-thiol peptidase.

Carboxypeptidase yscα cannot be due to the well-known carboxypeptidases yscY and yscS, neither can it be due to the newly detected carboxypeptidases yscγ, yscδ and yscε [11]. The experiments were carried out in a strain devoid of carboxypeptidase yscY and yscS activities. As expected no activity against the carboxypeptidase yscY substrate Bz-Tyr-4-nitroanilide could be detected in the membrane preparation nor could any activity be detected against the peptide Cbz-Gly-Leu (not shown), which is a substrate of carboxypeptidase yscY and of carboxypeptidase yscS [12]. The membrane preparation is free of activity against the substrates of carboxypeptidase yscγ, yscδ and yscε [7]. It should be noted, however, that during this search a Cbz-Phe-Leu splitting membrane associated carboxypeptidase activity was found (1.2 mU/mg; preliminary named carboxypeptidase yscλ), which is only partially inhibited by PMSF and which is orders of magnitude less sensitive to the action of mercury as compared to carboxypeptidase yscα.

Mutants of carboxypeptidase yscα will provide proof about the participation of this carboxypeptidase in the α-factor maturation process. The present knowledge of some characteristics of carboxypeptidase yscα should facilitate the identification of such mutants.

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