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Isolation procedure and some properties of the bromoperoxidase from the seaweed *Ascophyllum nodosum*

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A large-scale isolation procedure for bromoperoxidase from the brown alga *Ascophyllum nodosum* is described. The purified enzyme showed one major band (40 kDa) after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. The activity of the enzyme in the bromination of monochlorodimedone was studied. The rate of the reaction showed optima between pH 4.5 and pH 6.5, the position of which was determined by the concentration of both Br^- and H_2O_2. The enzyme was not inhibited by H_2O_2, azide or cyanide and only weakly by Br^-. The K_m for bromide of 12.7 mM is independent of pH (6.0–7.6). However, the K_m for H_2O_2 increased with increasing H^+ concentration. Bromoperoxidase was thermostable and was resistant to high concentrations of organic solvents such as methanol, ethanol and 1-propanol.

Introduction

It is becoming increasingly clear that nature produces a wide variety of halometabolites. These vary from the iodinated thyroid hormones [1] in mammals to toxic fluorinated fatty acids produced by certain plants [2]. A huge number of halometabolites are also produced by fungi [3], actinomycetes [4,5] and marine organisms [5,6].

In particular, algae produce a fascinating diversity of extraordinary compounds, including heterogeneous dihalide derivatives [6]. All these products show considerable antibacterial activity and may have a function against predation or act as messengers in systems involved in chemical defence [6]. Although very likely, only in a few cases has it actually been demonstrated that peroxidases are involved in the biosynthesis of these metabolites. The best studied haloperoxidase is that secreted by the fungus *Caldariomyces fumago* [7] and those present in red and green algae [6,8–14]. Some of the brown algae also contain peroxidase activity [15,16] and accumulate brominated compounds [17]. The peroxidases from *Laminaria digitata* [18] and *Ascophyllum nodosum* [19,20] have been partly purified and characterised. Most of the peroxidases of marine origin are markedly thermostable [11,15,21] and since these enzymes may be of potential value in the production of speciality chemicals or the biosynthesis of novel compounds of commercial interest [22], the reported thermostability may be of advantage. This prompted us to study the kinetic behaviour of the bromoperoxidases in more detail.

Since green or red algae cannot easily be obtained in Dutch coastal waters, we have restricted ourselves to the brown alga *A. nodosum*, which grows abundantly along the shoreline.

Materials and Methods

A. nodosum was collected by the authors along the Afsluitdijk, near Kornwerderzand, The Netherlands, towards the end of November. The seaweed was washed and stored frozen at −20°C.

The enzymic activity of bromoperoxidase in the

oxidation of Br^- into HOBr was measured at 25°C by following the conversion of monochlorodimedone ($\varepsilon = 20.2\ mM^{-1} \cdot cm^{-1}$ at 290 nm) into monochloromonobromodimedone ($\varepsilon = 0.2\ mM^{-1} \cdot cm^{-1}$ at 290 nm) [13,23]. The reaction mixture contained 100 mM KBr, 0.1 M potassium phosphate (pH 6.5), 0.2 M Na_2SO_4 and 50 μM monochlorodimedone and the reaction was started by the addition of 2 mM H_2O_2. Na_2SO_4 was present in the assay to avoid large changes in ionic strength due to variation of buffer ion concentration with pH. H_2O_2 solutions were prepared by dilution of a 30% stock solution of perhydrol (Merck, Darmstadt, F.R.G.). The concentration of H_2O_2 was determined spectrophotometrically with an absorption coefficient of $43.6\ M^{-1} \cdot cm^{-1}$ at 240 nm [24]. Protein was determined by the method of Lowry et al. [25] with bovine serum albumin as standard. Sephacryl S-200 and DEAE-Sephacel were from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical grade. 0.2 M Tris-sulphate buffer was made up by mixing Tris with concentrated sulphuric acid to pH 8.3. Spectrophotometric measurements were performed on a Cary-17 spectrophotometer. SDS-polyacrylamide gel electrophoresis was carried out with 10% gels as described by Maurer [26]. Standard proteins used for molecular weight determinations ranged in molecular weight from 20000 to 94000.

The samples were incubated for 30 min at 25°C in the presence of 1% sodium dodecyl sulphate and 5% (v/v) β-mercaptoethanol. Bromoperoxidase activity was detected on the gels by soaking them in 35 μM Phenol red and subsequently in a medium containing 2 mM H_2O_2, 0.1 M KBr and 0.1 M potassium phosphate (pH 6.5). When bromoperoxidase activity is present, Phenol red is brominated as detected by the formation of a brown colour. Alternatively, since this colour development did not last, the gels were immersed in 1 mM orthodianisidine and subsequently in 1 mM H_2O_2.

Purification of bromoperoxidase

Seaweed, chopped in a meat mincer, was stirred for 30 min in 2 vol. 0.2 M Tris-sulphate (pH 8.3). After centrifugation at $1000 \times g$ for 15 min the supernatant was removed and the pellet was again extracted with twice the volume of the same buffer. The resulting supernatant was removed and the precipitate was again extracted. The supernatants were combined, centrifuged at $14000 \times g$ for 25 min and concentrated to 20 l in an Amicon hollow-fibre apparatus with an H_5P_{10}-43HF cartridge for viscous solutions. Solid $(NH_4)_2SO_4$ was added to 80% saturation, the precipitate collected by centrifugation at $14000 \times g$ for 25 min and homogenized in a medium containing 60% ethanol, 0.2 M Tris-sulphate (pH 8.3). After centrifugation at $14000 \times g$ for 25 min, the upper phase was collected and further ethanol was added to a final concentration of 90%. The precipitate obtained after centrifugation at $14000 \times g$ for 25 min was dissolved in 0.1 M Tris-sulphate (pH 8.3) applied to a column of DEAE-Sephacel and eluted with 0.2 M potassium phosphate/0.5 M NaCl (pH 6.0). The fractions containing bromoperoxidase activity were pooled, dialysed against 0.1 M Tris-sulphate (pH 8.3) and applied to a column of Sephacryl S-200. Elution was performed with 0.1 M Tris-sulphate (pH 8.3) and the fractions containing the highest specific activity were pooled.

Results

Table I summarises the isolation procedure for the bromoperoxidase from the brown alga *A. nodosum*. The yield of the purified enzyme compared to the activity of the combined extracts was 10% and the specific activity was increased 100-fold in terms of μmol monochlorodimedone brominated per min per mg protein. The total amount of purified protein per kg of seaweed was about 5 mg.

At first sight the extraction procedure reported here for bromoperoxidase appeared not to be very effective: even after repeated extractions of a sample with Tris-sulphate buffer (pH 8.3), the same amount of bromoperoxidase activity was found after every extraction. Attempts to increase the efficiency of the extraction procedure were not successful. Neither addition of anionic, non-ionic, zwitterionic or cationic detergents to the extraction medium, nor variation of the ionic strength or polarity of this buffer increased the yield. Variation in pH, however, did have some effect. At pH values higher than 9, a considerably lower activity was found, whereas at low pH values no activity

TABLE I

PURIFICATION OF BROMOPEROXIDASE FROM *A. NODOSUM*

Fraction	Volume (l)	Protein (g)	Activity [a]	Specific activity [b]	Recovery (%)
Amicon hollow fibre concentrate	20	65.4	58750	0.9	100
Ammonium sulphate precipitate	4.5	27.2	60750	2.45	100
Ethanol precipitate in Tris-sulphate (pH 8.3)	1.4	–	60375	–	100
Eluate from DEAE-Sephacel column	0.170	0.477	15250	31.6	25
Eluate from Sephacryl S-200 column (main fraction)	0.008	0.0714	6250	87.5	10

[a] μmol monochlorodimedone brominated per min.
[b] μmol monochlorodimedone brominated per min per mg protein.

was released from the algae. Other methods to disrupt these algae, e.g., use of a mechanical mortar and sand, or a Waring Blendor, did not lead to additional release of enzyme.

Upon SDS-polyacrylamide gel electrophoresis, the bromoperoxidase preparation gave rise to one major band which stained for protein (Fig. 1A) and even under these denaturing conditions the enzyme catalysed the bromination of Phenol red to Bromophenol red (not shown). This reaction was specific for the peroxidase, since none of the marker proteins exhibited this reaction. From the gels it was possible to determine a relative molecular mass (M_r) of 40000. The minor bands of molecular mass higher than 40000 also stain for bromoperoxidase activity. Under non-denaturing conditions (in the absence of SDS), electrophoresis of the preparation on a polyacrylamide gel gradient of 6–30% showed three main bands, all of which stained for peroxidase activity and protein (not shown). In order to establish the molecular weight of the bromoperoxidase, the enzyme was applied to a Sephacryl S-200 column. However, all bromoperoxidase activity was found in the void volume of the column.

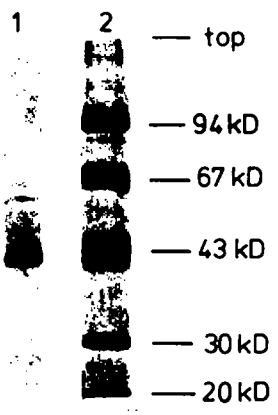

Fig. 1. Gel electrophoresis of bromoperoxidase under denaturing conditions, 1% sodium dodecyl sulphate. Lane 1, 2.6 μg bromoperoxidase; lane 2, standard proteins.

It has been demonstrated that the bromoperoxidase from the green alga *Penicillus capitatus* [12] and the red alga *Bonnemaisonia hamifera* [14] contain haem. The evidence that the peroxidases from the green alga *Enteromorpha linza* [10], and red alga *Cystoclonium purpureum* [8] are haemoproteins, is indirect, i.e., based mainly on the inhibition of the enzyme by cyanide and azide. Our purified enzyme preparation at a protein concentration of 10 mg/ml does not exhibit an absorption peak in the Soret region of the optical spectrum. Also, the brominating activity of the enzyme was not inhibited by 1 mM azide, whereas 0.1 mM cyanide inhibited the reaction only temporarily. After a lag time which was dependent upon the cyanide concentration, bromination of monochlorodimedone reoccurred (not shown). Since it has been demonstrated in Ref. 13 that HOBr has a higher affinity for CN^- than for monochlorodimedone and reacts with CN^- to yield CNBr, this observation demonstrates that even when CN^- does inhibit the enzymic activity of bromoperoxidase, the inhibition is incomplete and reversible.

Steady-state kinetics

Only limited studies [12,14] have appeared on the steady-state kinetics of the bromination reac-

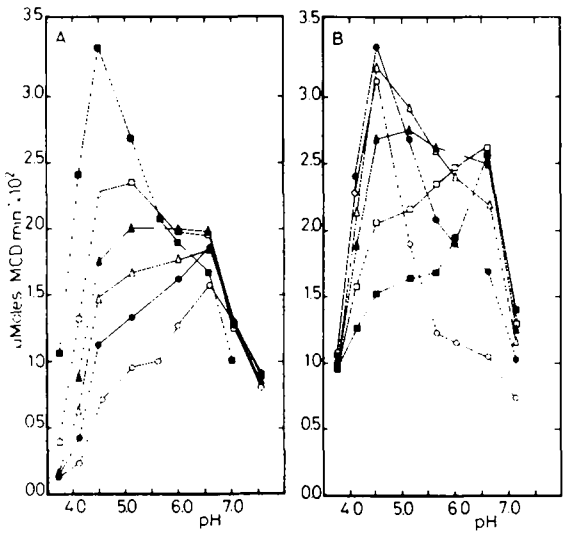

Fig. 2. (A) The pH dependence of the bromination reaction as a function of the H_2O_2 concentration. The reaction was started by the addition of H_2O_2 to the sample which contained 0.2 M potassium sulphate, 0.025 M potassium bromide, 0.1 M potassium citrate (pH 3.7–6.6) or 0.1 M potassium phosphate (pH 6.6–7.6), 50 μM monochlorodimedone and 0.09 μg/ml bromoperoxidase. ○——○, 0.1 mM H_2O_2; ●——●, 0.2 mM H_2O_2; △——△, 0.3 mM H_2O_2; ▲——▲, 0.5 mM H_2O_2; □——□, 1.0 mM H_2O_2; ■——■, 5 mM H_2O_2; (B) The pH-dependence of the bromination reaction as a function of the bromide concentration. The H_2O_2 concentration was 5 mM. [KBr] (mM): ○——○, 10; ●——●, 25; △——△, 50; ▲——▲, 100; □——□, 200; ■——■, 500.

tion catalysed by bromoperoxidase. It is, however, known that bromoperoxidase, like other haloperoxidases [27–29], shows a pH optimum in the bromination of monochlorodimedone.

Fig. 2 illustrates that the bromoperoxidase from *A. nodosum* also has distinct activity optima which, at a fixed concentration of Br^- (Fig. 2A), shifted to lower pH values when the concentration of H_2O_2 was increased. On the other hand, when the concentration of Br^- was increased, the optima shifted to higher pH values (Fig. 2B). It can be seen from Fig. 2A that high concentrations of H_2O_2 (1–5 mM) do not appreciably inhibit the enzyme.

Fig. 2B shows that Br^- at low pH values (pH 3.7–5.0) inhibited the reaction to a certain extent, whereas at pH 6.5–7 the enzyme did not show saturation with 10–20 mM Br^-, suggesting that the K_m for Br^- lies in that order of magnitude. Indeed, from Lineweaver-Burk plots (not shown) of the rate as a function of the bromide concentration it was possible to obtain a K_m value for bromide of 12.7 ± 3.0 mM (n = 13). This value, as well as the V_{max}, is independent of pH (6.0–7.6). It was not possible to obtain K_m values at lower pH values because of more pronounced inhibition by Br^-.

As Fig. 2A suggests, the affinity for H_2O_2 decreases when the pH is decreased. Indeed, as shown in Fig. 3, the logarithm of the K_m of bromoperoxidase for H_2O_2, obtained from Lineweaver-Burk plots of rates as a function of $[H_2O_2]$, increased linearly with pH. However, the V_{max} was not affected by pH (3.7–6.0). Like the other bromoperoxidases, the enzyme was not inhibited by chloride up to 0.5 M (not shown).

It has been known for a long time that some of these algal peroxidases are quite thermostable [11,15,21]. Fig. 4 shows that when the enzyme is kept at 50°C, the brominating activity was not affected for a long period of time. At higher temperatures the enzyme was rapidly inactivated, but at 70°C 38% of the activity still remained after 1 h of incubation. This denaturation is not reversible, since incubation of the inactivated

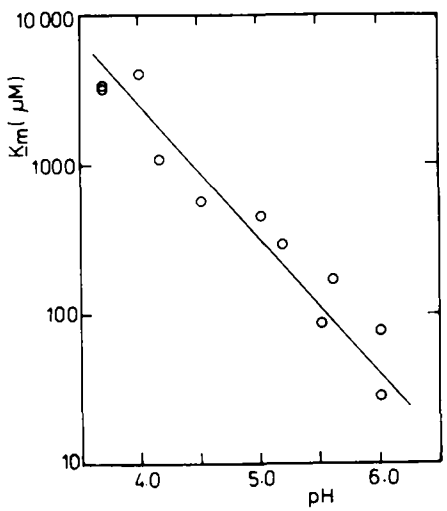

Fig. 3. pH-dependence of the K_m values for H_2O_2 of bromoperoxidase in the bromination reaction. The K_m values were calculated from Lineweaver-Burk plots. Conditions as in Fig. 2.

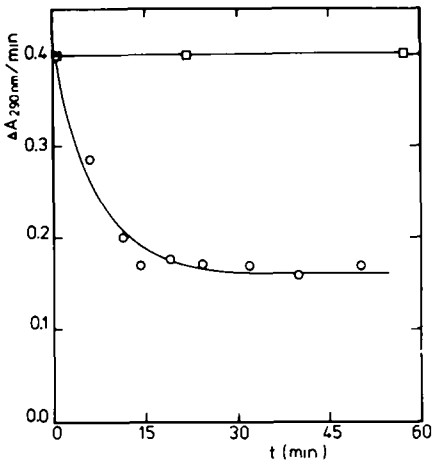

Fig. 4. Effect of temperature on the bromoperoxidase activity. Bromoperoxidase (0.07 mg protein/ml) was incubated in 0.1 M sodium acetate (pH 5.0) at 50°C (□———□) or 70°C (○———○). At variable time intervals, samples were taken and assayed for bromoperoxidase activity. Assay conditions: 0.1 M sodium acetate (pH 5.0); 0.2 M sodium sulphate; 10 mM potassium bromide; 50 μM monochlorodimedone and 1 mM H_2O_2. Temperature, 25°C.

bromoperoxidase at 0°C for 30 min did not restore the activity. The thermostability of the enzyme is also reflected in the effect of temperature on the initial rate of bromination. This rate was nearly linearly dependent on the temperature up to 70°C (not shown).

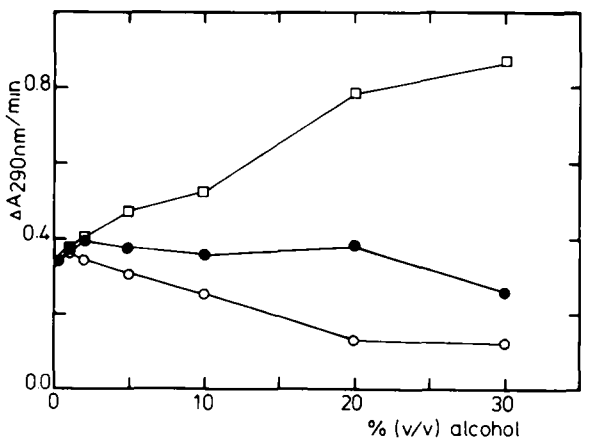

Fig. 5. Effect of alcohols on the bromoperoxidase activity. Assay conditions: 0.1 M potassium phosphate (pH 6.5), 25 mM potassium bromide, 50 μM monochlorodimedone and 1 mM H_2O_2. Protein concentration, 0.3 μg/ml. ○———○, methanol; ●———●, ethanol; □———□, 1-propanol.

In line with this thermostability, the enzyme was also resistant to organic solvents. As Fig. 5 shows, 30% (v/v) ethanol had little effect on the bromoperoxidase activity, whereas in the presence of 30% (v/v) methanol the activity decreased to 40% of its original value. Surprisingly, in 1-propanol an increase in the bromoperoxidase activity was found. A similar increase was observed when low concentrations of Tween-80, cholate or Triton X-100 were added to the reaction mixture (not shown).

Discussion

Our purified preparation of bromoperoxidase shows only one main protein band by SDS-gel electrophoresis which exhibited brominating activity. Considering the amount of protein present in our preparation, the enzyme should show an appreciable absorption in the optical absorption spectrum attributable to a haemoprotein. This was not observed by Vilter [20] and ourselves, and this may suggest that the peroxidase activity is not derived from an enzyme containing a haem prosthetic group. Others [14,16] apparently also came to this conclusion and have even considered the possibility that the bromoperoxidase is a flavoprotein. However, it has been reported [14,18] that specific flavoprotein inhibitors had no effect on the activity. Recently it was demonstrated [20] that the peroxidase from *A. nodosum* could be inactivated by dialysis at low pH and that the activity was specifically restored by addition of vanadium(V). We were able to confirm this observation and studies are in progress to assess the amount of vanadium in the bromoperoxidase. Also the lack of inhibition of the brominating activity by azide or cyanide suggests that this peroxidase is not a haemoprotein.

As in other haloperoxidases [27–29] the steady-state kinetics of the peroxidation of Br^- catalysed by the bromoperoxidase is rather complex. Like other haloperoxidases [27–30], the enzyme shows pH optima in the activity, which demonstrates that protonation steps are involved in the catalytic cycle. In myeloperoxidase and lactoperoxidase [27,30], the K_m measured for H_2O_2 increased with decreasing pH. In bromoperoxidase this is also found and this suggests that at low pH

an inhibitory protonated complex is formed in line with the decrease in activity at low pH values. This inhibition is not due to irreversible denaturation. When the enzyme was incubated at pH 4.0 and the activity was measured at neutral pH, the same activity was found as before incubation at low pH. Our gel-chromatography experiment on Sephacryl S-200 indicates that the molecular mass of bromoperoxidase is greater than 250000. It is conceivable that the enzyme is aggregated under these conditions, but it is also possible that the enzyme is tightly bound to alginate, a polysaccharide which is present in these algae [31]. This alginate is probably also responsible for the high viscosity found in the supernatants after extraction of the seaweed. Similar association of the peroxidase with polysaccharides has been observed with peroxidases from the red alga *B. hamifera* [14] and the brown alga *L. digitata* [18].

In line with Ref. 12, we have observed that bromoperoxidase is not inhibited by chloride and that chloride is not used as an electron donor. Considering the radius of chloride and bromide and their hydration number [32], the former observation is rather surprising. From a chemical point of view one would expect that the smaller chloride ion would act as an inhibitor, since it is likely to be bound to the active site. How this is avoided is not clear, but it is physiologically understandable in view of the concentration in seawater of chloride and bromide [6], 500 mM and 1 mM, respectively.

Acknowledgements

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