Localization of the thermosensitive X-prolyl dipeptidyl aminopeptidase in the vacuolar membrane of *Saccharomyces cerevisiae*

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Most of the X-prolyl dipeptidyl aminopeptidase activity of *Saccharomyces cerevisiae* was found to be associated with purified vacuolar membranes (specific activity approx. 75-times higher than in the protoplast lysate). The tonoplast-bound enzyme is thermosensitive. Another heat-resistant enzyme was found in the protoplast lysate. The tonoplast-bound thermosensitive enzyme shows an apparent K_m of 0.06 mM against L-alanyl-L-prolyl-*p*-nitroanilide while the heat-resistant enzyme shows an apparent K_m of 0.4 mM against the same substrate.

Yeast *Dipeptidyl aminopeptidase* *Proteinase* *Vacuole* *Membrane-bound proteinase* *Tonoplast*

1. INTRODUCTION

Yeast vacuoles can be identified by their contents of polyphosphate [1], basic amino acids [2], *S*-adenosyl-L-methionine [3] some proteinases and other hydrolytic enzymes (review [4,5]). Like animal lysosomes [6] yeast vacuoles appear to play an important role both in metabolite storage and intracellular digestion (review [7]).

In contrast to most of the known proteases from yeast which are soluble [8,9] X-prolyl dipeptidyl aminopeptidase activity (X-prolyl DPAPase) was found to be preferentially associated to the membrane fraction [10]. Interestingly enough, a membrane-bound heat-stable DPAPase activity has been proposed to be implicated in α-factor processing in normal yeast cells [11].

This paper shows that most of the membrane-bound X-prolyl DPAPase from *S. cerevisiae* is localized in the vacuolar membrane and that this activity is thermosensitive.

2. MATERIALS AND METHODS

2.1. *Strains and culture conditions*

The diploid wild type strain LBG 1022 of *S. cerevisiae* (kindly supplied by Dr A. Wiemken, ETH, Zurich) was grown in liquid medium containing 0.7% yeast nitrogen base with amino acids and 1% glucose in a rotatory shaker at 28°C. Cells were collected at the late exponential phase of growth before glucose exhaustion.

2.2. *Chemicals*

DEAE-dextran, dextran sulfate and Ficoll 400 were products of Pharmacia. Helicase and cytohelicase were from Industrie Biologique Française, Villeneuve la Garenne. Zimolyase 60000 was from Kirin (Japan). L-Alanyl-L-proline-*p*-nitroanilide was a product of Bachem, Switzerland.

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Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; X-prolyl DPAPase, X-prolyl dipeptidyl aminopeptidase

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2.3. *Preparation of yeast protoplasts*

Protoplasts were obtained from log-phase yeast cells pre-treated as in [12] and resuspended in 0.6 M sorbitol, 20 mM Mes–Tris (pH 6.0) containing 2.4% (w/v) lyophilized helicase, 5% (v/v) cytohelicase and 0.004% Zymolyase 60000. Conversion to protoplasts was usually complete within 45–60 min. Protoplasts were freed from lytic enzymes and debris by a 20 min centrifugation at 4000 × *g* over 0.6 M sucrose, 1.5% Ficoll, 20 mM Mops–Tris (pH 7.0) in a swing-out type of rotor. The pelleted protoplasts were washed twice with cold 0.6 M sorbitol, 20 mM Mops–Tris (pH 7.0) to eliminate Ficoll.

2.4. *Preparation of vacuoles and vacuolar membranes*

Protoplasts were disrupted as in [13] to liberate the intact vacuoles. The vacuoles were purified by sucrose-Ficoll gradients as follows: the protoplast lysate was mixed with an identical volume of a cold solution containing 0.6 M sucrose, 2.5% Ficoll, and 20 mM Mops–Tris (pH 7.0). Thereafter, 15 ml of the diluted protoplast lysate (phase II, middle) were charged over 10 ml of 0.6 M sucrose, 2.5% Ficoll and 20 mM Mops–Tris (pH 7.0) (phase III, bottom). Finally, 5 ml of 0.6 M sorbitol (phase I, upper) was charged over phase II and the whole centrifuged at 2500 × *g* in a swing-out type of rotor for 60 min. After centrifugation vacuoles floated in phase II, while lipid granules floated in phase I and protoplast and debris were found in the pellet. Phase I was carefully eliminated with a vacuum pump and phase II was collected and diluted to 6 vols with 0.6 M sorbitol and 20 mM Mops–Tris (pH 7.0). Diluted phase II was centrifugated for 20 min at 2700 × *g*. Lipid granules and contaminants remaining in the supernatant were removed by vacuum aspiration. The pellet of purified vacuoles was gently resuspended in a solution containing 0.6 M sorbitol and 20 mM Mops–Tris (pH 7.0), counted (haemocytometer) and centrifuged again at 7000 × *g* for 30 min.

Finally the pelleted vacuoles were resuspended in water, briefly sonicated and the vacuolar lysate centrifuged at 100000 × *g* for 60 min to obtain the vacuolar membranes and the soluble vacuolar content.

2.5. *Enzyme assays*

X-Prolyl dipeptidyl aminopeptidase was assayed as in [10]. α-Mannosidase was determined by the method of [14] modified as follows: the standard reaction mixture (0.4 ml) contained 25 mM sodium citrate (pH 6.5), 0.5% sodium deoxycholate, 0.125 mg/ml *p*-nitrophenyl-α-D-mannopyranoside and enzyme. The assay was performed at 30°C and the reaction was stopped by adding 0.1 ml 5% $ZnSO_4$ and 0.1 ml 6% $Ba(OH)_2$ [15]. After adding 0.4 ml of 0.2 M Na_2CO_3 and centrifugation, the absorbance of the liberated *p*-nitrophenol was determined at 405 nm in the clear supernatant.

Carboxypeptidase Y activity was measured by the method of [16] modified as follows: the standard reaction mixture (0.6 ml) contained 100 mM Tris–HCl (pH 7.6), 0.5% sodium deoxycholate, 1 mM benzoyl-L-tyrosine-*p*-nitroanilide dissolved in dimethylformamide (4.17% final conc.) and enzyme. The reaction was stopped and liberated *p*-nitroaniline measured as indicated above for α-mannosidase. NADPH-cytochrome *c* reductase [17], mitochondrial and plasma membrane ATPase [18], protease B [19] and α-glucosidase [20] were assayed by published methods. Protein was determined as in [21].

3. RESULTS

The combination of the DEAE-dextran procedure of [22] with glucose addition as indicated in [3] allowed us to isolate a highly purified vacuolar fraction from a concentrated protoplast suspension (1×10^9/ml). This minimized the volumes to handle during the purification steps. The distribution of subcellular fractions was monitored for both vacuolar and protoplast lysate by measuring the following marker enzymes: carboxypeptidase Y for the vacuolar sap [13,23], α-glucosidase for the cytosol compartment [20], α-mannosidase for vacuolar membranes [24], NADPH-cytochrome *c* reductase for microsomes [25], azide-sensitive ATPase for mitochondria [18] and vanadate-sensitive ATPase for plasma membranes [26].

Table 1 shows that the recovery of carboxypeptidase Y in the vacuolar fraction was about 36% and that its specific activity was increased 89-fold, while the recovery of α-mannosidase, a tonoplast marker [24], was about 15% and its increase in

Table 1

Distribution of marker enzyme activities and X-prolyl dipeptidyl aminopeptidase in vacuolar and protoplast lysates

Enzyme	Protoplast lysate		Vacuole lysate		Ratio (B/A)	Recovery (b/a × 100)
	Spec. act. (A[a])	Total act. (a[b])	Spec. act. (B[a])	Total act. (b[b])		
α-Glucosidase	104.0	73.1	3.5	0.01	0.05	0.02
Carboxypeptidase Y	12.3	8.65	1100.0	3.15	89.2	36.4
α-Mannosidase	6.5	4.53	230.0	0.67	36.1	14.8
NADPH-cytochrome *c* reductase	208.0	146.3	78.3	0.22	0.4	0.1
Azide-sensitive ATPase	51.8	35.7	23.3	0.06	0.3	0.2
Vanadate-sensitive ATPase	62.8	44.1	26.6	0.07	0.4	0.2
X-Prolyl dipeptidyl aminopeptidase	58.3	40.8	1900.0	5.43	32.7	13.3
Total protein (mg)	701		2.8			

[a] A and B (μkat/kg protein)
[b] a and b (nkat/s)

Assays were carried out as described in section 2

specific activity was 36-fold. The lack of quantitative correlation between the subcellular distribution of carboxypeptidase Y and α-mannosidase may result either from the existence of α-mannosidase in subcellular sites other than the vacuole or, alternatively, from partial inhibition of carboxypeptidase Y in the protoplast lysate by its cytoplasmic inhibitor [27]. The good correlation found between the yield of α-mannosidase activity and the yield of vacuoles favors this last explanation.

The recoveries of the 4 other marker enzyme activities in the vacuolar fraction were found to be less than 0.2% of their total activities as measured in the protoplast lysate. These results indicate that the vacuole fraction was virtually free from cytosolic contamination as well as from mitochondria and other membranous contamination.

X-Prolyl dipeptidyl aminopeptidase activity shows a very similar distribution in the subcellular fractions when compared with that of the tonoplast-bound [24] α-mannosidase, i.e., 33-fold enrichment and 13% recovery in the vacuolar lysate. Moreover, the vacuolar membrane fraction isolated from highly purified vacuoles contained 77 and 76%, respectively, of the vacuolar α-mannosidase and X-prolyl DPAPase activities. Less than 4% of the carboxypeptidase Y and protease B activities, known to be localized in the soluble compartment of vacuoles [13], were recovered in the vacuolar membrane fraction (table 2). Vacuolar membranes showed approx. 2–3-fold higher specific activity of X-prolyl DPAPase than that found in vacuolar lysates. This correlates well

Table 2

Distribution of X-prolyl dipeptidyl aminopeptidase and other vacuolar enzymes into the vacuolar sap and vacuolar membranes

Enzyme	Total activity		
	Whole vacuolar lysate	Vacuolar supernatant	Vacuolar membrane
Carboxypeptidase Y[a]	3.15	2.91	0.06
α-Mannosidase[a]	0.67	0.06	0.52
Protease B[b]	273.1	226.1	4.13
X-Prolyl dipeptidyl aminopeptidase[a]	5.43	0.97	0.90
Protein (mg)	2.8	1.9	0.9

[a] nkat/s
[b] units

Vacuoles were disintegrated as described in section 2 and the resulting vacuolar membranes and supernatant were recovered

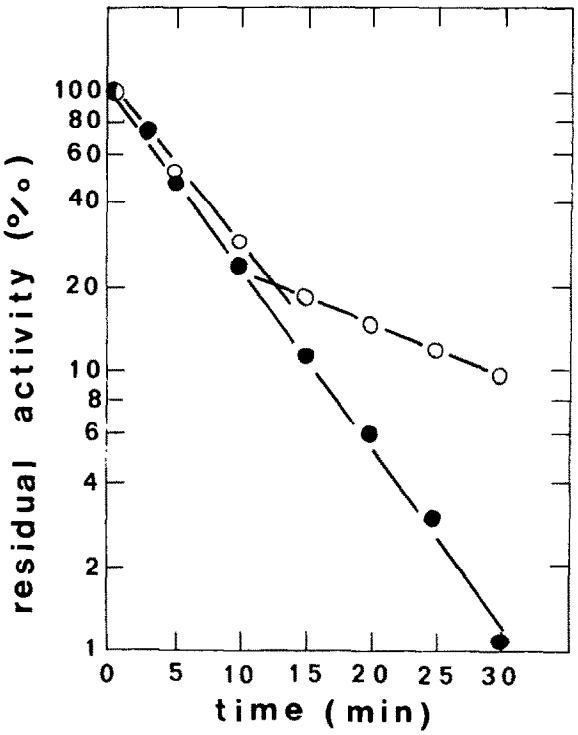

Fig.1. Heat inactivation of X-prolyl dipeptidyl aminopeptidase activity. Samples were preincubated at 60°C without substrate for the time indicated, quickly cooled and equilibrated at 37°C for 5 min before the assay. Enzymatic activity was measured as indicated in section 2. (○—○) Protoplast lysate; (●—●) purified vacuolar membranes.

with the enrichment here found for α-mannosidase in vacuolar membranes. These findings clearly suggest that most of the X-prolyl DPAPase from yeast is bound to the vacuolar membrane.

The recent implication of a membrane-bound heat-stable DPAPase activity in α-factor processing in yeast [11] led us to study the heat stability of X-prolyl DPAPase both in the protoplast lysate and the purified vacuolar membranes. The results in fig.1 show that in whole protoplast lysates two activities differing in heat-stability can be detected. These activities are likely to correspond to DPAPases A and B reported in [11]. Only the heat-labile X-prolyl DPAPase activity can be detected in purified vacuolar membranes. Moreover the two activities can be further distinguished by their affinity for the substrate L-alanyl-L-proline-*p*-nitroanilide. The apparent K_m value is 0.4 mM for

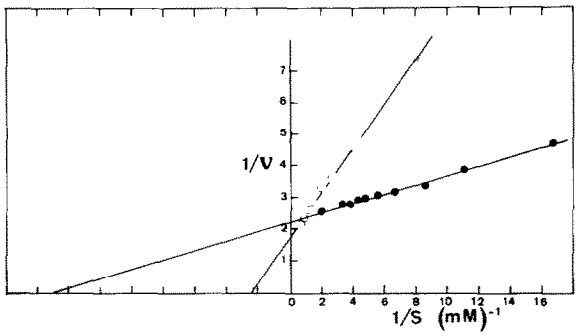

Fig.2. Lineweaver–Burk plot of the activity of X-prolyl dipeptidyl aminopeptidase against L-alanyl-L-proline-*p*-nitroanilide concentration. The enzymatic activity was measured at 37°C in 100 mM Hepes–Tris buffer (pH 7.0) and expressed as the increase in absorbance at 405 nm. (○—○) Heat-resistant X-prolyl DPAPase (protoplast lysate preincubated prior to substrate addition at 60°C for 30 min). K_m app., 0.4 mM. (●—●) Heat-labile X-prolyl DPAPase (purified vacuolar membranes). K_m app., 0.06 mM.

the heat-resistant activity (as measured in a protoplast lysate fraction preincubated at 60°C for 30 min prior to substrate addition, in order to inactivate the heat-labile activity) and 0.06 mM for the heat-labile activity bound to the purified tonoplasts (fig.2).

4. DISCUSSION

Results reported here indicate that most of the thermosensitive X-prolyl dipeptidyl aminopeptidase activity in *S. cerevisiae* is bound to the vacuolar membrane. First, the distribution of this activity in protoplasts and whole vacuoles is very similar to that found for α-mannosidase which has been shown to be localized in the vacuolar membrane [24]. Second, most of the α-mannosidase and X-prolyl DPAPase activities sediment with the vacuolar membranes after mild sonication of purified vacuoles whereas the soluble protease B and carboxypeptidase Y activities [13] remain in the soluble fraction (table 2). The small amount of activity which remains soluble after vacuole disruption and centrifugation may result from X-prolyl DPAPase liberation induced by sonication or it may correspond to other soluble DPAPase activities (see below). These results make the thermosensitive X-prolyl DPAPase a suitable marker enzyme for tonoplasts.

Although other DPAPase activities have been recently described for yeast [28] the activity localized preferentially in the vacuolar membrane appears to be the most abundant DPAPase present in yeast cells.

The functional significance of X-prolyl DPAPase associated with the yeast tonoplast is unknown. One possibility could be related to the fact that proteins localized inside the vacuole are synthesized as larger precursors which are processed before reaching their final destination ([29,30]; reviews [7,31]). The tonoplast-bound X-prolyl DPAPase may participate in the proteolytic processing of the precursors of vacuolar hydrolytic enzymes. A heat-stable form of DPAPase activity has been proposed to play a role in the processing of the mating α-factor in yeast [11,32]. However, it is unlikely that the tonoplast-bound X-prolyl DPAPase activity could participate in such processing because of its thermolability.

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