Vacuoles are not the sole compartments of proteolytic enzymes in yeast

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Localization in vacuoles, the lysosome-like organelle of yeast, was checked for several newly detected proteolytic enzymes. While aminopeptidase Co and carboxypeptidase S were found in vacuoles, proteinase D and proteinase E as well as a variety of other proteolytic activities detectable with the aid of chromogenic peptide substrates do not reside in this cell compartment.

Yeast *Mutant* *Compartmentation* *Vacuole* *Endopeptidase* *Exopeptidase*

1. INTRODUCTION

The vacuole has been proposed to represent the lysosome of the yeast cell [1]. In fact, most of the proteolytic enzymes previously found in yeast are localized in the vacuole [2–4]. The concept of the vacuole being a digestive cell compartment was supported by recent genetic studies. Mutants lacking the two vacuolar endoproteinases A and B exhibited a considerably lowered turnover of intracellular protein [5–8]. When using mutants which were devoid of one or more of the known proteinases, we were able to detect a variety of new proteolytic enzymes in yeast [9–16]. While some of the newly detected proteolytic activities are found in vacuoles, others are not. This finding raises the question of whether vacuoles are not the sole digestive compartment or whether the proteolytic enzymes not located in vacuoles serve highly specific functions.

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Abbreviations: Nan, 4-nitroanilide; Ac, acetyl; Bz, benzoyl; Cbz, benzyloxycarbonyl; MeO, methoxy; Suc, succinyl; Tos, tosyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid; n.d., not determined; unless otherwise stated amino acids were of L-configuration. Genetic markers: *pra1*, proteinase A deficiency; *prb1*, proteinase B deficiency; *prc1*, carboxypeptidase Y deficiency; *cps1*, carboxypeptidase S deficiency; *ade*, adenine auxotrophy

2. MATERIALS AND METHODS

2.1. *Chemicals*

Bz-Pro–Phe–Arg-Nan and Tos-Gly–Pro–Arg-Nan were a generous gift of Drs P. Ries and L. Svendsen, Pentapharm AG (Basel). All other chromogenic peptide substrates used were from Bachem AG (Bubendorf). α-Casein was from Sigma (Taufkirchen). [^{3}H]Formaldehyde (85 mCi/mol) was obtained from New England Nuclear (Dreieich). All yeast growth media were from Difco (Roth, Karlsruhe). Ficoll was purchased from Pharmacia (Freiburg). Sorbitol was from Roth. Pepstatin and chymostatin were from the Peptide Institute (Osaka). *Helix pomatia* juice was from IBF Société Chimique Pointet-Girard (Villeneuve-la-Garenne). Glusulase was obtained from Endo Laboratories (Garden City). Zymolyase 60000 was purchased from Seikagaku Kyogo (Tokyo). Aminopeptidase M and all other chemicals (of highest purity available) were obtained from Merck (Darmstadt).

2.2. *Yeast strains and growth conditions*

Strains 4545/III-2-1A (*a prc1*) [17] and ABYS1 (*a pra1 prb1 prc1 cps1 ade*) (B. Mechler and D.H.

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Wolf, unpublished) were used in these experiments. Strains were grown on YPD medium (1% yeast extract, 2% peptone, 2% glucose) for 16–17.5 h at 30°C until the glucose was exhausted.

2.3. *Preparation of vacuoles*

Spheroplasts were prepared as in [18] with some modifications. Cells were harvested by centrifugation, washed once with distilled water and thereafter with 0.1 M Tris–SO_4 buffer, pH 9.2. 12 g of cells (wet wt) were suspended in 0.1 M Tris–SO_4 buffer, pH 9.2 (5 ml/g of cells) containing mercaptoethanol (0.185 ml/g of cells). The suspension was gently shaken for 12 min at 30°C. After centrifugation for 5 min at 6000 rev./min (Sorvall centrifuge, SS34 rotor) cells were washed twice with 10 ml 1 M sorbitol dissolved in distilled water. Cells (11.2 g) were suspended in 1 M sorbitol (2.5 ml/g of cells), 5% *H. pomatia* juice, 0.5% glusulase and 0.04% Zymolase 60000. This mixture was shaken at 30°C up to 45 min. Spheroplasts formed were pelleted for 5 min at 6000 rev./min (Sorvall centrifuge, SS34 rotor) and washed twice with 100 ml 1 M sorbitol. Vacuoles were prepared combining published protocols of [19] and [20]. When vacuoles of strain 4545/III-2-1A (*a prc1*) were prepared, spheroplasts (10 g) were lysed by osmotic shock by suspending them in 12% Ficoll and 0.1 M sorbitol dissolved in 10 mM Mes–Tris buffer, pH 6.8 (10 ml/g of spheroplasts). Lysis was facilitated by gently pressing the mixture twice through a 50-ml syringe (step 1). The lysed spheroplasts (20 ml) were transferred into centrifuge tubes and overlayed with 7% Ficoll in 10 mM Mes–Tris buffer, pH 6.8 (18 ml). Centrifugation was done at 12000 rev./min for 35 min (Sorvall centrifuge, AS 4.13 rotor). The float containing the vacuoles was removed (step 2). The crude vacuoles were further purified by adjusting the float with a 13% Ficoll solution (in 10 mM Mes–Tris buffer, pH 6.8) to a final concentration of 10% Ficoll and subsequent centrifugation (20 min; 4500 rev./min, Sorvall centrifuge, AS 4.13 rotor) of 4 ml of this mixture on which a 7% Ficoll solution in 10 mM Mes–Tris buffer (pH 6.8) had been layered on top (step 3). The float appearing which contained the vacuoles was diluted with 0.5 M sorbitol in 5 mM Mes–Tris buffer, pH 6.8 (1:5, v/v), and layered on top of a cushion containing 25 ml of 0.25 M sucrose and 0.25 M sorbitol dissolved in 7.5 mM Mes–Tris buffer (pH 6.8) and centrifuged as above (step 4). The pellet containing the purified vacuoles was used for testing. As a reference, the lysate (step 1) was tested without any purification.

When strain ABYS1 was used for preparation of vacuoles, spheroplasts and spheroplast lysates were prepared and step 2 of the vacuole preparation was done as outlined above, except that 0.1 M sorbitol in the lysis buffer was omitted. The subsequent steps of the vacuole isolation of strain ABYS1 were modified as follows. The float of step 2 containing the vacuoles was removed and diluted 1:5 (v/v) with 0.5 M sorbitol dissolved in 5 mM Mes–Tris buffer (pH 6.8). The suspension was centrifuged for 15 min at 4500 rev./min (Sorvall centrifuge, rotor AS 4.13) (step 3). The pellet containing the vacuoles was resuspended in 0.5 M sorbitol dissolved in 5 mM Mes–Tris buffer (pH 6.8). The resuspended vacuoles (3 ml) were layered on top of a solution (6 ml) containing 0.25 M sucrose and 0.25 M sorbitol in 7.5 mM Mes–Tris buffer (pH 6.8). They were centrifuged for 15 min at 4500 rev./min (Sorvall centrifuge, rotor AS 4.13) (step 4). The pellet contained the purified vacuoles.

2.4. *Protein determination and enzyme assays*

Published methods were used to assay protein [21], α-mannosidase [22], aminopeptidase I [23], aminopeptidase Co [12], carboxypeptidase S [9], glucose-6-phosphate dehydrogenase [24] and [^{3}H]methylcasein splitting activity [25] at pH 7.3 in the presence of 20 mM $MgCl_2$. ATP (10 mM) effect on the [^{3}H]methylcasein splitting activity was tested in the presence of an ATP regenerating system (phospho*enol*pyruvate (10 mM) and pyruvate kinase (20 units)) [15]. Proteolytic cleavage of aminoterminally blocked peptidyl-4-nitroanilides was tested in two modifications [15]. One modification (A in table 3) measures proteolytic cleavage of the 4-nitroanilide bond by direct liberation of 4-nitroaniline, which is detected spectrophotometrically at 405 nm. The other modification (B in table 3) measures proteolytic cleavage of the peptide derivatives at a site other than the 4-nitroanilide bond. Visualization of the cut is possible after addition of aminopeptidase M, which liberates 4-nitroaniline from the aminoterminally unblocked peptidyl-4-nitroanilide generated. For test conditions and definition of units

see [15]. When strain 4545/III-2-1A was used in the experiments spheroplast lysis and tests were done in the presence of chymostatin (50 μg/ml) and pepstatin (50 μg/ml) to inhibit unspecific proteolysis due to the action of proteinase A and proteinase B.

3. RESULTS AND DISCUSSION

At first we investigated the intracellular localization of two of the newly detected exopeptidases, carboxypeptidase S [9,10] and aminopeptidase Co, an enzyme strongly dependent on Co^{2+} [12]. To allow determination of carboxypeptidase S activity without interference by the vacuolar carboxypeptidase Y, we isolated vacuoles from a mutant devoid of carboxypeptidase Y activity [9,17]. In all experiments described here α-mannosidase, an enzyme of the vacuolar membrane [22], and aminopeptidase I [2] were used as marker enzymes of the vacuole. Glucose-6-phosphate dehydrogenase activity was tested to allow calculation of cytoplasmic contamination of the vacuoles prepared [26]. When vacuoles were prepared, aminopeptidase Co and carboxypeptidase S were found to be enriched together with the two vacuolar marker enzymes (table 1). Proof that carboxypeptidase activity measured in the vacuole fraction was actually due to carboxypeptidase S and not due to carboxypeptidase Y, which might have appeared upon reversion of the *prc1* mutation, came from inhibition studies. The enzyme activity was completely inhibited by the chelating agent EDTA (not shown), which is a potent inhibitor of carboxypeptidase S but not of carboxypeptidase Y [9]. Zn^{2+} (10^{-4} M) nearly completely inhibited the Co^{2+}-stimulated Lys-Nan splitting activity in the vacuolar fraction (not shown), a typical characteristic of aminopeptidase Co [12].

Using chromogenic peptide substrates and [^{3}H]methylcasein, we have been able to uncover a variety of new proteolytic enzymes in mutants lacking 3 or 4 of the vacuolar proteinases – proteinase A, proteinase B, carboxypeptidase Y and carboxypeptidase S [11,15]. Two of these enzymes have been characterized in more detail. Proteinase D has been shown to represent the major activity in the mutant deficient in proteinases A and B and the carboxypeptidases Y and S. The enzyme splits Bz-Pro–Phe–Arg-Nan at some site other than the 4-nitroanilide bond [13]. Proteinase E is essentially the only enzyme cleaving Cbz-Gly–Gly–Leu-Nan

Table 1

Aminopeptidase Co and carboxypeptidase S activities in a cell lysate and the vacuole fraction of *Saccharomyces cerevisiae* strain 4545/III-2-1A

Enzyme tested	Specific activity (munits·mg^{-1}) Fraction		Ratio vacuole:lysate
	Lysate	Vacuole	
Aminopeptidase Co	1.24	25.00	20.2
Carboxypeptidase S	8.10	134.51	16.6
Aminopeptidase I	3.21	67.55	21.0
α-Mannosidase	0.24	4.98	20.8
Glucose-6-phosphate dehydrogenase	45.30	4.30	<0.1

Strain 4545/III-2-1A was grown, spheroplast lysate and vacuoles were prepared as outlined in section 2. Aminopeptidase Co was tested using 180–360 μg protein (spheroplast lysate) and 4.8–9.6 μg protein (vacuole lysate). Carboxypeptidase S was tested using 58–116 μg protein (spheroplast lysate) and 3.2–6.4 μg protein (vacuole lysate). Aminopeptidase I was tested using 90–180 μg protein (spheroplast lysate) and 7.2–14.4 μg protein (vacuole lysate). Protein included in the α-mannosidase test was as outlined for the aminopeptidase Co test. Glucose-6-phosphate dehydrogenase was tested using 9 and 18 μg protein (spheroplast lysate) and 4.8–9.6 μg protein (vacuole lysate)

in this mutant. It also cleaves Cbz-Ala–Ala–Leu-Nan. Cleavage occurs at the 4-nitroanilide bond [13,16]. We investigated whether proteinase D and proteinase E, as well as proteolytic activities easily detectable against other substrates tested [15] are located in the vacuole or whether proteolytic activities can be found outside of this digestive organelle. Results are summarized in table 2. As can be seen, a clear enrichment, together with the vacuolar marker enzyme α-mannosidase, is found for the Tos-Gly–Pro–Arg-Nan and Bz-Ile–Glu–Gly–Arg-Nan splitting activities in the presence of aminopeptidase M. When MeO-Suc–Ala–Ala–Pro–Met-Nan, Bz-Ile–Glu–Gly–Arg-Nan, and Tos-Gly–Pro–Arg-Nan were tested without aminopeptidase M added and Bz-Phe–Val–Arg-Nan was tested with and without aminopeptidase M, partial enrichment of proteolytic activity in the vacuole fraction indicates the presence of at least two different proteolytic enzymes active against each substrate, one of which is of vacuolar origin, the other not. No significant enrichment in the vacuolar fraction is found for activities which split Ac-Ala–Ala–Pro–Ala-Nan, MeO-Suc-Ala–Ala–Pro–Val-Nan, MeO-Suc-Ala–Ala–Pro–Met-Nan, Suc-Ala–Ala–Pro–Phe-Nan, Suc-Phe–Leu–Phe-Nan and Bz-Pro–Phe–Arg-Nan in the presence of aminopeptidase M, indicating that the major activity(ies) splitting these substrates is (are) not located in the vacuole. However, because the ratio of specific activity found in the lysate against all these substrates under the test conditions employed does not drop to the ratio found for the cytoplas-

Table 2

Proteinase activities in the cell lysate and the vacuole fraction of *Saccharomyces cerevisiae* strain ABYS1

Substrate tested	Enzyme	Specific activity (munits · mg^{-1})					
		Fraction				Ratio vacuole : lysate	
		Lysate		Vacuole			
		A	B	A	B	A	B
Ac-Ala–Ala–Pro–Ala-Nan	not characterized	0.63	6.40	0.02	5.65	0.03	0.88
Cbz-Gly–Gly–Leu-Nan	proteinase E	0.27	0.29	<0.02	<0.02	<0.07	<0.07
Cbz-Ala–Ala–Leu-Nan	proteinase E	0.31	0.63	<0.02	<0.02	<0.06	<0.03
MeO-Suc-Ala–Ala–Pro–Val-Nan	not characterized	n.d.	0.94	n.d.	1.38	–	1.47
MeO-Suc-Ala–Ala–Pro–Met-Nan	not characterized	0.4	3.88	1.66	3.98	4.15	1.03
Suc-Ala–Ala–Pro–Phe-Nan	not characterized	0.08	0.65	<0.02	1.20	0.25	1.85
Suc-Phe–Leu–Phe-Nan	not characterized	0.20	0.16	<0.02	0.18	0.10	1.13
Bz-Ile–Glu–Gly–Arg-Nan	not characterized	0.20	0.21	0.44	1.49	2.20	7.10
Bz-Phe–Val–Arg-Nan	not characterized	0.14	0.45	0.46	1.43	3.29	3.17
Bz-Pro–Phe–Arg-Nan	proteinase D	0.18	2.15	<0.02	1.92	0.11	0.89
Tos-Gly–Pro–Arg-Nan	not characterized	0.23	0.15	1.08	1.39	4.70	9.26
[^{3}H]Methylcasein	not characterized	631[a]		611[a]		0.97	
	α-mannosidase	0.13		1.26		9.70	
	aminopeptidase I	3.40		47.60		14.00	
	glucose-6-phosphate dehydrogenase	72.90		3.89		0.05	

[a] cpm · min^{-1} · mg^{-1}

Strain ABYS1 was grown, spheroplast lysate and vacuoles were prepared as outlined in section 2. For test of enzymes splitting chromogenic peptide substrates 29–348 μg protein (spheroplast lysate) and 4.2–21 μg protein (vacuole lysate) were included in the test. For measuring [^{3}H]methylcasein activity 160 μg protein (spheroplast lysate) and 60 μg protein (vacuole lysate) were included in the test. Protein included in the α-mannosidase, aminopeptidase I and glucose-6-phosphate dehydrogenase tests is outlined in the legend to table 1. (A) Test without addition of aminopeptidase M; (B) test in the presence of aminopeptidase M. For details see section 2

mic marker enzyme glucose-6-phosphate dehydrogenase (table 2), the possibility exists that the value calculated represents the presence of minor additional activities splitting these substrates which are located in the vacuole. Evidence has actually been presented that several enzymes splitting a given chromogenic substrate exist in yeast [11,15]. Also [^{3}H]methylcasein splitting activity [15] is not enriched in the vacuolar fraction. We had detected an ATP and polyvalent anion-stimulated caseinolytic activity [15]. The residual caseinolytic activity found in the vacuole fraction (table 2) is inhibited by ATP indicating the presence of the ATP-stimulated activity outside the vacuole. Clearly the two characterized enzymes proteinase D (activity found against Bz-Pro–Phe–Arg-Nan in the presence of aminopeptidase M) and proteinase E (activity found against Cbz-Gly–Gly–Leu-Nan and Cbz-Ala–Ala–Leu-Nan in the absence of aminopeptidase M) are not enriched in the vacuolar fraction, indicating their location outside of this digestive organelle.

The results presented show that the vacuole is not the sole locus of proteolytic enzymes. The question remains to be answered whether the proteolytic enzymes found outside the vacuole are actually digestive enzymes responsible for protein degradation or whether these proteinases serve highly specific functions. Recently two proteolytic enzymes have been found in mitochondria [27,28] which are involved in the specific event of processing of mitochondrial protein precursors.

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