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Chemical Modification of Lysine and Histidine Residues in *Gracilaria tenuistipitata* Bromoperoxidase: Effect on Stability and Activity

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ABSTRACT: Bromoperoxidase (BPO) from the Thai red seaweed *Gracilaria tenuistipitata* Chang et Xia has been chemically modified with iodoacetamide under defined experimental conditions yielding derivatives of native bromoperoxidase with enhanced catalytic activity. It has been shown that this modified reagent reacts with the ε-amino groups of 15 out of a total of 46 lysine residues and 9 from a total of 16 histidine residues of bromoperoxidase. Results obtained on kinetic parameters before and after modification are discussed in terms of their contributions to the mechanism of irreversible thermoinactivation and activity enhancement. The results presented in this study indicate that bromoperoxidase may acquire some new and useful characteristics related to stability and activity upon modification of specific side chains in their primary structures involving specific amino acids in this enzyme.

KEYWORDS: bromoperoxidase, lysine, histidine, kinetic, iodoacetamide, *Gracilaria*.

INTRODUCTION

The bioinorganic chemistry of vanadium has aroused much interest in recent years due to the curious roles and states of this metal in various lower organisms[1]. A particular example, which is attracting attention, is the presence of this metal in the active site of a family of haloperoxidases (HPO), a subgroup of peroxidases (EC.1.11.1). A role for the enzymes in the synthesis of some of the widespread natural halogenated compounds has been proposed[2]. The importance of these compounds for diverse pharmaceutical and industrial applications has been documented[3]. Catalytically active vanadium bromoperoxidase requires one equivalent of vanadium (V) per subunit of enzyme.

Using vanadium as the cofactor, these bromoperoxidases catalyze the two-electron oxidation of bromide and iodide by hydrogen peroxide in a substrate-inhibited bi-bi ping- pong mechanism[4]. The oxidized halogen intermediate can act to halogenate an appropriate organic substrate or oxidize a second equivalent of hydrogen peroxide to produce dioxygen in the singlet excited state[5]. It has also been established that bromination of organic substrates by vanadium bromoperoxidase proceeds through an electrophilic (i.e. Br^+) rather than a radical process[6] (Br^-).

These enzymes are found in many brown, in some red and in one green marine alga[7] and in the lichen *Xantoria parietina* (L.) Beltr.[8]. Recently, vanadium dependent haloperoxidases were also found in some fungi[9]. All the vanadium haloperoxidases isolated to date share some common features: they are composed of one or more subunits of relative molecular mass around 67 kDa; they can be inactivated by dialysis against EDTA at low pH; their activity is only restored by addition of vanadium (as vanadate) and they seem to have similar coordination of vanadium in the active site[10]. Vanadium haloperoxidases have been traditionally classified as chloro-, bromo- and iodo-peroxidases. Vanadium chloroperoxidases have only been detected in terrestrial organisms, whereas bromo- and iodo-peroxidases are dominant in the marine environment.

In biogeographic terms, the Eastern coast of Thailand falls within the warm temperate tropical region. This paper reports on the isolation and purification of bromoperoxidases from *G. tenuistipitata* Chang et Xia collected along this coast. 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. In this study iodoacetamide was used for modification of bromoperoxidase (BPO) from *G. tenuistipitata* to investigate the improvement of the catalytic properties, and a kinetic study was done to determine the modified amino acid residues that may participate in the enzymatic activity.

MATERIALS AND METHODS

Source of Enzyme

G. tenuistipitata, a red alga, was collected from shallow waters on the Eastern Thailand coast of

Banlangsok beach in Trad province, at the end of summer. The algae were transported to the laboratory and washed several times in ice-cold deionized water, drained and stored in units of 1 kg wet weight at 4°C before use.

Enzyme Extraction and Purification

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 pooled filtrate was centrifuged for 30 min at 16,000×g at 4°C to remove smaller particles. The 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 cold 100 mM Tris-HCl buffer, at 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-HCl buffer at pH 7.0. The dialyzed ammonium sulfate fraction was applied to a 5×20 cm DEAE-cellulose DE52 column equilibrated with 100 mM Tris-HCl pH 8.5 buffer. The column was washed with 500 ml of 100 mM Tris-HCl pH 8.5 buffer followed by a linear gradient of 1000 ml of 0 –1 M NaCl in 100 mM Tris-HCl pH 8.5 buffer. All the active fractions were pooled and submitted to a second chromatographic separation on a fast protein liquid chromatography (FPLC) system[11] 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.*[12] with bovine serum albumin (BSA) as a standard.

Molecular Mass Determination

The relative molecular mass was determined by FPLC/gel filtration chromatography on Superose 12 (LKB-Pharmacia) with a mobile phase of 50 mM Tris–HCl (pH 9.0). Standard proteins from Pharmacia (ribonuclease A, MW 13.7 kDa; chymotrypsinogen, MW 25 kDa; ovalbumin, MW 43 kDa; aldolase, MW 158 kDa; and catalase, MW 232 kDa) were used for the calibration of the column.

Modification of Free Amino Groups

Modification with iodoacetamide was done as determined in earlier experiments. The free amino groups were modified with iodoacetamide by incubating the enzyme with 50 mM iodoacetamide previously determined to be optimal for 0-24 h at 35°C for each incubation time. The modified sample was dialyzed extensively against potassium phosphate buffer (KPB) and the BPO activity was assayed by the bromination assay of monochlorodimedone (MCD). This enables the incubation time that produced optimal enzyme activity to be determined, namely 18 hours.

Enzyme Assay

Bromination activity was measured spectrophotometrically at 290 nm, using the molecular absorbance of MCD. 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[13] (molar extinction coefficient at 290 nm = 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.

Free Amino Acid Determination

The amino acid composition of the enzyme was determined by high performance liquid chromatography (HPLC) after hydrolysis. The enzyme (0.125 mg of protein/ml) was added with 1 ml of 6N hydrochloric acid in the vial. Oxygen in the vial was removed and nitrogen gas was purged into the vial by Pico-Tag vacuum station (Waters). The hydrolysis vials were kept at 110 °C for 24 h. Afterwards, the HCl was removed and 30 ml of redrying reagent (methanol:triethylamine:water = 2:1:1) were added to each vial for the neutralization of the samples. The samples were dried under vacuum and stored prior to analysis at -18 °C.

For determination of cysteine, the enzyme samples were oxidized with performic acid, prepared by mixing 19 volume of 97% formic acid with 1 volume of 35% hydrogen peroxide and left standing (covered) for 1 hour at room temperature. 10 µl of the performic acid solution were added to the enzyme and the reaction was allowed to occur at room temperature for 30 minutes. The performic oxidized enzymes were hydrolysed by hydrochloric acid. Ten µl of redried solution (methanol:triethylamine:water = 2:1:1) were added to the dried hydrolysates in the enzyme tubes and they were dried under vacuum to neutralize the enzymes before derivatization, following which the enzyme tubes were dried by Pico-Tag vacuum station. The dry enzymes were derivatised with 10 µl PITC derivatizing solution (methanol:triethylamine:phenyl-isothiocyanate:water = 7:1:1:1), after standing at room temperature for 10 min the reaction mixture was dried by Pico-Tag vacuum station.

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. Kinetic parameters of native and iodoacetamide-modified BPO.

Enzyme	MCD		KBr		H_2O_2	
	K_m (M)	V_{max} $(mU/ml)^{-1}$	K_m (M)	V_{max} $(mU/ml)^{-1}$	K_m (M)	V_{max} $(mU/ml)^{-1}$
Native	2.94×10^{-5}	4.17×10^{-2}	2.17×10^{-4}	4.76×10^{-2}	1.04×10^{-4}	3.39×10^{-2}
Modified enzyme	1.43×10^{-5}	1.28×10^{-1}	1.54×10^{-2}	1.29×10^{-1}	2.09×10^{-7}	7.14×10^{-2}

Results

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. The molecular weight of the purified enzyme as determined by FPLC/gel filtration was 70 kDa.

K_m and V_{max} values were determined for the native and modified BPO that had been incubated with iodoacetamide for 18 hours. As shown in Table 2 with MCD as a substrate, the K_m value of the modified enzyme was two fold less than that of the native enzyme while V_{max} value of the modified enzyme increased 3.07 fold. With KBr as a substrate, the K_m value of the modified enzyme was 71- fold more than that of the native enzyme while the V_{max} value of the modified enzyme increased 2.8 fold. With H_2O_2 as a substrate, the K_m value of the modified enzyme decreased 497.60 fold compared with the native enzyme while the V_{max} value of the modified enzyme increased 1.8 fold.

The native and iodoacetamide-modified BPO was 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 modified enzyme decreased to 86% and 11% at 12 and 60 h of incubation, respectively. The half-life values of the native and modified enzyme are shown in Table 3.

Amino acid analysis (Table 4) showed that the enzyme consists predominantly of acidic amino acids.

Table 3. The half-life of the native and iodoacetamide-modified BPO at pH 7.0 at two temperatures.

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

Table 4. Amino acid composition of native BPO.

Amino acid	Molar percentage
Cysteine	6.03
Aspartic acid	13.48
Glutamic acid	13.45
Serine	7.65
Glycine	11.87
Histidine	3.67
Arginine	2.07
Threonine	2.87
Alanine	4.87
Proline	3.64
Tyrosine	1.43
Valine	5.94
Methionine	3.89
Isoleucine	3.75
Leucine	4.48
Phenylalanine	4.39
Lysine	6.54

The amino acid compositions of bromoperoxidases have been determined from several organisms such as *Xantoria parietina* (L.) Beltr.[14], *Ceramium rubrum* (Hudson) C. Agardh var. *virgatum* C. Agardh[15], *Corallina pilulifera* Postels et Ruprecht [16], *Laminaria saccharina*

(Linnaeus) Lamouroux[17], *Ascophyllum nodosum* (Linnaeus) Le Jolis[18]. The most striking general characteristic of these analyses is the predominance of the amino acidic resides Asx (Asp + Asn) and Glx (Glu + Gln) over the basic residues (arginine and lysine).

BPO was predominantly composed of acidic amino acids, i.e. aspartic acid and glutamic acid. The cysteine residues might be in the form of a disulfide bridge, since they were not modified by iodoacetamide. Tromp *et al*.[19] studied bromoperoxidase from *A. nodosum* and indicated a very compact molecule of the enzyme, and suggested that all 16 cysteine residues in the enzyme participated in the formation of disulfide bridges.

Lysine at time zero was a 6.54 molar percentage. At 4, 6, 8, 12, 18 and 24 hours of incubation, the molar percentages of lysine were 5.47, 4.92, 4.37, 5.02, 4.38 and 3.05, respectively. These results showed that lysine decreased during incubation, suggesting that lysine in BPO reacts with iodoacetamide. The number of modified lysine residues was estimated as a function of incubation time. The total lysine in the BPO was estimated to be 45.82 residues per molecule of enzyme. Estimation of the modified lysine residues per molecule of BPO at 4, 6, 8, 12, 18 and 24 hours with iodoacetamide of incubation were 8.59, 9.59, 9.92, 10.64, 15.16 and 24.43, respectively. So the average number of the modified lysine residues that caused the highest activation of the enzyme activity for BPO should be 15 residues out of the total of 46 lysine residues, the number at 18 h of incubation.

Table 5. The number of iodoacetamide- modified amino acid residues in BPO during incubation.

Incubation time (h)	Modified lysine (residues/molecule)	Modified hisdidine (residues/molecule)
0	0	0
4	8.59	1.19
6	9.59	2.20
8	9.92	5.71
12	10.64	6.23
18	15.16	9.35
24	24.43	11.26

The total histidine in the BPO was estimated to be 16.32 residues per molecule of enzyme. Estimation of the modified histidine residues per molecule of BPO at 4, 6, 8, 12, 18 and 24 hours with iodoacetamide of incubation were 1.19, 2.20, 5.71, 6.23, 9.35 and 11.26, respectively. So the average number of the modified histidine residues that caused the highest activation of the enzyme activity for BPO should be 9 residues out of the total of 16 histidine residues, the number at 18 h of incubation. Iodoacetamide is known to react specifically with the thiol group of cysteine[20]. However, iodoacetamide also reacts with histidine, lysine and methionine residues. From amino acid analysis, cysteine and methionine residues were not found to be modified after modification.

Discussion

Modification of lysine and histidine residues was carried out using iodoacetamide and following the procedure described in the Materials and Methods section. The number of modified lysine and histidine residues for the proteins were found to be 15 in total 46 lysine residues and 9 in total 16 histidine residues. The modification of amino groups of the lysine and histidine residues alters the positive charge to a neutral charge. Thus, at pH 7.0, at which most of the experiments were carried out, there is a change of the charge at the modified residues. This alteration of the charge condition should introduce an increase in the amount of the repulsion within a single polypeptide chain protein causing an expansion in the macromolecule. These introduced groups may reduce the contact area with the water in the denatured state and, therefore, prevent it from incorrect refolding. Furthermore, the introduced groups attached to the (modified) lysine and histidine residues provide new opportunities for the occurrence of the hydrogen bonding.[21, 22]

Treatment of BPO with the modifier results in a dramatic enhancement of its catalytic potential as determined at 35°C. Concomitant with changes in the catalytic potential, we observed substantial alteration in kinetic parameters of these enzymes, including the decrease in the K_m and increase in the V_{max} for all substrates (Table 2). Enhancement of the catalytic potential of BPO is of importance in the context of the practical usefulness of a thermophilic enzyme. The dramatic enhancement in thermostability observed upon modification of BPO, suggests possibilities for making such an important change in a mesophilic protein. However, enhanced thermostability is proposed to be the result of reduction of irreversible thermoinactivation.

Enhancement of the catalytic potential of the enzymes presumably occurs as a result of an increase in flexibility. Introduction of new acetamide groups at positions where lysine residues are altered presumably includes one or more lysine residue(s) in the vicinity of the active site of the enzyme. It is possible that the neutrally charged environment provided by the introduced residues at the active site of the enzyme contribute to the observed outcome.

This laboratory has reported 2.06 and 497.60 fold catalytic activity for MCD and H_2O_2 of BPO, respectively, following reaction of iodoacetamide for 18 hours. Iodoacetamide react with amino groups with retention

of charge neutralization. At neutral pH, the amine functions of lysine residues are mostly protonated and thus positively charged. However, unprotonated lysines will exist in equilibrium with the charged form. The acetamide group introduced by the iodoacetamide reaction is small and electrically neutral. It cannot bring about any charge reversal and is too small to provide significant shielding of close lying groups. 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 enzyme to unfold at high temperatures.

Ugarova *et al*.[23] 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 (observed by circular dichroism) and increased thermostability. Stabilization was due to the degree of modification (i.e., the number of modified lysines), not to the nature of the modifier. Modification of all six lysines, however, actually reduced thermostability. It is clear that non cross-linking chemical modifications can benefit stability of enzymes. Tuengler and Pfleiderer[24] acetamidinated 17 of the 24 lysines of pig heart lactate dehydrogenase converting them to arginine-like structures. Modification increased the tolerance of the enzyme to heat, alkali, and tryptic digestion. The increases in half-life ranged from 5- to 50-fold depending on the elevated temperature used.

A five-fold increase in the apparent half-life of HRP at 65°C has resulted from acetic acid N-hydroxysuccinimide ester modification of three of the six lysines of the enzyme. This result is more modest than those summarized above and compares with stabilization of up to 23-fold obtained with bis-succinimides where up to five lysines were modified[25]. Ugarova *et al*.[23] concluded that stabilization resulted from the degree of modification rather than from the nature of the modifier.

Liu *et al*.[26] found that chemical modification of horseradish peroxidase (HRP) by phthalic anhydride which is the same lysine-specific reagent as bis-succinimides and acetic acid *N*-hydroxysuccinimide and glucosamine hydrochloride increased their thermostability (about 10- and 9-fold, respectively) and in turn also increased the removal efficiency of phenolics. The phthalic anhydride modified enzyme required lower dose of enzyme than native horseradish peroxidase to obtain the same removal efficiency. Both modified HRPs show greater affinity and specificity of phenol.

Iodoacetamide is used for modification of many enzymes as an alkylating agent to study the essential residues of enzymes. In this study, iodoacetamide was used to modify bromoperoxidase; the modified amino acid could be cysteine, methionine, histidine and lysine residues. The results of amino acid analysis showed that histidine and lysine but not cysteine and methionine residues decreased after modification with iodoacetamide, suggesting that histidine and lysine residues were modified. Thus, it might be possible that the modified histidine and lysine residues involved in the enhancement of the enzyme activity and maintenance of enzyme stability.

It has been proposed that histidine residue might involve in the prosthetic group of the enzyme; the nitrogen atom of histidine residues might bind to vanadium at the prosthetic group. Histidine residues might be involved in catalytic activity of the enzyme, since it seemed likely that the pKa of histidine residue was closed to the optimum pH. A steady-state kinetic study of bromoperoxidase from *A. nodusum*[4] showed that inhibition of enzymic activity at low pH was due to protonation of ionizable group. Since hydrogen peroxide was unable to bind to the native enzyme when this group was protonated, this functional group which could be a histidine or a water molecule was probably responsible for the pH-controlled binding of H_2O_2. Also the binding of bromide was found to be pH dependent.

The acetamide group introduced by the iodoacetamide reaction is small and electrically neutral. It cannot bring about any charge reversal and is too small to provide significant shielding of closelying groups. 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.

In this study, neutralization of charges was expected to improve the thermal stability and activity of the enzyme. Activity of amino-derivatized BPO has been described. Attachment of acetamide to BPO amino groups led to increased activity and stability. Iodoacetamides will react with certain free amino groups. The behavior of iodoacetamide-BPO shows that acetamidation of lysine and histidine residues benefits BPO activity and stability.

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