Enzyme and Microbial Technology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/enzmictec

Research Paper

Biosynthesis of gold and selenium nanoparticles by purified protein from *Acinetobacter* sp. SW 30

Sweety A. Wadhwani[a], Utkarsha U. Shedbalkar[b], Richa Singh[a], Balu A. Chopade[a,c,*]

[a] Department of Microbiology, Savitribai Phule Pune University, Pune 411007, Maharashtra, India
[b] Department of Biochemistry, The Institute of Science, Mumbai 40032, Maharashtra, India
[c] Dr. Babasaheb Ambedkar Marathwada University, Aurangabad 431004, Maharashtra, India

ARTICLE INFO

Keywords:
Lignin peroxidase
Purification
Specific activity
Acinetobacter
Gold nanoparticles

ABSTRACT

Synthesis of nanoparticles is an enzymatic reduction process in microorganisms. In the present study, a protein, lignin peroxidase has been purified by DEAE-Cellulose anion exchange chromatography and Biogel P-150 gel filtration chromatography from the cell suspension of *Acinetobacter* sp. SW30 responsible for the synthesis of gold nanoparticles (AuNP) and selenium nanoparticles (SeNP). The purified fraction has a specific activity of 29.4 U/mg/min with 959 fold purification. Native and SDS PAGE confirmed that purified lignin peroxidase is monomeric enzyme with 97.4 KDa molecular weight. The enzyme synthesized spherical crystalline AuNP (10 ± 2 nm) and amorphous SeNP (100 ± 10 nm). It has maximum activity at pH 2 and temperature 40 °C, with 1.0 mM Km value, when *n*-propanol was used as a substrate. Activity was completely inhibited by sodium thiosulphate and zinc sulphate. This is the first report on association of lignin peroxidase in the synthesis of AuNP and SeNP from *Acinetobacter* sp. SW30.

1. Introduction

Microorganisms, such as bacteria, fungi and algae, and their products play an important role in biosynthesis of nanoparticles. Enzymes, extracellular polysaccharides, DNA, rhamnolipids etc. are involved in biosynthesis of nanoparticles, [1–4], which lead to the formation of polydispersed nanoparticles owing to the presence of multiple organic components in cell suspension or cell free extract. Proteins may cover the surface of such bio-nanoparticles in its native form and may confer stability [2,5].

Microoragnisms produce metal nanoparticles either as the by-product of respiration process or as a survival mechanism against metal toxicity. It is a reduction process followed by capping with the proteins. It has been observed that nanoparticles synthesized by purified protein are monodispersed and smaller in size. Hence, purification and identification of proteins involved in the synthesis of metal nanoparticles are essential to predict the possible mechanisms of synthesis. Moreover, additional procedures for extraction of nanoparticles from microbial cells are not required which reduces recovery and purification cost [2].

Enzymes such as laccase, peroxidases, proteases, reductases and fibrinolytic enzyme (URAK) are known for the reduction of salts into insoluble elemental particles in microorganisms [2,3,6,7]. ABC transporter from thermophilic bacterium *Thermus scotoductus* SA-01 is found to reduce Au (III) through an electron shuttle mechanism involving a cysteine disulphide bridge [8]. In viable cells, Au reduction is a three-electron two-step reduction process from Au^{+3} to Au^{+} followed by its reduction to Au^{00}. The emergence of methylated Au^{+3} supported defence mechanism against metal toxicity [9,10]. Similarly, selenate reduction is also a two-step reduction process: (i) selenate (SeO_4^{-2}) to selenite (SeO_3^{-2}) catalyzed by selenate reductases and (ii) selenite to insoluble elemental selenium (Se^0) catalyzed by nonspecific selenite reductases, which predominantly include nitrite and sulphite reductases [11].

Acinetobacter is a diverse group of organisms present ubiquitously in nature [12–15]. They are commonly found in water, soil, foods and skin of humans and animals [16–19]. They have an excellent ability to form biofilm [19,20]. *Acinetobacter* can survive in extreme environmental conditions [21]. Bioremediation potential of *Acinetobacter* is well studied with respect to textile dyes, where an active involvement of lignin peroxidase was reported [25].

Acinetobacter sp. can synthesize various metal nanoparticles such as gold, silver, platinum and selenium [22–24]. Proteins responsible for PtNP production from *Acinetobacter calcoaceticus* has been purified [24]. In view of this background, the present work is focused on the purification and biochemical characterization of one of the proteins responsible for the synthesis of gold and selenium nanoparticles from

* Corresponding author at: Department of Microbiology, Savitribai Phule Pune University, Pune 411007, Maharashtra, India.
E-mail addresses: bachopade@gmail.com, chopade@unipune.ac.in (B.A. Chopade).

http://dx.doi.org/10.1016/j.enzmictec.2017.10.007
Received 19 June 2017; Received in revised form 11 October 2017; Accepted 18 October 2017

Acinetobacter sp. SW 30. Further purified protein has been used for the synthesis of gold and selenium nanoparticles and the characterization of gold and selenium nanoparticles is done by various analytical techniques such as TEM, EDX and SAED.

2. Materials and methods

2.1. Microorganism and culture conditions

The *Acinetobacter* sp. SW30 was isolated from activated sewage sludge and can synthesize gold nanoparticles (AuNP) and selenium nanoparticles (SeNP) [22,26]. The culture was routinely sub-cultured and maintained on Luria Bertani (LB) (HiMedia, India) agar at 4 °C and in glycerol stocks stored at −80 °C.

2.2. Preparation of crude enzyme

A loopful of culture of *Acinetobacter* sp. SW30 was inoculated into the 10 ml LB broth medium and incubated at 30 °C overnight to obtain the inoculum. The 250 ml LB broth was inoculated with 1% inoculum and incubated at 30 °C, 180 rpm for 24 h. Cells were harvested by centrifugation (10,000 rpm for 10 min at 10 °C) and washed thrice with sterile distilled water (D/W). Cell pellet was suspended in sterile D/W and incubated at 30 °C, 180 rpm. Supernatant was checked for laccase, lignin peroxidase, nitrate reductase and tyrosinase activity upto120 h with an interval of 24 h. The cells having maximum activity were used in further study. The cells were collected by centrifugation (10,000 rpm for 10 min at 10 °C) and re-suspended in 50 mM phosphate buffer (pH 7.4) and were disrupted by sonication (Q Sonicator) at 4 °C by keeping sonifier output at 50 amplitude and giving 10 strokes, each of 60 s, with 60 s interval (10,000 rpm for 10 min at 10 °C). The homogenate was centrifuged under cold conditions at 18,000 rpm for 20 min, the clear supernatant obtained was collected and used as a source of crude protein.

2.3. Protein estimation and enzyme activity

Protein content of supernatant was measured by Folin Lowry method [27], using bovine serum albumin as standard. The clear supernatant was checked for the presence of laccase, lignin peroxidase, nitrate reductase and tyrosinase enzymes. Laccase activity was measured at 420 nm for 1 min by incubating supernatant with 0.1% ABTS substrate prepared in sodium acetate buffer (0.1 M, pH 4). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmole of substrate.

Lignin peroxidase activity was determined at 310 nm for 1 min using *n*-propanol as a substrate. The reaction mixture contained 40 mM *n*propanol, 2 mM H_2O_2 and 50 mM tartaric acid to maintain the pH of 2. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmole of substrate.

Nitrate reductase activity of crude enzyme was determined by incubating enzyme with 10 mM potassium nitrate in the presence of 2 mM NADH for 2 min at room temperature. The reaction was stopped by adding 58 mM sulphanilamide (prepared in 3 M HCl) and 77 mM N' naphtyl ethylene diamine dihydrchloride (NEDD). The presence of pink coloured product was measured at 540 nm.

Presence of tyrosinase enzyme in the supernatant was determined by adding 10 mg/ml catechol in sodium phosphate buffer (0.1 M, pH 7.4) as substrate. The formation of product was measured at 265 nm for 1 min. Suitable blanks were included in all the enzymatic assays. Assays were performed in triplicate.

2.4. Purification of proteins

Step-1: DEAE-Cellulose anion exchange chromatography

The clear supernatant was loaded on pre-activated DEAE-Cellulose column (15 × 2.1 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.4). The column was washed with the same buffer and enzyme was eluted with 0.2, 0.4, 0.6, 0.8 and 1.0 N NaCl at a flow rate of 60 ml/h. Fractions showing peroxidase activities were pooled and dialysed against 50 mM potassium phosphate buffer (pH 7.4). Pooled fraction was loaded on new DEAE-Cellulose column under the same conditions used previously, eluted with 0.2-1.0N NaCl and fraction showing lignin peroxidase activity was dialysed against 50 mM potassium phosphate buffer (pH 7.4).

Step- 2: Biogel P-150 gel filtration chromatography

The dialysed fraction was concentrated in sucrose and loaded on a biogel column (75.5 × 0.9 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.4). Column was washed with the same buffer and void volume was determined using blue dextran (2 mg/ml in 5% glycerol). Enzyme was eluted using the same buffer at the flow rate of 6.6 ml/h. Fraction containing peroxidase activity was collected and stored at 4 °C until use.

2.5. Polyacrylamide gel electrophoresis (PAGE) analysis

Native and sodium dodecyl sulphate (SDS-PAGE) was performed using 4% stacking and 10% resolving gel in vertical gel electrophoresis unit (GeNei, Banglore, India). Both gels were silver stained and molecular weight of purified lignin peroxidase was determined using protein molecular weight broad range marker GeNei and Sigma (Pune) for native and SDS PAGE respectively.

2.6. Synthesis of nanoparticles from purified enzyme

The purified peroxidase of 300 µg/ml concentration was challenged with 1 mM concentrations of $HAuCl_4$ and Na_2SeO_3; incubated at 37 °C and observed for colour change. Synthesized nanoparticles were characterized by UV–vis spectrophotometry, TEM and EDX. The particle size was measured by image J software.

2.7. Determination of optimum pH and temperature

The effect of pH on activity of purified enzyme was studied within a pH range of 1–10 using

n-propanol as a substrate and 50 mM of the following buffers: HCl-KCl buffer (pH 1–3), acetate buffer (pH 4–5), phosphate buffer (pH 6–8), tris-HCl buffer (pH 9) and carbonate-bicarbonate buffer (pH 10). The optimum temperature of the enzyme was determined over the temperature range of 10–90 °C with *n*-propanol as substrate at its optimal pH value [28].

2.8. Effect of salts

Effect of addition of various salts such as $MgSO_4$, $CuSO_4$, NH_4SO_4, $HgCl_2$, $CaCl_2$, NaCl, K_2HPO_4, KH_2PO_4, $NaNO_3$, KNO_3, NH_4NO_3, $Na_2S_2O_3$, $ZnSO_4$ at the concentration of 25 mM on the enzymatic activity of lignin peroxidase was studied [28].

2.9. Substrate specificity

Substrate specificity of peroxidase was studied for different substrates, such as *n*-propanol, L-DOPA, guaiacol, hydroxyquinone, catechol and veratryl alcohol at 40 mM concentration and 2 mM H_2O_2 and 50 mM tartaric acid to maintain the pH of 2. The specific activity of enzyme was estimated through spectroscopic measurement of oxidation of substrates by enzyme at respective wavelength. Michaelis constant (Km) was determined by using *n*-propanol as a substrate with concentrations ranging from 0.1 to 65 mM using 2 mM H_2O_2 and 50 mM tartaric acid [28].