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A NEW METHOD FOR THE CYTOCHEMICAL DEMONSTRATION OF PHOSPHATASE ACTIVITIES IN YEASTS BASED ON THE USE OF CERIOUS IONS

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1. Introduction

The conventional method for the ultrastructural localization of phosphatase activities is based on the use of lead ions as a capturing agent for enzymatically liberated phosphate. However, some properties of lead ions are unsatisfactory and have given rise to a number of disadvantages concerning its use in enzyme cytochemistry [1]. Main problems were (a) the inhibitory effect of Pb$^{2+}$ on the activity of various enzymes, (b) difficulties in preparing stable incubation mixtures, and (c) presence of artificial staining deposits after incubation. For this reason a number of modifications have been developed, including the use of chelating agents to overcome precipitations, especially at high pH values, and the use of other phosphate capturing ions, which were eventually converted into lead phosphate after incubation [2–5]. However, these modifications still suffer from various disadvantages.

Cerium salts have also found an application in cytochemistry, particularly for the cytochemical demonstration of both intracellular and extracellular sites of oxidase activities [6,7]. The method is based on trapping of enzymatically produced H$_2$O$_2$ which leads to the formation of electron-dense deposits, presumably cerium perhydroxide [7]. Recent investigations in our laboratory have shown that cerous ions can also be used in phosphatase cytochemistry [8].

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The present paper presents the results of a cytochemical study on the ultrastructural localization of several phosphatase activities in the yeasts Hansenula polymorpha and Candida utilis, using Ce$^{3+}$-ions as a phosphate-capturing agent. The advantages of the method over the conventional lead salt method are discussed.

2. Materials and Methods

2.1. Organisms and cultivation

The experiments were performed with cells of *H. polymorpha* de Morais et Maya CBS 4732 and *Candida utilis*NCYC 321. The organisms were grown in 500 ml erlenmeyer flasks containing 100 ml of the basal medium, described previously [9]. *H. polymorpha* was grown at 37°C in the presence of 0.5% (v/v) methanol as the sole source of carbon, *C. utilis* was grown at 29°C on 0.3% (v/v) ethanol as the carbon source. Cells were harvested in the mid-exponential or in the stationary phase of growth.

2.2. Preparation of spheroplasts

Spheroplasts were prepared by treatment of suspensions of whole cells with "Zymolase" [10] for 10–30 min at 37°C according to the method of Osumi [11].

2.3. Cytochemical staining techniques

The cytochemical staining experiments were performed on spheroplasts, prefixed in 6% glutaraldehyde...
0.1 M Na-cacodylate buffer pH 7.2, containing 1 mM CaCl₂ and 1 mM MgCl₂, for 15 min at 0°C. Subsequently the cells were thoroughly washed with the cacodylate buffer to remove excess glutaraldehyde. All incubation mixtures for the cytochemical experiments were freshly prepared before use. Before incubation, glutaraldehyde-fixed spheroplasts were incubated for 15 min in the reaction mixture without enzyme substrate. For the demonstration of acid phosphatase (EC 3.1.3.2) the incubation mixture contained 0.1 M acetate buffer, pH 4.5 or pH 5.5, 1 mM CeCl₃, and 7 mM Na-β-glycerophosphate. Incubation was for 30 min at 37°C. Controls were performed in the absence of substrate or in the presence of 10 mM NaF as an inhibitor of acid phosphate activity.

The incubation mixture for the demonstration of glucose-6-phosphatase (EC 3.1.3.9) contained 0.1 M Tris-malate buffer, pH 6.6, 1 mM CeCl₃, and 2 mM glucose-6-phosphate. Incubation was for 30 min at 37°C. Controls were performed in the absence of substrate or in the presence of 60 mM sodium citrate as an inhibitor of glucose-6-phosphatase activity [12]. The incubation mixture for alkaline phosphatase (EC 3.1.3.1) and hexosediphosphatase (FBP-ase, EC 3.1.3.11) contained 0.1 M glycine-HCl buffer, pH 9.3, 1 mM CeCl₃, 4 mM MgCl₂ and 7 mM Na-β-glycerophosphate or 2 mM fructose-1,6-bis-phosphate (FBP). Incubation was for 45 min at 37°C. Controls were performed in the absence of substrate or in the presence of 0.5 mM AMP as an inhibitor of FBP-ase activity. In addition, incubations were performed in the absence of Mg²⁺ ions.

After the cytochemical incubations the spheroplasts were washed for 15 min in 0.1 M Na-cacodylate buffer, pH 6.0.

2.4. Postfixation

Postfixation was performed in a solution of 1% OsO₄ and 2.5% K₂Cr₂O₇ in 0.1 M Na-cacodylate buffer, pH 7.2, for 45 min at room temperature. After dehydration in a graded alcohol series the cells were embedded in Epon 812. Ultrathin sections were cut with a diamond knife and examined in a Philips EM 300.

3. Results

Initially the suitability of cerous ions for phosphatase cytochemistry was investigated in experiments aiming at the localization of FBP-ase. This enzyme was chosen since the difficulties of the lead method are most pronounced with its subcellular detection. Therefore, H. polymorpha and C. utilis were grown on methanol and ethanol, respectively, conditions which lead to high FBP-ase activities. Especially H. polymorpha was suitable in this respect since growth on methanol involves FBP-ase activities which are the highest encountered so far in yeasts (0.25 μmol/min/mg) [13]. Since the method involved the use of cerous ions, spheroplasts rather than whole cells were used because the cell wall of yeasts forms a permeability barrier for the penetration of this cation [6].

Freshly prepared incubation mixtures containing CeCl₃ and the substrates for the various phosphatases yield clear solutions both at acid and alkaline pH and were stable for several hours.

3.1. Localization of FBP-ase activity

After incubation of the glutaraldehyde-fixed spheroplasts with CeCl₃ in the presence of 2 mM FBP very fine granular staining deposits were observed in the cytosol of both organisms studied (Figs. 1, 2). The presence of reaction products was strictly dependent on the presence of Mg²⁺-ions in the incubation media; control experiments in the absence of Mg²⁺ invariably showed negative results (Fig. 3). After incubation in the presence of AMP as an inhibitor of FBP-ase activity about half the cells still showed a weak staining in the cytosol.

3.2. Localization of alkaline phosphatase activity

After incubation with CeCl₃ in the presence of β-glycerophosphate at pH 9.3 the reaction products were localized in the vacuolar region. However, in both yeasts used in this investigation the actual localization was dependent on the growth phase of the cells. In exponentially growing cells the staining deposits were mainly located along the inner surface of the tonoplast (Fig. 6). In addition, staining was also observed scattered throughout the vacuolar fluid. However, in cells from the stationary phase of growth the
Figs. 1 and 2. Demonstration of FBP-ase activity in the cytosol of exponentially growing cells of *H. polymorpha* (Fig. 1; 45 000×) and *C. utilis* (Fig. 2; 88 000×).

Fig. 3. Detail of a cell of *C. utilis*, after incubation for FBP-ase activity in the absence of Mg$^{2+}$-ions. Reaction products are not observed (48 000×).

Fig. 4. Section through a cell of *C. utilis*, after incubation for alkaline phosphatase in the presence of KCN as an inhibitor of enzyme activity, showing the absence of reaction products (42 000×).

Abbreviations in electron micrographs: m, mitochondrion; n, nucleus; p, peroxisome; v, vacuole.
reaction product was sometimes located at the cytoplasmic side of the vacuolar membrane (Fig. 5). Both possibilities were also observed in one cell (Fig. 5). However, cytoplasmic staining other than in association with the vacuolar membrane was not observed. Control experiments in the absence of substrate or in the presence of KCN as an inhibitor of alkaline phosphatase activity showed negative results (Fig. 4).

3.3. Glucose-6-phosphatase

Incubation of spheroplasts with CeCl₃ in the presence of 2 mM glucose-6-phosphate resulted in the formation of reaction products, which are exclusively located inside the vacuoles (Fig. 7). Generally large staining deposits were located against the inner surface of the vacuolar membrane, but staining was also frequently observed in the vacular fluid. The staining pattern did not differ in the two organisms studied. Control experiments, performed in the absence of substrate or in the presence of citrate as an inhibitor of glucose-6-phosphatase activities showed negative results (Fig. 8).
Figs. 7 and 8. Details of cells of *C. utilis*, showing the site of glucose-6-phosphatase activity (Fig. 7; 35 000×) and the absence of reaction products after incubations in the presence of citrate as an inhibitor of enzyme activity (Fig. 8; 50 000×).

Figs. 9 and 10. Demonstration of acid phosphatase activity, both at pH 5.5, in the vacuoles of exponentially growing cells of *C. utilis* (Fig. 9; 35 000×) and *H. polymorpha* (Fig. 10; 34 000×). The reaction products are mainly located along the inner surface of the tonoplast (arrows).

3.4. Acid phosphatase

After incubations with CeCl₃ in the presence of β-glycerophosphate at acid pH values 4.5 or 5.5) the reaction products were located in the vacuoles of both yeasts; the results were comparable to the pattern described for glucose-6-phosphatase. The main reaction product was located against the inner surface of the vacuolar membrane, whereas in addition staining deposits were sometimes present in the vacuolar fluid. (Figs. 9, 10). In addition, an increased density of the nuclear chromatine was observed, especially at low pH value (4.5). This was independent of the presence of substrate. Staining of the vacuoles was, however, not observed in control experiments in the absence of substrate or after incubation in the presence of NaF as an inhibitor of acid phosphatase activity.

It is well known that at alkaline pH values a precipitate of cerous hydroxyde may be slowly formed during incubation [7,8]. This precipitate cannot permeate through intact cell membranes and is easily
removed after a short wash with a weak acid buffer (see Section 2).

Diffusion of reaction products once formed was not observed in any of the experiments. In addition, we never observed the presence of artificial staining deposits in the different experiments.

4. Discussion

The use of Ce$^{3+}$ ions for the cytochemical demonstration of both intracellular and extracellular sites of H$_2$O$_2$-producing oxidase is well known [7,8]. The results of the present study show that Ce$^{3+}$ ions also can be successfully employed as a trapping agent for the localization of various phosphohydrolase activities in yeast cells. In the electron microscope cerous phosphate appeared as an intense staining, composed of very fine granular deposits which, after proper incubations, permitted an accurate localization of the sites of enzyme activity. However, prolonged incubations lead to overstaining, thus obscuring ultrastructural detail, a phenomenon which was also observed after long incubation from series of time-dependent incubations (not shown).

In general, the results of our cytochemical experiments are in good agreement with biochemical data, obtained with yeast cells on the enzymes studied. Fractionation studies on methanol-grown cells of the yeast H. polymorpha have shown that the FBP-ase activity was present in the non-particulate fraction [13] and therefore support our finding of FBP-ase activity in the cytosol. Using lead citrate as a capturing agent, Saito and Ogawa [14] showed that in rat liver cells FBP-ase activity is present in the cytoplasmic matrix of these cells. The reaction products were found to be located on fine filamentous materials of about 40 Å in diameter. The latter were not observed in our experiments. In agreement with our results was the observation that the presence of reaction product was strictly dependent on the presence of Mg$^{2+}$ ions in the incubation mixture. AMP, a specific inhibitor of FBP-ase activity, however, did not completely block enzyme activity in our experiments. This may be due to the fact that yeast FBP-ase shows a decreased sensitivity to AMP at higher pH values [15].

Tonino and Steyn-Parvé [16] have shown that in the yeast Saccharomyces carlsbergensis the distribution of a non-specific alkaline phosphatase was dependent on the age of the culture. They showed that in young cultures of this organism the major part of the enzyme was particle bound, whereas in older cultures the greater part of the enzyme was present in the soluble fraction. The biochemical experiments of Wiemken et al. [17] have shown that in Saccharomyces cerevisiae the alkaline phosphatase activity is partially bound to the vacuole of these cells. In cryosections of partly phosphatase-derepressed cells of S. cerevisiae Bauer and Sigarlakie [18] showed that alkaline phosphatase activity was mainly associated with the inner side of the tonoplasts. These data are in agreement with the results of our experiments. However, our results further indicate that the activity, present in the older cells outside the vacuole, still remains associated with the outer surface of the vacuolar membrane since any additional cytosolic reaction products were not observed. In addition, Wiemken et al. [17] also showed that glucose-6-phosphatase activity was exclusively present in these vacuoles which confirms the results of our cytochemical experiments.

Acid phosphatase is well-known as a constituent of the vacuole and, depending on conditions of growth, also in the cell wall of yeasts [16,19,20]. Working with phosphatase-derepressed cells of S. cerevisiae, Linnemans et al. [21] showed that acid phosphatase was localized in two layers of the cell wall. In addition, in acid phosphatase secreting protoplasts of this organism the enzyme was also present in the entire endomembrane system [22]. However, in repressed cells, during rapid growth on glucose [22], in which the synthesis of phosphatases is repressed [23], only an aspecific staining of the vacuoles occurred, probably due to the presence of polyphosphate. In methanol-grown cells of H. polymorpha and ethanol-grown C. utilis a specific staining of the vacuoles occurred, independent of the growth phase of the cells, which was absent after substrate-free incubations or incubations in the presence of NaF. Therefore, our results indicate that acid phosphatase is present in the vacuoles of both yeasts studied.

The results of our cytochemical experiments permit the conclusion that Ce$^{3+}$ ions are very suitable for phosphatase cytochemistry. Preliminary studies in rat liver [8] have indicated that with cerous ions the
localization of various phosphohydrolases is not different from results obtained with the lead salt method, but routinely a much better resolution is achieved. Since the use of Ce³⁺ ions allows stable incubation mixtures to be prepared at both acid and alkaline pH, our method may in future permit qualitative determination of phosphatase activities in situ under well-defined conditions. It remains to be determined to what extent cerous ions inhibit the activity of the various phosphatases studied here. Preliminary experiments on the effect in vitro of Ce³⁺ on FBP-ase activity in cell-free extracts of methanol-grown H. polymorpha have shown that Ce³⁺ in concentrations up to 1 mM do not inhibit this enzyme.

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