CHAPTER I

INTRODUCTION

1.1 Peroxidases and Haloperoxidases

Among all enzymes, peroxidases have propably attracted most attention of chemists, biologists and physiologists. Peroxidases (EC. 1.11.1) form a subgroup of the oxido-reductases that catalyse the oxidation of organic as well inorganic substrates by hydrogen peroxide. Haloperoxidases are the subgroup of peroxidase that are able to catalyse the insertion of halide ions; iodide, bromide and chloride, but not fluoride, into a suitable substrate in the presence of hydrogen peroxide for the formation of carbon-halogen bond of substrate resulting in halogenated products (1) as shown in the following reaction

$$2AH + 2X^- + H_2O_2 \xrightarrow{\text{Haloperoxidase}} 2AX + 2H_2O$$

A = organic substrate

X^- = halide ion (I^-, Br^- and Cl^- but not F^-)

AX = halogenated product

1.2 Type of Haloperoxidases

Haloperoxidases are divided into three groups according to their specificity for halide ion. The name of the haloperoxidase is determined by the most electronegative halide which can be oxidized; so chloroperoxidases are able to catalyze the oxidation of all halides except fluoride, bromoperoxidases can use bromide and iodide as electron donors and iodoperoxidases are specific for iodide and do not activated bromide and chloride.

1.3 Discovery of Haloperoxidases

Although peroxideases were known before 1900, the existence of haloperoxidases was established some decades later. In 1925, Sauvagean (2) found free bromine (Br_2) in marine algae and he reported that algae cells were able to convert fluorescein into tetra-brominated derivative, eosin. In 1926, he also found free iodine in extracts of red algae, suggestion that I_2 was present in living cells (3). After the discovery of iodinating and brominating enzymes in marine algae, some mammalian haloperoxidases were detected. In 1941, Agner (4) isolated myeloperoxidase from tuberlar empyema. Lactoperoxidase was purified from milk (5), and

mammals thyroid peroxidase was found (6). By 1960's three additional haloperoxidase were identified : a peroxidase was isolated from roots of Japanese radish plant (7), chloroperoxidase from fungus *Caldariomyces fumago* (8), was found to excrete and eosinophil peroxidase from human eosinophilic granulocytes (9). In the past fifteen years, the broad occurrence of haloperoxidases become apparent. Nowadays haloperoxidases are know from more than 100 sources including mammals (10), higher plants (11, 12), marine animals (13), sponges (14), algae (15-19), lichen (20), fungi (21, 22) and bacteria (23-25). Some well-characterized haloperoxidases are given in Table 1.

1.4 Types of Bromoperoxidases

Bromoperoxidases are divided into three classes : heme, eukaryotic non-heme, and bacterial non-heme bromoperoxidases. The heme type enzymes usually have protoporphyrin IX as prosthetic group (23, 25), and show halogenating activity as peroxidase and catalase activity. The reaction mechanism of these enzymes has been thoroughly investigated (38). Bromoperoxidases of the second class contain vanadium instead of heme (39) whereas the third class, the bacterial non-heme bromoperoxidases do not require metal ion or any other cofactor (40, 41). Types of bromoperoxidases are shown in table 2.

1.5 Sources of Bromoperoxidases from Marine Algae

Bromoperoxidases were found from different kinds and species of marine algae as shown in Table 3.

1.6 Prosthetic Group

1.6.1 Heme

Bromoperoxidases consist of prosthetic group and glycoprotein. Since heme-bromoperoxidaes display Soret peaks at 400-420 nm in the optical absorption spectrum, and the structure of heme prosthetic group has been studied extensively. Most of heme-enzymes contain ferriprotoporphyrin IX as prosthetic group except for bromoperoxidase from milk, lactoperoxidase which contains iron complex of 18-mercaptomethyl-2, 7, 12, trimethyl-3, 8-divinylporphyrin-13, 17-dipropionic acid (28).

Table 1 Some well-characterized haloperoxidases

Type trivial name	Source	Reference
Iodoperoxidase		
Horseradish peroxidase	Horseradish root	11
Turnip peroxidase	Turnip root	12
Thyroid peroxidase	Thyroid gland (mammals, birds)	26
Iodoperoxidase	Brown algae	27
Bromoperoxidase		
Lactoperoxidase	Milk, saliva, tears	28
Ovoperoxidase	Sea urchin eggs	29
Bromoperoxidase	Marine snail : *Murex trunculus*	30
Bromoperoxidase	Brown, green and red algae	31-34
Bromoperoxidase	Bacteria :	
	Streptomyces phaeochromogenes	23
	Pseudomonas aureofaciens	25
Chloroperoxidase		
Chloroperoxidase	Mold : *Caldariomyces fumago*	21
Myeloperoxidase	Neutrophillic granulocytes	35
Eosinophil peroxidase	Eosinophilic granulocytes	36
Bromoperoxidase	Alga : *Penicillus capitatus*	37

The structure and intimate interaction between heme and glycoprotein imparts a unique set of catalytic properties. The heme prosthetic groups has been shown to have dual role in the catalytic function, i.e.,

1. The metal (iron III) activates the heterolytic cleavage of H_2O_2 and stores one oxidizing equivalent.

2. The porphyrin regulates the oxidation-reduction potential and stores one oxidizing equivalent.

Table 2 Types and sources of bromoperoxidases

Type	Source	Prosthetic groups
Heme	Milk, saliva, tears :	
	Lactoperoxidase	Heme
	Sea urchia eggs	Heme
	Marine snail :	
	Murex trunculus	Heme
	Marine algae :	
	Rhodomela larix	Heme
	Penicillus lamourouxii	Heme
	Penicillus capitatus	Heme
	Bacteria :	
	Streptomyces phaechromogenes	Heme
	Pseudomonas aureofaciens	Heme
Eukaryotic non-heme	Brown algae :	
	Ascophyllum nodosum	Vanadium
	Chorda filum	Vanadium
	Laminaria saccharina	Vanadium
	Red algae :	
	Ceramium rubrum	Vanadium
	Corallina piluliferaa	Vanadium
	Corallina officinalis	Vanadium
	Lichen :	
	Xanthoria parietina	Vanadium
Bacterial non-heme	Bacteria :	
	Streptomyces aureofaciens	Unknown
	Streptomyces griseus	Unknown

Table 3 Sources of bromoperoxidase from marine algae

Rhodophyta (red)	**Chlorophyta (green)**	**Phaeophyta (brown)**
Amphiroa hancockii	*Bryopsis pennata*	*Ascophyllum nodosum*
Asparagopsis taxiformis	*Caulerpa racemosa*	*Chorda filum*
Bonnemaisonia hamifera	*Chaetomorpha linum*	*Dictyota divaricata*
Bryothamnion triquetrum	*Cladophorales* sp.	*Laminaria saccharina*
Ceramium rubrum	*Caldophoropsis membranacea*	*Lobophora variegata*
Chondria atropurpurea	*Codium isthmocladum*	
Chondria cnicophylla	*Cadium repens*	
Coelothrix irregularis	*Dictyosphaeria cavernosa*	
Coralline pilulifera	*Halimeda discoidae*	
Corollina officinalis	*Halimeda incrassate*	
Dasya pedicellata	*halimeda goreauii*	
Dasys sp.	*Halimeda opuntia*	
Euchewna isiforme	*Halimeda tuna*	
Galaxaura comans	*Penicillus capitatus*	
Galaxaura squalida	*Penicillus dumentosus*	
Gracilaria sp.	*Penicillus lamourouxii*	
Haloplegma duperreyi	*Rhipocephalus phoenix*	
Laurencia chondroides	*Udotea flabellum*	
Laurencia intricata	*Udotea wilsoni*	
Laurencia lata	*Ulva lactuca*	
Laurencia obtuse	*Valonia marophysa*	
Laurencia papillosa		
Laurencia poitei		
Liagora sp.		
Ochtodes secundiramea		

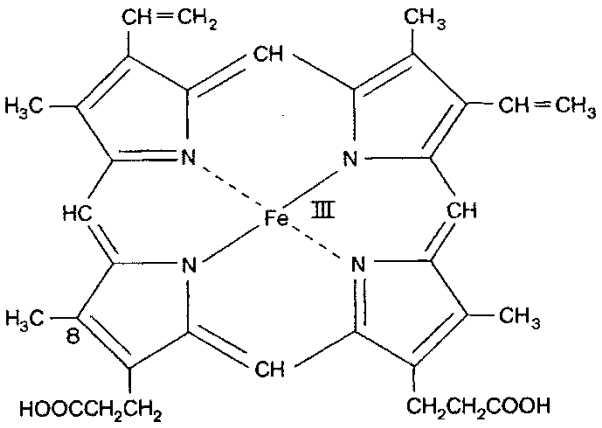


Figure 1 The heme component of bromoperoxidase-ferriprotophyrin IX

1.6.2 Eukaryotic Non-Heme

Non-heme bromoperoxidase does not have Soret band. The firstly found non-heme enzyme is lactoperoxidase from bovine milk (42), however, the specific activity in the oxidation of iodide is less than 1% of that reported for lactoperoxidase. Later, Vilter detected a fully active non-heme enzyme in *Ascophyllum nodosum* which was insensitive toward inhibition by cyanide but inactivated by dialysis against EDTA in a citrate/phosphate buffer pH 3.8 and reactivated when the enzyme was incubated with vanadium (V) whereas other metal ions were ineffective in the reconstitution of this enzyme (43, 44). These results were confirmed by de Beer et al. (39, 45) and the presence of vanadium in the enzyme was established by several physiochemical methods such as atomic absorption spectrophotometry, electron paramagnetic resonace.

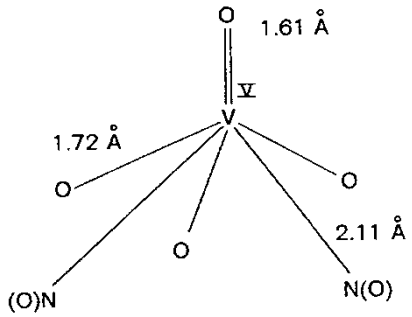


Figure 2 Proposed coordination of vanadium in native bromoperoxidase

Vanadium probably only serves to bind hydrogen peroxide and bromide. The vanadium coordinating amino acids have not been identified yet. However, it was proposed that the native bromoperoxidase might contain vanadium in the 5^{+} oxidative state and the highly electronegative ligands as oxygen or nitrogen should be coordinated to the vanadium ion (46, 47). In addition, it was also proposed from by X-ray absorption spectroscopy that vanadium ion coordinated to one oxygen atom at a short distance that vanadium ion coordinated to one oxygen atom at a short distance of 1.61 Å, three oxygen atoms at 1.72 Å andtwo nitrogen or oxygen atoms in a distorted octahedral symmetry. The oxygens might be derived from amino acid such as aspartic acid or tyrosine, and the nitrogents from histidine (48, 49).

1.6.3 Bacterial Non-Heme

Bacterial non-heme bromoperoxidase presents in *Streptomyces aureofaciens* (40, 50) and *Streptomyces griseus* (41) neither a heme group nor vanadium could be detected. The nature of the active site in these enzymes is still unknown. In these enzyme preparations, zinc as well as iron was detected, however, it is uncertain whether these metals are involved in the catalytic activity. Since iron and zinc are very common transition metals, nature may have selected those elements for a stabilizing function in bromoperoxidases.

1.7 Glycoprotein-Containing Bromoperoxidase

Besides prosthetic group, glycoprotein is another component of the enzyme. The glycoprotein plays an important role in determining the reaction of the enzyme and stabilization of reactive intermediate (38).

1.8 Catalytic Mechanism and Kinetic Properties

Bromoperoxidase is unique in catalyzing halogenation reactions. The mechanism of peroxidation catalyzed by bromoperoxidase has been studied intensively (51-55). The suggested catalytic mechanism involved three important enzyme-species; i.e., native enzyme and two intermediate forms where the substrates hydrogen peroxide and halide are bound to the enzyme. In the first step of the reaction sequence, hydrogen peroxide binds to the enzyme.

In heme-containing enzyme, the iron (III) of the heme group is oxidized to the ferryl state (IV) and an additional oxidizing equivalent is retained on the heme groups or on amino acid residue. During this process the oxygen-oxygen bond in the hydrogen peroxide

molecule is cleaved (38, 56). As native vanadium bromoperoxidasses containing vanadium in 5^+ state probably bind to hydrogen peroxide as a ligand. In the subsequent reaction, the halide ion is bound (54) bound (54) and oxidized by the enzyme-H_2O_2 intermediate.

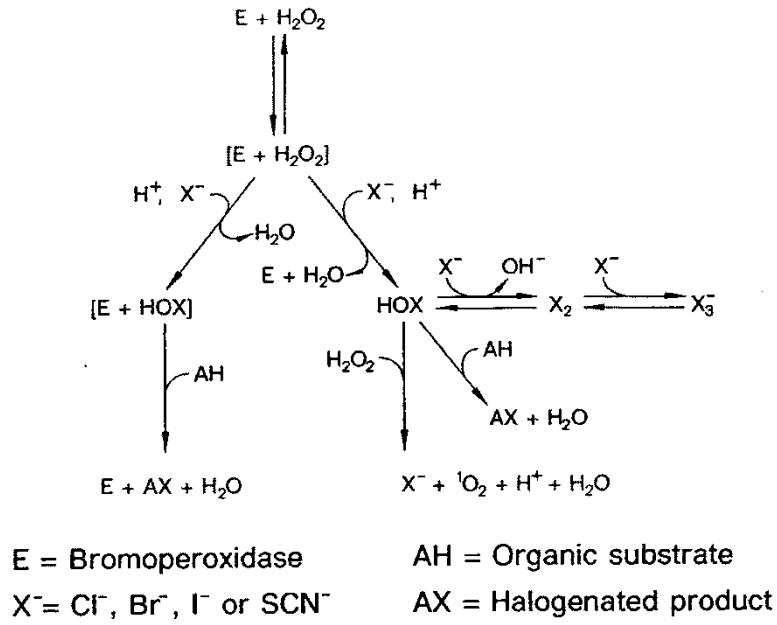


Figure 3 The enzymic mechanism of bromoperoxidase

It is still uncertain whether the organic substrate (AH) is halogenated by the oxidized halogen species (HOX) in solution or by the enzyme-HOX intermediate (57, 58). As depicted in Figure 3, the oxidized halogen species, hypohalous acid (HOX) in aqueous solution is in rapid equilibrium with molecular halogen (X_2 and trihalide ions (X_3^-) (59).

In the absence of an organic substrate the oxidized halogen species is able to react with hydrogen peroxide to yield singlet oxygen and halide (60, 61). Because of this side reaction, the nucleophil acceptor (AH) most compete with hydrogen peroxide for HOX (62). Measurement of the rate of enzyme production of oxidized bromine species (Br_2, Br_3^- and HOBr) and of the bromination rate of, 2-chlorodimedone showed that a ternary complex between an enzyme-halogenating intermediate and an organic substrate is not involved. This explains the lack of region-and stereospecificity as suggested by Itoh et al., (63).

1.9 Function of Bromoperoxidase

Enzyme-catalyzed halogenation is a common biological phenomenon by now. After the discovery of various bromoperoxidases in marine organisms, considerable research effort has been made to understand the function of these enzymes. Bromoperoxidases can react with broad spectrum of substrates (38). It is clear that if biologically relevant substrates vital to the survival of living cells are inactivated by enzymatic modification, or if the product of the reaction between the enzyme and biologically relevant substrate has toxic properties, then the enzymes will have potential for the destruction of pathogenic cells. Although the natural function of bromoperoxidases is still not known, these enzymes seem to be involved in the defence mechanism of their hosts.

1.9.1 Oxidative Agents

In the presence of halogenating enzyme, H_2O_2 and an appropriate halide or thiocyanate ion, halogenation, thiocyanation or oxidation can occur. Mammalian bromoperoxidase and lactoperoxidase is known to directly involve directly in the oxidative destruction of invading microorganisms, because the enzyme can produce hypobromous acid (HOBr) and hypothiocyanous acid (HOSCN) which both act as bactericidal agents (64).

It is believed that thiocyanate ion mediates its effects by reactions with sulfhydry (–SH) groups in proteins (65). In strain of *Streptococci,* Caslsson et al., (66) have indicated that glycolytic pathway and glyceraldehydes-3-phosphate dehydrogenase in particular appear to be the targets of lactoperoxidase. The inhibitory effect of lactoperxidase on microorganisms is shown in Table 4.

Table 4 *In vitro* antimicrobial activity of lactoperoxidase

Inhibited microorganism	Reference
Bacillus cereus	67, 68
Escherichia coli	69, 70
Pseudomonas fluorescens	71
Salmonella typhimurium	72
Streptococcus agalactiae	73
Streptococcus lactis	70
Streptococcus mitis	66
Streptococcus mutans	65, 66
Streptococcus salivarius	66
Streptococcus sanquis	66

1.9.2 Halometabolites

Whether bromoperoxidase fro microorganisms, plants and marine species also a direct role in destroying predators is still open to debate. It is known that halogen-containing compounds are physiologically more active than their non-halogenated counterparts (74). Halometabolites are especially abundant in the marine environment (75-77) which is obviously and ecological niche for halogenation. Since seawater contains 0.5 M Cl^- and 10^{-5} M Br^-, there are plenty of halogen anions available to insert into organic molecules. It was found that the large majority of brominated substances were been obtained from marine organisms. A highly brominated antibiotic (five bromine atoms per molecule) was isolated from the marine bacterium *Pseudomonas bromoutilis* (78). The marine worm *Balanoglossus biminiensis* produced large amounts of 2,6-dibromophenol as a defense mechanism toward predators (79). Fenical (80) indicated that a broad range of haloketones isolated from the red seaweed *Asparagois taxiformis* showed strong antimicrobial activity against a variety of microorganisms. The major compounds were 1,1,3,3,-tetrabromoacetone and tribromo-3-buten-2-one. Fenical (75) commented that in various species of red algae, halometabolite such as brominated phenols, including terpene, phenol were involved in chemical defense against parasitic microflora as well as being involved as feed detergents against invertebrate predators. Sheikh and Djerassi (81) had suggested that

bromophenols had a similar protective role in the marine tube worm, *Phoronopsis viridis*. McConnel and Fenical (82) had shown that the family *Bonne msisoniaceae* produced an array of C_7-C_9 ketones, alcohols and acids that appeared to be stored in specialized vesicular cells, referred earlier as sites of bromide for use as antimicrobial and antifeedant agents. For example, 1,3-dibromo-2-heptanone showed minimal inhibitory concentrations of 0.05 µg/ml against both *Staphylococcus aureus* and *candida albicans.* Hager (83) reported that, in studying extracts of 1,200 samples of marine microorganisms had repored a strong correlation between the antimicrobial activity of lipid extracts and their halometabolite content and it was found that *Asparagopsis taxiformis* produced 3-5% of its dry weight as halogenated methane derivatives, with bromoform ($CHBr_3$) being the major component. Halomethanes have been considered as potent biocides (77). Other halometabolites have been demonstrated to have chemical defense functions in marine organisms, i.e., the green algae, *Avrainvillea longicaulis* produced the brominated diphenylamine, avrainvilleol which has antifeedant activity (84).

Furthermore, halometabolites are found as intermediates in the biosynthesis of non-halogenated compounds, or complex halogenated compounds. Strong presumptive evidence has been accumulated that in introducing marine environment, brominated intermediates may be involved in the cyclization of isoprenoids to yield terpenes and in rearrangement reactions of linear ketones and terpenoids molecules (77).

1.10 Haloperoxidase Reactions

Enzymatic reactions often have three general characteristics : reversibility high product selectivity, and narrow substrate range. Haloperoxidases possess none of these characteristics. However, it is because haloperoxidases have such a broad substrate range so that the wide variety of *the following in vitro* reaction is possible.

1.10.1 Reaction with Alkenes

Haloperoxidases have been shown to catalyze reaction with a wide range of alkene substrates to from alpha, beta-halohydrins (Figure 4). In a systematic study of gaseous alkenes, it was demonstrated that isolated (e.g., propylene), conjugated (e.g., butadiene), and cumulative (e.g., allene) carbon-carbon double bond were reactive (85).

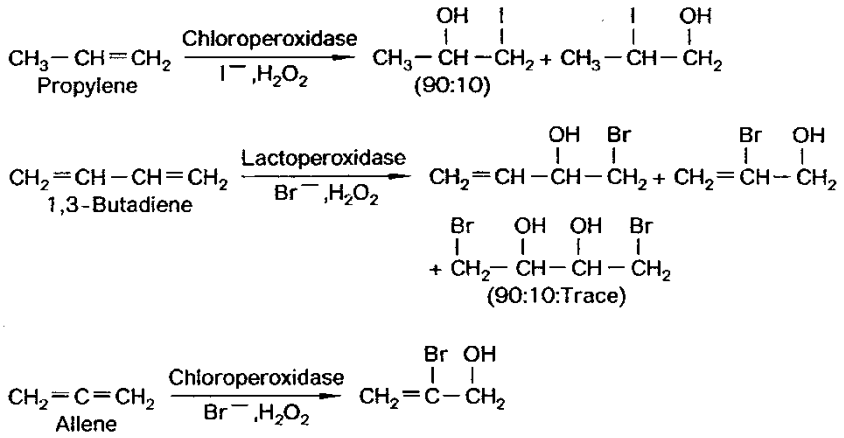


Figure 4 Reaction of haloperoxidases with alkenes to form alpha, beta-halohydrins.

Functional groups present on the alkene can lead to products other than halohydrins. Haloperoxidases form haloacetones from unsaturated carboxylic acid owing to the presence of adjacent carboxyl anion as shown in Figure 5 (86-89).

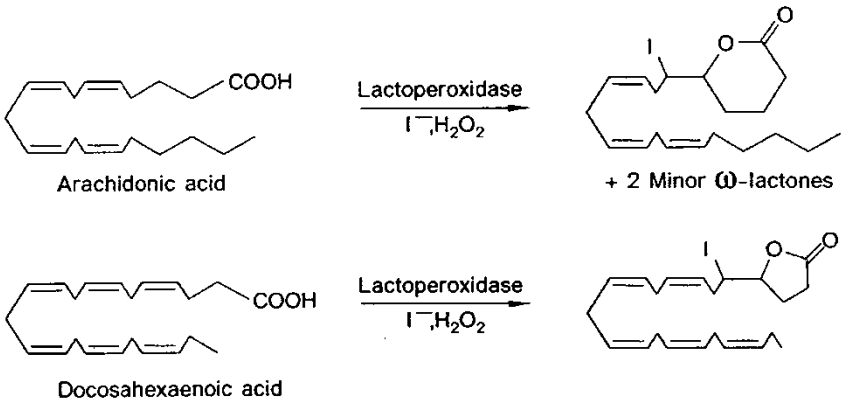


Figure 5 Reaction of haloperoxidases with alkenes to form haloacetones.

Halide anion effectively competes with hydroxyl ion for the presumed halonium ion intermediate; a dihalide product is formed instead of the expected halohydrin. The product distribution in such a reaction has been found to be dependent upon the halide ion (s) concentration in the reaction mixture (90, 91) as shown in Figure 6.

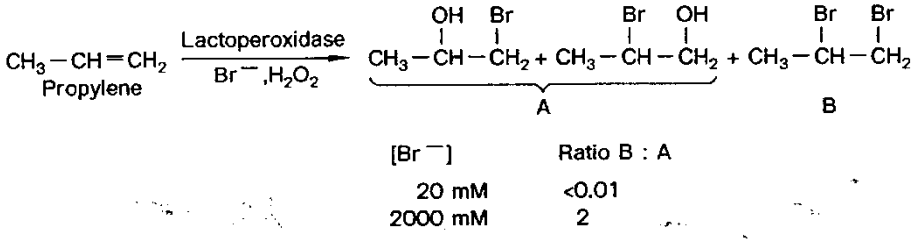


Figure 6 Reaction of haloperoxidases with alkenes to form alpha, beta-dihalides.

1.10.2 Reaction with Alkynes

With alkyne substrates, haloperoxidase-catalyzed reactions yield alpha-haloketones. These enzymes are thus distinguished by being able to react with both carbon-carbon double bonds and triple bonds.

The product distribution in the reaction with alkynes is dependent upon the halide ion(s) concentration in the reaction mixture. Increasing the halide ion concentration results in the formation of alpha, beta-dihalides (90, 92) as shown in Figure 7.

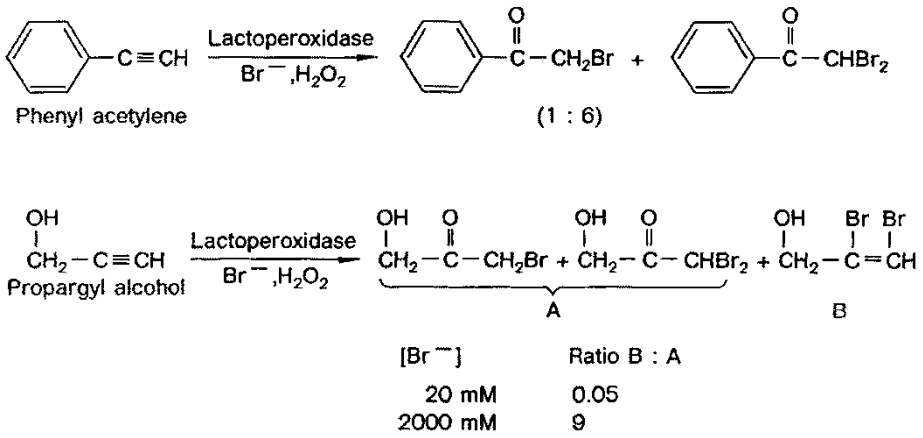


Figure 7 Reaction of haloperoxidases with alkyenes to form alpha, beta-haloketones and alpha, beta-dihalides.

1.10.3 Reaction with Cyclopropanes

The reaction between cyclopropanes and heloperoxidases yield alpha, gamma halohydrins (92) as shown in Figure 8.

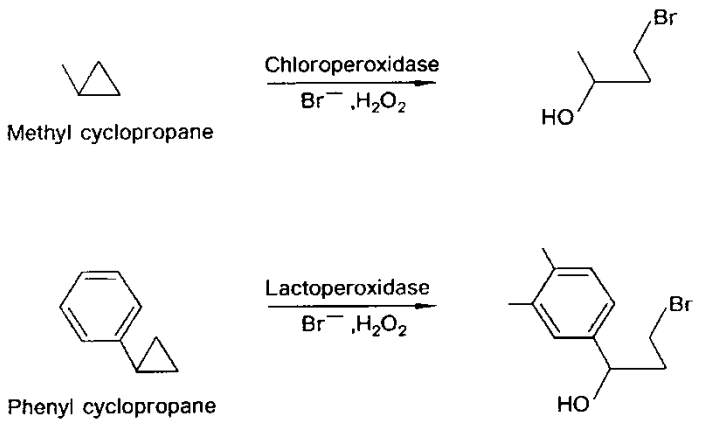


Figure 8 Reaction of haloperoxidases with cyclopropanes to form alpha, gamma-halohydrins.

1.10.4 Reaction with Phenols, Anilines and other Aromatics

A wide range of aromatic compounds are reasdily halogenated by haloperoxidases, i.e., phenols, anilines (and their respective O-and N-alkylated derivatives) and heterocyclic aromatics (93-97) as shown in Figure 9.

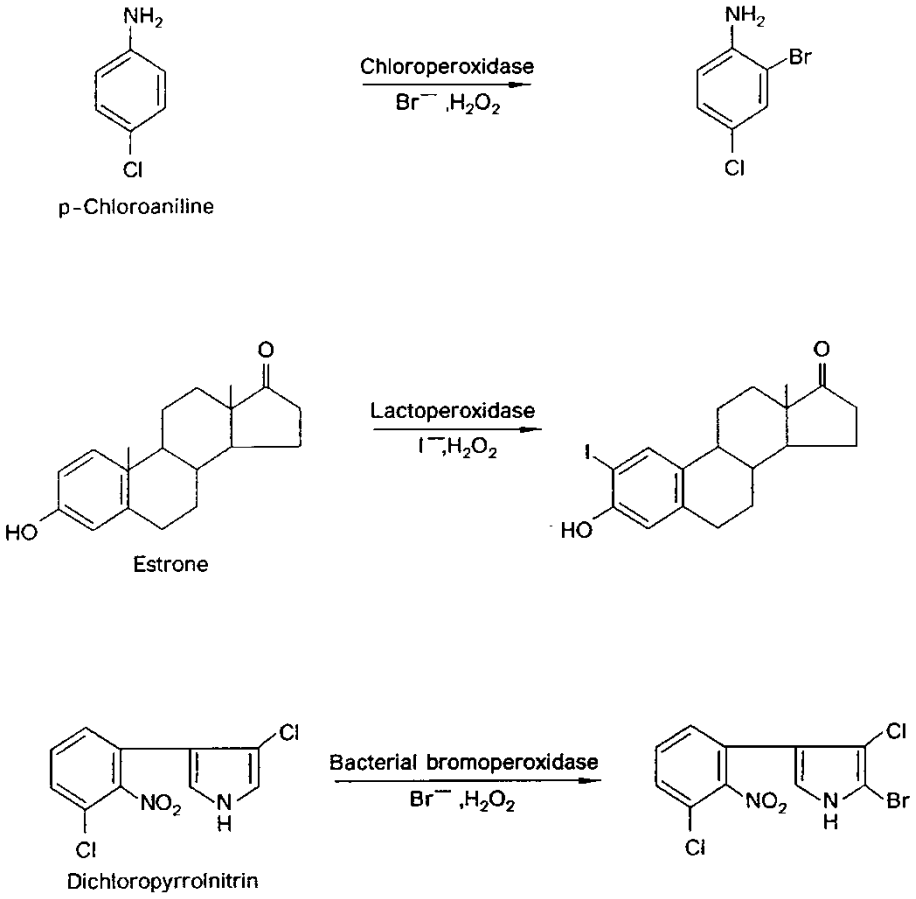


Figure 9 Reaction of haloperoxidases with phenols, aniline and other aromatics.

1.10.5 Reaction with Beta-Diketones and Beta-Ketoacids

A wide range of beta-diketones can be halogenated by haloperoxidases. The reactivity appears to be a function of percent enol content of the substrate in the reaction mixture. A simple ketone like 2-heptanone which has little enol content is unreactive, while a beta-diketone like monochlorodimedone (MCD) which is predominantly in the enol form in solution is a very reactive substrate. The MCD reaction is the standard assay used to assign the activity of a haloperoxidase enzyme. The beta-diketone substrates can range from monocyclic compounds to polycyclic steroids (98). The reactions are shown in Figure 10.

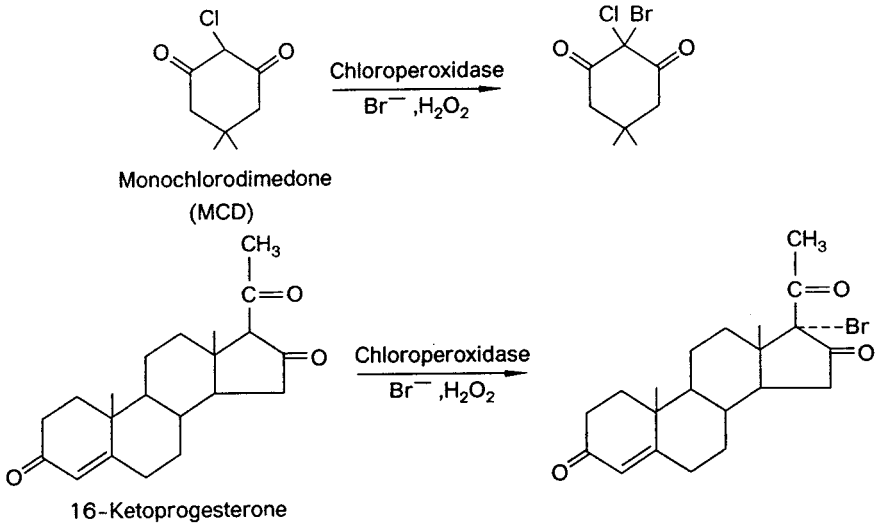


Figure 10 Reaction of haloperoxidase with β-diketones.

Beta-ketoacids readily decarboxylate upon halogenation with haloperoxidases (99, 100). The reaction are shown in Figure 11.

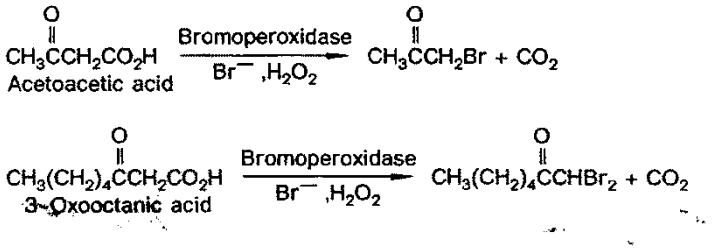


Figure 11 Reaction of haloperoxidase with β-diketoacids.

1.10.6 Reaction with Nitrogen Atoms

Haloamines are the primary products formed when haloperoxidases react with amine compounds, however many of these haloamines are unstable, and rapidly deaminate/decarboxylate, and thus freeing the halogen (101, 102). The reactions are shown in Figure 12.

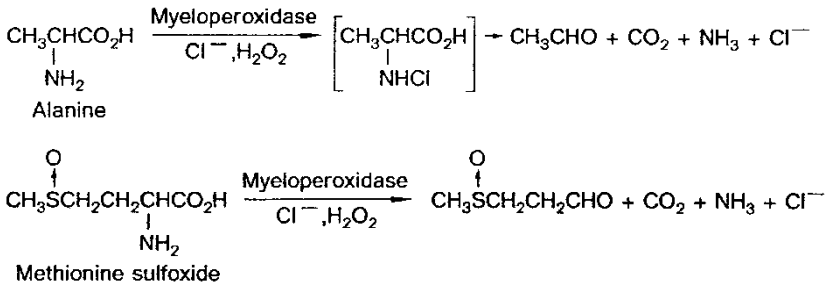


Figure 12 Reaction of haloperoxidase with nitrogen atom.

1.10.7 Reaction with Sulfur Atoms

Thiols (-SH) are oxidized by haloperoxidases to yield the sulfenyl halide (-SX) which in turn can react rapidly with excess thiol compounds to yield a disulfide (–S–S–) (103). Disulfides, like cystine; and dialkyl sulfides and methionine, are also oxidized by haloperoxidases in the presence of halide ions yielding sulfoxides (104, 105).

Thiocyanate (SCN^-) is a pseudo-halide and in the presence of hydrogen peroxide, most haloperoxidases will oxidize thiocyanate to hypothiocyanous acid (HOSCN) (38).

1.11 Applications for Haloperoxidases

The present commercial applications of haloperoxidase are only a tiny fraction of the established biocatalyst market. However, haloperoxidases have high potential uses for enzyme-catalysed halogenation, it can be speculated about wide range of potential commercial applications are as follows.

1.11.1 As a Synthetic Tool

Halogenation is a key step in the production of many industrial chemicals. In some cases, the end product contains halogen; in others, the halogenated chemical is an intermediate (Figure 14). For example, an enzymatic process for converting process for

converting propylene to propylene oxide via propylene halohydrin has been developed. Haloperoxidase was used to convert propylene to propylene halohydrin and then a second enzyme, halohydrin epoxidase was used to convert the halohydrin to propylene oxide and halide ion.

1.11.2 As a Pharmaceutical Agent

The antibacterial properties of hypohalous acid, haloperoxidases have been used as *in situ* antibacterial-generating agents. For example, lactoperoxidase in combination with thiocyanate ion and urea peroxide, as well as myeloperoxidase in combination with iodide ion have been formulated with powdered drug preparations to preserve the drug formulated with powdered drug prepartions to preserver the drug from bacterial decomposition (106, 107). These enzymes are possibly used as therapeutic agents if they can be delivered to the site of an infection or tumor by coupling the enzyme to a monoclonal antibody, the haloperoxidases could release their potent oxidant, hypohalous acid, and thus selectively destroy the invading agent or cell. Kelssler and Rosenbaum (108) have obtained a patent on the use of these enzymes as peroxidases in toothpastes, mouthwashes, and chewing gum as a method of rapidly killing oral bacteria to alleviate gingival disease and periodontal disease.

1.11.3 In Pollution Control

Slime growth is a major problem in processing of water from the pulp and paper mill and other industries, resulting in the fouling or plugging of pipes and filters. To overcome this problem, biocides are added to nonselective microorganisms. Hypohalous acid is one such biocide, and a paten has been issued in which a haloperoxidase, halide ion and H_2O_2 are added to the effluent (109).

1.11.4 For Analytical Diagnostics

The radioisotopes of iodide, ^{125}I and ^{131}I, and to a lesser extent the radioisotope of bromide, ^{77}Br and used to label proteins in order to study biological processes (110). Haloperoxidases provide a means by which the radioisotopes are rapidly, mildly, and stably attached to the protein molecules, yielding high specific radioactivity with retention of the protein's biological activity.

Haloperoxidase has also been used as a selective probe for measuring the level of a particular halide ion in a mixture of halide ions (111). The selective measurement of iodide ion in the presence of bromide, chloride, and fluoride ions could be applied by using other halide ion/haloperoxidase combinations.

In coupling enzyme assays, the enzymes were used to quantitate the amount of glucose in the blood by measuring the amount of glucose in the blood by measuring the amount of H_2O_2 produced in the presence of glucose oxidase (112), or in enzyme immunoassays, the enzymes were used to measure the presence of antigen/antibody complexes in which either species may be labeled with the enzyme (38).

1.12 Physicochemical Properties

The apparent molecular mass for these enzymes ranges from 42,000 Da for horseradish peroxidase (113) and chloroperoxidase (21) to 790,000 Da for the bromoperoxidase from *C. pilulifera* (114).

Several haloperoxidases contain subunits (i.e. multiple peptide chains). The bromoperoxidases from *P. capitatis* (17) and *P. aureofaciens* (25) are dimes having polypeptide chains of equal size. Thyroid peroxidase (26) and eosinophil peroxidase (36) consist of two peptide chains of unequal size. The molecular weight and subunits of some haloperoxidases are shown in Table 5.

Table 5 Apparent molecular weight and subunits of haloperoxidase glycoproteins

Enzyme	Apparent molecular weight	Subunits	Reference
Bromoperoxidase *(P. aureofaciens)*	158,000	77,000	25
Myeloperoxidase	146,000	57,000	115
		39,000	116
		11,000	
Bromoperoxidase *(P. capitatus)*	97,600	55,000	17
Thyroid peroxidase	93,000	60,000	117
		24,000	
Lactoperoxidase	85,000	-	43
Eosinophil peroxidase	71,000	58,000	37
		14,000	
Ovoperoxidase	70,000	-	29
Chloroperoxidase	42,000	-	21
Horseradish peroxidase	42,000	-	118

Most heme-containing haloperoxidases are glycoproteins. The percentage of carbohydrate present in these enzymes varies from 3% in myeloperoxidase (35) to 30% in chloroperoxidase (21). Only a few of the non-heme haloperoxidases contain sugar residues; e.g. in the chloroperoxidase from *C. inaequalis* a carbohydrate content of 9% was detected (119). Beside the percentage of various sugar monomers, little is known about the chemical nature of this carbohydrate. Carbohydrate content of some haloperoxidase are shown in Table 6.

Table 6 Carbohydrate content of haloperoxidases

Enzyme	Carbo-hydrate content (wt/wt)	Predominant sugars	Reference
Chloroperoxidase	13-17 %	Arabinose	21
(*C. Fumago*)		Glucosamine	120
Horseradis peroxidase	14-17 %	Glucosamine	118
Thyroid peroxidase	10 %	Mannose	117
		N-acetylglucosamine	
Lactoperoxidase	5-10 %	Glucosamine	121
		Galactosamine	122
Ovoperoxidase	4 %	Mannose	29
		N-acetylglucosamine	
Myeloperoxidase	3 %	Mannose, glucose	35
		N-acetylglucosamine	
Bromoperoxidase	0 %	–	17
(*P. capitatus*)			

Some structural characteristics and kinetics properties of non-heme bromoperoxidases are show in Table 7. The vanadium enzymes have comparable affinity for hydrogen peroxide, the non-heme bromoperoxidase from *S. aureofacien,* however, has a considerable lower affinity for this substrate. Furthermore, the bromoperoxidase from the lichen *X. parietina* has a much higher affinity for bromide than the algal enzymes. The specific activities of bromoperxidases from red seaweeds *C. rubrum* and *C. pilulifera* are lower than the values reported for the other enzymes.

Table 7 Structural characteristics and kinetic properties of some non-heme bromoperoxidase

Source	Molecular mass subunits (kDa)	Iso-electric point	pH optimum	K_m for H_2O_2 (M)	K_m for Br^- (M)	Reference
S. aureofaciens	32	3.5	4.3	8.0×10^{-3}	2.0×10^{-2}	40
X. parietina	65	4.5	5.5	8.7×10^{-4}	3.0×10^{-5}	20
C. rubrum	58	3.9	7.4	1.7×10^{-5}	2.0×10^{-3}	123
C. pilulifera	64	3.0	6.0	9.2×10^{-5}	1.1×10^{-2}	114
L. saccharina	64, 66	4.2	6.5	2.7×10^{-5}	1.0×10^{-3}	124
A. nodosum I	67	5.0	6.0	2.7×10^{-5}	1.5×10^{-2}	125
A. nodosum II	70	5.0	7.2	2.7×10^{-5}	6.7×10^{-3}	126

1.13 Amino Acid Composition of Haloperoxidase

The amino acid composition of many haloperoxidases have been determind. In Table 8, the amino acid composition of several non-heme haloperoxidases from different sources are shown in Table 8. The most striking general characteristic of these analysis is the predominance of the amino acidic residues Asx (Asp + Asn) and Glx (Glu + Gln) over the basic residues (arginine and lysine). The is in consistent with the low isoelectric points of these enzymes.

Although the non-heme bromoperoxidases show close agreement in amino acid composition (Table 8) nearly no immunological relationships were detected. Only the vanadium bromoperoxidase from the red seaweed *C. rubrum* was partially immunologically identical to the vanadium enzyme from *Ascophyllum* (128). Itoh et al., (129) showed that the bromoperoxidases from different *Coralline* algae are immunologically related.

Table 8 The amino acid composition of several non-heme haloperoxidase

Amino acid	Molar percentage							
	CPO[1] *P. pyrrocinia* (127)	BPO[2] *S. aureofaciens* (40)	BPO *X. parietina* (20)	BPO *C. rubrum* (123)	BPO *C. pilulifera* (114)	BPO *L. saccharina* (125)	BPO1 *A. nodosum* (126)	BPO II *A. nodosum* (126)
Asx	11.8	10.6	9.8	12.8	11.4	12.9	14.0	9.5
Thr	5.1	4.9	8.4	9.9	4.9	6.4	6.9	5.9
Ser	3.7	6.2	7.3	7.9	6.7	5.8	5.0	6.5
Glx	11.1	5.9	9.3	9.7	10.2	9.1	10.2	12.4
Pro	4.4	4.6	5.2	6.0	5.3	5.2	4.9	5.2
Gly	5.9	19.2	9.3	10.4	7.8	8.9	9.8	9.7
Ala	6.8	7.5	12.7	9.0	10.0	8.0	11.1	9.1
Val	7.1	5.8	6.1	5.8	6.5	5.5	6.2	6.2
Met	2.2	2.9	2.5	1.6	1.4	2.0	1.0	2.4
Ile	3.3	3.6	3.9	3.5	5.9	2.7	4.0	4.0
Leu	7.3	5.3	8.5	5.3	9.2	9.4	8.5	8.5
Try	4.6	2.4	3.4	6.4	2.2	3.1	1.7	3.1
Phe	5.8	5.1	5.8	2.8	5.7	3.5	5.7	7.0
Lys	5.9	4.4	2.0	2.2	4.3	3.7	1.6	1.7
His	5.1	3.3	1.3	0.5	1.4	1.7	1.2	1.0
Arg	5.7	4.3	3.0	1.8	5.1	4.7	4.0	4.0
Cys	0.3	n.d.	n.d.	n.d.	0.8	5.5	1.7	n.d.
Trp	3.6	n.d.	n.d.	n.d.	0.8	n.d.	n.d.	n.d.

1) Chloroperoxidase

2) Bromoperoxidase

n.d. = not determined

1.14 Thermostability of Bromoperoxidase

Bromoperoxidase from algae and other organisms seem to be thermostable. Heme-containing bromoperoxidases from *Penicillus capitatus, Penicillus lamourouxii* and *Rhipocephalous phoenix* were remarkably heat-stable (17). They resisted denaturation at 50-60^{O}C for 1 hour or more with no loss in activity. Vanadium-containing bromoperoxidase from *Ascophyllum nodosum* was thermostable, the enzyme activity was stable at 50^{O}C but rapid inactivated at 70 ^{O}C, i.e., 38^ of the activity remained after 1 hour. Vanadium-containing bromoperoxidase from *Xanthoria parietina* was remarkably thermostable, the enzyme activity was not affected upon inclubating at 50 ^{O}C and the enzyme activity slowly decreased at 60-80 ^{O}C (20). Bacterial non-heme bromoperoxidase from *Streptomyces aureofaciens* not containg vanadium or other metals ion : Zn (II), Fe (II), Cu (II) and Mn (II) was remarkably thermostable; i.e., the enzyme was slowly inactivated at 70 ^{O}C and the enzyme activity was not lost upon incubating at 50 ^{O}C for at least 4 hours (130).

Thermostability of the enzyme may result from the habitat of the organism, i.e., as bromoperoxidase from *Penicillus* sp. and *Rhipocephalus* sp. are shallow water species, and thus are exposed to a wide range of temperature. The enzyme from algae or organism that grows during summer or at high temperature condition contains enzyme that are remarkably thermostable.

1.15 Chemical Modification of Enzyme

Chemical modification is any transformation involving the formation or the rupture of a covalent or partially covalent bond of the enzymes under studied (131). Chemical modification has been successfully used to study the structure and function relationship of enzymes. The common approach is to evaluate the effect of a chemical modification of amino acid on the property or function of the enzyme. Modification of haloperoxidase was reported by Hori and Ikeda-Saito (132). They studied a photo-induced chemical modification of the chromophore group in myeloperoxidase. However, chemical modification of bromoperoxidase has not been reported, so it is interesting to study the effect of the chemical modification of brmoperoxidase has not been reported, so it is interesting go study the effect of the chemical modification on this enzyme.

1.16 Effect of Chemical Modification

There are a limited number of functional groups available in the side chains of amino acid residues in proteins for derivatization (133). These functional groups are easily derivatized to yield a wide variety of protein adducts as shown in Table 9.

Table 9 Amino acid side chains in proteins commonly modified (134)

Side chain	Commonly used modifications
Amino	Alkylation, acylation
Carboxyl	Esterification, amide formation
Disulfide	Reduction, oxidation
Imidazole	Oxidation, alkylation
Indole	Oxidation, alkylation
Phenolic	Acylation, electrophilic substitution
Sulfhydryl	Alkylation, oxidation
Thioether	Alkylation, oxidation
Guanidine	Condensation with dicarbonyls

The introduction of various functional groups into proteins can result in a variety of changes at the molecular level that can affect protein functionality as listed in Table 10 (135). Chemical modifications play a major role in altering the functional properties of proteins. It may be that reaction conditions alone (e.g., pH, solvent, ions, etc.) may be dominant factors in controlling the changes in the protein conformation. On the other hand, these could result from a series of interrelated changes in functional properties stemming from something as simple as change in the net surface charge of the protein.

Table 10 Summary of some consequences of chemical modifications of proteins (135)

1) Introduced reactive side chains, e.g., thiols, phosphate.
2) Altered surface charge.
A) Changed-pI prepare more negatively or positively charged proteins
B) Altered equilibrium between proteins subunits.
C) Induced more randomness in structure of protein from charge repulsions at surface.
D) Stabilized against thermal denaturation
E) Enhanced surface hydrophobicity of protein by exposing buried hydrophobic residues.
F) Increased amphiphilic behavior of proteins to more detergent-like
G) Modified proteins stabilized by intramolectular thiol-disulfide interactions.

1.17 Effect of Chemical Modification on Enzyme Property

Enzyme deactivations play an important role in biotechnological processes. The efficiency of these processes may be constrained by a rapid inactivation of the enzymes involved. Thus, considerable effort has been spent in elucidating enzyme deactivation mechanisms and in stabilizing the activity of enzymes. These studies provide fundamental physical insights into enzyme deactivation mechanisms and into the enzyme structure itself. Not only is information regarding the influence of chemical modification on enzyme activity is simultaneously presented. This is especially true since enzyme stability and activity are, in general, of opposing nature.

1.17.1 Effect of Chemical Modification on Enzymatic Activity

Chemical modification has been used to study the nature of the active site residues and to differentiated between those amino acids that participate in the catalytic act and those that are important in substrate binding. These investigations have also substantiated the existence of effector sites that control the overall reactivity of the enzyme molecule.

There are numerous structural features of proteins that influence their functionality. Many of these structural features depend upon the amino acid composition and sequence as shown in Table 11.

Table 11 Structure properties and interactions of polypeptide which influence their functionality (136)

Amino acid composition (major groups)
Amino acid sequence (segments/polypeptides)
Secondary/tertiary conformation (compact/coil)
Surface charge, hydrophobicity, polarity
Size, shape (topography)
Quaternary structures
Secondary interactions (intra-and inter-peptide)
Hydrogen bonding, ionic, van der waals, hydrophobic and electrostatic interactions
Disulfide/sulthydryl content
Environmental conditions (pH, O/R status, salts, temperature)

The enzyme catalysis that the has been studied through chemical modification includes reaction and substrate specificity, cooperativity and ionization behavior of the functional groups. Besides the use in these areas, this technique has been used to alter enzyme specificity systematically. These are many ways in which enzymatic specificity can be altered by chemical modification. It has been possible to alter the pH optima for the action of enzymes, to alter the relative reactivity of an enzyme toward different types of substrates, to change patterns of substrate inhibition and activation, to alter the type of reaction catalyzed by an enzyme. The effects of chemical modification on enzymatic specificity can be expressed through changes in K_m value for the action of the enzyme on different categories of substrates. In more radical modification, new active functional groups can be introduced in the structure of an existing enzyme and the catalytic behavior of the enzyme can be altered completely (137).

Once an enzyme molecule has been chemically altered, generally an analysis is performed to determine the efficacy with which the modified and native enzymes catalyze a particular reaction. The covalent transformation of amino acid side chains, particularly at the active site, is often manifested by dramatic changes in the specificity of the enzyme. Once this

effect has been noted, it is then necessary to correlate cause with effect, i.e., to elucidate the identity of the amino acid that has been altered. The importance of this exercise can not be over estimated since the ultimate point is the determination of those residues that play a pivotal role in enzyme specificity.

Examples of chemical modifications that affect the affect the enzyme activity are as follows.

1.17.1.1 Pepsin

Perlmann investigated the acetylation of porcine pepsin's zymogan, pepsinogen (138). N-Acetylimidazole was used to produce an acetylated pepsin that was only 40% as active toward hemoglobin as was the native species. However, the relative specific activity of the modified enzyme toward the synthetic substrate, N-acetly-DL-phenylalanyldiiodotyrosine was enhanced. Furthermore, the activity of the acetylpepsin toward the oxidized and reduced B chain of insulin was approximately equal to that of native pepsin. Since insulin is intermediate in molecular weight between the synthetic substrate and hemoglobin, Perlman concluded that acetylation of pepsin enhanced its activity toward small substrates while the activity toward large substrates was inhibited. The acetylated residue in pepsin was not identified. Herriott (139) concluded on chemical and spectral evidence that tyrosine acetylation might be responsible for the activity modulation.

1.17.1.2 Carboxypeptidase A

Carboxypeptidase A was acetylated with acetic anhydride or N-acetylimidazole resulting in modification of 2 of the 19 tyrosyl (140). Interestingly, esterase activity was almost entirely abolished. Diazotization with 5-diazo-1H-tetrazole resulted in the covalent modification of only one tyrosine residue and a concomitant 1.8-fold increase in esterase efficiency without a corresponding change in peptidase activity (141). When a great excess of this reagent was employed, a second tyrosine residue as well a histidine residue were modified. This derivatized carboxypeptidase A no longer possessed peptidase activity; however, esterase activity remained intact. Lipscomb was able to deduce the sites of chemical alteration based upon the crystal structure (142). Acetylation and diazotization resulted in the modification of tyrosine residues 198 and 248, though it was not clear which histidine residue was diazotized. Sokolovsky and

Vallec have suggested that elimination of peptidase activity corresponded to the modification of histidine (141).

1.17.1.3 Subtilisin

Vallee et al., (143) and Svendsen et al., (144) noted a increase in proteolytic activity of subtilisin Carlsberg upon nitration. Nitrated subtilisin Carlsberg hydrolyzed clupein six times more efficiently than the native enzyme. Iodination and succinylation of subtilisin resulted in a modified enzyme possessing a 6 and 7-fold enhancement in the activity, respectively.

Iodination, nitration, and succinylation of subtilisin Carlsberg serve to introduce negative charge which enhances the binding of clupein (a positively charged protein) without affecting the binding of casein of benzoyl tyrosine ethyl ester (neutral substrates). The negative charge resulted from the lowered pK_a of the iodo-or nitrotyrosine hydroxyl relative to that of the tyrosine hydroxyl, or from the presence of a free carboxyl group in succinylated tyrosine. The introduction of negative charge at certain regions in the binding site of subtilisin produces an enzyme with increased hydrolytic activity toward large positively charged substrates. Furthermore, specific bonds within these substrates are preferentially subject to hydrolysis in the presence of the modified enzyme.

1.17.1.4 Trypsin

Riordan et al., showed that acylation of trypsin with acetylimidazole under nondenatruing conditions resulted in no change in enzymatic activity toward peptide (casein) or ester (benzoylarginine ethyl ester., BAEE) substrates (145). Subsequently Trenholm et.,al (146) studies both the acetic anhydride and N-acetylimidazole-acetylated trypsin and likewise found that both hydrolyzed BAEE at rate comparable to that of the native enzyme. However, with N-α-p-toluenesulfonyl-L-arginine methyl ester (TAME) as substrate, the specific enzymatic activity of acetyltrypsin showed greater substrate activation than does the native enzyme at high TAME concentrations. Acetylation of trypsin increase the enzyme's activity toward both p-toluenesulfonylarginine amide (TAA) and benzamidine by a factor of two (146).

The enhanced activity of acetyltrypsin can be reversed by treatment with hydroxylamine and therefore Trenholm has proposed that tyrosines are the sites of modification. Chevalier et al., (147) reported that trypsin acetylated at 4 tyrosine residues possessed similar properties to the species produced by Trenholm.

1.17.1.5 Papain

Papain is probably the most extensively studied of all the cysteine proteinases. Much of the attention has centered around the functional role of the tryptophan residues. By a variety of chemical modification studies, such as N-bromosuccinimide (NBS) oxidation (148), photooxidation (149, 150), and alkylation (151-153), these tryptophans have been implicated to play an important functional role in papain's catalytic action. The NBS oxidation was originally reported to inactivate papain (154). However, later studies (155, 156) reported that papain's activity toward benzoylarginine amide derivatives was enhanced upon modification. One of the later studies (156) also noted that oxidized papain had increased peptidase activity but decreased esterase (N-benzoylarginine ethyl ester) and proteinase (casein, protamine) activities relative to the native enzyme.

1.17.1.6 Lysozyme

Imoto et al., (157) oxidized Trp-62 with ozone to form the N-formylkynurenine derivative of lysozyme. This species posesed 40% of the naïve cell lysis ability. However, it exhibits only 15% of the native enzyme's activity toward glycol chitin. The N-formyl kynurenine lysozyme was then deformylated by acid hydrolysis to provide kynurenine-62 lysozyme. Deformylation resulted in a species with increased cell lytic activity (80% of native enzyme) and glycol chitin cleavage activity (30% of native enzyme). These authors suggested that the relatively high catalytic efficacy of Kyn-62 lysozyme may be due to the ability of kynurenine to form a coplanar structure similar to an indole ring.

Davies and Neuberger (158) specifically inhibited lysozyme's lytic activity toward *M. lysodeikticus* by acetylating the six lysine residues of the enzyme. Although this physiologically important reaction was inhibited, the lytic activity toward the tetramer of N-acetylglucosamine [$(NAG)_4$] was unaltered. These two activities were differentially affected with other reagents as well. For example, the modification of seven of the eleven arginine residues with butanedione had a similar effect on the catalytic activity.

In modification of lysine residues by ethyl acetamidate which introduces an appendage of similar size to the acetyl group without affecting surface charge caused the hydrolytic activity of this derivatized enzyme remains unaffected toward $(NAG)_4$. In contrast, the

cell lysis activity is no longer inhibited, and, in fact, the activity is slightly increased relative to that of the native enzyme.

It has been shown that conversion of charged residues to uncharged derivatives selectively decreases the lytic activity of lysozyme toward *M. lysodeikticus,* whereas the chemical modificaition of the hydrophobic binding site disrupts the hydrolytic activity of lysozyme toward only small neutral substrates.

1.17.1.7 Fructose-1,6-Biphosphatase

Fructose 1,6-bisphosphatase was activated by the modification of specific cysteine residues. These enzyme derivatives include mixed disulfides with mercaptoethanol. Ethylmercaptan, N, N-diacetylcystamine, cystamine (159), CoA, and acyl carrier protein (160, 161). Modification with homocystine (162), Ellman's reagent (159), iodoacetamide, fluorodinitrobenzene (163), and p-hydroxymercuribenzoate (164) also enhanced enzymatic activity. Activation ranged from 1.8-fold with p-hydroxymercuribenzoate to more than 8-fold with homocystine. Interestingly, the inhibition response to AMP of fructose-1,6-bisphosphatase treated with p-chloromercuribenzoate (165) or cystamine (162) was greater than that of the native enzyme. Clearly, these studies indicate that one or more free cysteine residues may play an important role in fructose-1, 6-bisphophatase activation.

1.17.2 Effect of Chemical Modification on Enzyme Stability

Attempts have been made to determine the "principles of enzyme stability". The thermal stability of enzymes may be changed intrinsically or extrinsically. Enzyme stability may be changed intrinsically by alteration of single amino acid. Extrinsically, enzyme stability may be changed by the addition of suitable stabilizing effectors (cations, cross-links, peptides, ete.). One method of promoting stabilization is to constrain conformational fluctuations (166). This suppresses the unfolding of the enzyme and denaturation transitions. This may be brought about by multipoint attachment to matrix (167). Another approach is based on the effect of amino acid substitutions on enzyme stability. Argos et al., (168) have compared amino acid sequences for thermophilic and mesophilic molecules of ferredoxin, glyceraldelyde-3-phosphate dehydrogenase and lactate dehydrogenase. They show that Gly, Ser, Lys, and Asp residues in mesophiles are generally substituted, respectively; by Ala, Ala, Thr, Arg, and Glu residues in thermophiles.

They suggest that thermal stability can be achieved by the addition of many small changes throughout the molecule without substantially changing the backbone conformation.

Though these studies provide significant physical insights into enzyme stabilization and into the enzyme structure itself, they do not emphasize, or even indicate, the influence of modification on the specific activity of these enzymes. Besides, some modifications lead to enhanced stabilization, but in some cases is a decreased stabilization.

Enzymes may be stabilized, among other techniques, i.e., immobilization, chemical modification and by grafting to polysaccharides and synthetic polymers (169). The desired properties of the enzyme are usually to be stable to heat, to organic solvent and extreme pH. The chemical modification of enzymes leading to enhanced stability includes monofunctionally substituted proteins, reticulation by glutaraldehyde and other bifunctional agents and grafting to polysaccharides and synthetic polymers. The introduction of crosslinks leads to a modification of the tertiary structure and to a general stabilization of the native conformation. The sources of enhanced stability include hydrogen bonding salt bridges and hydrophobic interactions. All of these may contribute towards a subtle blending of interaction forces. The thermostability may be promoted by even small adjustments of these forces.

It has been observed that enzymes immobilized onto solid supports frequently show gratlyenhanced stabilities when compared with unmodified controls (169-172). Investigation of these findings has led to the conclusion that this enhanced stability is conferred by multipoint attachments rather than single-point one. An enzyme immobilized at multiple sites is thought to have a considerably reduced conformational flexibility (167, 170, 179) and while still quite capable of movement required for efficient catalysis, gross distortions of the enzyme structure that would normally cause denaturation are prevented by the covalent attachments to the inflexible solid support. Such increased stability is noticed both in cases of covalent attachment to solid support or in cases of microencapsulation or co-polymerization. It has also been encountered in enzymes subjected to dynamic immobilization (172). The latter involves the co-precipitation of the enzyme of interest with a biological polymer onto a membrane surface. Both this process and true co-polymerization (i.e., the entrapment of enzyme molecule by physical constraint. Different bifunctions have been used to stabilize enzymes ranging from diimidoesters (reacting with amino groups) and dithiols (reacting with activated, newly introduced-SH groups) and dithiols (reacting with activated, newly introduced-SH groups) to dicarboxylic, acids and diamines (previously activated with carbodiimide in the case of dicarbosylic acids using

carbodiimide-activated enzyme in the case of diaminces) (174, 179, 189). Diisocyanate cross-linking has also been reported (189). The stabilizations in these examples are achieved by cross-linking different parts of the enzyme molecule together in the active conformation. The braces provided by the added bifunctional prevent critical unfolding of the molecule. Molecular rigidification is achieved by cross-linking i.e. by the reaction of both functional groups of the bifunctional reagent with the enzyme backbone. This phenomenon must be distinguished from serendipitous stabilization achieved by single-point modification (175, 189).

Chemical modification of aminoacid residues of proteins by other than bifunctional reagents has proved useful in protein stabilization. A variety of reagents has been used to enhance enzyme stability by modifying protein functional groups, especially the free amino groups of notablylabile lysine residues. Such methods include reductive alkylation of amino groups by glyoxylic acid and sodium cyanoborohydride (181), amino group acetylation or guanidianation (182, 183) where the former neutralizes the positive charge-and succinic anhydride addition to amino groups resulting incharge reversal (182). Acetamidination is a mild modification procedure selective for lysine amino groups which preserves charge (179).

Chemical modifications could lead to artifical hydrophilization of the surface area of protein globules results in protein stabilization (186). The researchers have studied two distinct enzymes to verify the hydrophilization approach. The surface tyrosines of trypsin were converted to aminotyrosines resulting in enhanced stability against irreversible therminactivation (up to 100 times more stable than the native trypsin). Alpha-chymotrypsin was hydrophilized by acylating its available amino groups with anhydrides of aromatic carboxylic acids. After modification the thermostability of both enzymes was practically equal to that of proteases from thermophilic bacteria, among the most stable enzymes known.

Another form of chemical modification that has yielded surprising spin-off advantages in terms of stabilization is the addition of PEG (polyethyleneglycol) to enzyme molecules. This was originally carried out with a view to using lipase and other enzymes in organic solvents (187). It was reasoned that addition of the amphiphilic PEG would allow the enzyme molecule to be placed in a hydrophobic environment without gross unfolding and loss of activity. While this much was indeed achieved, it was also noticed that the PEG-modified lipase was considerably more stable in an aqueous solution than was the native form (187). This extra stability could be due to steric hindrance of protein unfolding by the covalentlylinked PEG. In other words, if the protein is prevented from unfolding by the bulk of the attached PEG molecule

it should become resistant to denaturation or inactivation, since unfolding of the protein is in a very real sense the basis of activity loss (190).

Proteins coupled to carbohydrates and glycoenzymes are known to be more stable than those free of carbohydrates (171, 188, 191). The rationale for this effect is that of altered enzyme-solvent interactions resulting in a tightening of chain folding and progressive exclusion of water molecules from the (relatively) hydrophobic core of the enzyme, to which water had been previously able to gain access. Such a hypothesis is suggested by Mozhaez et al., (186) that exclusion of water from the core of the enzyme globule results in a more compact hydrophobic nucleus with improved stability. It is likely that hydrophobic interactions are of overall importance in the maintenance of native protein conformation (186, 192, 193). Note also that Kellis et al., (193) have suggested the stabilization of proteins by filling internal holes with hydrophobic substitutions. Enzyme-carbohydrate couplings may also play a role in reduction of autolysis (171). Types of chemical modification used to stabilize enzymes are shown in Table 12.

It is necessary to quantify the influence of chemical modification on both the specific activity and the stability of enzymes. It would be of considerable assistance to have access to guiding principles and a predictive approach to tailor-make and to judiciously balance enzyme stabilization and enzyme specific activity. Examples do exist in the literature where both the initial specific activity and the stability of the enzyme are presented. For example, the cross-linking of exopencillinase by covalent attachment of bifunctional reagents to non-essential amino acid residues yields an enzyme with lower catalytic activity but enhanced stability (194). In addition, the methyl acetimidate modification of 17 from 24 lysine residues in pig heart lactate dehydrogenase yields an enzyme derivative with enhanced stability toward heat and alkaline denaturation (185). Other examples involving a kinetic study which include the compensation-like effect or trade-off that has been observed by analyzing enzyme deactivations by a series-type mechanism (195-197). A kinetic expression for loss of catalytic function with protein modification has been suggested (198). This expression relates the fractional enzyme activity with the relative concentration of groups (which are essential for activity at the site) modified. A presentation of enzyme stability data together with specific activity data would provide another reasonable avenue of understanding enzymes and enzyme deactivation processes.

Table 12 Types of chemical modification used to stabilize enzyme

Modification	Reference
Multiple-site immobilization	167, 171, 173
Dynamic immobilization*	172
Co-polymerization	170
Use of bifunctionals :	
Diimidoesters	174
Dithiols	175, 176
Dicarboxylic acids + carbodiimides	177
Diamines + carbodiimides	178, 179
Diisocyanates	180
Functional group modification :	
Glyoxylation of amino groups	181
Acetylation of amino groups	182
Guanidination of amino groups	183
Succinylation of amino groups	184
Acetaminidation of lysine-amino groups	185
Hydrophilization of surface groups	186
Enzyme-PEG couplings	187
Enzyme-carbohydrate couplings	188

* Co-precipitation of enzyme with biological polymer onto membrane surface

1.18 Iodoacetamide and Iodoacetic Acid

Iodoacetamide and iodoacetic acid have been used extensively in protein alkylations. The reaction are essentially irreversible, and not highly specific most commonly, initial modification is observed on cysteine. Alkylation of histidine residues with iodoacetamide and iodoacetic acid have been widely employed. The reaction may be rendered relatively specific for histidine by suitable choice of pH (246, 247). Reaction has been observed with ε-amino (248-250), phenolic hydroxyl groups, methionine residues and rarely carboxyl groups as well (251-254), however, the reaction is pH dependent and more slowly than with sulfhydryl group. Increased rate of reaction at high pH values (pH9) indicates that reaction is probably with RS^{-}. In

spite of the lack of high selectivity, the stability of the carboxamidomethyl derivatives and the ease which they can be identified and quantitated makes this a very useful modification (255, 256).

1.19 Aims of Study

Enzymes are crucial to many of the products and processes encountered throughout biotechnology. Their uses range from traditional or long-practice application. However, the problem of the long-term stability of the enzymes used in these processes and applications places an important limitation on their use.

Bromoperoxidase is one of the enzyme that has high potency to be used in many fields because it can react with many substrates to produce biotechnology halometabolite products that may be important in pharmaceutical and industrial chemistry field or produce hypophalous acid that is toxic to microorganisms. However the study for improvement of the enzyme activity and enzyme property that may be useful for development of the enzyme to be used in these fields had not been reported. In addition, the catalytic reaction of this enzyme although has been studied intensively however, it is not well understood. Chemical modification of proteins may be used to study the enzyme property, both at structural elucidation and at alteration of catalytic characteristics. In the study, the influence of some chemical modification has been studied to give some details of the enzyme properties, as well as enzyme catalytic activity and stability.

In this study, some chemical modifying reagents were used for modification of bromoperoxidase from Thai seaweed, *Polycarvernosa* sp. and it was found that iodoacetamide improved the property of the enzyme by enhacement of thermostability and activity. The aims of this study are :

1. To study the effect of some chemical modifying reagents on bromoperoxidase.
2. To develop the optimal condition for modification of bromoperoxidase by iodoacetamide for enhancement of thermostability and activity.
3. To study the thermostability and kinetic properties of the iodoacetamide-modified bromoperoxidase.
4. To determine the modified amino acid residues that may participate in the enhancemet of thermostability and activity of bromoperoxidase.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

The chemicals and reagents used in the experiments are listed in the Table 18. Most chemicals are analytical grade.

Chemicals and reagents used in the experiments

Name of Chemical	M.W.	Company
Acetonitrile HPLC grade	41.05	Riedel-de Haen
N-Acetylimidazole	110.12	Sigma
Acrylamide	71.10	Sigma
Amino acid standard solution	-	Sigma
Ammonium sulphate	132.14	Carlo Erba
Ammonium persulphate	228.70	May & Baker
Boric acid	61.83	Baker
Bovine serum albumin	67000	Sigma
Bromophenol bule	670.00	Sigma
N-Bromosuccinimide	177.99	Sigma
Citric acid	210.00	BHD
Coomassie brilliant blue G-250	854.04	Amresca
Coomassie brilliant blue R-250	859.00	Serve
Copper sulphate pentahydrate	249.68	Fluka
1,2-Cyclohexanedione	112.10	Sigma
L-Cysteic acid	169.20	Sigma
DEAE cellulose, pre-swollen (DE52)	-	Whatman
Diethyl barbituric acid	184.02	Fisher
Diethylpyrocarbonate	162.10	Sigma
Dipotassium hydrogen phosphate	174.18	Fluka

Chemicals and reagents used in the experiments (continued)

Name of Chemical	M.W.	Company
Disodium hydrogen phosphate	141.96	Merck
5,5′-Dithiobis (2-nitrobenzoic acid)	396.35	Sigma
DNP-lysine	366.77	BDH
Ethanol (absolute)	46.07	Riedel-de-Haen
1-Ethyl1-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	191.70	Sigma
Ethylene diamine tetraacetate (disodium salt) (EDTA)	336.20	Sigma
Formaldehyde	30.03	Merck
Formic acid	46.03	Carlo Erba
Gel bond	-	Sigma
Glycerol	9210	Carlo Erba
Glycine	75.07	Sigma
Gracial acetic acid	60.05	Merck
Hydrochloric acid	36.46	Carlo Erba
Hydrogen peroxide	34.02	Chemikit
Hydrogen peroxide 35%	34.02	Riedel-de Haen
2-Hydroxy-5-nitrobenzyl bromide	232.00	Sigma
Iodoacetamide	184.90	Sigma
Iodoacetic acid	171.90	Sigma
N, N-Methylene bisacrylamide	154.20	Sigma
2-Mercaptoethanol	78.13	
Methanol HPLC grade	32.00	Baker
Moleccular weight marker, Dalton VII	-	Sigma
Monochlorodimedone (MCD)	174.60	Sigma
Nitrogen gas	28.00	Thai Industrial Gas
O-Nitrophenylsulfenyl chloride	189.60	Sigma
Phramalyte pH 3-10	-	Sigma
Phenol red sodium salt	376.40	Sigma

Chemicals and reagents used in the experiments (continued)

Name of Chemical	M.W.	Company
Phenylisothiocyanate (PITC)	135.80	Pierce
Phosphoric acid	98.00	Carlo Erba
Pico-Tag diluent	-	Millipore
Potassium bromide	119.01	Merck
Potassium cyanide	65.12	Carlo Erba
Potassium dihydrogen phosphate	136.09	Riedel-de Haen
Potassium dichromate	294.91	Merck
Silver nitrate	169.87	Merck
Sodium acetate trihydrate	136.08	Merck
Sodium azide	65.01	Fluka
Sodium bicarbonate	84.01	Riedel-de Haen
Sodium carbonate	105.99	Fluka
Sodium chloride	58.44	Carlo Erba
Sodium dihydrogen phosphate dehydrate	156.01	Riedel-de Haen
Sodium dodecyl sulphate (SDS)	225.40	Sigma
Sodium hydroxide	40.00	Merck
D-Sorbitol	182.17	Fisher
N,N, N′, N′,-tetraethylen diamine (TEMED)	116.20	Eastman
Trichloroacetic acid	163.39	Carlo Erba
Triethylamine	101.19	Aldrich
2,4,6 Trinitrobenzenesulfonic acid	121.11	Sigma
Tris (hydroxymethyl) aminomethane HCl	181.90	Sigma

Gel filtration calibration kit for molecular weight determination from Pharmacia consisted of blue dextran (2,000,000), thyroglobulin (669,000), aldolase (158,000), boving serum albumin (67,000) and ovalbumin (45,000).

Isoelectric focusing standard kit from Sigma consisted of trypsinogen (pI-9.30), lentil lectin-basic band (pI-8.65), lentil lectin-middle band (pI-8.45), lentil lectin-acidic band (pI-8.15), myoglobin-basic band (pI-7.35), myoglobin-acidic band (pI-6.85), human carbonic

anhydrase B (pI-6.55), bovine carbonic anhydrase B (pI-5.85), β-lactoglobulin A (pI-5.20), soybean trypsin inhibitor (pI-4.55) and amyloglucosidase (pI-3.50).

2.1.2 Instruments

Conductance meter YSI model 35 (YSI, U.S.A.).

Electrophoresis set, mighty small gel caster (Hoeffer, U.S.A.).

Fast protein liquid chromatography (Pharmacia LKB., Sweden) with MonoQ 5/5, Superose12 16/50 columns.

Fraction collector model 7000 (LKB, Sweden).

Gel dryer (Bio-Rad, U.S.A.) with vacuum pump.

Hettich bench top centrifuge.

HPLC with UV 486 Turnable absorbance detector, Waters intelligen sample processor Waters 712 WISP, Waters temperature control module and Pico-Tage column 15 cm (Waters Division of Millipore, U.S.A.).

Micro Pro-dicon negative pressure microprotein dialysis/concentrator (Bio-Molecular Dynamic, U.S.A.).

Peristaltic pump model 2232 (LKB, Sweden).

pH meter model 145 (Corning, England).

Pico-Tag workstation (Waters Division of Millipore, U.S.A.) with Vacuum pump.

Power supply (Biohad, U.S.A.).

Sartoriur analytical banlance (Sartorium Werke, Gottingen Germany).

Speed Vac Plus SC110 (Savant, U.S.A.) with vacumme pump.

Speedfuge HSC 10K microcentrifuge (Sacant, U.S.A.).

Shimadzu UV visible recording spectrophotometer UV-160 with Circulating water (Haake FS, Germany) or Temperature control TCC controller (Shimadzu, Japan).

Shimadzu UV visible recording spectrophotometer UV-2100 (Shimadzu, Japan).

Mini-IEF (Bio-Rad, U.S.A.)

Sorvall superspeed refrigerated centrifuge model RC5 (Sorvall, U.S.A.).

Sorvall superspeed refrigerated centrifuge model RC28 (Dupont, U.S.A.).

Teflon hand homogenizer (Thomas, U.S.A.) with Sargent cone drive.

Vortex J.R. Mixer (Scientific Industries Inc., U.S.A.).

Water bath model SW1 (Julabo, West Germany).

2.2 Methods

2.2.1 Collection of Seaweed

Thai red seaweed, *Polycarvernosa* sp. was collected from the beach of Ban Lam Sok in Trad province. The seaweed was washed with tap water to remove sand and other contaminants, then washed with deionized water and stored frozen at -20°C before use.

2.2.2 Enzyme Assay

Brominating activity was measured spectrophotometrically (15) with 0.2 mM monochlorodimedone (MCD), 20 mM KBr, 2mM H_2O_2 and a suitable amount of enzyme in 30 mM potassium phosphate buffer (KPB) pH 5.5 for BPO1 or pH 7.0 for BPO2. The assay was started with enzyme and absorbance changes were measured against the substrate reagent blank. One unit (U) of bromoperoxidase activity is defined as the amount catalyzing the formation of 1 μmol bromochlorodimedon per minute. The enzyme activity was calculated as the following equation.

$$\text{Bromoperoxidase activity (unit/ml)} = \frac{\Delta A_{290}/\text{min} \times V}{\varepsilon \times v}$$

$\Delta A_{290}/\text{min}$ = the decrease in MCD absorbance at 290 nm per min

V = total volume of reaction mixture (ml)

ε = molar extinction coefficient of MCD at 25°C (2.16×10^4 M^{-1} cm^{-1})

v = volume of enzyme solution used (ml)

2.2.3 Purification of Bromoperoxidase from Seaweed

2.2.3.1 Crude extract

About on kilogram of frozen seaweed was thawed, washed with distilled water. The seaweed was added with 1,000 ml lit 20 mM Tris-HCl buffer pH 7.6 and kept at 4°C overnight. The seaweed was cut into small pieces and homogenates were filtered through a cheese cloth. Cell debris was removed by centrifugation at 8,000 x g for 15 min, the supernates were pooled together and kept at 4°C.

2.2.3.2 Ammonium Sulphate Precipitation

Ammonium sulphate was added to 60% saturation to the crude extract while the mixture was stirred at 0°C. After centrifugation at 12,100 x g for 30 min the precipitate was collected, redissolved in 20 mM Tris-HCl buffer pH 7.6 by using Potter homogenizer. The protein suspension was centrifuged at 12,100 x g for 30 min at 4°C, the pellet was discarded and the supernate was dialysed against the same buffer for 16 hours at 4°C.

2.2.3.3 DE52 Column Charomatography

DEAE-cellulose DE52 (Whatman DE-52) was preswollen in 20 mM Tris-HCl buffer pH 7.6, the DEAE-cellulose was allowed to settle and the fine particles in the supernate was removed by decantation. DEAE-cellulose was packed into the column (3.0 × 50 cm). The column was equilibrated with 20 mM Tris-HCl buffer pH 7.6 at the flow rate of 0.5 ml/min at 4°C. The dialysed sample of ammonium sulphate precipitate was clarified by centrifugation to remove insoluble materials and the resulting supernates were applied on to the column. The column was washed with the buffer until the conductivity of the effluent did not change and proteins were eluted with a gradient of 0-1.0 M NaCl in the same buffer (1,600 ml). Fractions of 4 ml were collected and assayed for protein by measuring the absorbancy at 280 nm and the brominating activity was assayed as described in section 2.2.2, and the concentration of NaCl was determined by conductivity weter (Beckman model RC-16C). Fractions containing bromoperoxidase activity were pooled separately as BPO1 and BPO2. The enzymes were dialysed and concentrated with Pro-dicon concentrator with 10,000 M.W. cutoff bag aginst 20 mM Tris-HCl buffer pH 7.6 at 4°C.

2.2.3.4 MonoQ Column Chromatography

The dialysed and concentrated enzymes, BPO1 and BPO2, from DE52 column were filtered through a 0.22 µm sterile filter. The MONOQ HR 5/5 column of Pharmacia FPLC system was equilibrated with 20 mM Tris-HCl buffer pH 7.6 at the flow rate of 1 ml/min. The enzyme sample was applied onto a MonoQ column, proteins were elucted with a linear gradient of 0-1.0 M NaCl in the same buffer. Fractions of 0.5 ml were collected and assayed for brominating activity. Fractions containing bromoperoxidase activity were pooled, dialysed and concentrated with Pro-dicon concentrator with 10,000 M.W. cut off.

2.2.4 Molecular Weight Determination by Superose 12 Column Chromatography

The molecular weight of BPO1 and BPO2 were estimated by molecular-sieve chromatography with a Superose 12 column of Pharmacia FPLC system. The column was equilibrated with 20 mM Tris-HCl buffer pH 7.6 at the flow rate of 0.5 ml/min. The column was calibrated with the standard proteins; thyroglobulin (M.W. = 699,000), ferritin (M.W. = 440,000), aldolase (M.W. = 158,000) bovine serum albumin (M.W. = 67,000), ovalbumin (M.W. = 43,000). The blue dextran (M.W. = 2,000,000) and DNP-lysine (M.W. = 366.70) were used to determine the void volume and total volume of the column, respectively.

2.2.5 Protein Determination Using Coomassie Brilliant Blue G-250

Protein determination was performed according to the method of Bradford (257). Three parts of solution A (85 mg of Coomassie Brilliant Blue G-250 in methanol : H_2O = 50 : 500 v/v) were mixed with two part of 2.17 M of phosphoric acid. 100 µg of bovine serum albumin.

2.2.6 Absorption Spectra Determination

BPO1 (14 µg/ml) and BPO2 (0.15 mg/ml) in 20 mM KPB pH 7.0 were measured at 25°C for absorption spectra in cuvettes of 1-cm path length with a Shimadzu UV visible recording spectrophotometer UV-160.

2.2.7 Non-denaturing Gel Electrophoresis

Non-denaturing gel electrophoresis was performed according to the modified method of Davis (257). The slab gel (15 × 10 × 0.1 cm) with 12% gel for separating gel and 3% for stacking gel.

2.2.8 Modification of BPO1 and BPO2 by Iodoacetamide

Reaction mixture consisted of BPO1 (14 µg) or BPO2 (116 µg) was reacted with 0.25 – 250 mM iodoacetamide in 20 mM KhPB pH 7.0 in the total volume of 1 ml. The control reaction mixture contained no iodoacetamide. The reaction mixtures were incubated at 35°C for 16-20 hours according to the method of Holeysovsky and Lazdunski (258). After incubation, the reaction mixture was dialysed to remove unreated iodoacetamide against the same buffer at 4°C

for 16 hours and enzyme activity was assayed an presented as percentage of relative activity compared to control at zero time.

2.2.9 Stability of BPO1, BPO2, Modified BPO1 and Modified BPO2 by Various Modified Reagents at 37°C

The native BPO1, BPO2, modified BPO1 and modified BPO2 by various modifying reagents at appropreciate concentrations were incubated at 37 °C in 20 mM KPB at different pH. The enzyme activity was assayed at various time intervals and presented as percentage of relative activity compared to enzyme activity at zero time.

The stability profile at 37 °C of the modified enzyme was compared to the native enzyme.

1.2.10 Effect of pH on Modification of BPO1 by Iodoacetamide

BPO1 (14 µg) was reacted with 50 mM iodoacetamide in 20 mM KPB pH 5.0, 7.0, 8.5 in the total volume of 1 ml. The control reaction mixture contained no iodoacetamide. The reaction mixture was incubated at 35 °C for 0-24 hours. After incubation at various intervals of 3, 6, 12, 18, 24 hours; the reaction mixture was dialysed against the same buffer at 4 °C for 16 hours and enzyme activity was assayed and presented as percentage of relation activity compared to control at zero time.

2.2.11 Effect of Temperature on Modification of BPO1 by Iodoacetamide

BPO1 (14 µg) was reacted with 50 mM iodoacetamide in 20 mM KPB pH 7.0 in the total volume of 1 ml. The control reaction mixture contained no iodoacetamide. The reaction mixture was incubated at 25, 35 and 45 °C for 0-24 hours. After incubation at various time intervals, the reaction mixture was dialysed against the same at 4 °C for 16 hours and the enzyme activity was assayed and presented as percentage of relative activity compared to control at zero time.

2.2.12 Iodoacetamide-Modified BPO1

BPO1 (14 µg/ml) was reacted with 50 mM iodoacetamide in 20 mM KPB pH 7.0 at 35 °C for 18 hours. After incubation, the enzyme was dialysed against the same buffer to

remove unreacted iodoacetamide at 4 °C for 16 hours. This modified enzyme was used for further studies.

2.2.13 Effect of Protease Inhibitor on BPO1 and Iodoacetamide-modified BPO1

The native BPO1 (14 µg/ml) and iodoacetamide-modified BPO1 (14 µg/ml) were reacted with 1 mM PMSF, apotinin (70 µg/ml) and leupeptin (50 µg/ml) in 20 mM KPB pH 7.0. The reaction mixtures were incubated at 25 °C for 12 hours, then the enzyme activities were assayed and presented as percentage of relative activity compared to the enzymes that contained no protease inhibitor.

2.2.14 Effect of Temperature on BPO1 and Iodoacetamide-Modified BPO1

The native BPO1 and modified BPO1 by iodoacetamide as described in the Methods section 2.2.24 were incubated in 20 mM KPB pH 7.0 at various temperatures from 30 to 80 °C for 10 min, then the enzymes were cooled at 4 °C in an ice bath. The enzyme activity was assayed and presented as percentage of relative activity compared to the enzymes at zero time.

2.2.15 Stability of BPO1 and Iodoacetamide-Modified BPO1 at 25 °C and 45 °C pH 7.0

The native BPO1 and iodoacetamide modified BPO1 as described in the Methods, section 2.2.24 were incubated at 25 and 45 °C in 20 mM KPB pH 7.0. The enzyme activity was assayed at various time intervals of and presented as percentage of relative activity compared to the enzyme activity at zero time.

The stability profile of the modified enzyme was compared to the native enzyme.

2.2.16 Stability of BPO1 and Iodoacetamide-Modified BPO1 at 25 °C and 4 °C, pH 5.5

The native BPO1 and iodoacetamide modified BPO1 as described in the Methods, section 2.2.24 were incubated in 20 mM KPB pH 5.5 at 25 °C and 4 °C. At various

time intervals the enzyme activity was assayed and presented as percentage of relative activity compared to the enzyme at zero time.

2.2.17 Effect of NaN_3 and KCN on BPO1 and Iodoacetamide-Modified BPO1

The native BPO1 and iodoacetamide-modified BPO1 as described in the Methods, section 2.2.24 were reacted with 1 mg/ml of Na_3N and 1 mg/ml of KCN in 20 mM KPB pH 7.0. The reaction mixtures was incubated at 25 °C for 12 hour, the dialysed against the same buffer at 4 °C for 16 hour and measured for the enzyme activity using MCD bromination assay as described in the Methods, section 2.2.2. The enzyme activity was presented as percentage of relative activity with the native enzyme in the absence of NaN_3 and KCN.

2.2.18 pH Activity of BPO1 and Iodoacetamide-Modified BPO1

The native BPO1 and iodoacetamide modified BPO1 as described in the Method section 2.2.24 were assayed for the enzyme activity in 0.1 M KPB at various pH from 3.5 to 8.5 at 25 °C. The enzyme activity was presented as percentage of relative activity compared to the highest enzyme activity.

The pH activity profile of the modified enzyme was compared to the native enzyme.

2.2.19 pH Activity of BPO2

The native BPO2 was assayed for enzyme activity in 0.1 M KPB at various pH from 4.0-10.0 at 25 °C. The enzyme activity was presented as percentage of relative activity compared to the highest enzyme activity.

2.2.20 pH Stability of BPO1 and Iodoacetamide-Modified BPO1

The native BPO1 and iodacetamide modified BPO1 as described in the Methods, section 2.2.24 were incubated in the universal buffer pH 2.0-11.00 at 25 °C for 15 min. the enzyme activities in different pH were assayed and presented as percentage of relative activity compared to the highest enzyme activity. The pH stability profile of the modified enzyme was compared to the native enzyme.

2.2.21 Effect of V_2O_5 on Modification of BPO1 by Iodoacetamide

BPO1 (14 µg/ml) in 10 mM Tris-HCl buffer pH 8.0 was prepared for five reactin mixtures as the following. The first reaction mixture used as the control consisted of BPO1 in the buffer and kept at 4 °C. The second reaction mixture consisted of BPO1 and 0.3 mM V_2O_5 incubated at 4 °C for 24 hours, then dialysed against the same buffer to remove the excess V_2O_5 at 4 °C for 16 hours. The third reaction mixture consisted of BPO1 added with 50 mM iodoacetamide. The reaction mixture was incubated at 35 °C for 18 hours and dialysed against the same buffer at 4 °C for 16 hours. The fourth reaction mixture consisted of BPO1 added with 0.3 mM V_2O_5 and prepared as the second reaction mixture, then the enzyme was added with 50 mM iodoacetamide and incubated at 35 °C for 18 hours, then dialysed a gainst the same buffer at 4 °C for 16 hours. The fifth reaction mixture consisted of BPO1, 0.3 mM V_2O_5 and 50 mM iodoacetamide. The reaction mixture was incubated at 35 °C for 18 hours and dialysed against the same buffer at 4 °C for 16 hours. The fourth reaction mixture consisted of BPO1 added with 0.3 mM V_2O_5 and prepared as the second reaction mixture, then the enzyme was added with 50 mM iodoacetamide and incubated at 35°C for 18 hours, then dialysed a gainst the same buffer at 4 °C for 16 hours. The fifth reaction mixture consisted of BPO1, 0.3 mM V_2O_5 and 50 mM iodoacetamide. The reaction mixture was incubated at 35°C for 18 hours and dialysed against the same buffer at 4 °C for 16 hours.

The enzyme activities of the reaction mixtures were assayed and presented as percentage of relative activity compared to control in the first reaction mixture.

2.2.22 Analytical Isoelectric Focusing of Iodoacetamide-Modified BPO1.1 and Iodoacetamide-Modified BPO1.2

2.2.22.1 Separation of BPO1.1 and BPO1.2 by Electrophoresis

BPO1 was separated by electrophoresis. The enzyme was applied onto the non-denaturing slab gel electrophoresis. After running, the gel was cut for long section with the cross section about 0.5 cm, then the gel was stained for activity using phenol red as substrate. After activity staining, two blue bands appeared, the band at R_f about 0.16-0.22 was BPO1.1 and the other was BPO1.2, R_f about 0.40-0.44. The bands of BPO1.1 and BPO1.2 on the gel were cut separately into the small pieces and eluted for the protein by electroeluter, and then concentrated by using speed-vac.

CHAPTER III

RESULTS

3.1 Purification of BPO1 and BPO2 from Seaweed

Bromoperoxidase from red seaweed, *Polycarvernosa* sp. (Figure 13) was extracted and precipitated by 0-60% ammonium sulphate saturation. The enzyme was purified by DEAE cellulose column chromatography and FPLC by using MonoQ and Superose12 column. The results of enzyme purification are given in Table 13. The crude extracts with pink colour had specific activity 4.80 mU/mg. 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 the specific activity was slightly increase. In DEAE-cellulose column chromatography, the bromoperoxidase activities were seperated into two peaks (Figure 14). The first peak (BPO1) was eluted at 0.16-0.32 M NaCl and the second peak (BPO2) was eluted at 0.35-0.55 M NaCl. The enzyme activities of both BPO1 and BPO2 were about 5.6 times more enzyme activity than BPO1.

BPO1 and BPO2 were further purified by using FPLC. In the MonoQ step BPO1 was eluted at 0.55-0.70 M NaCl (Figure 15), about 74% of proteins were eliminated and the specific activity increased about 2.6-fold, the % yield

Figure 13 Red seaweed, *Polycarvernosa* sp. from Lhaem Sok beach, Trad province.

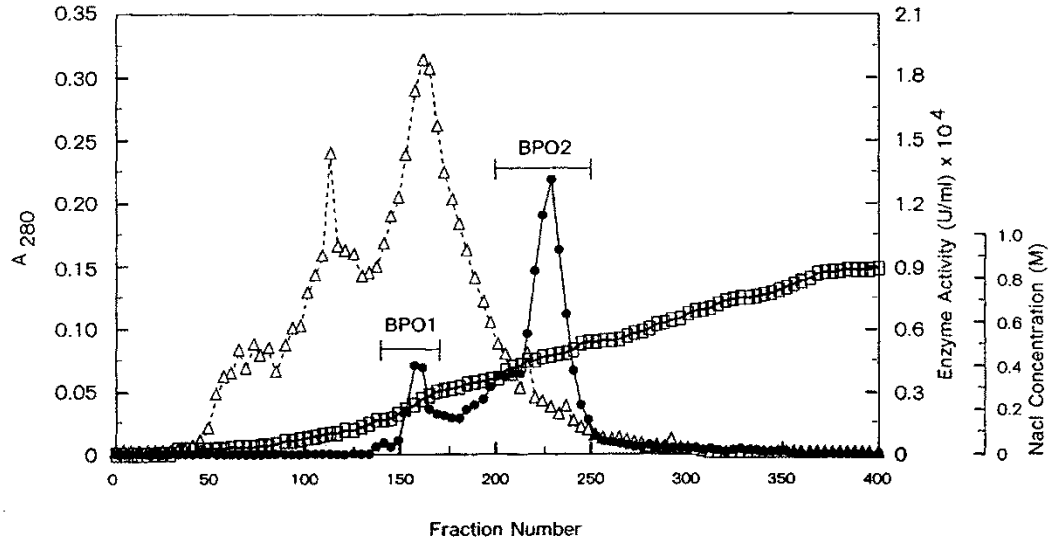


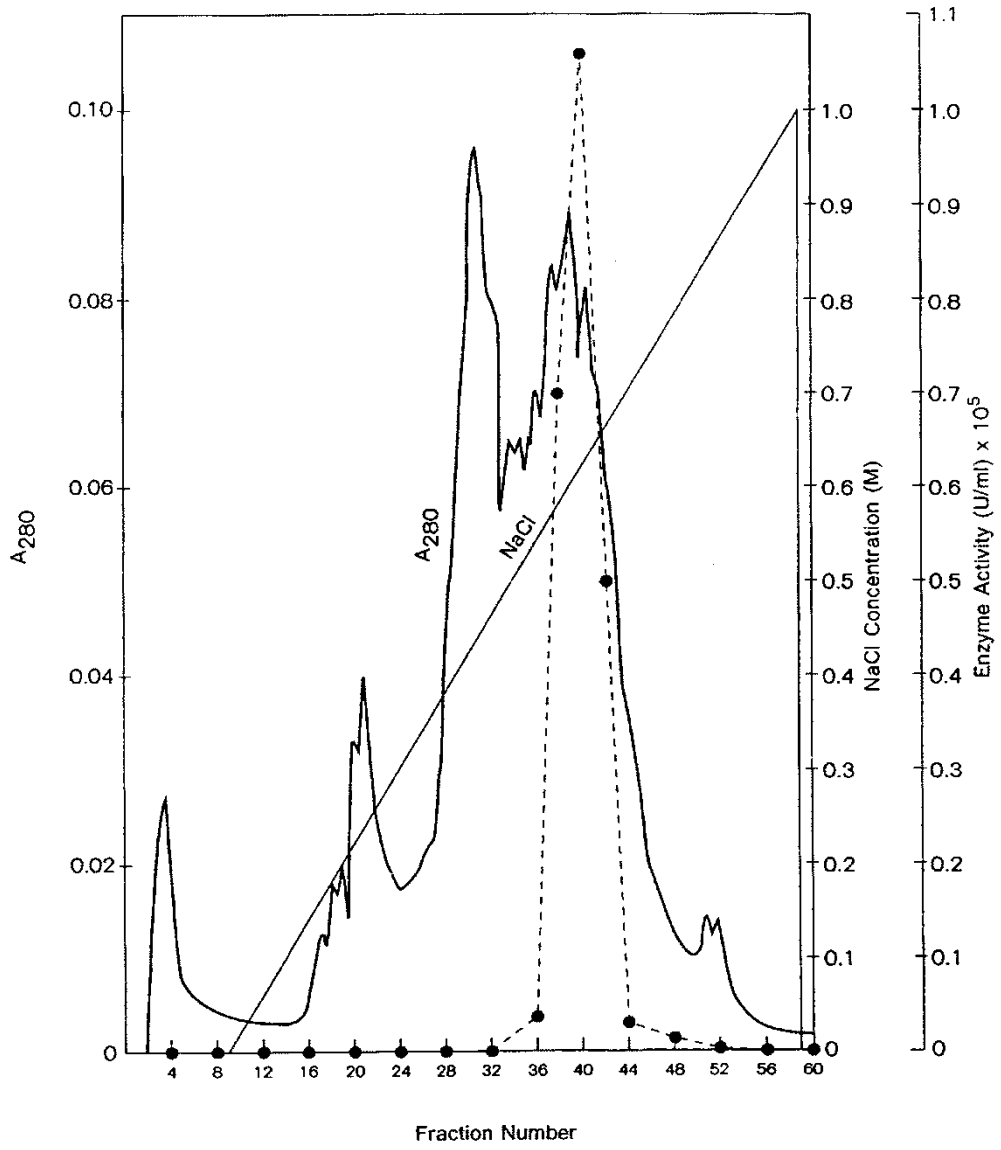
Figure 14 Elutionn profile of 0-60% amomonium sulphate precipitation from chromatography on DE-52 column showing the separation of the two bromoperoxidase, BPO1 and BPO2.

Dialysed 0-60% ammonium precipitate was applied to the DE52 column equilibrated with 20 mM Tris-HCl buffer pH 7.6 at the flow rate 0.5 ml/min. The column was washed with the same buffer and proteins were eluted with a gradient 0-1.0 M NaCl (□) (Methods, section 2.2.3.1). Fractions (4 ml) were measured for protein (Δ) and enzyme activity (●) using MCD bromination assay as described in the Methods, section 2.2.2.

Dialysed concentrated BPO1 from DE52 column was applied to the MonoQ column equilibrated with 20 mM Tris-HCl buffer pH 7.6 at the flow rate 1 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 as described in the Methods, section 2.2.2.

Figure 15 Chromatographic profile of BPO1 from DE52 column on MonoQ column

Dialysed concentrated BPO1 from DE52 column was applied to the MonoQ column equilibrated with 20 mM Tris-HCl buffer pH 7.6 at the flow rate 1 ml/min. Proteins in the column were eluted with a linear gradient 0-1.0 M NaCl in the same buffer (Methods, section 2.2.3.4). Fractions were measured for protein (A_{280}) and enzyme activity (●)using MCD bromination assay as described in the Methods, section 2.2.2.


0.10
0.08
0.06
0.04
0.02
0
A280
A280
NaCl
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
NaCl Concentration (M)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Enzyme Activity (U/ml) x 10^5
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Fraction Number

decrease about 32.2% compared to the DEAE cellulose step. BPO2 was eluted at 0.63-0.75 M NaCl (Figure 16), about 71.4% proteins were eliminated and specific activity increased about 2-fold, the % yield decreased about 42% compared to the DEAE cellulose step.

In further purification by using Superose 12 column on FPLC (Figure 17 and 30). For BPO1, the proteins were eliminated about 56.6%, the specific activity increased about 1.6 fold and % yield decreased by 31% compared to the MonoQ step. For BPO2, about 58.4% of proteins were eliminated, the specific activity increased about 1.9 fold and % yield decrease about 19.6% compared to the MonoQ step.

In purification of bromoperoxidase from 1 kg of seaweed, crude extract contained 402.35 mg protein with specific activity of 4.8 mU/mg. After purification by ammonium sulphate precipitation, DE52 MonoQ and Superose12 chromotography, the enzyme BPO1 had 1.68 mg protein with 12.89 fold purification and 5.38% yield; BPO2 had 0.82 mg protein with 68.89 fold purification and 14% yield (Table 13)

Figure 16 Chromatographic profile of BPO2 from DE52 column on MonoQ column

Dialysed concentrated BPO2 from DE52 column was applied to the MonoQ column equilibrated with 20 mM Tris-HCl buffer pH 7.6 at the flow rate 1 ml/min. Proteins in the column were eluted with a linear gradient 0-1.0 M NaCl in the same buffer (Methods, section 2.2.3.4). Fractions were measured for protein (A_{280}) and enzyme activity (●)using MCD bromination assay as described in the Methods, section 2.2.2.

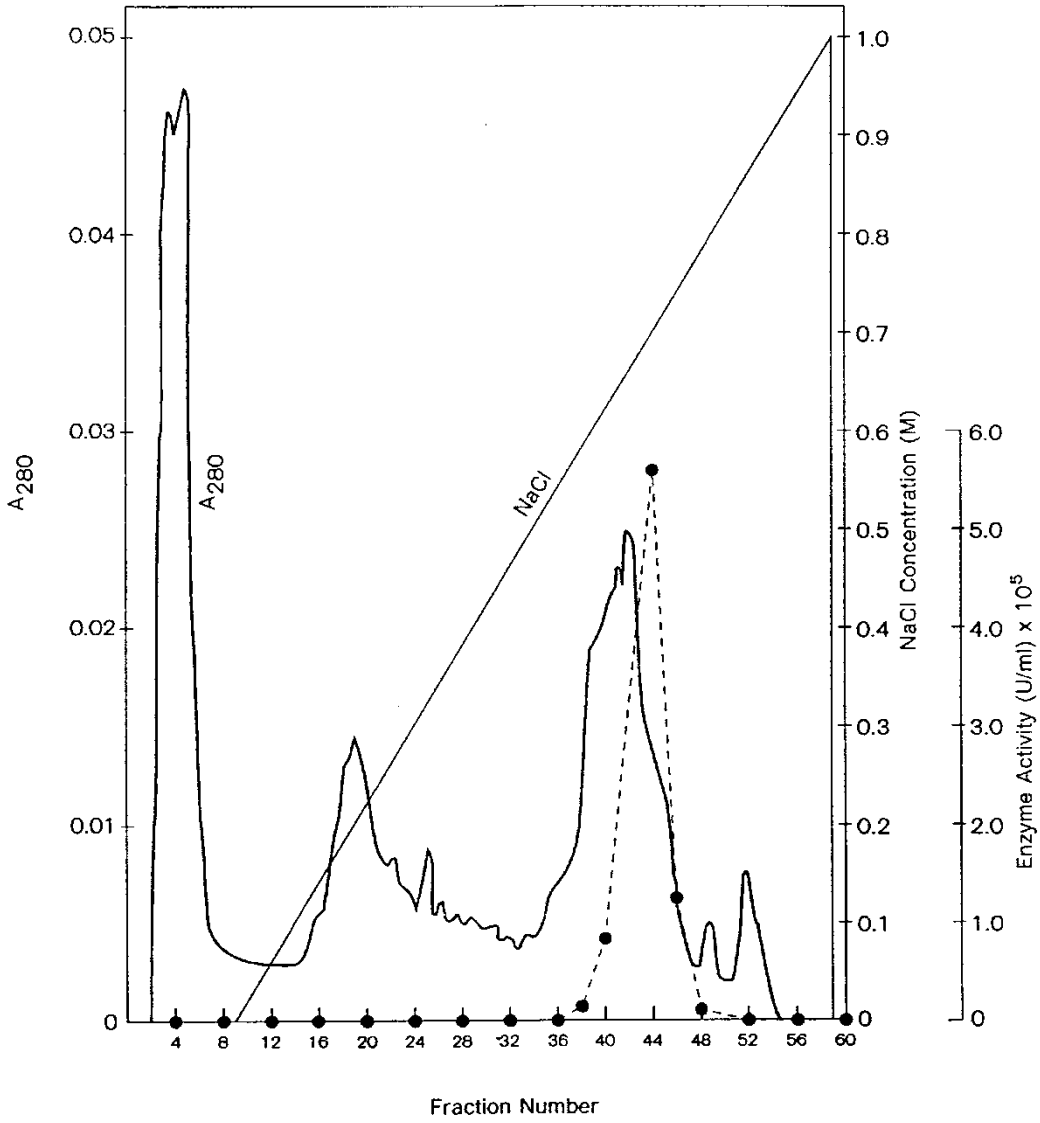

0.05
0.04
0.03
0.02
0.01
0
A280
A280
NaCl
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
NaCl Concentration (M)
6.0
5.0
4.0
3.0
2.0
1.0
0
Enzyme Activity (U/ml) x 10^5
4
8
12
16
20
24
28
32
36
40
44
48
52
56
60
Fraction Number

Figure 17 Chromatographic profile of BPO1 from MonoQ column on Superose12 column

Dialysed concentrated BPO1 from MonoQ column was applied to the Superose12 column equilibrated with 20 mM Tris-HCl buffer pH 7.6 at the flow rate 0.5 ml/min. After elution with the same buffer (Methods, section 2.2.3.5), fractions were measured for protein (—, A_{280}) and enzyme activity (○) using MCD bromination assay as described in the Methods, section 2.2.2.

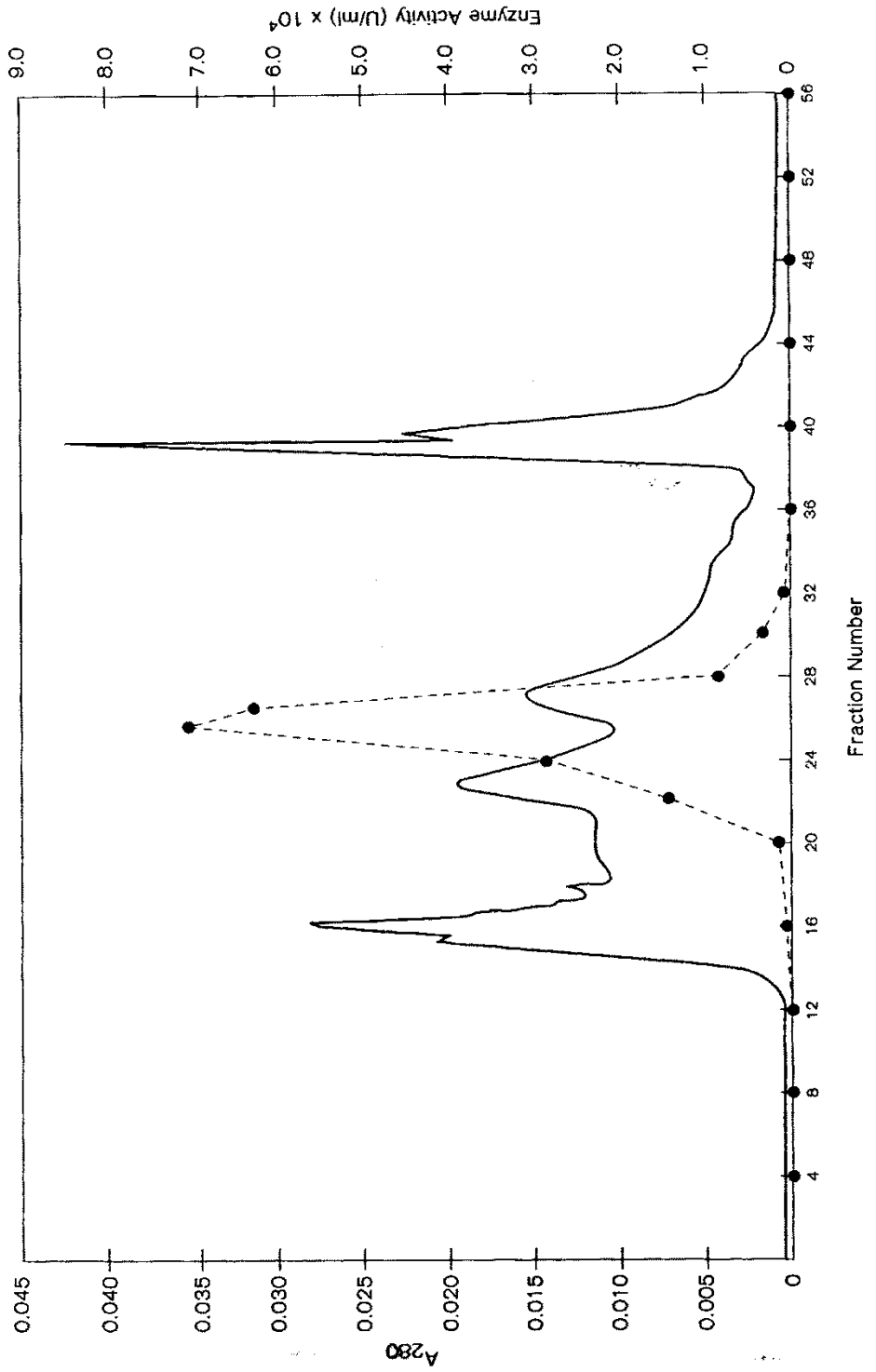
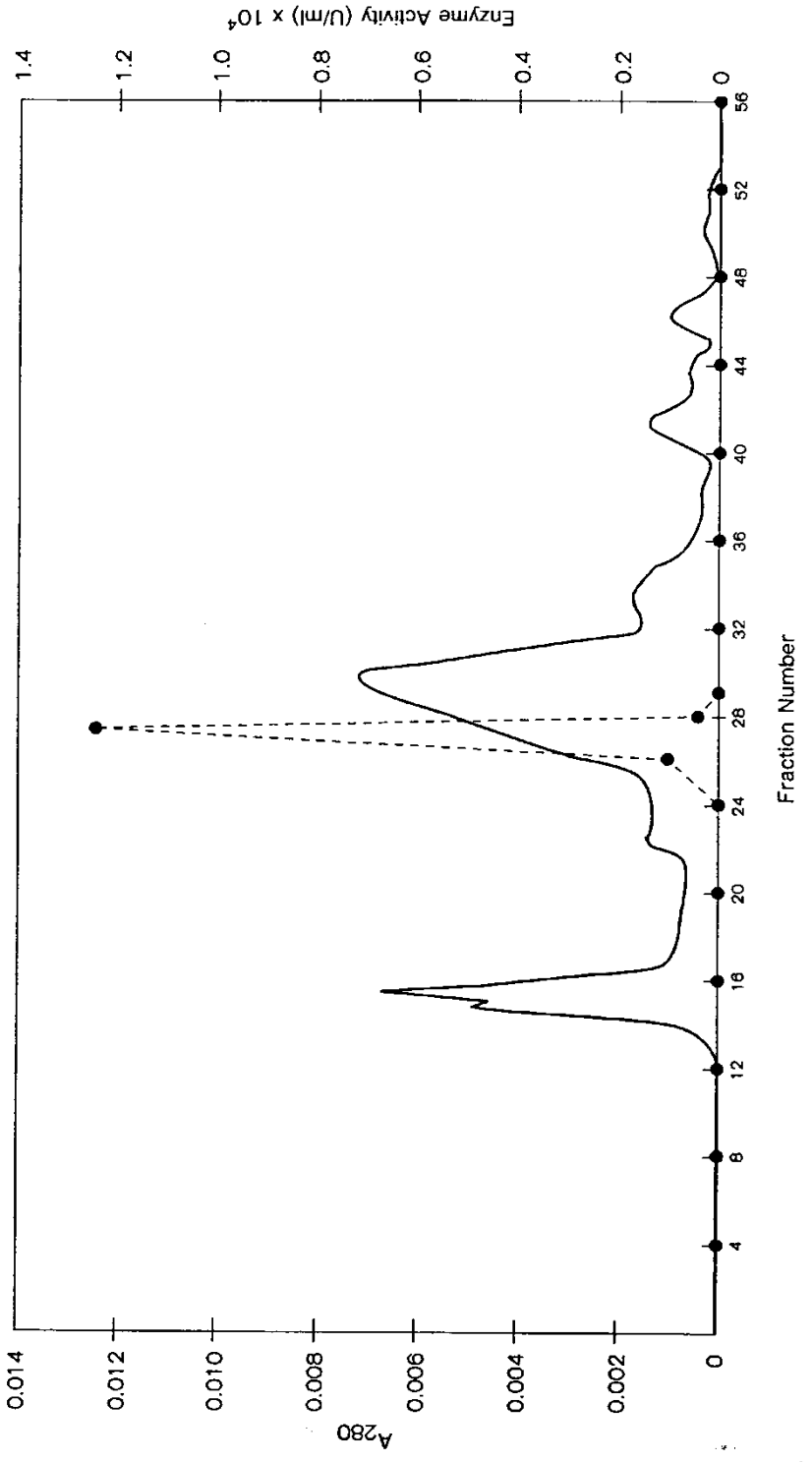

Enzyme Activity (U/ml) x 10⁴
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
A280
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
Fraction Number

Figure 18 Chromatographic profile of BPO2 from MonoQ column on Superose12 column

Dialysed concentrated BPO2 from MonoQ column was applied to the Superose12 column equilibrated with 20 mM Tris-HCl buffer pH 7.6 at the flow rate 0.5 ml/min. After elution with the same buffer (Methods, section 2.2.3.5), fractions were measured for protein (—, A_{280}) and enzyme activity (○) using MCD bromination assay as described in the Methods, section 2.2.2.


Enzyme Activity (U/ml) x 10⁴
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A280
0.014
0.012
0.010
0.008
0.006
0.004
0.002
0
4
8
12
16
20
24
28
32
36
40
44
48
52
56
Fraction Number

3.1 Purification of BPO1 and BPO2 from Seaweed

Table 13 Purification of bromoperoxdiase from seaweed, *Polycarvernosa* sp.

Step of Purification	**Total protein (mg)**	**Total activity (mU)**	**Specific activity (mU/mg)**	**Purification Fold**	**% yield**
Crude extract	402.35	1932.30	4.80	1.00	100.00
0-60%$(NH_4)_2SO_4$	351.85	1892.95	5.38	1.12	97.96
DE52					
BPO1	15.13	228.31	15.09	3.14	11.82
BPO2	6.88	581.52	84.52	17.61	30.10
MonoQ					
BPO1	3.87	151.20	39.07	8.14	7.77
BPO2	1.97	337.22	171.18	35.66	17.45
Superose 12					
BPO1	1.68	103.92	61.86	12.89	5.38
BPO2	0.82	271.08	330.58	68.87	14.03

3.2 Iodoacetamide Modification of BPO1 and BPO2

3.2.1 BPO1

Iodoacetamide at various concentrations from 0.25-250 mM were used to modify BPO1 at 35°C for 18 hours at pH 7.0. After incubation and dialysis, the enzyme activity was assayed. As shown in Figure 19, BPO1 activity increased as the concentrations of iodoacetmide increased. At 0.25, 2.5, 25 and 250 mM iodoacetamide, the enzyme activities were 215, 416, 410 and 485%, respectively. The results showed that at 25 mM iodacetamide the enzyme activity was activated to the highest activity and at the concentration of 250 mM the enzyme activity still remained at the highest activation. The results suggested that iodoacetamide might react with certain amino acid residues involving in the catalytic activity of the enzyme.

3.2.2 BPO2

In Figure 20, BPO2 was treacted with 0.025-250 mM iodoacetamide. It was shown that iodoacetamide inactivated the activity of BPO2 at high concentration. When the concentration of iodoacetamide increased, the enzyme activity decreased. At 0.25, 2.5, 25 and 250 mM iodoacetamide, the enzyme activities were 98, 97, 103 and 61%, respectively. The result suggested that iodoacetamide might react with certain amino acid residues and the modified amino acid residues might interfere the catalytic activity and caused the decrease in enzyme activity.

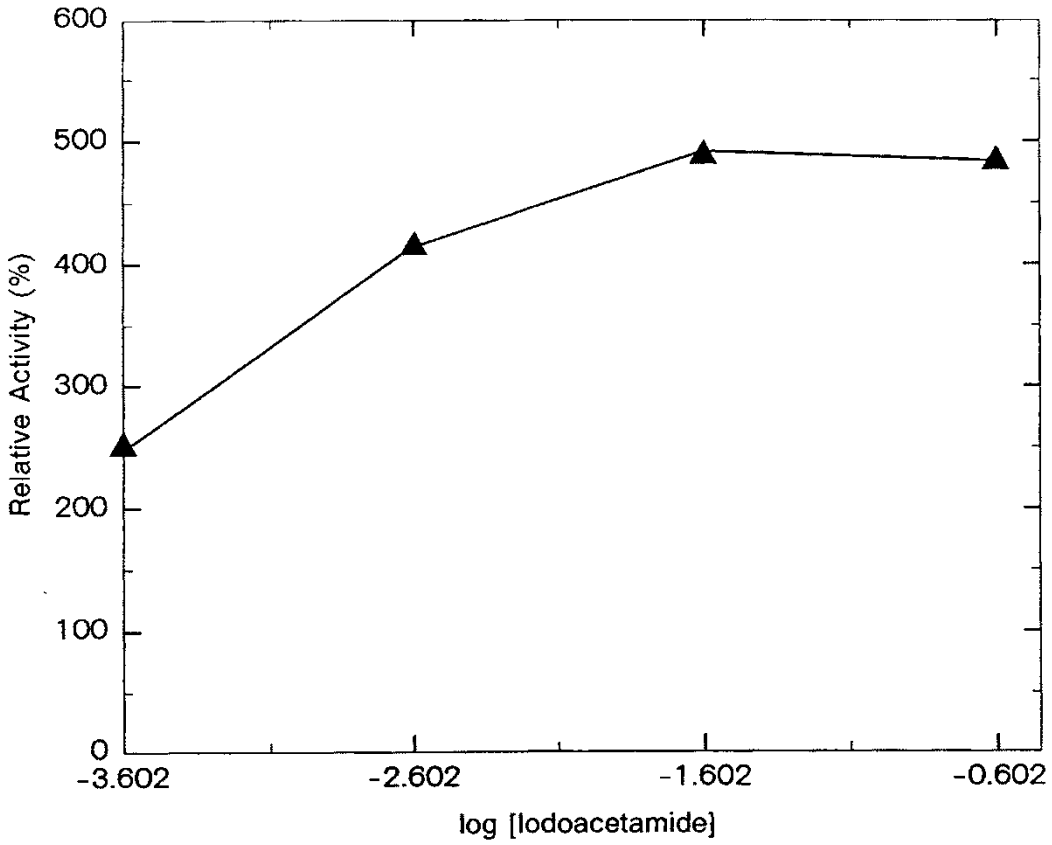


Figure 19 Effect of iodoacetamide on bromoperoxidase activity of BPO1.

BPO1 was added with 0.25-250 mM iodoacetamide in 20 mM KPB pH 7.0 and allowed to react at 35°C for 18 hours. After incubation, the reaction mixture was dialyzed and measured for the enzyme activity using MCD bromination assay as described in the Methods, section 2.2.2. The enzyme activity in various concentrations of iodoacetamide was compared as percentage of relative activity with the native enzyme in the absence of iodoacetamide at time zero.

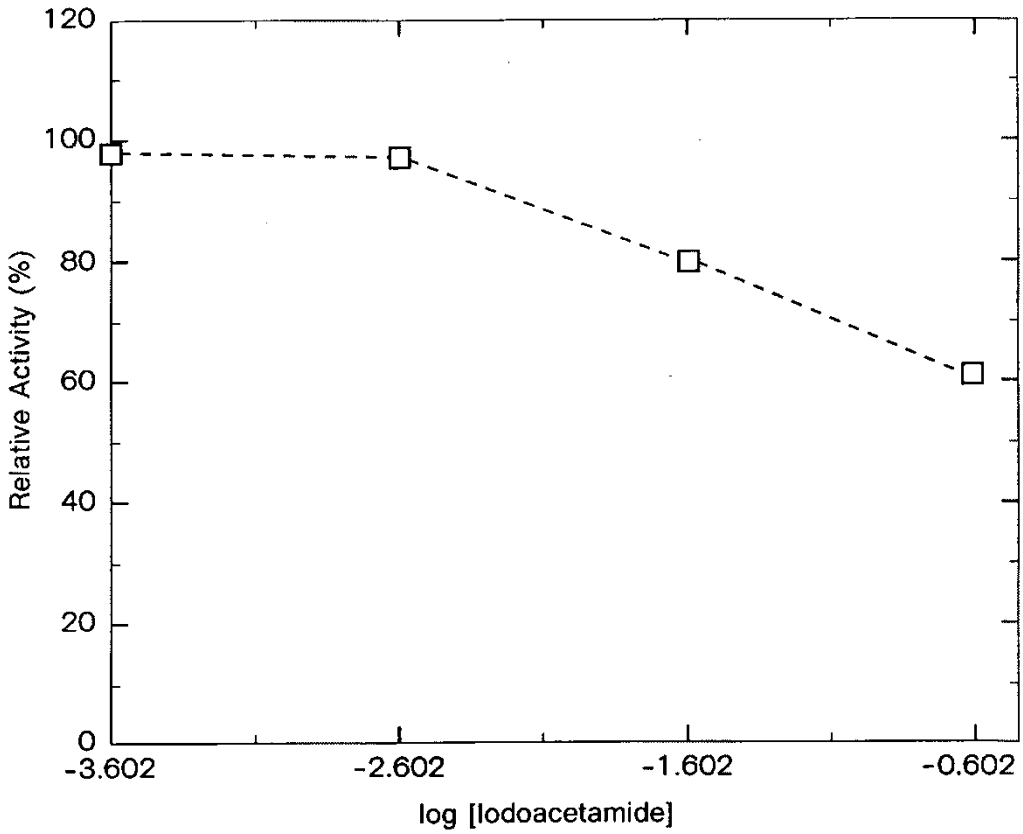


Figure 20 Effect of iodoacetamide on bromoperoxidase activity of BPO2.

BPO2 was added with 0.25-250 mM iodoacetamide in 20 mM KPB pH 7.0 and allowed to react at 35°C for 18 hours. After incubation, the reaction mixture was dialyzed and measured for the enzyme activity using MCD bromination assay as described in the Methods, section 2.2.2. The enzyme activity in various concentrations of iodoacetamide was compared as percentage of relative activity with the native enzyme in the absence of iodoacetamide at time zero.

3.3 Effect of Chemical Modification on BPO1 and BPO2

Form Figure 38-74, the effects of chemical modification on the enzyme activity of BPO1 and BPO2 were summarized in the Table 14.

Table 14 Effects of chemical modification on bromoperoxidase activity of BPO1 and BPO2

Modifying reagent	**Concentration (mM) of modifying reagent used for modification**		**% Relative activity**	
	BPO1	**BPO2**	**BPO1**	**BPO2**
TNBS	400.0	40.0	150	0
N-Acetylimidazole	20.0	48.8	101	100
NBS	5.0	24.4	0	7
HNBB	2.5	2.5	0	0
o-Nitrophenylsulfenyl chloride	13.9	13.9	29	99
DEP	173.0	173.0	102	55
1,2-Cyclohexanedione	1.09	1.09	98	95
EDC	1.0	195.0	0	42
Iodoacetic acid	50.0	50.0	0	0
Iodoacetamide	250.0	250.0	485	61

3.4 Number of Modified Amino Acid Residues by Modifying Reagents

From Absorption spectra of the modified BPO1 and BPO2, the number of the modified amino acid residues could be determined by the molar extinction coefficient. The number of the modified amino acid residues were summarized Table 15.

Table 15 Number of modified amino acid residues of BPO1 and BPO2 by modifying reagents

Specificity	**Modified reagent**	**Number of amino acid residues modified (residues/mole)**	
		BPO1	**BPO2**
Lysine	TNBS	31.92	45.57
Tryptophan	NBS	15.48	18.95
Tyrosine	N-Acetylimidazole	11.21	11.50

3.5 Stability of BPO1, BPO2, Modified BPO1 and Modified BPO2

The native BPO1, BPO2 and the modified BPO1, BPO2 by various modifying reagents were incubated at different time intervals at 37°C in different pH. The enzyme activities were assayed at various time intervals. The results represented the relative activity of the native and modified enzyme under different conditions.

3.6 Iodoacetamide Modification

3.6.1 BPO1

The native BPO1 and modified BPO1 by 50 mM iodoacetamide were incubated at 37°C for 0-144 hours. From Figure 21, it was shown that the native BPO1 was less stable than the iodoacetamide-modified BPO1. The enzyme activity of the native BPO1 was completely lost at 36 hours of incubation while the iodoacetamide-modified BPO1 still remained the enzyme activity of 38±4% relative activity at 144 hours of incubation. This suggested that iodoacetamide-modified the amino acid residues of BPO1 enhanced the enzyme stability.

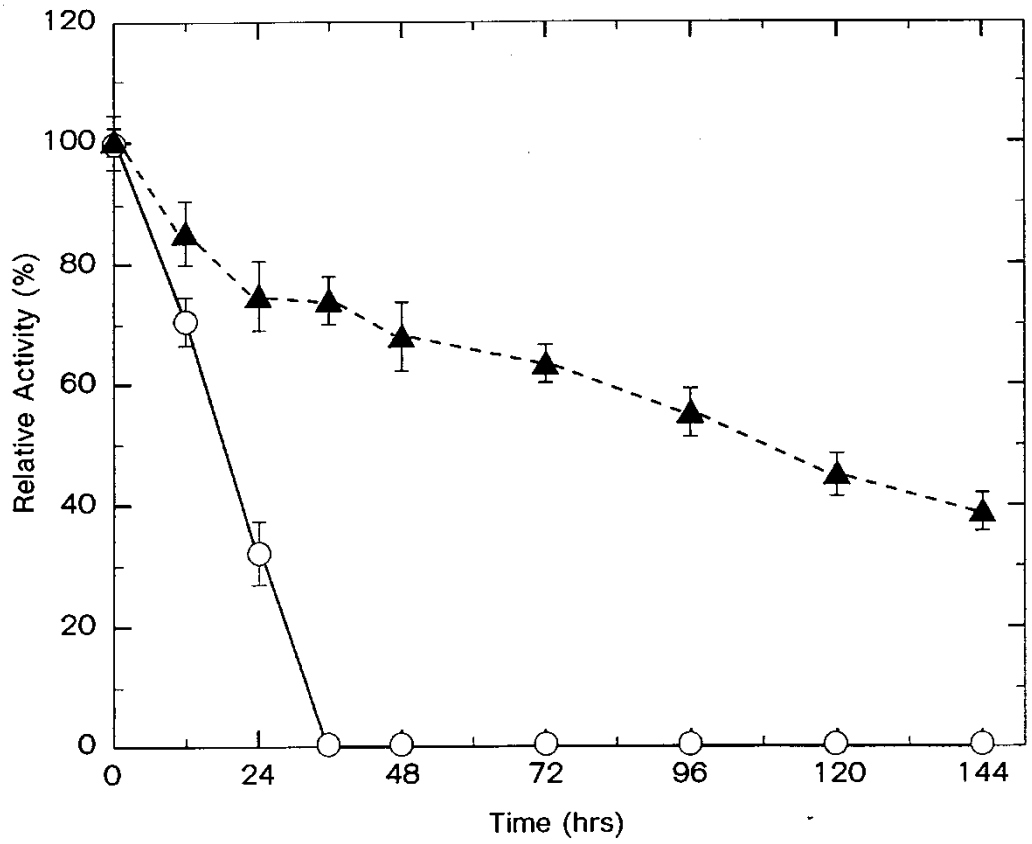


Figure 21 Thermostability of BPO1 and iodoacetamide-modified BPOI at 37°C, pH 7.0.

The native BPO1 (○) and modified BPO1 by 50 mM iodoacetamide (▲) were incubated in 20 mM KPB pH 7.0 at 37°C. During incubation, the enzyme activity was measured using the MCD bromination assay and compared as percentage of relative activity with the enzyme at time zero as decribed in the Methods, section 2.2.21.

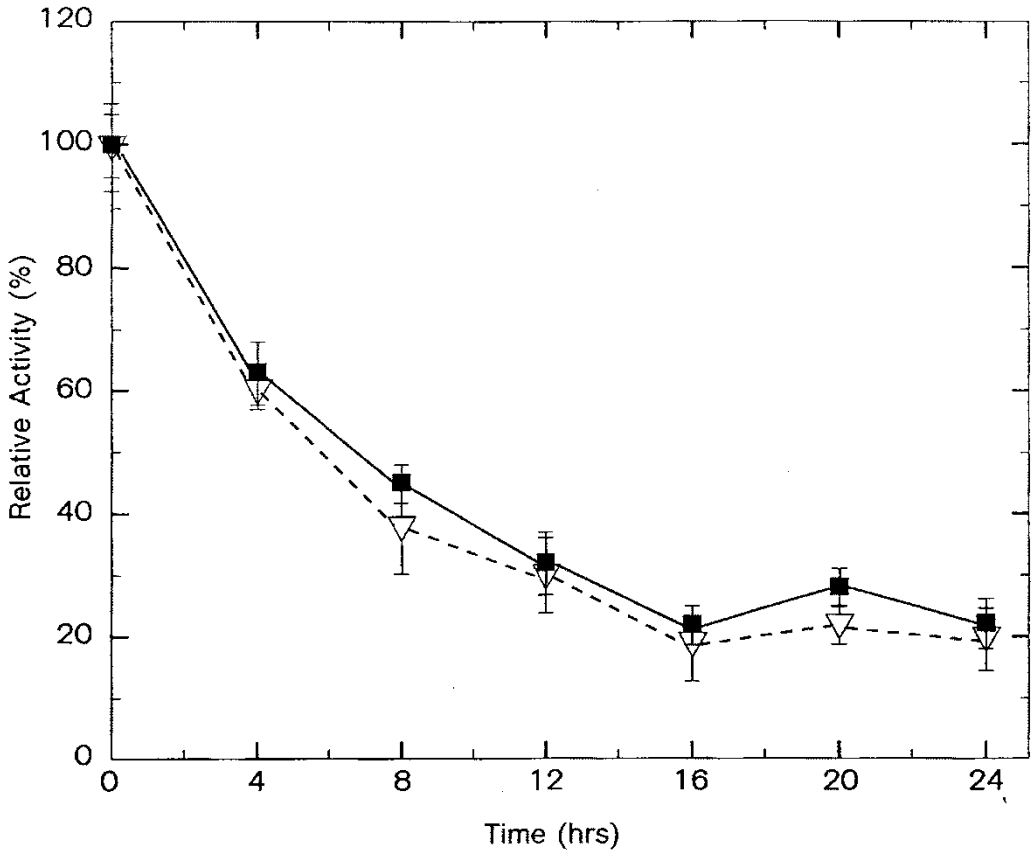


Figure 22 Thermostability of BPO2 and iodoacetamide-modified BPO2 at 37°C, pH 7.0.

The native BPO2 (■) and modified BPO1 by 50 mM iodoacetamide (▽) were incubated in 20 mM KPB pH 7.0 at 37°C. During incubation, the enzyme activity was measured using the MCD bromination assay and compared as percentage of relative activity with the enzyme at time zero as described in the Methods, section 2.2.21.

3.6.2 BPO2

As shown in Figure 22, the native BPO2 and modified BPO2 by 50 mM iodoacetamide showed similar stability profiles. The enzyme activities were 10±5 and 12±4% at 24 hours of incubation for the modified BPO2 and the native BPO2, respectively. This suggested that iodoacetamide-modified amino acid residues did not involve in the stability of BPO2

3.7 Half-Life Values Determination

From the stability profiles of the native and modified enzyme in Figure 75-94, the half-life values were estimated from the incubation time that caused 50% decrease in the enzyme activity. The half-life values were summarized in Table 16.

Table 16 Stability of the native and modified BPO1 and BPO2 by various modifying reagents at 37°C in 20 mM potassium phosphate buffer

Modifying reagent	pH	Half-life (hours)			
		BPO1		BPO2	
		Control	Modified	Control	Modified
TNBS	8.5	12.2±2.0	37.2±3.0	6.2±1.0	5.5±.0
N-Acetylimidazole	7.5	16.0±2.0	18.1±1.2	6.0±2.0	6.5±2.0
NBS	5.0	16.5±3.0	16.5±2.0	6.0±1.0	7.2±2.0
HNBB	5.0	15.0±2.0	5.2±2.0	5.8±1.2	3.0±0.5
o-Nitrophenylsulfenyl chloride	7.0	17.0±2.4	18.2±10	6.0±1.0	6.2±1.2
DEP	6.0	18.1±2.1	18.1±1.1	5.5±1.0	3.0±0.5
1,2-Cyclohexanedione	8.0	12.5±1.0	11.0±0.5	7.0±1.0	7.2±1.2
EDC	5.0	17.0±3.0	18.1±3.0	6.5±1.0	7.5±1.0
Iodoacetic acid	7.0	18.4±3.0	2.1±1.0	6.2±2.0	2.3±1.0
Iodoacetamide	7.0	18.4±2.0	108±6.0	6.1±1.4	5.8±1.0

3.8 Effect of Temperature on Iodacetamide Modification of BPO1

BPO1 was treated with iodoacetamide and incubated at different temperature as described in the Methods, section 2.2.23. The result in Figure 23 showed that the relative activity of the enzyme incubated at 35°C was higher than of the enzyme incubated at temperature 45°C and 25°C at all incubation times. The relative activity of the enzyme incubated at 45°C was higher than at 25°C. the enzyme activity at 35°C was activated to about 500% at 12-18 hours of incubation. The highest relative activity of enzyme incubated at 45°C was 425% at 12 hours and decreased to 405% at 18 hours of incubation. The enzyme incubated at 25°C was activated to 309% at 18 hours of incubation. The enzyme incubated at 25°C was activated to 309% at 18 hours of incubation. The enzyme incubated at 25°C was activated to 309% at 18 hours and decreased to 282% at 24 hours of incubation. The results suggested that temperature of 35°C might be optimal for iodoacetamide modification of the enzyme.

3.9 Effect of Temperature on BPO1 and Iodoacetamide-Modified BPO1

BPO1 and iodoacetamide-modified BPO1 were incubated at various temperatures from 30 to 80°C for 10 minutes. The native and modified enzyme showed similar temperature profile. The activities of the native enzyme were 100, 100, 83 and 9% while those of the modified enzyme were 100, 100, 90 and 25% at 30, 40, 50 and 60°C, respectively (Figure 24.) At temperature 70 and 80°C, activities of the native and modified enzyme were completely lost. The result showed that activities of both native and modified enzyme lost rapidly as the temperature increased to 60°C.

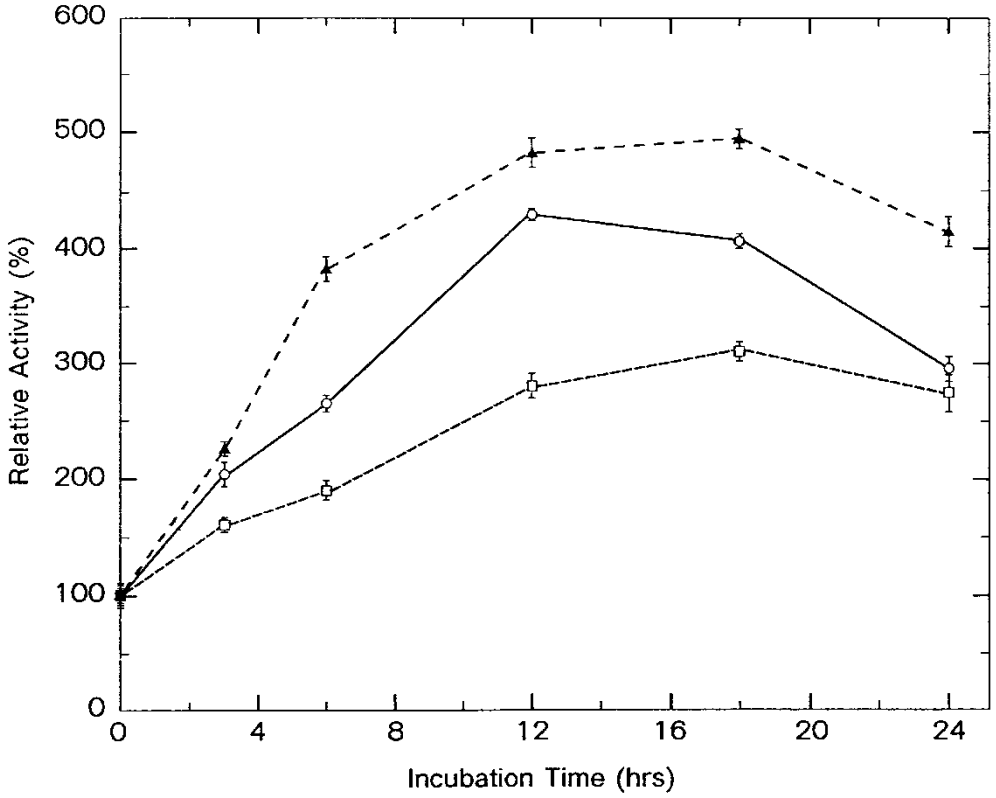


Figure 23 Effect of temperature on iodoacetamide in in 20 mM KPB pH 7.0.

The reaction mixture was incubated at 25 °C (□), 35°C (▲) and 45°C (○), at variable time intervals, the reaction mixture was dialysed and assayed for bromoperoxidase activity and compared as percentage of relative activity with the enzyme without iodoacetamide at time zero as described in the Methods, section 2.2.23.

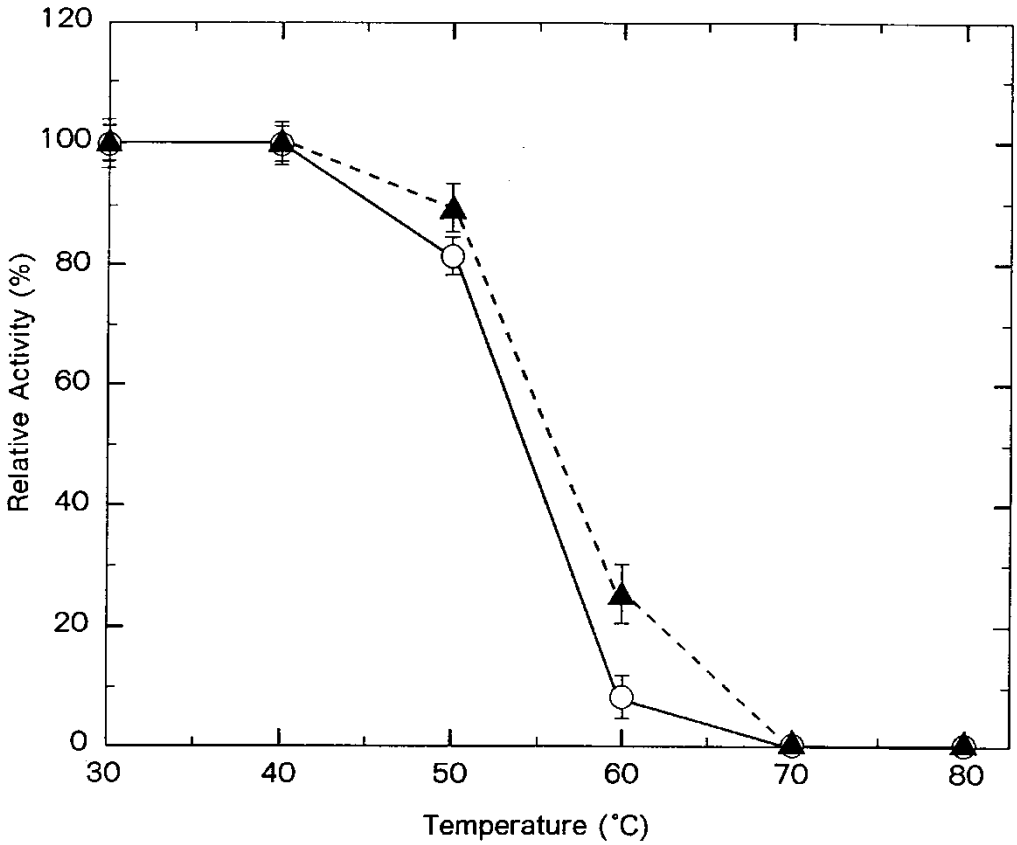


Figure 24 Effect of temperature on bromoperoxidase activity of BPO1 and iodoacetamide-modified BPO1.

The native BPO1 (○) and iodoacetamide-modified BPO1 (▲) were incubated in 20 mM KPB pH 7.0 at 30-80C for 10 minutes before the enzyme activity were assayed as described in the Methods, section 2.2.2. The enzyme activity was compared as percentage of relative activity with the enzyme at time zero as described in the Methods, section 2.2.26.

3.10 Themostability of BPO1 and Iodoacetamide-Modified BPO1 at 25°C and 45°C, pH 7.0

3.10.1 At Temperature 25°C

The native BPO1 and iodoacetamide-modified BPO1 were kept in 20 mM KPB, pH 7.0, at 25°C for various time interval before assaying for the remained activity. As shown in Figure 25, the modified enzyme was stable more than the native enzyme during storage for 144 hours. The activity of the native enzyme at 24 hours of storage was 71% and decreased to 20% at 144 hours of storage. The activity of modified enzyme was 92% at 24 hours of storage, and decreased to 66% at 144 hours of storage.

3.10.2 At Temperature 45°C

The native BPO1 and iodoacetamide-modified BPO1 was kept in 20 mM KPB, pH 7.0, at 45°C. The result in Figure 26, showed that the modified enzyme was slightly stable more than the native enzyme. Activity of the native enzyme were 60% and 31% at 12 and 24 hours of storage, respectively. The activity of the native enzyme was completely lost at 36 hours of storage. The activity of modified enzyme was 86% at 12 hours of storage and decreased to 11% at 60 hours of storage. The half-life values of the native and iodoacetamide-modified BPO1 were show in Table 17.

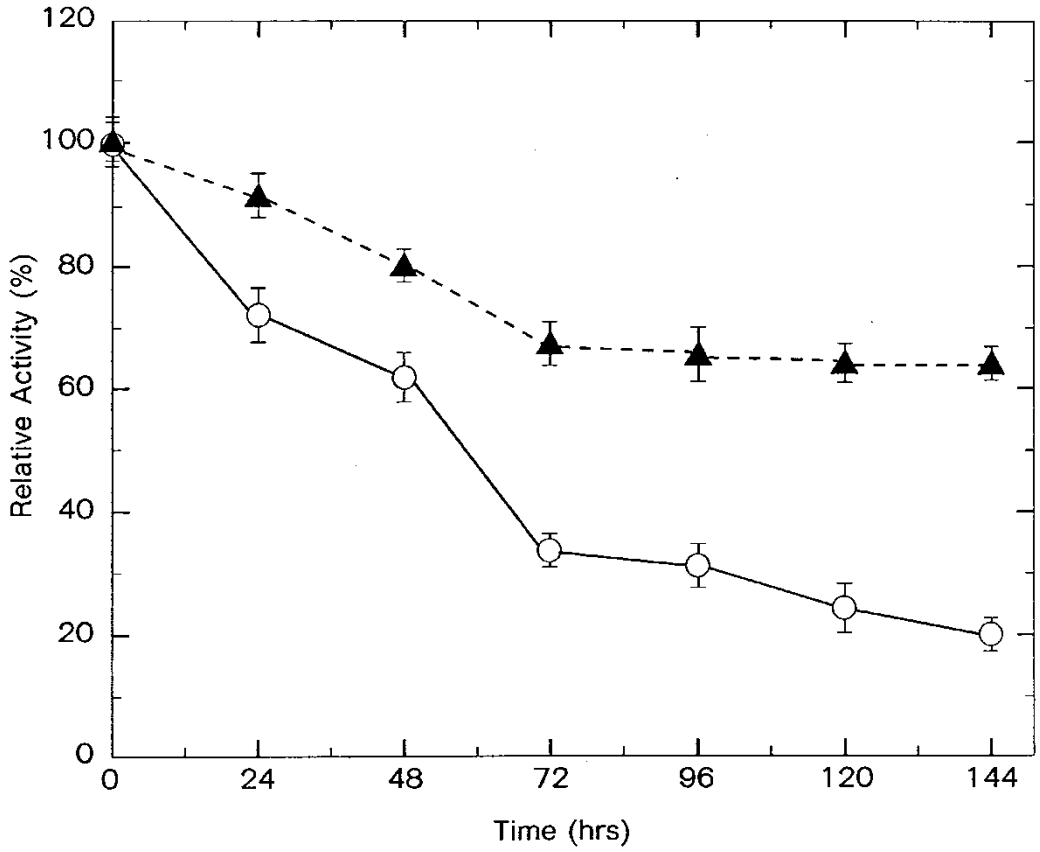


Figure 25 Thermostability of BPO1 and iodoacetamide-modified BPO1 at 25°C, pH 7.0.

The native BPO1 (○) and iodoacetamide-modified BPO1 (▲) were incubated in 20 mM KPB pH 7.0 at 25°C. During incubation, the enzyme activity was measured using the MCD bromination assay and compared as percentage of relative activity with the enzyme at time zero as described in the Methods, section 2.2.27.

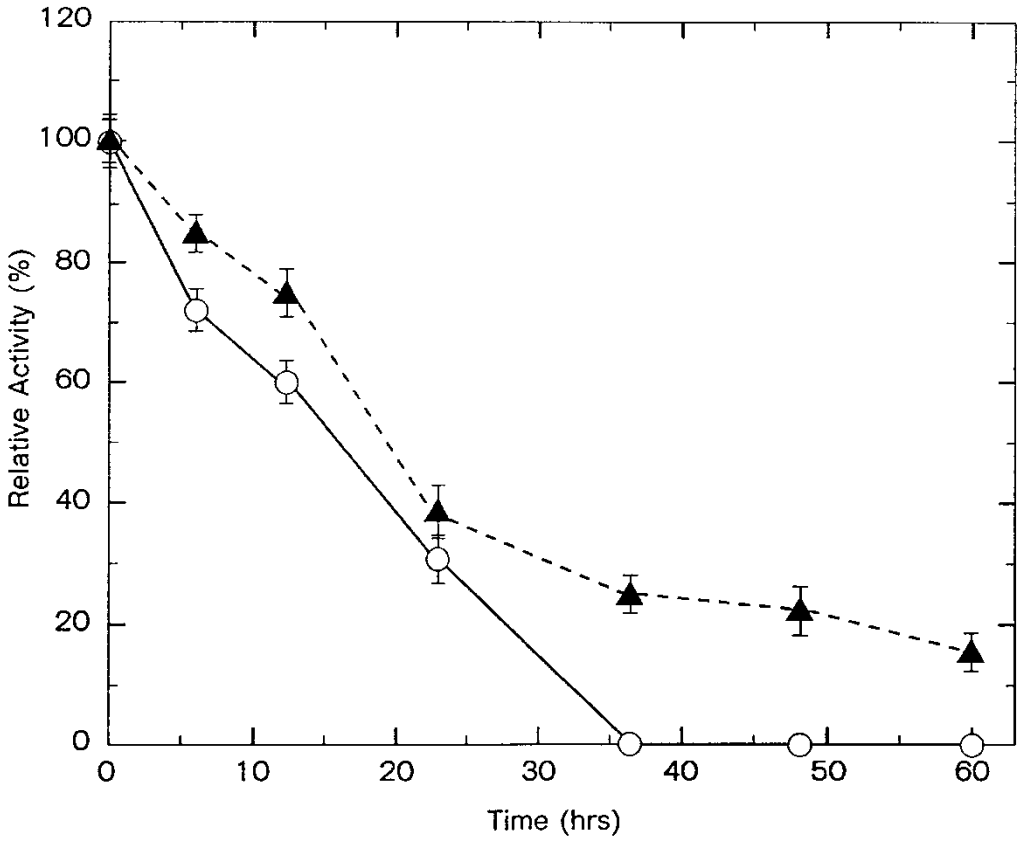


Figure 26 Thermostability of BPO1 and iodoacetamide-modified BPO1 at 45°C, pH 7.0.

The native BPO1 (○) and iodoacetamide-modified BPO1 (▲) were incubated in 20 mM KPB pH 7.0 at 45°C. During incubation, the enzyme activity was measured using the MCD bromination assay and compared as percentage of relative activity with the enzyme at time zero as described in the Methods, section 2.2.27.

Table 17 The half-life of the native BPO1 and iodoacetamide-modified BPO1 at pH 7.0 in different temperature

Temperature (°C)	Half-life (hrs)	
	BPO1	Iodoacetamide-modified BPO1
25	57	> 144
45	16	20

3.11 Thermostability of BPO1 and Iodoacetamide-Modified BPO1 at 25°C, pH 5.5

The native BPO1 and iodoacetamide-modified BPO1 were kept at 4°C and 25°C in 20 mM KPB, pH 5.5. As shown in Figure 27, the modified enzyme was more stable than the native enzyme during storage at 25°C, the activity of the modified enzyme remained unchanged for at least 9 days. This result was similar to those of both the native and modified enzyme stored at 4°C. The activity of the native enzyme stored at 25°C was 64% at 1 day of storage and decreased to 33 and 8% at 2 and 3 days of storage, respectively. At 4 days of storage the enzyme activity was completely lost. The half-life of the native enzyme in this condition was 36 hours.

3.12 Effect of Protease Inhibitor on BPO1 and Iodoacetamide-Modified BPO1

The native BPO1 and iodoacetamide-mdified BPO1 were treated with PMSF, apotinin and leupeptin as described in the Methods, section 2.2.25 and assayed for the enzyme activity after incubation at 25°C for 12 hours. The result in Table 18 showed that PMSF, aponitin and leupeptin had no significant effect on both the native and iodoacetamide-treated enzyme.

Table 18 Effect of protease inhibitors on BPO1 and iodoacetamide-modified BPO1

Protease inhibitor	Relative activity (%)	
	BPO1	Iodoacetamide-modified BPO1
PMSF	97 ± 6	95 ±7
Apotinin	104 ± 5	98 ± 3
Leupeptin	98 ± 6	102 ± 4
None	100 ± 3	100 ± 6

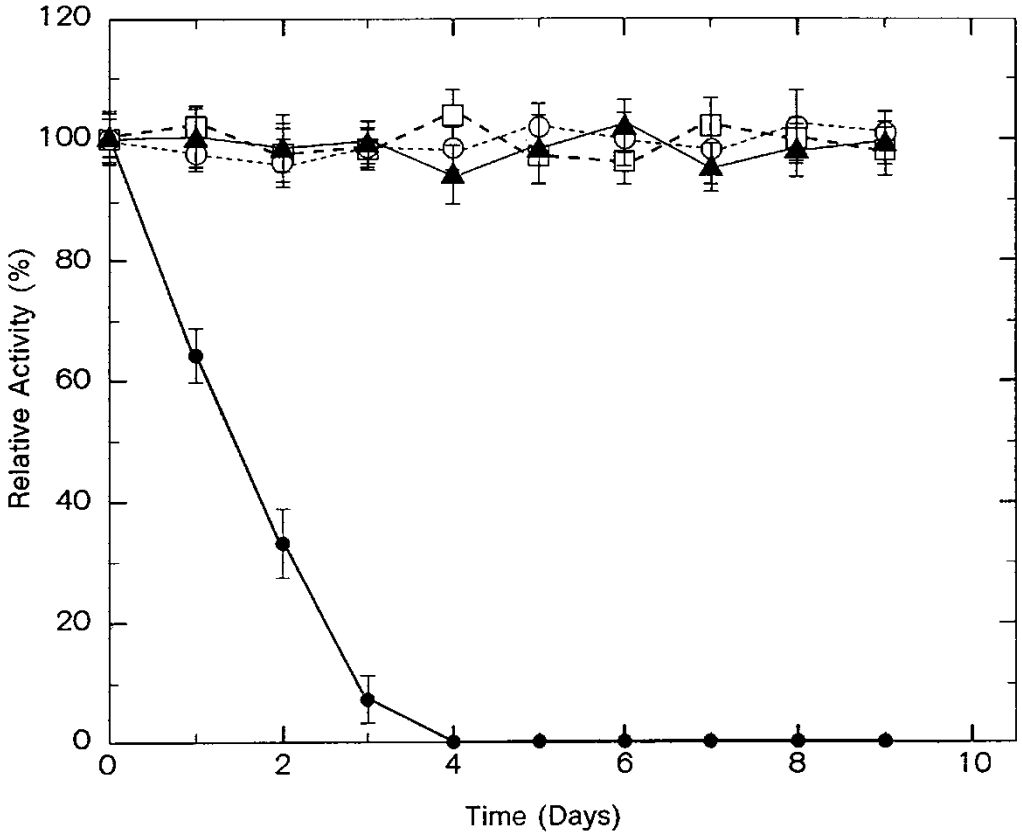


Figure 27 Thermostability of BPO1 and iodoacetamide-modified BPO1 at 25°C and 4°C, pH 5.5.

The BPO1 (○) was incubated at 25°C (●), 4°C (○) and iodoacetamide-modified BPO1 was incubated at 25°C (▲) and 4°C (□) in 20 mM KPB pH 5.5 at 45°C. During incubation, the enzyme activity was measured using the MCD bromination assay and compared as percentage of relative activity with the enzyme at time zero as described in the Methods, section 2.2.28.

3.13 Effect on NaN_3 and KCN on BPO1 and Iodoacetamide-Modified BPO1

BPO1 and iodoacetamide-modified BPO1 were treated with 1 mg/ml of NaN_3 and 1 mg/ml of KCN, and the enzyme activity were assayed. The result in Figure 28 showed that NaN_3 inactivated the native and modified-BPO1 to 6±3 and 3±2% activity, respectively. The enzyme activities in the presence of KCN were 4±1 and 5±2% respectively. However, when the NaN_3 and KCN treated enzyme were dialysed, the activityl was recovered. For NaN_3, the activities of the native and modified-BPO1 were 98±9 and 33±6%, respectively. For KCN, the activities of the native and modified-BPO1 were 99±4 and 66±7% respectively. This showed that the activity of the native enzyme was recovered more than that of the modified-BPO1 for both NaN_3 and KCN. The recovery of the native enzyme was nearly 100% for both KCN and NaN_3. The recovery of KCN treatment for the modified-BPO1 about 2 times higher than that of NaN_3. The result suggested that the modified-BPO1 was more susceptible to both NaN_3 and KCN than the native enzyme, and the modified-BPO1 was more susceptible to NaN_3 than KCN.

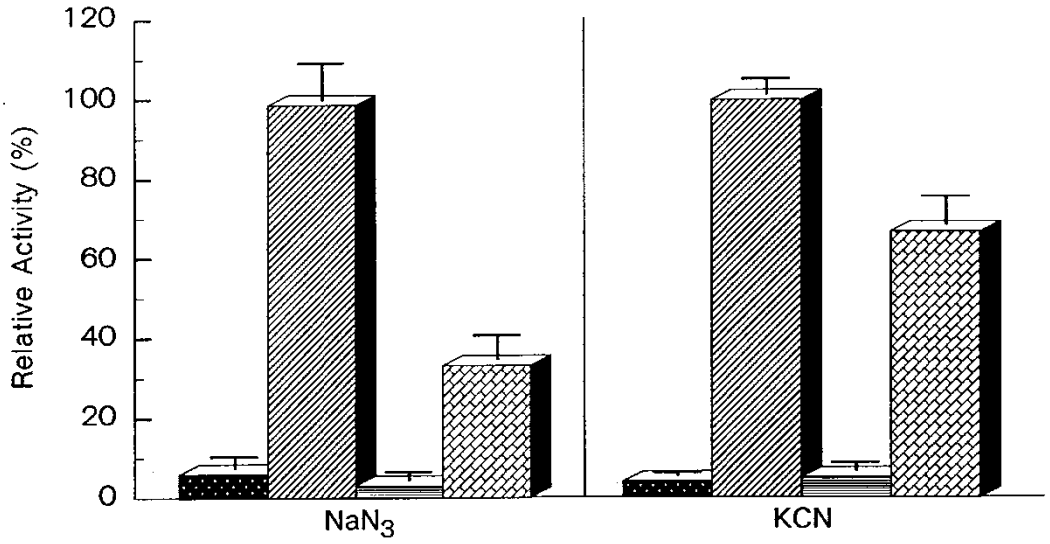


Figure 28 Effect of NaN_3 and KCN on bromoperoxidase activity of BPO1 and iodoacetamide-modified BPO1.

BPO1 and iodoacetamide-modified BPO1 were added with 1 mg/ml of NaN_3 and KCN in 20 mM KPB pH 7.0, and they were incubated at 25°C for 12 hrs. After incubation, the reaction mixture was dialysed against the same buffer at 4°C for 16 hrs and measured the enzyme activity using the MCD bromination assay as described in the Methods, section 2.2.2. The enzyme activity was compared as percentage of relative activity with the native enzyme in the absence of NaN_3 and KCN.

3.14 pH Activity of BPO1 and Iodoacetamide-Modified BPO1

The native BPO1 and iodoacetamide-modified BPO1 were assayed for enzyme activity in the buffer pH 3.5 – 7.5 as described in the Methods, section 2.2.30. Both the native and modified enzyme showed similar pH profile in acidic pH range with the optimum pH at 5.3 – 5.8 and 4.8 – 6.0, respectively (Figure 29). The modified enzyme seemed to have a slight wider pH optimum than the native enzyme.

3.15 pH Activity of BPO2

The native BPO2 was assayed for enzyme activity in the buffer pH 4.0 – 10.0 as described in the Methods, section 2.2.31. The native BPO2 showed high enzyme activity in neutral pH with optimum pH at 7.0 – 7.5 (Figure 30).

3.16 pH Stability of BPO1 and Iodoacetamide-Modified BPO1\

The native BPO1 and iodoacetamide-modified BPO1 were incubated in the universal buffer pH 2.0 – 11.0 and assayed for remained activity as described in the Method section 2.2.32. The result in Figure 31, showed that both native and modified-BPO1 were stable in acidic and neutral pH. Activity of the native enzyme rapidly decreased to 10% at pH 10 while the activity of the modified enzyme decreased stightly to about 80%. The result showed that the modified BPO1 was more stable in alkaline pH than the native enzyme.

3.17 Effect of V_2O_5 on the Modification of BPO1 by Iodoacetamide

The native BPO1 and the iodocetamide-modified BPO1 were prepared at different conditions as described in the Methods, section 2.2.33. The result in Figure 32 showed that V_2O_5 enhanced enhanced the enzyme activity of native and modified BPO1 to similar pattern; i.e., 267 and 315% respectively. The enzyme activity of BPO1 treated with V_2O_5 before iodoacetamide modification and BPO1 simultaneously added with V_2O_5 and iodoacetamide were also enhanced similarly; i.e, 505 and 520% respectively. This results confirmed that both V_2O_5 and iodoacetamide activated the activity of BPO1.

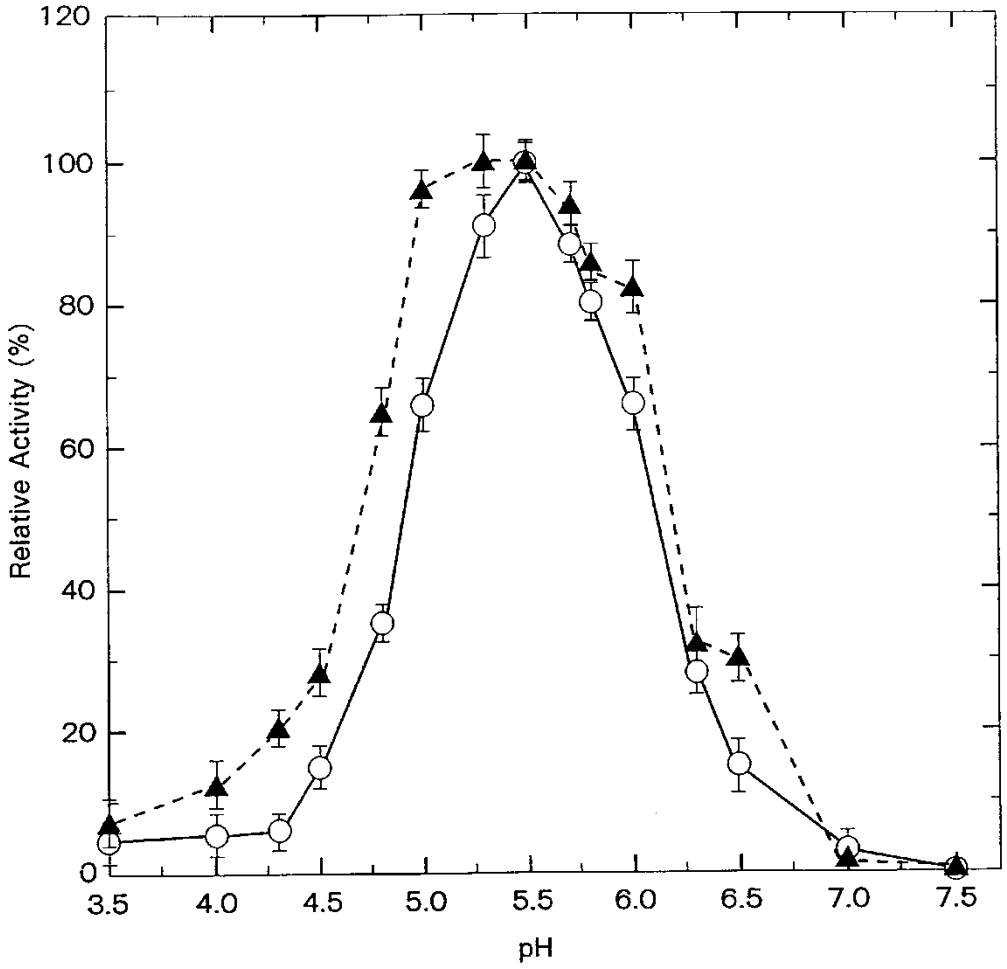


Figure 29 pH activity of BPO1 and iodoacetamide-modified BPO1.

The native BPO1 (○) and iodoacetamide-modified BPO1(▲) were assayed for bromoperoxidase activity in 0.1 M KPB with different pH from 3.5-7.5. The enzyme activity was compared as percentage of relative activity with the enzyme activity, as described in the Methods, section 2.2.30.

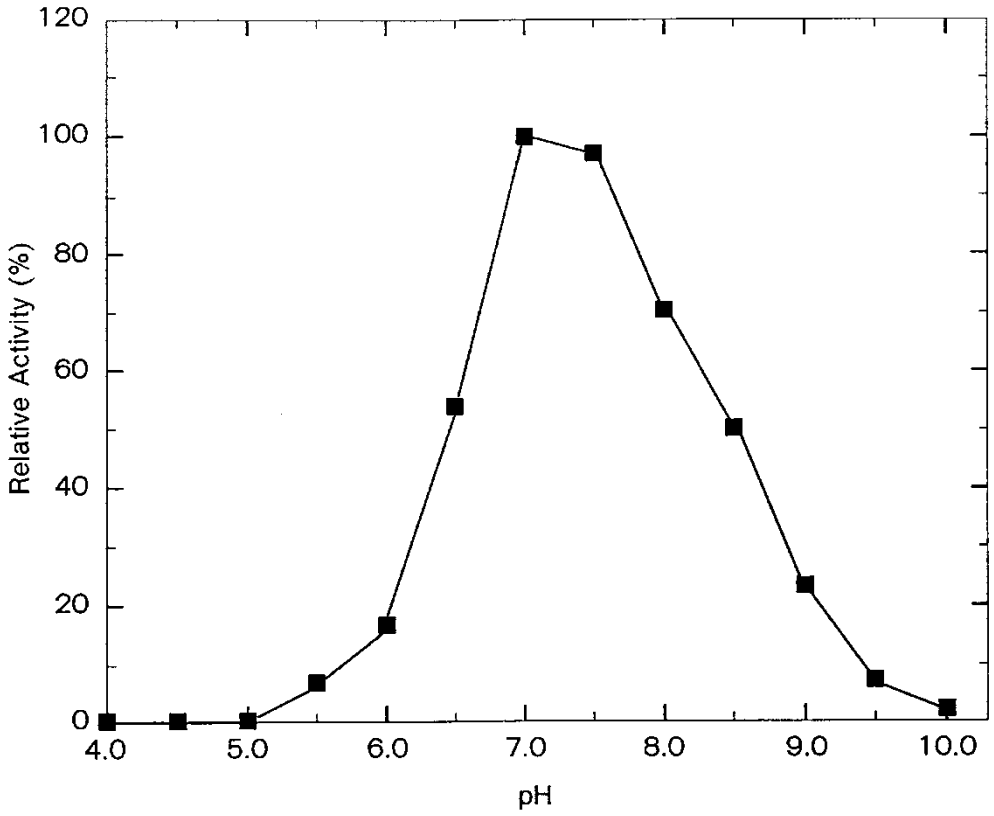


Figure 30 pH activity of BPO2.

The native BPO2 was measured for enzyme activity by MCD bromination assay as described in the Methods, section 2.2.2. in 0.1 M KPB with different pH from 4.0-10.0. The enzyme activity was compared as percentage of relative activity with the highest enzyme activity, as described in the Methods, section 2.2.31.

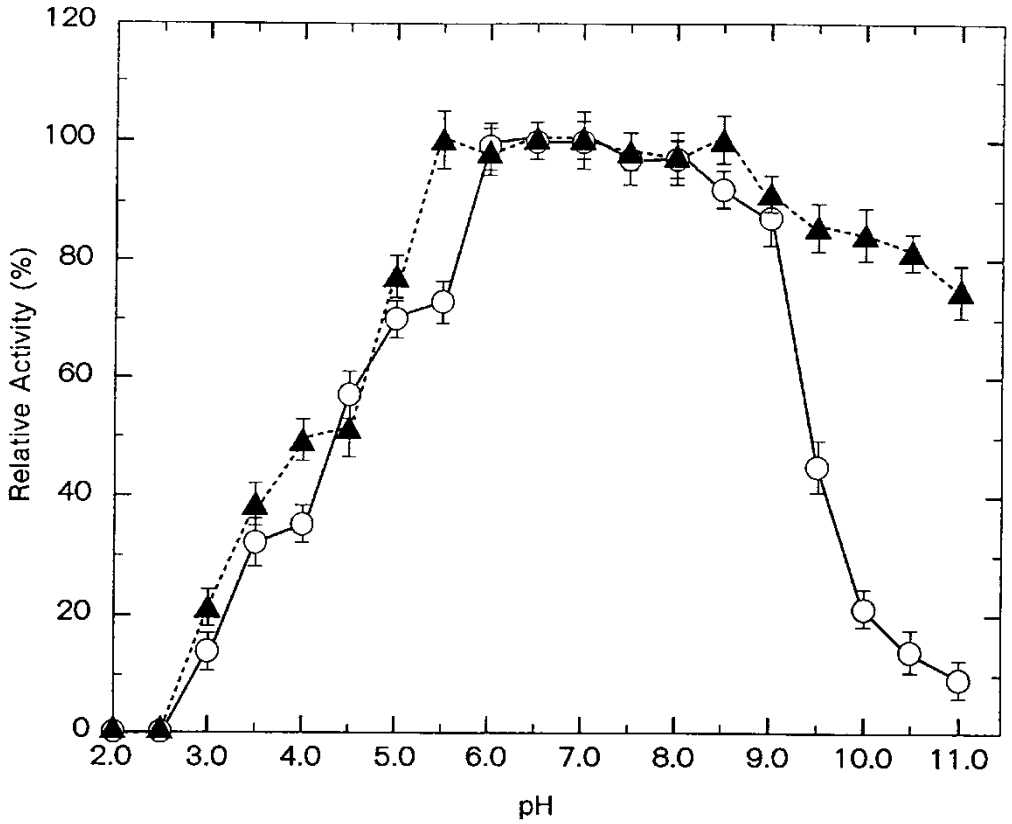


Figure 31 pH stability of BPO1 and iodoacetamide-modified BPOI at 37°C, pH 7.0.

The native BPO1 (○) and modified BPO1 Bby 50 mM iodoacetamide (▲) were incubated in the universal buffer pH 2.0-11.0 at 25°C for 15 min. During incubation, the bromoperoxidase activity in different pH were assayed and compared as percentage of relative activity with the highest enzyme activity, as described in the Methods, section 2.2.32.

Figure 32 Effect of V_2O_5 on the modified BPO1 by iodoacetamide in 10mM Tris-HCl buffer pH 8.0.

(A) BPO1 at 4°C

(B) BPO1 added with 0.3 mM V_2O_5 incubated at 4°C for 24 hours and dialysed against the same buffer

(C) BPO1 added with 50 mM iodoacetamide incubated at 35°C for 18 hours and dialysed against the same buffer

(D) The BPO1 from (B) added with 50 mM iodoacetamide incubated at 35°C for 18 hours and dialysed against the same buffer

(E) BPO1 added with 0.3 mM V_2O_5 and 50 mM iodoacetamide incubated at 35°C for 18 hours and dialysed to remove the excess and unreacted reagents against the same buffer

(F) The enzyme activity was measured using the MCD bromination and compared as percentage of relative activity with BPO1 at 4°C (A) as described in the method, section 2.2.33.

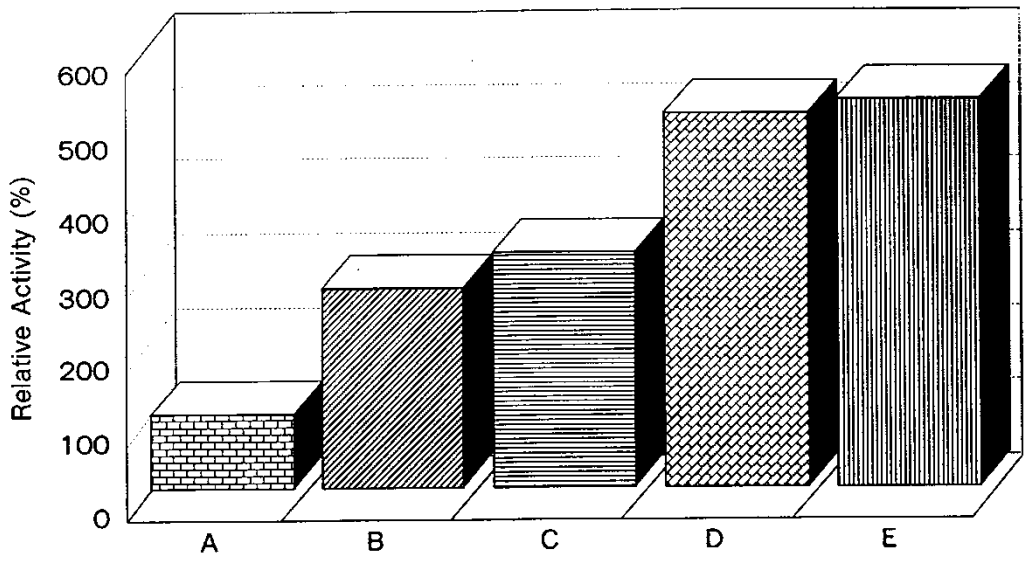

Relative Activity (%)
600
500
400
300
200
100
0
A
B
C
D
E

Table 19 Amino acid composition of BPO1.1 and BPO1.2

Amino acid	Molar percentage	
	BPO1.1	**BPO1.2**
Cysteine	3.25	8.81
Aspartic acid	11.51	15.46
Glutamic acid	14.17	12.74
Serine	8.53	6.77
Glycine	14.05	9.70
Histidine	4.25	3.09
Arginine	2.45	1.69
Threonine	3.65	2.10
Alanine	6.98	2.76
Proline	4.63	2.65
Tyrosine	1.35	1.52
Valine	5.69	6.19
Methionine	1.02	6.76
Isoleucine	4.10	3.40
Leucine	4.89	4.07
Phenylalanine	3.50	5.24
Lysine	6.02	7.07

3.18 Amino Acid Analysis of Iodoacetamide-Modified BPO1.1

BPO1.1 was eluted from the gel of non-denaturing gel after electrophoresis. The enzyme was dialysed and concentrated by speed-vac concentrator, then the enzyme was treated with iodoacetamide at 35°C, pH 7.0. At different time intervals, the enzyme mixture was withdrawn, dialyzed and concentrated. The enzyme was hydrolysed and the PTC-derivative samples were prepared for amino acid analysis by Pico-Tag HPLC system. Figure 116-120 were the HPLC profiles of amino acid analysis of hydrochloric acid hydrolysates of BPO1.1 at 4, 8, 12, 18 and 24 hours of incubation with iodoacetamide, respectively. Figures 122-126 were the HPLC profiles of performic acid oxidation prior to HCl hydrolysis of BPO1.1 t 4, 8, 12, 18 and 24 hours

of incubation with iodoacetamide, respectively. Figures 122-126 were the HPLC profiles of performic acid oxidation prior to HCl hydrolysis of BPO1.1 at 4, 8, 12, 18 and 24 hours of incubation with iodoacetamide, respectively. The results as shown in Figure 33 and 138 were percentage of some amino acid composition of the enzyme after modification at various after modification by iodoacetamide of glutamic acid, arginine, valine and phenylalanie. Glutamic acid at time zero was 17.81 molar percentage. At 4, 8, 12, 18 and 24 hours of incubation, the molar percentage of glutamic acid did not change significantly. The molar percentage of arginine, valine and phenylalanine also did not change during incubation with iodoacetamide. This suggested that iodoacetamide did not reat with these amino acids. Figure 34 showed the percentage of cysteine, methionine histidine and lysine of BPO1.1 after modification by iodoacetamide. From the result, it was shown that cysteine and methionine at time zero were 3.25 and 1.02 molar percentage respectively. At 4, 8, 12, 18 and 24 hours of inculation, the molar percentage of cysteine and methioninedid not change significantly. This showed that cysteine and methionine of BPO1.1 did not react with iodoacetamide. Histidine at time zero of incubation was 4.25 molar percentage. At 4, 8, 12, 18 and 24 hours of incubation time, the molar percentage of histidine was 3.37, 3.38, 3.48, 3.47 and 3.37 respectively. The results showed that histidine decreased during incubation, suggesting that histidine in BPO1.1 reacted with iodoacetamide. The number of the modified histidine was estimated from the molecular weight of the enzyme and amino acid. Molecular weight of BPO1.1 was 70,000 and the molecular weight of the average amino acid was 100, the total histidine of the native BPO1.1 or at time zero of incubation should be 29.75 residues per mole BPO1.1. The molar percentage of histidine after 4 hours of incubation was 3.37, so the remaining residues of histidine that was not modified were 23.59 residues per mole BPO1.1 The modified histidine were 29.75 - 23.59 = 6.16 residues per mole BPO1.1 IN the similar way, the modified histidine at 8, 12, 18 and 24 hour of incubation were 6.09, 6.44, 5.39 and 6.16 residues, respectively.

Form the results, it was shown that at 4 -24 hours after incubation, the number of histidine modified by iodoacetamide should be about 6 residues in a total of 30 histidine residues per mole of BPO1.1.

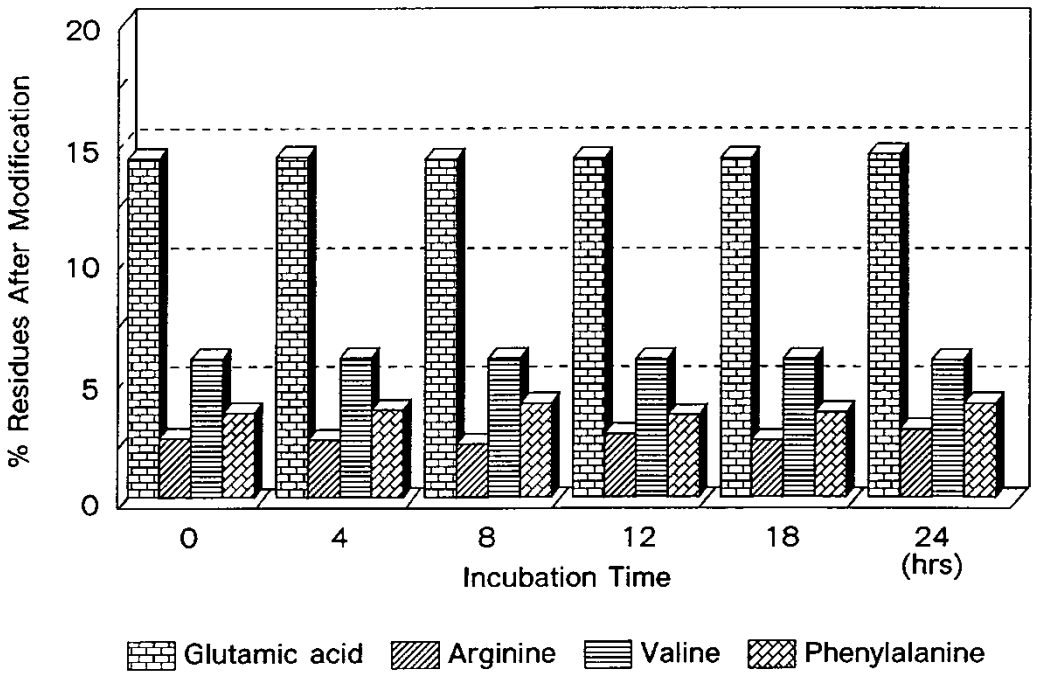


Figure 33 Percentage of glutamic acid, arginine, valine and phenylalanine residues of BPO1.1 after iodoacetamide modification.

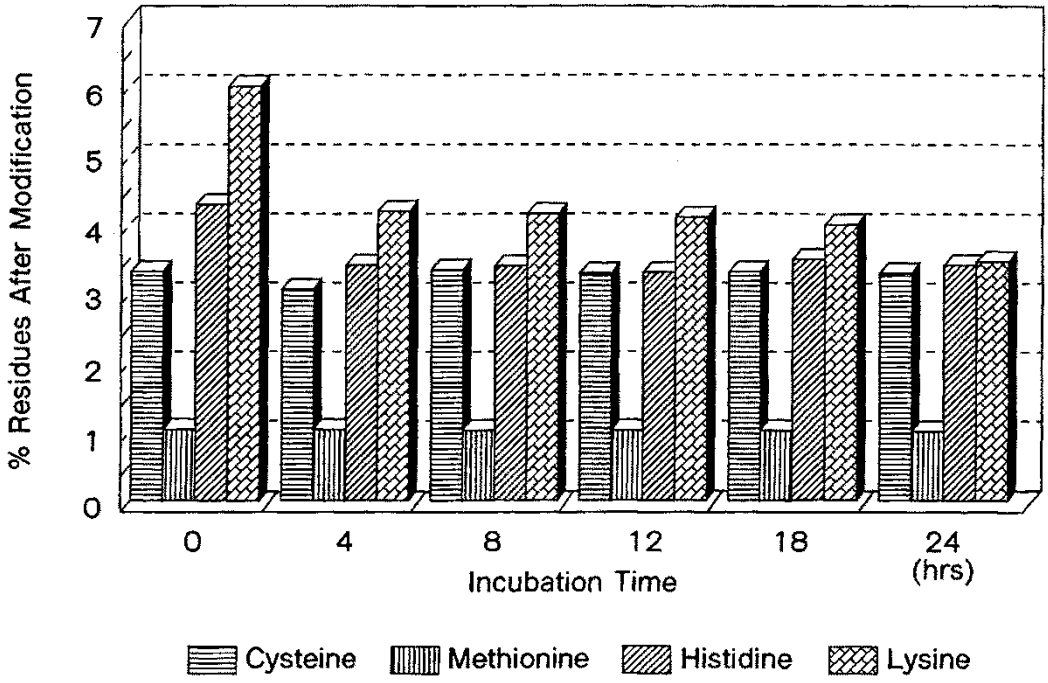


Figure 34 Percentage of cysteine, methionine, histidine, and lysine residues of BPO1.1 after iodoacetamide modification.

Lysine at time zero was 6.02 molar percentage. At 4, 8, 12, 18 and 24 hours of incubation, the molar percentages of lysine were 4.19, 3.42, 4.07, 3.98 and 3.48, respectively. This result showed that lysine decreased during incubation, suggesting that lysine in the BPO1.1 reacted with iodoacetamide. The number of modified lysine was estimated by the same method as that of the modified histidine. The total lysine in the BPO1.1 was estimated to be 42.14 residues per mole. The modified lysine per mole BPO1.1 at 4, 8, 12, 18 and 24 hours of incubation were 12.81, 19.46, 13.65, 14.28 and 17.78, respectively. So the average number of the modified lysine that caused highest activation of the enzyme activity for BPO1.1 should be 14.28 residues out of the total of 42 lysine residues at 18 hours of incubation.

3.15 Amino Acid Analysis of Iodoacetamide – Modified BPO1.2

BPO1.2 was eluted form the gel of non-denaturing gel after electrophoresis, the enzyme was dialysed and concentrated by speed-vac concentrator, then the enzyme was incubated with iodoacetamide at 35°C, pH 7.0. At different time intervals, aliquot of the reaction mixture was withdrown and dialysed and concentrated. The enzyme was hydrolysed and prepared for PTC-derivative sample for amino acid analysis by Pico-Tag HPL system. Figures 131-134 showed the HPLC profile of amino acid analysis of hydrochloric acid hydrolysate of BPO1.2, at 6, 12, 18 and 24 hours of incubation with iodoaceatamide. Figures 133-136 showed the HPLC profile amino acid analysis of the performic acid oxidation prior to HCL hydrolysis of BPO1.2 at 6, 18 and 24 hours of incubation with iodoacetamide. The results as shown in Figure 35 and 140 were the percentage of some amino acid composition of the enzyme after modification at various time intervals. Figure 36 showed the percentage of amino acid residues after modification by iodoacetamide of glutamic acid, argiinine, valine and phenylalamine. Glutamic acid at time zero was 14.12 molar percentage. At 6, 12, 18 and 24 hours of incubation, the molar percentage of glutamic acid did not change significantly. The molar percentage of arginine, valine and phenylalanine also did not change during incubation with iodoacetamide. This suggested that iodoacetamide did not react with these amino acid residues.

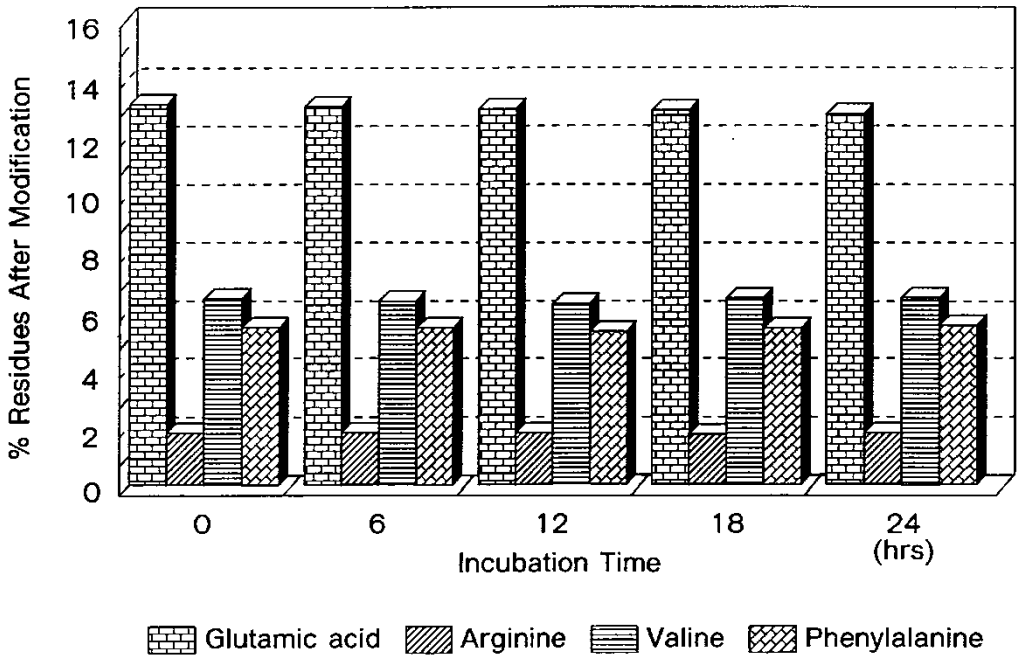


Figure 35 Percentage of glutamic acid, arginine, valine and phenylalanine residues of BPO1.2 after iodoacetamide modification.

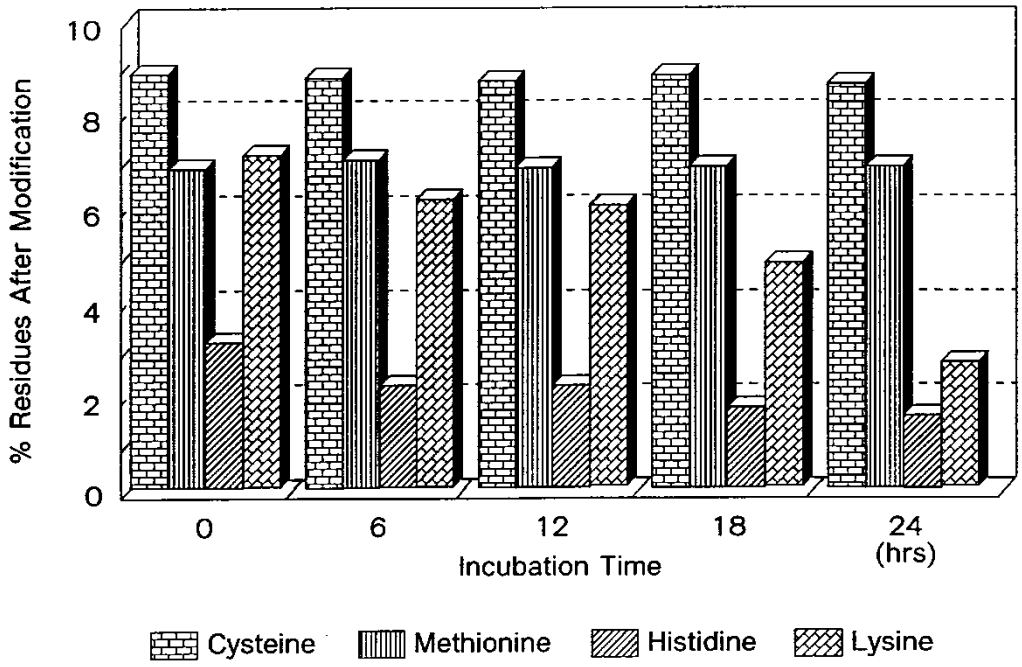


Figure 36 Percentage of cysteine, methionine, histidine, and lysine residues of BPO1.2 after iodoacetamide modification.

Figure 36 showed the molar percentange of cysteine methionine, histidine and lysine of BPO1.2 after modification by iodoaceamide. From the results, it was shown that cysteine and methionine at time zero were 8.81 and 6.76 molar percentage, respectively. At 6, 12, 18 and 24 hours of incubation, the molar percentage of cysteine and methionine did not change significantly. This showed that cysteine and methionine of BPO1.2 did not react with iodoacetamide.

Histidine at zero incubation time was 3.09 molar percentage. At 6, 12, 18 and 24 hours of incubation, the molar percentage of histidine were 2.20, 2.20, 1.74 and 1.61, respectively. The results showed that the molar percentage of histidine decreased during incubation, so histidine in BPO1.2 reacted with iodoacetamide. The number of the modified histidine, were estimated from molecular weight of the enzyme and amino acid, in the same way as that of BPO1.1. The total histidine of the native BPO1.2 or at time zero should be 21.69 residues per mole of BPO1.2. The molar percentage of histidine after 6 hours of incubation was 2.20, so the remaining residues of unmodified histidine should be 21.63-15.40 = 6.23 residues per mole BPO1.2. In the similar way, the modified histidine at 12, 18 and 24 hours of incubation were 6.23, 9.45 and 10.36 residues, respectively. From the results, it was shown that the modified histidine increased according to the incubation time. At the highest activation of the enzyme activity at 18 hours of incubation, the modified histidyl residues should be 9.5 residues out of the total 21.6 histidine residues.

Lysine at time zero was 7.07 molar percentage. At 6, 12, 18 and 24 hours of incubation, the molar percentage of lysine were 6.12, 5.98, 4.78 and 2.63, respectively. This results showed that lysine decresed during incubation, suggesting that lysine in BPO1.2, the modified lysine at 6, 12, 18 and 24 hours were 6.65, 7.63, 16.03 and 31.08 residues per mole of BPO1.2, respectively. From the results , it was shown that the modified lysine increased according to the incubation time. At the highest activation of enzyme activity at 18 hours of incubation, the modified lysine residues should be 16 residues out of the total 49.5 lysine residues per mole of BPO1.2.

The number of iodoacetamide modified amino acid residue of BPO1.1 and BPO1.2 were shown in the Table 20 and Figure 141.

Table 20 The number of iodoacetamide-modified amino acid residues of BPO1.1 and BPO1.2 during incubation

Incubation time(hrs)	**Modifird amino acid (residues/mole)**			
	Histidine		**Lysine**	
	BPO1.1	**BPO1.2**	**BPO1.1**	**BPO1.2**
0	**0.00**	**0.00**	**0.00**	**0.00**
4	**6.16**	**-**	**12.81**	**-**
6	**-**	**6.23**	**-**	**6.65**
8	**6.09**	**-**	**13.09**	**-**
12	**6.44**	**6.23**	**13.65**	**7.63**
18	**5.39**	**9.45**	**14.28**	**16.03**
24	**6.16**	**10.36**	**17.78**	**31.07**

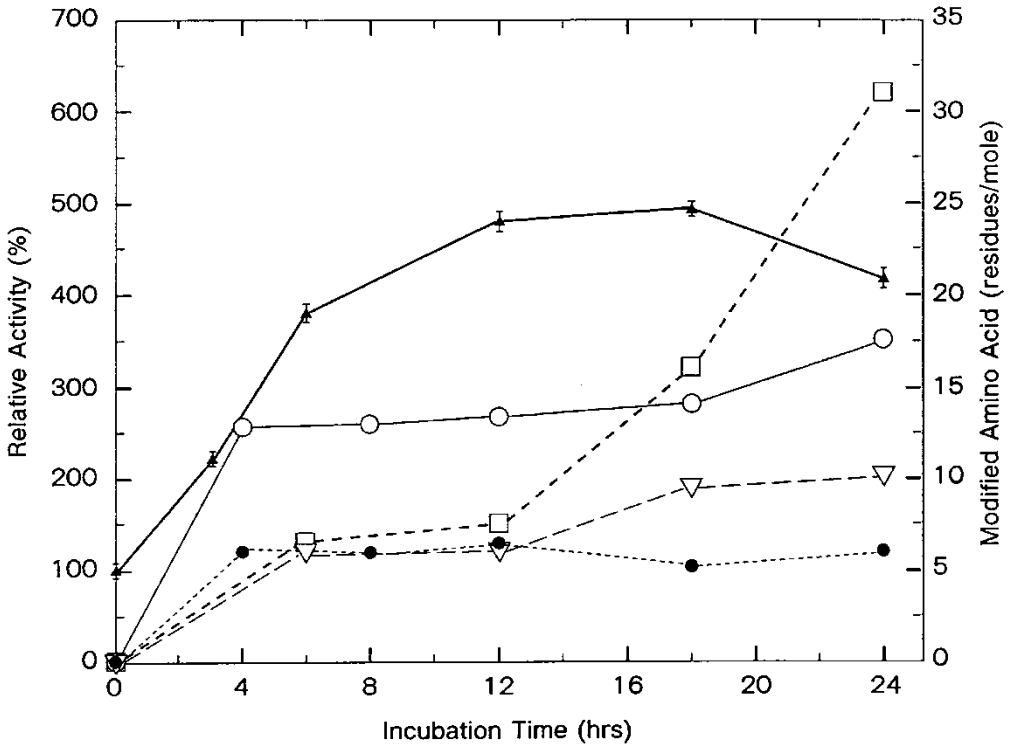


Figure 37 The relationship between the relative activity of BPO1 and the number of modified histidine residues and lysine residues of BPO1.1 and BPO1.2 during iodoacetamide modification.

▲bromoperoxidase activity of BPO1.1 during iodoacetamide modification

● modified histidine residues of BPO1.1

∇ modified histidine residues of BPO1.2

CHAPTER IV

DISCUSSION

4.1 Purification of Bromoperoxideses from seaweed

Bromoperoxideses used in this study were purified from red seaweed, *Polycarvernosa* sp., which was harvested from Lhaem Sok beach in Trad province. DE52 column chromatography was used for purification of bromoperoxidase. The suitable pH range for column chromatography was about 7.4-7.8. At pH 7.4, the resolution of 2 isoenzymes of the enzyme, BPO1 and BPO2 was poor while at pH 7.8 or higher BPO1 was not stable while BPO2 was not affected. So, in this step, pH 7.6 was chosen for isolation of the isoenzyme. The chromatographic profile showed that BPO1 was eluted befor BPO2, suggesting that BP01 had less negative charges than BPO2. In other studies, anion exchange chromatography was also used to isolate the isoenzyme of crude bromoperoxidess from *Strephomyces griseus* which contained three isoenzymes (259), and *Streptomyces griseus* which contained two isoenzymes (130).

DE52 column chromatography was suitable for purification of enzyme in large scale. In addition, DE52 column had taken part to eliminate the pigment and viscosity of the enzyme solution before further purification on ManoQ column by using FPLC.

In ManoQ column chromatography, which gave higher resolution than DE52, BPO1 was purified to 8.14 fold and still contained a little pink pigment while BPO2 was free from pigment. This suggested that the pigment had charge characteristic similar to that of BPO1.

Both BPO1 and BPO2 were further purified by using Superrose12 column. The pigment was separated from BP01, resulting the purified BPO1 with specific acitivity of 61.86 mU/mg with 12.89 fold purification. BPO2 was purified to 68.87 fold in Superrose 12 column. The molecular weight of BPO1 and BPO2 estimated from standard protein marker were 70,000 and 48,000, respectively.

As shown in Figure 32, BPO1 after purification by Superose 12 column contained two isoenzyme BPO1.1 and BPO1.2, which appeared as homogeneous band on non-denaturing gel electrophoresis. In this study, BPO1.1 and BP1.2 were not separated from each other by column chromatography, this might be due to similar characteristic between BPO1.1 and BPO1.2 (Figure 111). Amino acid composition of BPO1.1 and BPO1.2 as shown in

Table 19 also showed similar amino acid composition, except BPO1.2 contained more cysteine than BPO1.1. Results from Superose 12 also indicated that BPO1.1 and BPO1.2 had similar molecular weight. Thus, BPO1.1 and BPO1.2 had similar characteristics, i.e., charge, size and amino acid composition. BPO1 was purified by anion exchange chromatography and gel filtration, due to these similar characteristics, BPO1.1 and BPO1.2 did not separated from each other, however, both isoenzymes were separated as homogeneous on non-denaturing gel electrophoresis.

BPO2 showed a single homogeneous protein band in the electrophoresis pattern. BPO2 seemed to be different from the other proteins of crude extract in charge characteristic, so BPO2 was almost lastly eluted from DE52 column due to highly anionic characteristic, so BPO2 was purified with higher yield than BPO1. BPO2 differed from BPO1 in several aspects, i.e, charge characteristic size, electrophoresis pattern, optimum pH, pH stability, specific activity, thermo-stability and effect of chemical modification. These might come from the differences in amino acid composition, amino acid conformation of the enzyme.

Studies on chemical modification of BPO1 and BPO2 might elucidate the amino acid residues that might play role to the catalytic function of the enzyme in the view of the isoenzyme of bromoperoxidase. On the other hand, chemical modification changed the specificity and enzymatic properties that might be the advantages for the development for further sued in the biotechnology field.

The stability of both iodoacetic acid-modified BPO1 and BPO2 decreased, suggesting that the introduced negatively changed groups decreased stability of the enzyme.

4.2 Iodoacetamide Modification of BPO1 and BPO2

The reaction of Iodoacetamide with proteins depends on pH, temperature time and concentration of iodoacetamide. In this study, the reaction of the modification was carried out according to the method of Harada and Irie (256), Holeysosky and Lazdunski (258) The incubation time for complete reaction could be varied from 2-4 hours to 100 hours, and the temperature used in the reaction could be varied from 25 to 43 °C. Temperature higher than 40 °C might decompose certain modified amino acidic residues, such as S-carboxamidomethylmethionine sulfonium salt of the modified methionyl residue and caused cleavage of peptide bond at methionyl residue. The pH used for the reaction of modification was from 2-8.5, depending on the amino acid residues desired to modify.

Concentration of iodoacetamide used could be at about 2-3 to ten thousand molar excess. In this study, the concentration used was 0.25-250 mM, at 35 °C, pH 7.0 for 18 hours. The result in Figure 19 showed that maximal enhancement of BPO1 activity (500%) occurred at 25 mM iodoacetamide.

In contrast, BPO2 activity was inhibited by iodoacetamide modification This difference had led to a suggestion that BPO1 and BPO2 had different conformation different amino acid might participate in the activity and/or maintenance of the enzyme activity.

Themostability of iodoacetamide-modified BPO1 was different from BPO2, i.e. the modification resulted in increased stability of BPO1 but had no effect on BPO2. An increase enzyme activity of BPO1 might cause an increased enzyme stability in a similar way to the case of TNBS- modified BPO1 in which lysyl residues were modified; the modified lysyl residues might enhance the activity and stability of BPO1. These different effects between BPO1 and BPO2 might be due to the different structure and amino acid composition of the enzyme.

4.3 Effect of pH on Iodoacetamide Modification

Modification of amino acid residue in protein is pH dependent since the ionizable group of amino acid residue is involved. At pH 5.0, no increase in enzyme activity was observed, suggesting that iodoacetamide did not react with amino acid residue in the enzyme or reacted with amino acid residue that did not involve in the catalytic function. The probable amino acid residues that could be modified were residue and/or histidyl residue Horseradish peroxidase were inactivated by alkylation of both histidyl and methionyl residues, depending on the pH, from amino acid analysis it was assumed that the inactivation rate increased as the pH was raised from 7.0 to 7.9, this could be reflection of enzyme of enzyme inactivation through preferential alkylation of histidyl residues, while the increased inactivation rate at lower pH values might be a consequence of alkylating of mehionyl residues (260).

Modification at pH 7.0 caused an enhancement in enzyme activity. The amino acid residues that were susceptible to modification could be sulfhydryl group of cysteinyl, methionyl, histdyl and lysyl residues. From the result of DTNB modification, no product of TNB^{2-} was observed, suggesting that sulfhydryl group of cysteinyl residue might be in the form of disulfide bridge. The result of amino acid analysis (Figure 115-120) and Figure 127-131) also suggested that no cysteinyl residue was reacted with iodoacetamide. Result of amino acid analysis also showed that histidine and lysine were lost during modification with iodoacetamide but

methionine was not lost during iodoacetamide modification, thus histidyl and lysyl residues should react with iodoacetamide resulting in activation of the enzyme activity. The modified amino acid residues might be involved in acceletation of the catalytic reaction or changed the conformation of the enzyme to be more active form.

Modification of the enzyme at pH 8.5 increased the thermostability of the enzyme. At pH 8.5, the susceptible amino acid residues were similar to those at pH 7.0, i.e., cysteinyl, methionyl, histidyl and lysyl residue. The result that the activity of the modified enzyme was not enhanced but its stability increased suggested that the modified amino acid residues enhanced the stability of the enzyme under condition studied. On the other hand, it could be possible that the modified residues might cause an enhancement in enzyme activity, however the modified enzyme was rather unstable at alkaline pH.

The different effect of iodoacetamide modification at pH 5.0, 7.0 and 8.5 might be due to the different rate of modification of amino acid residues in the enzyme. The neutral pH was optimal for the iodoacetamide modification tor enhancement of the enzyme activity and stability under condition studied.

4.4 Effect of Temperature on Iodoacetamide Modification of BPO1

The results on iodoacetamide modification at different temperatures in Figure 46 showed that the activity of the modified BPO1 was highest at 35°C. Increased temperature might increase the chemical reaction of modification, however, high temperature might also denature the native enzyme and might decompose the modified residues. So optimum temperature should be determined in modification of the enzyme. The results suggested that at 45°C the native BPO1 might be denatured during incubation and some modified residues might be decomposed. Although the native BPO1 was stable, but the reaction of iodoacetamide with amino acid residues was slow at temperature 25°C, so the enzyme activity was activated to a lesser extent than those at 35 and 45°C.

4.5 Effect of Temperature on BPO1 AND Iodoacetamide-Modifed BPO1

From the results (Figure 24), the native enzyme and the modified enzyme had similar pattern of temperature profile. It might be that at low temperature, the modified amino acid residues might be participated int the maintenance of the tertiary structure but when the temperature increased, the modified residues of the modified enzyme might be decomposed and

could not participate in the maintenance of the tertiary structure, resulting in similar decay rate between native and modified enzyme.

4.6 Effect of pH on Activity and Stability of Native and Modified BPO1

The native BPO1 and iodoacetamide-modified BPO1 showed pH optimum in the acidic pH range, about 5.3-5.7 (Figure 29). Most of non-heme bromoperoxidase have pH optimum at 4.3-7.4 (19-20). The acidic optimum pH about 5.5 of the modified enzyme suggested that protonation steps might be involved in the catalytic reaction. The modified enzyme showed wider range of optimal pH than the native enzyme. pH optimum in the bromination reaction might be varied according ton the concentration of substrates, H_2O_2 and Br^-. Bromoperoxidase from *A. nodosum* showed distinct activity optima which, at affixed concentration of Br^-, shifted to lower pH values when the concentration of H_2O_2 was increased and this effect caused the higher K_m. On the other hand when the concentration of Br^- was increased the optima pH shifted to higher pH values (20). The decreased enzyme activity at low pH might be due to the formation of reversible inhibitory protonated complex. For iodoacetamide-modified enzyme, the shift of optimum pH to lower pH than the native enzyme suggested that the inhibitory protonated complex was formed less than in the naïve enzyme.

The native BPO1 and iodoacetamide-modified BPO1 were stable at pH 6-9, the activities of both native and modified enzyme decreased at pH lower than 6. However, at pH higher than 9.0, native BPO1 was rapidly denatured while the modified BPO1 was still stable; activity of the modified BPO1 decreased to about 20% at pH 11.0 (Figure 31). It could be possible that the modified enzyme contained the modified residues with more hydrophobic than the native enzyme, the modified hydrophobic residues might maintain the enzyme conformation from denaturation in the extreme pH. This result had led ton a suggestion that hydrophobic interaction increased pH stability of the enzyme.

4.7 Thermostability of native BPO1 and Iodoacetamide-Modified BPO1

The native BPO1 lost activity very rapidly at 45°C. Thermostability of BPO1 was similar to both bromoperoxidase from red alga *Ceramium rubum* which was not stable at high temperature (123). The iodoacetamide-modified BPO1 resulted in improving thermostability of the enzyme. Modification caused and enzyme increased in the half-life more than 2-fold and 25°C and 37°C, and the half-life of the modified enzyme was slightly increased at 45°C. This

suggested that the modified enzyme might have modified residues that enhance the maintenance of the tertiary structure, probably through increasing the rigidity of the conformation. However, the modified residues might be decomposed when the temperature increased.

Thermostability of the enzyme was found to be dependent on pH (Table 20). The half-life of the native enzyme at pH 5.5 was less than the half-life at pH 7.0, suggesting that at low pH value the native enzyme was less stable, apparently due to effect of charge density. The modified enzyme showed the enhancement of themostability at pH 5.5 and 7.0, 25°C when compared to the naïve enzyme. This suggested that at low pH value, certain modified residues might have hydrophobic property and thus the hydrophobic interaction stabilized the enzyme from acidic pH denaturation.

4.8 Effect of V_2O_5 on the Modification of BPO1 by Iodoacetamide

Tris-HCl buffer pH 8.0 was used in studying activation effect of V_2O_5. Lower pH decreased the activating effect of V_2O_5 while the higher pH caused unstability of the enzyme. Tris-HCl buffer was used instead of KPB to prevent the effect of phosphate which is an anolog of V_2O_5. The results showed that naïve and iodoacetamide-modified enzyme were equally activated to about 300%. The results in this study (Figur 104) suggested that enhancement of the enzyme activity by V_2O_5 might be due to the binding of V_2O_5 to the apoenzyme and the enhancement of the enzyme activity by iodoacetamide might be resulted from iodoacetamide modified holoenzyme. In addition, iodoacetamide modification did not interfere the binding between V_2O_5 and enzyme. The resulted suggested that iodoacetamide might modify the amino acid residues not involving in the catalytic site or the site that V_2O_5 bound to the enzyme.

CHAPTER V

CONCLUSIONS

1. Bromoperoxidase was extracted and purified from Thai red seaweed *Polycarvernosa* sp. and two isoenzymes, BPO1 and BPO2 were separated in DE52 column chromatography.
2. After purification by fast protein liquid chromatography, the specific activity of BPO1 and BPO2 was 61.86 and 330.58 mU/mg, with 12.89 and 68.87 purification fold, respectively.
3. Both BPO1 and BPO2 were non-heme enzymes and they were activated about 2.5 and 3.5 fold by V_2O_5, respectively.
4. Chemical modification of BPO1 by TNBS and iodoacetamide coused on enhancement of the enzyme activity at 37°C, pH 7.0.
5. Chemical modification of BPO1 by NBS, HNBB, p-nitrophenylsulfenyl chloride, EDC and iodoacetic acid inactivated the enzyme activity, none of these chemical modifying agents caused an enhancement in enzyme stability.
6. Chemical modification of BPO1 by N-acetylimidazole, DEP,1,2-cyclohexanedione had no effect on the enzyme activity and none of these chemical modifying agents caused an enhancement of the enzyme stability at 37°C, pH7.0.
7. Chemnical modification of BPO2 by TNBS, NBS, HNBB, DEP, EDC, iodoacetic acid iodoacetamide inactivated the enzyme activity. These chemical modifying reagents did not enhance the enzyme stability.
8. Chemical modification of BPO2 by N-acetimidazole, o-nitrophenylsulfenyl chloride, 1, 2, - cyclohexanedione not change the enzyme activity and none of these modifying reagents caused an enhancement of the enzyme stability.
9. The optimal condition for iodoacetamide modification of BPO1 that caused the highest enzyme activity (500% relative activity) was at pH 7.0, 35°C for 18-20 hours.
10. The half-life values of iodoacetamide-modified BPO1 at 25, 37 and 45°C were more than 144, 108 and 20 hour, respectively while the half-life values of the native BPO1 were 57, 18, and 16 hours, respectively.
11. Activity of iodoacetamide-modified BPO1 did not change significantly for at least 9 days in pH 5.5 at 25 °C while the activity of the native enzyme was completely lost after 4 days.
12. The optimum pH of native and iodoacetamide-modified BPO1 were found to be similar, i.e., at pH range 5.0-5.5.

13. The modified enzyme was more stable at pH 8.5-11.0 than the native enzyme.
14. The activity of BPO1 during modification could be activated about 2 fold by $V_2 O_5$ 10 mm Tris-HCI buffer pH 8.0.
15. K_m values of iodoacetamide-modified BPO1 were 1.43 x 10^5, 1.54 x 10^2 and 9.09 x 10^7M for MCD, KBr and H_2O_2, respectively, while K_m values of the native BPO1 were 2.94 x 10^5, 2.17 x 10^4 and 1.00 x 10^4 M respectively.
16. BPO1 contained two isoenzymes , BPO1.1 and BPO1.2 when it was separated by non-denaturing gel electrophoresis.
17. pI values of BPO1.1 and BPO1.2 were 5.50 and 5.25 respectively while pI values of the iodoacetamide-modified BPO1.1 and iodoacetamide-modified BPO1.2 were 5.40 and 5.20, respectively.
18. BPO1.1 and BPO1.2 composed of 25.68 and 28.22 molar percentage, of acidic amino acid respectamied.
19. Incubation with iodoacetamide at 35 °C, pH 7.0 for 18 hours, BPO1.1 contained 6 residues of modified histidine from the total of 30 residues and contained 14 residues of modified lysine from the total of 42.8 residues.
20. Incubation with iodoacetamide at 35°C, pH 7.0 for 18 hours, BPO1.2 contained 9.5 residues of modified histidine from the total of 21.6 residues and contained 16 residues of modified lysine from the total of 49.5 residues.
21. The amount of cysteine in iodoacetamide-modified BPO1.1 and BPO1.2 did not alter from the native enzyme.
22. TNBS-modified BPO1 enhanced the enzyme activity to 150% relative activity and also enhanced the stability for about 3 times while DEP-modified BPO1 did not change the enzyme activity and stability.
23. Results from chemical modification study of BPO1 and BPO2 suggested that the carboxyl group and tryptophanyl residues might be involved in the catalytic activity.
24. Results from chemical modification study of BPO1 and BPO2 suggested that the tyrosyl and arginyl residue might not be involved in the catalytic activity and stability of the enzyme.
25. The modified lysyl and histidyl residues of BPO1 might be involved in the enhancement of the enzyme activity and stability.

CHAPTER VI

BIBLIOGRAPHY

1. Neidleman SL, Geigert S. Biological halogenation : Roles in nature, potential in industry. Endeavour New Series 1987; 11 : 5-15.
2. Sauvageau C. Localization of bromine in a Floridian alga (Antithamnionella sarniensis Lyle). Compt Rend Acas Sci [Paris] 1925; 181 : 841-843.
3. Sauvageau C. On a note of Chemin and Legendre on the existence of free iodine in *F. doubletti.* Copmt Rend Acad Sci [Paris] 1926; 183:1006-1007.
4. Agener K. Verdoperoxidase. Acta Physiol Scand 1941; 2, suppl 8 : 5-62.
5. Theorell H. Akeson A. Purified milk peroxidase. Arkiv Kemi Min Geol 1943; 17B : 6.
6. Dempsey EW. Fluorescent and histochemical reactions in the rat thyroid gland at different state of physiological activity. Endocrinolvgy 1944; 34 : 27-28.
7. Morita Y, Kameda K. Phytoperoxidase VIII. A new peroxidase of Japanese radish. Mem Res Inst Food Sci Kyoto 1957; 12 : 1-13.
8. Show PD, Hager LP. An enzymatic chlorination reaction. J Am Chem Soc 1959; 81 : 1011-1012.
9. Archer GT, Air G, Jackas M, Morell DB. Studies on rat eosinophil pexoxidase. Biochim Biophys Acta 1965; 99 : 96-101.
10. Bolscher BGJM, Wever R. The nitrosyl compounds of ferreous animal haloperoxidases. Biochim Biophys Acta 1984; 791 : 75-81.
11. Keilin D, Hartree EF. Purification of horse-radish peroxdase and comparison of its properties with those of catalase and methaemoglobin. Biochem J 1951; 49 : 88-104.
12. Hosoya T. Turnip peroxidase I. Purification and physicochemical properties of multiple components in turnip peroxidase. J Biochem (Tokyo) 1960; 47 : 369-381.
13. Ahern Tj, Allen GG, Medcalf DG. New bromoperoxidase of marine origin partial purification and characterization. Biochim Biophys Acta 1980; 616 : 329-339.
14. Theiler RF, Siuda JS, Hager LP. In : Drugs and food from the Sea Myth or Reality : Edited by Kaul PN, Sinderman CJ. The University of Oklahoma, Norman, Oklahoma, 1978 : 153-169.
15. Manthey JA, Hager LP. Purification and properties of bromoperoxidase. J Biol Chem 1981; 256 : 11232-11238.

16. Yamaka H, Itoh N, Murakami S, Izumi Y. New bromoperoxidase from coralline algae that brominates phenol compounds. Agric Biol Chem 1985; 49 : 2961-2967.

17. Wever R, Plat H, de Boer E. Isolation procedure and some properties of the bromoperoxidase from the seaweed *Ascophyllum nodosum.* Biochim Biophys Acta 1985; 830 : 181-186.

18. Plat H, Krenn BE, Wever R. The bromoperoxidase from the lichen *Xanthoria purietina* is a novel vanadium enzyme. Biochem J 1987; 248 : 277-279.

19. Morris DR, Hager LP. Chloroperoxidase I. Isolation and properties of the crystalline glycoprotein. J Biol chem. 1966; 241 : 1763-1768.

20. Hunter-Cevera JC, Sotos L. Screeing for a new enzyme in nature haloperoxidase production by death-valley dematiaceous hyphomycetes. Micro Ecol 1986; 12 : 121-127.

21. van Pee' K-H, Lingens F. Purification and molecular and catalytic properties of bromoperoxidase from *Streptomyces phacochromogenes*. J Gen Microbiol 1985; 131 : 1911-1916.

22. Wiesner W, van Pee' K-H, Lingens F. Purification and properties of bromoperoxidase from *Pseudomonas pyrrocinia*. Biol Chem Hoppe-Seyler 1985; 366 : 1085-1095.

23. van Pee'. K-H, Linegens F. Purification of bromoperoxidase from *Pseudomonas aureofaciens*. J Bact 1985; 161 : 1171-1175.

24. Taurog A. Thyroid peroxidase-catalyzed iodination of thyroglobuline; inhibition by excess iodide. Arch Biochem Biophy 1970; 139 : 212-220.

25. Murphy MJ. O'h Eocha C. Partial purification and characterization of an iodide peroxidase from *Enteromorpha linza*. Biochem J 1970; 119 : 21-22.

26. Nichol AW, Angel LA, Moon T, Clezy PS. Lactoperoxidase haem, an iron porphyrin thiol. Biochem J 1987; 247 : 147-150.

27. Deits T, Farrance M, Kay ES, et al. Purification and properties of ovoperoxidase, the enzyme responsible for hardening the fertilization membrane of the sea urchia egg. J Biol Chem 1984; 254 : 13525-13533.

28. Jannun R, Coe El. Bromoperoxidase from the marine snail, *Murex truneulus*. Comp Biochem Physiol 1987; 88B : 917-922.

29. Murphy MJ, O'h Eocha C. Peroxidase activity in the brown alga *Laminaria digitata.* Phytochemistry 1973; 12 : 2645-2648.

30. Baden DG, Corbett MD. Bromoperoxidase from *Penicillu capitatus, Penicillus lamourouxii* and *Rhipocephalus phonix*. Biochem J 1980; 187 : 205-211.

31. Murphy MJ, O'hEocha C. Peroxidase from the red alga *Cystoclonium purpureum*. Phytochemistry 1973; 12 : 55-59.

32. Pederson M. A brominating and hydroxylating peroxidase from the red alga. *Cystoclonium purpureum.* Physiol Plant 1976; 37 : 6-11.

33. Bakkenist AR, Wever R, Vulsma T, Plat H, van Gelder BF.Isolation procedure and some properties of myeloperoxidase from human leucocytes. Biochim Biophys Acta 1978; 524 : 45-54.

34. Bolscher BG, Plat H, Wever R. Some properties of human eosinophil peroxidase, a comparison with other peroxidases. Biochim Biophys Acta 1984; 784 : 177-186.

35. Manthey JA, hager LP. Characterization of the catalytic properties of bromoperoxidase. Biochemistry 1989; 28 : 3052-3057.

36. Neidleman SL, Geigert J. Biologenation : Principles, basic roles and applications. 1 st ed. Chichester : Ellis Horwood, 1986.

37. de Boer E, van Kooyk Y, Tromp MGM, Plat H, Wever R. Bromoperoxidase from *Ascophyllum nodosum*: 9 novel class of enzymes containing vanadium as a prosthetic group ? Biochim Biophys Acta 1986; 869 : 48-53.

38. Krenn BE, Plat H, Wever R. Purification and some characteristics of a non-haem bromoperoxidase from *Streptomyces aureofaciens*. Biochim Biophys Acta 1988; 952 : 255-260.

39. Zeiner R, van-Pee'K-H, Lingens F. Purification and partial characterization of multiple bromoperoxidases from *Streptomyces griseus.* J Gen Microbiol 1988; 134 : 3141-3149.

40. Dumontet C, Rousset B. Identification, purification and characterization of a non-heme lactoperoxidase in bovine milk. J Biol Chem 1983; 258: 14166-14172.

41. Vilter H. Peroxidases from Phaeophyceae 3. Catalysis of halogenation by peroxidase from *Asscophyllum nodosum* (L) Le-Jol. Bot Mar 1983; 26 : 429-435.

42. Vilter H. Peroxidase from phaeophyceae : a vanadium (V)-dependent peroxidase from *Ascophyllum nodosum*. Phytochemistry 1984; 23 : 1387-1390.

43. de Boer E, Boon K, Wever R. Electron paramagnetic resonance studies on conformational states and metal ion exchange properties of vanadium bromopersoxidase. Biochemistry 1988; 27 : 1629-1635.

44. Vilter H, Rechar D. ^{51}V NMR investigation of a vanadate (V)-dependent peroxidase from *Ascophyllum nodosum* (L.) Le Jol. Inorg Chem Acta 1987; 136 : L7-L10.

45. de Beer E, Keijzers CP, Klassen A-AK, et al. ^{14}N-Coordination to VO^{2+} in reduced vanadium bromoperoxidase an electron spin echo study. FEBS Lett 1988; 235 : 93-97.

46. Hormes J, Kuetgens U, Chauvistre R, et al. Vanadium K-edge absorption spectrum of bromoperoxidase from *Ascophyllum nodonum*. Biochim Biophys Acta 1988; 956 : 293-299.

47. Arber JM, de Boer E, Gamer CD, Hasnain SS, Wever R. Vanadium K-edge X-ray absorption spectroscopy of bromoperoxidase from *Ascophyllum nodosum*. Biochemistry 1989; 28 : 7968-7973.

48. van Pee' K-H, Sury G, Lingens F. Purification and properties of a nonheme bromoperoxidase *Streptomyces aureofacieus.* Biol Chem Hoppe-Seyler 1987; 368 : 1225-1232.

49. Everett RR, Kanofsky JR, Butler A. Mechanistic investigation of the novel non-heme vanadium bromoperoxidase : Evidence for singlet oxygen formation. J Biol Chem 1990; 265 : 4908-4914.

50. Yamada H. Itoh N, Izumi Y. Chloroperoxidase-catalyzed halogenation of trans-cinnamic acid and its derivatives. J Biol Chem 1985; 260 : 11962-11969.

51. de Boer E, Wever R. The reaction mechanism of the novel vanadium-bromoperoxidase. A steady state kinetic analysis. J Biol Chem 1988; 263 : 12326-12332.

52. Clague MJ, Keder NL, Butler A. Biomimics of vanadium bromoperoxidase : vanadium (V)-schiff base catalyzed oxidation of bromide by hydrogen peroxide. Inorg Chem 1993; 32 : 4754-4761.

53. Morrison M, Schonbaum GR. Peroxidase-catalyzed halogenation. Ann Rev Biochem, vol 45. Edited by Snell EE, Boyer PD, Meister A, Richardson CC. Palo Alto California : Annual Review Inc, 1976 : 861-888.

54. Lambeir AM, Dunford HB. A steady state kinetic analysis of the reaction of chloroperoxidase with peracetic acid, chloride, and 2-chlorodimedone. J Biol Chem 1983; 258 : 13558-13563.

55. Dunford HB, Lambeir AM, Kashem MA, Pickard M. On the mechanism of chlorination by chloroperoxidase. Arch Biochem Biophys 1987; 252 : 292-302.

56. Lewin M. Bromine and its compounds. Edited by Jolles ZE. Chapter 8 London : Ernest Benn, 1966.

57. Kanofsky JR. Singlet oxygen production by chloroperoxidase-hydrogen peroxide-halide systems. J Biol Chem 1984; 259 : 5596-5600.

58. Kanafsky JR, Hoogland H, Wever R, Weiss SJ. Singlet Oxygen production by human eosinophils. J Biol Chem 1988; 263 : 9692-9696.

59. Franssen MCR, Jansma JD, Van der plas HC, De Boer E, wever R. Enzymatic bromination of barbituric-acid and some of its derivatives. Bioorg chem. 1988; 16 : 352-363.

60. Itoh N, Hasan AK, Izumi Y, Yamada H. Substrate specificity, regiospecificity and stereospecificity of halogenation reactions catalyzed by non-heme-type bromoperoxidase of *Corallina pilulifera*. Eur J Biochem 1988; 172 : 477-484.

61. Thomas EL, Bates KP, Jefferson MM. Peroxidase antimicrobial system of human saliva : requirements for accumulation of hypothiocyanite. J Dent Res 1981; 60 : 785-796.

62. Thomas EL, Pera KA, Smith KW, Chwang AK. Inhibition of *Streptococcus mutans* by the lactoperoxidase antimicrobial system. Infect Immun 1983; 39 : 767-778.

63. Carlsson J. Iwami Y, Yamada T. Hydrogen peroxide excretion by oral *Streptococci* and effect of lactoperoxidase-thiocyanate-hydrogen peroxide. Infect Immun 1983; 40 : 70-80.

64. Zajak M, Bjorck L, Claesson O. Antibacterial effect of the lactoperoxidase system against *Bacillus cereus.* Milchwissenschaff 1981; 36 : 417-418.

65. Tenovuo J, Makinen KK, Sievers G. Antibacterial effect of lactoperoxidase and myeloperoxidase against *Bacillus cereus.* Antimicrob Agents Chemother 1985; 27 : 96-101.

66. Thomas EL, Aune TM. Susceptibility of *Escherichia coli* to bactericidal action of lactoperoxidase, peroxide, and iodide or thiocyanate. Antimicrob Agents Chemother 1978; 13 : 261-265.

67. Marshall VME, Reiter B. Comparison of the antibacterial activity of the hypothiocyanite anion towards *Streptococcus lactis* and *Escherichia coli*. J Gen Microbiol 1980; 120 : 513-516.

68. Reiter B, Harnulv BG. The preservation of refrigerated and uncooled milk by its natural lactoperoxidase system. Dairy Ind Internet 1982; 47 : 13-19.

69. Purdy MA, Tenovuo J, Pruitt KM, White Jr WE. Effect of growth phase and envelope structure on susceptibility of *Salmonella typhimurium* to the lactoperoxidase-thiocyanate-hydrogen peroxide system. Infect Immum 1983; 39 : 1187-1195.

70. Mickelson MN. Antibacterial action of lactoperoxidase-thiocyanate-hydrogen peroxide on *Streptococcus agalactiae*. Appl Environ Microbiol 1979; 38 : 821-826.

71. Neidleman SL, Geigert J. Volatile halogenated pollutants. The biological contribution. Trends Biot 1985; 3 : 193-194.

72. Fenical W. Halogenation in Rhodophyto-Review. J Phycol 1975; 11 : 245-259.

73. McConnel OT, Fenical W. Halogenated metabolites – including favorsky rearrangement product- - from the red seaweed *Bonnemaisonia nootkana.* Tetrahedron Lett 1979; 48 : 4159-4162.

74. Fenical W. Natural products chemistry in the marine environment. Science 1982; 215 : 923-928.

75. Burkholder PR, Pfister RM, Leitz FH. Production of a pyrrole antibiotic by a marine bacterium. Appl Microbiol 1966; 14 : 649-653.

76. Ashworth RB, Cormier MJ. Isolation of 2,6-dibromophenol from the marine hemichordate, *Balanoglossus biminiensis.* Science 1967; 155 : 1558-1559.

77. Fenical W. Polyhaloketones from the red seaweed *Asparagopsis taxiformis.* Tetrahedron Lett 1974; 51/52 : 4463-4466.

78. Sheikh YM, Djerassi C. 2,6-Dibromophenol and 2,4,6, tribromophenols-antiseptic secondary metabolites of *Phoronopsis viridis*. Experientia 1975; 31 : 265-266.

79. McConnel OT, Fenical W. Halogen chemistry of the red algae *Bonnemaisonia.* Phytochemistry 1980; 19 : 233-247.

80. Hager LP. Mother nature like some halogenate compounds. Basic Life Sci 1982; 19 : 415-429.

81. Sunn HH, Paul VJ, Fenical W. Avrainvilleol, a brominated diphenylmethen derivature with feeding deterent properties from the tropical green aalga *Availled Longicaulis*. Phytochemistry 1983; 22 : 743-745.

82. Geigert J, Neidleman SL, Dalietos DJ, Dewitt SK. Haloperoxidase: Enzymatic synthesis of α, β-halohydrins from gasrous alkenes. Appl Environ Microbiol 1983; 45 : 366-374.

83. Boeynaems JM, Hubbard WC. Transformation of arachidonic acid into an iodolactone by the rat thyroid. J Biol Chem 1980; 255 : 9001-9004.

84. Boeynaems JM, Regan D, Hubbard WC. Lactoperoxidase-catalyzed iodination of arachidonic acid : Formation of macrolipids Lipid 1981; 16 : 246-249.

85. Boeynaems JM, Watson JT, Oates JA, Hubbard WC. Iodination of decosahexaenaic acid by lactoperoxidase and thyroid gland *in vitro* : Formation of an iodolactone. Lipid 1981; 16 : 323-327.

86. Turk J, Henderson WR, Klebanoff SJ, Hubbard WC. Iodination of arachidonic acid mediated by eosinophil peroxidase, myeloperoxidase and lactoperoxidase. Identification and comparison of products. Biochem Biophys Acta 1983 ; 751 : 189-200.

87. Geigert J, Neidleman Sl, Dalietos DJ, Dewitt SK. Novel haloperoxidase reaction : Synthesis of dihalogenated products. Appl Environ Microbiol 1983; 45 : 1575-1581.

88. Neidleman SL, Geigert J. The enzymatic synthesis of heterogeneous dihalide derivatives : A unique biocatalytic discovery. Trends Biotechnol 1983; 1 : 21-25.

89. Geigert J, Neidleman SL, Dalietos DJ. Novel haloperoxidase substrates. Alkynes and cyclopropanes. J Biol Chem 1983; 258 : 2273-2277.

90. Corbett MD, Chipko BR, Batchelor AO. The action of chloride peroxidase on 4-chloroaniline. Biochem J 1980; 187 : 893-903.

91. Loo TL, Burger JW, Adamson RH. Bromination of phthalien dyes by the uterus of the dogfish, *Squalus acanthias*. Proc Soc Exp Biol Med 1964; 114 : 60-63.

92. Matkovics B, Rakonczay, Rajki SE, Balaspiri L. Steroids. XII Iodination of aromatic steroids by peroxidase. Steroidologia 1971; 2 : 77-79.

93. van Pee' KH, Lingen F. Detection of bromoperoxidaase in *Streptomyces phaeochromogenes*. FEBS lett 1984; 173 : 5-8.

94. Neidleman SL, Cohen AI, Dean. Enzymatic bromination of the thiazole ring. Biotechnol Bioeng 1969; 11 : 1227-1232.

95. Neidleman SL, Diaassi PA, Junta B, Palmere RM, Pan SC. The enzymatic halogenation of steroids. Tetrahedron Lett 1966; 44 : 5337-5342.

96. Theiler R, Cook JC, Hager LP, Siuda JF. Halohydrocarbon synthesis by bromoperoxidase. Science 1978; 202 : 1094-1096.

97. Beissner RS, Guilford WJ, Coates RM, Hager LP. Synthesis of brominated heptanoues and bromoform by a bromoperoxidase of marine origin. Biochemistry 1981; 20 : 3724-3731.

98. Grisman MB, Jefferson MM, Metton DF, Thomas EL. Chlorination of endogenous amines by isolated neutrophils ammonia-dependent bactericidal cytotoxic and cytolytic activities of the chloramines. J Biol Chem 1984; 259 : 10404-10413.

99. Tsan MF. Myeloperoxidase-mediated oxidation of methionine and amino acid decarboxylation. Infect Immun 1982; 36 : 136-141.

100. Silverstein RM, Hage LP. The chloroperoxidase-catalyzed oxidation of thiols and disulfides to sulfenyl chlorides. Biochemistry 1974; 13 : 5064-5073.

101. Stelmazynska T, Zgliczynski JM. Oxidatiion of cystine by hydrogen peroxide catalyzed by horseradish peroxidase. Acta Biochem Polenica 1963; 10 : 371-378.

102. Tsan MF. Myeloperoxidase-mediated oxidation of methionine. J Cell Physiol 1982; 111 : 49-54.

103. Bjorck KEL. Pharmaceutical preparation with antibacterial effect. Swedish Pat. SE, 425,593. 1982.

104. Hasegawa E, Kobayashi T. Method for recovering myeloperoxidase and pharmaceutical composition containing myeloperoxidase as major constituent U.S. Pat. 4, 379, 141. 1983.

105. Kessler JH, Rosenbaum KS. Bactericidal method U.S. Pat. 4, 476, 108. 1984.

106. Ordorff SA. Enzymatic catalyzed diocide system U.S. Pat. 4, 370, 199. 1983.

107. Iman A. Investigation into the asymmetric distribution of proteins in human milk-fat-globule-membranes. J Biochem 1986; 99 : 143-152.

108. Promptmas C. Haloperoxidases and their analytical diagnostic applications. Ph.D. Thesis in Biochemistry. Faculty of Graduate Studies. Mahidol University, 1994.

109. Tang B. Application of Bromoperoxidase in enzyme-couple assay. M. Sc Thesis in Biochemistry. Faculty of Graduate Studies. Mahidol University, 1992.

110. Hoyle MC. High resolution of peroxidase-indoleascetic acid oxides isoenzymes from horseradish by isoclectric focusing. Plant Physiol 1977; 60 : 787-193.

111. Itoh N, Izumi Y, Yamada H. Characterization of nonheme type bromo-peroxidase in *Corallina pilluifera* J Biol Chem 1986; 261 : 5194-5200.

112. Merrill DP. Purification of human myeloperoxidase by concanavalin A-Sepharose affinity chromatography Preparative Biochem 1980; 10 : 133-150.

113. Olsen RL, Little C. Studies on the subunits of human myeloperoxidase. J Biol Chem 1984; 222 : 701-709.

114. Rawitch AB, Taurog A, Chernoff SB, Dorris ML. Hog thyroid peroxidase physical, chemical, and catalytic properties of the high purified enzyme. Arch Biochem Biophys 1979; 194 : 244-257.

115. Albara S, Yamashita H, Mori E, Kato M, Morita Y. Isolation and characterization of five neutral isoenzymes of horseradish peroxidase. J Biochem 1982; 92 : 531-539.

116. Liu TN, M'Timkutu T, Geigert T, et al. Isolation and characterization of a novel nonheme chloroperoxidase. Biochem Biophys Res Comm 1987; 142 : 329-333.

117. Hashimoto A, Pickard MA. Chloroperoxidases from *Caldavimyces* (=Leptoxyphium) cultures: glycoproteins with variable carbohydrate content and isoenzymic forms. J Gen microbial 1984; 130 : 2051-2058.

118. Rombauts WA, Scroeder WA, Morrison M. Bovine lactoperoxidase. Patial characterization of the further purification protein. Biochemistay 1969; 6 : 2965-2977.

119. Carlstrom A. Physical and compositional investigations of the subfractions of lactoperoxidase. Acta Chem Scandinavia 1969; 23 : 185-202.

120. Krenn BE, Plat H, Wever R. The bromoperoxidase from the red algae *Ceramium rubrum* also contain vanadium as prosthetic groups. Biochim Biophys Acta 1987; 912 : 287-291.

121. de Boer E, Tromp MGM, Plat H, Krenn BE, Wever R. Vanadium (V) as an essential element for haloperoxidase activity in marine brown algae : Purification and characterization of vanadium (V)-containing bromo-peroxidase from *Laminaria succharina.* Biochim Biophys Acta 1986; 872 : 104-115.

122. Wever R, Krenn BE, Offenberg H, De Boer E, Plat H. Structure and function of vanadium containing bromoperoxidases. Prog Clin Biol Res 1988; 247 : 477-493.

123. Krenn BE, Tromp MGM, Wever R. The brown alga *Ascophyllum nodosum* contains two different vanadium bromperoxidase. J Biol chem. 1989; 264 : 19287-19292.

124. Weisner W. van Pee' K-H, Lingen F. Purification and characterization of novel bacterial non-heme chloroperoxidase from *Pseudomonas pyrrocinia*. J Biol Chem 1988; 263 : 13725-13732.

125. Krenn BE, Izumi Y, Yamada H, Wever R. A comparison of different (vanadium) bromoperoxidases; the bromoperoxidase from *Corallina pilulifera* is also a vanadium enzyme. Biochim Biohys Acta 1989; 998 : 63-68

126. Itoh N, Hassan AKMO, Izumi Y, Yamada H. Immunoloical properties of bromoperoxidase in *Corallina* algae. Biochem Internat 1987; 15 : 27-33.

127. Weng M, Pfeifer O, Krauss S, Lingens F, Van Pee' K-H. Purification, characterization and comparison of two non-haem bromoperoxidase from *Streptomyces aureofaciens* ATCC 10762. J Gen Microbiol 1991 ; 137 : 2539-2546.

128. Milstein S, Cohen LA. Concurrent general-acid and general-base catalysis of esterification. J Am Chem Soc 1970; 92 : 4377-4382.

129. Hori H, Ikeda Saito M. Photochemically modified myeloperoxidase, with optical spectral properties analogous to those of lactoperoxidase; retains its original catalytic activity. Biochemistry 1990; 29 : 7106-7112.

130. Mem GE, Feeney RE. Chemical modification of proteins. San Francisco : Holden-Day Inc, 1971.

131. Feeney RE. In : Food proteins. Improvement through chemical and enzymatic modification. Edited by Feey RE, Whitaker FR. Washiton, D.C. : American Chemical Society, 1977 : 3-36.

132. Richardson T, Kester JJ. Chemical modification that affect nutritional and functional properties of proteins. J Chem Educ 1984; 61 : 325-331.

133. Kinsella JE. Protein structure and functional properties : Emulsification and flavor binding effects In : Food protein deterioration : Mechanism and functionality. Edited by Cherry JP. Washiton, D.C. American Chemical Society, 1987 : 301-306.

134. Kaiser ET, Lawrence DS, Rokita SE. The chemical modification of enzymatic specificity. Ann Rev Biochem. 1985; 54 : 565-595.

135. Perlmann GE. Acetylation of pepsin and pepsinogen. J Biol Chem 1966; 241 : 153-157.

136. Herriott RM, Northrop JH. Crystalline acetyl derivatives of pepsin. J Gen Physiol 1934; 18 : 35-67.

137. Simpson RT, Riordan JF. Vallee BL. Functional tyrosyl residues in the active center of bovine pancreatic carboxypeptidase A. Biochemistry 1963; 2 : 616-622.

138. Sokolsvsky M, Vallee BL. Azocarboxypeptidase : functional consequences of tyrosyl and histidyl modification. Biochemistry 1967; 6 : 700-708.

139. Lipscomb WN, Reeke GN, Hartsuck JA, Quiocho FA, Bethge pH. Structure of Carboxypeptidase A [CPA]. VIII. Atomic interpretation at 0.2 nm resolution, a new

study of the complex of glycyl-L-tyrosine with CPA, and mechanistic deductions. Phil Tran Roy Soc London Ser B 1970; 257 : 177-214.

140. Sokolovsky M, Riordan JF, Vallee BL. Tetranitromethane, A reagent for the nitration of tyrosyl residues in proteins. Biochemistry 1966; 5 : 3582-3589.

141. Johansen JT, Ottesen M, Svendsen I. Chemical derivatives of subtilisin Carlsberg with increased proteolytic activity. Biochim Biophys Acta 1967; 139 : 211-214.

142. Trenholm HL, Spomer WE, Wootton JF. Substrate activation of trypsin. The effect of enzyme acetylation. J Am Chem Soc 1966; 88 : 4281-4282.

143. Trenholm HL, Spomer WE, Wootton JF. The effects of acetylation upon the activity of trypsin toward ester and amide substrates. Biochemistry 1969; 8 : 1741-1749.

144. Chevallier J, Yon J, Spotorno G, Labouesse J. Influence of aetylation of tyrosines on the activity of N-episilon-acetyltrypsin. Eur J Biochem 1968; 5 : 480-486.

145. Lowe G, Whitworth AS. A Kinetic and fluorimetric investigation of papain modified at tryptophan-69 and -177 by N-bromosuccinimide. Biochem J 1974; 141 : 503-515.

146. Jori G, Galiazzo G. Proflavine-sensitived selective photooxidation of the tryptophyl residues in papain. Photochem Photobiol 1971; 14 : 607-619.

147. Jori G, Gennari G, Toniolo C, Scoffone E. Probing the topography of proteins in solution by phytosensitized oxidation. The catalytic region of papain. J Mol Biol 1971; 59 : 151-168.

148. Mole JE, Horton HR. A Kinetic analysis of the enhanced catalytic efficiency of papain modified by 2-hydroxy-5-nitrobenylation. Biochemistry 1973; 12 : 5285-5789.

149. Chang SM, Horton HR. Structure of papain modified by reaction with 2-chloromethyl-4-nitrophenyl N-carbobenzoxyglycinate. Biochemistry 1979; 18 : 1559-1563.

150. Evans BLB, Knopp JA, Horton HR. Effect of hydroxynitrobenzylation of tryptophan-177 on ractivity of active site cysteine-25 in papain. Arch Biochem Biophys 1981; 206 : 362-371.

151. Steiner RF. Varying luminescence behavior of the different tryptophan residues of papain. Biochemistry 1971; 10 : 771-778.

152. Kirschenbaum DM. The enhancement of the enzymatic activity of papain by reaction with N-bromosuccinimide. Biochim Biophys Acta 1971; 235 : 159-163.

153. Loffler HG, Schneider F. Kinetic and chemical study of succinyl papain. Z. Naturforsch 1972; 27 : 1490-1497.

154. Imoto T, Fujioto M. Yagishita K. The role of tryptophan-62 in the enzymic reaction of lysozyme. J. Biochem 1974; 76 : 745-753.

155. Davies RC, Neuberger A. Modification of lysine and arginine residues of lysozyme and the effect of enzymatic activity. Biochim Biophys Acta 1969; 178 : 306-317.

156. Pontremoli S, Traniello S, Enser M, Shapiro S, Horecker BL. Regulation of Fructose diphosphatase activity by disulfide exchange. Proc Natl Acad Sci USA 1967; 58 : 286-293.

157. Nakashivma K, Pontremoli S, Horecker BL. Activation of rabbit liver fructose disphosphatase by coenzyme and acyl carrier protein. Proc Natl Acad Sci USA 1969; 64 : 947-951.

158. Nakashima K, Horecker BL, Traniello S, Pontremoli S. Rabbit liver and rabbit kidney fructose diphosphatase : catalytic properties of enzyme activated by coenzyme A and acyl carrier protein. Arch biochem Biophys 1970; 139 : 190-199.

159. Nakashima K, Horecker BL, Pontremoli S. Regulatory sulfhydryl groups and activation by homocystine in liver fructose diphosphatase. Arch Biochem Biophys 1970; 141 : 579-587.

160. Pontremoli S, Luppis B, Wood WA, Traniello S, Horecker BL. Fructose diphosphatase from rabbit liver. 2. Changes dinitrofluorobenzene. J Biol Chem 1965; 240 : 3464-3468.

161. Pontremoli S, Luppis B, Traniells S, Rippa M, Horecker BL. Fructose diphosphatase from rabbit liver IV. Sulfhydryl groups and their relation to the catalytic activity. Arch Biochem Biophys 1965; 112 : 7-15.

162. Little C, Sanner T, Pihl A. Stimulation of fructose 1,6-diphosphatase by sulfhydryl reagent and interaction between the different enzymic site in the molecule. Eur J Biochem 1969; 8 : 229-236.

163. Mozhaev VV, Martinek K. Structure stability relationship in proteins-New approachs to stabilizing enzymes. Eur Microb Technol 1984; 6 : 50-59.

164. Martinek K, Mozhaeu VV, Berezin IV. In : Enzyme Engineering : Future directions. Edited by Wingard LB, Berezin Jr Iv, Klyorov AA. New York : Plenum Press, 1980 : 3-54.

165. Argos P, Rossman MG, Grau JM, Zuber H, Frank G, Tratschin JD. Thermal stability and protein structure. Biochemistry 1979; 18 : 5693-5730.

166. Schmid RD. Oxidoreductases-present and potential applications in technology. Process Bio 1979; 14 : 2.

167. Mozhaev VV, Siksnis VA, Torchilin VP, Martinek K. Operational Stability of copolymerization enzymes at elevated temperatures. Biotechnol Bioeng 1985; 25 : 1937-1945.

168. Klibanov AM. Enzyme stabilization by immobilization. Anal Biochem 1979; 93 : 1-25.

169. Gianfreda L, Greco G. The stabilizing effect of soluble macromolecules on enzyme performance. Biotechnol Lett 1981; 3 : 33-38.

170. Mosbach K. Immobilized enzymes. Methods in Enzymolgy, vol 44. Edited by Mosbach K. New York and London : Academic Press, 1976 : 3-7.

171. Trubtskoy VS, Torchilin VP. Artificial and natural theromostabilization of subunit enzymes. Do they have similar mechanism?. Int J Biotech 1985; 17 : 661-663.

172. Torchilin VP, Maksimeako AV, Simirnov VN, Berezin IV, Martinek K. Principles of enzyme stabilization. V. The possibility of enzyme self stabilization under the action of potentially reversible intramolecular cross-linkdages of different length. Biochim Biophys Acta 1979; 568 : 1-10.

173. Duncan RJS, Weston PD, Wrigglesworth R. A new reagent which may be used to introduce sulfhydryl groups into proteins, and its use in the preparation of conjugates for immunoassay. Analyt Biochem 1983; 132 : 68-73.

174. Torchilin VP, Trubetskoy VS, Omel' yanenko KG, Martinek KJ. Stabilization of subunit enzyme by intersubunit crosslinking with bifunctional reagents-studies with bifunctional reagents-studies with glyceralehyde-3-phosphate dehydrogenase. J Mol Catal 1983; 19 : 291-301.

175. Dziember-Gryszkiewicz E, Maksimenko AV, Torchinlin VP, Ostrowski WS. Stabilization of human prostatic acid phosphatase by cross-linking with diamines. Biochem Internat 1983; 6 : 627-633.

176. Torchilin VP, Maksimenko AV, Smirnov VN, Berezin IV, Klianov AM, Martinek K. The principles of enzyme stabilization. III. The effect of the length of intra-molecular cross-linkages on thermostability of enzyme. Biochem Biophys Acta 1978; 522 : 277-283.

177. Klemes Y, Citri N. Cross-linking preserves conformational Changes induced in penicillinase by its substrates. Biochem J 1980, 187 : 529-532.

178. Melix-Nubarov NS, Mozhaev VV, Siksnis S, Martinek K. Stabilitzation via hydrophilization-stabilization of alpha-chymotrypsin by reductive alkylation with glyoxylic acid. Biotechnol Lett 1987; 9 : 725-730.

179. Cupo P, El-Deiry WS, Whitney PL, Awad WM Jr. Stability of acetylated and superguanidinated chymotrypsinogens. Arch Biochem Biophys 1982; 216 : 600-604.

180. Cupo P, El-Deiry WS, Whitney PL, Awad WM Jr. Stabilization of proteins by guanidination. J Biol Chem 1980; 225 : 10828-10833.

181. Hollecker M, Creighton TE, Effect on protein stability of reversing the charge on amino groups. Biochim Biohys Acta 1982; 701 : 395-404.

182. Tuengler P, Pfleiderer G. Enhanced heat, alkaline and tryptic stability of acetamidinated pig heart lactate dehydrogenase. Biochim Biophys Acta 1977; 484 : 1-8.

183. Mozhaev VV, Siksnis VA, Melik-Nubarov NS. et al., Protein stabilization via hydrophilization Covalent modification of trypsin and alpha-chymotrypsin. Eur J Biochem 1988; 173 : 147-154.

184. Inada Y, Takahashi K, Yoshimoto T, Ajuna A, Matsushima A, Saito Y. Application of polyethyleneglycol-modified enzymes in biotechnological processes : organic solvent-soluble enzymes. Trends Biotechnol 1980; 4 : 190-194.

185. Marshall JJ, Rabinowitz ML. Enzyme stabilization by covalent attachment of carbohydrate. Arch Biochem Biophys 1975; 167 : 777-779.

186. Torchilin VP, Tishchenko EG, Smirnov VN. Enzyme immobilization on bio-compatible carriers : II. Immobilizatiion of α-chymotrypsin on polyvinylpyrrolidone. Bioorg Khim 1976; 2 : 399-405.

187. Mozhaev VV, Martinek K. Inactivation andreactiation of proteins (enzymes). Eur Microb Technol 1982; 4 : 299-309.

188. Christensen TB. Vegarud G. Birkenland AJ. Stabilization of enzymes by glycosylation. Process Bio 1976; 11 : 25-26.

189. Kennedy JH, Kricka LJ, Wilding P. Protein-protein coupling reactions and the applications of protein conjugates. Clin Chim Acta 1976; 70 : 1-31.

190. Kellis JTJr, Nyberg K, Sali D, Fersht AR. Contributiion of hydrophobic inter-actions to protein stability. Nature 1988; 333 : 784-786.

191. Klemes Y, Citri N Catalytic and conformational properties of cross-linked derivatives of penicillinase. Biochim Biophys Acta 1979; 567 : 401-409.

192. Henley JP, Sanada A. A mathematical analysis of enzyme stabilization by a series-type mechanism : Influence of chemical modifiers. Biotechnol Bioeng 1984; 26 : 959-969.

193. Henley JP, Sanada A. Serie-type enzyme deactivations. Influence of inter-mediate activity on deactivation kinetics. Eur Microb Technol 1984; 6 : 35-41.

194. Henley JP, Sanada A. In : Proceedings of the fifth symposium on bio-technology for fuels and chemicals. Edited by Scott CD. New York : Wiley, 1984 : 277-283.

195. Sanada A, Henley JP. Biotechnology reports : Effect of chemical modification on enzymatic activities and stabilities. Biotechnol Bioeng 1986; 28 : 256-268.

196. Henley JP, Sanada A. Categorization of enzyme deactivations using a series-type mechanism. Eur Miceob Technol 1985; 7 : 50-60.

197. Monod J, Wyman J, Changeux JP. On the nature of allosteric transitions : a plausible model. J Mol Biol 1965; 12 : 88-118.

198. Kosland DE, Nemethy JrG, Filmer D. Comparison of experiment binding data and theoretical models in proteins containing subunits. Biochemistry 1966; 5 : 365-385.

199. Lett D, Chase Jr T. Chemical modification of mushroom tyrosinase for stabilization to reaction inactivation. Adv Exp Med Biol 1974; 42 : 314-328.

200. Klemes Y, Citri N. Cross-linking preserves conformational changes induced in penicillinase by its substrates. Biochem J 1980; 187 : 529-532.

201. Shick AF, Singer SJ, Nazaki Y, Tanford C. The solubility of amino acid and related compounds in adequeous urea solution. J Biol Chem 1963; 238 : 4074-4081.

202. Zaborsky OR. In : Enzyme Engineering. Vol 2. Edited by Pye EK, Wingard JrLB. New York : Plenum Press, 1974 : 115-122.

203. Abe T, Nihara T, Tanaka A, Fukui S. Influence of long-chain primary alcohol on cholesterol oxidase from *Schizophyllum commune.* Biochim Biophys Acta 1983; 749 : 69-76.

204. Fornaini G, Leoneini G. Segni P, Calabria GA, Dacha M. Relationship between age and properties of human and rabbit erythrocyte glucose-6-phosphate dehydrogenase. Eur J Biochem 1969; 7 : 214-222.

205. Yoshida A. Glucose-6-phosphate dehydrogenase of human erythrocytes. I. Purification and characterization of normal (B^+) enzyme. J Biol Chem 1966; 241; 4966-4976.

206. Nickle EC, Solomon RD, Torchia TE, Wriston JC Jr. Chemical modifications of *Escherichia coli* L-asparaginase and their effect on plasma clearance rate and other properties. Biochim Biophys Acta 1982; 704 : 345-352.

207. Eaton JW, Boraas M, Etkins NL. Catalase activity and red cell metabolism. Adv Exp Med Biol 1972; 28 : 121-131.

208. McMahon S, Stern A. The interrelationship of superoxide dismutase and peroxidatic enzymes in the red cell. Biochim Biophys Acta 1979; 566 : 253-258.

209. Cohen G, Hochstein P. Generation of hydrogen peroxide in erythrocytes by hemolytic agents. Biochemistry 1964; 3 : 895-900.

210. Cohen G, Hochstein P. *In vivo* generation of H_2O_2 in mouse erythrocytes by hemolytic agents. J Pharmacol Exp Ther 1965; 147 : 139-143.

211. Marks PA, Szeinberg A. Substances stimulating glucose catabolism by the oxidative ractions of the pentose phosphate pathway in human erythrocytes. J Clin Invest 1961 ; 40 : 914-924.

212. Kirkman HN. Glucose-6-phosphate dehydrogenase from human erythrocytes I. Further purification and characterization. J Biol Chem 1962; 237 : 2364-2370.

213. Okuyama T, Sataka K. On the preparation and properties of 2,4,6-trinitro-phenyl-amino acids and-peptides. J Biochem (Tokyo) 1960; 47 : 454-466.

214. Goldfarb AR. A kintic study of the reactions of amino acids and peptides with trinitrobenzenesulfonic acid. Biochemistry 1966; 5 : 2570-2574.

215. Habeeb AFSA. Determination of free amino groups in proteins by trinitro-benzenesulfonic acid. Anal Biochem 1966; 14 : 328-336.

216. Fields R. The measurement of amino groups in proteins and peptides. Biochem J 1971; 124 : 581-590.

217. Field R. The rapid determination of amino groups with TNBS. In : Enzyme structure part B. Methods in Enzymology, vol 25B. Edited by Hir CHN, Timasheff SN. New York and London : Academic Press, 1972 : 464-468.

218. Stadtman ER. In : The mechemism of enzyme action. Edited by McElroy WD, Glass B. Baltimore, Maryland : John Hopkins Press, 1954 : 581.

219. Riodan JF, Vallee BL. Acetylcarboxypeptidase. Biochemistry 1963; 2 : 1460-1467.

220. Ramachandran LK. Elastsidin- a mixture of three proteins. Biochem Biophys Res Comm 1962; 6 : 443-448.

221. Ramachandran LK, Witkop B. N-Bromosuccinimide Cleavage of peptides. In : Enzyme structure. Methods in Enzymology, vol 11. Edited by Hirs CHW. New York and London : Academic press, 1967 : 283-299.

222. Okada Y, Onone K, Nakashima S, Yamamura Y. Studies on enzyme-antienzyme systems II. N-Bromosuccinimide modified bacterial α-amylase. J Biochem (Tokyo) 1963; 54 : 477-483.

223. Koshland DEJr, Karkhanis YD, Latham HG. An environmentally-sensitive reagent with selectivity for the tryptophan residue in proteins. J Am Chem Soc 1964; 86 : 1448-1450.

224. Horton HR, Kashland DEJr. A highly reactive colored reagent with selectivity for the tryptophanyl residue in proteins, 2-hydroxy-5-nitrobenzyl bromide. J Am Chem Soc 1965; 87 : 1126-1132.

225. Scoffone E, Fontana A, Rocchi R. Selective modification of the tryptophan residue in peptide and protein using sulfenyl halides. Biochem Biophys Res Commun 1966; 25 : 170-174.

226. Scoffone E, Fontana A, Rocchi R. Sulfenyl halide as modifying reagents for polypeptide and proteins. I. Modification of tryptophan residues Biochemistry 1968; 7 : 971-979.

227. Fontana A, Scoffone E, Benassi CA. Sulfenyl halide as modifying reagents for polypeptide and proteins. II Modification of cysteinyl residues. Biochemistry 1968; 7 : 971-979.

228. Fontana A, Veronese FM, Scoffone E. Sulfenyl halides as modifying reagents for polypeptides and protein III. Azobenzene-2-sulfenyl bromide, a selective reagent for cysteinyl residues. Biochemistry 1968; 7 : 3901-3905.

229. Riddles PW, Blakeley RL., Zerner B. Title Ellman's reagent: 5,5-dithiobis (2-nitrobenzoic acid)-a reexamination. Anal Biochem 1979; 94 : 75-81.

230. Parker AJ, Kharasch N. The scission of the surfur-surfur bond. Chem Rev 1959; 59 : 583-628.

231. Ovádi J, Keleti T. Efect of diethylpyrocarbonate on the conformation and enzyme activity of d-glyceraldehyde-3-phosphate dehydrogenase. Acta Biochim Biophys Acad Sci Hung 1969; 4 : 365-378.

232. Melchior WBJr, Fahrney D. Ethoxyformylation of proteins. Reaction of ethoxyformic anhydride with α-chymotrypsin, pepsin and pancreatic ribonuclease at pH 4. Biochemistry 1970; 9 : 251-258.

233. Mile EW. Modification of histidyl residues in proteins by diethylpyro-carbonate. In : Enzyme structure part E. Methods in Enzymology, vol 47. Edited by Hirs CHW. New York and London : Academic Press 1977 : 431-468.

234. Brustein Y, Walsh KA, Neurath H. Evidence of an essential histidine residue in thermolysin. Biochemistry 1974; 13 : 205-210.

235. Vincent JP, Schweitz H. Lazdunski M. Structure-function relationships and site of action of apamin, a neurotoxic polypeptide of bee venom with an action on the central nervous system. Biochemistry 1975; 4 : 2521-2525.

236. Leskovac V, Pavkov-Periein D. Evidence for a histidine and a cysteine residue in the substrate-binding site of yeast alcohol dehydrogenase. Biochem J 1975; 145 : 581-590.

237. Holbrook JJ, Ingram VA. Ionic properties of an essential histidine residue in pig heart lactate dehydrogenase. Biochem J 1973; 131 : 729-738.

238. Kumagai T, Utagawa T, Yamada H. Studies on tyrosine phenol lyase modification of essential histidyl residue by diethylpyrocaronate. J Biol Chem 1975; 250 : 1661-1667.

239. Toi K, BynumE, Norris E, Itans HA. Studies on the chemical modification of arginine 1. The reaction of 1,2-cyclohexanedione with arginine and arginyl residues of protein. J Biol Chem 1967; 242 : 1036-1043.

240. Khorana HG. Carbodiimides. II. The reaction of sulfonic acids with carbo-diimides. A new method of preparation of sulfonic anlydrides. Can J Chem 1953; 31 : 585-588.

241. Hoare DG, Koshland DE Jr. Amethod for the quantitative mofidication and estimation of carboxylic acid groups in proteins. J Biol Chem 1967; 242 : 2447-2453.

242. Kurzer F, Douraghi-Zadeh K. Advances in the chemistry of carbodiimides. [Review] Chem Rev 1967; 67 : 107-152.

243. Gurd FRN. Carboxymethylation. In : Enzyme structure. Methods in Enzymology, vol 11. Edited by Hir CHW. New York and London : Academic press, 1967 : 532-541.

244. Harris CM, HillRL. The carboxymethylation of human metmyoglobin. J Biol Chem; 244 : 2195-2203.

245. Szenezuk A, Connell GE. The reaction of iodoacetamide with the active center of γ-glutamyl transpeptidase. Biochem Biophys Acta 1965; 105 : 352-367.

246. Kravchenko NA, Kleopina GV, Kaverzneza ED. Investigation of the active sites of lysozyme. Carboxymethylation of the imidazole group of histidine and the ε-aminogroup of lysine. Biochim Biophys Acta 1964; 92 : 412-414.

247. Heinrikson RL. On the alkylation of amino acid residues at the active site of ribonuclease. J Biol Chem 1966; 241 : 1393-1405.

248. Vithayathil PJ, Richards F. Modification of the Methionine residue in the peptide component of ribonuclease-S. J Biol Chem 1960; 235 : 2343-2351.

249. Cohen LA. Group-specific reagents in protein chemistry. Ann Rev Biochem 1968; 37 : 695-726.

250. Vallee BL, Riordan JF. Chemical approaches to the properties of active sites of enzymes. Ann Rev Biochem 1969; 38 : 733-794.

251. Takahashi K, Stein WH, Moore S. The identification of a glutamic acid residue as part of the active site of ribonuclease T-1. J Biol Chem 1967; 242 : 4682-4690.

252. Goren HJ, Glick DM, Barnard EA. Analysis of carboxymethylated residues in proteins by an isotopic method, and its application to the bromoacetate-ribonuclease reaction. Arch Biochem Biophys 1968; 126 : 607-623.

253. Harada Y, Iric M. Alkylation of ribonuclease from *Aspergillus saitoi* with iodoacetate and iodoacetamide. J Biochem 1973; 73 : 705-716.

254. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of ptorein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72 : 248-254.

255. Davis BJ. Disc electrophoresis II. Method and application to human serum proteins. Ann N Y Acad Sci 1964; 121 : 404-427.

256. Riordan JF, Wacker WEC, Vallee BL. N-Acetylimidazole : A reagent for determination of “free” tyrosyl residues of proteins. Biochemistry 1965; 4 : 1758-1765.

257. Ellman Gl. Tissue sulfhydryl groups. Arch Biochem Biophys 1959; 82 : 70-77.

258. Yu H, Whittaker JW. Vanadate activation of bromoperoxidase from *Corallina officinalis*. Biochem Biophys Res Commun 1989; 160 : 87-92.

259. Chaijaroonporn T. Purification and characterization of bromoperoxidase from Thai seaweeds *Polycarvernosa* sp. MSc. Thesis in Biochemistry. Faculty of Graduate Studies, Mahidol University, 1990.

260. Weinryb I. Alkylation of horseradish peroxidase with iodoacetate. Arch Biochem Biophys 1968; 124 : 285-291.