A NEW X-PROLYL-DIPEPTIDYL AMINOPEPTIDASE FROM YEAST ASSOCIATED WITH A PARTICULATE FRACTION

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1. Introduction

Proteases play an important role in cellular regulation. In yeasts, ⩾7 proteolytic enzymes have been characterized, most of which are located in the vacuole, but their involvement in specific regulatory processes is still unclear (reviews [1,2]).

Studies carried out with mutants lacking one [3] or several [4,5] of the known proteases have led to the idea that other proteolytic activities must exist in yeast to account for the many biological events which seem to be associated with proteolysis. The existence of several new proteolytic activities has been reported in a mutant lacking proteinase B, carboxypeptidase Y and carboxypeptidase S [6].

Here, we report the results obtained in the search of new proteases in yeasts. We have found an activity which releases *p*-nitroaniline from substrates having the structure X-prolyl-*p*-nitroanilide (X-Pro-*p*NA, where X stands for L-alanyl of glycyl) but no *p*-nitroaniline was liberated from L-proline-*p*-nitroanilide as substrate. Thus, in this respect our enzyme resembles the dipeptidyl peptidase IV (EC 3.4.14.-) widely distributed among human tissues [7–9]. The yeast enzyme needs the presence of a free amino group for revealing its activity and thus is of aminopeptidase type. Before a wider study of substrate specificity is made we propose that it could be named as 'yeast X-prolyl-dipeptidyl aminopeptidase' or shortly yeast dipeptidyl aminopeptidase.

The activity has been found both in a strain having all known proteases and also in a yeast mutant lacking protease A, protease B and carboxypeptidase Y.

Contrary to most of the known proteases, the new activity is associated with a particulate fraction obtained after centrifugation of crude extracts at 100 000 × *g*. This property allows a clear separation from other aminopeptidase activities [10–12] described in yeast.

2. Materials and methods

L-Alanyl–L-proline-*p*-nitroanilide (L-Ala–L-Pro-*p*NA) was obtained from Bachem (Switzerland) as well as *N*-benzyloxycarbonyl-L-alanyl–L-proline-*p*-nitroanilide (*N*-CBZ-L-Ala–L-Pro-*p*NA). Glycyl–L-proline-*p*-nitroanilide tosylate (Gly–L-Pro-*p*NA) was a product from The Protein Research Foundation (Japan). L-Leucine–*p*-nitroanilide (Leu-*p*NA), L-lysine-*p*-nitroanilide (Lys-*p*NA), L-alanine-*p*-nitroanilide (Ala-*p*NA), L-proline-*p*-nitroanilide (Pro-*p*NA), 2-(*N*-morpholino) ethanesulfonic acid (Mes) and *N*-2-hydroxyethylpiperazine ethanesulfonic acid (Hepes) were all obtained from Sigma (USA). Yeast nitrogen base with amino acids was from Difco Labs. D-Glucose was a product of Merck (FRG) and all the other products were of the highest purity available.

2.1. *Yeast strains and growth conditions*

Three yeast strains were used in this work, *Saccharomyces cerevisiae* 1022 kindly supplied by Dr A. Wiemken (ETH Zurich), *Saccharomyces cerevisiae* X 2180-1B and a mutant derived from it, strain 20B-12 were generously supplied by Dr E. W. Jones [4,5].

Saccharomyces cerevisiae 1022 is a diploid wild-type strain. *Saccharomyces* X 2180-1B (α, *trp1, gal2, SUC*) is the parent strain of 20B-12 (α, *trp1, gal2, SUC, pep4-3*). Gene symbols are: α mating type allele, *trp1* requirement for tryptophan, *gal2* inability to ferment galactose and *SUC* ability to ferment sucrose. The *pep4-3* is a pleiotropic mutation that lowers the levels of

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proteinases A, B and carboxypeptidase Y to 10%, 7% and 3% of the corresponding wild-type activities [5].

Yeasts were grown in liquid medium containing 0.7% yeast nitrogen base with amino acids and 1% glucose, incubated in a rotatory shaker at 28°C and harvested when glucose had been exhausted from the medium.

2.2. *Preparation of crude extracts and differential centrifugation*

The method in [13] was applied by using the following conditions: cells were harvested by centrifugation, washed once with NaCl 0.9% and resuspended in 50 mM potassium acetate buffer (pH 5.0). To 1 g wet wt cells, 2.5 ml buffer containing 75 μl of a 25% solution of octylic alcohol in ethyl alcohol as antifoam and 7.5 g glass beads (0.45–0.50 μm diam.) were added and the mixture was shaken for 30–45 min.

Crude extracts were recovered by floating them with the aid of a 2 M sucrose solution and subjected to a 15 min centrifugation at 10 000 × *g* in a Sorvall RC-2B centrifuge. The turbid supernatant fluid (S 10 000 × *g*) was removed and centrifugated again for 90 min at 100 000 × *g* using the 75 Ti rotor of a Beckman ultracentrifuge and the corresponding supernatant (S 100 000 × *g*) and pellet (R 100 000 × *g*) were used for enzymatic analysis.

2.3. *Enzyme assays*

The new proteinase activity of yeast described here was assayed using the *p*-nitroanilides derivatives of L-Ala–L-Pro and Gly–L-Pro peptides as substrates by a modification of the method in [14] for the dipeptidyl peptidase IV of human tissues. The substrates were dissolved in 25% methanol aqueous solution in 3 mM final conc. and stored at 4°C. The incubation mixture contained 200 μl enzyme plus water, 250 μl Hepes–Tris buffer 400 mM (pH 7.0) and 50 μl X-Pro-*p*NA substrate (3 mM). The blank and standard tubes contained water and *p*-nitroaniline instead of enzyme, respectively. All tubes were incubated at 37°C and the reaction stopped by adding 500 μl 5% $ZnSO_4$ and 100 μl 7.5% $Ba(OH)_2$ [15]. The mixture was centrifugated for 10 min in a Beckman microcentrifuge and the A_{405} of the clear supernatant was measured in a Pye Unicam spectrophotometer.

One unit of X-prolyl-dipeptidyl aminopeptidase is defined as the amount of enzyme which liberates 1 μmol *p*-nitroaniline from the substrate in 1 min at 37°C in our assay conditions.

Aminopeptidases I and II were assayed essentially as in [11] using Leu-*p*NA and Lys-*p*NA as substrates, respectively.

2.4. *Protein determination*

Protein was estimated according to [16] using crystalline bovine serum albumin as standard.

3. Results

The distribution along the different cellular fractions of the X-prolyl-dipeptidyl aminopeptidase activity, as well as that of the two main yeast aminopeptidases are found in table 1 for *Saccharomyces cerevisiae* 1022. The results obtained show that ~40% of the X-prolyl-dipeptidyl aminopeptidase activity present in the crude extract is associated with a particulate fraction (R 100 000 × *g*) of the cell extract, the specific activity of the enzyme being the highest there and reaching a 3-fold purification with respect to the crude extract. To the contrary, the distribution of the two main yeast aminopeptidases shows the already known fact that both are located mainly in the soluble fraction (S 100 000 × *g*) of a cell extract.

Electron microscopic examination of the pellet obtained after centrifugation at 100 000 × *g* (R 100 000 × *g*) stained with uranyl acetate showed the presence of polyribosomal-like particles as well as membranes and mitochondrial debris.

X-Prolyl-dipeptidyl aminopeptidase activity can be detected directly in a yeast crude extract. Further, no activation was achieved by preincubation at pH 5.0 for several hours. This property clearly differentiates this new activity from the proteinases A, B and carboxypeptidase Y already described in yeast [17].

Table 2 shows the distribution of X-prolyl-dipeptidyl aminopeptidase activity in cellular fractions obtained from the haploid wild-type strain X2180-1B and from a mutant derived from it, *Saccharomyces* 20B-12, which has very low levels of protease A, B and carboxypeptidase Y with respect to the wild-type strain. It can be seen that the levels of X-prolyl-dipeptidyl aminopeptidase are very similar in both strains, showing that the *pep4-3* mutation does not affect the new proteolytic activity described here.

We have made a first attempt to characterize X-prolyl-dipeptidyl aminopeptidase regarding substrate

Table 1
Distribution of proteolytic activities in cellular fractions of *Saccharomyces cerevisiae* 1022

	Vol. (ml)	Total prot. (mg)	Total act. (units)	Spec. act. (mU/mg)	Yield (%)	Purification (-fold)
(A) X-Prolyl-dipeptidyl aminopeptidase (substrate: L-Ala–L-Pro-*p*NA)						
Crude extract	60	1488	3.12	2.1	100	1.00
S 10 000 × *g*	48	605	2.05	3.4	66	1.62
S 100 000 × *g*	46	368	0.55	1.5	17.6	0.70
R 100 000 × *g*	46	198	1.35	6.8	43.4	3.20
(B) X-Prolyl-dipeptidyl aminopeptidase (substrate: Gly–L-Pro-*p*NA)						
Crude extract	60	1488	0.17	0.11	100	1.00
S 10 000 × *g*	48	605	0.11	0.18	63.5	1.60
S 100 000 × *g*	46	368	0.03	0.08	18.2	0.73
R 100 000 × *g*	46	198	0.07	0.34	40.1	3.00
(C) Aminopeptidase I (substrate: L-Leu-*p*NA)						
Crude extract	60	1488	5.22	3.5	100	1.00
S 10 000 × *g*	48	605	3.90	6.4	74.5	1.80
S 100 000 × *g*	46	368	3.31	9.0	63.4	2.60
R 100 000 × *g*	46	198	0.37	1.8	7.0	0.50
(D) Aminopeptidase II (substrate: L-Lys-*p*NA)						
Crude extract	60	1488	1.08	0.73	100	1.00
S 10 000 × *g*	48	605	0.91	1.51	84.3	2.1
S 100 000 × *g*	46	368	1.15	3.12	106	4.3
R 100 000 × *g*	46	198	0.01	0.08	1.5	0.1

Yeast cells were grown and crude extracts obtained as in section 2. The activity of X-prolyl-dipeptidyl aminopeptidase against L-alanyl–L-propyl-*p*-nitroanilide (A) and against glycyl–L-prolyl-*p*-nitroanilide (B) as well as the activity of aminopeptidase I (C) and aminopeptidase II (D) were determined as in section 2 in fractions obtained after differential centrifugation

Table 2
Distribution of yeast X-prolyl-dipeptidyl aminopeptidase activity in cellular fractions

Cellular fraction	Spec. act. (mU/mg)		Yield (%)		Purification (-fold)	
	A	B	A	B	A	B
Crude extract	2.8	2.4	100	100	1.00	1.00
S 10 000 × *g*	3.1	2.2	60	67	1.11	0.92
S 100 000 × *g*	1.2	0.8	15	12	0.43	0.33
R 100 000 × *g*	7.8	7.2	42	41	2.78	3.00

Cell growth and differential centrifugation were carried out as in section 2. Enzyme activity was determined with L-alanyl–L-prolyl-*p*-nitroanilide as substrate: (A) *Saccharomyces* X 2180-1B; (B) *Saccharomyces* 20B-12

specificity and the results obtained are summarized in table 3. The new proteolytic activity associated with the particulate fraction did not recognize the substrate with its N-terminal group blocked by a benzyloxycarbonyl group. This leads to consider it as an aminopeptidase, excluding the possibility of being any residual activity of the carboxypeptidase S already described [6]. The enzyme does not recognize L-Pro-*p*NA as substrate and the relative activity towards L-Ala-*p*NA is very low, indicating the necessity of $\geqslant 2$ amino acids in the N-terminal end of the bond to be broken. For this reason and before a wider study of substrate specificity is made, we propose to call the enzyme X-prolyl-dipeptidyl aminopeptidase or simply dipeptidyl amino peptidase.

Table 3
Relative activity of X-prolyl-dipeptidyl aminopeptidase from *Saccharomyces cerevisiae* 1022 against various substrates

Substrate	Final conc. (mM)	Rel. act. (%)
L-Ala–L-Pro-*p*Na	0.3	100
N-CBZ-L-Ala–L-Pro-*p*NA	0.3	0
L-Leu-*p*NA	1.0	20
L-Lys-*p*NA	1.0	3
L-Pro-*p*NA	0.3	0
L-Ala-*p*NA	1.0	3
Gly–L-Pro-*p*NA · Tos[a]	0.3	5

[a] Although the specific activity of the enzyme for this substrate seems to be very low, the distribution of activity in cellular fractions (table 1) is very similar for L-Ala–L-Pro-*p*NA and for Gly–L-Pro–*p*NA indicating that probably the same enzyme acts upon both substrates but with different affinity

The particulate fraction (R 100 000 × *g*) obtained as in section 2 was used as enzyme preparation. Activity against the substrates tested was determined by measuring the liberation of *p*-nitroanilide and is expressed as munits/mg protein. Activity towards L-alanyl–L-proline–*p*-nitroanilide was taken as 100

4. Discussion

Here we describe a new proteolytic activity in the yeast *Saccharomyces cerevisiae* which we propose to call yeast X-prolyl-dipeptidyl aminopeptidase or simply dipeptidyl amino peptidase according to the chemical structure of the substrates it hydrolyzes. The activity was found to be present in a mutant strain lacking protease A, B and carboxypeptidase Y [5] and could not be activated by prolonged incubation at pH 5.0 contrary to the above proteases [17]. These two facts together demonstrate that dipeptidyl amino peptidase is different from proteases A, B and carboxypeptidase Y described in yeast.

The situation concerning aminopeptidases in yeast is far from being clear: four activities have been separated after starch gel electrophoresis [10], one of which is quite well characterized and has been called 'vacuolar aminopeptidase' [10] or aminopeptidase I [11]. Unfortunately, there is a great discrepancy in the nomenclature of the 3 remaining aminopeptidases [11,12], one of which seems to be a dipeptidase. In any case, all of them are located in the soluble fraction (S 100 000 × *g*) of a cell extract while X-prolyl-dipeptidyl aminopeptidase is undoubtedly associated with a particulate fraction which by electron microscopic examination shows the presence of polyribosomal-like particles and membrane debris. The only proteolytic activity of yeast so far described which seems to be associated with a membranous fraction is carboxypeptidase S [2]. The fact that X-prolyl-dipeptidyl aminopeptidase needs the presence of a free N-terminal group for revealing its activity shows that it is not a residual activity of carboxypeptidase S.

Thus, our results clearly indicate that X-prolyl-dipeptidyl aminopeptidase is a new yeast proteinase. To our knowledge this is the first yeast aminopeptidase described to be associated with a particulate fraction of a cell extract.

Studies to further characterize this activity and to define its cellular localization are in progress. These studies will help to elucidate its physiological role which according to its particulate nature could be associated with membrane translocation.

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