Isolation and Purification of Mn-Peroxidase from Azospirillum brasilense SP245

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Abstract—Homogenous Mn-peroxidase of a 26-fold purity grade was isolated from a culture of Azospirillum brasilense Sp245 cultivated on a medium containing 0.1 mM pyrocatechol. The molecular weight of the enzyme is 43 kD as revealed by electrophoresis in SDS-PAAG. It was shown that the use of pyrocatechol and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonate) at concentrations of 0.1 and 1 mM as inductors increased the Mn-peroxidase activity by a factor of 3.

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INTRODUCTION

Diazotrophic Azospirillum bacteria known for synthesizing biologically active substances, which positively affect plant growth and development, are used as model objects to study plant–bacterial associations. It has been recently shown that Azospirillum possesses phenoloxidase activity [1–3]. Screening of a variety of Azospirillum strains for the presence of phenoloxidases revealed Mn-peroxidase activity [3].

For the first time, Mn-peroxidases (MnPs, EC 1.11.1.13) were isolated from Phanerochaete chrysosporium [4]. The enzyme demonstrated oxidative activity only in the presence of Mn^{2+}. Later, it was shown that the enzyme catalyzed peroxide-dependent oxidation of Mn^{2+} to Mn^{3+}, which, in turn, appeared to be a strong oxidant. MnP is an enzyme with a molecular weight of 42–47 kD, which is usually located in extracellular space [5].

The enzyme is often used as a natural oxidant in contemporary biotechnologies, such as pulp and textile bleaching and wastewater and natural pool treatment against biocides, dyes, and xenobiotics. Although MnPs of several species of basidiomycetes have been isolated and well studied, bacterial MnPs have been poorly investigated. To date, only one study, which deals with the isolation and purification of MnPs from Bacillus pumilus and Paenibacillus, has been published [6]. The high oxidative capacity of MnPs, as well as their multifunctionality in different organisms, makes this enzyme interesting for both applied and basic research.

The present study was aimed at the isolation and purification of extracellular MnPs from Azospirillum brasilense Sp245.

EXPERIMENTAL

Bacteria Cultivation Conditions. Strains of A. brasilense Sp245 from the microbial collection of the Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, were used as an object of our study. The bacteria were cultivated in 250-mL Erlenmeyer flasks on a liquid medium with the following content: 0.1 g/l of KH_{2}PO_{4}, 0.4 g/l of K_{2}HPO_{4}, 0.1 g/l of NaCl, 0.002 g/l of Na_{2}MoO_{4} \cdot 7H_{2}O, 0.2 g/l of MgSO_{4} \cdot 7H_{2}O, 0.02 g/l of FeSO_{4} \cdot 7H_{2}O, 5 g/l of malic acid, 1.7 g/l of NaOH, 1.0 g/l of NH_{4}Cl, 0.02 g/l of CaCl_{2}, MnSO_{4} \cdot 5H_{2}O at a concentration of 1 mM, and pH 6.8. A 12-h culture grown on the same medium was used as an inoculation material. The bacteria were cultivated at 37°C for 35 h.

Induction of MnP Activity. To induce MnP activity, the medium was supplemented with syringaldazine (Acros Organics, United States), 2,6-dimethoxyphenol (Acros Organics, United States), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS, Sigma, United States), and pyrocatechol (Acros Organics, United States) at concentrations of 0.1, 0.5, and 1 mM.

Estimation of the MnP Activity. The activity of extracellular MnPs was measured spectrophotometrically at each stage of the study, using a Specord M40 spectrophotometer (Carl Zeiss, Germany), by the rate of oxidation of 2,6-dimethoxyphenol (ε = 30.5 M^{-1} cm^{-1}) at 30°C [7]. The reaction mixture (2 ml) contained 50 mM sodium tartrate buffer, pH 4.5, 1 mM 2,6-dimethoxyphenol, 1 mM MnSO_{4} \cdot 5H_{2}O, and an enzyme preparation. The reaction was initiated by the introduction of 100 μl of 1 mM H_{2}O_{2}. A unit change in
the absorption at a 468-nm wavelength for 1 min was considered to correspond to 1 unit of enzymatic activity. The specific enzymatic activity was expressed as units per mg of protein. The protein concentration was estimated by the Bradford method.

**Enzyme Purification.** A 36-h bacterial culture medium, containing 0.1 mM pyrocatechol as an inducer, was centrifuged at 10000 g for 20 min at 4°C. The obtained supernatant was desalinated on a G-25 column against buffer A (0.025 M sodium acetate buffer, pH 5.0). A release of protein fractions was registered using a Uvicord S-II detector (LKB, Sweden) at a λ = 280-nm wavelength. The collected active fractions were purified by HPLC using a HPLC SmartLine 5000 device (Knauer, Germany). Separation was performed on an anion-exchange TSK Bioassist Q column (Tosohaas, United States) preequilibrated with buffer A. Proteins were eluted with NaCl concentration gradient in buffer A at a 1 ml/min flow rate using photometric detection at a λ = 280-nm wavelength. Fractions, which possessed Mn-peroxidase activity, were dialyzed against water and used for further investigations.

**Electrophoresis.** The homogeneity of the obtained enzyme preparation, as well as its molecular weight, were estimated by electrophoresis in denaturing gels (SDS-PAAG) according to the method of Laemmli using protein molecular weight standards (Fermentas, Latvia) containing β-galactosidase (116 kDa), bovine serum albumin (66.2 kD), ovalbumin (45.0 kD), lactate dehydrogenase (35.0), REase Bsp98I (25.0 kD), β-lactoglobulin (18.4 kD), and lysozyme (14.4 kD). Electrophoresis in non-denaturing PAAG was performed according to the same method, but the stage of boiling or adding SDS and mercaptoethanol were omitted. The protein bands were stained with a solution containing silver.

**RESULTS AND DISCUSSION**

Cultivation of *A. brasilense* Sp245 strains on a standard malate-containing medium in the presence of 0.094 mg/l of Mn²⁺ without inductors revealed low MnP activity, which did not exceed 1.2 units/mg by the oxidation of 2,6-dimethoxyphenol. The increase in the concentration of MnSO₄ · 5H₂O to 1 mM resulted in an increase in the MnP activity to 2.6 units/mg (Fig. 1). The given data are typical of bacteria in the stationary phase of growth (36-h culture), while for 24-h cultures, lower enzymatic activity is commonly observed (data are not shown). It was previously shown that the increase in the manganese concentration in the medium led to an increase in the yield and activity of fungal MnPs [8].

It is known that synthesis of extracellular enzymes significantly depends on the culture medium content. Usually, high concentrations of complex organic substances in a medium lead to more intensive bacterial growth and an increase in production of extracellular enzymes. However, the opposite effect was observed for the studied extracellular MnPs. The introduction of a yeast extract to the medium resulted in a sharp decrease in the level of enzymatic activity. Therefore, it was not used any further.

Based on the suggestion that bacterial MnPs, similarly to fungal enzymes, are also inducible enzymes, the cultivation medium was supplemented with different concentrations of aromatic substrates in order to increase MnP activity. It was shown that the introduction of phenol compounds, such as syringaldazine, 2,6-dimethoxyphenol, ABTS, and pyrocatechol increased MnP activity. The data on the effect of phenol compounds on MnP activity are shown in Fig. 1. An increase in the Mn-peroxidase activity of *A. brasilense* Sp245, which were cultivated in the presence of inductors, was observed after 36 h similarly to the one was observed for bacteria grown in a regular malate-containing medium. The highest level of MnP activity, which exceeded the control values by a factor of 3, was observed after the introduction of pyrocatechol and ABTS at concentrations of 0.1 mM and 1 mM, respectively (Fig. 1).

Based on the data obtained, *A. brasilense* Sp245 were further cultivated in a medium supplemented with 0.1 mM pyrocatechol and 1.0 mM MnSO₄ · 5H₂O for 36 h.

At the first stage of enzyme purification, the supernatant was freed from low-molecular-weight substances by gel filtration on a G-25 Sephadex column preequilibrated with 0.025 M sodium acetate buffer, pH 5.0. The main obstacle was to separate the enzyme from the pigment, which was formed as a result of the enzymatic oxidation of the inducer. The pigment was only totally removed at the next stage of purification.