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Study on Activity and Stability of Native and Chemical Modified Bromoperoxidase

Jirasak Kongkiattikajorn[1]* and Pintip Ruenwongsa[2]

ABSTRACT

Chemical modification of enzymes is widely used as a tool for studying localization of individual amino acids, their participation in the maintenance of the native conformation and for their catalytic activity and stabilization. In this study, two isozymes of bromoperoxidase (BPO); BPOI, BPOII; were isolated and purified from *Gracilaria* sp. using fast protein liquid chromatography (FPLC) method. The isozymes were characterized and modified by various chemical modifying reagents. The effects of the chemical modification on catalytic activity and stability of BPOI were studied. BPOI was inhibited by 2-hydroxy-5-nitrobenzyl bromide (HNBB), 2-nitrophenylsulfenyl chloride (NPS), N-bromosuccinimide (NBS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). The amino groups of the enzyme were modified with iodoacetamide to change the enzyme's structure character and to increase its hydrophobicity. This modification improved the enzyme specific activity and increased the thermostability. Chemical modification suggested involvement of Trp, Asp/Glu in the catalytic site of the enzyme due to the decrease of enzyme activity of the modified enzyme. The 2, 4, 6-trinitrobenzenesulfonic acid modification increased the catalytic activity and the iodoacetamide- modified BPOI also showed greater thermal stability and catalytic activity. The improvements of catalytic properties are related to the changes of the hydrophobicity of substituted groups of lysine residues of BPOI.

Key words: bromoperoxidase, chemical modification, activity, stability

INTRODUCTION

Many species of marine macro-algae contain a variety of halogenated secondary metabolites (Niedleman and Geigert, 1986). A halogenating enzyme, haloperoxidase, is considered to participate in their syntheses in the presence of halides and hydrogen peroxide. Haloperoxidases catalyze the oxidation of halides (X^-) in the presence of hydrogen peroxide to the corresponding hypohalous acid (HOX), which can in turn react with a suitable nucleophilic acceptor (AH) to give rise to a halogenated compound (AX) (de Boer *et al*.,1986).

$$H_2O_2 + H^+ + X^- + \text{Enzyme} \rightarrow HOX + H_2O + \text{Enzyme}$$

$$HOX + AH \rightarrow AX + H_2O$$

Specific halogenation may, however, occur via a halogenating enzyme intermediate:

$$\text{Enzyme.HOX} + AH \rightarrow \text{Enzyme} + H_2O + AX$$

This group of enzymes is named

[1] School of Bioresources and Technology, King Mongkut's University of Technology Thonburi, Bangkok 10150, Thailand
[2] Department of Biochemistry, Mahidol University, Thailand
* Corresponding author, e-mail: jirasak.kon@kmutt.ac.th

according to the most electrophilic halide they are able to oxidize, e.g., bromoperoxidases (BPO) is able to oxidize both bromide and iodide. Three classes of haloperoxidases have been identified, two of which contain heme or vanadium and a third class of bacterial origin which does not contain a prosthetic group. The most well defined members are the heme-containing haloperoxidases, e.g., chloroperoxidase from *Caldariomyces fumago* (Shaw and Hager, 1959). The vanadium-containing haloperoxidases were initially isolated from seaweeds, the first being the bromoperoxidase from *Ascophyllum nodosum* (Vilter, 1984). The first terrestrial vanadium haloperoxidase, also a bromoperoxidase, was isolated from the lichen *Xanthoria parietina* (Plat *et al*., 1987).

Over the years, several techniques have been developed to ameliorate this loss of catalytic function, including lyophilization in the presence of lyoprotectants and excipients such as KCl, crown ethers, cyclodextrins and molecular imprinters (Lee and Dordick, 2002), the use of site-directed mutagenesis and directed evolution, or chemical modification (Davis, 2003). Chemical modification has now reemerged as a powerful complementary approach to site-directed mutagenesis and directed evolution (DeSantis and Jones, 1999). Chemical modification of horseradish peroxidase (HRP) surface has been performed to improve its stability. Acetic acid N-hydroxysuccinimide ester (Miland *et al*., 1996a) and bifunctional N-hydroxysuccinimide ester (Miland *et al*., 1996b) were successfully employed to modify HRP to increase HRP's stability in organic solvents.

Liu *et al*. (2002) reported that modification of HRP by phthalic anhydride improved HRP's stability and catalytic activity in aqueous buffer. Also O'Brien *et al*. (2003) reported that phthalic anhydride modification enhanced HRP's stability in DMF and THF. In this study, we investigated the important roles of various amino acid residues, in either binding of substrates or in the catalytic activity of the enzyme, by chemical modification in addition to investigating the activity and stabilization of the modified enzyme.

MATERIALS AND METHODS

Source of enzyme

Red algae, *Gracilaria* sp., were collected from shallow water of the eastern Thailand coast, at Banlangsok beach in Trad province. The algae were transported to the laboratory and washed several times in ice cold deionized water, drained and stored in 1 kg wet weight portions at 4°C before use. Each 1kg portion of algae was suspended in 2000 ml of 100 mM phosphate buffer, pH 7.0, and processed for 5 min in a Waring homogenizer. The homogenates were pooled and filtered through cheesecloth. The residue was resuspended in 100 ml of the same buffer and homogenized and filtered again. The filtrate was centrifuged for 30 min at 16,000 × g at 4°C.

Enzyme purification

The concentrated cell-free extract was brought to 60% saturation with solid ammonium sulfate. The solution was stirred overnight at 4°C and centrifuged at 16,000 × g for 30 min. After centrifugation, the pellet was resuspended in 250 ml of cold 100 mM Tris buffer pH 7.0. Any remaining undissolved precipitate was removed by centrifugation at 16,000 × g for 15 min and the resuspended pellet was dialyzed against 100 mM Tris buffer pH 7.0. The dialyzed ammonium sulfate fraction was applied to a 5×20 cm DEAE-cellulose DE52 column equilibrated with 100 mM Tris pH 8.5 buffer. The column was washed with 500 ml of 100 mM Tris pH 8.5 buffer followed by a linear gradient of 1000 ml of 0–1,000 mM NaCl in 100 mM Tris pH 8.5 buffer. Fractions containing activity were pooled, concentrated and submitted to a second chromatographic separation on a fast

protein liquid chromatography (FPLC) system with a Mono Q HR 5/5 column. The column was equilibrated with 20 mM Tris-HCl (pH 8.0) and the protein eluted with a gradient of 0–1 M NaCl in the same buffer. The protein was eluted at 0.25–0.60 M NaCl. Further purification was carried out with Superose 12 (LKB-Pharmacia) gel filtration by eluting with a solution of 50 mM Tris-HCl (pH 9.0). The purified enzyme was stored at -20°C. Protein content was determined by the method of Lowry *et al*. with bovine serum albumin (BSA) as a standard (Lowry *et al*., 1951).

Enzyme assay

Bromination activity was measured spectrophotometrically at 290 nm, using the molecular absorbance of monochlorodimedone. The standard assay mixture contained 0.1 M phosphate buffer (pH 6.0), 2 mM H_2O_2, 50 mM KBr at 25°C. The decrease in absorbance at 290 nm ($\varepsilon = 1.99 \times 10^4\ M^{-1}\ cm^{-1}$ (van Pée and Lingens, 1985) upon bromination of the MCD enol was monitored over time on a Shimadzu UV-Vis spectrophotometer. Kinetic data were obtained under steady state conditions by changing the concentrations of reactants. One unit of bromoperoxidase is defined as the amount of enzyme required to form 1 μmol of bromochlorodimedone in one minute.

Molecular mass determination

The relative molecular mass was determined by FPLC/gel filtration chromatography on Superose 12 (Pharmacia) with a mobile phase of 50 mM Tris–HCl (pH 9.0). Standard proteins, with Mr 13.7, 25, 43, 158, and 232 kDa, were used for the calibration of the column.

Chemical modification

The purified enzyme BPOI was preincubated with different concentrations of chemical modifiers namely 2, 4, 6-trinitrobenzenesulfonic acid (TNBS), iodoacetamide, 2-hydroxy-5-nitrobenzyl bromide (HNBB), 2-nitrophenylsulfenyl chloride (NPS), N-bromosuccinimide (NBS), N- acetylimidazole (NAI), diethylpyrocarbonate(DEPC), 5,5′-dithiobis(2-nitrobenzoic acid)(DTNB), 1,2 cyclohexanedione and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) at room temperature under appropriate conditions. The relative activities were determined with respect to the controls.

RESULTS

Purification of bromoperoxidase

The results of the purification procedures are summarized in Table 1. The highest specific BPO activity observed after the Superose 12 gel column step was 61.86 and 330.58 mU/mg for BPOI and BPOII, respectively. The two purified haloperoxidases show bromoperoxidase activity, but the specific activity of BPOII is 5.34 fold of that of BPOI (specific activities: BPOI 61.86 mU/mg; BPOII 330.58 mU/mg). The purified enzymes are colourless and their absorption spectra in the visible range are featureless.

The properties of the enzyme are summarized in Table 2. The molecular weight of the purified enzyme as determined by FPLC/gel filtration was 70 and 48 kDa. The effect of pH on the bromoperoxidase activity was analyzed by carrying out assays at different pHs ranging from 4.5 to 10 at 50°C. The enzymes were stable in a pH range of 5.0–9.5 and 5.5–9.0 after 24 hr preincubation at 4°C, for BPOI and BPOII, respectively. Studies on the effect of temperature on the enzymes were also performed. The enzyme activity increased with temperature to reach an optimum at 50°C and 45°C, for BPOI and BPOII, respectively. The enzyme was stable at 45°C after 30 min incubation and 20% of maximal activity was detected at 60°C after 30 min incubation.

The K_m values for H_2O_2 and Br^-, determined by Lineweaver-Burk plots, for BPOI

Table 1 Summary of purification of bromoperoxidases from red algae, *Gracilaria* sp.

Purification step	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Yield(%)	Purification (fold)
Crude extract	1932.30	402.35	4.80	100.00	1.00
0-60% Saturation (NH4)2SO4	1892.95	351.85	5.38	97.96	1.12
DE52					
BPOI	228.31	15.13	15.09	11.82	3.14
BPOII	581.52	6.88	84.52	30.10	17.61
MonoQ					
BPOI	151.20	3.87	39.07	7.77	8.14
BPOII	337.22	1.97	171.18	17.45	35.66
Superose12					
BPOI	103.92	1.68	61.86	5.38	12.89
BPOII	271.08	0.82	330.58	14.03	68.87

Table 2 Properties of bromoperoxidase from red algae, *Gracilaria* sp.

Property	BPOI	BPOII
Molecular weight	70kDa	48kDa
Optimum pH	5.5	7.0
pH stability	5.0-9.5 for 24 hr at 4°C	5.5-9.0 for 24 hr at 4°C
Optimum temperature	50°C	45°C
Temperature stability	45°C for 30 min	45°C for 30 min
Metal ion requirement	Required	Required
Inhibitor	EDTA, NaN_3, KCN	EDTA, NaN_3, KCN
K_m(H2O2)(M)	1.00×10^{-4}	2.47×10^{-5}
K_m(MCD)(M)	2.94×10^{-5}	8.52×10^{-4}
K_m(Br-)(M)	2.17×10^{-4}	4.72×10^{-5}
Bromoperoxidase activity comparing to dialysed enzyme(%)		
- Dialysed enzyme	100	100
- Dialysed enzyme + EDTA*	0	0
- Control (Non-dialysed enzyme)	207	220
- Dialysed enzyme + V^{5+}*	250	350
- Control + NaN_3*	8.5	7.0
- Control + KCN*	5.5	4.3

*Concentration of the reagent was 1 mM.

are larger than those for BPOII, showing that BPOI has a lower affinity for the two substrates while the K_m values for MCD, for BPOI is less than that for BPOII, showing that BPOI has higher affinity for MCD. Influence of metal ions on the activity of the enzyme revealed that the bromoperoxidase requires metal ions for its activity. The bromoperoxidase treated with EDTA showed inhibition at 1.0 mM of EDTA. Incubation with 1.0 mM EDTA treated enzyme showed that V^{5+} could restore and activate the activity of the treated enzyme (Table 2), indicating that the bromoperoxidase needs metal ions for expressing full activity. The metal ion could play a role in protein folding or in catalysis. Most of bromoperoxidases are known to be metal ion dependent enzymes. Moreover, NaN_3 and KCN which are heme inactivate reagents could inhibit reactivity of both BPOI and BPOII.

Chemical modification

Chemical modification as shown in this study was only of BPOI activity (data not shown for chemical modification of BPOII). It was found that HNBB, NPS and NBS (for Trp), and EDC (for Asp and Glu) inhibited the enzyme activity of BPOI (Table 3). These results showed that the amino acid residues which were modified are involved in catalytic activity, suggesting that Trp, Asp and Glu are important for affinity and catalysis activity of the BPOI. Further investigation is needed to find out which amino acids are involved in native conformation, substrate binding and catalytic reaction of the enzyme.

The half-life values of the native and iodoacetamide-modified BPOI were estimated from the incubation time that caused 50% decrease in the enzyme activity. The half-life values are summarized in Table 4.

The native and iodoacetamide-modified BPOI was kept in phosphate buffer pH 7.0 at 25°C and 45°C for various time intervals before assaying for the remaining activity. At 25°C, the results showed that the modified enzyme was more stable than the native enzyme during storage for 144 hr. The activities of the native enzyme and modified enzyme decreased to 20 and 66% at 144 hr, respectively. At 45°C, the modified enzyme was also more stable than the native enzyme. The activity of the native enzyme was completely lost at 36 hr and that of the modified enzyme decreased to 86 and 11% at 12 and 60 hr of incubation,

Table 3 Reaction conditions for the chemical modification of bromoperoxidase (BPOI) with different group-selective reagents and effects of reagents on enzyme activity.

Target residue	Reagent	Protein: reagent (mol:mol)	Reaction condition	pH	Activity(%)*
Lys	TNBS	1:200	6-24 hr, RT	8.0	150 ± 9.3
Lys	Iodoacetamide	1:500	6-24 hr, RT	7.5	485 ± 11.6
Trp	HNBB	1:200	1 hr, RT	5.5	1.0 ± 0.5
Trp	NPS	1:200	4 hr, RT	7.0	29 ± 3.6
Trp	NBS	1:200	1 hr, RT	5.0	0.5 ± 0.2
Tyr	NAI	1:200	4-6 hr, RT	7.5	101 ± 7.4
His	DEPC	1:500	1 hr, RT	6.0	102 ± 5.4
Cys	DTNB	1:200	2-6 hr, RT, dark	7.0	99 ± 7.8
Arg	1,2 Cyclohexanedione	1:50	4-6 hr, RT	8.0	98 ± 9.2
Glu/Asp	EDC	1:100	3 hr, RT, dark	5.0	0.5 ± 0.2

* The percentage values given refer to control samples under the same reaction conditions and are means ± S.D. of at least three independent experiments.

respectively. The half-life values of the native and modified enzymes are shown in Table 5.

DISCUSSION AND CONCLUSIONS

The purification of enzymes extracted from marine algae is a difficult task, since the composition of the raw material is complex and the presence of polyphenols and tannins in the extracts complicate the process. In our studies with *Gracilaria* sp. we extracted, purified and characterised two enzymes. Interestingly, modification of the indole group of tryptophan residues with HNBB, NBS and NPS brought about a large loss of BPOI activity. These results may indicate involvement of the tryptophan residues in the enzyme binding sites. The enzyme has, however, other tryptophan residues as well. The tryptophan residues are important also for the activity of BPOI (Messerschmidt and Wever, 1996). Another amino acid required for BPO activity is glutamic acid and/or aspartic acid (Butler, 1999). Modification of these amino acids by EDC could inhibit the enzyme activity. These findings would suggest the presence of glutamic acid and /or aspartic acid residues in close proximity to or inside the binding site of BPOI. A change in the microenvironment surrounding the tryptophan residues and/or carboxyl residues changes enzyme activity because of a change in the relative orientation or distance between the prosthetic group and the tryptophan residues and/ or carboxyl residues leading to a decrease in the efficiency of energy transfer (Ligtenbarg *et al*., 2003). This also denotes that the distance between the prosthetic group and the tryptophan residues is increased and might indicate that the modified tryptophan residues and /or carboxyl residues became more exposed and located in a more polar environment.

Vanadium-dependent haloperoxidases were shown to be thermostable (de Boer *et al*., 1987). Recently, it was found that the analysis of the three-dimensional structure of BPO from *C. pilulifera* suggested that one calcium ion per subunit is bound in a loop at the top of the active-site cleft (Littlechild and Garcia-Rodriguez, 2003). The finding of a calcium ion in the enzyme molecule is specific for the *Corallina* BPOs which

Table 5 The half-life of the native and iodoacetamide-modified BPOI at pH 7.0 at different temperatures.

Temperature (°C)	Half-life (hr)	
	Native	Modified BPOI
25	57	>144
45	16	20

Table 4 The half-life (hours) of the native and modified bromoperoxidase (BPOI) at 37°C.

Modifying reagent	pH	Half-life (hr)	
		Native	Modified enzyme
TNBS	8.0	12.2 ± 1.7	37.2 ± 3.0
Iodoacetamide	7.5	18.4 ± 1.9	108 ± 5.7
HNBB	5.5	15.0 ± 1.8	5.2 ± 1.9
NPS	7.0	17.0 ± 2.4	18.2 ± 1.0
NBS	5.0	16.5 ± 3.0	16.5 ± 2.0
NAI	7.5	16.0 ± 1.7	18.4 ± 1.2
DEPC	6.0	18.1 ± 2.1	18.1 ± 1.2
DTNB	7.0	17.5 ± 1.4	17.7 ± 1.5
1,2-Cyclohexanedione	8.0	12.5 ± 1.0	11.0 ± 0.5
EDC	5.0	17.0 ± 2.6	18.1 ± 2.9

suggests an important role in the enzyme' s structure and the importance of calcium and other metal ions for protein stability (Garcia-Rodriguez *et al*., 2005).

Enzyme activity and stability could be increased by chemical modification. Chemical modification have been reported as a technique used to overcome the problem of substrate partition. Modi *et al*. (1995) reported an increase of substrate affinity of cytochrome P450 when the heme was replaced with a heme dimethyl ester. An increment of both surface and active site hydrophobicities, through covalent coupling of poly(ethylene)glycol in surface free amino groups, and the methyl esterification of heme propionates, increased the specific activity and the substrate range of cytochrome c for the oxidation of polycyclic aromatic hydrocarbons (Tinoco and Vazquez-Duhalt, 1998).

In the present report, a mild and selective procedure is described for modification of e-amino groups of lysine residues in bromoperoxidase (BPOI). Observed changes in thermal stabilities and catalytic properties of the enzyme brought about by the modification were studied. The role of chemical modification on the amino acid residues was studied and it was concluded that the modified lysine residues in this enzyme could contribute to the enhancement of enzyme stability and activity. The catalytic enhancement was attributed to an increased affinity for substrates in the modified protein. The substituted groups might attribute to increased hydrophobic interaction and maintain the active site. It appears that this simple method may provide a very useful strategy for giving proteins some new and useful characteristics related to stability and catalytic activity.

From the results, it is suggested that the reactive modified lysine helps enhancing the catalytic rate instead of mediating a substrate-induced conformational change. The iodoacetamide modified enzyme seemed to suffer from higher temperature more than the native enzyme as a result of increased hydrophobicity which affects the specific activity. This study reveals the potential roles of a modified lysine in BPOI. The roles of reactive modified lysine in BPOI is currently being investigated.

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