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Increased Thermostability and Activity of Chemically Modified Bromoperoxidase from the Red Alga *Gracilaria tenuistipitata*

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ABSTRACT: Chemical modification of proteins 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 stabilization. Amino groups of bromoperoxidase from the red seaweed *Gracilaria tenuistipitata* Chang & Xia (collected in Eastern Thailand, at Ban Laem Sok beach in Trad province) were modified with iodoacetamide to change its structure and to increase its hydrophobicity. The effect of the chemical modification on substrate affinity and catalytic activity were studied. Chemical modification of the enzyme improved the specific activity up to 5 times. In addition, the chemical modification slightly increased the solvent concentration at which the enzyme was catalytically active. The thermostabilities of native and modified bromoperoxidase were assayed. The chemical modification of bromoperoxidase increased its thermostability after incubation at 25°C for 144 h (about 3.3 fold); the modified form retained 20% of its activity after incubation at 45°C for 60 h. The optimum pH activity is 5.5 for both native and modified forms of the enzyme. The modified enzyme could tolerate a higher temperature than the native enzyme. This biocatalytic behavior could be attributed to the increased hydrophobicity of the enzyme. On the other hand, the chemical modification of the enzyme altered its hydrophobic characteristic and affected the specific activity.

KEYWORDS: bromoperoxidase, chemical modification, activity, stability, *Gracilaria.*

INTRODUCTION

Many species of marine macro-algae contain a variety of halogenated secondary metabolites[1]. 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 to give rise to a halogenated compound[2] (AX).

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

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

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 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 haem or vanadium and a third class of bacterial origin which does not contain a prosthetic group. The most well defined members are the haem containing haloperoxidases, e.g., chloroperoxidase from *Caldariomyces fumago*[3]. The vanadium-containing haloperoxidases were initially isolated from seaweeds the first being the bromoperoxidase from *Ascophyllum nodosum* (Linneus) Le Jolis[4]. The first terrestrial vanadium haloperoxidase, also a bromoperoxidase, was isolated from the lichen *Xanthoria parietina*[5]. 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[6], the use of site-directed mutagenesis and directed evolution, or chemical modification[7]. Chemical modification has now reemerged as a powerful complementary approach to site-directed mutagenesis and directed evolution[8]. Chemical modification of horseradish peroxidase (HRP) surface has been performed to improve its stability. Acetic acid N-hydroxysuccinimide ester[9] and bifunctional N- hydroxysuccinimide ester[10] were successfully employed to modify HRP to increase HRP's stability in organic solvents.

Liu et al.[11] reported that modification of HRP by phthalic anhydride improved HRP's stability and catalytic activity in aqueous buffer. Although O'Brien et al.[12] 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, we investigated the activity and stabilization of the modified enzyme. These are the first reports on these subjects.

Materials and Methods

Source of Enzyme

The red alga, *Gracilaria tenuistipitata* Chang & Xia, was collected from shallow water at the Eastern Thailand coast, at Ban Laem Sok 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 1 kg portion of algae was suspended in 2000 ml of 100 mM phosphate buffer, pH 7.0, and processed for five minutes in a Waring homogenizer. The resulting homogenates were pooled and filtered through cheesecloth. The residue was resuspended in 100 ml of the same buffer and homogenized and filtered again. The pooled filtrate was centrifuged for 30 min at 16,000 x 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 x g for 30 min. After centrifugation, the pellet was resuspended in 250 ml cold 100 mM Tris buffer, pH 7.0. Any remaining undissolved precipitate was removed by centrifugation at 16,000 x 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 5x20 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 and concentrated. All the active fractions were pooled 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 1 M NaCl in the same buffer. The protein was eluted at 0.25 – 0.6 M NaCl. Further purification was carried out with a gel filtration Superose 12 (LKB-Pharmacia). The elution was carried out 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. [13] with bovine serum albumin (BSA) as a standard.

Enzyme Assay

Bromination activity was measured spectrophotometrically at 290 nm, using the molecular absorbance of monochlorodimedone[14]. The standard assay mixture contained 0.1 M phosphate buffer (pH 6.0), 2 mM H_2O_2, 50 mM KBr. The decreases of the absorbance of monochlorodimedone (S_{290} =20.1 mM^{-1} cm^{-1}) were measured at 25°C. Kinetic data were obtained under steady state conditions by changing the concentrations of reactants.

Chemical Modification

The purified enzyme was preincubated with different concentrations of chemical modifiers namely 2, 4, 6- trinitrobenzenesulfonic acid (TNBS), iodoacetamide, 2-hydroxy-5-nitrobenzyl bromide (HNBB), 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 dimethylamino propyl) carbodiimide (EDC) at room temperature under appropriate conditions. The relative activities were determined with respect to the controls.

Results

Purification of Bromoperoxidase

Purification of bromoperoxidase from *G. tenuistipitata* was carried out by the FPLC method. The purification method gave a 5.38% yield with a specific activity of 61.86 mU/mg and 12.89-fold purification. Purification results are summarized in Table 1.

Chemical Modification

Out of ten chemical modifiers used in this study, HNBB, NPS and NBS (for Trp), and EDC (for Asp and

Table 1. Purification of bromoperoxidase.

Purification step	Total activity(mU)	Total protein(mg)	Specific activity(mU/mg)	Yield(%)	Purification(fold)
Crude extract	1,932.30	402.35	4.80	100.00	1.00
0-60% (NH_4SO_4)	1892.95	351.85	5.38	97.96	1.12
DE52	228.31	15.13	15.09	11.82	3.14
MonoQ	151.20	3.87	39.07	7.77	8.14
Superose12	103.92	1.68	61.86	5.38	12.89

Table 2. Reaction conditions for the chemical modification of bromoperoxidase with different group-selective reagents and effects of reagents on enzyme activity.

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

Glu) inhibited the enzyme activity (Table 2). 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 substrate binding and catalysis activity of the BPO. Further investigation needs to be done to find out which amino acids are involved in native conformation of the enzyme, substrate binding and catalysis reaction of the enzyme.

The half-life values of the native and modified enzyme were estimated from the incubation time that caused 50% decrease in the enzyme activity. The half-life values are summarized in Table 3.

Table 3. The half-life (hour) of the native and modified bromoperoxidase at 37 °C.

Modifying reagent	pH	Half-life* (h)	
		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

*The half-life (hour) of the native and modified bromoperoxidase under the same reaction conditions and are means ± S.D. of at least three independent experiments.

The native and iodoacetamide-modified BPO were kept in phosphate buffer pH 7.0 at 25 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 h. The activity of the native enzyme and modified enzyme decreased to 20% and 66% at 144 h. At 45 °C, the modified enzyme was more stable than the native enzyme. The activity of the native enzyme was completely lost at 36 h and the modified enzyme decreased to 86% and 11% activity at 12 and 60 h of incubation, respectively. The half-life values of the native and modified enzyme are shown in Table 4.

Table 4. The half-life of the native and iodoacetamide-modified BPO at pH 7.0 in different temperatures.

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

Discussion and Conclusion

Interestingly, modification of indole group of tryptophan residues with HNBB, NBS and NPS brought about a large loss of BPO activity. These results may indicate involvement of the tryptophan residues in acceptor binding sites. The enzyme has, however, other tryptophan residues as well. The tryptophan residues are important also for the activity of BPO. Another amino acid required for BPO activity is glutamic and/or aspartic acid. Modification of these amino acids, EDC; (1-ethyl-3-(3 dimethylamino propyl) carbodiimide)), could inhibit the enzyme activity. These findings suggest the presence of glutamic and/or aspartic acid residues in close proximity to or inside the binding sites of BPO. We consider it significant that inactivation of BPO both by tryptophan reactive and carboxylic acid reactive reagents is protected against by either donor or acceptor substrate of the enzyme. Thus, the presence in the enzyme of this tryptophan- and carboxylic acid- region would explain protection of the enzyme by its substrates against the action of both tryptophan and carboxylic acid modifying reagents. It is suggested that the reactive modified

lysine helps enhance the catalytic rate instead of mediating a substrate-induced conformational change.

This laboratory has reported 3.05 and 5.87 -fold thermostabilization at 37 °C of BPO following reaction of TNBS and iodoacetamide, respectively. It is interesting that such a similarity should arise in the BPO -stabilizing abilities of two types of amino-specific monofunctionals. Both TNBS and iodoacetamide react with amino groups with leading to charge neutralization. At neutral pH, the amine functions of lysine residues are mostly protonated and thus positively charged. For iodoacetamide or TNBS modification, an amide cannot carry a charge and thus the positive charge of the original amino group is lost. Neutralization of the repulsion of like charges may be responsible for the stabilization observed here: A decreased number of like charges will lessen the tendency of the protein to unfold at high temperatures. There is an alternative explanation. Certain lysine is thought to interact with the prosthetic group of BPO. It is unlikely to react with TNBS or iodoacetamide and any such reaction would likely cause inactivation. Neutralization of positive charges within such hydrophobic sequences may well have a thermostabilizing effect. Ugarova et a1.[15] studied thermostability of HRP following modification of its lysine amino groups with a variety of carboxylic acid anhydrides and TNBS. Some of these compounds reversed the positive charge on the lysine. These chemical treatments led to restricted conformational mobility and increased thermostability. In this study, the modified lysine of BPO might reduce flexibility correlated with enhanced stability. In addition, stabilization might be due to the degree of modification (i.e., the number of modified lysines), not to the nature of the modifier. It is clear that chemical modifications can benefit stability of enzymes other than BPO. Tuengler and Pfleiderer[16] acetamidinated 17 of the 24 lysines of lactate dehydrogenase converting them to arginine- like structures. Modification increased the tolerance of the enzyme to heat, alkali, and tryptic digestion[16]. The increases in half-life ranged from 5- to 50-fold depending on the elevated temperature used. Melik-Nubarov and colleagues[17] used glyoxylic acid and sodium cyanoborohydride to perform reductive alkylation of up to 10 amino groups in alpha-chymotrypsin. The enzyme was dramatically stabilized against heat: Modified forms tolerated 60°C 1000-fold better than did the native molecule. The effect was ascribed to hydrophilization of the protein surface, resulting in decreased contact between water and nonpolar clusters[17]. Six of the 14 lysines lie close to hydrophobic residues on the protein surface[18]. Mozhaev et al.[18] later carried out acylation (using carboxylic acid anhydrides) and reductive alkylation (with aliphatic aldehydes) of the enzyme. They obtained a wide range of "hydrophilized" and "hydrophobized" derivatives. The stabilized enzyme forms had identical fluorescence spectra to the native chymotrypsin, indicating that the stabilization was not due to conformational effects.

More than 2.5 fold and 5.87 fold increase in the apparent half-life of BPO at 25 °C and 37°C, respectively, and 4.85 fold increase in the activity have resulted from iodoacetamide modification of lysines of the BPO. This was a remarkable result from such simple chemical alterations with low molecular weight compounds. Increasing stability and activity might correlate with increasing the neutralized groups of lysine whether this hydrophobilization was due to the nature of the modifier itself or to the number of lysines modified by the compound.

Obviously, the iodoacetamides are more successful as chemical stabilizers and increase activities of BPO than TNBS. One would expect that neutralization of charges would improve the activity and ability of a protein to tolerate high temperature. In this study, activity of amino-derivatized BPO has been described. The behavior of TNBS or iodoacetamide modified BPO shows that of modified lysine residues benefits BPO stability and activity. The lysyl modified BPO is more thermostable and active than the native BPO.

The iodoacetamide or TNBS modified enzymes could suffer from higher temperature more than native enzyme catalytic reactions. This biocatalytic behavior could be attributed to the increased hydrophobicity of the enzyme. On the other hand, the chemical modification of the enzyme altered its hydrophobicity characteristic, thus affecting the specific activity. This study represents the potential roles of a conserved active site modified lysine in BPO and it has been being investigated roles of reactive modified lysine on BPO.

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