Yeast Permeabilization as a Tool for Measurment of *in situ* Enzyme Activity: Localization of Alkaline Phosphatase

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The biochemical and ultracytochemical localization of alkaline phosphatases in permeabilized cells of *Saccharomyces cerevisiae* 257 has been studied. The treatment with non-ionic surfactant Triton X-100 allows the penetration of the substrate into intact yeast cells and thus provides detailed detection of the enzyme activity in ultracytochemical studies.

Introduction

The determination of enzyme activities in whole yeast cells is hampered by the cell natural permeability barrier to substrates and products. A number of permeabilization procedures allow the enzyme activities to be measured under conditions resembling the *in vivo* situation, i. e., *in situ* (King *et al.*, 1991; Laouar *et al.*, 1996). Previously we have shown that the permeabilization of the yeast cells with the nonionic surfactant Triton X-100 provided high efficiency in determination of acid and alkaline phosphatases (Galabova *et al.,* 1993; Christova *et al.,* 1996). It has been shown that Triton X-100 increased, at the conditions applied the permeability of the cell to *p*-nitrophenyl phosphate, the phosphatase artificial substrate. Furthermore, the detergent action did not affect intracellular structures such as vacuoles (Galabova *et al.,* 1996). Thus, the permeabilization of yeast cells with Triton X-100 allowed the determination of enzymes, which are retained in the cells and remained in natural surroundings.

The *Saccharomyces cerevisiae* alkaline phosphatases (ALPases) are products of two structural genes (*PHO8* and *PHO13*). The *PHO8* gene product phosphatase [EC 3.1.3.1] is a non-specific alkaline phosphatase (Toh-e *et al.*, 1976) and is usually localized in the yeast vacuole (Klionsky and Emr, 1989). The enzyme, product of *PHO13* [EC 3.1.3.41], is a monomeric phosphate – irrepressible enzyme attacking *p*-nitrophenyl phosphate only (Attias and Bonnet, 1972). So far, there is no data available about the location of this enzyme.

The work described in the present paper arose from the attempts to use cell permeabilization with Triton X-100 as an way to receive more information about the alkaline phosphatase activity and its localization in *Saccharomyces cerevisiae* cells. Biochemical analysis of the localization of ALPase was carried out as a supporting part of the study.

To avoid an interference of acid phosphatases, a strain defective of constitutive and repressible forms of acid phosphatases was used.

Materials and Methods

Strain and growth conditions. S. cerevisiae strain was kindly provided by Prof. P. Venkov from the Institute of Molecular Biology (IMB), Bulgarian Academy of Science (IMB culture collection: 257). It was obtained from the Prof. A. Hinnen collection (AH:220, *MATa* leu2–3 leu2–112 his3 trp1 *pho3 pho5*). The strain was grown in 500 ml Erlenmeyer flasks with 100 ml medium under slow rotary agitation at 28°C. The medium contained 4% (w/v) Bacto-yeast extract, 2% (w/v) Bacto-pepton and 2% (w/v) glucose. Growth was followed by the change in the absorbance of the culture at 570 nm (A_{570}).

Enzyme assay. Alkaline phosphatase activity was assayed with *p*-nitrophenyl phosphate (*p*NPP) as substrate. The reaction mixture contained 0.1 ml

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enzyme sample, 0.1 ml 2 mM *p*NPP and 0.1 ml 50 mM Tris [Tris(hydroxymethyl)aminomethane]-HCl buffer (pH 8.4). After incubation at 37 °C for 15 min, the reaction was terminated with 1 ml 0.2 M NaOH and the absorbance of the *p*-nitrophenylate ion (*p*NP) at 410 nm was measured, using a molar absorption coefficient of 16200 $M^{-1}cm^{-1}$. One enzyme unit (U) was defined as the amount of enzyme releasing 1 *n*mol nitrophenol per min.

Assay for P_i. The method of Bencini *et al.* (1983) was used when *o*-phospho – DL – serine (Ser-P_i) was tested as a substrate. One unit of enzyme activity was defined as 1 *n*mol of inorganic phosphate (P_i) liberated from the substrate per min at 37 °C.

Protein was determined by the method of Bradford, using bovine serum albumin as a standard.

Permeabilization procedure was done as described in previous works (Galabova *et al.*, 1993, 1996). Briefly, exponentially grown cells (A_{570} of 1.8 = 2.98 ± 0.16 g dry cells/l) were centrifuged (6,000 x *g*, 10 min), washed twice and cell pellet was resuspended in Tris-HCl buffer (0.1M, pH 8.4). To 9 ml of suspension, 1 ml 1M solution of Triton X-100 was added. Assays were incubated with gentle shaking for 20 min at room temperature, centrifuged (3,000 x *g*, 10 min) and pellet was resuspended in a buffer to 10 ml.

Preparation of subcellular fractions. Cell disintegrates of an exponentially-grown culture was prepared as described in previous paper (Galabova *et al.*,1996) and were subjected to differential centrifugation at 3,000 x *g* (10 min, 0 °C), 11,000 x *g* (40 min, 0 °C), and 108,000 x *g* (2 h, 0 °C). The last centrifugation was done in Beckman LM ultracentrifuge (SW 55 T_i rotor). Supernatant and pellet fractions were separated and tested for the presence of alkaline phosphatase activity and protein content.

DEAE-chromatography. A sample of supernatant of centrifugation at 3,000 x *g* was dialyzed against 20 mM Tris-HCl buffer, pH 8.4 (buffer A) and was loaded on a DEAE-cellulose (DE-52) column (1.8 x 10 cm) equilibrated with the same buffer. Elution of the ALPase was achieved by a linear NaCl gradient (0–0.6 M, 60 ml) in the initial buffer. Fractions of 1.2 ml were collected at a flow rate of 25 ml.

Electron microscopy and ultracytochemistry. The samples, treated and non-treated with Triton X-100 cells were fixed preliminary in cold 6.25% (v/v) glutaraldehyde for 10 min at 4 °C and then washed with cacodylate buffer (0.1 M, pH 7.2). The cells were post-fixed in 0.25% (v/v) glutaraldehyde and 3% (v/v) formaldehyde in 0.1 M cacodylate buffer containing 0.22 M sucrose (buffer B) for 1 h at room temperature. To demonstrate alkaline phosphatase activity, the samples were incubated in assay mixture for 30 min at 28 °C after preliminary fixation. The assay mixture contained 1.4 ml 0.2 M Tris-maleate buffer (pH 8.4), 0.22 M sucrose, 2 ml 0.1 M *p*NPP, 2.6 ml 1 mM M_g SO_4 and 4 ml saturated lead citrate solution. The final pH was adjusted to 8.4 with either NaOH or HCl. After assay incubation the samples were washed with buffer B and post-fixed as described above. The controls were cells incubated in assay mixture where the enzyme substrate *p*NPP was omitted. All samples were dehydrated in a graded series of ethanol solutions and embedded in Durcupan (Fluka). The ultrathin sections were cut with a Reicher-Young ultramicrotom. Observations were carried out on a Carl Zeiss EM 10C electron microscope.

Results

DEAE-chromatography. Our previous studies on strain *S.cerevisiae* 257 revealed the existence of two known alkaline phosphatase activities, a nonspecific alkaline phosphatase encoded by the *PHO8* gene and a specific *p*-NPP phosphatase encoded by the *PHO13* gene. As shown on Fig. 1 alkaline phosphatase activities appeared as two peaks in the chromatography on DEAE cellulose, measured as *p*NPP activity and as one peak when *o*-phospho – DL – serine (Ser-P_i) was used as substrate. It is known that the specific alkaline phosphatase is active only to *p*NPP and the activity is strongly dependent on the presence of Mg^{2+} ions (Attias and Bonnet, 1972). To improve the distinction of both phosphatase activities and to receive more reliable data of the biochemical investigations, the phosphamino acid was included as an additional substrate for nonspecific ALPase. The rate of hydrolysis of Ser-P_i compared to *p*NPP was lower, and increased with the purification of the enzyme (data not shown). No other activities toward *p*NPP have been registered on DE-52 chromatography.

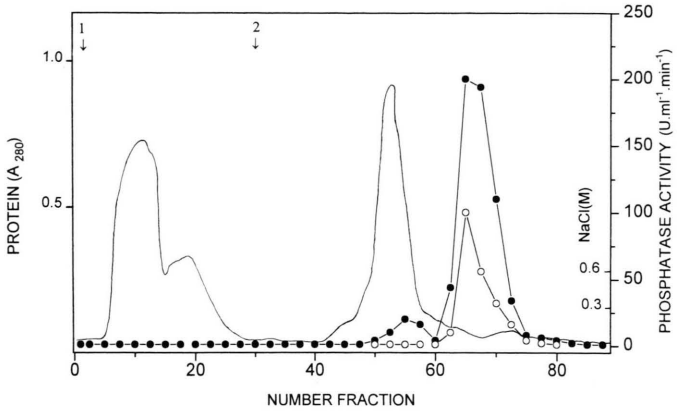

Fig. 1. Elution profiles of ALPase from DEAE-cellulose column. The samples loaded are described in Methods. ---, A_{280}; o---o, Ser-P_i-ALPase activity; •---•, pNPP – ALPase activity; 1- start of the elution with 20 mM Tris/HCl, pH 8.4, containing 0.1 M NaCl; 2- start of the salt gradient (0.1–0.6 M NaCl, 60 ml) in Tris/HCl buffer, pH 8.4.

Biochemical localization of phosphatase activities. Suspension of disrupted cells was subjected to differential centrifugation and phosphatase activities were determined in pellet and supernate fractions at each centrifugation. The results presented in Table I show that the highest specific phosphatase activity was found in the 100,000 x *g* pellet (membrane-associated fractions). The increase of specific *p*NPP-ase and Ser-P_i-ase activities when compared to the initial activities is more than 200-fold and 500-fold, respectively. This indicated that the specific and nonspecific alkaline phosphatase activities are located in vacuoles and their membranes.

Ultracytochemical localization of pNPPase activities. The observations of ultrathin sections clearly showed the reaction product in the form of combined electronically fine and, in some cases, relatively coarse and dense lead phosphate precipitates (Fig. 2 a,b). Such precipitates in form of amorphous irregular granules localized on the surface of the vacuolar membranes as well as inside the vacuoles can be observed (Fig. 2 a,b). Reaction products on the vacuolar inclusions were established as well. In a few cells electron-dense granules were located on cell walls as can be seen in Fig. 2 a. Single deposits of the reaction product were found in the cytoplasm and cytoplasmic membranes (Fig. 2 b). Specific deposition of the reaction product within the control specimens (Fig. 2 c) and in non-treated with Triton X 100 cells as well (Fig. 2 d) can not been observed.

Table I. Distribution of *p*NPPase and Ser(P_i)ase activities in fractions after differential centrifugation.

Fraction	Specific phosphatase activity* nmol $P_i \cdot (mg\ protein)^{-1}$	
	*p*NPPase	Ser(P_i)ase
Crude extract	91.4	12.7
3,000 x g		
Pellet	133.4	68.2
Supernatant	107.0	63.7
11,000 x g		
Pellet	87.1	24.3
Supernatant	135.2	39.4
100,000 x g		
Pellet	1941.1	1517
Supernatant	162.7	81.2

* Data (three determinations) were pooled to give ± SEM of within 10–15%. Mean values are given.

Discussion

Under the conditions used, the cytochemical distinction of both phoshatases in *S. cerevisiae* 257, a non- specific ALPase and *p*NPP-specific ALPase, was not possible. Two observations indicated, however, that the registered activity is mainly non-specific ALPase: i) specific ALPase presents in the yeast cells as a minor activity compared to the non-specific ALPase (Fig. 1) and, ii) at the concentrations of Mg^{2+} used the activity of specific ALPase is a low. It is well established that *pho8* ALPase is a vacuolar enzyme (Wiemken *et al.*, 1979). There was, however, some discrepancy as to association of ALPase with the vacuolar membrane. By some reports the enzyme does not appear to be membrane bound (Uchida *et al.*, 1985) while cytochemical analysis reported by other authors (Clark *et al.*, 1982) revealed that the enzyme associates exclusively with the vacuolar

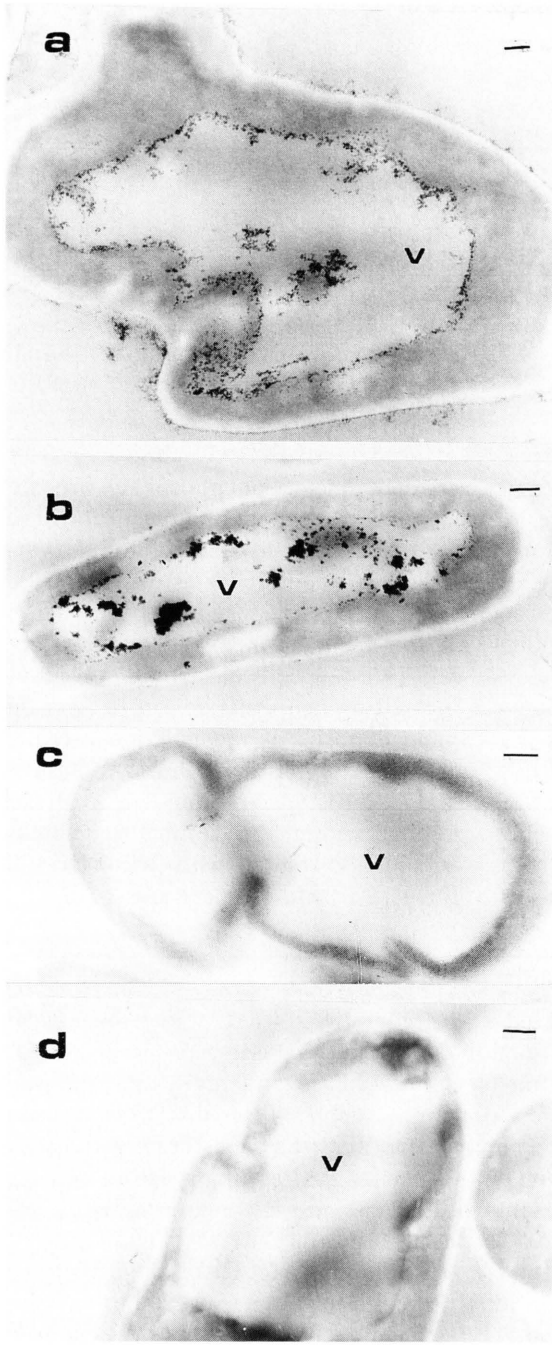

Fig. 2. A cytochemical reaction for alkaline phosphatase activity in permeabilized (a, b, c) and non-permeabilized (d) *S. cerevisiae* 257 cells. V, vacuole. All marker bars, 0.2 μm.
(a) Reaction products in vacuole , on vacuolar membrane, on cell wall, and in cytoplasm.
(b) Reaction products in vacuole , on vacuolar membrane; single reaction deposits in cytoplasm and cytoplasmic membrane.
(c) Control incubation without an enzyme substrate (pNPP).
(d) Cells non-treated with Triton X-100.

membrane. ALPase activity has been detected also in microglobules inside the nucleus, in the cytoplasm, in the membrane vesicles, and in the periplasm (Vorisek, 1989). Biochemical analysis of the fractionated cell disintegrates conducted in this study revealed that the *p*NPP phosphatase activity as well as the Ser-Pi phosphatase activity were present mainly in the membrane associated pellet. Cytochemical observations of the permeabilized with Triton X-100 cells show a presence of specific

depositions of the reaction product mainly associated with the vacuolar membrane and inside the vacuoles. There is a possibility that the observed ALPase activities on the cell membranes are due to partial solubilization of the vacuole membrane ALPase situated close to the cell surface. On the other hand, Cheng *et al.* (1971) noted that the fixation in glutaraldehyde causes a shift of ALPase from the periplasmic space to the cell surface.

It has been reported (Fishman *et al.,* 1980) that surfactants can suppress the exoenzyme formation. Cherepova and Spasova (1996) found that the detergent Lubrol W_1 (0.5 and 1%) reduced the acid phosphatase activity in some bacteria. Our results indicate, however, that the treatment of yeast cells with 0.1% Triton X-100 neither suppresses and nor inhibits ALPase.

Vorisek *et al.*(1985) have found that the prefixation of the yeast cells in cold glutaraldehyde allowed the penetration of the substrate into intact cells of *S. cerevisiae* and maintained a significant activity of the dipeptidyl aminopeptidase and alkaline phosphatase as well. Experiments carried out in this work to use prefixation in cold glutaraldehyde as the only "permeabilizing" procedure revealed that no ALPase in cells nontreated with Triton X-100 is present as found out from ultracytochemical observations.

The results from the present study show that the permeabilization of yeast cells with Triton X-100 extends the experimental possibilities to measure alkaline phosphatase activities *in situ*. The procedure allows to determine the localization of alkaline phosphatases in the cells of *S. cerevisiae*.

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Attias J. and Bonnet J. L. (1972), A specific alkaline *p*-nitrophenylphosphatase activity from baker's yeast. Biochim. Biophys. Acta **268**, 422–430.

Bencini D. A., Wild J. R. and O'Donovan G. A. (1983), Linear one-step assay for the determination of orthophosphate. Analyt. Biochem. **132**, 254–258.

Cheng K. J., Ingram J. M. and Costerton J. W. (1971), Interactions of alkaline phosphatase and the cell wall of *Pseudomonas aeruginosa*. J. Bacteriol. **107**, 325–336.

Cherepova N. and Spasova D. (1996), Ultrastructural localization of acid phosphatase in some bacteria, after treatment with Lubrol W_1. Microbios **88,** 199–204.

Christova N., Tuleva B. and Galabova D. (1996), Dependence of yeast permeabilization with Triton X-100 on the cell age. Biotechnol. Techn. **10,** 77–79.

Clark D. W., Tkacz J. S. and Lampen J. O. (1982), Asparagine-linked carbohydrate does not determine the cellular location of yeast vacuolar nonspecific alkaline phosphatase. J.Bacteriol. **152**, 865–873.

Fishman J., Rottom S. and Citri N. (1980), Preferential suppression of normal exoenzyme formation by membrane modifying agents. J. Bacteriol. **141**, 1435–1438.

Galabova D., Tuleva B. and Balasheva M. (1993), Phosphatase activity during growth of *Yarrowia lipolytica*. FEMS Microbiol. Letters **109,** 45–48.

Galabova D., Tuleva B. and Spasova D. (1996), Permeabilization of *Yarrowia lipolytica* cells by triton X-100. Enzyme Microb. Technol. **18,** 18–22.

King A. T., Davey M. R., Mellor I. R., Mulligan B. J., and Lowe K. C. (1991), Surfactant effects on yeast cells. Enzyme Microb. Technol. **13**, 148–153.

Klionski D. J. and Emr S. D. (1989), Membrane protein sorting: biosynthesis, transport and processing of yeast vacuolar alkaline phosphatase. EMBO J. **8,** 2241–2250.

Laouar L., Lowe K. C., and Mulligan B. J. (1996), Yeast responses to nonionic surfactants. Enzyme Microb.Technol. **18,** 433–436.

Toh-e, A., Nakamura, H. and Oshima, Y. (1976), A gene controlling the synthesis of non specific alkaline phosphatase in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **428**, 182–192.

Vorisek J., Schwencke J. and Kotyk A. (1985), Electron cytochemical reaction for dipeptidyl aminopeptidase in the yeast *Saccharomyces cerevisiae*. Histochem. J. **17,** 535–537.

Vorisek J. (1989), Ultracytochemical localization of the vacuolar marker enzymes alkaline phosphatase, adenosine triphosphatase, carboxypeptidase Y and aminopeptidase reveal new concept of vacuole biogenesis in *Saccharomyces cerevisiae*. Histochemistry **92,** 421–432.

Uchida E., Ohsumi Y. and Anraku Y. (1985), Purification and properties of H^+-translocating, Mg^{2+}-adenosine triphosphatase from vacuolar membranes of *Saccharomyces cerevisiae*. J. Biol. Chem. **260**, 1090–1095.

Wiemken A., Schellenberg M. and Urech K. (1979), Vacuoles: the sole compartments of digestive enzymes in yeast (*Saccharomyces cerevisiae*)? Arch. Microbiol. **123**, 23–35.