ISOLATION AND CHARACTERIZATION OF A BROMOPEROXIDASE FROM *PLOCAMIUM CARTILAGINEUM*

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF A BROMOPEROXIDASE FROM *PLOCAMIUM CARTILAGINEUM*

by Heidi Amato

A bromoperoxidase from the red algae *Plocamium cartilagineum* was investigated. The bromoperoxidase was isolated using a combination of chromatographic techniques including gel permeation and hydrophobic interaction chromatography. Electrophoresis techniques were utilized for the determination of the molecular weight and isoelectric point.

Efficient recovery of bromoperoxidase from *Plocamium cartilagineum* requires the presence of 1 M NaBr and a 14 day "aging"period to become fully active. During the "aging" period the enzyme forms aggregates larger than 5,000 kDa which may be a requirement for enzymatic activity. Unlike most characterized bromoperoxidases, the enzyme is highly unstable and lost enzymatic activity when it came into contact with a variety of standard chromatographic media and extraction methods. It is similar to other bromoperoxidases with subunit molecular weight of 70 kDa and a pI of 6.0.

Future work in this lab will utilize the bromoperoxidase in chemoenzymatic reactions.

ACKNOWLEDGMENTS

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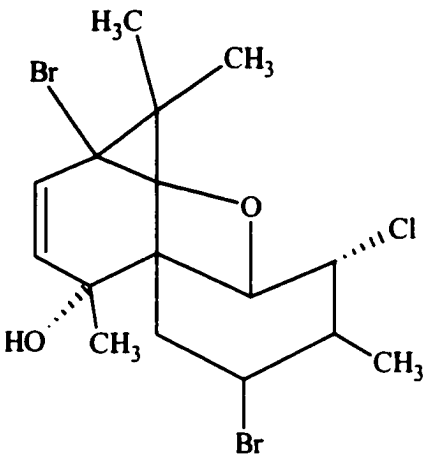
CHAPTER 1

INTRODUCTION

A myriad of halogenated natural products have been isolated from marine plants and animals.[1] The oceans contain halogens in fairly high concentrations (chloride 0.5 M, bromide 1 mM, iodide 1 μm) thus it is not unexpected that marine organisms incorporate halogens into their metabolic chemistry. Nature provides for the survival needs of organisms and it has been suggested that the halogenated natural products enhance the survival of those organisms. There are at least three major roles that the halogenating enzymes and their products play: (1) intermediates in physiological activities, (2) biological defense mechanisms, and (3) devices to synthesize complex natural products. The defensive roles of halogenated natural products that furnish the organism with a survival advantage are in the forms of antimicrobial agents, toxins, antifeedants, and as catalysts in the formation of defensive structures.[1-3] The biosynthesis of halogenated natural products in sizeable amounts is of concern to some because this may be a natural cause for the depletion of the ozone layer.[4]

Halogenated natural products vary greatly in their structures and properties (Figure 1). In most cases, the biological roles that these compounds play in cellular processes are not understood. The great diversity of these compounds suggests that these roles are far more complex beyond what little we know about them. Most of the halogenated natural products have been obtained from marine sources, with algae being the major contributor. Terrestrial sources such as lichen, fungi, and microorganisms also make a small contribution.[5]



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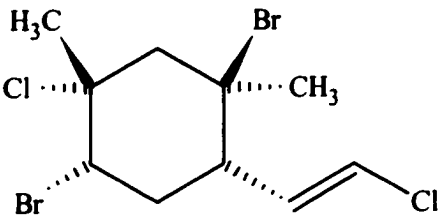
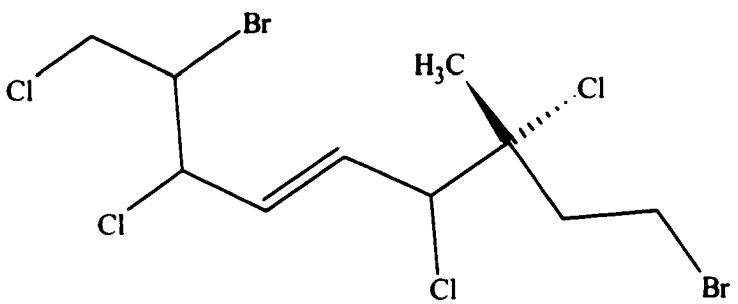


Plocamium cartilagineum



Plocamium sp.

Figure 1. Examples of halogenated natural products from marine algae.[1]

It has been well documented that marine algae are sources of vast numbers of organic halogenated compounds.[4-9] The chemical classes represented are low molecular weight ketones, aromatic compounds, hydrocarbons, indoles, and mono-, sesqui-, and diterpenes. Three of the halogens (Cl, Br, and I, but not F) have been found in natural products extracted from red algae. Red algae are among the most interesting group of organisms that produce halogenated natural products because of the diversity in the natural products that have been extracted from them.[5]

A. Haloperoxidases

The halogens in natural products are incorporated biosynthetically by enzymes into organic compounds produced by marine organisms, plants, and, animals. The majority of the halogens are stored in the ionic form (Cl^-, Br^-, and I^-). These ionic forms can then be integrated into organic halogenated compounds.[2] Halide ions selectively diffuse across cell membranes to be utilized for metabolic processes. The halides are often stored in specialized cells where the concentration of halides has been estimated to be up to 30 times the concentration found in neighboring cells.[5]

The halogens in natural products are believed to be incorporated by enzymes known as haloperoxidases. These enzymes catalyze the oxidation of a halide by hydrogen peroxide and this results in the halogenation of an organic substrate. The oxidized halide (e.g., bromonium ion) acts as an electrophile which attacks the organic substrate resulting in a substitution or addition. The halide is not always found in the final product as it may be used as part of an intermediate in the biosynthesis of the actual metabolite.[5]

There are three types of haloperoxidases and their names are based on the most electronegative halide that the haloperoxidase is able to oxidize: chloroperoxidase (Cl^-, Br^-, and I^-), bromoperoxidase (Br^- and I^-), and iodoperoxidase (I^- only).

The insertion of halogens into organic substrates by haloperoxidases can be represented by a generalized equation:

$$\text{substrate} + \text{halide} + H_2O_2 \xrightarrow{\text{haloperoxidase}} \text{halogenated substrate} + H_2O$$

The halide may be bromide, chloride, or iodide.

Although the enzyme catalyzes the reaction to produce the halogenated substrate, an enzyme frequently requires a non-protein component called a cofactor for catalysis. The cofactor may serve as an intermediate substrate or as a catalyst for an oxidation-reduction reaction. The cofactor may be a metal ion or an organic molecule. Tightly bound cofactors, known as prosthetic groups, are often bound to the enzyme by covalent bonds. Heme is an example of a prosthetic group. A prosthetic group is chemically altered during the enzymatic reaction and must be converted back to its initial state before it will perform its function again.[10]

Many marine algae contain bromoperoxidases.[5] They can be classified not only by the halide that is oxidized, but by the metal cofactor present. Two types of haloperoxidases have been identified: heme and nonheme. Heme haloperoxidases, generally chloroperoxidases, contain iron as a cofactor. Although chloride is found in much higher concentrations than bromide in sea water, very few chloroperoxidases have been reported from marine algal sources. Nonheme haloperoxidases, generally

bromoperoxidases, often contain vanadium as the metal cofactor.[11] Bromoperoxidases containing vanadium are only the second known examples of enzymes which contain this transition metal as a cofactor. Since vanadium is the second most abundant transition metal in the ocean, it is not surprising that vanadium containing enzymes have been found in marine algae.[3]

Marine bromoperoxidases are typically non-heme enzymes and are the most common in the marine algae that have been reported. Some algae that contain bromoperoxidase do not produce halogenated compounds and thus the enzymes may play another role in the metabolism of the algae.[5]

The following table lists a few examples of bromoperoxidases from marine algae and their physical characteristics. It is important to note the relative similarities in the characteristics from the different sources.

Table 1 Examples of bromoperoxidases and their physical characteristics.

Source		Optimal pH	Molecular Weight	pI	Halide Selectivity
Penicillus capitatus[7]	green algae	4	56,500	-	I and Br
Penicillus lamourouxii[7]	green algae	4	48,000	-	I and Br
Rhipocephalus phoenix[7]	green algae	5.5	60,000	-	I and Br
Corallina officinalis[9]	red algae	6.5	64,000	3.9	-
Ascophyllm nososum[3]	brown algae	6.0	97,000	5.0	-
Xanthoria parietina[3]	orange-red lichen	5.5	65,000	-	-
Corallina pilulifera[6]	algae	6.0	64,000	3.0	I and Br

B. Isolation of Bromoperoxidases

Typical reported purification procedures of bromoperoxidase begin with homogenization of the algae in the chosen buffer system followed by centrifugation. The pellet is then collected and subsequently purified by differing techniques. These techniques include: centrifugation, ammonium sulfate precipitation, ion exchange chromatography, organic solvents, preparative gel electrophoresis, gel filtration, dialysis, and hydrophobic interaction chromatography.[7,12-14] The stability of the bromoperoxidases examined previously is surprisingly high under many of these conditions. Vanadium bromoperoxidases have very similar physical characteristics. They are all acidic and have similar amino acid compositions. The average molecular weight of a subunit ranges from 65,000 to 70,000 Da. Two, four, or twelve subunits make up the quaternary structure of a complete enzyme. The isoelectric points of the bromoperoxidases that have been studied are between 3 and 5.[4-9] For example, the subunit of the vanadium bromoperoxidase from *Corallina officinalis* has a molecular weight of 64 kDa and there are 12 subunits in the native enzyme.[9] The bromoperoxidase from *Penicillus capitatus* possesses a monomic subunit with a molecular weight of 56 kDa and a dimer structure for the native enzyme.[13]

In order to obtain an optimal catalytic activity from the bromoperoxidase, certain conditions must be maintained. Among these are the concentrations of the buffer, the halide, and hydrogen peroxide. The pH of the buffer is important and the buffer is usually aqueous, but no one buffer system seems to be more advantageous than another. Most bromoperoxidases are quite stable in the presence of organic solvents such as methanol dissolved in the buffer system. The purification procedure for the bromoperoxidase from

Ascophyllum nodosum utilizes an ethanol precipitation step.[4] Once the optimal conditions of the buffer system are ascertained,[5] bromoperoxidases tend to be very stable in solution under refrigeration. The optimal pH range for most bromoperoxidases is between pH 5.0 and 7.0.[5] The activity of the enzyme decreases drastically (about 50%) at 1 pH unit on either side of the optimum pH. The bromide concentration required for optimal enzymatic activity varies among the different sources of bromoperoxidase. If too high, the bromide concentration usually acts as an inhibitor to the enzymatic reaction, but some bromoperoxidases are still active at higher concentrations.[15] While the bromoperoxidase from *Xanthoria parietina* is inhibited by high concentrations of bromide (50 mM), the bromoperoxidase from *Penicillus capitatus* maintains its activity even at bromide concentrations above 1.5 M. The concentration of hydrogen peroxide needs to be cautiously monitored. A high concentration will decrease the activity of the enzyme, but a minimum concentration of hydrogen peroxide needs to be present to oxidize the halide quickly enough for use by the enzyme.[5]

Marine bromoperoxidases have been investigated as potential chemoenzymatic reagents.[1] They react with organic substrates to produce halogenated reaction products. The studies show that bromoperoxidases are capable of halogenating a large variety of substrates. When alkenes react with bromoperoxidases the primary products are bromohydrins, but dibromides and epoxides are also found. Aromatic compounds, such as phenols, that are susceptible to electrophilic aromatic substitution are highly reactive substrates for bromoperoxidases and react readily to produce brominated products in high yields. The reaction of monochlorodimedone (MCD) with

bromoperoxidase to produce 2-chloro-2-bromodimedone is used as a quantitative assay to track enzymatic activity in fractions (Figure 2).[5]

C. *Plocamium cartilagineum*

Plocamium cartilagineum, is a red algae that can be found in geographically distinct areas around the world. *P. cartilagineum* produces large quantities of halogenated acyclic monoterpenes (Figure 3). A remarkable fact is that chloride and bromide are incorporated into natural products produced by *P. cartilagineum* at approximately the same ratio. The ratio of chloride to bromide in sea water is 500 to 1. Nature has optimized the catalyst to preferentially incorporate bromide over chloride into the algae. Another noteworthy fact is that many of the natural products isolated from *P. cartilagineum* contain chiral halogens.[5]

Interestingly, sea hares (*Aplysia californica* and others) consume enormous quantities of *Plocamium* and concentrate the halogen containing compounds in their digestive glands where the compounds remain with little degradation. Sea hares have few predators which may be a reflection of the high concentration of halogenated compounds stored in them.[2,3]

D. Prior Work

A bromoperoxidase from *P. cartilagineum* has been isolated from prior work in this laboratory. It was determined that to obtain maximum activity from the bromoperoxidase in *P. cartilagineum*, procedures had to be followed that were distinctly

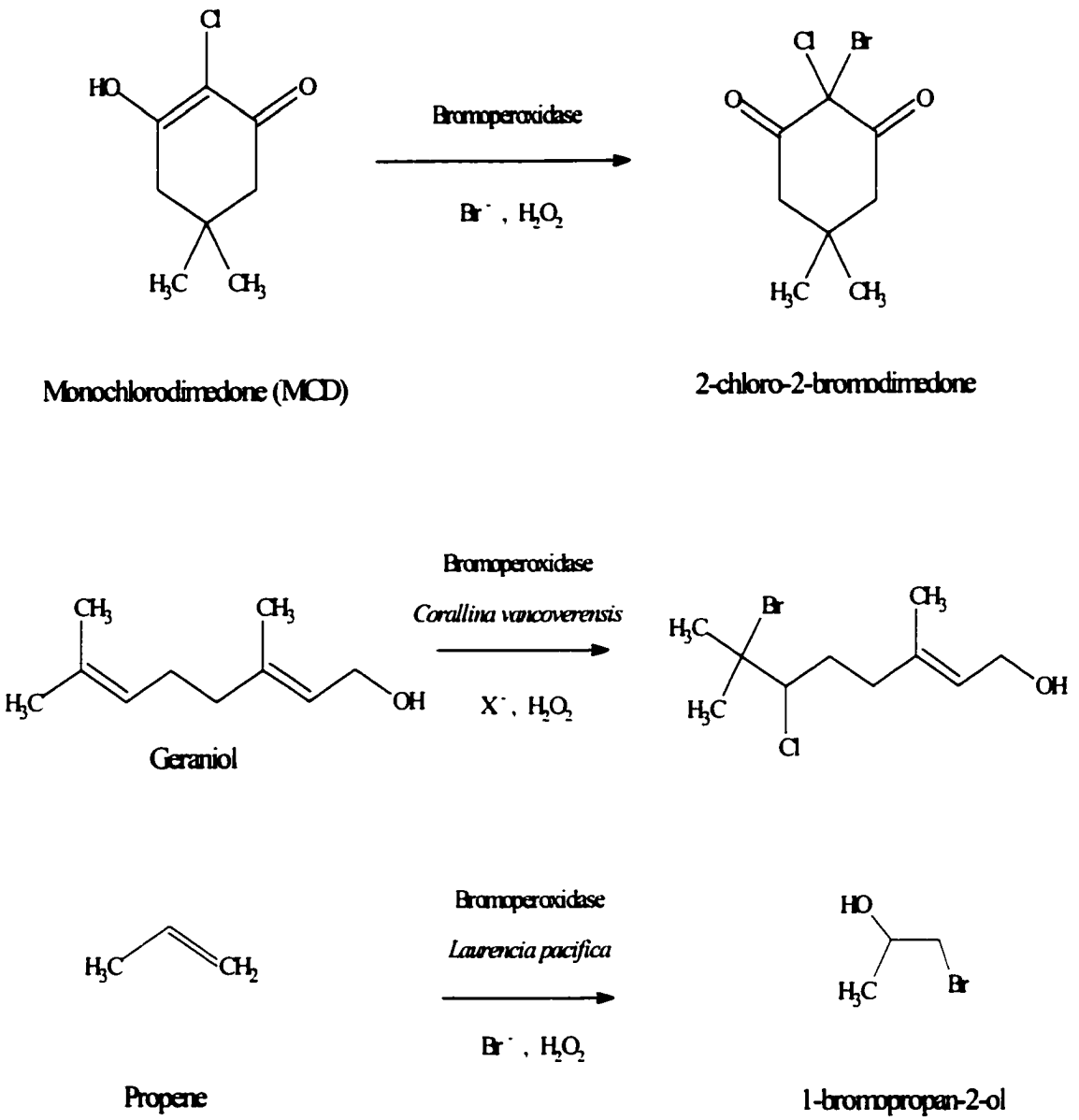


Figure 2. Reactions of bromoperoxidase.[5]

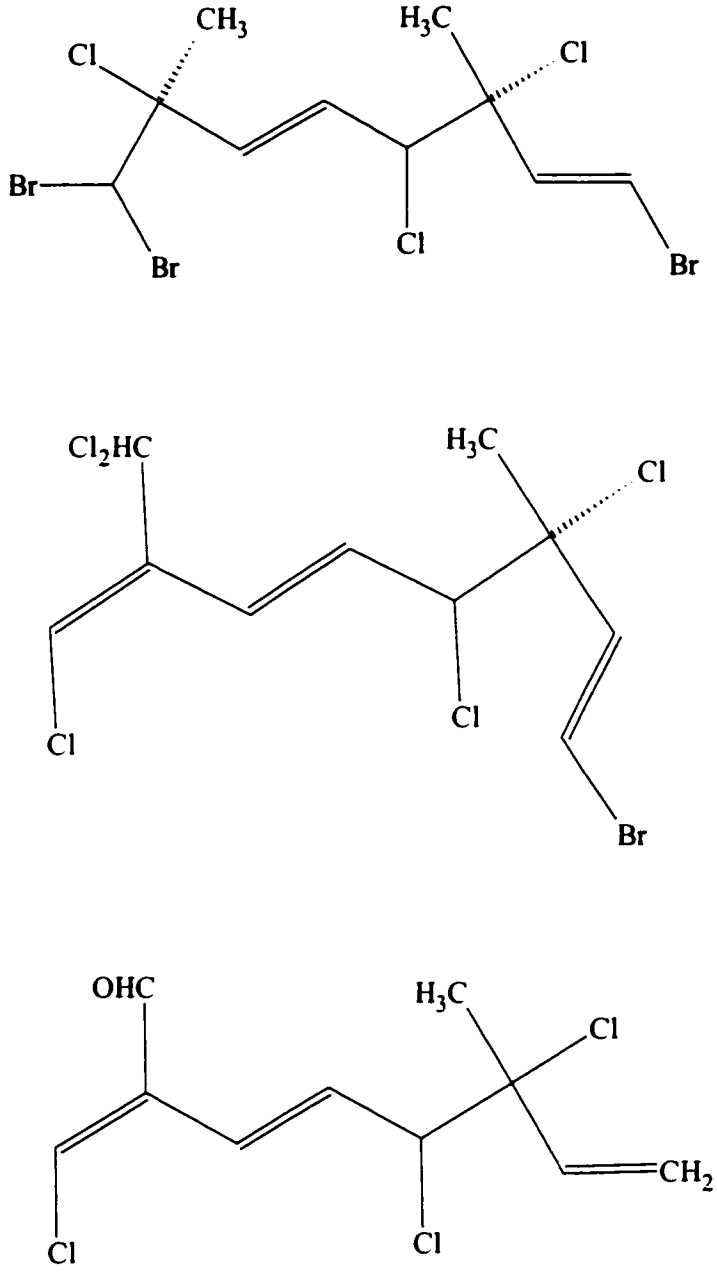


Figure 3. Examples of halogenated acyclic monoterpenes from *Plocamium cartilagineum*.[5]

different from other reported purification procedures.[3] In particular, good recovery of bromoperoxidase activity required the addition of 1 M sodium bromide to the extraction buffer, and an aging period of fourteen days at 4°C. The aging period is required because the activity of the enzyme is extremely low at the time of extraction. This could be due to a disruption in the tertiary or quaternary structure of the enzyme followed by renaturation of the enzyme during the aging period. Another explanation is the removal of a cofactor from the active site during homogenization of the enzyme which is restored slowly. The activity then increases, reaches its maximum at fourteen days, and then declines rapidly. The removal of the sodium bromide completely arrests the emergence of activity and thus no activity can ever be recovered from the enzyme in its absence. Lower concentrations of sodium bromide will produce lower activity levels from the bromoperoxidase. Addition of sodium bromide after the initial extraction initiates no change in the activity of the enzyme.

E. Rationale for this Project

The production of many industrial chemicals requires as an important step, the halogenation of a precursor molecule. The halogenated chemical may be the final product or an intermediate in the process. Although the chemically initiated halogenation step is typically more economically favorable, the enzyme-mediated halogenation may be able to carry out halogenation reactions that are difficult or impossible through a chemical reaction. In addition, the use of bromoperoxidases to carry out chemoenzymatic reactions is much more environmentally benign than traditional methods.

For this study *P. cartilagineum* was collected intertidally from two areas off the

coast of California: Davenport and La Jolla. There were three collection sites at La Jolla: Camino de la Costa, Casa Beach, and Bird Rock. *Plocamium cartilagineum* is important in that it is a bromoperoxidase-containing algae that has chiral halogens in its metabolites.[5] Because of this, the enzyme(s) from *Plocamium cartilagineum* may be capable of stereospecific halogenation.

The purpose of this project was to develop a simple and efficient method to isolate the bromoperoxidase in *P. cartilagineum*. This was accomplished through the use a minimal number of steps. The algae was ground and aged fourteen days to allow the enzyme to reach maximum activity. Numerous methods to purify the enzyme were attempted. Most attempts failed and led to a loss of enzymatic activity. The most useful method was an initial cleanup step through a Sepharose CL-6B gel filtration column that was followed by ultrafiltration to concentrate the enzyme. The final purification step used hydrophobic interaction chromatography to separate the enzyme of interest from other proteins in the extract.

CHAPTER 2

EXPERIMENTAL

The bromoperoxidase from the marine alga *Plocamium cartilagineum* was obtained by extraction of plants collected from tidal pools along the coast of California. The bromoperoxidase was extracted from the algae and "aged" for two weeks for the enzyme to reach maximum activity. The enzyme was purified through a number of steps. The enzyme was purified for two reasons: (1) to characterize the bromoperoxidase and (2) to use the enzyme in future work.

A. Collection of Marine Alga *Plocamium cartilagineum*

Plocamium cartilagineum (*Rhodophyta, Plocamiaceae;* Figure 4) was collected intertidally from three locations (Figure 5) along the coast of La Jolla, CA between January 25, 1991 and March 28, 1999: Camino de la Costa, Bird Rock, and Casa Beach. *P. cartilagineum* from Davenport Landing (Santa Cruz, CA) was likewise collected. The collected samples were bagged, labeled with the collection site and date, then immediately stored on ice until transferred to the laboratory where they were stored at -10°C until studied.

B. Chemicals and Supplies

All chemicals used in this study were reagent grade. Tris[hydroxymethyl]aminomethane (Tris base), glycine, tetramethylethylenediamine (TEMED), ammonium persulfate ($(NH_4)_2S_2O_8$), agarose Zero M_r, acrylamide, bis

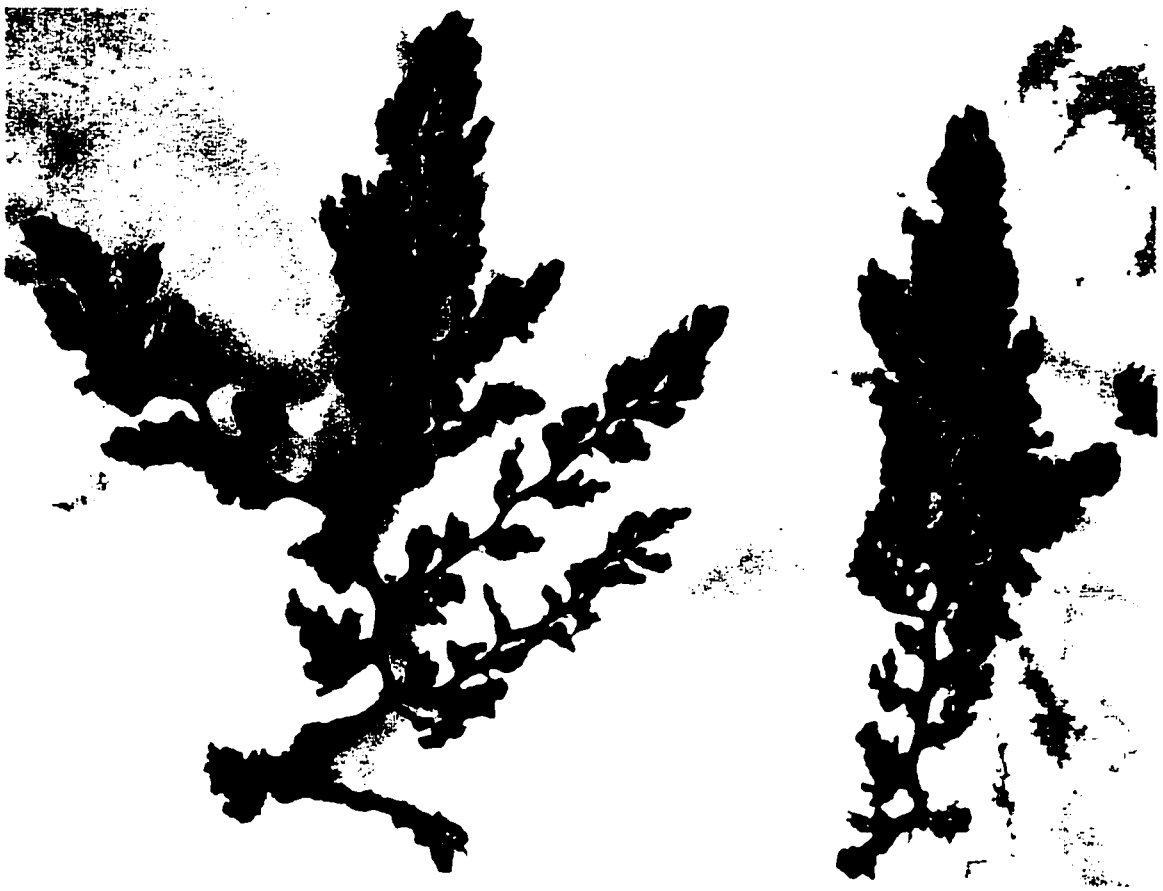


Figure 4. *Plocamium cartilagineum*

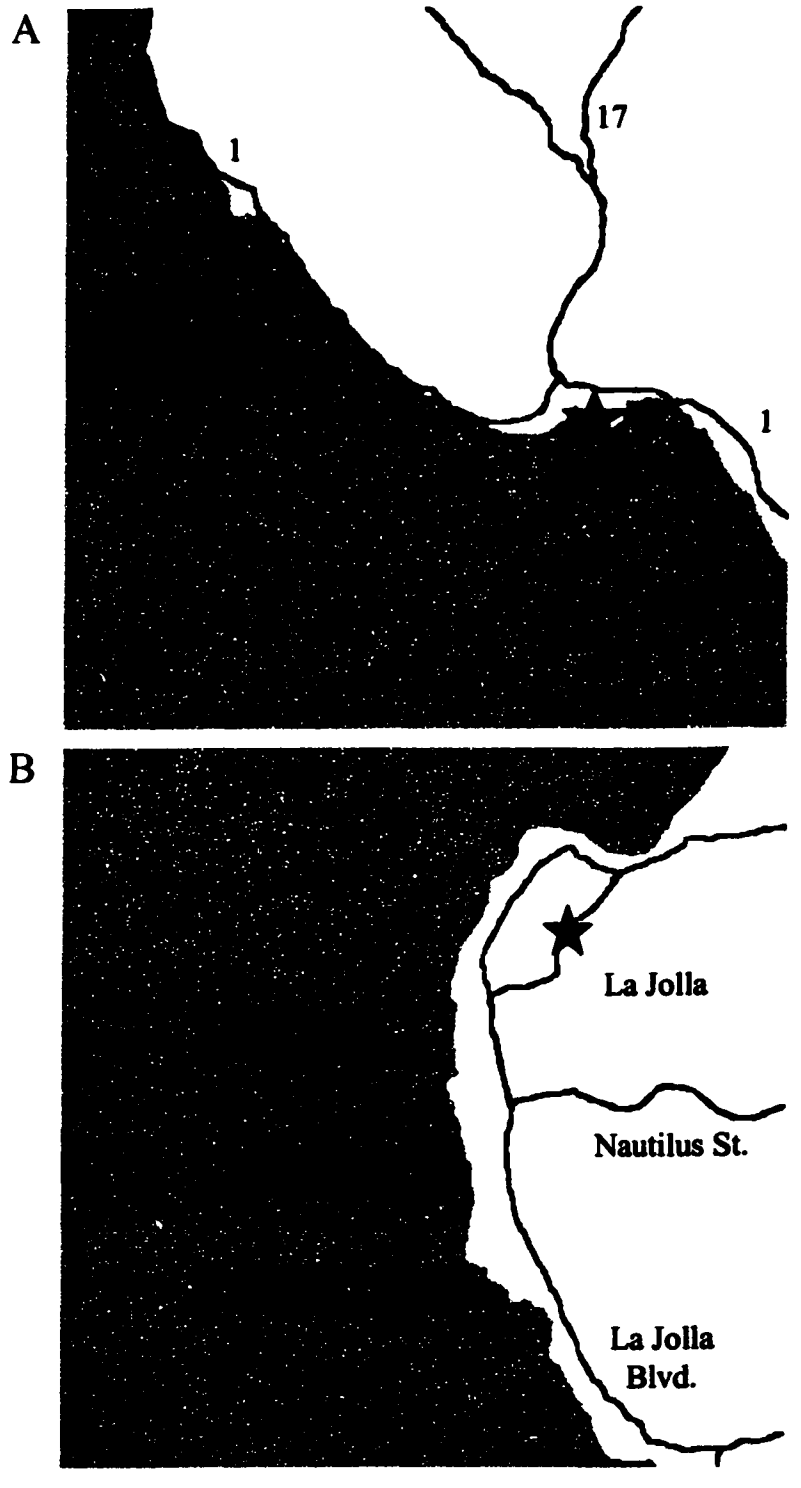


Figure 5. Maps of collection sites for *Plocamium cartilagineum.* Map A is Davenport landing north of Santa Cruz, CA. Map B shows the three La Jolla collection sites near San Diego, CA.

acrylamide, and sodium dodecyl sulfate (SDS) were purchased from BioRad. Ammonium sulfate ($(NH_4)_2SO_4$), and sodium phosphate (Na_3PO_4) were purchased from Sigma. Sodium bromide (NaBr) and potassium phosphate (K_3PO_4) were purchased from Aldrich. Urea was purchased from J.T. Baker. All other chemicals were purchased from Fisher Scientific.

C. Extraction of *Plocamium cartilagineum*

Frozen *Plocamium cartilagineum* was quickly thawed, blotted between paper towels, and weighed. For a typical extraction, 10 g of algae were refrozen at -10°C then ground in a cold mortar and pestle (-10°C) with 20 ml of phosphate buffer (50 mM pH 5.5) and 1.0 M NaBr. A pinch of sand was added for abrasion. The algae was then manually ground into a paste. The paste was centrifuged at 5000 rpm at room temperature for 10 minutes to sediment the thick, heavy pellet to the bottom of the centrifuge tube. The pellet was discarded. The pink to red/orange supernatant was collected, stored at 4°C for 14 days, and monitored periodically by phenol red assay. After 14 days, the supernatant was again assayed with phenol red to measure the activity (see below). If the amount of activity was sufficient to proceed to purification then further processing was begun. The criteria for sufficient activity was a complete color change of the assay solution from yellow to purple within one minute after the addition of enzyme.

D. Phenol Red Assay

Phenol red was used as a qualitative assay to track the activity of the enzyme

through the purification procedure. The assay solution consisted of 50 mM phenol red, 50 mM NaBr, 50 mM phosphate buffer (pH 5.5), and 1.0 mM H_2O_2. The assay was performed by the addition of 25 µl of enzyme solution to 4.0 ml of the yellow assay solution. If bromoperoxidase was present, the assay solution would become yellow/red and then finally purple as the phenol red was brominated to become bromphenol blue (Figure 6). The color change could take from 15 seconds to 24 hours and was dependant on the quantity of bromoperoxidase present. An active fraction would complete the color change of the assay from yellow to purple in less than 30 seconds (++++). A moderately active fraction would change color in 5 to 30 minutes (+++), a slightly active fraction in 12 hours (++), and extremely low activity level in a fraction could take 24 hours for a partial color change to occur (+). The assay solution was used to test column fractions for activity and as a "stain" for polyacrylamide and agarose gels to track the migration of the enzyme through the gel.

E. Monochlorodimedone Assay

The monochlorodimedone (MCD) assay was used to quantitate bromoperoxidase activity. The assay was 50 mM phosphate buffer (pH 5.5), 50 mM NaBr, 5.0 mM H_2O_2, and 80 μM MCD. The assay was performed at ambient temperature using a diode-array spectrophotometer (HP model 8452A). The absorbance was monitored at 290 nm using a single cell kinetics mode. The determination of the enzymatic activity was ascertained by a diminution in the absorbance as MCD was brominated to become 2-chloro-2-bromodimedone (Figure 2). One unit of enzymatic activity is defined as

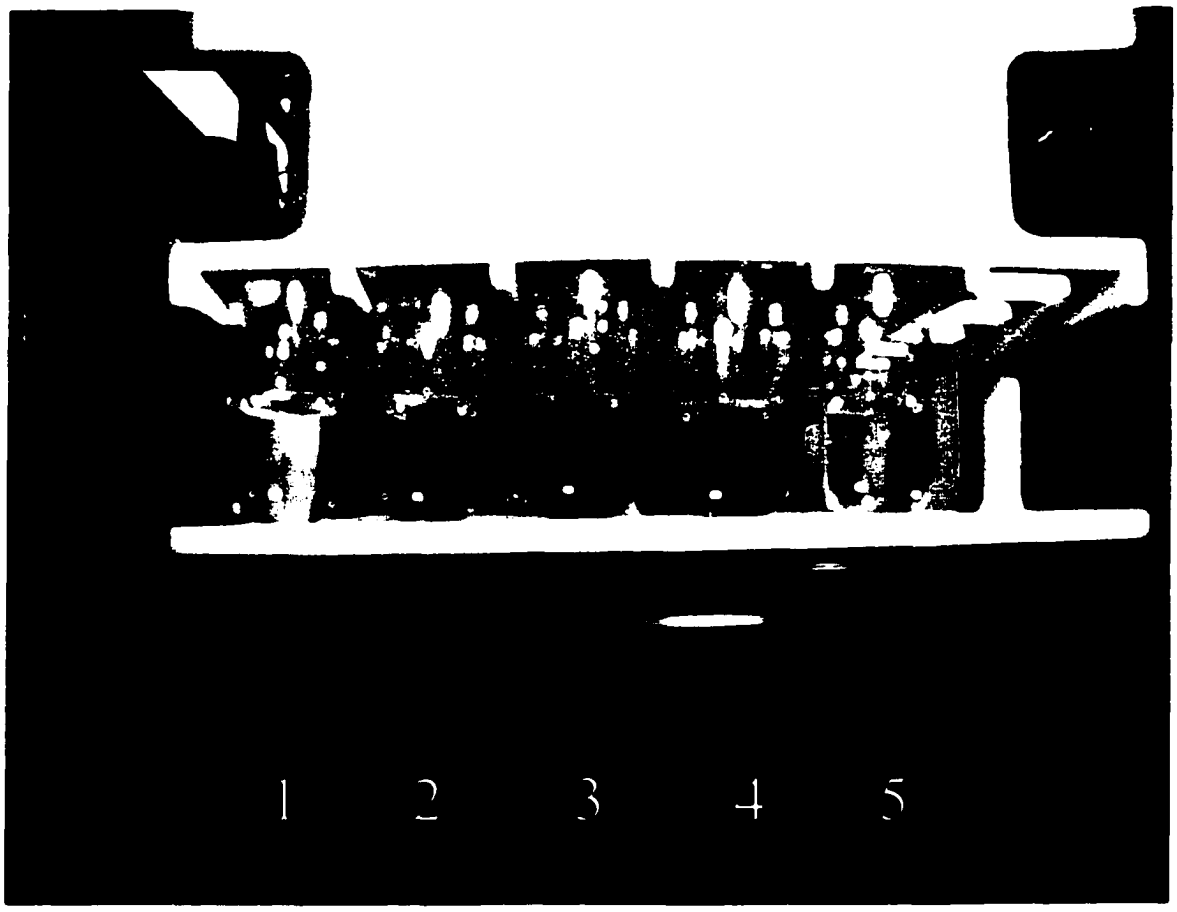


Figure 6. Phenol red assay of column fractions containing active enzyme after 2 minutes. Tubes 1 and 5 show the color of the assay prior to the addition of enzyme and contain little or no enzyme. Tubes 2 and 4 show the intermediate transition color. Fractions 2 and 4 contain enzyme and will be added to the fraction from tube 3. In an additional 3 minutes tubes 2 and 4 developed to the endpoint of the assay. Tube 3 shows the end point of the assay. This fraction contained most of the enzyme. An active fraction would complete the color change in less than 30 seconds (++++), a moderately active fraction in 5 to 30 minutes (+++), a slightly active fraction in 12 hours (++), and an extremely low activity level in a fraction would change in 24 hours or longer (+).

the amount of enzyme required to convert one μmol of monochlorodimedone to 2-chloro-2-bromodimedone in one minute.[16]

F. Electrophoresis

Vertical slab gel electrophoresis was performed on a Hoefer SE 250 mini gel electrophoresis unit with 6.0% T polyacrylamide gels using the Laemmli system. Gels (0.75 mm) were run at 15 mA/gel constant current at ambient temperature. The gels were stained for protein with Pierce Gelcode™ stain overnight and destained with water. The molecular weight of the protein was determined by boiling the purified sample in 2X treatment buffer that contained 7.0 M urea. The reduced enzyme was run in an acrylamide gel against the relative mobility of urease, jack bean (272 kDa and 545 kDa), albumin, bovine serum (66 kDa and 132 kDa), and α-lactalbumin (14 kDa).

G. Isoelectric Focusing (IEF)

The isoelectric point of the enzyme was determined by using a Bio Rad Model 111 mini IEF cell flat bed isoelectric focusing unit. Two microliter samples of protein and standard solutions were applied to the gel. Ampholites of pH range 3-10 in 0.5% agarose were focused at 60 V for 15 minutes then 150 V for 2 hours at ambient temperature. The pI of the enzyme was estimated with pI marker proteins (Sigma kit for isoelectric focusing range 3.6 - 6.6).

H. Column Chromatography

Gel permeation chromatography was carried out in a 2 X 60 cm Sepharose CL-6B column with gravity flow. Two ml of the crude enzyme extract was applied to the column. Two ml fractions were collected at 3 minute intervals with gravity feed at 4°C in 50 mM phosphate buffer (pH 5.5) containing100 mM NaBr. The fractions were assayed for activity with phenol red. The void volume of the column (38 ml) was determined with blue dextran (2,000 kDa).

Hydrophobic interaction chromatography using pentyl agarose (Sigma) was used as a final purification step. The column media was equilibrated with 50 mM phosphate buffer (pH 5.5) and 100 mM NaBr at 4°C . The enzyme was eluted with an increasing step gradient of NaCl in the equilibration buffer. NaCl was increased by 0.1 M with each step and the enzyme eluted when 0.2 M NaCl was washed through the column as indicated by the phenol red assay.

I. PHast Gels

PHast™ gel system (APBiotech) polyacrylamide electrophoresis gels ran on 4-15% T gradient gels using the preprogramed separation protocol for native proteins. Four microliter samples of enzyme and standard were applied to the gel. The gels were stained with Coomassie blue R-350 stain solution to detect proteins down to 50 ng/band.

The 4-15% gradient gels are capable of separating native proteins up to 700 kDa. Although these gels did not work for separating the enzyme and determining a molecular weight, the results did demonstrate the relative purity of the enzyme solution.

J. Additional Unsuccessful Purification Methods

The following chart is an outline of the techniques that were tried, but were unsuccessful in isolating the enzyme. The technique either inactivated the enzyme or failed to remove it from other contaminants. Full details can be found in the Results and Discussion section.

Table 2

Method	Conditions	Results
Column Chromatography		
t-Butyl Hydrophobic Interaction Chromatography (HIC)	Batch and column runs Ambient and 4°C	Initial run successful, but not reproducible
Decyl agarose HIC	Ambient and 4°C Change in ion to elute	No results to minimal amounts of activity
Phenyl agarose HIC	Ambient and 4°C Change in ion to elute	No results to minimal amounts of activity
Propyl Agarose HIC	Ambient and 4°C Change in ion to elute	Moderate amounts of activity, but not always reproducible
Ion exchange (anion and cation)	0.1 to 1.0 M NaBr and NaCl	Extremely weak activity in some samples
Extraction		
Urea 6 M	Addition to buffer and polyacrylamide gels	Decrease in enzyme activity, but no separation on the gel
Triton X	0.5 to 5 % solutions	Aggregates still present
Glycerol	0.5 to 5 % solutions	Aggregates still present
Tween 80, 40, 20	0.5 to 5 % solutions	Aggregates still present
Brij 30	0.5 to 5 % solutions	Aggregates still present

Electrophoresis		
Vertical slab gel electrophoresis	0.5 to 1.0 % gels	No migration of enzyme into the gel
Other Methods		
Hydroxyapatite		No enzymatic activity
Ethanol and methanol precipitations	Iced and -20°C	No enzymatic activity

CHAPTER 3

RESULTS AND DISCUSSION

A. Collection and Comparative Enzyme Activity

P. cartilagineum from three coastal collection sites in La Jolla (Bird Rock, Camino de la Costa, and Casa Beach) and one from Santa Cruz (Davenport Landing) were compared for enzyme activity. *P. cartilagineum* from Bird Rock and Casa Beach was selected over samples collected from Camino de la Costa or Davenport. Preliminary activity studies of samples from Camino de la Costa consistently exhibited significantly diminished activity levels from those collected from the other two La Jolla collection sites although two of the samples from Camino de la Costa did have enzymatic activity that surpassed the other two sites. Camino de la Costa samples gathered from 1993 to 1998 were assayed for activity with the MCD assay against samples from the other two La Jolla sites (Figure 7). Time of year, color of the algae extract (which could vary in color from bright pink to red/orange), predatory inhabitants, and the growth cycle of the algae were examined to investigate any similarities between environmental conditions and activity of the enzyme. No conclusion could be made. It is possible that other conditions that were not monitored could be responsible for the reduction in enzyme activity: these include water temperature, salinity, or pollution.

While collections from Davenport possessed a high degree of enzymatic activity, samples from this site were not used in this study; inconsistency with the enzyme extraction was weighed against the activity. For example, centrifugation was required after

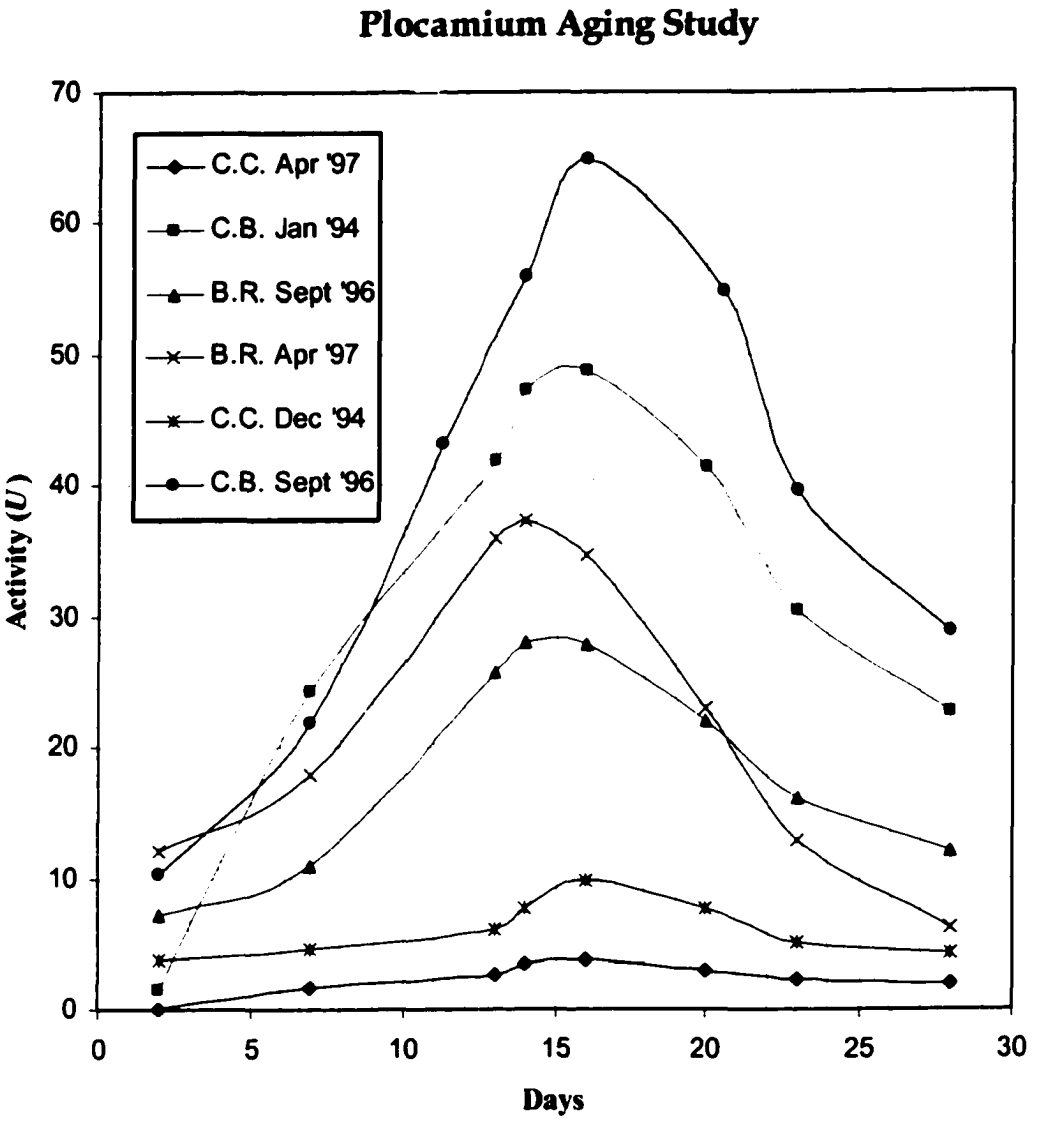


Figure 7. MCD assay aging study of bromoperoxidase from Casa Beach (C.B.), Bird Rock (B.R.), and Camino de la Costa (C.C.). The samples shown are representative of the samples found at that site. Enzyme from a frozen algae sample was extracted with 1 M NaBr in phosphate buffer. Samples were stored at 4°C and assayed using MCD (described in experimental). A unit of activity is the amount of enzyme catalyzing the reaction of 1 μmol of monochlorodimedone to product in one minute.

ammonium sulfate precipitation and dialysis to extract the enzyme completely; the pellet was thicker and had "stickier" consistency than the La Jolla sample pellets. Due to the deviations in the behavior of the enzyme extract from Northern CA, no further purification procedures were attempted on the samples from this site.

While there is enzymatic consistency among the different collection sites, there is also variability in the activity and possibly in the taxonomy. This latter variable could be the reason for the difference in the behavior of the enzyme extract from Davenport during the purification process. No connection could be made to determine the differences in the enzymatic activity and the collection sites of *P. cartilagineum*. Environmental conditions, life cycle, or age of the algae are all plausible causes for the significant impact on the enzymatic activity between the samples from the same site and the same collection bag. Mynderse *et al* extensively studied *P. cartilagineum* from La Jolla.[15] Individual plants were tagged and collected at different times of the year. Variations were found in the amounts and in the composition of the monoterpenes extracted from the algae. No evidence was found for seasonal or sexual variations. Thus, it is not surprising that variability in enzyme activity is also observed.

B. Extraction Methods and "Aging" the Enzyme

Frozen *P. cartilagineum* was weighed, thawed, cleaned and frozen again prior to grinding. Grinding the frozen sample insured a uniform consistency of the enzyme extract. Some samples were refrozen in liquid nitrogen after cleaning and then ground. The extremely low temperature of the liquid nitrogen reduced the time required to grind the

algae. No appreciable decrease could be noted in the activity of the extract after processing the algae in this manner, but it is unknown if the extremely low temperature had an effect on the enzymatic activity. It should be noted that samples from the same collection bag could not be compared directly, since samples prepared in the same manner could have significantly dissimilar activities.

The sample was ground to a fine powder, followed by the addition of 50 mM phosphate buffer containing 1.0 M NaBr to process the sample into a paste. If the NaBr was omitted from the buffer, the enzyme would only reach a small percentage of its potential activity. If the concentration of the NaBr was less than 1.0 M, the activity of the enzyme did not reach an optimum level. Sodium bromide was also found to have a key role in the "aging" process which is described below.

After grinding, the sample was centrifuged to pellet the solids. The supernatant was pipetted off and stored at 4°C for two weeks to age. During the aging period, the samples were monitored periodically for activity using the phenol red assay. The aging period was required for the enzyme to achieve maximum activity (Figure 7). The period required for the enzyme to reach the maximum activity level was surprisingly consistent, irrespective of the sample collection date, the collection site, or the amount of activity that the enzyme sample contained. Over 150 samples were ground and "aged" for this project. All samples were periodically tested for enzymatic activity during the "aging" period. In nearly every case, the enzyme had only minimal activity on the day it was ground and always required the two week aging period to obtain maximum activity.

In addition to facilitating the extraction of the bromoperoxidase, NaBr was also

found to play an important role in the "aging" of the enzyme. In one representative sample a 10 g sample was ground in 50 mM phosphate buffer, divided into three fractions, and NaBr was added to the concentrations listed below. The samples were aged at 4°C for fourteen days and assayed with MCD. The results are shown below in the following chart.

Table 3

Sample•Casa Beach 4/27/97	Conditions	Activity (U)
Fraction 1 (⅓ of sample)	Aged with 1.0 M NaBr	79.93
Fraction 2 (⅓ of sample)	Aged with 0.5 M NaBr	27.81
Fraction 3 (⅓ of sample)	Aged with 0 M NaBr	4.88

All subsequent purification steps involved 0.1 M NaBr in 50 mM phosphate buffer (pH 5.5). The reduction of the bromide concentration from 1.0 M to 0.1 M after the enzyme becomes fully active does not have a negative effect on the activity. The total removal of bromide from the buffer results in the inactivation of the enzyme. The following chart shows the results of an MCD activity study of three fractions from a single sample that were dialyzed overnight against different NaBr concentrations at 4°C.

Table 4

Sample•Casa Beach 10/28/96	Conditions	Activity (U)
Fraction 1 Post $(NH_4)_2SO_4$ precipitation	Fraction 1 dialyzed against 1.0 M NaBr in 50 mM phosphate	53.47
Fraction 2 Post $(NH_4)_2SO_4$ precipitation	Fraction 1 dialyzed against 100 mM NaBr in 50 mM phosphate	49.96
Fraction 3 Post $(NH_4)_2SO_4$ precipitation	Fraction 1 dialyzed against 50 mM phosphate	3.46

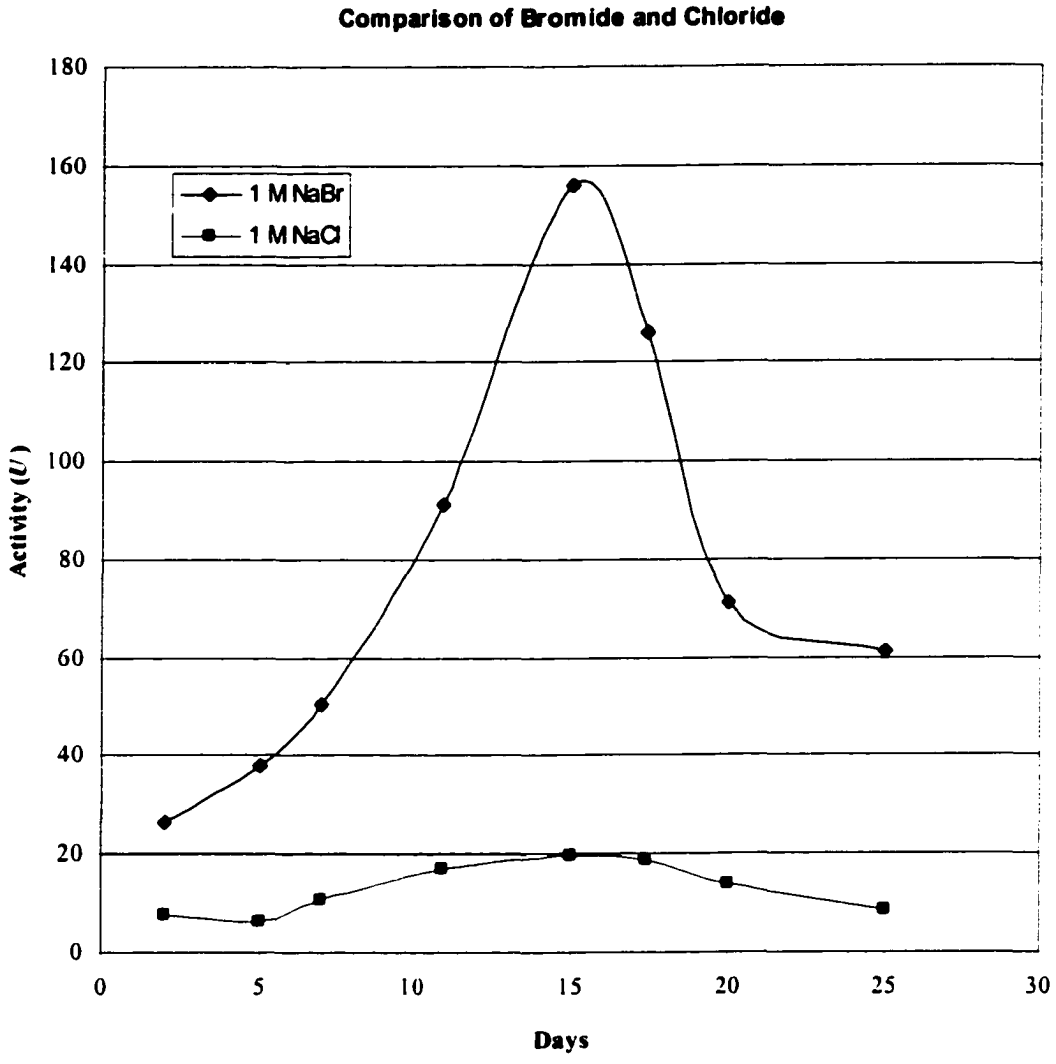


Figure 8. An aging study of bromoperoxidase comparing NaBr and NaCl. A 10 g sample was divided and aged with 1 M NaBr or 1 M NaCl for 25 days. Activity was determined using the MCD assay. A unit of activity is the amount of enzyme catalyzing the reaction of 1 μmol of monochlorodimedone to product in one minute.

NaCl was used in the same concentrations in place of NaBr in the grinding buffer and then in place of NaBr after the two week incubation (Figure 8). A sample of algae was ground to a fine powder, divided into two samples, and suspended in aging buffer as follows. Sample 1 contained 1 M NaBr in 50 mM phosphate buffer and sample 2 contained 1 M NaCl in 50 mM phosphate buffer. The enzyme in sample 2 never obtained any appreciable activity . The removal of bromide from the buffer always resulted in the total loss of activity from the enzyme.

To eliminate or reduce the formation of aggregates in the extract solution a number of detergents (Triton X, Tween, and Brij) and a solubilizing reagent (glycerol) were investigated. The detergents were added to the aging buffer and added to the sample when the algae was ground. To confirm that it was the reagent having an effect on the activity of the bromoperoxidase, a large (25 g) sample of algae was ground and separated into multiple fractions. Each fraction was in 50 mM phosphate buffer and 1 M NaBr containing one of the following detergents. Triton X, Brij 30, or Tween 80, 40, 20 were added to 0.5% and 5% v/v. Glycerol was added to make solutions of 5% and 50% v/v. One fraction was treated with only phosphate buffer and 1 M NaBr as a control. The samples were aged for two weeks and assayed for activity with phenol red (Table 5). The glycerol samples had activity that were comparable to the control sample. The other samples were completely inactive. The glycerol fraction and the control were run on a 4% T polyacrylamide electrophoresis gel then stained with phenol red. The two samples were still in the loading wells, indicating the presence of large aggregates.

A large (25 g) sample of *Plocamium* was ground and aged in phosphate buffer

Method	Conditions	Activity Detergent in Aging Buffer	Activity Detergent Added After Aging	Aggregates Present
Triton X	0.5 % and 5 %	—	+	Yes
Brij 30	0.5 % and 5 %	—	++	Yes
Tween 20,40,80	0.5 % and 5 %	—	++	Yes
Glycerol	5 % and 50 %	++++	++++	Yes
Control	No Detergent	++++	++++	Yes

Table 5. Comparison of detergents present in extraction buffer during aging or added at 14 days after formation of aggregates. Activity of extract: very active (color change in less than 30 seconds,++++), moderately (color change in 5 to 30 minutes,+++), slightly (color change in 12 hours,++), very slight (color change in 24 hours or more,+), and no activity (-).

with 1 M NaBr. A fraction of the sample was run on a 4% T polyacrylamide electrophoresis gel to confirm the presence of aggregates prior to further tests. The aggregated sample was divided into fractions for further examination with the detergents and solubilizing agent. Each fraction was treated with a single detergent and stored at 4°C overnight. The fractions were assayed for activity with phenol red. Active fractions were run on a 4% T polyacrylamide gel and subsequently stained with phenol red to determine the relative molecular weight of the enzyme from a particular fraction. Triton X was added to fractions at 0.5% and 5% v/v. Aged fractions never showed more than extremely low levels of activity when compared to the control (Table 5). Aggregates were present in the gel and compared to those of the control. Glycerol was added to fractions at 5% and 50% v/v. Aged fractions compared favorably in activity to the activity found in the control, but the aggregates were still present in the gel. Tween 80, 40, and 20 were added at 0.5% and 5% v/v. Fractions showed diminished activity levels when compared to the control and aggregates were not dissociated. Brij 30 was added to fractions at 0.5% and 5% v/v. Fractions showed decreased activity levels when compared to the activity level of the control, but the aggregates were still present.

The two week period required for the enzymatic activity to peak may be due to the temporary inactivation of the enzyme, followed by a slow reconstitution to regain the catalytic activity. Possible explanations could be the removal or change of the cofactor in the enzyme, which might then return to the catalytic site slowly. Quaternary structure has been observed to be important in other algal bromoperoxidases such as from *Corallina officinalis* and *Corallina pilulifera*.[6,9] Thus, another explanation for the behavior of the

Plocamium enzyme is the disruption of quaternary structure which returns to the native state over two weeks. To our knowledge, this "aging" effect has not been observed with any other haloperoxidases, and is in general an unusual behavior for the enzyme.

Bromide is required for the bromoperoxidase to be active and must be present in the buffer in which the enzyme is extracted and aged. The bromide may act as a chaotrope and aid in the extraction of the enzyme from the algae. Large aggregates form during the aging process and in our project the aggregates have so far not been broken apart by the detergents and solubilizing reagents that have been tried. The aggregates are different from the multimers that form the quaternary structure of the enzyme. The multimeric form protects the substrate and keeps it in a hydrophobic environment. The aggregates may create a favorable environment that may be closer to the conditions within the cell. Only one set of conditions was used (e.g. phosphate buffer) which may not be optimal.

The different areas of the thallus (undifferentiated plant body) may contain different concentrations of the bromoperoxidase. A study of the bromoperoxidase from *Ascophyllum nodosum* determined that the enzymatic activity was localized in the receptacles (fruiting bodies). Bromoperoxidase activity could hardly be detected anywhere else in other parts of the alga.[4] When *P. cartilagineum* for this project was collected, usually several different plants were bagged at the site of collection and eventually frozen upon arrival to the lab. When a sample is defrosted to be ground, there is no way to determine what portion of the plant is taken from the bag or if a combination of samples from the same site are ground together into one sample. This may explain the difference in activity levels of the enzyme from algae taken from the same bag.

C. Chromatography

Prior to chromatography, the aged sample was precipitated with 80% $(NH_4)_2SO_4$ to concentrate the protein sample to be loaded onto the column. The $(NH_4)_2SO_4$ solution was centrifuged to precipitate the protein. The supernatant was discarded and the deep red pellet was resuspended in a minimum amount of phosphate buffer containing 100 mM NaBr and dialyzed overnight (MWCO 10 K) against 50 mM phosphate buffer (pH 5.5) with 100 mM NaBr.

1. Gel Filtration Chromatography

Sepharose CL-6B was the first column in the purification process. The entire dialyzed fraction from the $(NH_4)_2SO_4$ precipitation was applied to the top of the media. The length of time the enzyme was aged prior to $(NH_4)_2SO_4$ precipitation determined the number of fractions collected that contained enzymatic activity. If the $(NH_4)_2SO_4$ precipitation fraction was applied to the gel filtration column prior to the end of the two week incubation period (7 to 10 days), the column yielded enzyme-containing fractions from the void volume of 2,000 kDa to a much smaller molecular weight that was never determined. Twenty seven of the 46 fractions collected contained enzyme beginning with fraction 1 (void volume) to fraction 39. Precipitating the enzyme and applying it to the gel filtration column at the end of the two week incubation period allowed all of the active fractions to be collected in two to three 2 ml fractions that eluted with the void volume along with blue dextran (2,000 kDa). Once the elution pattern of the enzyme was determined, it was easily tracked on later columns without blue dextran due to its faint

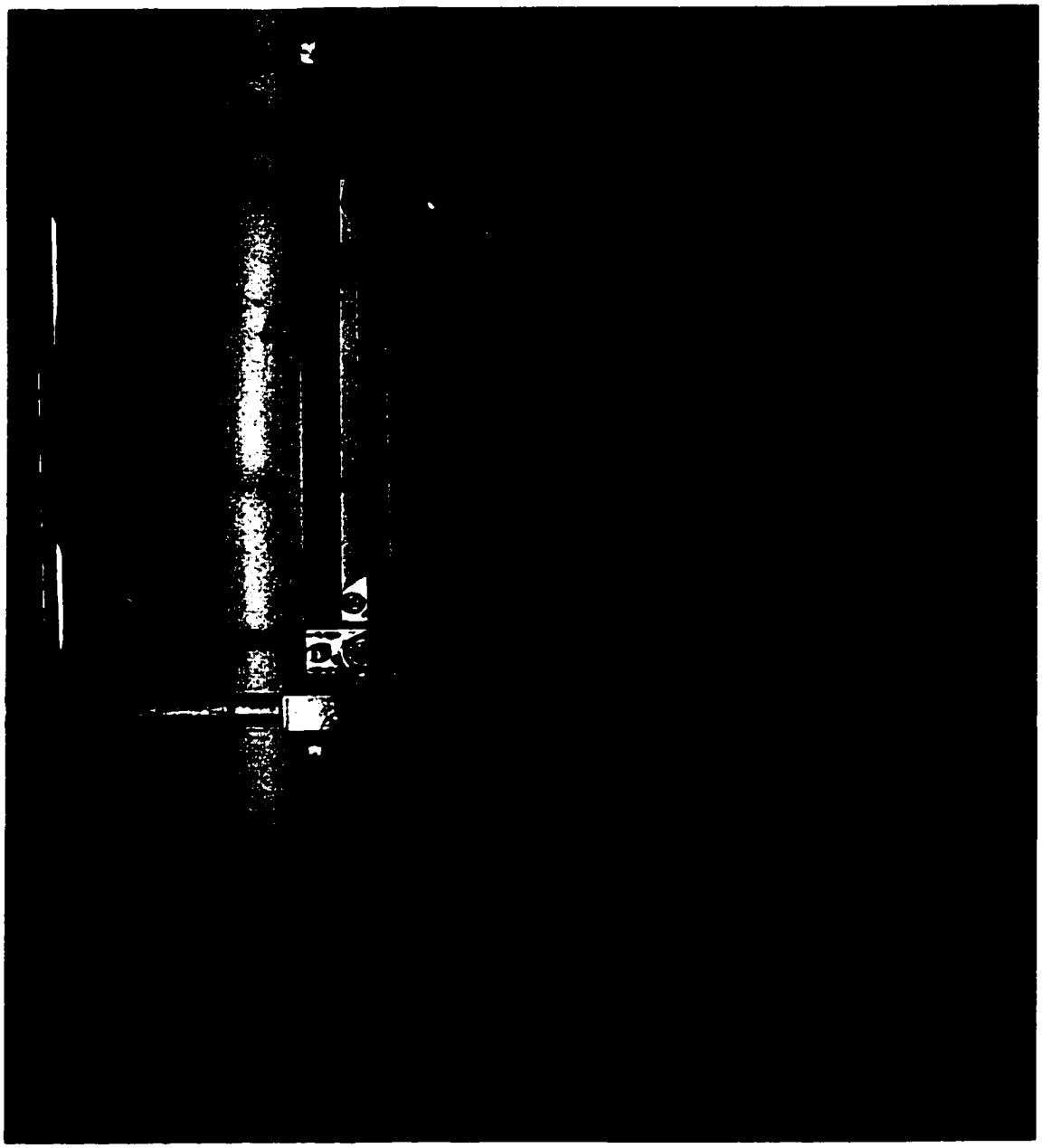


Figure 9. Sepharose CL-6B chromatography column. The faint pink/brown band near the cross bar behind the column contains the bromoperoxidase. The bright pink band is phycoerythrin.

pink/brown color in contrast to the white color of the Sepharose CL-6B (Figure 9). The bright red/pink band is assumed to be phycoerythrin.

The active fractions from the gel filtration media were concentrated with ultrafiltration (MWCO 10,000) on ice using no stirring. The losses at this step were evident as there was considerable amount of a red/brown colored paste that remained on the filter after the concentrated enzyme extract was removed from the ultrafiltration chamber. The red/brown paste that adhered to the filter was scraped from the filter and tested for enzymatic activity with the phenol red assay (Table 8). The immediate color change of the assay demonstrated the large amount of enzyme that had precipitated and hence was not recovered in solution. All further chromatography trials were run with a post ultrafiltration enzyme solution.

Centricon concentrators (Millipore) with molecular weight cut-offs of 10 kDa, 30 kDa, and 100 kDa were tried as a method to concentrate the enzyme. The pores of the concentrators became clogged completely with the red/brown paste before the process was complete. The speed of the centrifuge was lowered to avoid clogging the pores, but this process never worked to completion.

2. Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) was used as a final step in the purification procedure. Several supports were utilized and four yielded active fractions. A Macro-Prep *t*-Butyl HIC support (Bio Rad) column was equilibrated with 2 M or 1.5 M $(NH_4)_2SO_4$ or NaCl in phosphate buffer. The sample was applied to the column and eluted

Method	Equilibration Conditions	Results
t-butyl (first column)	2.0 M $(NH_4)_2SO_4$	++++
(subsequent columns)	2.0 M $(NH_4)_2SO_4$	—
	1.5 M $(NH_4)_2SO_4$	—
	2.0 M NaCl	—
	1.5 M NaCl	—
Decyl Agarose	100 mM NaBr room temperature	—
	100 mM NaBr 4°C	—
Phenyl Agarose	100 mM NaBr room temperature	++
(Eluted in wash)	100 mM NaBr 4°C	++
Propyl Agarose	100 mM NaBr room temperature	+++
(Not reproducible)	100 mM NaBr 4°C	+++
Boronic Acid	200 mM ammonium acetate pH 8.5	—

Table 6. Comparison of hydrophobic interaction chromatographic methods. All solutions are 50 mM phosphate buffer pH 5.5 unless otherwise indicated. Activity of extract: very active (color change in less than 30 seconds,++++), moderately (color change in 5 to 30 minutes,+++), slightly (color change in 12 hours,++), very slightly (color change in 24 hours or more,+), and no activity (—).

with a decreasing linear gradient of $(NH_4)_2SO_4$ or NaCl from the equilibration concentration to 0 M containing 100 mM NaBr. In succeeding trials, the NaBr was increased to 500 mM in an attempt to keep the enzyme active. Fractions were collected and assayed for activity using phenol red. The macro-Prep *t*-Butyl HIC support eluted very active fractions the first time it was used after equilibrating the column with 2.0 M $(NH_4)_2SO_4$, but despite repeated attempts with the original packed column and replacing the column with new media, the results could never be duplicated (Table 6). Following an unsuccessful column chromatography experiment, the media itself was placed into phenol red assay solution to test if the enzyme had been irreversibly bound to the column. No activity was observed. This procedure of placing the media into phenol red assay solution was utilized for all subsequent attempts with media that failed to produce active enzyme fractions. This result suggested that the enzyme was not irreversibly bound to the media, but that the enzyme was inactivated by the column media.

Other HIC media, including boronic acid (Pierce) and decyl, phenyl, and propyl agarose (BioRad), were tested (Table 6). The decyl, phenyl, and propyl agarose columns were all equilibrated with phosphate buffer and 100 mM NaBr. The enzyme sample was applied to these columns, washed, and eluted with an increasing NaBr gradient of 100 mM to 1 M in phosphate buffer. The columns were run at room temperature and then later at 4°C to facilitate desorption from the column matrix. The decyl agarose column never eluted active fractions. This could be the result of the loss of a portion of the enzyme that adhered to the media. Phenyl agarose yielded somewhat active fractions, but the enzyme eluted in the initial wash fractions with other contaminants (as determined by gel

electrophoresis). Propyl agarose yielded moderately active fractions, but the results were not always reproducible and the activity of the bromoperoxidase was not as high as the fractions from the pentyl agarose column. Boronic acid was equilibrated with 200 mM ammonium acetate (pH 8.8). The pH of the sample was increased to 8.5, mixed with the supplied binding buffer and loaded onto the column. Elution was accomplished with 200 mM sorbitol. The collected fractions were tested for activity with phenol red. There were no active fractions. The results suggest the less hydrophobic media (propyl and pentyl) do not inactivate the enzyme. The longer arm of the more hydrophobic decyl agarose media either holds on to the enzyme tightly, or some interaction with the media itself leads to inactivation of the enzyme.

3. Ion Exchange Chromatography

Anion and cation exchange chromatography were also tried as a final purification step. CM Sephadex and CM Sepharose and DEAE Sephadex and DEAE Sepharose (Sigma) are cation and anion exchangers on dextran or agarose, respectively. The partially purified sample was loaded onto the prepared column, washed with an increasing gradient of 100 mM to 400 mM NaBr or NaCl in phosphate buffer with the cation exchanger or Tris-HCl with the anion exchanger. A phenol red assay indicated extremely weak activity in the fractions eluted with NaBr as shown in table 7. Sodium bromide was added to the post column fractions to bring the concentration up to 1 M to recover the activity. The fractions were stored at 4°C for 48 hours and assayed periodically for activity. No additional activity was recovered.

Method	Elution Conditions	Results
Cation Exchange		
CM Sephadex	100 mM - 400 mM NaCl	—
	100 mM - 400 mM NaBr	+
CM Sepharose	100 mM - 400 mM NaCl	—
	100 mM - 400 mM NaBr	+
Anion Exchange		
DEAE Sephadex	100 mM - 400 mM Tris-HCl	—
DEAE Sepharose	100 mM - 400 mM Tris-HCl	—

Table 7. Comparison of ion exchange media. Cation exchange media was eluted with either NaCl or NaBr in 50 mM phosphate buffer. Anion exchange was eluted in Tris-HCl. Activity of extract: very active (color change in less than 30 seconds,++++), moderately (color change in 5 to 30 minutes,+++), slightly (color change in 12 hours,++), very slight (color change in 24 hours or more,+), and no activity (-). The results indicate the activity collected from all combined fractions.

Fraction	Volume (ml)	Activity (U)	Protein (mg)	Spec. Act. (U / mg)	Recovery (%)	Purification (fold)
Crude Extract	20	39	46	0.84	100	1
Ammonium Sulfate	1.5	34	35	0.97	87	1.2
Ultracentrifugation	1.5	24	19	1.3	71	1.5
Eluate from pentyl agarose	2	8	2	4.2	20	5

Table 8. Purification of bromoperoxidase from *Plocamium cartilagineum*.

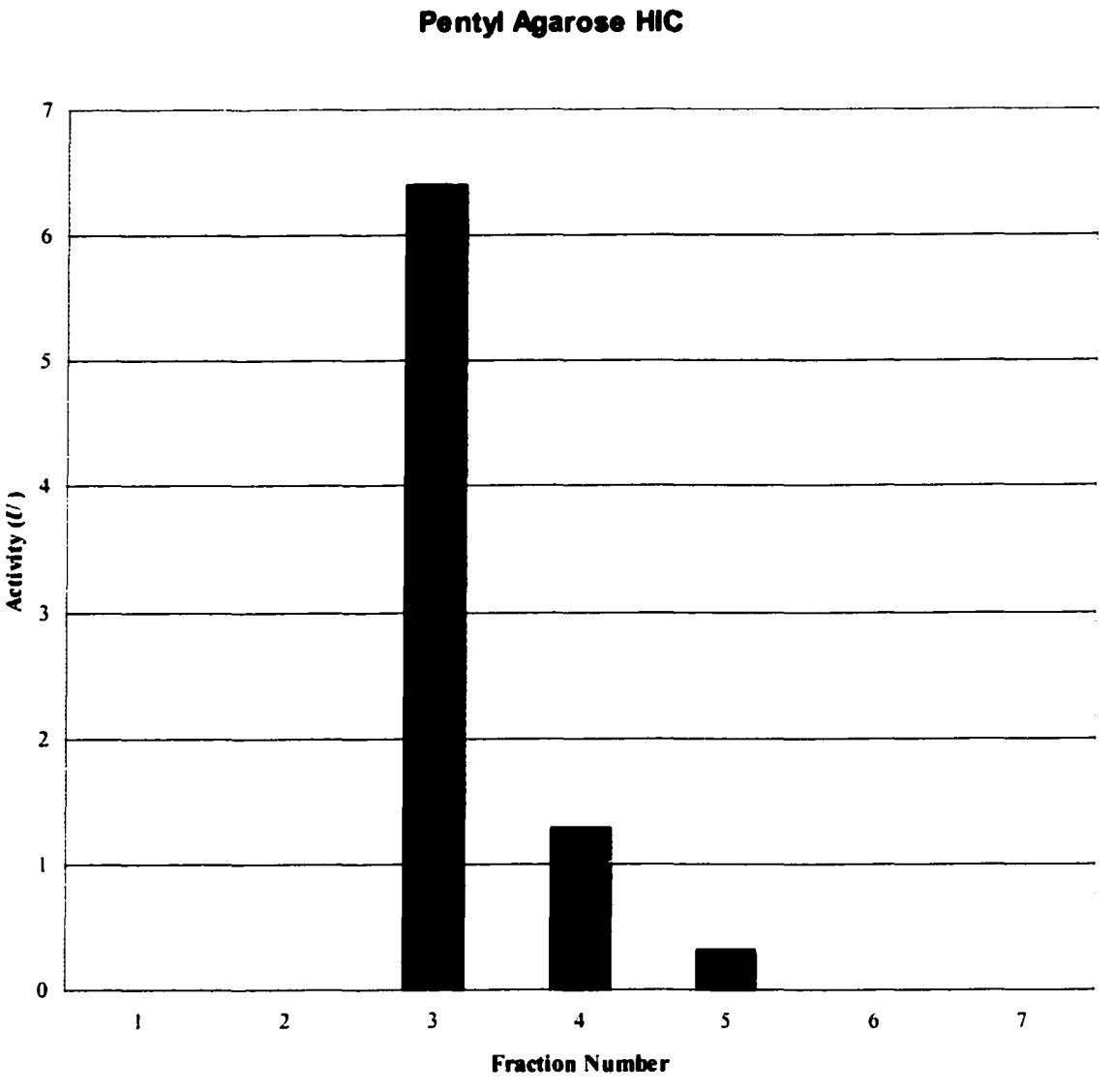


Figure 10. Activity of fractions assayed with MCD from a pentyl agarose HIC column. The protein was loaded onto a 1 X 1 cm column with gravity flow. The column was equilibrated with 50 mM phosphate buffer with 100 mM NaBr. One ml fractions were collected with an increasing step gradient (100 mM/step) of NaCl. The fractions were assayed with MCD. A unit of activity is the amount of enzyme catalyzing the reaction of 1 μmole of monochlorodimedone to product in one minute.

4. Summary of Chromatography Results

The HIC media pentyl agarose yielded the only consistent elution pattern with fractions that exhibited adequate activity to proceed to subsequent analysis of the enzyme (Figure 10). Once purified with this step, the enzyme had to be used or assayed immediately or significant loses in activity could be measured overnight. The following table demonstrates the losses observed if the bromoperoxidase is not used immediately after purification.

Table 9

Sample•Casa Beach 07/23/93	Conditions	Activity (U)
post pentyl agarose HIC	30 minutes	9.49
post pentyl agarose HIC	16 hours	2.79

The gel filtration chromatography suggests the formation of the aggregates are essential under these conditions for the enzyme to be active. The increase in activity of the enzyme coincides with the formation of the aggregates. The specificity of the enzyme for varied functional groups on HIC media is not surprising because the aggregation indicates the presence of a hydrophobic exterior on the quaternary structure of the protein. The interaction with the media is dependant upon the pattern and size of the hydrophobic patches on the enzyme. Small changes in the nonpolar groups of the HIC media can have a large effect on the ability of the enzyme to interact with them. The interaction of the enzyme with the matrix of the column seems to affect or inactivate a key aspect required for the activity of the *P. cartilagineum* bromoperoxidase.

A

B

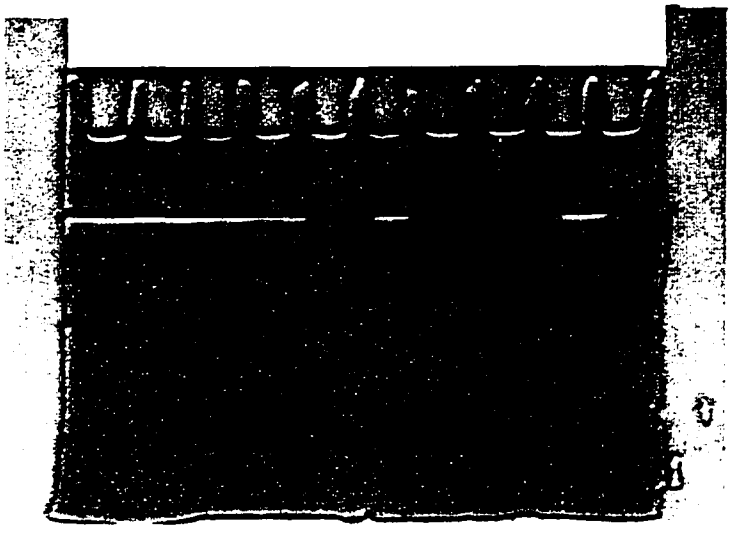


Figure 11. Polyacrylamide gel electrophoresis of bromoperoxidase. Both gels are 6% T and have identical samples loaded onto them. Lanes 3 and 6 are a crude enzyme extract 20 days after grinding. Lane 4 is albumin, bovine serum (66 kDa and 132 kDa). Gel A was stained with Coomassie Blue. Gel B was stained with phenol red assay solution. Note the stained protein in the loading wells and the sensitivity of the phenol red assay as a stain.

D. Electrophoresis

Polyacrylamide gel electrophoresis using a standard Laemmli buffer system was utilized to determine the molecular weight of the enzyme and establish the relative purity of the extract solution. Fifteen mA per gel constant current was applied for approximately 60 minutes until the tracking dye reached the bottom of the gel. The size and hydrophobicity of the aggregated enzyme made gel electrophoresis difficult. In order to track its location on the gels with phenol red, the enzyme was in its native state (Figure 11). The fully active enzyme would not migrate into the polyacrylamide gel; the gels were prepared as low as 4% T to facilitate migration into the polyacrylamide by making the pore size as large as possible, but the enzyme always failed to migrate into the gel.

Vertical agarose mini gels of 0.5% and 1% were poured and run as an alternative to polyacrylamide to take advantage of the larger pore size available with agarose. The protein size ranges for the 0.5% and 1% gels are >5,000 kDa and 1,000 to 5,000 kDa respectively. The native gels were prepared with the standard Laemmli buffer system without SDS. Twenty mA constant current was applied for 2 hours. The enzyme did not enter into the agarose, hence the molecular weight of the aggregated enzyme must be greater than 5,000 kDa. The acrylamide and agarose gels were stained with phenol red assay solution. In all cases, a purple band appeared at the top of the gel in the wells of the stacking gel indicating the presence of the enzyme only in the loading well.

Three to four weeks after grinding, the enzyme extract still possessed a considerable measure of activity (80% to 50% respectively). Running a 6% T native

polyacrylamide gel with the crude extract at that time would generate a gel with several active enzyme bands when stained with phenol red assay solution (Figure 11). The molecular weight of the active enzyme band(s) could be small (approximately 70 kDa) to very large (over 200 kDa). The enzyme fragments were never consistent from one gel to another so the determination of the size for an individual subunit or the quaternary structure could not be determined using a native gel. The fragments that developed after three weeks were probably the result of degradation of the aggregates from protease or microbial contamination. The grinding procedure did not take place under sterile conditions nor were any inhibitors used to stop bacterial or enzymatic degradation of the peroxidase. Sodium azide has been shown to be a competitive inhibitor of bromoperoxidase.[6,12] A protease inhibitor, N-tosyl-$_L$-phenylalanine chloromethyl (TPCK), was used in previous work in this lab and found to have no effect on the aging process of the enzyme. The enzyme was collected and ground without processing to preserve the activity of the enzyme.

An attempt was made to purify the enzyme with preparative gel electrophoresis (Figure 12) Polyacrylamide plug gels from 10 to 15% T were poured under separating gels from 4% to 6% T. Urea was added to the stacking and separating gels. The depth of the separating gel was reduced from 6 to 2 cm. A stacking gel was poured on top of the separating gel. Voltages were varied from 30 to 300 volts.

At less than 15% T, the plug gel would absorb the tracking dye prior to it passing through the channel. The separating gel had to be poured with a low % T and a thickness no greater than 2 cm. Due to the large molecular weight of the aggregates, if the thickness

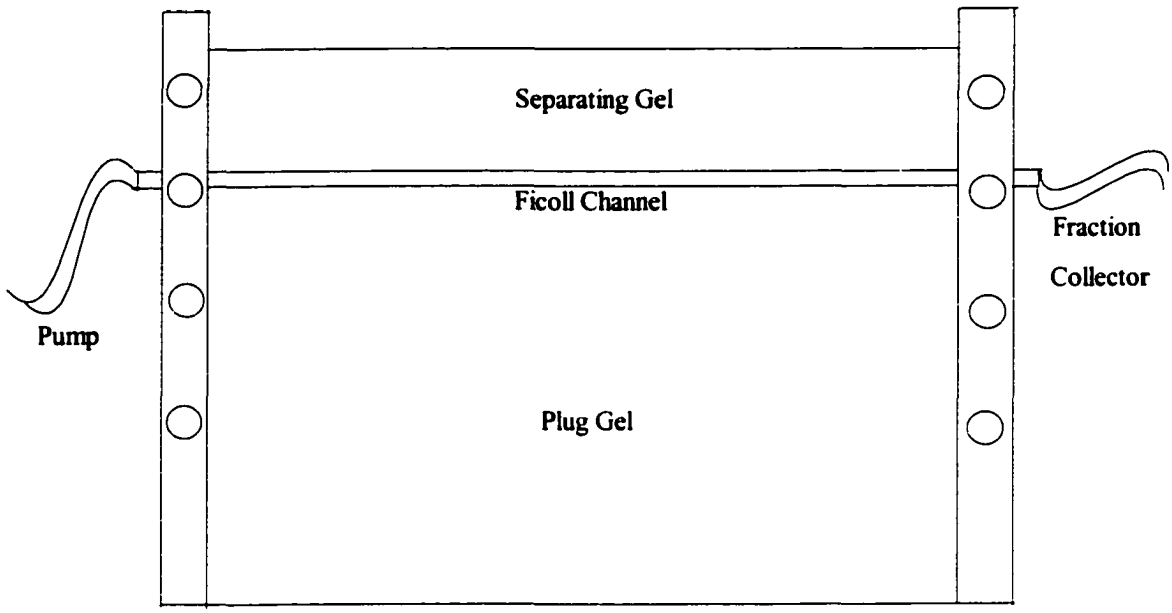


Figure 12. Diagram of a preparative gel electrophoresis apparatus. The crude enzyme extract was applied to the top of the separating gel. 300 volts, constant voltage was applied to the gel. The channel was formed with 50% w/v ficoll. The proteins eluted from the separating gel and were removed from the apparatus in 50 mM phosphate buffer with 100 mM NaBr pumped through the channel at 1 ml/min. Fractions were collected every 3 minutes and assayed for activity with phenol red.

of the separating gel was greater than 2 cm, the enzyme would not pass through the stacking gel and into the channel to be collected for assay with phenol red. At 2 cm and low % T the separating gel would collapse into the channel before the enzyme could pass through it. Some collected fractions, when tested with phenol red, indicated the presence of enzyme in extremely small quantities. The phenol red assay required 8 to10 hours to begin a color change (++) and the assay was never fully changed to purple. These fractions were not reproducible and did not contain enough enzyme for further purification studies.

Although the native gel electrophoresis was unsuccessful in the determination of the size of the individual subunit for the native enzyme, it was useful in the final phases of the purification procedure to indicate the success or failure of the purification process (Figure 13). The Phast Gels exhibited no stained bands with Coomassie Blue R-250 other than the enzyme which had not entered the acrylamide. Similarly, the 6% mini gels indicated the same by demonstrating that the purification process isolated the enzyme. Phast Gels have an advantage over vertical gel electrophoresis in the small amount of protein that can be detected and visualized by the Phast Gel. This can be as low as a 10 nanograms per band.

The size of the subunit for the enzyme (70 kDa) was ultimately determined by denaturing the enzyme in 6 M urea, 2 M SDS, and boiling the sample for 5 minutes. The enzyme was run on a 6% T polyacrylamide gel against molecular weight markers (urease, jack bean, lactalbumin, and albumin, bovine serum) to determine the molecular weight of the subunit (Figures 13 and 14). This is consistent with other peroxidases that have been

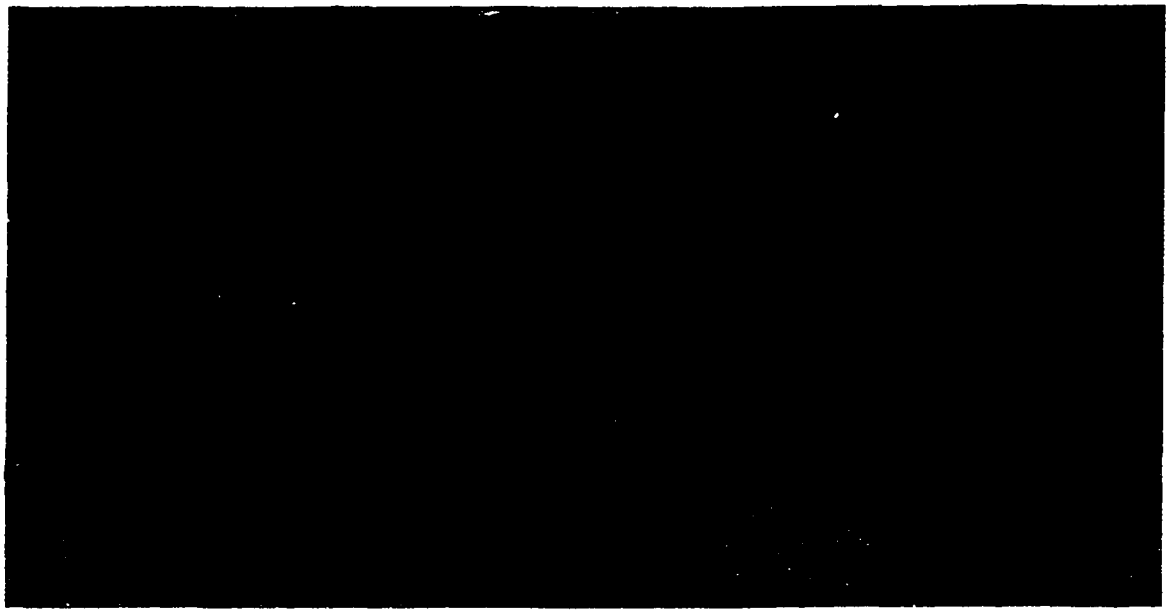


Figure 13. Polyacrylamide gel electrophoresis of bromoperoxidase on a 6% T gel. Lane 1: purified and denatured bromoperoxidase; Lane 2 and 6: crude enzyme extract 21 days after grinding; Lane 3: urease (272,000 and 545,000); Lane 4: albumin (66,000 and 132,000); Lane 5: aggregates of bromoperoxidase; Lane 7: lactalbumin (14,000).

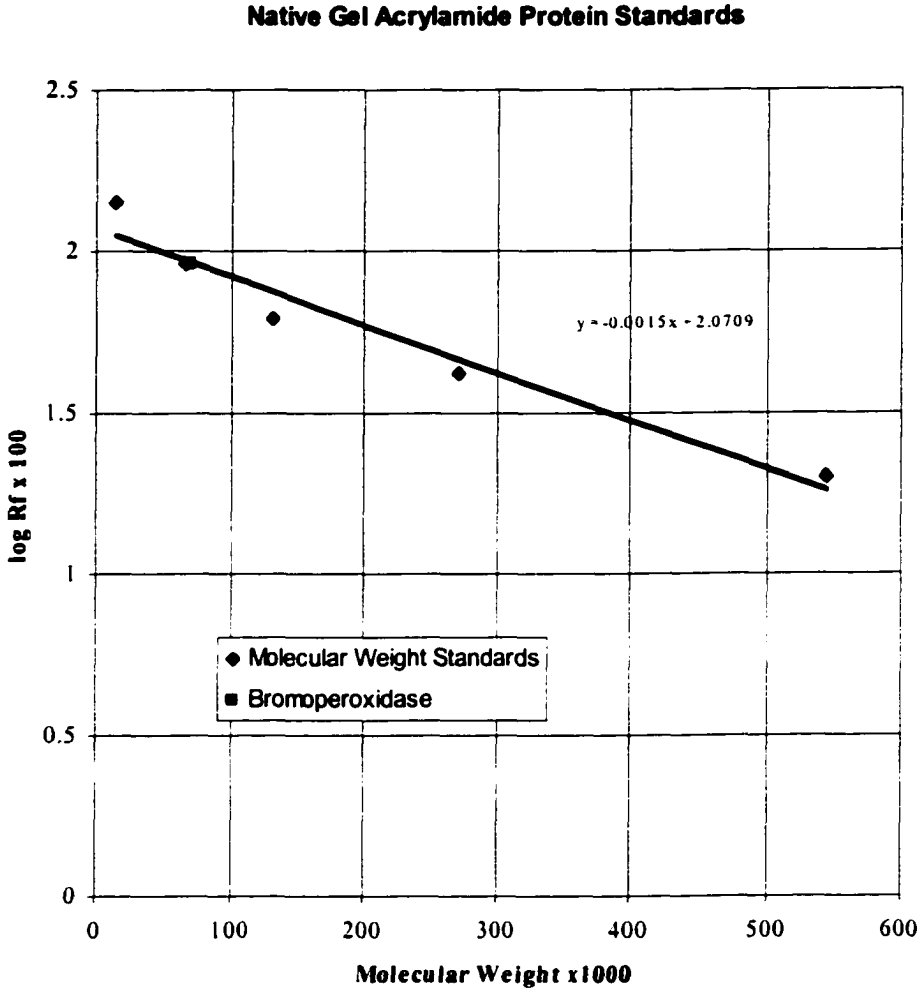


Figure 14. Analysis of molecular weight standard on polyacrylamide gel electrophoresis. The gel was calibrated with urease (272,000 and 545,000), lactalbumin (14,000) , and albumin, bovine serum(66,000 and 132,000). The molecular weight of the bromoperoxidase is 70 kDa.

reported in literature that range from 48 to 97 kDa (Table 1). The majority of peroxidases reported have a molecular weight within a few daltons (±) of 65 kDa.[1-5]

The 0.5% agarose gel showed that the size of the aggregates of the enzyme at optimum activity is greater than 5,000 kDa. After two weeks, the activity begins to decline as the aggregates begin to dissociate into smaller aggregates that will migrate into polyacrylamide gels. Small aggregates found in polyacrylamide gels were 140 kDa and 280 kDa indicating the formation of dimers and tetramers. Examples in published literature state the required formation of multimers for bromoperoxidase activity.[4-9]

E. Isoelectric Focusing (IEF)

The isoelectric point (6.0) was determined with flatbed isoelectric focusing. IEF standards were used to produce a standard curve: amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.6), carbonic anhydrase II (pI 5.4), and carbonic anhydrase I (pI 6.6). The distance the enzyme migrated from the anode was measured and compared to the standard curve (Figures 15 and 16). An IEF gel was stained with Pierce Gelcode™ stain to determine total protein. An identical gel was stained with phenol red assay solution to verify the position of the enzyme in the gel.

F. Other Attempted Purification Methods

The organic solvents methanol, ethanol, and butanol were tried as precipitation techniques for the enzyme extract. Twenty five ml of solvent was slowly added over 20 minutes to 1 ml of the sample in glass beaker in an ice bath containing dry ice. The

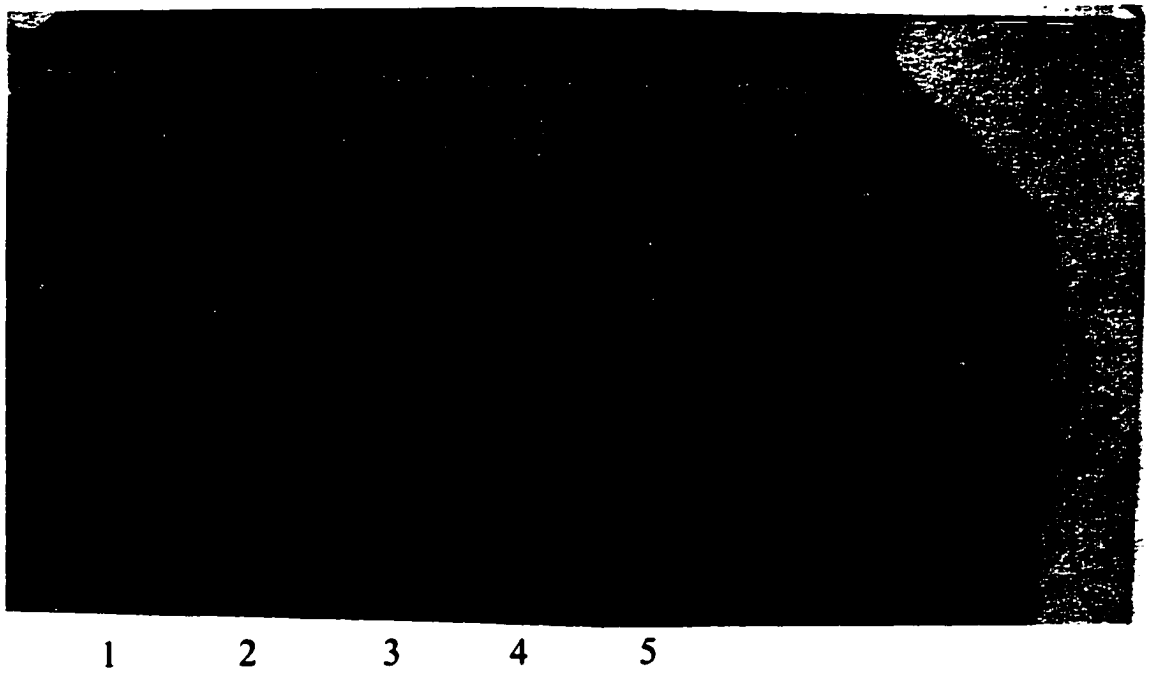


Figure 15. Agarose isoelectric focusing gel of bromoperoxidase. Lane 1: carbonic anhydrase I (pI 6.0); Lane 2: purified bromoperoxidase in box (pI 6.0); Lane 3: amyloglucosidase (pI 3.6); Lane 4: carbonic anhydrases II (pI 5.4); Lane 5; trypsin inhibitor (pI 4.6).

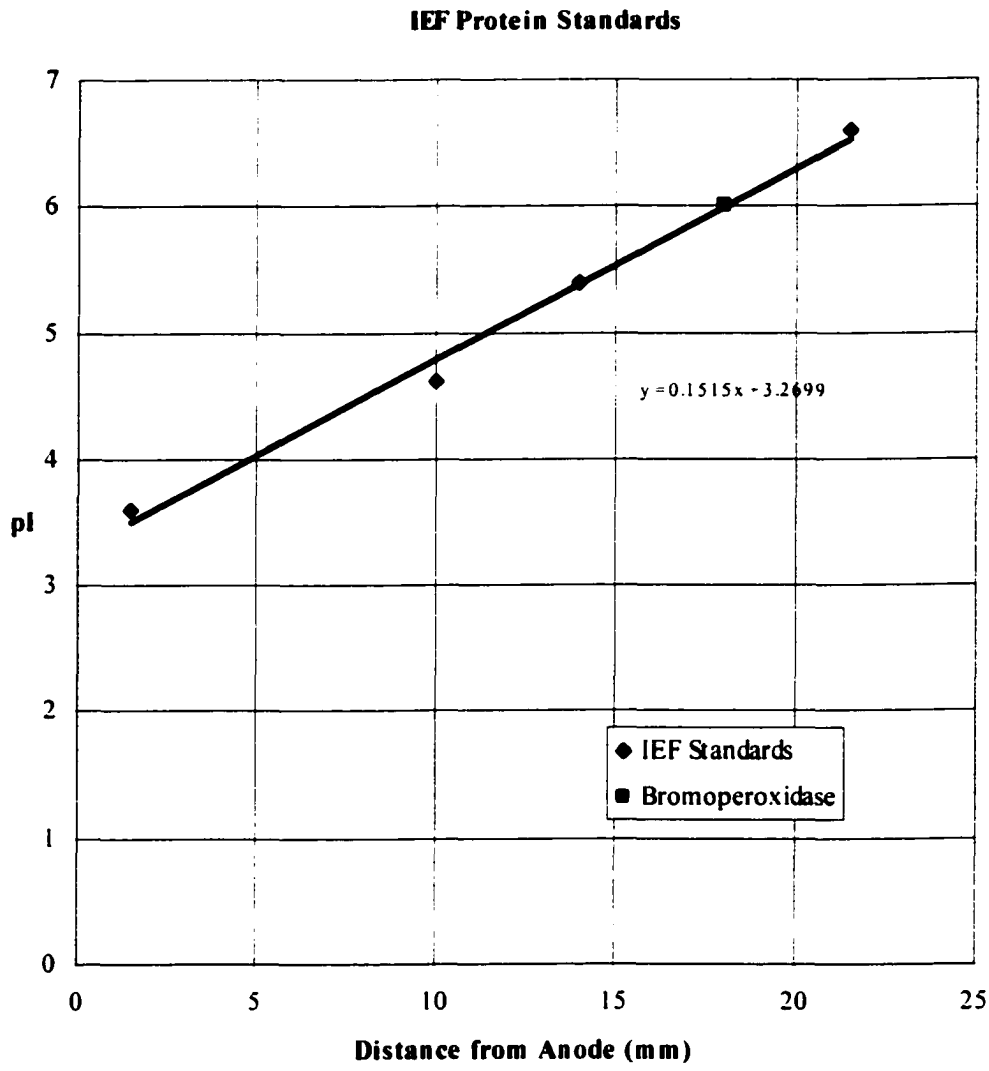


Figure 16. Analysis of pI markers on agarose isoelectric focusing gel. The gel was calibrated with amylogucosidase (pI 3.6), trypsin inhibitor (pI 4.6), carbonic anhydrase II (pI 5.4), and carbonic anhydrase I (pI 6.6). The pI of the bromoperoxidase is 6.0.

solution was slowly stirred with a magnetic stirrer. The solution equilibrated in the ice bath for 30 minutes before the pellet was spun down and collected. The pellet was resuspended in phosphate buffer with 1 M NaBr and assayed with phenol red for activity. The methanol and ethanol precipitated fractions contained no activity. When the enzyme was added to butanol, an emulsion formed that would not break up. The solution was centrifuged and a pellet was collected that contained a very small amount of activity. The following chart shows the activity of the enzyme after precipitation with organic solvents at 0°C and resuspension in 50 mM phosphate buffer with 100 mM NaBr.

Table 10

Organic Solvent	Conditions	Activity from Pellet
Methanol	0°C Precipitation	—
Ethanol	0°C Precipitation	—
Butanol	0°C Precipitation	++

Hydroxyapatite was used to attempt to purify the protein. The pI of the protein was not known at the time of the use of this column so two schemes were followed: one scheme for basic proteins and another for acidic proteins. For the basic protein assumption, the column was equilibrated with 1 mM phosphate buffer pH 6.8. The elution procedure was a step gradient from 0 M to 1 M NaCl. For the acidic protein assumption, the column was equilibrated in 1 mM NaCl and eluted with a gradient of 10 mM to 300 mM phosphate buffer pH 6.8. Fractions were collected from both columns and assayed for activity with phenol red. All of the fractions were absent of activity. The fractions were stored at 4°C for 48 hours and tested periodically for activity with phenol red. No activity

was ever observed with the assay.

Total protein concentration for each of the fractions during the purification procedure was ascertained with a micro BCA (bicinchoninic acid) protein assay reagent kit (Pierce).

The pI of the protein could not be determined from the hydroxyapatite column since neither column produced fractions containing activity. The pI (6.0) was determined with isoelectric focusing. This corresponds to published acidic pI values of bromoperoxidases from other algae (Table 1).

CONCLUSION

A fast and efficient purification procedure has been developed for the analysis and the characterization of the bromoperoxidase from *Plocamium cartilagineum*. The procedure involved manual grinding and extraction of the frozen algae in 50 mM phosphate buffer (pH 5.5) and 1 M NaBr followed by a two week aging period at 4°C. The crude bromoperoxidase was precipitated by $(NH_4)_2SO_4$, dialyzed overnight, then chromatographed using Sepharose CL-6B, which removed most of the contaminants. After concentration by ultrafiltration, the enzyme was purified by hydrophobic interaction chromatography using pentyl-agarose media. Electrophoresis indicates the monomeric subunits to have a molecular weight of 70 kDa, but in order to be at optimal activity, the enzyme forms large aggregates of over 5,000 kDa. The pI of the bromoperoxidase was determined to be 6.0 by isoelectric focusing.

The bromoperoxidase from *Plocamium* is very specific in its requirement for bromide, but not chloride, for the enzyme to develop optimal activity. Withholding NaBr from the buffer at any time during the extraction or purification will lead to irreversible inactivation of the enzyme.

The "aging" period of two weeks which was required by the enzyme to reach maximum activity was very consistent throughout 150 samples assayed during this project. This is a unique attribute for the *Plocamium* bromoperoxidase, and has not been reported for other bromoperoxidases from algae or other sources. The low activity of the enzyme at the time of extraction may be due to a change in the status of the metal cofactor (temporary loss or change in oxidation state), or a conformational change of the enzyme,

followed by a slow reconstitution to the active form.

The actual level of enzyme activity varied from sample to sample, and no correlation could be made between the collection site, the time of year, appearance of the sample, or other environmental factors. However, it was found that different parts of the thallus could contain significantly different levels of enzyme activity.

The hydrophobic nature of the enzyme created challenges in the purification procedure that have not been observed with other bromoperoxidases. The greatest enzyme activity was associated with the formation of large aggregates (>5,000 kDa), which made purification of the enzyme difficult. A wide variety of detergents or the chaotropic agent urea did not break down the aggregates, and led to loss of enzyme activity. Native gels did exhibit 140 kDa and 280 kDa bands, which indicate the formation of dimers and tetramers of the 70 kDa subunits of the enzyme.

Unlike other bromoperoxidases that have been reported, the *Plocamium* enzyme was highly unstable and was readily inactivated when it came into contact with a variety of chromatographic media.

Chemoenzymatic reactions using bromoperoxidases have and advantage over traditional halogenation methods in organic synthesis. Enzymatic reactions do not require the large amounts of hazardous material that classic methods require and are considered environmentally benign, yet enzymatic reactions are very simple to carry out. Future work in this lab will be to try to control the stereochemistry of the reactions with *Plocamium cartilagineum* to produce chiral products.

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