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Research Article

Cyanobacterium *Oscillatoria* sp. peroxidase active at alkaline pH and high stability under chemical stresses

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Abstract - Cyanobacterial peroxidase enzyme extracted from *Oscillatoria* sp. SWU121 was purified from crude extract by 20-80% ammonium sulfate precipitation, DEAE cellulose ion-exchange chromatography, and Sephadex G-100 size exclusion chromatography. The purified *Oscillatoria* sp. peroxidase (OsPOX) exhibited a specific activity of 6106.63 mmol.min^{-1}.mg.protein^{-1}, while purification fold and yield were 17.45 and 34.70%, respectively. The OsPOX showed single band of protein on native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The molecular weight was 45 kD similarly determined by gel filtration and SDS-PAGE suggesting that the OsPOX contained only one subunit. The apparent K_m and V_{max} values of the enzyme against phenol were 930 mM and 8078.25 mmol.min^{-1}.mg protein^{-1}, respectively. The temperature and pH optimum for OsPOX were 30ºC and pH 9.0, respectively. However, it was stable at 10 – 70ºC and pH 7.0 - 11.0. The presence of metal ions such as Na^+, $Mn^{2+}$and Fe^{3+} enhanced peroxidase activity. On the other hand, Zn^+, Cd^{2+} and Hg^{2+} strongly inhibited the enzyme activity. SDS and EDTA reduced the peroxidase activity at 10 mM (20%). The OsPOX was found to be stable in the presence of urea. The affinity of the enzyme was highest for gallic acid, followed by ascorbic acid, phenol and cafeic acid. This finding showed that OsPOX was active at alkaline pH and stable in presence of urea.

Keywords: Purification, characterization, peroxidase, cyanobacterium *Oscillatoria* sp.

1. Introduction

Cyanobacteria, blue green algae are microorganisms producing organic compounds and oxygen by photosynthesis (Stainier and Cohen, 1977; Grigorieva and Shestakov, 1982; Incharoensakdi and Laloknam, 2005; Rai *et al*., 2000). Because they produce free toxic oxygen therefore, they have to find out the mechanism to protect their lives. Since reactive oxygen species (ROS), e.g., superoxide radical, hydroxyl radical, and hydrogen peroxide are toxic to growth of cyanobacteria, their efficient removal is essential. For detoxification cyanobacteria and other living cells adapt various approaches including peroxidases and superoxide dismutase enzymes. Under stress conditions, ROS are generated in the cellular components, including DNA, protein and lipid membrane (Bertelsmann *et al*., 2007; Kumar and Sarla, 2008; Sakamoto and Komagata, 1996). Cyanobacteria can protect them from the damaging effects of oxidative stress by action of antioxidative agents like superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), low molecular weight antioxidants, e.g., ascorbic acid, glutathione (GSH) and phenolic compounds (Bertelsmann *et al*, 2007; Kumar and Sarla, 2008). It was reported that NaCl enhanced activity of superoxide dismutase and peroxidase, in *Zizyphus mauritiana* (Kumar and Sarla, 2008).

Peroxidase (E.C. 1.11.1.7; donor: hydrogen-peroxide oxidoreductase) are enzymes that catalyze hydrogen peroxide as the electron acceptor to reduce a number of oxidative reactions, found in bacteria (Sakamoto and Komagata, 1996; Zamocky, 2004; Welinder, 1992), fungi (Zamocky, 2004; Welinder, 1992; Heinfing *et al*., 1998), animals (Bertelsmann *et al*., 2007; Nelson, 1994) as well as plants (Kavitha *et al*., 2008; Al-Senaidy and Ismael, 2011; Zheng *et al*., 1999; Chen *et al*., 2010; Miranda *et al*., 2002; Fatima *et al*., 2007; Shannon *et al*., 1966).

Plant, e.g., horseradish and carrot are reported that a source of peroxidase as well as *Avicennia marina*, date palm leaves, *Pleurotus eryngii*, Tartary buckwheat bran, and Ivy gourd (Heinfing, 1998; Nelson *et al*., 1994; Kavitha *et al*., 2008; Al – Senaidy and Ismael, 2011; Zheng *et al*., 1999; Chen *et al*., 2010; Miranda *et al*., 2002; Fatima *et al*., 2007; Shannon *et al*., 1966; Kongwithtaya

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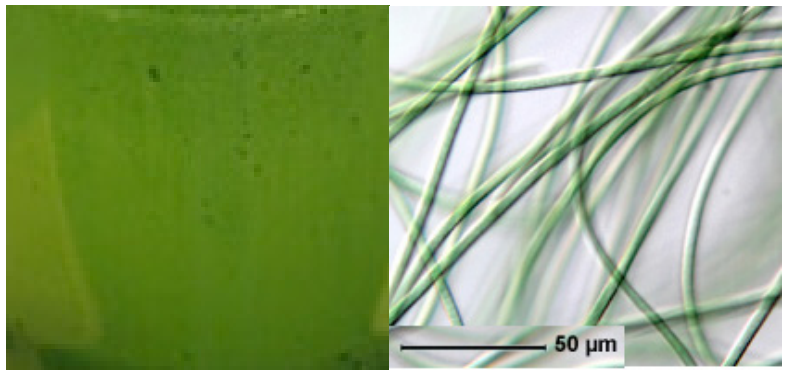
et al., 2010). Especially horseradish peroxidase is commonly used as a commercial source for peroxidase production (Miranda *et al*., 2002; Fatima *et al*., 2007; Shannon *et al*., 1966; Kongwithtaya *et al*., 2010). These enzyme groups can be found in various plant organelles (vacuoles and tonoplast) and tissues (plasmalemma and cell wall) which involved in plant hormone regulation, defense mechanisms, and control of biological function (Heinfing, 1998; Nelson *et al*., 1994; Kavitha *et al*., 2008). The peroxidase enzyme is not only found in plants but also green algae and cyanobacteria (Takeda *et al*., 1998; Regelsberger *et al*., 1992; Kachensuwan *et al*., 2012).

We had been studied to find the peroxidase activity from cyanobacteria, *Oscillatoria* sp. SWU121, *Nostoc* sp., *Synechocystis* sp. PCC6803, *Synechococcus* sp. PCC7942, and *Tolypothrix* sp. All cyanobacteria samples showed peroxidase activity using a mixture of 4-amino antipyrine, phenol and hydrogen peroxide as substrate, so *Oscillatoria* sp. SWU121 had the highest peroxidase activity and found that 20–80% cut of using ammonium precipitation showing high activity and yield (Kachensuwan *et al*., 2012).

Oscillatoria sp. SWU121 is filamentous cyanobacteria isolated from Sansab canal, Bangkok, Thailand and growth well under normal and alkaline pH condition (Laloknam *et al*., 2014). We have been reported that *Oscillatoria* sp. SWU121 peroxidase activity like plant peroxidase, e.g. an Ivy gourd ammonium sulfate precipitant peroxidase had optimum pH and temperature as 7.0 and 45°C, respectively (Kongwithtaya *et al*., 2010). In this paper we describe the purification step and initial characterization of some properties of cyanobacterium *Oscillatoria* sp. peroxidase active at alkaline pH.

2. Methodology

Materials: *Oscillatoria* sp. SWU121 was isolated from Sansab canal, Bangkok, Thailand. Ammonium sulfate, sodium chloride, DEAE cellulose, Sephadex G-25 and Sephadex G-100 were from Pharmacia Fine Chemicals (Piscataway, NJ, USA) and Comassie brilliant blue G-250, 4-aminoantipyrine (4-AAP) and hydrogen peroxide (H_2O_2) were from Fluka Chemie AG (Buchs, Switzerland). All other chemicals used were of analytical grade.



A B

Figure 1. Culture of 10-day *Oscillatoria* sp. SWU121 (A) and cell morphology of *Oscillatoria* sp. SWU121 (B).

Growth and crude sample preparation: cyanobacterium *Oscillatoria* sp. SWU121 was grown under continuously white light (30μEm2s-1) in 100 ml of BG_{11} medium, pH 7.6 at 28°C. The growth was measured by estimation fresh weight of cyanobacteria for 30 days. The harvested cyanobacterial cells were washed three times with 50 mM phosphate buffer, pH 7.0 at room temperature. The fifteen gram of wet sample was resuspended in 50 ml of phosphate buffer as a sonicating buffer. The cyanobacterial cells were disintegrated by sonicator at 30% amplitude using cycles of 20s on and 10s off for 10 min. The cell extracts were centrifuged at 5,000xg for 15 min, at 4°C, and the supernatant was designated as crude enzyme which was used to determine peroxidase activity. Crude extract was also stored at 4°C until used.

Detection of peroxidase activity: Peroxidase activity was measured by colorimetric method following a modified Wright and Nicell (1999). The reaction mixture (5 ml in total) was contained 50 mM phosphate buffer pH 7.0 (2mM 4–AAP, 2mM phenol and 4 mM hydrogen peroxide). The mixture was incubated at room temperature (30°C), for 10 min. The reaction was then started by adding 0.05 ml of crude enzyme, and the initial increase in absorbance was monitored at 400 nm using UV/VIS – Spectrophotometer Model Jenway 6405 (Jenway, UK). One unit of peroxidase activity was defined as the amount of the enzyme consuming 1 μmol of hydrogen peroxide per minute under the assay conditions. Protein was determined by Bradford method using bovine serum albumin as a standard (Bradford, 1976).

Purification steps of cyanobacteria *Oscillatoria* sp. peroxidase: All steps of enzyme purification were carried out at 0-4°C. Solid ammonium sulfate was added to crude extract to 0-20% saturation, left for 4 h and after that centrifuged and the precipitate was discarded. The supernatant was brought to 80% ammonium sulfate saturation. The precipitate was collected by centrifugation (10,000xg, 20 min), dissolved in 50 mM phosphate buffer, pH 7.0, and dialyzed for 24 h against the same buffer with repeated changes of buffer. The dialyzed protein was concentrated by osmosis against solid sucrose and loaded

onto DEAE- cellulose column (3x42cm) pre-equilibrated with 50 mM phosphate buffer, pH 7.0. The enzyme was eluted first by the above buffer and then by a linear gradient of 0.0 to 0.5 M NaCl with a flow rate of 30 $mL.h^{-1}$. Fractions of 3.0 mL each were collected and analyzed for protein at 280 nm and for activity of peroxidase. The active fractions giving clear cut activities for peroxidase were pooled separately and again concentrated against sucrose. Active peroxidase concentrated fraction was applied to a Sephadex G-100 column pre-equilibrated with 50 mM phosphate buffer, pH 7.0 for 10 h. The enzyme was eluted with same buffer at a flow rate of 15 $mL.h^{-1}$. The active fractions of 3.0 mL each were pooled and concentrated using sucrose and used as purified peroxidase. The concentration of protein at every step was determined by the method of Bradford (1976), using bovine serum albumin (BSA) as a standard. Molecular weight of the partially purified enzyme was determined by gel filtration on a Sephadex G-100 column and SDS-PAGE.

Optimum pH, optimum temperature and kinetic: The purified cyanobacterial peroxidase was determined for optimum pH at pH range of 4 – 11 and optimum temperature at range of 4 – 100°C, respectively. Kinetics was studied using phenol as substrate. The inset is the Lineweaver–Burk plot for K_m and V_{max} estimation.

pH and thermal stability: The purified cyanobacterial peroxidase was incubated at various temperature (0 – 100°C) for 1 h prior to assay, and then peroxidase was assayed at room temperature (30°C) using phenol as a substrate. The purified cyanobacterial peroxidase was incubated at various pH (4 – 11) for 1 h prior to assay, and then peroxidase was investigated at optimal temperature using phenol as a substrate.

Effect of some chemicals on peroxidase activity: Cyanobacterial peroxidase (0.5 $U.mL^{-1}$) was incubated with different concentration of urea (0.0 – 2.0 M), SDS (0.0 – 10.0 mM), and cations (5, 50, and 500 mM of Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Cr^{3+}, Fe^{2+}, Fe^{3+}, Mg^{2+}, Hg^{2+}, Ca^{2+}, K^{+}, Na^{+} and Mn^{2+}) for 1 h in 50 mM phosphate buffer, pH 6.0 at 45°C. Peroxidase activity was determined after each incubation period. The activity of the untreated enzyme was considered as control (100%) for calculating percent activity.

Substrate specificity: To study the substrate specificity of the peroxidase, Gallic acid, Cafeic acid, Ascorbic acid, and Cathecien were also tested to compare with phenol. The products of enzyme reaction were detected absorption spectra, which were identified by comparison with the spectra of authentic compounds.

3. Results and Discussions

***Purification of Oscillatoria* sp. SWU121.** The cell culture of *Oscillatoria* sp. SWU121 were grown as described in "Methodology" for 30 days, and then the cells were collected by centrifugation. The cell culture and cell morphology was shown in Figure 1. The initial specific activity in the crude extract was 349.95 units.mg $protein^{-1}$. Elution profile of peroxidase activity (POX) and protein on DEAE-cellulose column chromatography through NaCl gradient was shown in Figure 2. Protein was distributed randomly throughout all the fractions, whereas peroxidase activity was recorded for 110-150 fractions. Fractions with peroxidase activities were pooled and passed separately through Sephadex G-100 column (1.7x58 cm.) Peroxidase fractions were pooled at fraction number 80-100 (Fig. 3). The peroxidase enzyme was purified up to 17.45 fold with 34.70% recovery (Table 1). The specific activity of purified peroxidase was 6106.63 units.mg $protein^{-1}$. The protein staining showed equal peroxidase activity band (Fig. 4). Molecular weight of peroxidase as determined by gel filtration and SDS-PAGE was found to be similar value 45 kDa (Fig.5).

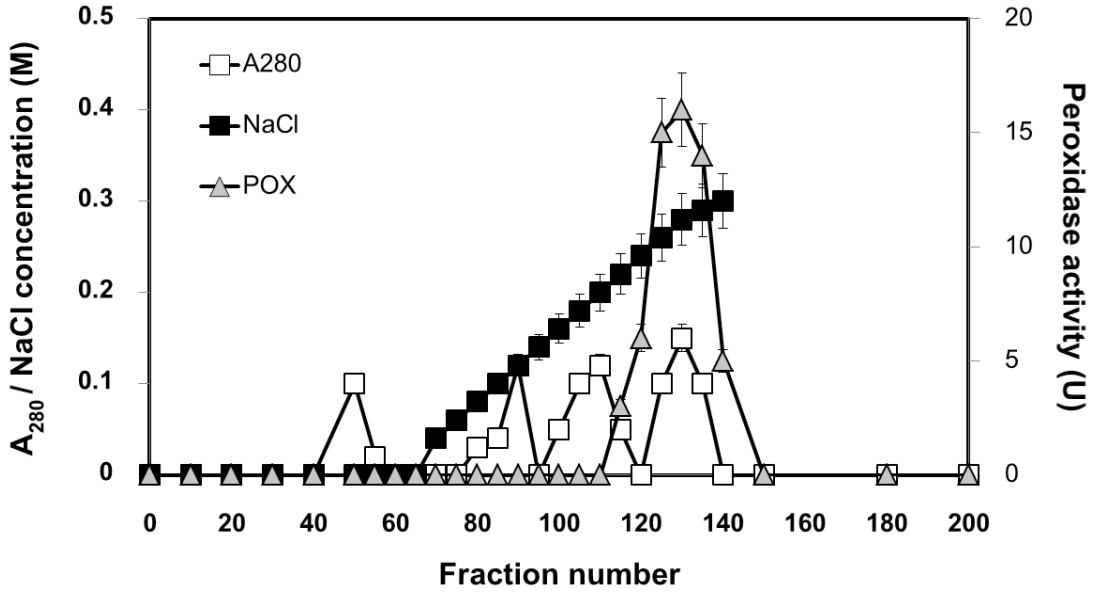


Figure 2. Profile of DEAE-cellulose column chromatography of peroxidase from *Oscillatoria* sp. at pH 7.5.

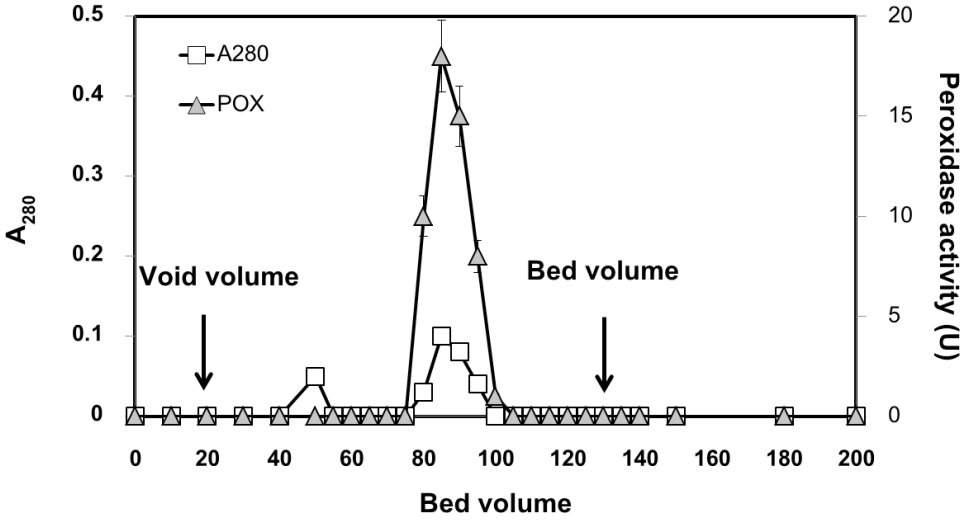


Figure 3. Profile of Sephadex G-100 column chromatography of peroxidase from *Oscillatoria* sp. at pH 7.5.

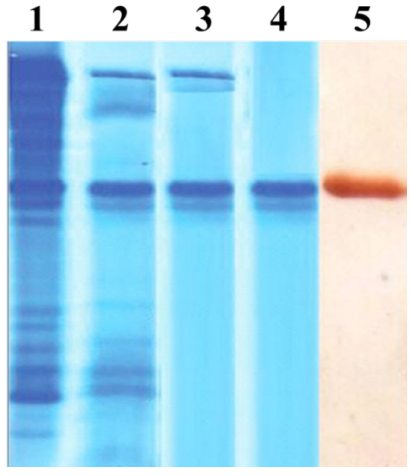


Figure 4. Non-denaturing PAGE and activity staining of peroxidase from different steps of purification. Lane: 1) Crude enzyme (20 μg); 2) $(NH_4)_2SO_4$ fraction (5 μg); 3) DEAE-cellulose pool (2 μg); 4) Sephadex G-100 pool (1 μg); 5) Peroxidase activity staining.

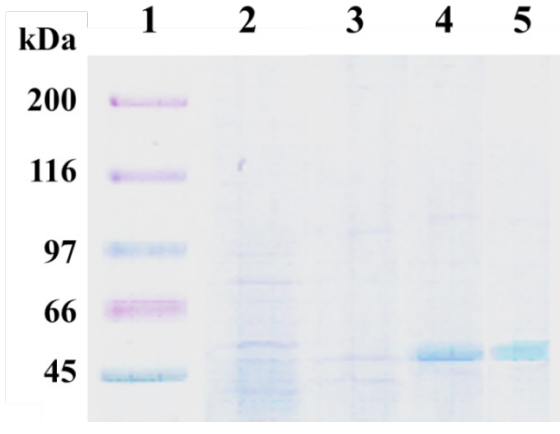


Figure 5. SDS-PAGE analysis of purified *Oscillatoria* sp. peroxidase from different steps of purification. Lane: 1) Protein molecular weight markers (myosin: 200 kD, b-galactosidase: 116 kD, phoshorylase: 97 kD, BSA: 66kD and ovabumin: 45 kD); 2) Crude enzyme (20 μg); 3) $(NH_4)_2SO_4$ fraction (5 μg); 4) DEAE-cellulose pool (2 μg); 5) Sephadex G-100 pool (1 μg).

Optimum pH: Purified peroxidase from *Oscillatoria* sp. showed a pH optimum of 9.0 and stability at pH ranged of 7.0 – 10.0 (Fig. 6). Enzyme stability within range of pH (7.0 – 10.0) suggested its function in both acidic and basic environments, like peroxidase in rice (Zheng *et al*., 1999), tomato (Vernwal *et al*., 2006), soybean (Wright and Nicell, 1999), coconut (Al – Senaidy and Ismael, 2011) and strawberry (Becana and Lotassa, 2007). The cyanobacterial peroxidase has an optimum pH of 9.0 with phenol as substrates, different to plant peroxidase, e.g., the bitter gourd and horseradish had optimal pH as 6.0 using o-dianisidine-HCl as substrate (Regelsberger *et al*., 2002). The buffers used (all at 50 mM) were: glycine-HCl (pH 3), sodium acetate (pH 4 and 5), phosphate, (pH 6 and 7), Tris-HCl (pH 8) and glycine-NaOH (pH 9).

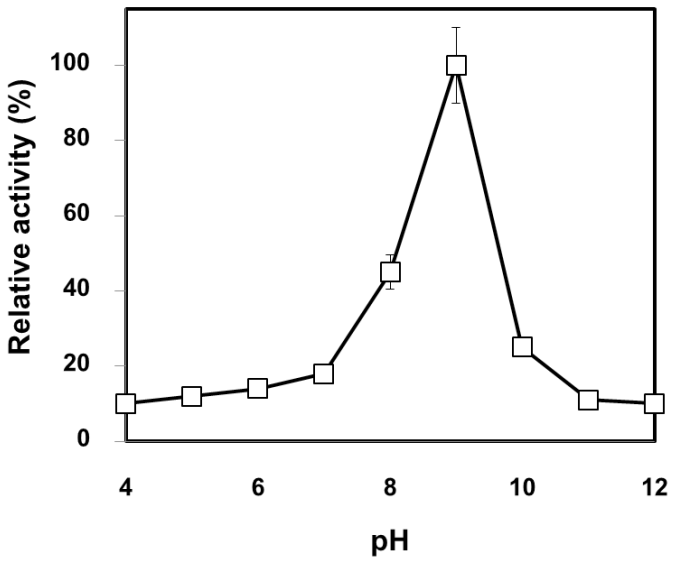


Figure 6. The effect of pH on the activity of purified peroxidase from *Oscillatoria* sp.

Effect of Temperature: The optimal temperature of purified peroxidase was 45°C. The enzyme showed stability temperature range from 10 – 70°C but with reduced activity to 20% (Fig. 7). The effect of temperature had been reported in bitter gourd (Veda and Dwivedi, 2011), cotton (Singh *et al*., 2010), strawberry (Becana and Lotassa, 2007) and coconut (Al – Senaidy and Ismael, 2011). It was also suggested that the enzyme active sites denatured at higher temperature (Becana and Lotassa, 2007).

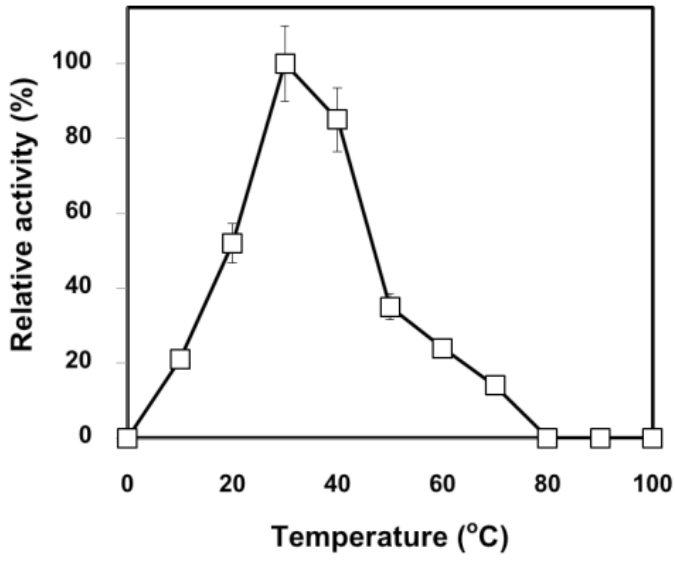


Figure 7. Effect of temperature on the activity of purified peroxidase from *Oscillatoria* sp.

***Kinetic of Oscillatoria* sp. *peroxidase*:** with phenol as a substrate the K_m and V_{max} values for OsPOX were 930 mM and 8078.25 mmol.min^{-1}.mg protein^{-1}, respectively (Fig. 8). Lower substrate specificity was reported in K_m values for peroxidase from tree legume, bitter gourd, Ivy gourd, Araucaria seeds, and horseradish root (Fatima *et al*., 2007; Shannon *et al*., 1966; Kongwithtaya *et al*., 2010; Regelsberger *et al*., 2002; Becana and Lotassa, 2007; Veda and Dwivedi, 2011; Vitali *et al*., 1998).

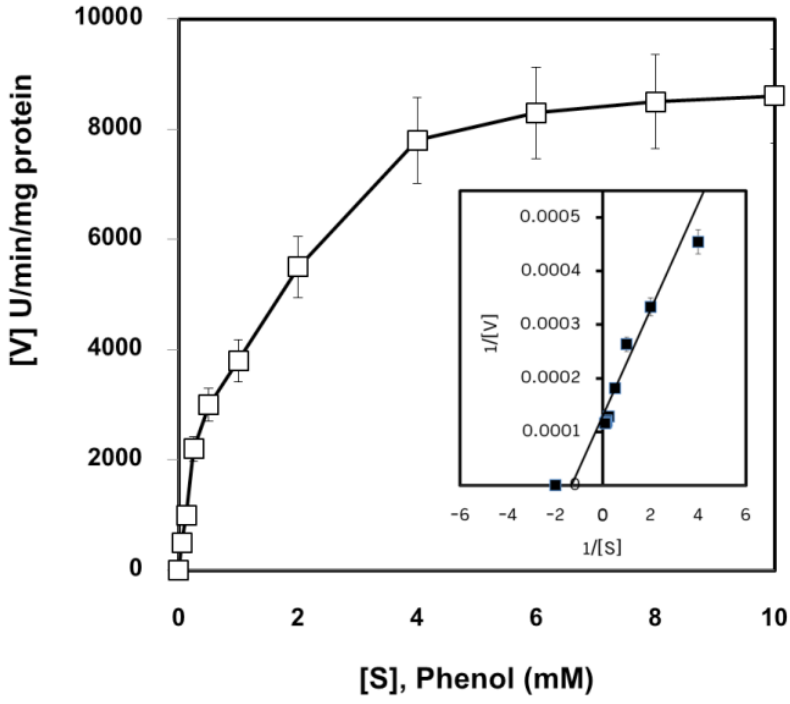


Figure 8. Michaelis-Menten curve of purified *Oscillatoria* sp. peroxidase. Inset: Lineweaver Burk double reciprocal plot of the effect of concentration of phenol on the initial velocity of peroxidase from *Oscillatoria* sp.

pH and thermal stability: *Oscillatoria* sp. peroxidase remained 50% of the initial activity after incubation at pH 4.0 and 8.0 (data not shown). However, the purified enzyme retained 80% of the initial activity after incubation at 60ºC (data not shown) as reported earlier about thermo stability of enzyme (Regelsberger *et al*., 2002).

***Effect of some chemicals on peroxidase activity from Oscillatoria* sp.:** Urea, SDS and cations were examined for peroxidase activity of OsPOX. There was no change in activity of peroxidase at increasing concentration of urea (Fig. 9). SDS, an anionic detergent, this indicated that enzyme retained its activity at wide range of SDS (0 – 10 mM), and it was 80% activity remained at 6 mM SDS (Fig. 10). Also, EDTA, an ion chelating agent on the activity of peroxidase, showed enzyme activity remained at range of EDTA concentrations (0 – 10 mM). Although, EDTA had no effect on peroxidase activity and at 4 mM EDTA, peroxidase activity has 80% of activity remained (Fig. 11). Monocation, K^+ had no effect but Na^+ showed increasing trend for peroxidase activity. It was also enhanced under condition contained Mn^{2+} and Fe^{3+}. This some heavy metal acts as cofactor for enzyme activity (Melda *et al*., 2010; Ogawa *et al*., 2004; Ihsan, 2008; Ajila and Prasada, 2008). However, enzyme activity was strongly inhibited by Zn^{2+}, Cd^{2+} and Hg^{2+} (Table 2). This might be suggested that heavy metals precipitated the protein and denatured enzyme protein (Ogawa *et al*., 2004; Ihsan, 2008).

Substrate specificity of peroxidase: Different compounds were tested as hydrogen donor substrates for the enzyme. The OsPOX showed activity with different substrates with variable optimum absorption spectrum (Table 3). Gallic acid, cafeic acid, ascorbic acid, and cathecien had optimum absorption spectrum at 400 nm while glucose and fructose no absorption spectrum could be detected. OsPOX had high specificity for gallic acid followed by cathecien, ascorbic acid, and cafeic acid, respectively (Table 3).

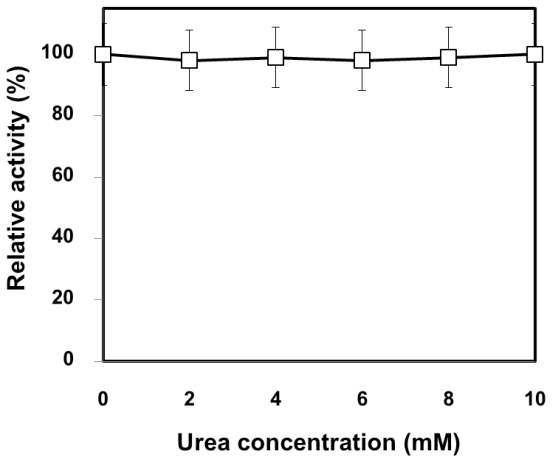


Figure 9. Effect of urea concentration on purified *Oscillatoria* sp. peroxidase activity.

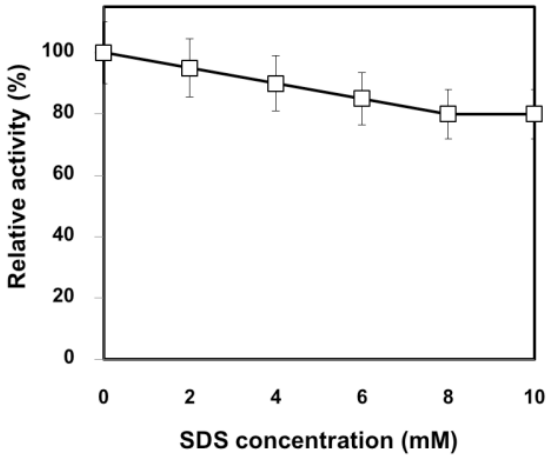


Figure 10. Effect of SDS concentration on purified *Oscillatoria* sp. peroxidase activity.

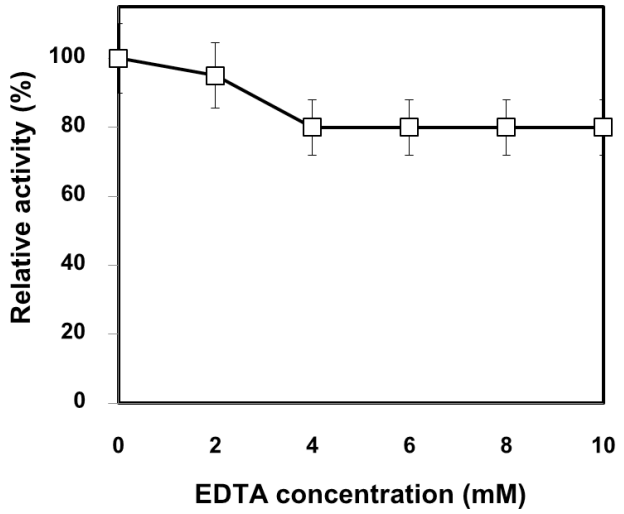


Figure 11. Effect of EDTA concentration on purified *Oscillatoria* sp. peroxidase activity.

Table 1. Enzyme yield and specific activity of purified *Oscillatoria* sp. peroxidase.

Step	Enzyme Yield (%)	Specific activity (units.mg protein^{-1})	Purity (fold)
Crude	100	349.95	1.00
20 – 80 % $(NH_4)_2SO_4$	60.00	1427.80	4.08
DEAE-cellulose	45.25	3586.99	10.25
Sephadex G-100	34.70	6106.63	17.45

Table 2. Effect of cations on purified peroxidase activity from *Oscillatoria* sp.

Cation Type	Relative *Oscillatoria* sp. peroxidase at various concentrations* (%)		
	5 μM	50 μM	500 μM
Na^+	120	150	180
K^+	98	94	87
Mg^{2+}	107	210	221
Ca^{2+}	107	116	124
Mn^{2+}	185	124	105
Fe^{2+}	108	105	96
Ni^{2+}	103	96	65
Cu^{2+}	95	80	55
Zn^{2+}	100	72	37
Cd^{2+}	100	70	34
Hg^{2+}	80	62	25
Cr^{3+}	100	82	75
Fe^{3+}	156	185	207

*Control is purified *Oscillatoria* sp. peroxidase activity without adding of cations which had peroxidase activity is 100 U/mg protein.

Table 3. Effect of substrates on the absorption spectrum and activity of *Oscillatoria* sp. peroxidase.

Substrate	Conc. (mM)	Max absorption spectrum (nm)	Specific activity (mmol.min^{-1}.mg protein^{-1})
Phenol	2	500	3644.13
Gallic acid	2	400	5170.00
Cathecien	2	400	4134.15
Ascorbic acid	2	500	3868.52
Cafeic acid	2	400	2679.24
Glucose	2	ND	-
Fructose	2	ND	-

ND: no detected

The OsPOX was fractionated in 20-80% $(NH_4)_2SO_4$ saturation. These results were in close agreement with previous works (Vernwal *et al*., 2006; Singh *et al*., 2010), who have reported that peroxidase was fractionated adequate amount in 35-75% ammonium sulfate saturation. Gel filtration and SDS-PAGE showed that OsPOX gave a molecular weight of 45 kDa. The peroxidase in *Zizyphus mauritiana* Lamk was predicted molecular weight of 36.3 kDa (Singh *et al*., 2010). Several workers have purified peroxidases from different sources yielding enzyme with a range of molecular weights like purified a Mn peroxidase with 23.08 % recovery with a fold purification of 5.8 (Ihsan, 2008). SDS and native PAGE of the purified peroxidase showed a single band of Coomassie Blue R 250. After heating at 60°C, and separation on SDS gel system, the enzyme maintained its ability to oxidize phenol and to

develop red bands on the gel. Treatment with 2-mercaptoethanol did not yield additional bands, indicating that the enzyme consisted of a single polypeptide chain. The molecular mass calculated by electrophoresis was 45 kDa, which corroborated very well when enzyme was separated with gel filtration.

5. Conclusions

In this work, *Oscillatoria* sp. SWU121 was found to be a value source for a peroxidase enzyme. The enzyme showed wide range of thermal and pH activity. It was stable in urea and slightly decreased in EDTA and SDS. OsPOX utilized some phenolic compound as substrate. The OsPOX might be used or developed to determine phenolic compound from unknown samples by using enzymatic method.

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