Biochem. J. (1987) 248, 277–279 (Printed in Great Britain)

The bromoperoxidase from the lichen *Xanthoria parietina* is a novel vanadium enzyme

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A novel bromoperoxidase was isolated from the lichen *Xanthoria parietina*. The enzyme contained vanadium, which is essential for enzymic activity. Under denaturating conditions the preparation showed a single protein band with an M_r of 65000. Thermal-denaturation studies showed that this bromoperoxidase could tolerate high temperatures. The affinity of the enzyme for its substrate bromide is high; the K_m for bromide was 29 μM. Excess halides (50 mM) inhibited enzymic activity considerably.

INTRODUCTION

In the last few years evidence has accumulated that the transition metal vanadium is essential for the catalytic activity of several bromoperoxidases from marine organisms and that these enzymes contain vanadium(V) as a prosthetic group [1–5].

The function of these bromoperoxidases is the formation of a fascinating diversity of halogenated organic compounds, which appear to participate in biological defence and communication systems [6]. Hitherto, the only two vanadium-containing enzymes reported in terrestrial prokaryotic organisms were the N_2-fixing enzymes nitrogenase from *Azotobacter chroococcum* [7] and nitrogenase from *Azotobacter vinelandii* [8].

We therefore checked whether the presence of vanadium-containing bromoperoxidases was restricted to the marine environment, or whether this newly discovered class of enzymes occurs also in terrestrial organisms. Since lichens are known to be rich in halogenated compounds [9], we tested *Xanthoria parietina*, a symbiosis of the green alga *Trebouxia* and an ascomycete [9]. This is an orange–red lichen common in coastal areas of The Netherlands. We demonstrate here that a bromoperoxidase is present in this lichen, and we show that the purified enzyme contains vanadium, which is essential for enzymic activity. Purification data and some of the properties of this novel enzyme are given. A preliminary report of part of this work was published previously [10].

MATERIALS AND METHODS

X. parietina was scraped from rocks, stones and walls near Hoorn during the summer, and stored frozen at −20 °C.

DEAE-Sephacel was obtained from Pharmacia (Uppsala, Sweden) and monochlorodimedone from Sigma Chemical Co. (St. Louis, MO, U.S.A.). H_2O_2 was from Merck (Darmstadt, Germany). Other reagents used were of analytical grade.

During purification, the brominating activity of the enzyme was followed by measuring the conversion of monochlorodimedone ($\epsilon = 20.2\ mM^{-1} \cdot cm^{-1}$) into monochlorobromodimedone ($\epsilon = 0.2\ mM^{-1} \cdot cm^{-1}$) at 290 nm [1]. The activity is expressed in μmol of monochlorodimedone brominated/min. The assay mixture contained 0.1 M-sodium acetate buffer, pH 5.5, 0.1 mM-KBr, 50 μM-monochlorodimedone and 1 mM-H_2O_2. Protein content was determined by the method of Bradford [11], with bovine serum albumin as a standard. Gel electrophoresis was done under mildly denaturing and under strongly denaturing conditions in 8% polyacrylamide slab gel, as described by Laemmli [12]. Standard proteins (Pharmacia) used for determinations of M_r values ranged in M_r from 14400 to 94000. Protein staining was done with Coomassie Brilliant Blue R-250. The sample was incubated for 30 min under mildly denaturing conditions at 25 °C in the presence of 0.2% SDS. Under strongly denaturing conditions the sample was incubated for 10 min at 100 °C in the presence of 1% SDS and 5% (v/v) 2-mercaptoethanol. Bromoperoxidase activity on the gels was detected by soaking them in a solution containing 1 mM-*o*-dianisidine, 1 mM-KBr and 0.1 M-sodium acetate buffer, pH 5.5, and subsequently in 1 mM-H_2O_2. When bromoperoxidase is present, *o*-dianisidine is brominated, which can be detected by the development of brown bands [1].

The vanadium content of the purified enzyme was determined with a Hitachi 180-80 Zeeman polarized atomic absorption spectrophotometer.

RESULTS AND DISCUSSION

For purification of the bromoperoxidase, the lichen was homogenized in a Waring blender in 0.2 M-Tris/sulphate buffer, pH 8.3. After centrifugation, the supernatant was removed and the extraction of the lichen with Tris buffer was repeated four times. The supernatants were combined, adjusted to 80% saturation with $(NH_4)_2SO_4$ and centrifuged again. The pellet was homogenized in 0.2 M-Tris/sulphate buffer, pH 8.3, in 60% (v/v) ethanol, centrifuged and subsequently dialysed against 50 mM-Tris/sulphate buffer, pH 8.3. This extraction procedure with 60% ethanol resulted in the removal of organic material to which the enzyme normally remained bound. However, a partial loss of activity was observed, which could be restored by the addition of vanadium(V).

Then the supernatant was applied on to a DEAE-Sephacel column, equilibrated with 50 mM-Tris/sulphate buffer, pH 8.3, and eluted with 0.1 M-Tris/sulphate

Table 1. Purification of bromoperoxidase from *X. parietina*

For experimental details see the text. One unit of activity is defined as 1 μmol of monochlorodimedone brominated/min. Protein content was determined by the method of Bradford [11].

Fraction	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (units/mg)	Recovery (%)
Combined extracts from 100 g of lichen	2000	134	287	2.1	100
Eluate from 1st DEAE-Sephacel column	190	7.6	190	25	66
Eluate from 2nd DEAE-Sephacel column	115	2.3	100	79	62

buffer, pH 8.3, containing 0.1 M-Na_2SO_4. After further dialysis against 50 mM-Tris/sulphate buffer, pH 8.3, this step was repeated, and subsequently the enzyme was concentrated in an Amicon concentration apparatus with a PM-30 membrane. The yield of purified enzyme was 2.3 mg, with a specific activity of 79 units/mg of protein (Table 1). The purified enzyme did not show absorption bands in the optical absorption spectrum. This phenomenon was also observed for other vanadium-containing bromoperoxidases [1–4,13].

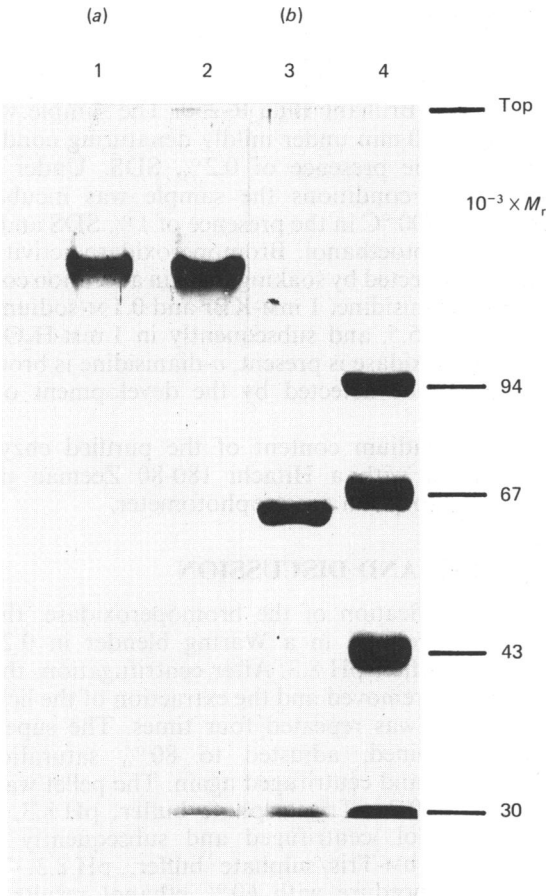

Fig. 1. Polyacrylamide-gel electrophoresis of bromoperoxidase

(*a*) Mild denaturing conditions (30 min at 25 °C, with 0.2% SDS): lanes 1 and lane 2, 11 μg of bromoperoxidase stained for activity and protein respectively. (*b*) Strong denaturing conditions (10 min at 100 °C, with 1% SDS/5% 2-mercaptoethanol): lane 3, 11 μg of bromoperoxidase stained for protein; lane 4, standard proteins of low M_r.

Gel electrophoresis was done under mildly denaturing and under strongly denaturing conditions. Upon staining for bromoperoxidase activity under mildly denaturing conditions, one major band was observed, which stains for both brominating activity and protein (Fig. 1). Under strongly denaturing conditions the enzyme lost its activity and the preparation showed one protein band with an M_r of 65000 (Fig. 1) and a minor band at the front of the gel.

Like the vanadium-containing bromoperoxidase from *Ascophyllum nodosum* [1], the purified enzyme from *X. parietina* was remarkably thermostable. The enzymic activity was not affected when the enzyme was incubated at 50 °C, and only slowly decreased at higher temperatures (Fig. 2). This is not surprising if one takes into account the habitat of the organism, i.e. rocks and (during the summer) heat. These growth conditions probably also explain why the enzyme is only active at extremely low bromide concentrations (1–100 μM). The affinity of this bromoperoxidase for its substrate bromide is very high; the K_m for bromide, determined from Eadie–Hofstee plots (not shown), was 28 μM at pH 5.5,

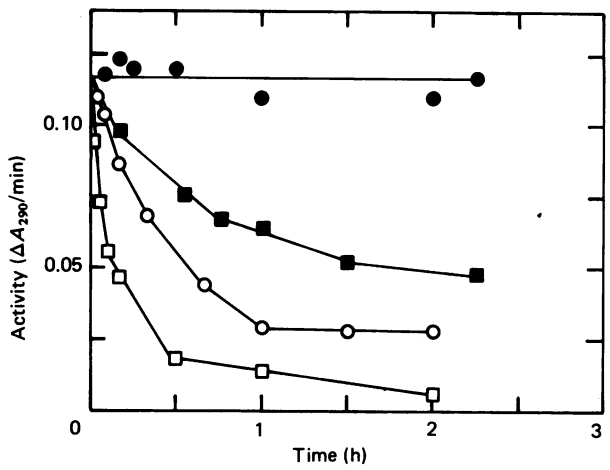

Fig. 2. Effect of temperature on the bromoperoxidase activity

Bromoperoxidase (35 μg/ml) was incubated in 0.1 M-sodium acetate buffer, pH 5.5, at 50 °C (●), 60 °C (■), 70 °C (○) and 80 °C (□). At various time intervals, samples were taken and assayed for bromoperoxidase activity. The assay mixture contained 0.1 M-sodium acetate buffer, pH 5.5, 0.1 mM-KBr, 50 μM-monochlorodimedone, 1 mM-H_2O_2 and 75 ng of the bromoperoxidase/ml, and the assay temperature was 25 °C.

and must be considered to be the lowest value reported so far for all known peroxidases. The K_m for H_2O_2 at pH 5.5 was 870 μM, and this is a normal value. Excess bromide (50 mM) inhibited the enzymic activity considerably, which effect was also observed for the halides fluoride and chloride (results not shown).

Like other haloperoxidases [6], the bromoperoxidase from *X. parietina* showed a pH optimum in activity (pH 5.5; results not shown) that demonstrates that protonation steps are involved in the catalytic cycle. The bromoperoxidase from *X. parietina* could be inactivated by dialysis of the enzyme against 0.1 M-citrate/phosphate buffer, pH 3.8, containing 1 mM-EDTA. This procedure removes vanadium from vanadium-containing bromoperoxidases from marine organisms [1–4,13]. As in these enzymes, the lost activity could be restored by addition of excess vanadate. Other metal ions were tested for re-activation of the enzyme, but were not effective [0.1–1.0 mM-$NiCl_2$, -$Ce(SO_4)_2$, -$CdSO_4$, -$CoCl_2$, -$CuSO_4$, -H_2MoO_4, -$MnCl_2$, -$MgSO_4$, -Nb_2O_5, -$ZnSO_4$ and -$FeSO_4$].

These experiments also suggest the presence of vanadium in bromoperoxidase from *X. parietina*. Indeed, it was shown with atomic absorption spectrophotometry that the purified bromoperoxidase contained 10 nmol of vanadium/mg of protein. According to Fig. 1, one subunit is present with an M_r value of 65000. This would correspond to a vanadium content of 0.6 mol/mol of subunit. That vanadium is present in less than stoichiometric amounts is not without precedent. This phenomenon has been observed before with the bromoperoxidases from *A. nodosum* [3], *Laminaria saccharina* [2] and *Ceramium rubrum* [4], and is ascribed to loss of vanadium during the isolation procedure. In this context it may be noted that the reconstituted enzyme indeed contained stoichiometric amounts of vanadium. It is not yet clear whether the bromoperoxidase in *X. parietina* is present in the algal part or in the fungal one. It is obvious, however, that vanadium-containing bromoperoxidases not only occur in the marine environment, but are also found in terrestrial eukaryotic organisms.

We gratefully acknowledge the support by D.S.M. N.V., Geleen, The Netherlands, and Dr. H. Sipman, Botanischer Garten and Botanisches Museum, Berlin, Germany, for the determination of the lichen. This work is part of the research programme of the Netherlands Foundation for Chemical Research (S.O.N.) and was made possible by financial support from the Netherlands Technology Foundation (S.T.W.).

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Received 27 July 1987; accepted 9 September 1987