ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 9, Issue, 01, pp.45401-45405, January, 2017

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

FORMATION OF 3',3'',5',5''-TETRABROMOPHENOL SULFONPHTHALEIN FROM PHENOLSULFONPHTALEIN CATALYZED BY CHLOROPEROXIDASE OF *CALDARIOMYCES FUMAGO*

[1]López-Hernández, J. F., [2]Flores-Vela, A. I. and [3,*]Márquez-Rocha, F. J.

[1]Instituto Tecnológico Superior de los Ríos, Balancán, Tabasco, CP 86930
[2]Centro Mexicano para la Producción más Limpia, CP. 07738
[3]Centro Regional para la Producción más Limpia, Instituto Politécnico Nacional, Tabasco, CP 86691, México

ARTICLE INFO

Article History:
Received 10th October, 2016
Received in revised form
08th November, 2016
Accepted 13th December, 2016
Published online 31st January, 2017

Key words:

Haloperoxidase,
Chloroperoxidase,
Bromination,
Bromophenol blue,
Catalytic properties.

ABSTRACT

Chlorination of organic molecules is catalyzed by chloroperoxidase in the presence of hydrogen peroxide, few reports have been published on bromination of organic compounds catalyzed by chloroperoxidase. In this work, chloroperoxidase was used to catalyze the bromination of organic compounds in the presence of hydrogen peroxide and sodium bromide. The reaction mixture contain phenol red, NaBr, hydrogen peroxide and chloroperoxide, the reaction was followed by spectrophotometry and HPLC measurements at 590 nm. Chloroperoxidase catalyze the transformation of phenolsulfonphthalein (Phenol Red; PR) to 3',3'',5',5''- tetrabromophenolsulfonphthalein (Bromophenol Blue; BPB), under mild reaction conditions. The product of the reaction was characterized by HPLC-MS and FTIR. The reaction follows a Michaelis-Menten equation for NaBr, H_2O_2, and Phenol Red at different concentrations each. Maximum specific reaction rate has a values between 6.6 to 7.62 μM^{-1} BPB formed $min^{-1}mg^{-1}$protein. Halogenation of phenol red with I, and Cl ions in the presence of chloroperoxidase was not detected. The main product of the reaction was Bromophenol blue, identified by HPLC-MS and FTIR analyses. Chloroperoxidase catalyze bromination reactions on organic compounds such as phenol red, thymol blue and cresol red. These capabilities might be used to produce a high value compounds.

Citation: López-Hernández, J. F., Flores-Vela, A. I. and Márquez-Rocha, F. J. 2017. "Formation of 3',3'',5',5''-tetrabromophenol Sulfonphthalein from Phenolsulfonphtalein catalyzed by Chloroperoxidase of *Caldariomyces fumago*", *International Journal of Current Research*, 9, (01), 45401-45405.

INTRODUCTION

Halogenation of organic compounds in the presence of halide ions and peroxides, such as H_2O_2 is catalyzed by haloperoxidase enzymes. Three classes of haloperoxidases have been described: haeme, vanadium and those without haeme group, enzymes which act in the presence of hydrogen peroxide for the halogenation of organic compounds (Novak and Littlechild, 2013). These enzymes have gained the attention, due to their ability for the halogenation of organic compounds for industrial use, many of these reactions are carried out by regioselective oxidation of organic compounds (Zhang *et al*., 2013). The chloroperoxidase [CPO, EC 1.11.1.10] is a versatile heme-containing enzyme, containing a ferriprotoporphyrin IX as the prosthetic group, which has the advantage of self-regeneration at the end of the catalytic cycle. CPO is capable of the oxidation of chloride, bromide and iodide ions (donors for halogenation reactions). Fluoride is not a substrate for chloroperoxidase, however is an inhibitor of the halogenation reaction. In addition, to catalyze halogenation reactions, also exhibits peroxidase, catalase and cytochrome P450-like activities (Hofrichter and Ullrich, 2014), oxidation of indol (Zhang *et al*., 2013), epoxidation and hydroxylates activated substrates such as organic sulfides and olefins (Hofrichter and Ullrich, 2014) and halogenation of flavanones and flavones (Yaipakdee and Robertson, 2001), highly steroselective chlorination-cyclization in antibiotic biosynthesis (Renirie *et al*., 2008; Bernahardt *et al*., 2011), chlorination of bisbiphenils (Speicher *et al*., 2003). Catalytic bromination reactions are generally carried out by vanadium bromoperoxides (V-BrPO), which recognize a variety of organic compounds as substrates, using hydrogen peroxide as an oxidant of bromide (Butler and Carter, 2004). Few reports on the bromination of organic compounds by CPO have been published, nevertheless CPO might be utilize Br instead of Cl. In the present work, the chloroperoxidase from *Caldariomyces fumago* was used to catalyze the bromination of phenolsulfonphthalein to 3'3''5'5''-tetrabromophenolsulfon phthalein under standard reaction conditions.

Corresponding author: Márquez-Rocha, F. J.
Centro Regional para la Producción más Limpia, Instituto Politécnico Nacional, Tabasco, CP 86691, México.

MATERIALS AND METHODS

Chemicals: Purified Chloroperoxidase (CPO) [EC 1.11.1.10] from the marine fungus *C. fumago* 89362 Commonwealth

Mycological Institute, Kew, Surrey, UK, was pursued from Sigma-Aldrich, Cat. 9055-20-3. Hydrogen peroxide, bromophenol blue, phenol red, buffer salts and methanol were obtained from Sigma-Aldrich Co. (St. Louis Missouri).

Performance of the Bromination of Phenolsulfonphthalein catalyzed by Chloroperoxidase

The catalytic reaction to transform phenol red (PR) to bromophenol blue (BPB) was followed by a spectrum measurement of a λ range from 350 to 650 nm, in a spectrophotometer DR/4000 Hach, Hach Co. (Loveland, Colorado). The mixture standard reaction with a volume of 2 ml, contain acetate buffer 40 mM, pH 3.5, 37.5 μM phenol red, 0.75 mM of NaBr, 0.5 mM H_2O_2 and 7.8 nM of CPO (an extinction coefficient of ε_{403nm} = 75,300 $M^{-1}cm^{-1}$ was used). The reaction was carried out at 30°C. Sequential picks were obtained at intervals of 90s.

Kinetics of the Bromination of phenol red catalyzed by CPO

The mixture standard reaction was used as base mixture reaction to perform kinetic studies. The effect of the substrates concentration, one at a time, on the formation of bromophenol blue was determined at concentration intervals of NaBr 0 – 3 mM, H_2O_2 0 – 1 mM, and phenol red 5 – 150 μM. The amount of bromophenol blue produced was determined at 590 nm using an extinction coefficient of ε_{590} = 80,944 M^{-1} cm^{-1}, for 2 min at 30°C, followed in a spectrophotometer DR/4000 Hach, Hach Co. (Loveland, Colorado) at 590 nm. Optimal pH of the reaction was determined by changing the pH of the reaction buffer from 3 to 11. Temperature and pH stability were carried out after the end of the reaction by fixing different temperatures or pH during 12 h.

HPLC and FTIR analyses of the products of the reaction

The products of the reaction were extracted with dichloromethane before been analyzed by LC-MS and IR. Mass spectra analysis was performed in a LC/MS system, Hewlett-Packard, MSD, model 1100 and the Infrared spectra in a High performance Gold Infinity FTIR, Mattson, with DGT detector. Elution conditions were fixed in an Agilent 1100 series HPLC system (Palo Alto, CA), using a C_{18} Zorbax 300 SB column (Agilent, Palo Alto, CA) eluted with a methanol:water gradient (30-70 to 70:30) at 0.8 ml/min. Substrate and product detection was carried out using a MWD multi-wave detector (Agilent, Palo Alto, CA) coupled to the HPLC system, using two λ at 440 nm and 590 nm. After 8 min of reaction 20 μl of the mix reaction were injected to the HPLC system.

RESULTS AND DISCUSSION

Spectral properties of the peroxidative bromination of phenolsulfonephthalein catalyzed by chloroperoxidase

The change of the absorbance (ΔA) at 590 nm is due to the incorporation of four bromide atoms to 3',3'',5',,5'' positions of the molecule of phenol red to produce 3',3'',5',5''-tetrabromophenolsulfonphthalein (BPB), λ_{max} 588-590 nm. No reaction was detected without CPO, NaBr or H_2O_2 and no halogenation activity was observed when use KCl or KI instead of NaBr. After some 90s of the beginning of the reaction, a new pick at 590 nm appeared, which increase as the reaction proceeds (Figure 1). The spectrum obtained was similar to the formation of BPB catalyzed by a transition-metal-ion-grafted mesoporous silicate material (Walker *et al*, 1997).

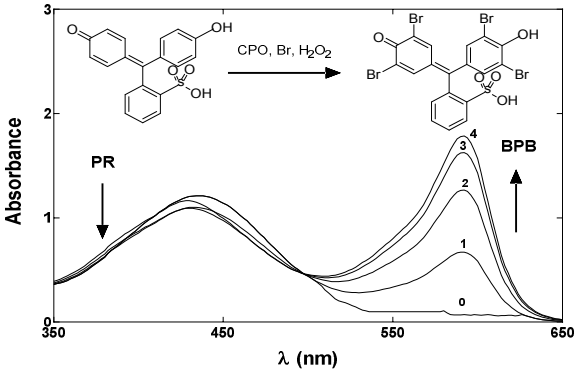

Figure 1. Absorbance spectra of the bromination reaction from 350 – 650 nm. Phenol red absorb at 440 nm and bromophenol blue at 590 nm. Numbers means 90s of difference on the reaction course

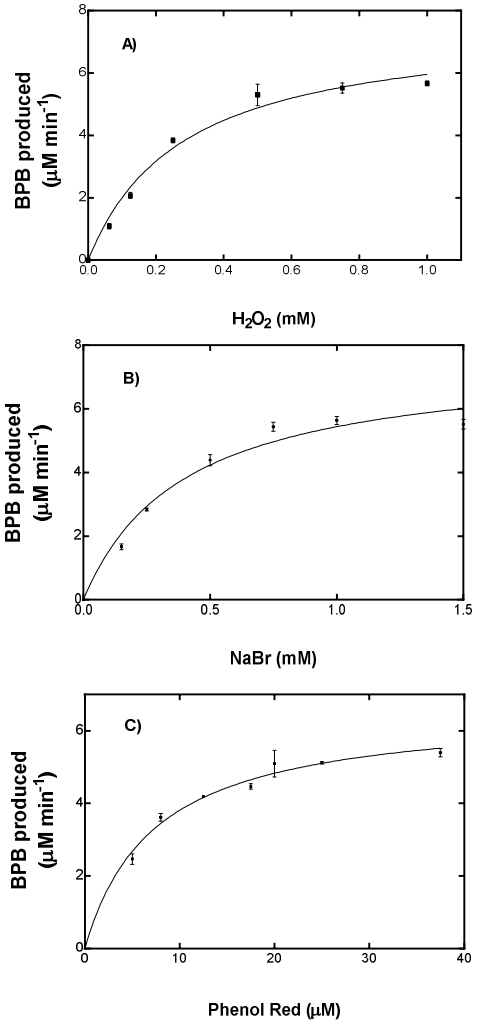

Figure 2. Time course of the bromination reaction. A) Hydrogen peroxide was used as substrate from 0-1 mM. B) NaBr at different concentration 0-1.5 mM. C) Phenol red from 0-40 μM

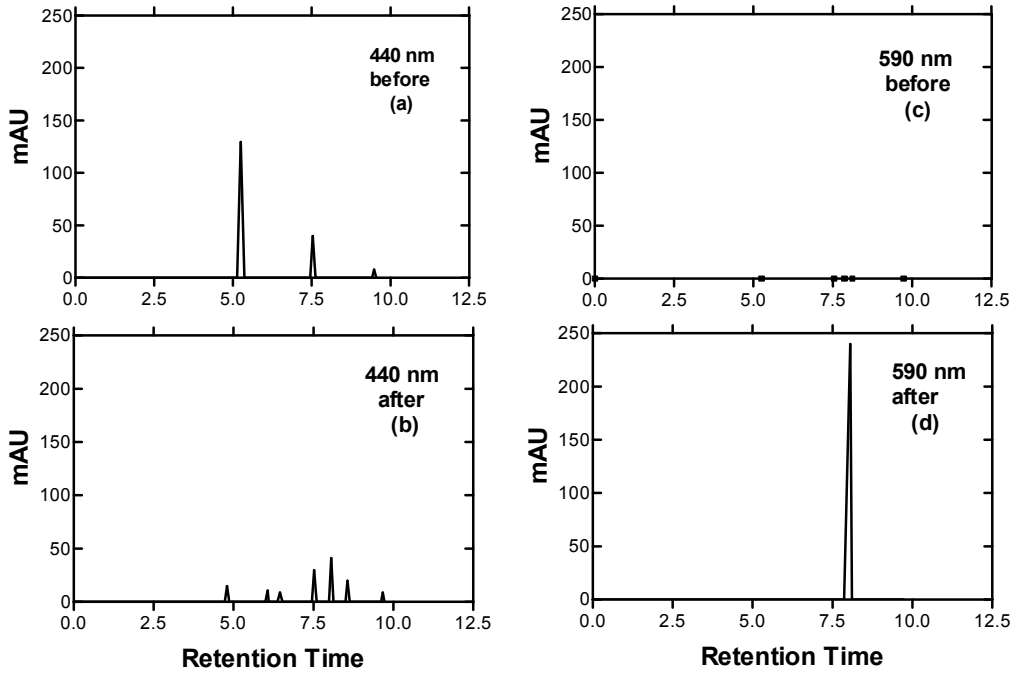

Figure 3. Detection of product formation by HPLC after the bromination of phenol red by the CPO. a, c) before the reaction monitored at 440 and 590 nm, respectively. b, d) after 8 min of reaction monitored at 440 and 590 nm, respectively

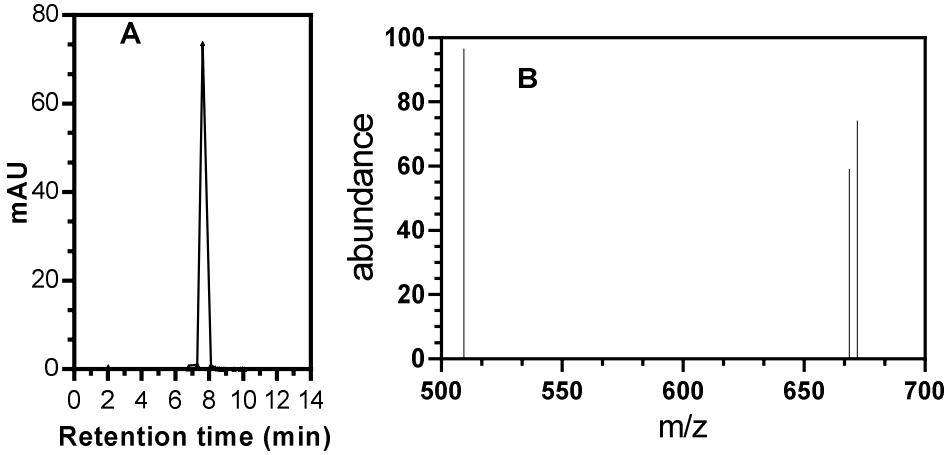

Figure 4. Determination of product formation by HPLC-MS. A) retention time of the main product. B) Major molecular ion peaks obtained

Table 1. Kinetic parameters of chloroperoxidase-catalyzed Bromination of phenol red

Kinetic Parameter	Value
$Vmax_{H2O2}$	7.62 ± 0.33
K_{H2O2}	0.28 ± 0.03
$Vmax_{Br}$	7.60 ± 0.38
K_{Br}	0.40 ± 0.05
$Vmax_{PR}$	6.60 ± 0.20
K_{PR}	7.33 ± 0.71

V_{max} units: BPB formed μM min^{-1} mg^{-1}protein,
K_{H2O2}, K_{Br}: mM, K_{PR}: μM

Table 2. Kinetic parameters of Thymol blue and Cresol Red bromination by CPO

Substrate	Parameter	Value
Thymol blue	$Vmax_{TB}$	4.42 ± 0.25
	K_{TB}	3.81 ± 0.03
Cresol Red	$Vmax_{CR}$	5.53 ± 0.38
	K_{CR}	4.50 ± 0.38

V_{max} units: bromothymol bromocresol formed μM min^{-1} mg^{-1}protein, K_{TB}, K_{CR}: μM

Kinetic analyses

The effect of H_2O_2 concentration (0-1.0 mM) on the CPO activity was assayed with a concentration of phenol red of 25 μM (Fig. 2a). The maximum reaction rate ($V_{maxH2O2)}$ for H_2O_2 as first substrate of CPO a value of 7.62 ± 0.33 BPB μM formed min^{-1} mg^{-1} protein CPO was calculated, with a 95 % confidence and a Km_{H2O2} of 0.28 ± 0.03 mM, the reaction fitted well for a Michaelis-Menten equation with a R^2= 0.985. Data were analyzed from a total number of 21 values, 7 for X coordinate and 3 replicates, kinetic values are summarized in Table 1. Bromination of phenol red reaction at different concentrations of NaBr from 0 to 1.5 mM (Fig. 2b) was also carried out. The reaction at different concentrations of NaBr was also analyzed by fitting to a Michaelis-Menten equation with a R^2 = 0.97, the value for maximum reaction rate (V_{maxBr}) was calculated by a double reciprocal plot (Linewaever-Burk linearization plot) a value of V_{maxBr} = 7.6 ± 0.24 BPB formed μM min^{-1} mg^{-1} protein CPO and a Km_{Br} of 0.4 ± 0.05 mM for sodium bromide were obtained, with a 95 % of confidence. Data were analyzed from a total number of 21 values, 7 for X coordinate and 3 replicates. The transformation rate of phenol red to BPB dependents of the concentration of the PR as substrate (Fig.2c), maximum reaction rate for PR concentration (V_{maxPR}) was 6.6 ± 0.2 BPB μM $min^{-1}mg^{-1}$ protein CPO, and a K_{mPR} of 7.33 ± 0.71 μM PR (Table 1). The fitting value for Michaelis-Menten equation was R^2= 0.997, data were analyzed from a total number of 15 values, 5 for X coordinate and 3 replicates of Y coordinates and a 95 % of confidence. Among the catalytic properties of CPO, the optimum pH was between 3.0 – 4.0, above pH 4.0 the biocatalytic activity of CPO decreased, and above pH 5 no activity of CPO can be detected. The bromophenol blue formed has stability between pH 3-5.0 and temperature of 10 to 35°C for at least 20 h. CPO may formed an intermediate compound [CPO-H_2O_2] as initial mechanistic sequence of bromination, followed by the formation of an intermediate [CPO-O-BR] through an electrophilic bromination reaction. It is not clear if phenol is oxidized as one intermediate, because not evidence exist of its formation during halogenation by CPO. One intermediate [CPO-O-BR] react with PR to join one molecule of Br to PR, the sequential reactions occur to brominate PR into 4 positions. In the first place, the activation of CPO by its oxidation in the presence of H_2O_2. According to Manoj and Hager, 2008), the organic substrate is oxidized before the halogenation happens, however there are not evidence it occur.

Products of the reaction

In the beginning of the reaction the peak corresponding to PR in HPLC appear at 5.2 min of elution at 440 nm using an UV detector (Figure 3a), after 8 min of reaction the peak observed at 5.2 min of elution disappear (Figure 3b). The same mixture reaction measure at 590 nm, at the beginning of the reaction not peak at 8 min of elution was detected (Figure 3c), however at the end of the reaction a major peak at 590 nm was detected (Figure 3d). The peak obtained at 8 min of elution at the end of the reaction was used for LC-MS analyses, the mixture after 8 min of reaction was extracted with dichloromethane (1:1 v/v) before been analyzed by LC-MS spectrum (Fig. 4a). Mass spectra analyses revealed the presence of molecular ion peaks at 668, 670, 672 m/z, and major ion at 510 m/z indicating the presence of four bromide atoms, the mass spectrum was similar to the reported mass spectrum of BPB by US Secretary of Commerce (http://pubchem.ncbi.nlm.nih.gov/rest/pug_view/data/key/814812_1). The bromination of Thymol has been reported for vanadium-bromoperoxidase from *Ascophyllum nodosum* (Sabuzi *et al*., 2015), but has not been reported for CPO or other heme-containing haloperoxidase. Infrared spectra (High Performance Gold Infinity FTIR, Mattson, with DGT detector) analyses OF BPB showed a characteristic transmission picks at 617, 820, 1191, 1339 and 2900-3000 cm^{-1}, which correspond to C-Br, C-O, O-S=O, and C=C conjugated bonds, all of them found into the BPB molecule.

Conclusion

Chloroperoxidase catalyze reactions that join Br ion to organic compounds such phenol red, as an electron-rich organic molecules, CPO is a versatile biocatalyst, with reaction of oxidoreductase, catalase and bromination, but no chlorination or iodination on PR. CPO use thymol blue and cresol red to produce bromothymol blue (V_{maxTB} 3.8±0.25 μM min^{-1} mg^{-1} protein, K_{mTB} 2.5 μM) and bromocresol purple (V_{maxCR} 4.7±0.36 μM $min^{-1}mg^{-1}$ protein, K_{mCR} 4.1±0.0.8 μM) respectively, Table 2. The capability of CPO to halogenate organic compounds might be used to produce a high value compounds instead of chemical synthesis, procedures that conduct toward the green chemistry.

Acknowledgments

This work was supported by IPN, Federal budget.

REFERENCES

Batler, A. and Carter, F. J. N. 2004. The role of vanadium bromoperoxidase in the biosynthesis of halogenated marine natural products. *Nat. Prod. Rep*., 21: 1-9.

Bernhardt, P. Okino, T. Winter, J. M. Miyanaga, A. and More, B. S. 2011. A stereoselective vanadium-dependent chloroperoxidase in bacterial antibiotic biosynthesis. *J. Am. Chem. Soc*., 133: 4268-4270.

Hofrichter, M. and Ullrich, R. 2014. Oxidations catalyzed by fungal peroxygenases. *Curr. Opin. Chem. Biol*., 19: 116-125.

Manoj, K.M. and Hager, L.P. 2008. Chloroperoxidase, a janus enzyme. *Biochemistry*, 47(9): 2997-3003.

Novak, H. and Littlechild, J. 2013. Marine enzymes for biocatalysis: Sources, biocatalytic characteristics and bioprocesses of marine enzymes. Wood head Publishing Series in Biomedicine. Pp 89-106. Doi: 10.1533/9781908818355.1.89.

Renirie, R. Dewilde, A. Pierlot, C. Wever, R. Hober, D. and Aubry J. M. 2008. Bacterial and virucidal activity of the alkalophilic P395D/L241V/T343A mutant of vanadium chloroperoxidase. *J. Appl. Microbiol*., 105: 264-270.

Sabuzi, F. Churokova, E. Galloni, P. Wever, R. Hollmann, F. Floris, B. and Conte, V. 2015. Thymol bromination – A comparison between enzymatic and chemical catalysis. *Eur. J. Inorg. Chem*., 2015: 3519-3525.

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

Walker, J. V. Morey, M. Carlsson, H. Stucky, G. D. and Butler, A. 1997. Peroxidative halogenation catalyzed by transition-metal-ion-grafted mesoporous silicate materials. *J. Am. Chem. Soc*., 119: 6921-6922.

Yaipakdee, P. and Robertson, L. W. 2001. Enzymatic halogenation of flavanones and flavones. *Phytochemistry*., 57: 341-347.

Zhang, R. He, Q. Chatfield, D. and Wang, X. 2013. Paramagnetic nuclear magnetic resonance relaxation and molecular mechanics studies oh the chloroperoxidase-indole complex: insights into the mechanism of chloroperoxidase-caltalyzed regioselective oxidation of indole. *Biochemistry,* 52(21): 3688-3701.

****