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Presence of a Dipeptidyl Aminopeptidase III in *Saccharomyces cerevisiae*

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The soluble fraction of a cell extract of *Saccharomyces cerevisiae* was found to contain hydrolytic activity toward arginyl-arginyl-β-naphthylamide (Arg–Arg–βNA). Most of the contaminating Arg–βNA hydrolysis activity was removed by diethylaminoethyl (DEAE)-cellulose column chromatography and the enzyme was partially purified. It is very similar in various properties to mammalian dipeptidyl aminopeptidase III (DAP III), although it differs slightly in some respects.

Keywords dipeptidyl aminopeptidase III; cellular peptidase; non-mammalian cell; *Saccharomyces cerevisiae*

In mammalian cells, four dipeptidyl aminopeptidases have been identified. Among them, dipeptidyl aminopeptidase III (DAP III), discovered and called alkaline dipeptide naphthylamidase by Ellis and Nuenke, degrades not only arginyl-arginyl-β-naphthylamide (Arg–Arg–βNA) but also some homo-oligopeptides longer than tetrapeptides.[1] One of the most striking findings was that the enzyme exhibits N-terminal Tyr–Gly releasing activity toward enkephalins.[2] Recently, we purified DAP III from human placenta[3] and showed that hydrolysis of enkephalin, but not that of Arg–Arg–βNA, by the enzyme was strongly dependent on the presence of Co^{2+}.[4] In this study we extended our search for the presence of this enzyme to non-mammalian cells and found that yeast exhibits hydrolytic activity toward Arg–Arg–βNA. The results obtained in this study indicate that the enzyme can be classified as a DAP III.

Materials and Methods

Materials All substrates except Cbz–Arg–Arg–βNA, chromatographic resins and protease inhibitors were obtained as indicated previously [3,4]. Cbz–Arg–Arg–βNA was purchased from Bachem Feinchemikalien Co., Budendorf. All other chemicals were of the highest purity available from Wako Chemicals Co., Japan.

Cultivation *Saccharomyces cerevisiae*, strain IFO 1466, was kindly provided by Dr. K. Igarashi, Faculty of Pharmaceutical Sciences, Chiba University, Japan. Growth media were obtained from Difco, Roth, Karlsruhe, FRG. The yeast was grown aerobically on the complete medium (1% yeast extract, 2% peptone and 2% glucose) for 30 h at 28 °C to the late logarithmic phase. Cells were harvested by centrifugation at 10000 ***g*** for 10 min and then washed twice with 10 mM Tris–HCl, pH 8.0.

Enzyme Assay The reaction mixture for the determination of Arg–Arg–βNA hydrolytic activity contained 1 mM substrate, 20 mM Tris–HCl, pH 8.0, and the enzyme in a final volume of 0.2 ml. After incubation for 60 min at 37 °C, the β-naphthylamine released was estimated by the method of Goldbarg and Rutenburg.[5] In order to determine aminopeptidase activity, the reaction was performed in the presence of 1 mM Arg–βNA.

Enzyme Purification A cell extract of the yeast was prepared by grinding with alumina (100 g alumina/50 g frozen cells). The resulting cell paste was extracted with 100 ml of 10 mM Tris–HCl, pH 8.0. The alumina and cell debris were removed by centrifugation at 10000 ***g*** for 10 min, and then the supernatant was further centrifuged at 105000 ***g*** for 60 min. The soluble fraction obtained was fractionated with ammonium sulfate (40—60%), and the precipitate formed was dissolved in 10 mM Tris–HCl, pH 8.0. After dialysis against the buffer, the solution was applied to a diethylaminoethyl (DEAE)-cellulose column (Whatman DE-52; 2.5 × 30 cm), and the proteins were eluted with a linear gradient of 0 to 0.4 M NaCl in 10 mM Tris–HCl, pH 8.0 (600 ml in total). The fractions of eluate containing Arg–Arg–βNA hydrolytic activity were combined. Solid ammonium sulfate was added to 40% saturation, and then the mixture was applied to a butyl-Toyopearl 650M column (1.5 × 5 cm) which had been equilibrated with a 40% saturated ammonium sulfate solution in 10 mM Tris–HCl, pH 8.0. The column was washed with the buffer, and the bound proteins were eluted with a linear gradient of 40 to 0% ammonium sulfate in 10 mM Tris–HCl, pH 8.0 (200 ml in total). The active fractions were pooled, dialyzed against 10 mM Tris–HCl, pH 8.0, and then applied to a DEAE-Toyopearl 650S column (1.5 × 5 cm). Elution was carried out with a linear gradient of 0—0.3 M NaCl in 10 mM Tris–HCl, pH 8.0 (200 ml in total). The fraction containing hydrolysis activity toward Arg–Arg–βNA was used as the partially purified enzyme preparation. The overall yield of the enzyme was calculated to be 18.7%, indicating 91.0-fold purification. The protein concentration of the effluent from chromatographic columns was monitored at 280 nm and that of pooled fractions was determined by the method of Lowry *et al.*[6]

Results and Discussion

Enzyme Purification A preliminary survey of the yeast homogenate and soluble fraction showed that at least 95% of the hydrolytic activity toward Arg–Arg–βNA detected in the homogenate was present in the soluble fraction. Before the enzyme purification, we analyzed the activities toward Arg–βNA and Arg–Arg–βNA in the soluble fraction. High Arg-aminopeptidase activity was present (22.6 nmol/min/mg protein), and the activity was almost completely inhibited on the addition of 0.5 mM bestatin (0.14 nmol/min/mg protein). On the other hand, the activity toward Arg–Arg–βNA was very low (1.94 nmol/min/mg protein), but about one half of the activity remained in the presence of

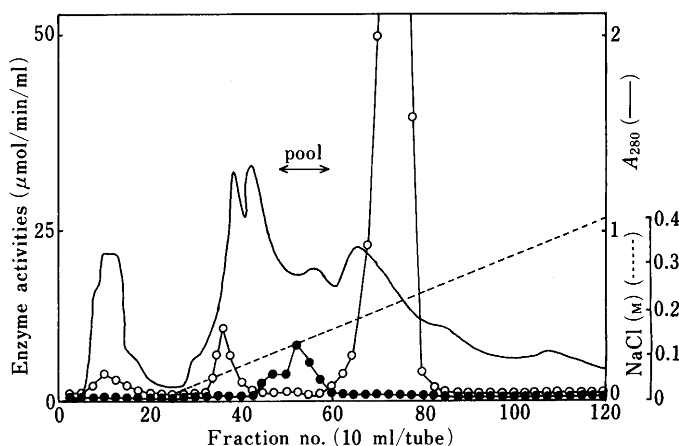

Fig. 1. Separation on DEAE-Cellulose of Enzymes Active on the β-Naphthylamides of Arg and Arg–Arg

The column was equilibrated with 10 mM Tris–HCl (pH 8.0). Elution was performed with a linear gradient of 0—0.4 M NaCl in 10 mM Tris–HCl (pH 8.0). Absorbance was monitored at 280 nm (——). DAP III activity was measured using Arg–Arg–βNA as a substrate in the presence of 0.5 mM bestatin (●). Aminopeptidase activity was measured with Arg–βNA as a substrate by a method similar to that used for measuring DAP III activity in the absence of bestatin (○). The horizontal line with arrowheads indicates the pooled fractions.

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TABLE I. Summary of Purification of Yeast DAP III

Step	Protein (mg)	Arg–Arg–βNA hydrolytic activity				Arg–βNA hydrolytic activity	
		Total activity (μmol/min)	Specific activity (nmol/min/mg)	Yield (%)	Purification (-fold)	Total activity (μmol/min)	Specific activity (nmol/min/mg)
Post-ribosomal sup.	1420	1.50	1.06	100	1	31.8	22.4
$(NH_4)_2SO_4$ fractionation	586	1.53	2.61	102	2.46	24.1	41.1
DE-52 cellulose	170	1.21	7.11	80.6	6.71	0.2	1.18
Butyl-Toyopearl	18.5	0.32	17.3	21.3	16.3	0.012	0.64
DEAE-Toyopearl	2.9	0.28	96.5	18.7	91.0	0.000	—

Starting material: 90.0 g wet weight cells.

bestatin (1.02 nmol/min/mg protein). The results indicate that the release of β-naphthylamine from Arg–Arg–βNA involved two processes, stepwise degradation by Arg-aminopeptidase and direct degradation by DAP. Arg–Arg–βNA and Arg–βNA hydrolytic activities were assayed in each fraction obtained from a DEAE-cellulose column. As shown in Fig. 1, Arg–Arg–βNA hydrolysis activity was eluted in fractions 44 to 58 and was well separated from Arg-aminopeptidase activity. The enzyme was further purified by sequential column chromatographies and the complete elimination of aminopeptidase activity was successfully accomplished (Table I). On thin layer chromatography of the reaction products obtained with the final enzyme preparation, it was found that only Arg–Arg was released from Arg–Arg–βNA (data not shown).

Catalytic Properties The effects of pH and various inhibitors on the activity of the enzyme were examined, with Arg–Arg–βNA as a substrate. The optimal pH for enzyme activity was around 8.0, considerable activity being observed in the pH range of 7.6—8.0. The IC_{50} values for effective inhibitors are shown in Table II. The enzyme was strongly inhibited by some sulfhydryl reagents, PCMB and DTNB. Metallochelators, ethylenediaminetetraacetic acid (EDTA) and *o*-phenanthroline, also inhibited the enzyme, the inhibition by EDTA being greater than that by *o*-phenanthroline. Little or no inhibition was seen in the presence of serine protease inhibitors (diisopropylfluorophosphate and phenylmethylsulfonyl fluoride) or microbial protease inhibitors (amastatin, antipain, bestatin, chymostatin, leupeptin, pepstatin and phosphoramidon). These results suggest that the enzyme is clearly distinguishable from other peptidases reported for yeast.[7] Table III shows relative rates of hydrolysis of six dipeptidyl-βNAs and N-terminal modified Arg–Arg–βNA by the enzyme. The highest activity was observed toward Arg–Arg–βNA among the dipeptidyl-βNAs examined, whereas Cbz–Arg–Arg–βNA was not hydrolyzed at all. As can be seen, Gly–Pro–βNA was not hydrolyzed by the enzyme. The involvement of a DAP in pro-α-factor processing has been reported for yeast cells.[8] The enzyme is associated with membranes, and its main characteristic is that it releases X-Pro from peptides. Therefore, the DAP examined in this study is clearly distinguishable from the latter enzyme. The hydrolytic activities of some exopeptidases are known to be stimulated by Co^{2+} *in vitro*. A carboxypeptidase involved in enkephalin processing requires Co^{2+} for maximal activity.[9] In yeast cells, an aminopeptidase, called aminopeptidase Co, is only active in the presence of Co^{2+}.[10] In our previous study, we showed the requirement of Co^{2+} for maximal enkephalin hydrolysis by human placental DAP III.[4] On the addition of 0.5 mM Co^{2+} to the reaction mixture, enkephalin hydrolysis was stimulated about 11-fold. The stimulatory effect on human placental DAP III was only detected with enkephalin pentapeptides and Tyr–Gly–Gly–Phe as substrates, and Arg–Arg–βNA hydrolysis was not sensitive to Co^{2+}. In this study, we tested the ability of Co^{2+} to stimulate the hydrolysis of Arg–Arg–βNA by the enzyme obtained in this study. In contrast with the case of the human placental DAP III, Co^{2+} stimulated Arg–Arg–βNA hydrolysis. Maximal stimulation of about 4.3-fold was observed in the presence of 0.5 mM Co^{2+}. No other divalent metal ion tested could substitute for Co^{2+}. The other metal ions slightly (Ca^{2+}, Mg^{2+}, Mn^{2+}) or strongly (Ni^{2+}, Fe^{2+}, Zn^{2+}) inhibited the enzyme. Although detailed data are not presented, enkephalin hydrolysis by the yeast enzyme was also stimulated by Co^{2+}. About 10-fold stimulation was observed in the presence of

TABLE II. IC_{50} Values for Effective Modifiers of Yeast DAP III

Inhibitor	IC_{50} values
Sulfhydryl reagents	
PCMB[a]	0.32 μM
DTNB[b]	8.0 μM
IAA[c]	4.1 mM
Metal chelators	
EDTA[d]	28 μM
o-Phenanthroline	81 μM

a) *p*-Chloromercuribenzoic acid. *b*) 5,5′-Dithiobis-(2-nitrobenzoic acid). *c*) Iodoacetic acid. *d*) Ethylenediamine tetraacetic acid. The enzyme was preincubated with each inhibitor in 20 mM Tris–HCl, pH 8.0, at 0 °C before the assay. The control values were obtained with Arg–Arg–βNA as a substrate in the absence of any modifier.

TABLE III. Substrate Specificities of Yeast DAP III

Substrate	Relative activities (%)
Arg–Arg–βNA	100
Ala–Arg–βNA	69.2
Ala–Ala–βNA	45.2
Leu–Gly–βNA	30.8
Lys–Ala–βNA	20.5
Ser–Tyr–βNA	0.02
Gly–Gly–βNA	0
Gly–Phe–βNA	0
Gly–Pro–βNA	0
Cbz–Arg–Arg–βNA	0

The relative activities were calculated with respect to the hydrolytic activity toward Arg–Arg–βNA.

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0.5 mM Co^{2+}. In many respects the yeast Arg–Arg–βNA hydrolytic enzyme resembles mammalian DAP III. However, some differences were noted, as follows. 1) The yeast enzyme optimally hydrolyzed Arg–Arg–βNA in the neutral region around pH 8.0, while the mammalian DAP III exhibited a marked preference for a somewhat alkaline region, at around pH 9.0.[1,3,11] 2) Although the yeast enzyme most rapidly hydrolyzed Arg–Arg–βNA among various dipeptidyl-βNAs, it showed a broad substrate specificity. Mammalian DAP III exhibited a high selectivity for Arg–Arg–βNA.[3,12] 3) With Arg–Arg–βNA as a substrate, the enzymes were strongly inhibited by metal chelators and sulfhydryl reactive reagents. The IC_{50} values for *o*-phenanthroline, PCMB and DTNB were closely similar to each other. On the other hand, the enzymes showed a clear difference in their response to EDTA.[3] The yeast enzyme shows a higher sensitivity to EDTA. The IC_{50} value for EDTA for the mammalian enzyme was calculated to be 7.2 mM. 4) Although Co^{2+} stimulated Arg–Arg–βNA hydrolysis by the yeast enzyme, that by the mammalian enzyme was not stimulated by any concentration of Co^{2+} tested.[4] Although we have presented evidence of the presence of DAP III in yeast cells, the molecular nature of the enzyme remains obscure, and will be the subject of further investigation.

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