Simultaneous isolation of the yeast cytosol and well-preserved mitochondria with negligible contamination by vacuolar proteinases

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Disruption of yeast spheroplasts by DEAE-dextran in isoosmotic conditions allows isolation of relatively undamaged subcellular fractions from yeast. The preservation of mitochondria and vacuoles permits the simultaneous isolation of the cytosol with negligible contamination by vacuolar proteinases and therefore, virtually eliminates proteolytic artefacts.

Yeast *Proteinase* *Vacuole* *Cytoplasm* *Proteolysis* *Mitochondria*

1. INTRODUCTION

Studies of cytoplasmic and mitochondrial enzyme systems are seriously hampered by the simultaneous presence of vacuolar proteinases released during cell disruption by the usual mechanical methods. Cross-contamination of the cytosol by soluble enzymes liberated by other damaged organelles is also a major problem.

Because of our interest in the study of proteinases characteristic of the cytoplasmic fraction it became necessary to obtain the cytoplasmic sap uncontaminated by proteinases of vacuolar origin. Here, we describe a novel and simple method which greatly limits cross-contamination of the cytoplasmic fraction by both vacuolar and soluble mitochondrial enzymes. Disruption of yeast spheroplasts from strain 1022 in isoosmotic conditions is based on methods for vacuole isolation in [1,2]. The cytosol thus obtained was found to be virtually free of the vacuolarly located proteinases A and B [3,4]. Two other vacuolar proteinases, carboxypeptidase Y and aminopeptidase V showed only low activity. In addition, L-lactate dehydrogenase (flavocytochrome b_2 [5]), an enzyme present in the intermembrane space in mitochondria [6], was found to be practically absent from the soluble cytoplasmic sap obtained in our conditions, indicating that the external mitochondrial membrane is undamaged. Moreover, excellent preservation of the cytochrome *c* content was found in mitochondria isolated from another yeast strain grown in conditions favoring mitochondrial development.

As shown here, this method can be applied to other yeast strains after a brief exploration of the conditions adequate for the DEAE-dextran-mediated isoosmotic rupture of the corresponding spheroplasts. The cytoplasmic sap thus obtained would be equivalent to that obtained from an isogenic, pleiotropic mutant lacking all vacuolar proteinases, ribonuclease and other hydrolases.

2. MATERIALS AND METHODS

Helicase and cytohelicase were obtained from IBF, Zymolyase 60000 from Kirin (Takasaki), Azocoll from Calbiochem Behring (France) and hemoglobin and phenyl methyl sulfonyl fluoride

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(PMSF) from Merck (FRG). Antipain is a product from the Protein Research Foundation (Japan). L-leucine-*p*-nitroanilide (LeuNA), lysine-*p*-nitroanilide (LysNA), L-lactate, NADH, α-ketoglutarate, N-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA), *p*-nitrophenyl α-D-glucoside, *p*-nitrophenyl phosphate, pepstatin A and buffer substances were supplied by Sigma (St Louis MO). DEAE-dextran, dextran-sulfate and Ficoll were products from Pharmacia (France). All other products were of the highest purity available.

2.1. *Yeast strains and growth conditions*

Saccharomyces cerevisiae 1022, a diploid wild-type strain, was supplied by Dr A. Wiemken (ETH, Zürich). It was grown aerobically at 28°C in a synthetic liquid medium containing 2% glucose [7] and harvested at the late exponential phase of growth before glucose exhaustion. *Saccharomyces cerevisiae* 777-3A, a haploid strain, α, ade1 op1 [8,9] was supplied by Dr P. Pajot (CGM, Gif-sur-Yvette). It was grown aerobically at 28°C in YP Gal medium (1% yeast extract, 1% bactopeptone, 2% galactose). The culture was collected at 4.5 g wet wt cells/l.

2.2. *Preparation of crude cytoplasmic extracts*

Crude extracts were obtained by brief sonication of a spheroplast pellet resuspended into 20 mM Mops–Tris buffer (pH 7.0). After centrifugation at 100000 × *g* 45 min at 4°C the supernatant was used as a crude cytoplasmic extract.

2.3. *Preparation of spheroplasts*

Cells (strain 1022, 1 g wet wt) washed once with 0.9% NaCl were pre-treated [10] at 30°C for 10 min with 5 ml 0.6 M sorbitol containing 10 mM EDTA, 5 mM dithiothreitol and 100 mM Tris (pH 8.0). Thereafter, cells were washed once with 0.6 M sorbitol, 20 mM 2-(*N*-morpholino)ethane sulfonic acid (Mes) (pH 6.0) and 1 g wet wt cells resuspended in 2.5 ml 0.6 M sorbitol, 1% (w/v) cytohelicase (2.5% helicase can be used alternatively), 0.02% (w/v) zymolyase 60000 and 20 mM Mops–Tris (pH 7.0). Although after 40 min incubation at 30°C conversion into spheroplasts was usually complete, incubation was continued for an additional 10 min. Spheroplasts were freed of the lytic enzymes by a 15 min centrifugation at 2700 × *g* over 0.6 M sucrose, 1.5% Ficoll, 20 mM Mops–Tris (pH 7.0) in a swing-out type of rotor and washed twice with cold buffer 1 [0.6 M sorbitol, 20 mM Mops–Tris (pH 7.0)] to eliminate Ficoll.

2.4. *Isoosmotic lysis of spheroplasts by DEAE-dextran*

The spheroplasts were disrupted by combining the procedures in [1,2]. The spheroplasts pellet was resuspended into cold buffer 1 and adjusted to 5×10^8 spheroplasts/ml; 50 μg DEAE-dextran/10^8 spheroplasts was added to this suspension and after 1 min at 0°C, 12.5 μg dextran-sulfate/10^8 spheroplasts was added. Finally, after a further 1 min at 0°C, glucose was added to 50 mM final conc. and the suspension incubated at 30°C. Rupture of spheroplasts and vacuole liberation followed by phase-contrast microscopy was usually 80% after 10–15 min. Thereafter, the lysate was rapidly chilled. Longer incubations are to be avoided to prevent premature rupture of the liberated vacuoles.

2.5. *Preparation of the purified cytoplasmic extract and the crude mitochondrial pellet*

Unless otherwise indicated all centrifugations were carried out at 2°C. The cold isoosmotic lysate of spheroplasts was centrifuged for 15 min at 1000 × *g* in the HS.4 rotor (swing-out type) of an RC.2B Sorvall centrifuge. The pellet (P_1) containing vacuoles, spheroplasts and cellular debris was retained. The supernatant (S_1), which does not contain vacuoles, was then centrifuged for 10 min at 12000 × *g* to obtain the crude mitochondrial pellet (P_{12}). The supernatant (S_{12}) was finally centrifuged for 45 min at 100000 × *g*. The supernatant (S_{100}) was carefully freed of the upper lipid layer by vacuum aspiration and recovered without removing a small fluffy layer resting over the ribosomal pellet. The purified cytoplasmic extract thus obtained was used as such or dialyzed against 20 mM Mops–Tris (pH 7.0) to eliminate the sorbitol when necessary.

2.6. *Preparation of the purified mitochondrial fraction*

2.6.1. From strain 1022

Purification is carried out starting with the crude P_{12} mitochondrial pellet essentially as in [11] for the enzymatic method.

2.6.2. From strain 777-3A

Spheroplasts from strain 777-3A were obtained by suspending the unwashed cells (0.4 g wet wt/ml) in 1.2 M sorbitol, 30 mM Mops–Tris (pH 7.0), 2.5% (w/v) Helicase, 0.04% zymolyase 60000 (1 mg/g wet wt). Conversion to spheroplast (controlled by phase contrast microscopy) was complete after 30 min at 30°C, but incubation was continued for an additional 10 min. Spheroplasts were harvested by centrifugation, washed once, and resuspended in cold 1.2 M sorbitol, 30 mM Mops–Tris (pH 7.6) and adjusted to 1×10^9 spheroplasts/ml. Then, in the cold, DEAE-dextran was added (25 μg/10^8 spheroplasts) and, after 1 min at 0°C, Dextran-sulfate (6 μg/10^8 spheroplasts). Finally, after a further 1 min at 0°C the spheroplast suspension was added of an equal volume of 0.1 M sorbitol, 50 mM glucose, 0.2% BSA and 10 mM Tes–Tris (pH 7.6) pre-warmed at 30°C. Rupture of spheroplasts was complete after 3 min at 30°C. Thereafter, the cold lysate was centrifuged for 15 min at 1000 × g in the HS-4 rotor, the supernatant saved and centrifuged for 15 min at 12000 × g to obtain the mitochondrial pellet.

2.7. *Low-temperature spectrophotometry*

Determination of the cytochrome spectra was carried out with a frozen mitochondrial suspension at the temperature of liquid nitrogen [12].

2.8. *Preparation of the purified vacuolar fraction*

The pellet P_1 from the first centrifugation of the isoosmotic homogenate was carefully resuspended in 0.6 M sucrose, 2.5% Ficoll, 20 mM Mops–Tris (pH 7) (3 ml/g wet wt of the original cell pellet) and then to it was added an equal volume of 0.6 M sorbitol and 20 mM Mops–Tris (pH 7). This suspension was topped with 5 ml 0.6 M sorbitol, 20 mM Mops–Tris (pH 7.0) and centrifuged in the HS-4 rotor (RC-2B Sorvall centrifuge) at 2500 × g for 60 min at 2°C. After vacuum aspiration of the upper phase the lower phase containing the vacuoles was removed and poured over 6 vol. 0.6 M sorbitol and 10 mM Mops–Tris (pH 7.0). The diluted vacuoles were centrifuged for 20 min at 2700 × g at 2°C in a swing-out rotor. The quasi-transparent pellet of vacuoles was resuspended in 10 mM Mops–Tris (pH 7) and briefly sonicated to obtain the vacuolar extract.

2.9. *Enzyme assays*

Proteinase A was assayed using denatured hemoglobin as in [13] with the activation step and modifications in [14]. One unit of enzymatic activity corresponds to the amount of trichloroacetic acid soluble peptides liberated after 1 h incubation at 37°C, equivalent to 1 g bovine serum albumin. Proteinase B was measured using Azocoll by a modification [15] of the method in [16]. One unit of proteinase B activity corresponds to a change of 1 A unit at 520 nm/h in a 1-ml final vol. after trichloroacetic acid precipitation. Activation of proteinase B with pepsin was performed as indicated in [15]. Carboxypeptidase Y was assayed essentially as in [17] as modified in [14]. One unit of activity corresponds to 1 μmol p-nitro-aniline produced/min, assuming a molar absorbance of 9900 [18]. Aminopeptidases II and V activities were determined essentially as in [19], modified as in [14]. Units of enzymatic activity are defined as for carboxypeptidase Y. L-lactic dehydrogenase was assayed with L-lactate using ferricyanide as acceptor as in [5]. L-glutamate dehydrogenase was measured with NADH as in [20]. Alkaline phosphatase assay and units were as described in [21]. α-Glucosidase assay and units were as in [22].

3. RESULTS AND DISCUSSION

3.1. *Comments on the isoosmotic rupture method*

Rapidity in obtaining spheroplasts is important for good preservation of the vacuoles after the isoosmotic lysis. Also, complete digestion of cell wall residues is essential for an efficient isoosmotic lytic step. A further 10 min incubation after 100% conversion into spheroplasts (phase-contrast microscopic observation) is enough to fulfill the requirement discussed above. Ficoll and sucrose interfere when present during the lytic step. Thus, when spheroplasts are purified by sedimentation through sucrose and Ficoll the spheroplast pellet obtained should be washed twice with sorbitol before the treatment with DEAE-dextran. Finally, removing the vacuoles from the isoosmotic homogenate by centrifugation in a swing-out rotor at low speed, is important to avoid vacuole rupture and thus, proteinase contamination of the homogenate.

3.2. *Determination of cross-contamination of the subcellular fractions*

The contamination of the cytoplasmic extract with vacuolar proteases is minimal as seen in table 1. The vacuolar endoproteinases A and B are virtually absent. It is important to note that these activities have been determined after 'activation' of the extract [14,15] to eliminate inhibition by their peptidic cytoplasmic inhibitors. The same is true for the mitochondrial fraction. Perhaps the most valuable marker enzyme for determining soluble vacuolar contamination of a given subcellular fraction is carboxypeptidase Y. Interference by its polypeptidic inhibitor can be eliminated by measuring enzyme activity in the presence of BTPNA and dimethylsulfoxide [23]. This eliminates the need for a previous 'activation' step. Therefore, the value of 5.3% found for the carboxypeptidase Y contamination of the cytoplasmic extract probably represents the most reliable estimate of the vacuolar sap contamination. Aminopeptidase V is not as reliable since its Leu·NA substrate is also slowly hydrolyzed (even in the presence of 0.5 mM Zn^{2+}) by the cytoplasmically located aminopeptidase II. Therefore, the value of 16.3% found with this enzyme for the vacuolar contamination of the cytoplasmic extract is excessively high. Conversely, the assessment of vacuolar contamination by measuring proteinase B or A activity gives abnor-

Table 1

Distribution of enzyme activities in yeast extracts and subcellular fractions from strain 1022

Enzyme (spec. act.)	Crude cytoplasmic extract	Purified cytoplasmic extract		Mitochondrial extract	Vacuolar extract		Total recovery[b] (%)
		Spec. act.	Vacuolar[a] contamination (%)		Spec. act.	Enrichment[b]	
α-Glucosidase[c]	3.40	3.31	–	n.d.	n.d.	–	91
L-Glutamic dehydrogenase[d] (NADH)	1.21	5.22	–	1.3	Traces	–	421
L-Lactic dehydrogenase[e] (Cyt b_2)	7.9	0.9	–	19.0	–	–	21
Alkaline phosphatase[c]	260.4	48.9	12.0	42.5	21 969	84	85
Aminopeptidase II[f]	4.11	5.12	–	0.32	>0.05	–	104
Aminopeptidase V[f]	3.7	0.94	16.3	0.07	750	203	32
Protease A[g]	7.50	Traces	>0.3	0.27	158.54	21	48
Protease B[g]	1.60	0.07	2.8	n.d.	230.1	144	71
Carboxypeptidase γ[f]	0.36	0.03	5.3	n.d.	51.9	144	76

n.d. = not detected

[a] Contamination of the cytoplasmic extract (total activity) is expressed by considering the total activity of the corresponding vacuolar proteinase found in the crude extract as 100%

[b] Calculated with reference to the crude extract. For most of the vacuolar proteinases these values are influenced both by the yield of vacuoles and by the presence of their cytosolic inhibitors in the crude extract

[c] The activities of α-glucosidase and alkaline phosphatase are expressed as μmol *p*-nitrophenol . min^{-1} . mg $protein^{-1}$

[d] The activity of L-glutamic dehydrogenase was measured with NADH and expressed as nmol NADH oxidized . min^{-1} . mg $protein^{-1}$

[e] The activity of L-lactic dehydrogenase was measured with potassium ferricyanide as acceptor and is expressed as nmol ferricyanide reduced . min^{-1} . mg $protein^{-1}$

[f] Activities of aminopeptidases II, V and carboxypeptidase Y are expressed as μmol *p*-nitroanilide . min^{-1} . mg $protein^{-1}$

[g] Activities of proteinases A and B were measured directly for the vacuolar extracts and after activation (see section 2) in all other extracts. Proteinase A activity is expressed in units . mg $protein^{-1}$. Proteinase B activity is expressed in units . mg $protein^{-1}$

mally low values. This is due to the fact that proteinase B activity is partly inactivated during the activation step [14]. The same is probably true for proteinase A.

Negligible contamination of the cytoplasmic extract by soluble mitochondrial enzymes is also indicated by the very low activity of L-lactic dehydrogenase found in the cytoplasmic extract. This indicates good preservation of the mitochondria external membrane during DEAE-dextran-mediated isoosmotic lysis since this enzyme is located in the intermembrane space in mitochondria [6]. Conversely, the mitochondrial extract shows no contamination by cytoplasmic soluble enzymes or the vacuolar proteinases B and carboxypeptidase Y. A weak activity of aminopeptidase V is detected. The values found for aminopeptidase II and proteinase A open the question of their presence in mitochondria.

Contrary to the cytoplasmic α-glucosidase and aminopeptidase II activities which show the same specific activity in crude and purified cytoplasmic extract, the specific activity of glutamate dehydrogenase is increased in the purified cytoplasmic extract. This fact also alters the recovery value. This, as will be shown here, is mainly due to the absence of proteolytic degradation. However, non-specific activation by polyols [24] may also play a role in this increase because of the presence of 0.6 M sorbitol. Recovery values are influenced both by the yield of the subcellular fractions and, in the case of the vacuolar proteinases, by the presence of their cytoplasmic inhibitors in the crude extract.

3.3. *Stability of NAD-linked glutamic dehydrogenase (GDH) in crude and pure cytoplasmic extracts*

As a further test of the vacuolar contamination of the pure cytoplasmic extract obtained after DEAE-dextran disruption, we decided to test the stability of endogenous GDH. The NAD-linked, cytoplasmically located enzyme [25] has been found to be extremely sensitive to both proteinases A and B in crude cytoplasmic yeast extracts [26]. Table 2 indicates that this enzyme activity completely disappears from crude cytoplasmic extracts after 4 h incubation at either 37°C or 30°C. Degradation was strongly diminished in the presence of pepstatin A and antipain, the specific inhibitors of proteinases A and B, respectively [13]. Contrary to this, the enzyme showed excellent stability in the cytoplasmic extract under similar conditions. Good preservation of enzyme activity in the pure cytoplasmic extract was found at 4°C over 3 days while in the crude cytoplasmic extract only 45% of the original activity was found at this time.

Table 2

Thermal stability of the glutamic dehydrogenase activity in crude and pure cytoplasmic extracts

	Residual activity[a] (%)			
Incubation at 37°C	0 h	1 h	1 h + inhibitors[b]	4 h
Crude cytoplasmic extract	100	34	60	n.d.
Pure cytoplasmic extract	100	66	67	39
Incubation at 30°C				
Crude cytoplasmic extract	100	58	103	n.d.
Pure cytoplasmic extract	100	96	96	94
Incubation at 4°C	Day 0	Day 1	Day 2	Day 3
Crude cytoplasmic extract	100	92	90	87
Pure cytoplasmic extract	100	67	53	45

[a] GDH–NADH activity determined as indicated in section 2. The specific activity in the fresh crude and cytoplasmic extracts was considered as 100%

[b] Incubation run in the presence of 1 μg/ml of each antipain and pepstatin A

n.d. = not detected

Crude cytoplasmic extract corresponds to that obtained after mechanical rupture of yeast cells. Pure cytoplasmic extract is defined as that obtained after isoosmotic disruption of spheroplasts by DEAE-dextran method described here

3.4. *Spectral characteristics of mitochondria isolated after DEAE-dextran disruption of yeast spheroplasts (strain 777.3A)*

To get a further insight into the characteristics of mitochondria obtained by the DEAE-dextran disruption, we decided to estimate their cytochrome content by low temperature spectra. Mitochondrial fractions were obtained from strain 777-3A (see section 2). The low temperature spectra of the crude P_{12} mitochondrial pellet (not shown) performed as in [12] indicate excellent preservation of the cytochrome *c* content. Using the tangent between 495 and 572 nm as a baseline reference, the ratio of cytochrome *c*: cytochrome *b* is 2.167 and the ratio of cytochrome c_1: cytochrome *b* is 1.014. These results contrast with the spectra of mitochondria obtained after mechanical disruption of cells which show them to be partially cytochrome *c*-depleted [12]. However, it should be stressed that using the DEAE-dextran disruption method, the yield of mitochondria was found to be low. Thus, only 2.8 mg mitochondrial protein was obtained from 4.5 g wet wt cells.

Curiously enough, most of the mitochondria were pelleted in the first 1000 × *g*, 15 min centrifugation. Recovery of mitochondria from this pellet by washing (without EDTA) was poor. The sedimentation of mitochondria at low speed found here, is comparable to the results in [27] with normal rat liver, where a sub-fraction of endoplasmic reticulum closely associated with mitochondria could be isolated from low speed pellets. Thus, our results may indicate that an important portion of yeast mitochondria is also associated with or trapped by the endoplasmic reticulum after DEAE-dextran rupture of yeast spheroplasts.

It is important to note that although liberation of mitochondria from strain 777-3A was not done in strictly isoosmotic conditions, mitochondria appear to be unharmed as indicated above. However, in these conditions, vacuole preservation is less efficient. We have determined that about 15% of the vacuoles burst, as indicated by the measure of the carboxypeptidase Y contamination of the corresponding cytoplasmic fraction. Nevertheless, this value is quite low when compared to results obtained by mechanical disruption.

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