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Role of Modified Lysine Residues on Enzyme Kinetic of Bromoperoxidase

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ABSTRACT

Bromoperoxidase (BPO) from Thai red seaweed *Gracilaria* sp. has been purified and chemically modified with iodoacetamide under defined experimental conditions yielding derivatives of native bromoperoxidase. The modification increased the catalytic activity of the enzyme at concentrations of iodoacetamide up to a level of 50 mM. In order to investigate the molecular mechanism of the activation, the differences changes between native and modified enzymes were studied using kinetics methodology. The modified BPO showed greater affinity and maximal velocity for different substrates than native BPO. It has been shown that this modification reagent reacts with the ε-amino groups of the 15 out of the total 46 lysine residues of bromoperoxidase. The substrate affinity of modified BPO increased with the increases of substituents at ε-amino groups of lysine of the enzyme. The chemical modification of BPO increased its affinity for substrate monochlorodimedone (MCD) and hydrogen peroxide (about 2- and 110-fold, respectively) and also increased the maximal velocity with MCD, KBr and hydrogen peroxide (about 3-, 2.7 and 21-fold, respectively). The improvements of catalytic properties are also related to the changes in the number of ε-amino groups modified. The modification might involve neutralization of positive charges residues in BPO. 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 amino acid side chains.

Key words: bromoperoxidase, lysine, kinetic, iodoacetamide

INTRODUCTION

Recent studies have revealed the existence of some types of non-heme haloperoxidase: vanadium-containing algal bromoperoxidases from *Corallina* (Itoh *et al*., 1985) and *Ascophyllum* (Vilter, 1984), the bacterial chloroperoxidase having no essential metals from *Pseudomonas* and *Streptomyces* (Weisner *et al*., 1988). Although these haloperoxidases have been interesting as novel metal or non-metal enzymes, the co-existence of other metals such as iron (Itoh *et al*., 1986), the relatively low and varying content of metal ions which do not coincide with the number of subunits of the enzyme (Krenn *et al*., 1989), and their reaction mechanisms, especially in non-metal enzymes. Bromoperoxidase from *Corallina pilulifera*, a representative of coralline algae, was first characterized by Itoh *et al.* (1985) and it was classed as a non-heme iron bromoperoxidase because of its high iron content (Itoh *et al*., 1986). However, recent studies have

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shown that the enzyme contains another essential metal for bromination activity, vanadium (Krenn *et al*., 1989).

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 from *Gracilaria* sp. to investigate the improvement of the catalytic and kinetic properties and to identify the modified amino acid residues that may participate in the enzymatic mechanism.

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 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.

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 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. 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 0-1 M NaCl in the same buffer. The protein was eluted at 0.5 – 0.7 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 8.0). 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).

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 8.0). Standard proteins from Pharmacia (ribonuclease A, Mr 13.7 kDa; chymotrypsinogen, Mr 25 kDa; ovalbumin, Mr 43 kDa; aldolase, Mr 158 kDa; and catalase, Mr 232 kDa) were used for the calibration of the column.

Iodoacetamide modification study

BPO was added with 0.25-250 mM iodoacetamide in 20 mM potassium phosphate buffer pH 7.0 and allowed to react at 35°C for 18 h. After incubation, the reaction mixture was dialyzed and measured for the enzyme activity using MCD bromination assay. The enzyme activity in various concentrations of iodoacetamide was compared as percentage of relative activity with the native enzyme without iodoacetamide.

Modification of free amino groups of BPO

Free amino groups of BPO were

modified with 50 mM iodoacetamide in 20 mM potassium phosphate buffer pH 7.0 and allowed to react at 35°C for 24 h. The modified sample was dialyzed extensively against PBS and the BPO activity was assayed by the bromination assay of MCD.

Enzymatic assays

Haloperoxidase activity was monitored using total volume of the enzyme reaction 1.0 ml, a mixture of 48 mM monochlorodimedone (MCD), 8.8 mM H_2O_2, 100 mM KBr and an appropriate amount of enzyme in 100 mM sodium phosphate, pH 6.0 at room temperature. The decrease in absorbance at 290 nm ($\mu = 1.99 \times 10^4$ 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. The control contained enzyme sample in the buffer, MCD and KBr but omitted H_2O_2. 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.

Steady-state kinetic analysis

The initial rates, v_0, plotted as a function of MCD, KBr or hydrogen peroxide concentration were fit to a Michaelis–Menten equation. The apparent kinetic parameters K_m^{app} as well as V_{max} were obtained from primary double reciprocal plots.

Free amino acid determination

Free amino acids were determined according to the method of Benjakul and Morrissey (1997) using an amino acid analyzer (Waters 2690 Alliance).

RESULTS AND DISCUSSION

Bromoperoxidase from red seaweed, *Gracilaria* sp., was extracted and precipitated by 0-60% ammonium sulphate saturation. The enzyme was purified by DEAE cellulose column chromatography and FPLC by using Mono Q and Superose 12 column. The results of enzyme purification are given in Table 1. In step of 0-60% ammonium sulphate saturation precipitation, about 87% of total protein in crude extracts was precipitated and the bromoperoxidase activity was slightly lost and specific activity was slightly increase. In DEAE-cellulose column chromatography, the bromoperoxidase activities were separated into two peaks (Figure 1). The first peak (BPOI) was eluted at 0.16-0.32 M NaCl and the second peak (BPOII) was eluted at 0.35-0.55 M NaCl.

BPOI was further purified by using FPLC. In the Mono Q step, BPOI was eluted at 0.55-0.70 M NaCl (Figure 2), about 74% of total proteins was eliminated and specific activity about 2.6-fold, the % yield decrease about 32.2% compared to the DEAE cellulose step.

In further purification by using Superose 12 column on FPLC (Figure 3) for BPOI, the

Table 1 Purification of bromoperoxidase (BPOI) from red algae, *Gracilaria* sp.

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% $(NH_4)_2SO_4$	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

protein were eliminated about 56.6%, the specific activity increased about 1.6 fold and % yield decreased by 31% compared to the Mono Q step. 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.

The K_m and V_{max} of native and modified BPO were determined. With MCD as a substrate, K_m value of the modified enzyme was 2 fold less than that of the native enzyme while Vmax value of the modified enzyme increased 3.07 fold. With KBr as a substrate, K_m value of the modified enzyme was 71 fold higher than that of the native enzyme while V_{max} value of the modified enzyme increased 2.8 fold. With H_2O_2 as a substrate, K_m value of the modified enzyme decreased 110 fold when compared with the native enzyme while V_{max} value of the modified enzyme increased 1.8 fold. From Table 2, we can see that K_m of the modified BPO for MCD and H_2O_2 was lower than that of the native enzyme and that V_{max} for all substrates of the modified BPO were greater than that of the native BPO. These results indicate that the chemical modification of BPO increased the substrate affinity and rate of reaction but not for affinity for KBr.

The enzyme was hydrolysed by hydrochloric acid for determination of amino acid composition. For determination of cysteine and methionine, the enzyme was oxidized with performic acid prior to HCl hydrolysis. The results of amino acid analysis are shown in Table 3. The results showed that the enzyme contained predominately of acidic amino acids.

Lysine at time zero was 6.54 molar percentage. At 4, 6, 8, 12, 18 and 24 h of incubation with iodoacetamide, the molar percentages of lysine were 5.47, 4.92, 4.37, 5.02, 4.38 and 3.05, respectively (Table 4). This results showed that lysine decreased during incubation, suggesting that some lysine in BPO reacted with iodoacetamide. The number of modified lysine was estimated. The total lysine in the BPO was estimated to be 45.82 residues per mole. The modified lysine per mole BPO at 4, 6, 8, 12, 18

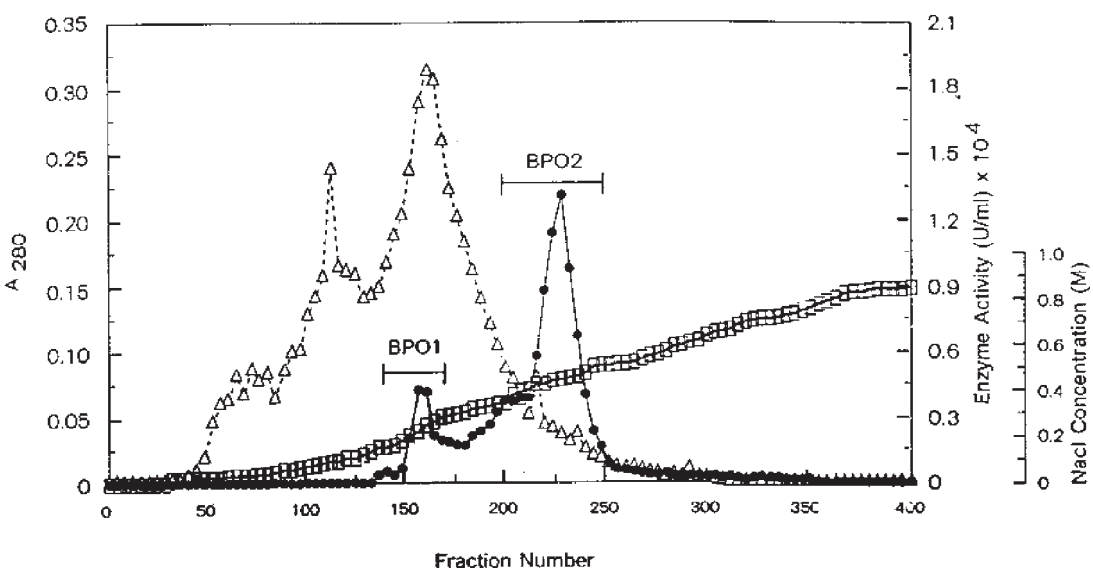

Figure 1 Elution profile of 0-60% ammonium sulphate precipitate from chromatography on DE 52 column showing the separation of the two bromoperoxidase, BPOI and BPOII.

and 24 h 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 that caused highest activation of the enzyme activity for BPO should be 15 residues out of the total of 46 lysine residues at 18 h of incubation.

From this study, it can be suggested that the modified amino acid residues by iodoacetamide might be attributed to the catalytic activity of BPO. It has been proposed that lysine residue was with in the active site of the vanadium BPO (Littlechild and Garcia-Rodriguez, 2003). The results show that the ε-amino groups of lysine modification can increase the specific activity for MCD and H_2O_2 (Table 2).

Biocatalytic behavior could be attributed

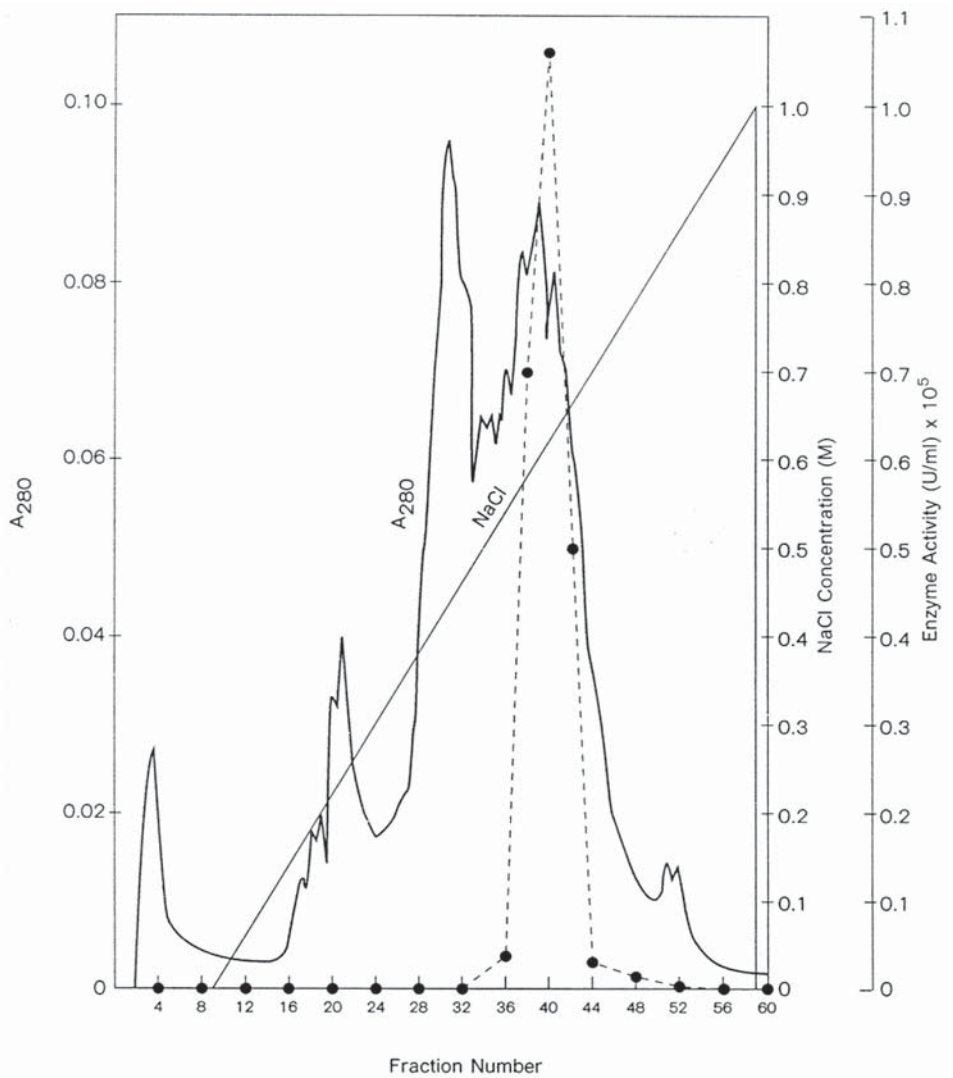

Figure 2 Chromatographic profile of BPOI on MonoQ column. Dialysed concentrated BPO from DE52 column was applied to the MonoQ column equilibrated with 20 mM Tris-HCl buffer pH 8.0 at the flow rate 1ml/min. Proteins in the column were eluted with a linear gradient 0-1.0 M NaCl in the same buffer. Fractions were measured for protein (—, A_{280}) and enzyme activity (--•--) using MCD bromination assay.

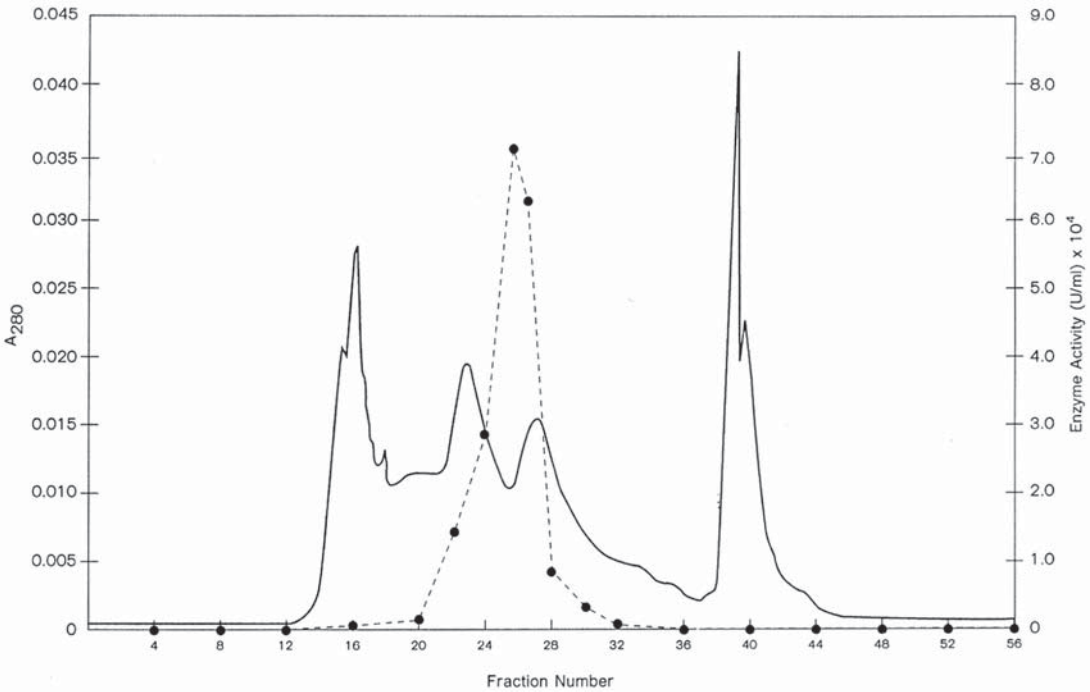

Figure 3 Chromatographic profile of BPOI from MonoQ column on Superose12 column. Dialysed concentrated BPOI from MonoQ column was applied to the Superose12 column equilibrated with 20 mM Tris-HCl buffer pH 8.0 at the flow rate 0.5 ml/min. Proteins in the column were eluted with a linear gradient 0-1.0 M NaCl in the same buffer. Fractions were measured for protein (—, A_{280}) and enzyme activity (--•--) using MCD bromination assay.

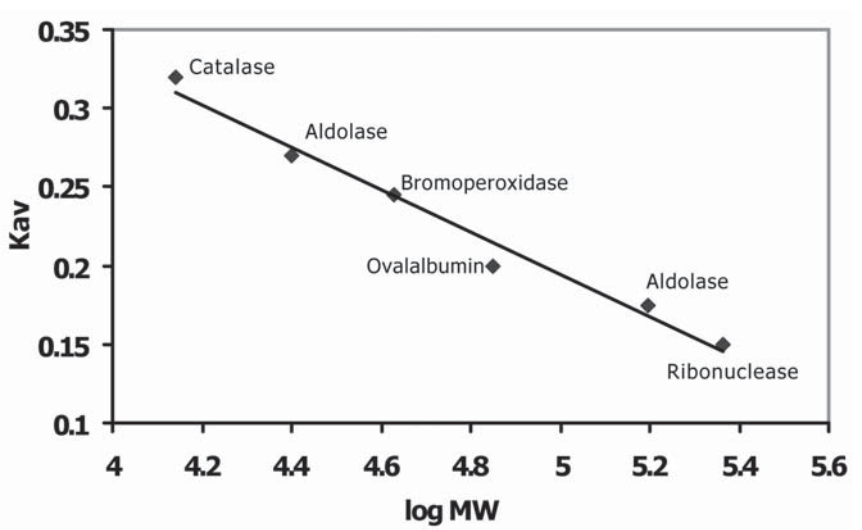

Figure 4 Molecular weight estimation for bromoperoxidase (BPOI) using Superose12 filtration. The K_{av} of bromoperoxidase is compared to that of standard proteins with known molecular weights.

to the electron character of the substituted group and /or hydrophobicity increase of active site. The chemical modification of the amino group altered its electron balance affecting the specific activity of the enzyme. When the ε-amino groups of lysine of this enzyme are modified by an alkylating group, acetamide, the hydrophobic interaction is expected to be increased, increasing enzyme activity. Thus, the results obtained indicate that it is possible to increase both affinity and catalytic activity of BPO by an increase in rigidity from the increased hydrophobic interaction. In the active site of *C. pilulifera* VBPO, there are several hydrophobic patches and charged residues that could provide binding sites for the organic substrates. Only three hydrophilic residues and no charged residues except those involved in the vanadate binding are observed within 7.5 Å from the vanadate O^4 oxygen in the *A. nodosum* structure (Weyand *et al*., 1999). These residues could affect the substrate specificity and stereoselectivity of the reaction.

This study might represent the case that changes in the hydrophobic interaction has led to changes in enzyme properties.

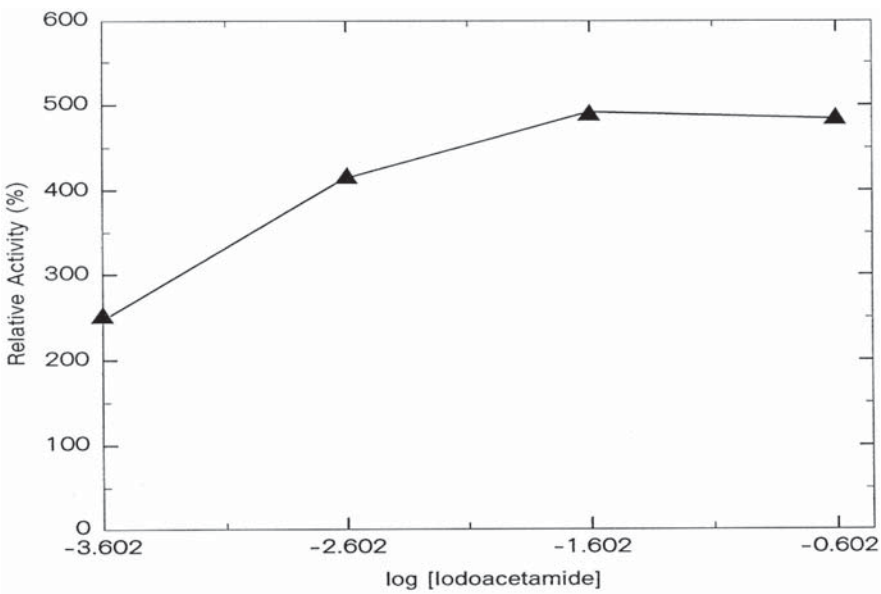

Figure 4 Effect of iodoacetamide on bromoperoxidase activity. BPOI was added with 0.25-250 mM iodoacetamide in 20 mM potassium phosphate buffer pH 7.0 and allowed to react at 35°C for 18 h. as described in materials and methods. The enzyme activity in various concentrations of iodoacetamide was compared as percentage of relative activity with the native enzyme without iodoacetamide.

Table 2 Kinetic parameter of native and iodoacetamide-modified bromoperoxidase (BPOI).

Enzyme	Substrate					
	MCD		KBr		H_2O_2	
	K_m(M)	V_{max}(M min^{-1})	K_m(M)	V_{max}(M min^{-1})	K_m(M)	V_{max}(M min^{-1})
Native	2.94×10^{-5}	4.17×10^{-8}	2.17×10^{-4}	4.76×10^{-8}	1.04×10^{-4}	3.39×10^{-8}
Modified	1.43×10^{-5}	1.28×10^{-7}	1.54×10^{-2}	1.29×10^{-7}	9.09×10^{-7}	7.14×10^{-8}

Table 3 Amino acid composition of bromoperoxidase.

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	4.385
Lysine	6.54

Table 4 The number of iodoacetamide modified amino acid residues of bromoperoxidase during incubation.

Incubation time (h)	Modified lysine (residues/mole)
0	0
4	8.59
6	9.59
8	9.92
12	10.64
18	15.16
24	24.43

CONCLUSIONS

The effect of iodoacetamide-modified amino groups of BPO on substrate affinity and catalytic activity were studied. It was shown that chemical modification of amino groups of lysine residues improved the substrate affinity . The results presented in this communication clearly indicate that BPO may acquire some new and useful characteristics related to activity upon modification of specific side chains and the conformation change of the modified enzyme might be further investigated. Such site-directed modification may complement site-directed mutagenesis involving specific amino acid replacement in this enzyme.

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