Purification and characterization of Mn-peroxidase from *Musa paradisiaca* (banana) stem juice

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Mn-peroxidase (MnP), a biotechnologically important enzyme was purified for the first time from a plant source *Musa paradisiaca* (banana) stem, which is an agro-waste easily available after harvest of banana fruits. MnP was earlier purified only from the fungal sources. The enzyme was purified from stem juice by ultrafiltration and anion-exchange column chromatography on diethylamino ethylcellulose with 8-fold purification and purification yield of 65%. The enzyme gave a single protein band in SDS-PAGE corresponding to molecular mass 43 kDa. The Native-PAGE of the enzyme also gave a single protein band, confirming the purity of the enzyme. The UV/VIS spectrum of the purified enzyme differed from the other heme peroxidases, as the Soret band was shifted towards lower wavelength and the enzyme had an intense absorption band around 250 nm. The *K*ₘ values using MnSO₄ and H₂O₂ as the substrates of the purified enzyme were 21.0 and 9.5 µM, respectively. The calculated *k*ₘₐₜ value of the purified enzyme using Mn(II) as the substrate in 50 mM lactate buffer (pH 4.5) at 25°C was 6.7 s⁻¹, giving a *k*ₘₐₜ/*K*ₘ value of 0.32 µM⁻¹s⁻¹. The *k*ₘₐₜ value for the MnP-catalyzed reaction was found to be dependent of the Mn(III) chelator molecules malonate, lactate and oxalate, indicating that the enzyme oxidized chelated Mn(II) to Mn(III). The pH and temperature optima of the enzyme were 4.5 and 25°C, respectively. The enzyme in combination with H₂O₂ liberated bromine and iodine in presence of KBr and KI respectively. All these enzymatic characteristics were similar to those of fungal MnP. The enzyme has the potential as a green brominating and iodinating agent in combination with KBr/KI and H₂O₂.

**Keywords:** Mn-peroxidase, Metalloenzyme, *Musa paradisiaca*, Banana, Plant peroxidase, heme-enzyme, Bromination reaction.

Manganese peroxidase (E.C.1.11.1.13) first reported¹ in the lignolytic culture of a fungus *Phanerochaete chrysosporium* is a ferric heme enzyme. It has also been shown to be present in a number of fungal strains²-⁴ and is a biotechnologically important enzyme⁵,⁶. The crystal structure of MnP from *P. chrysosporium* has been solved⁷-⁹. Kinetic and spectroscopic characterization of the oxidized intermediates of MnP, MnP(I) and MnP(II) have been done¹⁰. Following catalytic scheme for the catalysis by MnP has been suggested.

\[
\begin{align*}
\text{MnP} + \text{H}_2\text{O}_2 & \rightarrow \text{MnP(I)} + \text{H}_2\text{O} \quad \text{… (1)} \\
\text{MnP(I)} + \text{Mn(II)} & \rightarrow \text{MnP(II)} + \text{Mn(III)} \quad \text{… (2)} \\
\text{MnP(II)} + \text{Mn(II)} & \rightarrow \text{MnP} + \text{Mn(III)} \quad \text{… (3)} \\
\end{align*}
\]

H₂O₂ oxidizes the enzyme by two electrons to form MnP(I) which is oxyferryl porphyrin cation radical [Fe⁴⁺ = O P]⁺. Mn(II) or phenolic compounds can serve as reductants for the MnP(I) and form MnP(II) which is an oxyferryl chemical species [Fe⁴⁺ = O P], one electron oxidized form of the enzyme. For the reduction of MnP(II) to the MnP, Mn(II) is absolutely essential¹¹,¹². The enzyme generated Mn(III) is stabilized by chelators, such as oxalate which are secreted by the fungi¹³,¹⁴. The Mn(III) chelator complex oxidizes lignin substructures and aromatic pollutants¹⁵,¹⁶.

The MnPs have been only reported so far¹-⁴,¹⁵,¹⁶ from fungal species and no MnP has been purified from any other source¹⁷. In this communication, we report purification of MnP from *Musa paradisiaca* (banana) stem juice, a plant source which also contains peroxidase and lignin peroxidase¹⁸. *M. paradisiaca* stem is an agro-waste which is easily available after harvest of banana fruits. The enzymatic characteristics like *K*ₘ, *k*ₘₐₜ, pH and temperature optima have also been determined. The enzyme in combination with H₂O₂ liberates bromine and iodine in presence of KBr and KI, respectively.
Materials and Methods

Chemicals
DEAE-Cellulose was obtained from Sigma Chemical Co., St. Louis, USA. MnSO₄, NaCl and sodium acetate were from Merck Ltd., Mumbai (India) and lactic acid, sodium lactate, malonic acid, sodium malonate, oxalic acid, sodium oxalate and H₂O₂ were from s.d. fine chem Ltd., Mumbai (India) and used without further purification. The chemicals, including the protein molecular weight markers used in SDS-PAGE and Native-PAGE analysis of the purified enzyme were procured from Bangalore Genei Pvt. Ltd., Bangalore (India).

Enzyme purification
*Musa paradisiaca* stem was collected from the local garden. The enzyme was isolated by washing the stem with milli Q water, cutting it into small pieces, crushing the pieces in mortar with pestle, extracting the juice by keeping the pieces in four layers of cheese cloth and squeezing it. The juice was centrifuged using Sigma refrigerated centrifuge (model 3K30, Germany) at 4000 g for 20 min at 4°C to remove the cloudiness. The clear juice (340 ml) was concentrated using Amicon concentration cell model 8200 and ultrafiltration membrane PM-10 with molecular wt. cut-off value of 10 kDa. The concentrated crude enzyme solution (4 ml) was dialyzed against 4 L of 10 mM sodium acetate buffer (pH 6.0) for 24 h over three changes of the buffer.

The dialyzed crude enzyme solution (5 mL) was loaded on to a DEAE-cellulose (column size 1 cm × 33 cm) equilibrated with 10 mM sodium acetate buffer (pH 6.0) at the flow rate of 16 ml/h. The bound protein was washed with 100 ml of the same buffer and eluted with linear gradient of NaCl (0-1 M) in the same buffer (100 ml + 100 ml with 1 M NaCl). The 4.0 ml fractions were collected and analyzed for MnP activity as described above and for protein concentration using Lowry’s method²⁰. The active fractions were combined, concentrated using Amicon concentration cell model 8200 and ultrafiltration membrane PM-10. The concentrated enzyme sample was stored in the fridge at 4°C.

Enzyme assay
The activity of MnP was determined spectrophotometrically¹⁹ by monitoring the absorbance change at λ = 240 nm due to the formation of Mn(III) lactate and using the molar extinction coefficient value of 65.00 M⁻¹cm⁻¹. The reaction solution (1 ml) consisted of 50 µM MnSO₄, 50 µM H₂O₂ and a suitable aliquot of the enzyme solution in 50 mM sodium lactate buffer (pH 4.5) at 25°C. One enzyme unit transformed 1 µM of the substrate into the product under specified assay condition. UV/VIS spectrophotometer (Model U-2000, Hitachi, Japan) which was fitted with electronic temperature control unit was used for spectrophotometric measurements. The least count of the absorbance measurement was 0.001 absorbance unit.

SDS-PAGE and Native-PAGE analysis of the enzyme
The purity of the enzyme preparation was checked by SDS-PAGE as described previously²¹. The separating gel was 12% acrylamide in 0.375 M Tris-HCl buffer (pH 8.8) and stacking gel was 5% acrylamide in 0.063 M Tris-HCl buffer (pH 6.8). The enzyme was visualized by silver staining. The molecular weight markers were phosphorylase (97.4 kDa), bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.1 kDa) and lysozyme (14.3 kDa). The gel was run at a constant current of 20 mA.

The Native-PAGE was done using the reagent kit supplied by Bangalore Genei Pvt. Limited, Bangalore (India). The resolving gel was 8% acrylamide in 0.39 M Tris-HCl buffer of (pH 8.8) and the stacking gel was 5% acrylamide in 0.068 M Tris-HCl buffer (pH 6.8). The ovalbumin (43 kDa) was used as reference protein. The proteins were located by silver staining.

UV/VIS spectral studies
The UV/VIS spectra of native enzyme were recorded using the same spectrophotometer as used in case of the enzyme assay. The enzyme solution consisted of 1 ml of 0.038 µM enzyme in 100 mM sodium acetate acetic acid buffer (pH 5.0).

Determination of *K*ₘ, *k*ₐₜ and *pH* and temperature optima
The *Kₘ* value for Mn(II) was determined by measuring the steady-state velocities of the enzyme-catalyzed reaction at different concentrations of Mn(II) ions at a fixed saturating concentration of H₂O₂ and drawing double-reciprocal plots. The same procedure was adopted for determination of *Kₘ* value for H₂O₂. The *Kₘ* and *k*ₐₜ values were calculated by the linear regression analysis of the data points of double-reciprocal plots. The *pH* optimum of purified enzyme was determined by measuring the steady-state velocity of enzyme-catalyzed reaction in solutions of varying *pH* in the range 3.0 to 6.0 using 50 mM lactic
acid/sodium lactate buffer and plotting a graph of the steady-state velocity vs pH of the reaction solutions. The temperature optimum was determined by measuring the steady-state velocity of enzyme-catalyzed reaction in solutions in the range 15 to 35°C and plotting against the temperature.

**Effect of chelator molecules**

The effect of Mn(III) chelator molecules, such as lactate, malonate and oxalate was determined by measuring the enzyme activity at different concentrations of Mn(II) ions in presence of buffers of chelating carboxylic acids with their sodium salts. The initial velocity of Mn(III) formation was monitored by absorbance change at $\lambda = 270$ nm, as molar extinction coefficients values of Mn(III) complexes with chelators are known at this wavelength$^{23}$. The amount of Mn(III) produced was calculated using molar extinction coefficient values of 5500, 3500, 8500 M$^{-1}$cm$^{-1}$ for Mn(III)oxalate, Mn(III)lactate and Mn(III)malonate, respectively. The steady-state velocity of enzyme catalyzed reaction in µmol.min$^{-1}$ was plotted against [MnSO$_4$] in µM.

**Liberation of bromine or iodine in presence KBr or KI and H$_2$O$_2$ by the enzyme**

The experiment was performed following the method described previously$^{24}$ in which liberated halogens form trihalide complex with halogen ions which can be monitored spectrophotometrically. The absorption spectra of tribromide and triiodide formed in the reaction mixture (1 ml) consisting of 20 mM KBr or KI, 0.1 mM H$_2$O$_2$ and suitable amount of the enzyme in 20 mM sodium succinate/succinic acid buffer (pH 3.0) at 30°C were recorded using the UV/VIS spectrophotometer as mentioned in the enzyme assay section.

**Results and Discussion**

**Enzyme purification**

Although MnP has been purified from a number of fungal sources, namely *Aspergillus niger*$^{25}$, *A. terreus* LD$^{-1}$,$^{26}$ *Coriolus versicolor*,$^{27}$ *Ceropriopsis subvertmispora*,$^{28}$ *Nematalona frowardi*,$^{29}$ *Phanerochaete chrysosporium*,$^{20}$ *Phanerochaete sordida*,$^{30}$ and *Pleurotus ostreatus*$^{31}$, in this study, the MnP was purified for the first time from a non-fungal source (*Musa paradisiaca*). The purification of MnP from *M. paradisiaca* clarified juice involved ultrafiltration and anion-exchange chromatography on DEAE-cellulose and the results are summarized in Table 1. The enzyme elution profile is shown in Fig. 1. An eight-fold purification with 65% yield was achieved.

MnP from *P. chrysosporium* has been studied extensively. It has been purified by two different research groups using two different methods. The procedure reported by Gold and Glenn$^{20}$ involved precipitation by acetone, removal of lignin peroxidase by passing through DEAE-Sepharose column, fractionation of the eluent by blue agarose and then gel filtration on Sephadex G-100 column. The other procedure$^{32}$ involved ultra-filteration, DEAE-Sepharose column chromatography and chromatofocussing on PBE-94 column. The purification procedure of the enzyme reported in the present study was simple, as compared to that reported for MnP from most of the fungal sources. Moreover, *M. paradisiaca* stem after harvest of banana fruits is an agro-waste conveniently available.

**Table 1—Purification of Mn-peroxidase from *Musa paradisiaca* stem juice**

<table>
<thead>
<tr>
<th>Steps</th>
<th>Total volume (ml)</th>
<th>Protein (mg/ml)</th>
<th>Activity (U/ml)</th>
<th>Specific activity (U/mg)</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Purification fold</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>340</td>
<td>0.35</td>
<td>0.96</td>
<td>0.27</td>
<td>0.27</td>
<td>32.640</td>
<td>1</td>
<td>...</td>
</tr>
<tr>
<td>Concentrated sample (Amicon concentration cell unit using PM10)</td>
<td>4</td>
<td>0.62</td>
<td>2.146</td>
<td>3.46</td>
<td>2.48</td>
<td>8.58</td>
<td>12.80</td>
<td>100</td>
</tr>
<tr>
<td>After dialysis</td>
<td>5</td>
<td>0.76</td>
<td>1.82</td>
<td>2.39</td>
<td>3.8</td>
<td>9.10</td>
<td>8.85</td>
<td>69</td>
</tr>
<tr>
<td>DEAE-Cellulose column chromatography</td>
<td>15</td>
<td>0.06</td>
<td>0.16</td>
<td>2.26</td>
<td>0.9</td>
<td>2.40</td>
<td>8.37</td>
<td>65</td>
</tr>
</tbody>
</table>

![Fig. 1—Elution profile of Mn-peroxidase from the DEAE column](image-url)
**SDS-PAGE and Native-PAGE analysis**

The results of SDS-PAGE and Native-PAGE analysis are shown in Fig. 2. SDS-PAGE showed a single protein band (Fig. 2a, lane 3), clearly indicating that enzyme preparation was pure and the molecular mass determined was 43 kDa. The purity of the enzyme was further confirmed by the Native-PAGE (Fig. 2b, lane 2).

**UV/VIS spectral studies**

UV/VIS spectrum of the MnP purified from *Musa paradisiaca* stem is shown in Fig. 3. There was appreciable absorption around 406 nm, the wavelength of Soret band reported in case of MnP of *P. chrysosporium*\(^{23}\). The mM extinction coefficient of the purified enzyme at 406 nm was 132.8 mM\(^{-1}\) cm\(^{-1}\), which compared well with that reported for MnP of *P. chrysosporium* (129.3 mM\(^{-1}\) cm\(^{-1}\)). Moreover, the UV/VIS spectrum of the enzyme was affected by the addition of H\(_2\)O\(_2\) and the enzyme activity was inhibited by sodium azide. All these observations indicated that the purified enzyme was a heme peroxidase. The Soret band in case of the purified enzyme seemed to be shifted at 386 nm with mM molar extinction coefficient value of 189.47 mM\(^{-1}\) cm\(^{-1}\). This was not surprising, as the lowest wavelength at which Soret band has been reported in the literature is 370 nm for Mn (III) protoporphyrin (IX)\(^{13}\). Moreover, the purified enzyme showed strong absorption band around 250 nm. Thus, UV/VIS spectrum of the MnP purified from *M. paradisiaca* juice was unusual and requires separate detailed investigation.

**K\(_m\), k\(_{cat}\) and pH and temperature optima**

The calculated K\(_m\) values for Mn\(^{2+}\) and H\(_2\)O\(_2\) were 21.0 and 9.5 \(\mu\)M, respectively, from the double-reciprocal plots shown in Fig. 4a and b using MnSO\(_4\).\(^{33}\)
and H₂O₂ as the variable substrates, respectively. MnP from P. chrysosporium has been most extensively studied.\(^{23}\) \(K_m\) value (21.0 \(\mu\)M) of the MnP purified from M. paradisiaca stem juice was lower than that for MnP from P. chrysosporium using Mn(II) as the variable substrate (\(K_m\) 41 \(\mu\)M). The \(K_m\) value for H₂O₂ with the purified enzyme was also lower than (\(K_m\) 39 \(\mu\)M) MnP from P. chrysosporium\(^{23}\). The \(K_m\) values for Mn(II) and H₂O₂ as the substrates for the purified MnP were also lower than the corresponding values reported for MnPs purified from other fungal species\(^{34}\).

The calculated \(k_{cat}\) value for the purified MnP using Mn(II) as the substrate in 50 mM lactate buffer (pH 4.5) at 25°C was 6.7 s\(^{-1}\) giving a \(k_{cat}/K_m\) value 0.32 \(\mu\)M\(^{-1}\) s\(^{-1}\), whereas the calculated corresponding value\(^{23}\) for MnP purified from P. chrysosporium is 5.14 \(\mu\)M\(^{-1}\) s\(^{-1}\) on the basis of \(k_{cat}\) and \(K_m\) values reported in the literature\(^{23}\).

Thus, \(k_{cat}/K_m\) value of the purified enzyme from M. paradisiaca MnP was lower than the value calculated for the MnP of P. chrysosporium\(^{23}\).

The MnP showed pH optimum of 4.5 (Fig. 5a) which was found to be similar to that reported for the MnP of P. chrysosporium\(^{23}\). The pH optima for the MnPI, II and III isozymes of P. sordida are 4.5, 4.0 and 4.5, respectively\(^{30}\), whereas for MnP purified from A. terreus LD-I\(^{26}\) has shown pH optimum value of 12.5. The optimum temperature of MnP was 25°C (Fig. 5b) which was near to that reported for MnP of P. chrysosporium\(^{23}\) (28°C), but was different from that reported (37°C) for the MnP of A. terreus LD-I\(^{26}\).

**Effect of chelator molecules**

The effect of Mn(III) chelator molecules malonate, lactate and oxalate on the M. paradisiaca MnP activity, where steady-state velocities were plotted against the concentration of Mn(II) in presence of buffers of various chelating carboxylic acids is shown in Fig. 6. It was evident from the figure that the steady-state turnover rate of M. paradisiaca MnP was dependent on the chelator molecules (oxalate, lactate and malonate) like MnP of P. chrysosporium\(^{23}\).

**Liberation of bromine or iodine in presence KBr or KI and H₂O₂ by the enzyme**

Similar to the MnP of P. chrysosporium, the MnP of M. paradisiaca showed liberation of bromine and iodine in presence of KBr and KI, respectively with H₂O₂ (Fig. 7). Figure 7a shows the spectrum of the solution containing the purified enzyme, 20 mM KBr, 0.1 mM H₂O₂ in 20 mM sodium succinate/succinic acid, pH 3.0. The corresponding spectrum using KI in place of KBr is shown in Fig. 7b. The spectrum in Fig. 7a for tribromide complex showed absorption maximum which was identical to that reported for the

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**Fig. 5**—Effect of pH (a) and temperature (b) on the activity of enzyme [(a) Reaction solution (1 ml) consisted of 0.48 \(\mu\)M of the enzyme, 50 \(\mu\)M MnSO₄, 50 \(\mu\)M H₂O₂ in 50 mM lactate buffer at 25°C. The pH of the buffer was varied from 3.0 to 6.0; and (b) The composition of reaction solution was same as (a), except that pH of the buffer was fixed at 4.5, the enzyme was 0.61 \(\mu\)M and the temperature was varied from 15 to 35°C].

**Fig. 6**—Effect of Mn(III) chelator molecules on the activity of enzyme [The reaction solution (1 ml) consisted of 0.33 \(\mu\)M enzyme, 50 \(\mu\)M H₂O₂ in 20 mM buffers of chelators of pH 4.5 at 25°C and MnSO₄ concentration was varied. 10 \(\mu\)l of 1 mg/ml enzyme stock was used. Succinic acid-sodium succinate (○); Oxalate in succinic acid (✦); Lactic acid-sodium lactate (■); Malonic acid-sodium malonate (▲)].
tribromide complex obtained using MnP of  
P. chrysosporium with absorption maximum at 266 nm. Similarly, the spectrum in Fig. 7b was also similar to that reported for triiodide complex obtained with MnP of  
P. chrysosporium having absorption maxima at 285 nm and 353 nm.

In conclusion, MnP which was earlier isolated only from the fungal sources was purified for first time from a conveniently available plant source  
M. paradisiaca stem juice. Also, the purification procedure was simple than that reported for the MnPs from fungal sources.

The enzyme has the potential as a green brominating and iodinating agent in combination with KBr/KI and H₂O₂.

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References
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Fig. 7—Liberation of Br₂ and I₂ in presence of KBr and KI respectively by the enzyme and H₂O₂ [(a) The reaction solution (1 ml) consisted of 1.13 µM enzyme, 20 mM KBr, 100 µM H₂O₂ in 20 mM sodium succinate buffer, pH 3.0 at 30°C. The reaction solution had the same composition as (a), except the enzyme was 0.23 µM and 20 mM KBr was replaced by 20 mM KI]

[Diagram of absorption spectra]
<table>
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<th>Reference</th>
<th>Title / Authors</th>
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