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ORIGINAL ARTICLE

N-Oxidation of arylamines to nitrosobenzenes using chloroperoxidase purified from *Musa paradisiaca* stem juice

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Abstract

N-Oxidation of arylamines to their corresponding nitrosobenzenes using a new chloroperoxidase purified from *Musa paradisiaca* stem juice has been examined. The enzymatic characteristics of the stem chloroperoxidase using 4-chloroaniline as substrate were determined. The K_m values for 4-chloroaniline and H_2O_2 were 770 μM and 154 μM respectively, while the pH and temperature optima were 4.4 and 30°C respectively. The substrate specificities of the enzyme for the arylamines 3,4-dichloroamine, *p*-aminobenzoic acid, *p*-toluidine, *p*-anisidine, *m*-anisidine, *p*-aminophenol, *o*-aminophenol and *m*-aminophenol have been characterized. The feasibility of using concentrated *M. paradisiaca* stem juice for the specific conversion of 4-chloroaniline to 4-chloronitrosobenzene has been demonstrated. This enzyme can be used for the *N*-oxidation of other arylamines.

Keywords: *Peroxidase,* N*-oxidation, metalloenzyme,* Musa paradisiaca*, arylamine, nitrosobenzene*

Introduction

Chloroperoxidase (EC 1.11.1.10) (Morris & Hager 1966; Kuhnel et al. 2006) is a versatile biocatalyst for organic synthetic reactions (Spreti et al. 2004). In addition to halogenation (Hager et al. 1966) and classical peroxidation reactions (Thomas 1970), it catalyzes typical reactions of catalases and monooxygenases and is the most promising enzyme for synthetic applications (Hofrichter & Ullrich 2006). Chloroperoxidase is the catalyst of choice in oxygen transfer reactions of a variety of organic compounds, e.g. *N*-oxidation (Corbett et al. 1980), *S*-oxidation (Colonna et al. 1992; Allenmark & Andersson 1996), epoxidation (Hager et al. 1998; Zhu & Wang 2005), hydroxylation (Hu & Hager 1999; Ullrich & Hofrichter 2007), oxidation of alcohols (Kiljiunen & Kanerva 2000) and indole (Van Deurzen et al. 1996). In all of these reactions, chloroperoxidase from *Caldariomyces fumago*, a marine fungus, has been used (Sanfilippo et al. 2004; La Rotta Hernandez et al. 2005). Recently, a heme-containing chloroperoxidase from *Agrocybe aegerita* has been purified and studies on its potential as a biocatalyst in synthetic organic chemistry have been initiated (Ullrich et al. 2004; Ullrich & Hofrichter 2005). Although chloroperoxidase has been detected in plants (Speicher et al. 2003), the enzyme has not been purified until recently. We have purified a chloroperoxidase from *Musa paradisiaca* (plantain) stem juice (Yadav et al. 2009) and have initiated studies on its potential as a biocatalyst in organic synthetic reactions. The present communication reports the *N*-oxidation of arylamines to their corresponding nitrosobenzenes using the stem juice chloroperoxidase.

Materials and methods

Dimedone (1,1-dimethyl-3,5-cyclohexane) was from Acros Organics (Geel, Belgium). All other chemicals were either from Merck Ltd (Mumbai, India) or S.D. Fine Chemicals Ltd (Mumbai, India) and were used without further purification. The activity of the chloroperoxidase was assayed spectrophotometrically (Corbett et al 1978) using 4-chloroaniline as the substrate and monitoring the formation of 4-chloronitrosobenzene by the absorbance change at λ=320 nm, using a molar extinction coefficient

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value of 10 300 M^{-1} cm^{-1}. The assay solution consisted of 4 mM 4-chloroaniline, 0.6 mM H_2O_2 and a suitable aliquot of the enzyme in 20 mM potassium phosphate buffer pH 4.4 at 30°C. The reaction was initiated by the addition of H_2O_2. One enzyme unit is the amount of the enzyme which converts 1 μM of the substrate per minute into the product under the specified assay conditions.

A Hitachi (Tokyo, Japan) U-2000 UV/VIS spectrophotometer fitted with electronic temperature control was used for spectrophotometric measurements. The lowest absorbance measurement was 0.001 absorbance unit.

The chloroperoxidase from *M. paradisiaca* stem juice was purified by a method developed in our laboratory (Yadav et al. 2009). This consisted of washing the *M. paradisiaca* stem with MilliQ water, cutting it into small pieces, crushing the pieces in a mortar with a pestle, extracting the juice by placing the pieces in four layers of cheese cloth and squeezing it. The juice was centrifuged using a Sigma (Osterode, Germany) refrigerated centrifuge model 3K30 at 4000***g*** for 20 min at 4°C to remove the cloudiness of the juice. The clear juice was concentrated using an Amicon (Amicon Division, W.R. Grace & Co-Conn, Beverly, MA, USA) model 8200 concentration cell and a PM 10 ultrafiltration membrane (molecular weight cut-off of 10 kDa). The concentrated crude enzyme solution was dialyzed against a 1000 times excess of 10 mM sodium acetate buffer pH 6.0 for 24 h with three changes of buffer. The dialyzed crude enzyme solution was loaded on 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^{-1}. The bound protein was washed with the same buffer and the protein eluted with a linear gradient of 0–1 M NaCl in the same buffer (100 mL + 100 mL with 1 M NaCl). Fractions of 4.0 mL were collected and analyzed for protein concentration (Lowry et al. 1951) and chloroperoxidase activity. The active fractions were combined, concentrated using an Amicon (Amicon Division, W.R. Grace & Co-Conn, Danvers, MA, USA) model 8200 concentration cell and then a model 3 concentration cell with PM 10 ultrafiltration membranes. The concentrated enzyme sample was stored at 4°C until required.

The homogeneity of the purified enzyme was tested by SDS-PAGE analysis (Weber & Osborn 1969). 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 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 constant current of 20 mA.

The pH optimum for *N*-oxidation activity of the chloroperoxidase was determined by measuring the steady-state velocity of the enzyme-catalyzed reaction using 4-chloroaniline as substrate in solutions ranging from pH 3.0 to 6.5 in 20 mM potassium phosphate buffer at 30°C. The temperature optimum was determined by measuring the steady-state velocity as above at temperatures in the range 20–45°C.

The substrate specificities of the purified enzyme for the substituted arylamines were determined by measuring the steady-state velocities of the enzyme-catalyzed reactions using substituted arylamines as the variable substrates and monitoring the formation of the corresponding nitroso compounds spectrophotometrically by absorbance measurement at λ=320 nm. The K_m values for various substituted arylamines were determined from double-reciprocal plots (Engel 1977).

In order to test the feasibility of larger-scale enzymatic conversion of 4-chloroaniline to 4-chloronitrosobenzene, 200 mL of 2 mM 4-chloroaniline in 0.1 M potassium phosphate buffer pH 4.4 at 25°C was treated with 200 μL of the concentrated *M. paradisiaca* stem juice containing 4.7 U enzyme mL^{-1} and H_2O_2 was added in four steps, 0.5 mM in each step at intervals of 10 min. The reaction solution was left for 1 h and then extracted twice with 200 mL of diethyl ether. Diethyl ether was removed by evaporation at room temperature and the residual solid was recrystallized from methanol. The purity of the product was confirmed by the appearance of a single peak in HPLC (model M-600E chromatograph and Spherisorb C18 column, 5 μm

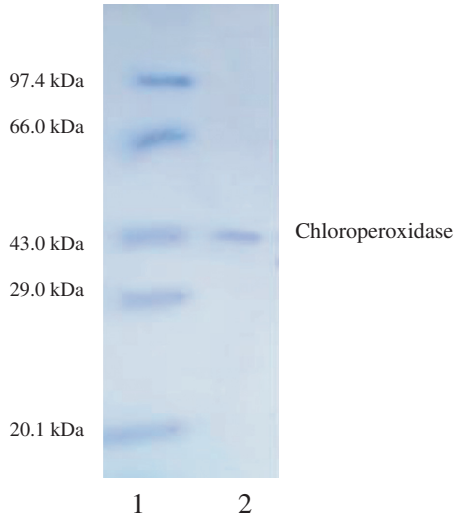

Figure 1. Results of SDS-PAGE analysis of the purified chloroperoxidase: lane 1, molecular weight markers; lane 2, purified enzyme.

film, 4.6 mm × 250 mm (Waters, Milford, MA, USA); methanol–water solvent (7:3, v/v) at a flow rate of 1 mL min^{-1}). The product was identified as 4-chloronitrosobenzene by IR (3100 FT-IR spectrometer; Varian Inc Corporate, Palo Alto, CA, USA) and ^{1}H and ^{13}C NMR (AL300 FT NMR spectrometer; JEOL Ltd, Tokyo, Japan).

Results and discussion

SDS-PAGE analysis of the purified enzyme is shown in Figure 1. The appearance of a single polypeptide band in lane 2 in which the purified chloroperoxidase had been applied indicated that the enzyme was pure. The Michaelis–Menten and double-reciprocal plots for the purified chloroperoxidase using 4-chloroaniline as the variable substrate at a fixed, enzyme-saturating concentration of H_2O_2 are shown in Figure 2. The equivalent plots using H_2O_2 as the variable substrate at a fixed enzyme-saturating concentration of 4-chloroaniline are given in Figure 3. The calculated K_m values for 4-chloroaniline and H_2O_2 were 770 μM and 154 μM respectively. The reported (Corbett et al. 1978) K_m value for 4-chloroaniline using *C. fumago* chloroperoxidase is 810 μM, which is similar to that calculated for the chloroperoxidase from the *M. paradisiaca* stem juice.

In order to assess the potential of *M. paradisiaca* stem juice chloroperoxidase in the transformation of arylamines to their corresponding nitroso compounds, the specificity towards 3,4-dichloroaniline, *p*-aminobenzoic acid, *p*-toluidine, *p*-anisidine, *m*-anisidine, *p*-aminophenol, *o*- aminophenol and *m*-aminophenol was tested. Double-reciprocal plots were drawn in all these cases and the K_m and k_{cat} values

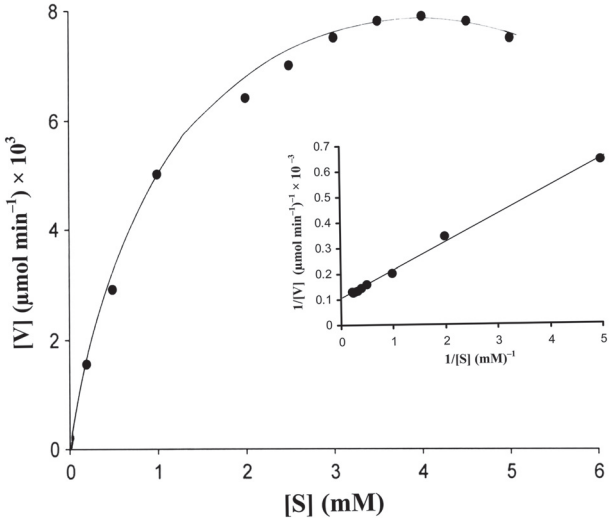

Figure 2. Michaelis–Menten and double-reciprocal plot (insert) for the chloroperoxidase of *Musa paradisiaca* stem juice using 4-chloroaniline as the variable substrate.

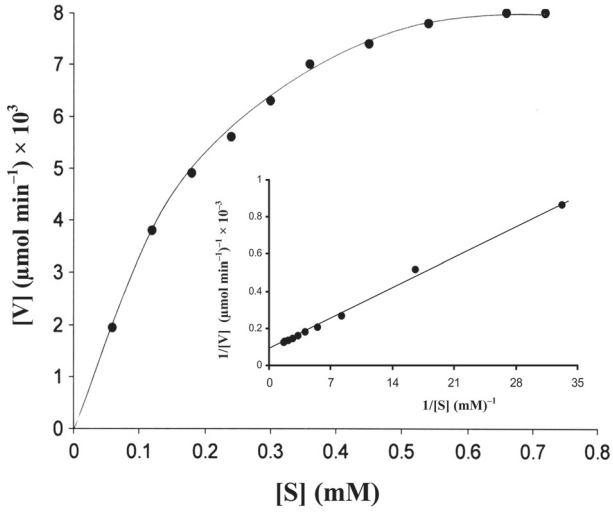

Figure 3. Michaelis–Menten and double-reciprocal plot (insert) for the chloroperoxidase of *Musa paradisiaca* stem juice using H_2O_2 as the variable substrate.

calculated. The results are summarized in Table I. All of the substituted arylamines tested were converted into their corresponding nitroso compounds, which were monitored spectrophotometrically by measuring the absorbance change at λ=320 nm.

In order to find the optimum conditions for the enzymatic transformations, the pH and temperature optima of the *M. paradisiaca* stem chloroperoxidase were determined using 4-chloroaniline as substrate (Figures 4 and 5 respectively). This showed that the pH and temperature optima for this transformation were 4.4 and 30°C respectively. The pH optimum of the chloroperoxidase from *C. fumago* also is also 4.4 (Corbett et al. 1978).

The feasibility of enzymatic transformation of 4-chloroaniline to 4-chloronitrosobenzene using the crude preparation of chloroperoxidase of *M. paradisiaca* stem juice has been demonstrated. The product extracted using diethyl ether gave a single peak by HPLC analysis and IR (KBr, ν_{max} (cm^{-1}): 1622, 2924 cm (C-H aromatic), 1622 (C=C), 1492 (N=O), 1437 (C=C), 1279 (C-N), 910 (C-N), 822 (1,4

Table I. K_m and k_{cat} values of the purified enzyme for different substrates.

Substrate	K_m (μM)	k_{cat} (s^{-1})
H_2O_2	154	0.36
4-Chloroaniline	770	0.35
3,4-Dichloroaniline	1000	0.56
p-Aminobenzoic acid	44	0.57
p-Toluidine	50	0.59
p-Anisidine	133	0.48
p-Aminophenol	132	0.4
o-Aminophenol	36	0.54
m-Aminophenol	125	0.29

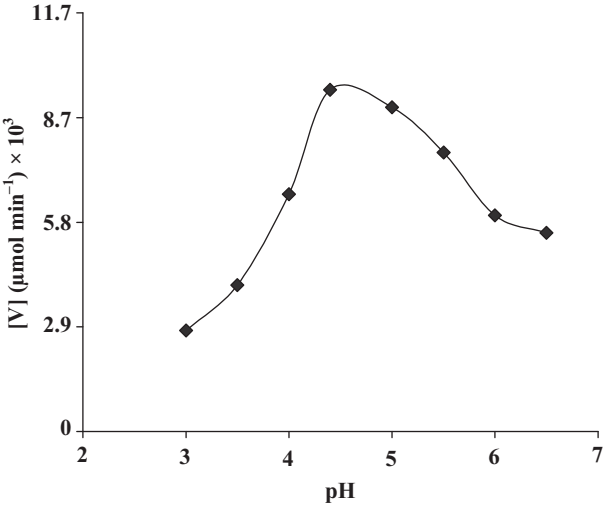

Figure 4. Variation of the activity of the chloroperoxidase of *Musa paradisiaca* stem juice with reaction pH.

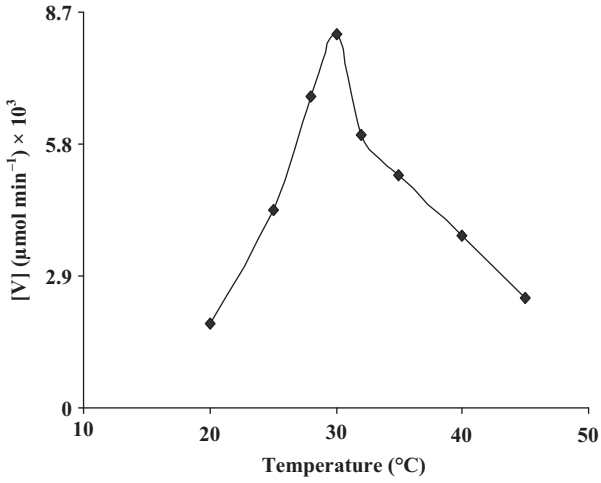

Figure 5. Variation of the activity of the chloroperoxidase of *Musa paradisiaca* stem juice with reaction temperature.

disubstition in benzene). ^{1}H NMR (300 MHz, DMSO, δ (ppm): 6.48 (2H, d, J=8.4 Hz), 6.99 (2H, d, J=8.4 Hz)) and ^{13}C NMR (75 MHz, DMSO, δ (ppm): 115.2, 118.7, 128.5, 147.7 (phenyl ring)) confirmed the identity of 4-chloronitrosobenzene. No other products were detected and 53 mg of 4-chloronitrosobenzene was obtained from 87 mg of 4-chloroaniline using 0.94 U enzyme, which was equivalent to 0.16 mg of the pure enzyme.

Elucidating the detailed kinetics and mechanism of the transformation of 4-chloroaniline to 4-chloronitrosobenzene catalyzed by chloroperoxidase from *M. paradisiaca* stem juice requires extensive studies. However, the mechanism suggested for the transformation of 4-chloroaniline to 4-chloronitrosobenzene catalyzed by chloroperoxidase from *C. fumago* (Corbett et al. 1978, 1980) may be considered operative. According to this mechanism, the enzyme-bound oxygen of chloroperoxidase (compound **I**) is directly incorporated into the arylamine substrate as shown in Scheme 1. A further two-electron abstraction from hydroxylamine leads to the arylnitroso compound. This proposal is based on the peroxidation mechanism for chloroperoxidase-catalyzed *N*-oxidation of arylamines (Doerge & Corbett 1991) and is supported by the accepted mechanism for cytochrome P450-catalyzed oxygenation of numerous substrates (Urlacher & Eiben 2006; Urlacher & Schmid 2006).

In conclusion, this communication reports the transformation of arylamines into their corresponding nitroso compounds using a new chloroperoxidase from *M. paradisiaca* stem juice. A single product is formed using even the crude enzyme preparation, which indicates the synthetic significance of the chloroperoxidase isolated from a convenient source using a simple procedure.

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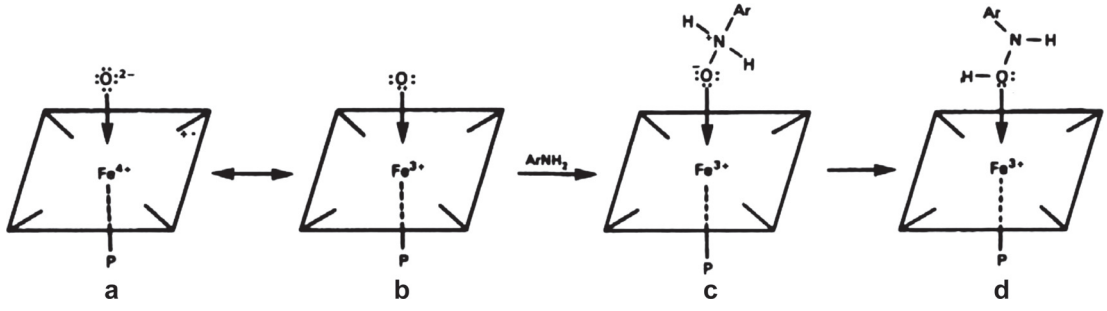

Scheme 1. Proposed mechanism for halide-independent *N*-oxidation (Corbett et al. 1980). (a) Tetravalent iron porphyrin π-cation radical resonant form of chloroperoxidase (compound **I**); (b) trivalent iron oxene resonant form of chloroperoxidase compound **I**; (c) arylamine bound to chloroperoxidase compound I. (d) arylhydroxyamine ligand of compound **I**. P represents the sixth axial ligand of chloroperoxidase.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Allenmark SG, Andersson MA. 1996. Chloroperoxidase catalysed asymmetric synthesis of a series of aromatic cyclic sulfoxides. Tetrahedron Asymm 7:1089–1094.

Colonna S, Gaggers N, Casella L, Carrea G, Pasta P. 1992. Chloroperoxidase and hydrogen peroxide: an efficient system for enzymatic and enantioselective sulfoxidations. Tetrahedron Asymm 3:95–106.

Corbett MD, Chipco BR, Baden DG. 1978. Chloroperoxidase catalysed oxidation of 4-chloroaniline to 4-chloronitrosobenzene. Biochem J 175:353.

Corbett MD, Chipko BR, Batchelor AO. 1980. The action of chloride peroxidase on 4- chloroaniline: *N*-oxidation and ring halogenation. Biochem J 187:892–903.

Doerge DR, Corbett MD. 1991. Peroxygenation mechanism for chloroperoxidase catalyzed *N*-oxidation of arylamines. Chem Res Toxicol 4:556–560.

Engel PC. 1977. Enzyme kinetics: The steady-state approach. London: Chapman and Hall.

Hager LP, Morris DR, Brown FS, Eberwein H. 1966. Chloroperoxidase. II. Utilization of halogen anions. J Biol Chem 241: 1769–1777.

Hager LP, Lakner FJ, Basavapathruni A. 1998. Chiral synthons via chloroperoxidase catalysis. J Mol Catal B: Enzym 5:95–101.

Hofrichter M, Ullrich R. 2006. Hemethiolate haloperoxidases: versatile biocatalysts with biotechnological and environmental significance. Appl Microbiol Biotechnol 71:276–288.

Hu S, Hager LP. 1999. Highly enantioselective propargylic hydroxylations catalyzed by chloroperoxidase. J Am Chem Soc 121:872–873.

Kiljiunen E, Kanerva LT. 2000. Chloroperoxidase catalysed oxidation of alcohols to aldehydes. J Mol Catal B: Enzym 6:163–172.

Kuhnel K, Blankenfeldt W, Terner J, Schlichting I. 2006. Crystal structure of chloroperoxidase with its bound substrates and complexed with formate, acetate and nitrate. J Biol Chem 281:23990–23998.

La Rotta Hernandez CE, Lutz S, Liese A, Bon EPS. 2005. Activity and stability of *Caldariomyces fumago* choloroperoxidase modified by reductive alkylation, amidation and cross-linking. Enzyme Microb Technol 37:582–588.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Morris DR, Hager LP. 1966. Chloroperoxidase. I. Isolation and properties of crystalline glycoprotein. J Biol Chem 241: 1763–1768.

Sanfilippo C, D'Antona N, Nicolosi G. 2004. Choloroperoxidase from *Caldariomyces fumago* is active in the presence of an ionic liquid as co-solvent. Biotechnol Lett 26:1815–1819.

Speicher A, Heisel R, Kolz J. 2003. First detection of a chloroperoxidase in bryophytes Phytochemistry 62:679–682.

Spreti N, Germani R, Incani A, Savelli G. 2004. Stabilization of chloroperoxidase by polyethylene glycols in aqueous media: kinetic studies and synthetic applications. Biotechnol Prog 20:96–101.

Thomas JA. 1970. Chloroperoxidase: classical peroxidatic catalatic and halogenating forms of the enzymes. J Biol Chem 245:3129–3134.

Ullrich R, Hofrichter M. 2005. The haloperoxidase of the agaric fungus *Agrocybe aergerita* hydroxylates toluene and naphthalene. FEBS Lett 579:6247–6250.

Ullrich R, Hofrichter M. 2007. Enzymatic hydroxylation of aromatic compounds. Cell Mol Life Sci 64:271–293.

Ullrich R, Nuske J, Scheibner K, Spantzel J, Hofrichter M. 2004. Novel haloperoxidase from the agaric basidiomycete *Agrocybe aegerita* oxidizes aryl alcohols and aldehydes. Appl Environ Microbiol 70:4575–4581.

Urlacher VB, Eiben S. 2006. Cytochrome P450 monoxygenases: perspective for synthetic application. Trends Biotechnol 24: 324–330.

Urlacher VB, Schmid RD. 2006. Recent advances in oxygenase catalysed biotransformations. Curr Opin Chem Biol 10:156–161.

Van Deurzen MPJ, Van Rantwijk, Sheldon RA. 1996. Synthesis of substituted oxindole by chloroperoxidase catalysed oxidation of indols. J Mol Catal B: Enzym 9:163–173.

Weber K, Osborn M. 1969. The reliability of molecular weight determinations by dodecyl sulfate–polyacrylamide gel electrophoresis. J Biol Chem 244:4406–4412.

Yadav P, Sharma JK, Singh VK, Yadav M, Yadav KDS. 2010. Purification of chloroperoxidase from *Musa paradisiaca* stem juice. Process Biochem (in press).

Zhu G, Wang P. 2005. Novel interface-binding chloroperoxidase for interfacial epoxidation of styrene. J Biotechnol 117: 195–202.