Purification of Chloroperoxidase from Musa paradisiaca Stem Juice

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ABSTRACT: Chloroperoxidase from Musa paradisiaca stem juice has been purified to homogeneity using a concentration obtained by ultrafiltration and anion exchange chromatography on diethylaminoethyl (DEAE) cellulose. The purified enzyme gave a single protein band in SDS-PAGE analysis corresponding to molecular mass of 43 kDa. The native PAGE analysis result has also given a single protein band, confirming the purity of the enzyme. The purified enzyme was chlorinated and brominated with monochlorodimedone, the substrate used for measuring the halogenating activity of chloroperoxidases. The \( K_m \) and \( k_{cat} \) values using monochlorodimedone as the substrate were 20 \( \mu \)M and 1.64 s\(^{-1} \), respectively, giving a \( k_{cat}/K_m \) value of \( 8.2 \times 10^4 \) M\(^{-1} \) s\(^{-1} \). The pH and temperature optima of the chlorinating activity were 3.0 and 25\( ^\circ \)C, respectively. The \( K_m \) values for the peroxidase activity using pyrogallol and \( \text{H}_2\text{O}_2 \) as the variable substrates were 89 and 120 \( \mu \)M, respectively. The pH and temperature optima of the peroxidase activity using pyrogallol as the substrate were the same as the pH and temperature optima of the halogenating activity. The peroxidase activity of the enzyme is competitively inhibited by sodium azide, indicating that it is a hemeperoxidase different from nonheme peroxidases.

INTRODUCTION

Chloroperoxidase [1] (E.C.1.11.1.10), a heme-thiolate haloperoxidase, is the most versatile enzyme [2,3] in the group of peroxidases. In addition to halogenation and peroxidation reactions, it catalyzes typical reactions of catalases and monooxygenases and is recognized as the most promising enzyme for synthetic applications [2,4,5]. Chloroperoxidase is the catalyst of choice in oxygen transfer reactions of a variety of organic compounds, e.g., N-oxidation [6], S-oxidation [7,8], epoxidation [9], hydroxylation [10], oxidation of alcohols [11], and indole [12]. These reactions have been performed with high activity and selectivity using chloroperoxidase. Chloroperoxidase from Caldariomyces fumago has been used in most of the reported synthetic applications [13–30]. Heme-containing chloroperoxidases from other sources have not been available for synthetic applications since long. Recently, chloroperoxidase from an agaric basidiomycete, Agrocybe aegerita, has been purified [31] and studies on its potential as a biocatalyst in organic synthesis have been initiated [32,33]. Indications are that chloroperoxidase is present in other fungal strains and is a versatile biocatalyst with biotechnological and environmental significance [34]. Although the presence of chloroperoxidase in plants has been detected [35], this enzyme has not been purified from...
a plant source. Keeping in view the applications of chloroperoxidase in synthetic organic chemistry, there is a need to identify new sources of chloroperoxidases and to assess their potential in synthetic organic chemistry.

Our observation on the presence of peroxidase activity and ligninperoxidase activity [36] in the juice of Musa paradisiaca stem has prompted us to test it for the chloroperoxidase activity [1,31]. The analysis has shown the presence of chloroperoxidase activity, which has been purified and characterized. The results of studies are reported in this communication. This is the first chloroperoxidase purified from a plant source, the juice of M. paradisiaca stem, which is an agrowaste available at relatively low cost. The purification process is simpler than those reported for the other chloroperoxidases [1,31].

MATERIALS AND METHODS

Chemicals

Pyrogallol was obtained from Sigma (St Louis, MO) and dimedone (1,1-dimethyl-3,5-cyclohexane) from Acros Organics (Geel, Belgium). All other chemicals were either from Merck (Mumbai, India) or from s.d. fine chem. (Mumbai, India) and were used without further purification. Monochlorodimedone and dichlorodimedone were prepared by using a previously reported method [37].

Determination of Peroxidase Activity

The peroxidase activity of the enzyme was determined spectrophotometrically [38] using pyrogallol as the substrate and monitoring the formation of purpurogallin at \( \lambda = 430 \text{ nm} \) using a molar extinction coefficient value of \( 2.47 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1} \). The reaction solution contained 0.5 mM pyrogallol, 0.45 mM \( \text{H}_2\text{O}_2 \) in 20 mM potassium phosphate buffer pH 4.8 at 25°C, and a suitable aliquot of the enzyme sample in a final volume of 1 mL. The reaction was initiated by addition of \( \text{H}_2\text{O}_2 \). The inhibition of the peroxidase activity of the purified enzyme by \( \text{NaN}_3 \) was studied by drawing double reciprocal plots in the presence of 0, 10, and 20 mM concentrations of sodium azide using guaiacol as the variable substrate.

Studies of Halogenating Activity

The halogenating activity of the enzyme was determined using monochlorodimedone as the substrate [37] and monitoring the conversion of monochlorodimedone to dichlorodimedone by a decrease in the absorbance at \( \lambda = 278 \text{ nm} \) using the molar extinction coefficient value of 12,200 \( \text{ M}^{-1} \text{ cm}^{-1} \). The reaction solution contained monochlorodimedone 0.12 mM, potassium chloride 20 mM, hydrogen peroxide 2 mM in 100 mM potassium phosphate buffer pH 2.75, and a suitable aliquot of the enzyme at 25°C in a final volume of 1 mL.

During chloroperoxidase purification, the chloroperoxidase activity was determined using 4-chloroaniline as the substrate and monitoring the increase in the absorbance at \( \lambda = 320 \text{ nm} \) due to formation of 4-chloronitrosobenzene [39] using the molar extinction coefficient value of 10,300 \( \text{ M}^{-1} \text{ cm}^{-1} \). The protein concentration was determined by using the Lowry method [40]. A UV–vis spectrophotometer Hitachi (Japan) model U-2000, which was fitted with a temperature control unit, was used for spectrophotometric measurements. Each data point is an average of three measurements, and standard deviation was less than 5%.

Purification of the Chloroperoxidase

M. paradisiaca stem was collected from the Botanical Garden, Department of Botany, D. D. U. Gorakhpur University, Gorakhpur, India. The enzyme was isolated by washing the stem of M. paradisiaca stem with milli Q water, cutting it into small pieces, crushing the pieces in a mortar with a pestle, extracting the juice by keeping the pieces in four layers of cheese cloth, and squeezing it. The juice was centrifuged using a Sigma-refrigerated centrifuge (Germany) model 3K 30 at 4000 \( \times g \) for 20 min at 4°C to remove the cloudiness of the juice. The clear juice about 850 mL was concentrated to 10 mL using an Amicon concentration cell model 8200 and ultrafiltration membrane PM 10. The concentrated crude enzyme solution 10 mL was dialyzed against 10 L of 10 mM sodium acetate buffer pH 6.0 for 24 h over three changes of the buffer. Five milliliters of the dialyzed crude enzyme solution was loaded on a diethylaminoethyl (DEAE) cellulose column size 1 cm \( \times 33 \text{ cm} \) equilibrated with 10 mM sodium acetate buffer pH 6.0 at the flow rate of 15 mL/h. The bound protein was washed with 100 mL of the same buffer, and the protein was eluted with a linear gradient of sodium chloride 0–1 M in the same buffer (100 mL + 100 mL with 1 M NaCl). Five-milliliter fractions were collected and analyzed for the chloroperoxidase activity using 4-chloroaniline as the substrate [39] and for the protein concentration using the Lowry method [40]. The active fractions were combined, concentrated using the Amicon concentration cell model 8200 and then model 3 with ultrafiltration.
membranes PM 10. The concentrated enzyme sample was stored at 4° C.

**SDS-PAGE and Native PAGE Analysis of the Enzyme**

The homogeneity of the enzyme preparation was checked by SDS-PAGE using the method of Weber and Osborn [41]. The separating gel was 12% acrylamide in 0.375 M Tris–HCl buffer pH 8.8, and the stacking gel was 5% acrylamide in 0.063 M Tris–HCl buffer 6.8. The enzyme was visualized by staining with coomassie Blue R-250. The molecular weight markers were phosphorylase (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and lysozyme (14.3 kDa). The gel was run at a constant current of 20 mA.

The native polyacrylamide gel electrophoresis was done using the reagent kit supplied by Bangalore Genei (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 of pH 6.8. The molecular weight marker used was ovalbumin (43 kDa). The proteins were located by silver staining.

**HPLC Analysis of the Reaction Product of the Enzyme and Monochlorodimedone in the Presence of H₂O₂ and KCl**

The reaction solution 1 mL contained 0.1 mM monochlorodimedone, 20 mM KCl, 2 mM H₂O₂, 0.02 IU of the enzyme in 50 mM phosphate buffer pH 2.75 at 30° C. The control experiment was performed with same constituents except KCl. The reaction was allowed to occur for 20 min, and the products were extracted thrice using 1 mL n-hexane each time. 20 μL of the extract was injected in the Waters HPLC model 600E using spherisorb C18 5 UV, 4.5 mm x 250 mm column. The eluent phase was a methanol–water mixture in the ratio 70:30 (v/v) at the flow rate of 1 mL/min. The detection was made using the Waters UV detector model 2487 at λ = 278 nm.

**Detection of Chlorine Liberated by the Enzyme Action in the Presence of H₂O₂ and KCl**

This was done by using the method of Libby et al. [42]. The reacting solution 50 mL contained 2 mM H₂O₂, 20 mM KCl, 0.96 IU of the enzyme (determined by 4-chloroaniline as the substrate) in 0.1 M phosphate buffer pH 2.75 at 30°C in a 100-mL conical flask fitted with a rubber cork having an inlet and an outlet syringe needles. N₂ gas was bubbled for 20 min through the reaction solution, and the outlet gas was passed into 50 mL of 50 mM NaOH solution kept in icebath. The spectrum of the NaOCl formed was recorded in the wavelength region 400–220 nm, and it was compared with the spectra of standard solution of NaOCl and of a solution in which chlorine water was added in 50 mM NaOH.

**RESULTS AND DISCUSSION**

The results of the purification procedure of the chloroperoxidase from the juice of *M. paradisiaca* stem are summarized in Table I. It involved a concentration of the juice using ultrafiltration, dialysis, and anion exchange chromatography on DEAE cellulose. The enzyme binds to DEAE cellulose at pH 6.0 in a 10 mM sodium acetate/acetic acid buffer and is eluted by a NaCl gradient in the range 125–400 mM. The activity elution profile of the enzyme is shown in

<table>
<thead>
<tr>
<th>S. N.</th>
<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>1</td>
<td>Crude enzyme</td>
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<td>0.35</td>
<td>0.096</td>
<td>0.27</td>
<td>27.95</td>
<td>81.9</td>
<td>1</td>
<td>100</td>
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<tr>
<td>2</td>
<td>Concentrated (Amicon concentration cell unit using PM10)</td>
<td>10</td>
<td>0.62</td>
<td>2.146</td>
<td>3.46</td>
<td>6.23</td>
<td>21.5</td>
<td>12.8</td>
<td>26.3</td>
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<tr>
<td>3</td>
<td>Dialyzed</td>
<td>13</td>
<td>0.76</td>
<td>1.82</td>
<td>2.39</td>
<td>9.88</td>
<td>23.66</td>
<td>8.85</td>
<td>28.9</td>
</tr>
<tr>
<td>4</td>
<td>DEAE cellulose column</td>
<td>20</td>
<td>0.08</td>
<td>0.18</td>
<td>2.25</td>
<td>1.6 (4.16)</td>
<td>3.6 (9.36)</td>
<td>8.33 (8.33)</td>
<td>(11.4)</td>
</tr>
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Since only 5 mL of 13 mL dialyzed sample was loaded on the DEAE cellulose column, the values given in brackets are calculated on the basis of 13 mL of dialyzed sample.
Purification of Chloroperoxidase from *Musa paradisiaca* stem juice

Figure 1  Elution profile of the chloroperoxidase activity from the DEAE cellulose column: activity (●) and protein (▲).

Fig. 1. All the chloroperoxidase active fractions were combined and concentrated as mentioned in the section Purification of the Chloroperoxidase. The concentrated enzyme sample was analyzed by SDS-PAGE and native PAGE analysis, and the results are shown in Fig. 2. The SDS-PAGE analysis gave a single protein band. The calculated molecular mass from the SDS-PAGE analysis shown in Fig. 2(a) of the purified enzyme was 43.0 kDa, which was in the same range as the molecular masses reported for chloroperoxidases of *C. fumago* 42 kDa [1] and *A. aegerita* 46 kDa [31]. The results of native PAGE analysis of the purified enzyme shown in Fig. 2(b) also gave a single protein band, confirming the purity of the enzyme preparation. The reported purification procedure for the chloroperoxidase of *M. paradisiaca* stem juice is simpler than the purification procedures of chloroperoxidases from the culture filtrates of *C. fumago* [1] and *A. algerita* [31]. It is obvious from Table I that the specific activity of the enzyme has not increased after anion-exchange chromatography as expected on the basis of the SDS-PAGE analysis of the crude and purified enzyme, indicating that the enzyme has lost some activity during the anion exchange chromatography step. The anion exchange chromatography has been performed at pH 6.0, at which the enzyme is unstable as shown in our later pH stability studies on the activity of this enzyme, indicating that enzyme should not be exposed to pH 6.0 during the purification procedure.

The color of the purified concentrated enzyme (0.8 mg/mL) was light reddish brown, characteristic of heme peroxidases. The UV-vis spectrum of the purified enzyme is shown in Fig. 3. The spectrum has been recorded by fixing the lamp change at 350 nm cannot be ignored. Even if the spectrum portion below 350 nm is ignored, there is neither absorbance maximum at λ = 403 nm as reported in the case of *C. fumago* chloroperoxidase [1] nor the absorbance maximum at λ = 420 nm as reported in the case of *A. aegerita* chloroperoxidase [31]. However, there is an appreciable absorbance value at λ = 403 nm, giving the molar extinction coefficient value of 76.1 mM$^{-1}$ cm$^{-1}$, which compares well with the molar extinction coefficient value of €$_{403}$ = 75.3 mM$^{-1}$ cm$^{-1}$ reported for chloroperoxidase of *C. fumago*. The enzyme preparation was repeated three times, but every time SDS-PAGE and native PAGE analysis of the enzyme using silver staining showed that the enzyme was pure. Addition of H$_2$O$_2$ changed the spectrum of the enzyme, and sodium azide inhibited the activity of the purified enzyme competitively with a KI value of 10 mM, indicating that it is heme chloroperoxidase [43] different from nonheme chloroperoxidase [44]. The explanation of unusual UV–vis spectrum of the purified enzyme needs extensive studies, which has not been undertaken in this communication.

The chlorinating and brominating activities of the chloroperoxidase purified from *M. paradisiaca* stem juice are shown in Fig. 4 in which a decrease in the absorbance at λ = 278 due to chlorination or bromination of monochlorodimedone is plotted against time. The rate of bromination of monochlorodimedone was 2.8 times faster than the rate of chlorination. It is worth noting that in the case of chloroperoxidase of *C. fumago*, the rate of bromination of monochlorodimedone has been reported [37] to be two times faster than the rate of chlorination. The chlorination of monochlorodimedone to dichlorodimedone in the presence of KCl, H$_2$O$_2$, and the enzyme has been further confirmed.
Figure 2 Results of SDS-PAGE analysis of the purified chloroperoxidase. (a) Lane 1: molecular weight markers, lane 2: concentrated crude enzyme; lane 3: purified enzyme. (b) Lane 1: ovalbumin (marker protein); lane 2: purified enzyme (8 μg). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

by the HPLC analysis of the reaction product. The results are shown in Fig. 5. Figure 5a shows the HPLC analysis of the n-hexane extract of the reaction products formed in the presence of KCl, H₂O₂, monochlorodimedone, and the enzyme. The peak at retention time 4.99 min coincides with the peak of dichlorodimedone as shown in Fig. 5b, where the retention time is 4.97 min. The peak with retention time is 5.58 min in Fig. 5a coincides with the peak of monochlorodimedone as shown in Fig. 5c, where the value of the retention time is 5.53 min. The retention time of the product in the absence of KCl as shown in Fig. 5d is 5.58 min, which also coincides with the retention time of monochlorodimedone, indicating that it has not been converted to dichlorodimedone in the absence of KCl. All these compounds have been detected at λ = 278 nm, which is λ_{max} for monochlorodimedone at pH 2.75. The ratios of the peak areas at retention time 4.97 and 5.53 min corresponding to dichlorodimedone and monochlorodimedone, respectively, do not represent the concentrations of these two components because their molar extinction coefficient values at λ = 278 nm are far apart, i.e., 190 and 1000.
Figure 5 Results of the HPLC analysis of the enzyme-catalyzed chlorination of monochlorodimedone: (a) product with the enzyme, H$_2$O$_2$, and KCl, (b) dichlorodimedone standard sample, (c) monochlorodimedone standard sample, and (d) a product with the enzyme, H$_2$O$_2$ without KCl.

12,200 M$^{-1}$ cm$^{-1}$, respectively [37]. The analysis of the ratios of the areas of the peaks taking molar extinction coefficients into account gives the concentration ratio of dichlorodimedone to monochlorodimedone as 50:3 in Fig. 5a.

The chloroperoxidase activity of the purified enzyme was further confirmed by the liberation of Cl$_2$ in a reaction medium containing 20 mM KCl, 2 mM H$_2$O$_2$ in 100 mM phosphate buffer pH 2.75, and 0.05 IU of the enzyme in 50 mL of the reaction solution. N$_2$ gas was bubbled in the reaction mixture, and the gas evolved was trapped in 50 mL of 50 mM NaOH solution, and spectrum of the solution was recorded along with the spectrum of the reaction product of Cl$_2$ water and NaOH and also the spectrum of standard solution of NaOCl. The results are shown in Fig. 6. Figure 6, curve a, shows the spectrum of the solution in which Cl$_2$ gas has been trapped in cold and dilute NaOH, Fig. 6, curve b, shows the spectrum of the reaction of Cl$_2$ water with cold and dilute NaOH, and Fig. 6, curve c, shows the spectrum of the standard solution of NaOCl. The similarity in the spectrum clearly confirms that in the presence KCl, H$_2$O$_2$ at pH 2.75, the purified enzyme liberates chlorine, showing that the purified enzyme is a chloroperoxidase.

Figure 6 Results of the enzyme-catalyzed liberation of chlorine from KCl: (a) Spectra of NaOCl (0.71 mM) formed by the reaction of cold dilute NaOH and Cl$_2$ liberated by the enzymatic reaction of H$_2$O$_2$ and KCl at pH 2.75. (b) Spectra of NaOCl (0.59 mM) formed by the reaction of Cl$_2$ water with cold and dilute NaOH. (c) Spectra of standard solution of NaOCl (0.77 mM). All the spectra were recorded with reference to 50 mM NaOH.

The Michaelis–Menten and double reciprocal plots were drawn using monochlorodimedone as the variable substrate at the fixed saturating H$_2$O$_2$ and KCl concentrations (figure not shown). The calculated $K_m$
value of the purified enzyme using monochlorodimedone as the substrate was 20 μM and the $k_{\text{cat}}$ value was 1.64 s$^{-1}$ giving a value of $k_{\text{cat}}/K_m$ 8.2 × 10$^4$ M$^{-1}$ s$^{-1}$. Michaelis–Menten and double reciprocal plots were also drawn using H$_2$O$_2$ as the variable substrate keeping the concentrations of monochlorodimedone and KCl at fixed saturating values, but the Michaelis–Menten curve was not hyperbolic at lower concentrations of H$_2$O$_2$, which did not allow the determination of the $K_m$ value for H$_2$O$_2$ of the purified enzyme for its chlorinating activity.

The peroxidase activity of the purified enzyme, which does not need the presence of halogen anions, was determined using pyrogallol as the variable substrate (0.06–0.6 mM) at the fixed saturating concentration of 0.45 mM of H$_2$O$_2$. The Michaelis–Menten and double reciprocal plots were drawn (not shown here). The calculated $K_m$ and $k_{\text{cat}}$ values were 89 μM and 26.56 s$^{-1}$, respectively, giving a $k_{\text{cat}}/K_m$ value of 29.84 × 10$^4$ M$^{-1}$ s$^{-1}$. The calculated $K_m$ value for H$_2$O$_2$ of the purified enzyme for the peroxidase activity was 120 μM.

Since chloroperoxidase is the most promising enzyme for synthetic applications [2–30,45–47], the optimal pH and temperature conditions for its catalytic functions for both halogenating and peroxidase activities were determined. The pH optimum of the purified enzyme for the halogenating activity is 3.0 as shown in Fig. 7, which is nearer to the pH optimum 2.75 of halogenating activity of $C.\ fumago$ chloroperoxidase [1]. The pH optimum of the peroxidase activity using pyrogallol as the substrate has also been found to be 3.0, the same as the pH optimum of its halogenating activity. This finding is different from the pH optimum reported [43] for the peroxidase activity of $C.\ fumago$ chloroperoxidase, which is in the range of 4.0–7.0 depending on the substrate used. The pH stability data for the purified enzyme is shown in Fig. 8. It is obvious from the figure that at pH 2.75 it loses 85% activity in 1 h, and it is completely inactivated in 24 h. At pH 4.4, the enzyme also loses activity with time and is completely inactivated after 2.5 h. However, the enzyme exposed to pH 6.0 has low activity but it remains constant for 24 h. According to Fig. 7, the chloroperoxidase activity should be low at pH 6.0 that is expected but the enzyme activity assay for chloroperoxidase has been done at pH 4.4 in each case and therefore the initial activity at time zero should be same in all the three cases but in the case of the enzyme exposed pH 6.0 it is not. It seems that enzyme exposed to pH 6.0 does not equilibrate to pH 4.4 in the time duration used for activity determination. van Rantwijk and Sheldon [2] have reported that the $C.\ fumago$ chloroperoxidase is irreversibly inactivated at pH > 6.0, and, therefore, it should be used below pH 5.0. The possibility that the enzyme preparation has multiple isoforms of the enzyme, some of which are irreversibly inactivated at pH 6.0, does not seem applicable because results obtained so far have not indicated the presence of isoforms of the enzyme. The temperature optimum of the chlorinating activity of the purified enzyme is 25°C as shown in Fig. 9, which is the same as the temperature optimum of the peroxidase activity.

This communication reports the purification of a chloroperoxidase from a plant source, $M.\ paradisiaca$
stem juice, for the first time. The source *M. paradisiaca* stem is an agrowaste available at relatively low cost, and the purification procedure is simpler than those used for the purification of other chloroperoxidases [1,31]. Indications are (unpublished results) that the reported chloroperoxidase may prove to be an alternative of *C. fumago* chloroperoxidase [31] as a biocatalyst in a variety of reactions used in synthetic organic chemistry.

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