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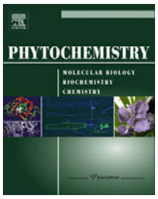

Bromoperoxidase activity and vanadium level of the brown alga *Ascophyllum nodosum*

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ABSTRACT

Vanadium-dependent peroxidase activity in extracts of *Ascophyllum nodosum* growing in the intertidal region close to Roscoff/France, and algal vanadium levels, followed approximately similar seasonal variation, as deduced from a study lasting from April 2005 to March 2006. High peroxidase (PO) activity was found in extracts obtained from algae collected in between midwinter to spring [~100–190 U per g dry mass (dm), triiodide assay] with a maximum in April. Periods of reduced PO activity lasted from summer to early winter (~50–90 U per g dm). High vanadium levels (1.5–2.2 mg kg^{-1} dm) were found in algae collected from midwinter to spring, whereas reduced levels (0.6–1.4 mg kg^{-1} dm) were found in summer to early winter.

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1. Introduction

The brown alga *Ascophyllum nodosum* contains vanadium-dependent peroxidases that are able to catalyze the oxidation of iodide, bromide, thiocyanate, or thioethers if exposed to H_2O_2 and appropriate substrates (Vilter, 1984; Butler and Walker, 1993; Walker and Butler, 1996; ten Brin et al., 1998; Everett et al., 1990). Optimum pH for H_2O_2 utilization by these enzymes lie in the range of 6.2 [bromoperoxidase (BPO) isoenzyme I, A.n.I] and 7.2 (BPO isoenzyme II, A.n.II) (Krenn et al., 1989). Elevated temperature and organic co-solvents exert surprisingly small effects on catalytic BPO activity of A.n.I (de Boer et al., 1987). Significantly less is known about the chemistry of isoenzyme A.n.II. It has been classified as glycoprotein that tends to loose its vanadium co-factor faster than A.n.I (Krenn et al., 1989). Both enzymes have considerable potential for the development of sustainable bromination chemistry, although neither is biotechnologically available at the moment. The parent seaweed grows abundantly in intertidal regions along shorelines world wide. A procedure for extracting substantial amounts of bromoperoxidases from the alga has been developed (Vilter, 1994). Its efficiency is guided by effectiveness of plant tissue disruption, alginate and polyphenol separation, but also by the amount of BPO present in the macroalga. Earlier reports had pointed to seasonal PO activity variation, which gradually fell along the sequence of investigated months April > March > July > September (Vilter et al., 1983). Based on an elevated demand of bromoperoxidases for the pursuit of homogeneous oxidation catalysis, the knowledge about a preferred season for *A. nodosum* harvest and thus enzyme purification recently became a major concern. This issue was addressed in a 12-month survey of extractable PO activity from *A. nodosum*, and determination of algal vanadium level referring to a location that was selected for future supply. The major results of this investigation pointed to distinct seasonal variation by a factor of ~4 in PO activity (triiodide assay), and ~3.5 in algal vanadium level (ICP-MS and adsorptive stripping voltammetry). Maximum values were found in January–May, minima between June and December.

2. Results and discussion

2.1. Plant material and extract formation for PO activity tests

A. nodosum was collected from April 2005 to March 2006 in close proximity to the Station Biologique Roscoff/Brittany (France).

Abbreviations: AdSV, adsorptive stripping voltammetry; A.n.I. and A.n.II, bromoperoxidase isoenzymes I and II from *Ascophyllum nodosum*; BPO, bromoperoxidase; dm, dry mass; HMDE, hanging mercury drop electrode; ICP-MS, inductively coupled plasma mass spectrometry.

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The material was immediately packed in polypropylene bags and shipped to Kaiserslautern. It was washed (H_2O), frozen, and freeze-dried for 2 days. Metal contamination was rigorously excluded. The PO activity of this material was equivalent to a sample shipped via air freight and truck while consistently being kept at dry ice temperature. This control provided evidence that no relevant PO activity loss had occured using the first shipping option (tested for April 2005). A sample collected at the intertidal pier of Dunstaffnage Marine Laboratory, Oban, Scotland in May 2004 was immediately frozen and subsequently freeze-dried for 2 days. It was included in the study for supplementing data with information obtained from an additional site.

Lyophylized algae were homogenized to 1 µm particle size in a milling process performed below 30 °C in order to minimize PO activity loss. The material was subjected to a liquid–liquid partitioning process, which was followed by dialysis versus Tris-buffered aq. $NaVO_3$ solution, for restoring catalytic activity of bromoperoxidases (Vilter, 1994). PAGE lanes (Fig. 1) of dialyzed extracts were stained for PO activity showing A.n.I and A.n.II as major components. Protein concentrations were routinely measured via UV/vis spectroscopy (Layne, 1957). Comparable activity data were obtained by strictly adhering to standardized protocols. The origin of PO activity spotted in PAGE lanes between the two BPO isoenzymes remained unclear. Control experiments conducted with an equine heart cytochrome C (Experimental), for approximating reactivity of algal cytochromes, showed no PO activity in a triiodide assay.

2.2. PO activity

PO activity in extracts of *A. nodosum* was determined via triiodide assay (Björkstén, 1968; Vilter et al., 1983) in phosphate-buffered solutions (pH 6.2) (Table 1). Initial rates were calculated from slopes of linear correlations derived from time dependent evolution of the I_3^- band at $\lambda = 350$ nm due to enzyme-catalyzed oxidation of I^- with H_2O_2. Correction terms were applied for adequately addressing the triiodide-iodide/iodine equilibrium in aqueous solutions under selected conditions. UV/vis spectra were recorded with a double beam spectrophotometer at 20.0 ± 0.5 °C, in order to correct for I_3^- formation from uncatalyzed background reaction.

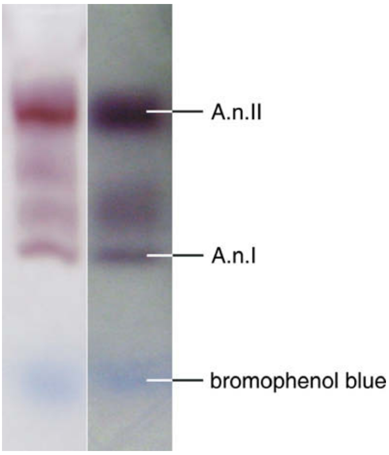

Fig. 1. Polyacrylamide gel electrophoresis (1 h, 18 mA; non-denaturating conditions) of bromoperoxidase fractions extracted from *A. nodosum* collected at Roscoff (France; left) and Oban (Scotland; right). Both lanes: 5 µl from a solution of 80 µl dialyzed extract (see above and Experimental), 20 µl H_2O (bidest.), saccharose (40 mg) and a solution of bromophenol blue in [0.1% (w/w) in H_2O (bidest)]. Staining was performed in a solution prepared from phosphate buffer (100 ml), H_2O_2 (10 ml), aq. KI (100 µl) (for concentrations of the three reagents, see triiodide test), and 1,4-phenyldiammonium dichloride (100 mg).

Table 1
PO activity (triiodide assay, phosphate buffer, pH 6.2) of extracts from *A. nodosum*

Entry	Harvest	*V* (ml)[a]	U (ml^{-1})	U (mg^{-1})[b]	U (g^{-1} dm)[c]
1	April 2005[d]	220	86 ± 7	210 ± 15	190 ± 13
2	May 2005[d]	195	78 ± 6	230 ± 19	150 ± 12
3	June 2005[d]	200	26 ± 2	56 ± 5	52 ± 5
4	July 2005[d]	165	40 ± 3	90 ± 6	66 ± 5
5	August 2005[d]	305	19 ± 2	76 ± 6	58 ± 5
6	September 2005[d,e]	240	37 ± 3	160 ± 13	89 ± 7
7	October 2005[e]	200	30 ± 2	57 ± 4	60 ± 5
8	November 2005[e]	170	53 ± 4	130 ± 11	90 ± 7
9	December 2005[d,e]	275	32 ± 2	111 ± 8	88 ± 7
10	January 2006[d,e]	235	67 ± 5	170 ± 12	160 ± 11
11	February 2006[d]	240	41 ± 3	140 ± 11	98 ± 7
12	March 2006[d]	220	63 ± 6	200 ± 18	140 ± 12
13	May 2004[f]	200	42 ± 3	140 ± 11	84 ± 7

[a] Volume of dialyzed extract in ml from 100 g of lyophylized *A. nodosum*.
[b] Protein mass was derived from UV/vis spectroscopically concentration measurement (Layne, 1957).
[c] PO activity referenced versus dry mass (dm) of freeze-dried algae.
[d] Collected at Roscoff/France.
[e] Mean value from two independent preparations.
[f] Collected at Oban/Scotland.

PO activities showed seasonal variations by a factor of ~4 (Table 1). The maximum value of 190 ± 13 U g^{-1} dry mass (dm) was obtained for specimen collected in April (Table 1, entry 1). In the following month PO activity slightly decreased. This trend continued in June (52 ± 5 U g^{-1}dm) and reached a level that prevailed until August (Table 1, entries 2–5). In the succeeding months a moderate increase in PO activity and a steep ascent in January 06 (160 ± 11 U g^{-1} dm) was noted leading to a high activity phase until the end of the study in March 06 (140 ± 12 U g^{-1} dm) (Table 1, entries 6–12). Different PO activities were obtained for algae collected at different sites (Oban/Scotland versus Roscoff/France) and in different years (May 2004 versus May 2005) (Table 1 entries 2 and 13).

April harvest of *A. nodosum* provided the most active BPO preparation (Table 1, entry 1). Earlier studies on fertile and on sterile organisms had shown that vegetative and reproductive parts of the algae contained approximately equivalent amounts of PO activity (Vilter et al., 1983). In view of this background it was refrained from exploring, e.g., conceptacles separately for obtaining extracts with adequate PO strengths for future applications in oxidation catalysis.

The specific PO activity obtained from homogenized April specimen of *A. nodosum* was 210 ± 15 U mg^{-1}, as determined on the basis of UV/vis spectroscopic approximation of protein concentration (Layne, 1957). In order to compare this value to activity reported for purified A.n.I (de Boer and Wever, 1988; Meister Winter and Butler, 1996; Martinez et al., 2001) in the monochlorodimedone assay (Hager et al., 1966; Wagner et al., 2008), bromoperoxidase isoenzymes were separated and purified by hydrophobic interaction chromatography on Phenyl Sepharose™ 6 FF and subsequent size exclusion chromatography on Sephacryl™ S-300 HR. Subjecting such preparations to triiodide (693 U mg^{-1} for A.n.I and 889 U mg^{-1} for A.n.II; both at pH 6.3) and MCD assays (172 U mg^{-1} for A.n.I and 117 U mg^{-1} for A.n.II; both at pH 6.5) afforded scaling factors of 4.0 for A.n.I and 7.6 for A.n.II, in order to numerically interconvert activity data from the two assays as a rough approximation. If expressed in MCD units, BPO activity of ~25–48 U mg^{-1} thus were estimated for the April extract. A.n.I preparations that were typically applied in mechanistic, kinetic, and synthetic investigations ranged from 69–132 U mg^{-1} (MCD assay at pH $\leqslant$ 6.0) (de Boer and Wever, 1988; Meister Winter and Butler, 1996; Martinez et al., 2001). These data showed that extracts obtained by lyophylization, liquid/liquid partitioning and dialysis versus Tris-buffered $NaVO_3$ solutions of algal material collected in April 2005 afforded enzyme preparations with notable PO strengths. Such preparations are considered useful for conducting syntheses of organobromine compound under sustainable conditions in future projects. For transformations that take profit from more specific BPO selectivities (Tschirret-Guth and Butler, 1994), purification of isoenzymes A.n.I or A.n.II will, as a matter of fact, be inevitable.

2.3. Vanadium analysis

Quantitative vanadium analysis in the expected range of mg kg^{-1} dry mass of biological samples (Seiler, 1995) has been performed previously via thermal neutron activation analysis (Blotcky et al., 1979), atomic absorption spectroscopy (AAS) (Sperling et al., 2000), inductively coupled plasma optical emission spectroscopy (ICP-OES) (Hu and Coetzee, 2007), ICP mass spectrometry (ICP-MS) (Knüttel et al., 1992; Łobiński et al., 2006), or adsorptive stripping voltammetry (AdSV) (Sander and Henze, 1996). In view of more general issues associated with the former three methods, ICP-MS was selected for vanadium trace-level analysis in *A. nodosum*. Since masses of $^{51}V^{+}$ and $^{35}Cl^{16}O^{+}$ were indistinguishable under chosen conditions, and certified vanadium standards prepared from macroalgae as biological matrix were not available at the time the study was performed, AdSV of vanadium-derived 2,5-dichloro-1,4-dihydroxy-3,6-benzoquinone (chloroanilic acid) complex was selected as supplementary method for data validation.

The issue of quantitative plant material degradation and organic matrix disruption was addressed as follows. Lyophylized algae (Section 2.1) was homogenized using a metal-free ball mill, to furnish a powder that was digested with a mixture of suprapure HNO_3 and H_2O_2 in a high pressure mineralizer. In an initial screen, vanadium levels of 1.7 ± 0.1 mg kg^{-1} dm (ICP-MS) and 1.65 ± 0.07 mg kg^{-1} dm (AdSV) were determined by digesting 53, 107, 153, 204, and 257 mg of powdered April harvest of *A. nodosum* with 2 ml of 65% (w/w) HNO_3 and 1 ml of 30% (w/w) H_2O_2 at 130 bar and 280 °C (Table 2, entry 1). Digestions prepared from ~250 mg of algal powder under such conditions tended to remain turbid and therefore were not further considered. Since vanadium levels measured via ICP-MS and AdSV were similar, ClO^{+} formation was assumed not to significantly interfere. The averaged vanadium level was close to concentrations determined via AAS in *A. nodosum* collected at Helgoland/Germany in 1981 (1.6 mg kg^{-1} dm) and Knock/Germany in 1998 (2.0 mg kg^{-1} dm) (Sperling et al., 2000). Parameter assessment for AdSV provided a detection limit of 0.16 ng V ml^{-1}, a limit of quantitation of 0.67 ng V ml^{-1}, a recovery of 90–110%, and reproducibility of 1.7%. For ICP-MS, a detection limit of 0.12 ng V ml^{-1}, limit of quantitation of 0.16 V ng ml^{-1}, recovery of 91–97% of V, and reproducibility of 3.7% was found. Analytical parameter were thus slightly in favor for AdSV. ICP-MS, on the other hand, provided additional information, for instance, on concentration of ^{55}Mn (18 ± 1 mg kg^{-1} dm), ^{59}Co (0.22 ± 0.01 mg kg^{-1} dm), ^{60}Ni (0.68 ± 0.05 mg kg^{-1} dm), ^{63}Cu (1.6 ± 0.7 mg kg^{-1} dm), ^{66}Zn (55 ± 2 mg kg^{-1} dm), ^{75}As (54 ± 3 mg kg^{-1} dm), ^{114}Cd (0.14 ± 0.01 mg kg^{-1} dm), ^{203}Pb (0.16 ± 0.01 mg kg^{-1} dm), and ^{238}U (0.71 ± 0.03 mg kg^{-1} dm) in *A. nodosum*. All values were similar to those reported previously for *Fucus vesiculosus* (Truus et al., 2001).

Table 2
Vanadium levels in *A. nodosum* collected at Roscoff (France)

Entry	Harvest	V (ICP-MS) (mg kg^{-1} dm)[a]	V (AdSV) (mg kg^{-1} dm)[a]
1	April 2005	1.7 ± 0.3	1.7 ± 0.3
2	May 2005	1.9 ± 0.3	1.5 ± 0.2
3	June 2005	0.9 ± 0.2	0.9 ± 0.2
4	July 2005	0.9 ± 0.2	0.9 ± 0.2
5	August 2005	0.6 ± 0.1	0.6 ± 0.2
6	September 2005	1.1 ± 0.2	1.1 ± 0.2
7	October 2005	1.3 ± 0.2	1.3 ± 0.2
8	November 2005	1.5 ± 0.2	1.3 ± 0.2
9	December 2005	1.3 ± 0.2	1.5 ± 0.2
10	January 2006	1.9 ± 0.3	2.3 ± 0.4
11	February 2006	1.5 ± 0.2	1.6 ± 0.3
12	March 2006	1.6 ± 0.3	1.4 ± 0.2

[a] From 3 independent *A. nodosum* digestions.

ICP-MS and AdSV analysis of the remaining 11 samples (Table 2, entries 2–12) pointed to seasonal variation of the algal vanadium level. Low concentrations were determined for June–December harvest (0.6–1.3 mg kg^{-1} dm) (Table 2, entries 3–9), which was followed by a maximum in January (1.9–2.3 mg kg^{-1} dm) (Table 2, entry 10), and a high level plateau for *A. nodosum* collected in February–March (Table 2, entries 11–12). The latter values were similar to vanadium levels measured for April and May 2005 samples (Table 2, entries 1–2). The use of ICP-MS and AdSV furnished in all instances comparable results.

Ocean water is ~42 nM in vanadium (Jeandel et al., 1987). Since measured concentrations in *A. nodosum* significantly exceeded vanadium levels added from this background in the course of lyophylization, it is obvious that the alga accumulates the metal. Interestingly, seasonal vanadium levels were paralleled by PO activity variation (Fig. 2). If protein concentration of solutions applied for PO activity measurements referred exclusively to A.n.I apoenzyme, and quantitative loading with the co-factor, i.e. one vanadium atom per subunit (Weynand et al., 1999) had occurred, vanadium levels of 0.6–0.9 mg kg^{-1} dm would have been predicted for *A. nodosum*. If compared to vanadium levels determined in the present study, it may well be that a notable fraction of the metal is bound to bromoperoxidase apoenzymes.

3. Concluding remarks

The data of the present study suggest that the season between midwinter to early spring is the preferred period of the for collecting *A. nodosum* in order to obtain most significant yields of vanadium-dependent bromoperoxidases from specimen growing in the intertidal region close to Roscoff/France. This suggestion is in line with earlier reports, although methods for enzyme extraction from the organism have been considerably improved since then. The fact that the algal vanadium level approximately correlated with the extractable PO activity was noteworthy but should not be overemphasized. The PO activity referred to in the present work relates to reactivity available from the organism under selected work up conditions thus including apoenzyme reconstitution via dialysis against Tris-buffered $NaVO_3$ solution. PO activity inside the organism prior to digestion, however, might be different from the values given in Table 1. A discussion of this topic, however, has to await results from studies on vanadium distribution in *A. nodosum*, which was not the objective of the present work.

4. Experimental

4.1. General remarks and instrumentation

A. nodosum was Lyophylized ground in a Retsch ZM1 centrifugal mill using a sieve of 1.5 mm pore size. The resulting green powder was stored at −28 °C. Extraction of enzymes was enhanced by using a Chemineer Greerco W250 colloid mill with cooling system. Dialysis was performed with a Visking Dialysis Membrane from ROTH® (molecular weight cut-off: 14000). Isoenzyme patterns of extracts were verified qualitatively via Colorless Native Polyacrylamide Gel Electrophoresis (CN-PAGE) using a MINI Vertical Dual Plate Electrophoresis Unit from ROTH®. Enzyme kinetics and UV/

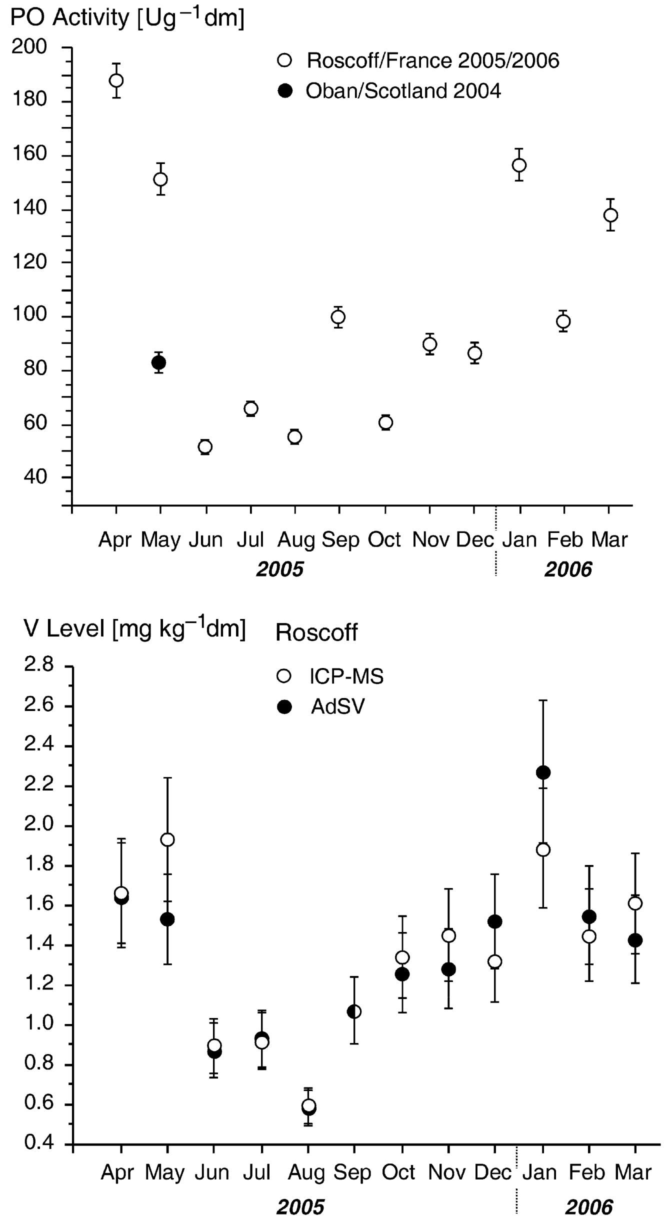

Fig. 2. Seasonal variation in PO activity of extracts of *A. nodosum* (top) and vanadium level of the algae (bottom; see text and Tables 1 and 2; dm = dry mass).

vis spectra were recorded with a Varian Cary 100 Conc double beam spectrophotometer.

ICP-MS were measured with a Perkin–Elmer-Sciex Elan 6000 connected to a Perkin–Elmer AS 90 autosampler. Adsorptive stripping voltammetry was performed using a 746 VA Trace-Analyzer equipped with a multimode electrode, Pt-auxiliary electrode, Ag/AgCl/KCl (3 M) reference electrode, a stirring device, 695 VA Autosampler, automatic sampling devices 700 Dosino and 685 Dosimat (Metrohm, Switzerland).

4.2. Plant material

A. nodosum was collected littoral and sublittoral near the Station Biologique Roscoff, France at 48°43′N, 3°58′W approximately in the middle of the month from April 2005 to March 2006, and at Dunstaffage Marine Laboratory, Oban, Scotland at 56°28.9′N, 5°30′W in May 2004. The former samples were identified by one of the authors (H.V.). The latter were identified by Dr. Küpper, head of Dunstaffage Marine Laboratory, The Scottish Association for Marine Science, Oban, Scotland.

4.3. Extracts from A. nodosum

Freeze-dried (2 days), finely ground *A. nodosum* (100 g) added to a solution of PEG 1500 (584 g) and 1-thioglycerole (10 ml) in H_2O (2 l). Stirring was continued (10 min) to furnish a suspension that was passed at −5 °C through the colloid mill having a gap adjustment of 40 μm. The gap adjustment was consecutively reduced to 1 μm in two succeeding runs conducted at –5 °C to afford a green slurry that was poured into a stirred solution of K_2HPO_4 (584 g) and KOH (6.68 g) in H_2O (1.34 l). The slurry was stirred for 30 min. Phase separation was achieved by centrifugation (2000 rpm, 5 min). The bright green upper phase (1 l) was separated and added to a solution of $MgSO_4 \times 7\ H_2O$ (400 g) in H_2O (800 ml). The solution was stirred for 30 min and the phases were allowed to separate (30 min). The bottom phase (1.00 l) was separated and mixed with a solution of PEG 1500 (50 g) in H_2O (50 g). The mixture was stirred for 30 min and the phases were allowed to separate (30 min). The bottom phase (820 ml) was separated and poured into a solution of PEG 1500 (164 g), Tris(hydroxymethyl)aminomethane (3.94 g) and $(NH_4)_2SO_4$ (492 g) in H_2O (820 ml) to afford a mixture that was stirred for 30 min. The phases were allowed to separate (30 min). To the upper phase (395 ml) was added cold (1 °C) acetone (1.20 l) to afford a suspension, which was centrifuged (4000 rpm, 5 min). The obtained solids were transferred into a dialysis tube by elutriation with aqueous (*aqua bidest.*) Tris-buffer (500 ml, 1.65 mM) and were dialyzed against aqueous (*aqua bidest.*), $NaVO_3$-containing (20 μM) Tris-buffer (5.00 l, 1.65 mM) in a cooling chamber at 4 °C. The bath was renewed every 24 h for 3 d. Tube contents were filtered to afford a clear solution ($\sim$220 ml) of bromoperoxidases, which was stored at 3 °C.

4.4. Triiodide assay

Reference and sample cuvettes (10.0 mm) were charged with phosphate buffer (3.00 ml, 132 mM K_2HPO_4, 33.9 mM citric acid monohydrate, pH 6.2), and stock solutions of KI (100 μl, 200 mM) and H_2O_2 (100 μl, 26.6 mM) and thermostated at 20 ± 0.5 °C. Water (100 μl) was pipetted into the reference cuvette and the contents were mixed. Enzyme solution (100 μl) was pipetted into the sample cuvette to the stirred solution followed by immediate monitoring of triiodide absorption ($\lambda = 350$ nm) as a function of time. A control experiment on activity of a cytochrome [equine heart cytochrome C, 400 μg/ml; Fluka, $\geqslant$90% purity (gel electrophoresis), no activity provided] under otherwise identical conditions, showed no catalytic activity, even in instances where undiluted samples were used that corresponded to a 200 fold higher enzyme concentration, if compared to A.n.I preparations used to spectrophotometrically follow I_3^- formation.

4.5. Monochlorodimedone assay

Bromoperoxidase activity for oxidation of Br^- was measured at 22.0 ± 0.5 °C by following the conversion of monochlorodimedone (MCD) ($\varepsilon = 20.1\ mM^{-1}\ cm^{-1}$ at 290 nm) into bromochlorodimedone ($\varepsilon = 0.2\ mM^{-1}\ cm^{-1}$ at 290 nm) with the aid of a UV spectrometer (Hager et al., 1966). Reference and sample cuvettes (10.0 mm) were charged with stock solutions of MCD (750 μl, 200 μM), phosphate buffer (750 μl, 400 mM K_2HPO_4, pH 6.5), KBr (750 μl, 400 mM) with Na_2SO_4 (800 mM) and H_2O_2 (750 μl, 8 mM). Water (6 μl) was pipetted into the reference cuvette. Contents were mixed. Enzyme solution (6 μl) was pipetted into the sample cuvette. Contents were mixed and subjected to UV/vis photometric analysis.

4.6. Reagents for quantitative vanadium analysis

Ultra pure H_2O (min 18 MΩ) and supra pure HNO_3 [65% (w/w), Merck] were used for operations described in the following sections. Acetate buffer (pH 4.6) was prepared from a solution of sodium acetate (82.0 g, 1 mol, p.a., Merck, Germany) in H_2O (500 ml) and HOAc (57.2 ml, 1 mol, suprapure, Merck, Germany). Water was added to a total volume of 1000 ml. A stock solution (1 mM) was prepared from chloroanilic acid (0.2909 g, p.a., Riedel-de-Haen) and H_2O.

Vanadium standard for adsorption voltammetry (1 μg vanadium ml^{-1}) was prepared from a standard solution (50 μl, 1.000 g vanadium per liter in 1 M HNO_3, Bernd Kraft, Duisburg, Germany), HNO_3 (1 ml) and H_2O in a quartz volumetric flask (50 ml).

Multielement standards [(i) H_2O, (ii) 5 ng vanadium ml^{-1}, (iii) 10 ng V ml^{-1}, (iv) 20 ng V ml^{-1}, and (v) 50 ng V ml^{-1}] for ICP-MS were prepared in quartz volumetric flasks (50 ml) from HNO_3 (0.5 ml), H_2O and multielement standard solution VI [(i) 0 μl, (ii) 25 μl, (iii) 50 μl, (iv) 100 μl, and (v) 250 μl; Merck, 30 cations in different concentrations, including 10 mg l^{-1} of vanadium].

Rhodium standard for ICP-MS (1 μg rhodium ml^{-1}) was prepared from a standard solution (50 μl, 1.000 g rhodium per liter in 1 M HNO_3, Bernd Kraft, Duisburg, Germany), HNO_3 (0.5 ml) and H_2O in a quartz volumetric flask (10 ml).

4.7. Vanadium analysis

Lyophilized *A. nodosum* (5–7 g) was homogenized in a metal-free ball mill (MM 301, Retsch, Haan, Germany) to furnish a dry powder. A fraction (0.100–0.150 g) of this material was placed in a dried high pressure mineralizer (Anton Paar-Physica, Graz, Austria) and charged with HNO_3 [2 ml, 65% (w/w)], suprapure, Merck, Germany) and H_2O_2 [1 ml, 30% (w/w), suprapure, Merck, Germany]. The mixture was allowed to rest, until the initial reaction had ceased (~14 h). The high pressure mineralizer was afterwards closed according to the manufacturer's instruction. Quantitative decomposition of the organic matrix was achieved using an initial pressure of 100 atm and the following temperature profile: ~20 → 100 °C (30 min), 100 °C (10 min), 100 → 150 °C (30 min), 150 °C (10 min), 150 → 200 °C (30 min), 200 °C (10 min), 200 → 280 °C (30 min), 280 °C (60 min), 280 → 0 °C (60 min). Nitrous gases were removed from the clear solution by placing the reaction vessel in an ultrasound bath for 10 min. Water was added directly into the vessel until a volume of 10 ml was obtained. The solution was mixed in order to obtain a homogenous solution, which was used for ICP-MS and adsorption voltammetry.

Vanadium analysis via adsorptive stripping voltammetry. A solution of digested alga (0.5 ml, see first paragraph of Section 4.7) was pipetted into the cell of the VA-Processor. Acetate buffer (9.5 ml) and chloroanilic acid stock solution (0.5 ml) were added. The solution was purged with N_2 (99.999% purity) for 5 min. Voltammograms were recorded using HMDE/Pt/KCl (3 M)/AgAgCl electrodes, differential pulse polarographic mode, –50 mV amplitude voltage, −250 mV adsorption potential, −450 mV peak potential, and 180 s adsorption time. Calibration was performed by two consecutive additions (50 μl) of vanadium standard prepared above.

Vanadium analysis via ICP-MS. A solution of digested alga (1.0 ml, see first paragraph of Section 4.7) was pipetted into a polypropylene tube. Internal rhodium standard (100 μl see above) and H_2O were added to afford a clear solution (total volume of 10 ml), which was administered via peristaltic pump (flow rate 1 ml min^{-1}) and cross-flow nebulizer into a Scott-chamber of the ICP-MS (Plasma: 1000 W of RF power, 15 l min^{-1} of plasma gas flow, 0.9 ml min^{-1} of auxiliary gas flow, 0.9 ml min^{-1} nebulizing gas flow). Peak hopping scan and dual detector mode were applied with the dwell time set to 10 ms/AMU. Analysis was performed for the atomic mass 51 AMU on the basis of calibration lines obtained using the multielement reference prepared above.

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