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Purification and characterisation of vanadium haloperoxidases from the brown alga *Pelvetia canaliculata*

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Abstract

Two enzymes characterised as iodoperoxidases (PcI and PcII), with vanadium-dependent activity, have been purified from the brown alga *Pelvetia canaliculata* (L.) Decne et Thur. (Fucaceae, Phaeophyceae), collected in the Northern Portuguese coast, at Viana do Castelo. The relative molecular masses were 166 kDa for PcI and 416 kDa for PcII, as determined by gel filtration. SDS–PAGE shows that PcI has just one band corresponding to a subunit of 66 kDa, while PcII shows four bands (66, 72, 157 and 280 kDa). The following kinetic parameters have been determined from a steady-state analysis of the oxidation of iodide by H_2O_2: PcI, $pH_{opt} = 6.0$, $K_M(I^-) = 2.1$ mM, $K_M(H_2O_2) = 110$ μM, $K_i(I^-) = 127$ mM; and PcII, $pH_{opt} = 6.5$, $K_M(I^-) = 2.4$ mM, $K_M(H_2O_2) = 20$ μM and $K_i(I^-) = 69$ mM. These iodoperoxidases are thermostable, as also observed for vanadium bromo- and chloroperoxidases.

Keywords: *Pelvetia canaliculata*; Fucaceae; Vanadium-dependent haloperoxidases; Iodoperoxidases; Vanadium in biology

1. Introduction

The number of cases in which vanadium has been found to occur in biology and appears to play a significant role has increased in recent years (Siegel and Siegel, 1995). A particular example, which is attracting attention, is the presence of this metal in the active site of a family of haloperoxidases (HPO), a subgroup of peroxidases (EC.1.11.1). For the vanadium chloroperoxidase, structural details are available (Messerschmidt and Wever, 1995). The enzymes are found in an increasing number of marine (algae) and terrestrial (lichen and fungi) organisms (Vilter, 1995), and a role for them in the synthesis of some of the widespread natural halogenated compounds has been proposed (Neidleman and Geigert, 1986). The importance of these compounds for diverse pharmaceutical and industrial applications has been documented (Kirk, 1991; Littlechild, 1999).

In the Fucaceae family of the brown algae (Phaeophyta), vanadium dependent HPO activity was previously reported for *Ascophyllum nodosum* (L.) Le Jolis, *Fucus serratus* (L.), *Fucus spiralis* (L.), *Fucus vesiculosus* (L.) and *Pelvetia canaliculata* (L.) Decne. et Thur from Northern Europe, and for *Fucus distichus* (L.) from Californian coast (Vilter, 1995). *Ascophyllum nodosum*, the most studied source of vanadium halo-

Abbreviations: BrPO, Bromoperoxidase; EDTA, Ethylene Diamine Tetra Acetic acid; HPLC, High Performance Liquid Chromatography; HPO, Haloperoxidase; IPO, Iodoperoxidase; Pc, *Pelvetia canaliculata*; PEG, PolyEthylene Glycol; PVDF, PolyVinyl DiFluoride; SDS–PAGE, Sodium Dodecyl Sulphate–PolyAcrylamide Gel Electrophoresis; Tris, Tris-(hydroxymethyl)aminomethane.

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peroxidases, contains two bromoperoxidases (Krenn et al., 1989b) and shares with *P. canaliculata* the singularity of having an associated endophytic fungus, the ascomycete *Mycosphaerella ascophylli* Cotton, which is omnipresent in *A. nodosum* but not in *P. canaliculata* (Kohlmeyer and Kohlmeyer, 1972). Iodoperoxidase and bromoperoxidase activity has been observed previously in crude extracts of *P. canaliculata* (de Boer Tromp et al., 1986; Wever et al., 1991; Vilter, 1995), but this was not further characterised.

Although all the vanadium HPO examined so far exhibit common basic features, it is not yet understood which factors determine why some of these enzymes catalyse the oxidation of iodide only and others also the oxidation of bromide and chloride. Work carried out in our laboratories has identified a possible trend of activity in that the enzyme forms extracted from species collected in the Portuguese coast seem to have lower oxidation potential than the same species collected in northern locations (Almeida et al., 1997), but this can be due to a lower content of the enzyme in the algae and the lower sensitivity of the bromide oxidation test, see below. Note that, so far, no chloroperoxidase activity has been detected in algae, although X-ray structure data does not eliminate such possibility (Weyand et al., 1999).

In biogeographic terms, the Portuguese coast falls within the warm temperate Mediterranean–Atlantic region (Lüning, 1990) and presents particular environmental features due to the confluence of North Atlantic and Mediterranean–North African influences. Nevertheless, it is still possible to find there many of the colder temperate algae, such as *P. canaliculata* which has a European North Atlantic distribution (Lüning, 1990) with the northern limit in Northern Norway and the southern limit in the Central Portugal (Ardré, 1969), where it is usually present in the lower eulitoral zone of open to sheltered locations, along with other cold temperate Fucaceae such as *F. spiralis, F. vesiculosus* and *A. nodosum*. The present report on the isolation and characterisation of haloperoxidases from *P. canaliculata* collected in the Portuguese coast, near the southern limit of its geographic distribution, is a contribution for the study of the role of vanadium in these enzymes.

2. Results and discussion

The *P. canaliculata* harvested in September and December showed haloperoxidase activity; however, for specimens collected in August, no activity was observed. This observation suggests a seasonal haloperoxidase activity for this species as it was also observed for *A. nodosum* (Vilter and Glombitza, 1983; Wever et al., 1985) and for the *Corallina* species (Itoh et al., 1996), which was later shown to be due to variation in availability of vanadium.

The purification of enzymes extracted from marine algae is a difficult task, since the composition of the raw material is complex and the presence of polyphenols and tannins in the extracts complicates the process. A preliminary study of extraction procedures using three different methods was carried out. The most efficient method — as far as yield and purity of samples are concerned — was method C (see Section 3), an adaptation of the method proposed by Krenn et al. (1989b) for the extraction of the external surface HPO from *A. nodosum*. In our studies with *P. canaliculata*, we extracted, purified and characterised two enzymes, independent of the extraction procedure used. Previously, the presence of more than one HPO in the same algae was observed only in *A. nodosum*, one located inside the thallus (bromoperoxidase I), particularly around the conceptacles, and the other (bromoperoxidase II) present at the thallus surface (Krenn et al., 1989b). These results suggest that the presence of more than one isoenzyme in algae may be a common feature, as is found in terrestrial plants (Jackson et al., 1996). However, the presence of the two isoenzymes apparently on the thallus surface (the extraction, in method C, was achieved just by washing the alga) is a new feature, since the location of the enzymes in the alga is different from that in *A. nodosum*, as confirmed by histochemical tests. The existence of different isoforms of haloperoxidases may be the result of an adaptation to environmental conditions; for instance, salinity, light conditions or presence of other marine organisms or substances (Almeida et al., 1998).

It is not yet known whether the two bromoperoxidases extracted from *A. nodosum* originate from the same gene. As in *P. canaliculata*, the two iodoperoxidases differ in their binding to ConA-sepharose, which implies a different glycosylation pattern.

The two purified haloperoxidases show mainly iodoperoxidase activity, but the specific activity of PcI is half of that of PcII (specific activities: PcI 55 U/mg; PcII 105 U/mg); their activity as a bromoperoxidase is very low (ca. 5% of the IPO activities). It is, therefore, proposed that they are iodoperoxidases, but according to the currently accepted nomenclature, these enzymes are classified as bromoperoxidases since they also promote the bromination of monochlorodimedone. However, as stated above, this test is much less sensitive than the formation of I_3^- (the molar absorbance of I_3^- is about 10^3 times higher than that of monochlorodimedone), and for low enzyme concentrations, the bromination activity may not be detected or only barely detected, as was the case of this work.

The purified enzymes are colourless and their absorption spectrum in the visible range is featureless.

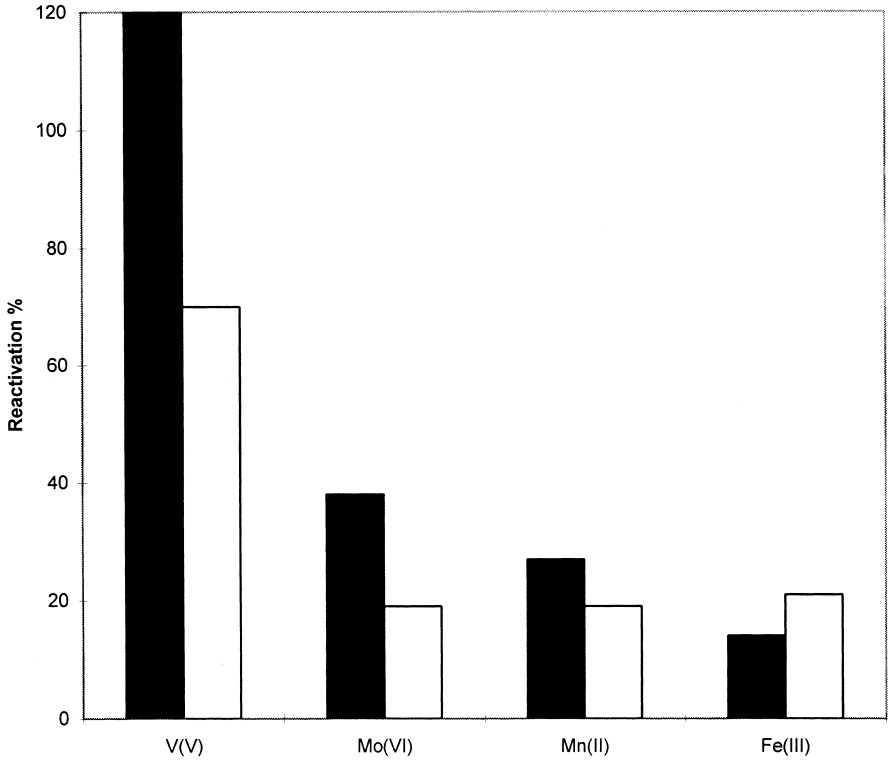

Fig. 1. Reactivation of PcI and PcII enzymes by different transition metals (activity value before inactivation was considered as 100%): ■ PcI; □ PcII.

As Fig. 1 shows, both enzymes are reactivated by vanadium (V). However, the PcII enzyme was difficult to deactivate and it was not possible to get it completely deactivated; even after a very thorough and extensive dialysis about 25% of the initial activity persisted. For the PcI enzyme, the deactivation was quite easy and the enzyme exhibited less than 2% of the initial activity.

Reactivation was also quite difficult for the PcII enzyme, and only 70% of the initial activity was achieved. This behaviour was also observed for the vanadium bromoperoxidase from *C. pilulifera* even after extensive dialysis (Sheffield et al., 1993).

The values of activity found, after reactivation, with the three other metal ions tested are significantly lower than the values obtained for vanadium (V). This result supports the idea that vanadate is essential for catalytic activity.

The molecular mass of the PcI enzyme is 166 kDa. Since under denaturating conditions only one subunit of 66 kDa (Fig. 2) is detected, the enzyme is probably dimeric. For the PcII enzyme, the situation is more complex. Molecular mass determination by gel filtration gave a molecular mass of 416 kDa, which is higher than the usual values for haloperoxidases from Fucacae. However, similar results were found before (Krenn et al., 1989a) for bromoperoxidases extracted from other algae. It is likely that the enzyme forms aggregates easily, but it is also possible that the enzyme is tightly bound to alginates (Sheffield et al., 1993). SDS–PAGE experiments (Fig. 2) show the presence of four subunits of different molecular mass (66, 72, 157 and 280 kDa), the lower molecular mass bands being dominant. This is an unusual behaviour since, so far, all the haloperoxidases that have been studied

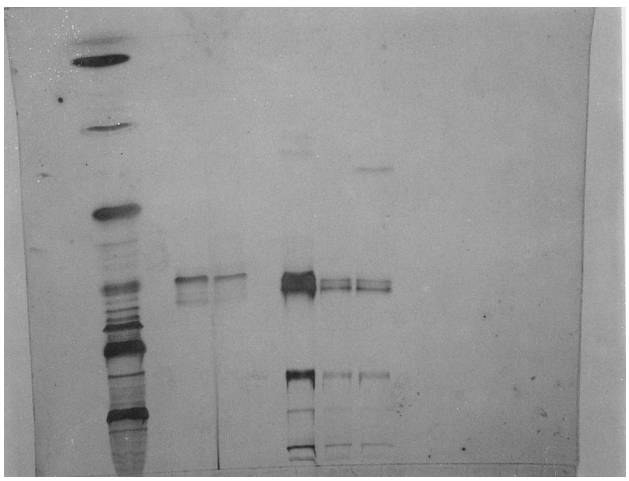

Fig. 2. SDS–PAGE in acrylamide linear gradient (5–15%). Lane 1: PcII; Lane 2: PcI; Lane 3: Standards. Gel stained by the silver nitrate method. Conditions: SDS 1% (w/v), β-mercaptoethanol 5% (v/v), boiling at 100°C for 3 min.

show only one subunit. Given the considerable resistance to denaturation of these proteins and the fact that the intensity of the bands diminishes with the increase of molecular mass, we decided to apply extreme incubation conditions in repeated denaturating electrophoresis in order to establish if those bands were or not true aggregates of the lower subunits. Even in extreme conditions, such as exposure to 6 M guanidine–HCl or prolonged boiling, the electrophoretic profile was not changed (not shown). If the higher molecular mass bands correspond to dimers or tetramers of the 66 kDa subunit, they must be strongly stabilised by interactions which are maintained under the extreme conditions used.

SDS–PAGE experiments also showed that both enzymes (PcI and PcII) stain for enzymatic activity on the gel in the presence of SDS when the samples are not heated. This behaviour clearly shows the resistance of these enzymes to chemical denaturation in the presence of SDS, as previously observed by Krenn et al. (1989a,b).

Another important aspect is the fact that the electrophoretic profile remains the same when the samples are prepared in the presence or absence of β-mercaptoethanol. This result suggests that the two HPO enzymes do not have disulphide bridges between the subunits.

Both subunits of 66 kDa from the two enzymes (PcI and PcII) give a positive glycoprotein test, whereas the subunits 157 and 280 kDa of the PcII gave negative results. This means that such residues do not have or have only very reduced affinity for concanavalin A, supporting the idea that they are strongly associated.

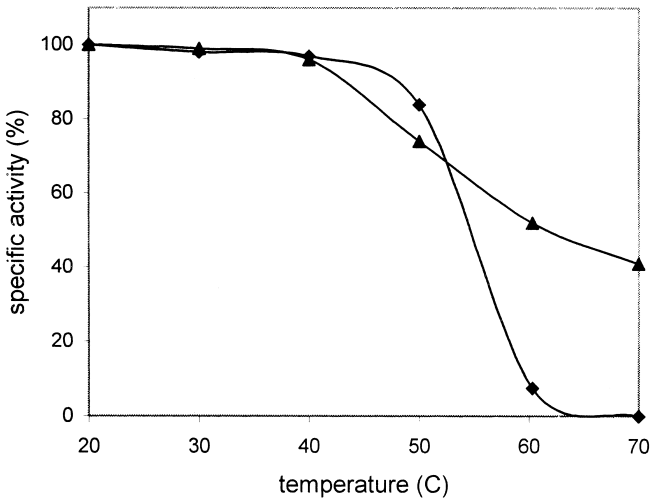

Fig. 3. Thermostability of the two iodoperoxidases. Samples of the enzymes (PcI and PcII) were incubated, in buffer, for 1 h at the given temperature after which enzymatic activity was measured as described in materials and methods.

The thermostability was also investigated — both enzymes remain active up to 40°C, but for PcI, the activity decreases abruptly at 60°C and it is completely lost at 70°C. PcII is more stable: 40% of the initial activity still remains at 70°C (Fig. 3).

The optimal pH for the activity of these two enzymes was initially determined in two buffers: phosphate (pH 5.7–7.8) and acetate (pH 4.0–5.6) in order to cover a convenient range of pH values. For the PcI enzyme, an appreciable difference of enzymatic activity was observed, around pH 5.5, upon changing the buffer. This was already observed by Sheffield, for the bromoperoxidase from *C. pilulifera* (Sheffield et al., 1993). Therefore, a third buffer system (citrate–phosphate) covering the same region, was used (pH 5.2–6.8) to determine the optimal pH, which occurs at pH 6.0. The activity of the PcII enzyme was not affected by the buffer system. Maximal activity was found at pH 6.5 in either phosphate or citrate–phosphate buffers. The difference is less than that in case of the two bromoperoxidases (I and II) extracted from *A. nodosum* (for which the optimal pH values are 6.0 and 7.3, respectively). Interestingly, the optimum pH for PcI is the same as for bromoperoxidase I, which confirms the similarities between these enzymes.

These results, together with the studies of deactivation/reaction, seem to imply a difference in the bonding of the vanadium in these two enzymes.

The K_M values for I^- and H_2O_2, determined by Lineweaver–Burk plots, for PcII are larger than those for PcI, showing that PcII has a lower affinity for the two substrates (Table 1). Inhibition by the iodide substrate was observed at high iodide concentration. The inhibition pattern was quite different for the two enzymes (Table 1).

In conclusion, the two iodoperoxidases from *P. canaliculata* are vanadium enzymes, but while PcI shows characteristics similar to those of other haloperoxidases, especially to bromoperoxidase I of *A. nodosum*, PcII enzyme differs considerably, with different carbohydrate content, as judged by the lower affinity for the concanavalin-A, high molecular mass, higher resistance to total inactivation and lower affinity for hydrogen peroxide and iodide.

One may wonder whether these particular results are associated with the *habitat* of the species. Only a more complete study on the origin of these isoenzymes, their

Table 1
Kinetic parameters of iodoperoxidases from *Pelvetia canaliculata*

Enzyme	$K_M(H_2O_2)$ (μM)	$K_M(I^-)$ (mM)	$K_i(I^-)$ (mM)	pH_{opt}
PcI	110	2.1	127	6.0
PcII	200	2.4	60	6.5

location in the plant and their structure, and a comparison with HPO of the same algae collected at other sites, will enable us to elucidate further the role played by these enzymes.

3. Experimental

Pelvetia canaliculata was harvested from the rocky intertidal zone in the northern coast of Portugal (Praia do Norte near Viana do Castelo, 41°40′N; 8°50′W), in August, September and December. After collection, the algae were transported to the laboratory where they were thoroughly washed with distilled water, chopped and stored frozen until required (except for method C, see below).

For the extraction and purification of HPO of *P. canaliculata*, three different methods, A, B, and C, were tested.

Method A used the strategy previously described by Wever et al. (1985) for the purification of bromoperoxidase from *A. nodosum*, with slight modifications, such as the elimination of the concentration step.

Method B used the purification schemes based on a two-phase aqueous system (Vilter, 1983) which, in our case, was constituted by 15% (w/v) polyethyleneglycol 1500 (PEG) and 20% (w/v) K_2HPO_3. After centrifugation at 5000 × *g* for 20 min, the upper phase was collected and the proteins precipitated by adding the same volume of acetone. The resulting pellet was collected after a second centrifugation (10,000 × *g*, 20 min) and then resuspended in 50 mM Tris–HCl, (pH 9.0). In order to reactivate the HPO, the extract was dialysed overnight against 2 mM $NaVO_3$ in 8.2 mM Tris–HCl (pH 8.3). The next step consisted of a hydrophobic interaction chromatography, performed on a Phenyl–Sepharose resin (Pharmacia) with a decreasing linear gradient elution (30–0%) (w/v) of $(NH_4)_2SO_4$ in 50 mM Tris–HCl (pH 9.0). The enzyme was further purified by HPLC/gel filtration (Shimadzu system) on a pre-packed Superdex 200HR 10/30 column (Pharmacia), equilibrated and eluted with 100 mM NaCl in 50 mM Tris–SO_4 (pH 9.0).

Method C was an adaptation of a method proposed by Krenn et al. (1989b). Intact fresh algae were washed in 20 mM Tris–SO_4 (pH 8.3) for 1 h, and the extract was centrifuged (7000 × *g*, 20 min) in order to remove sand, cell debris and other impurities. Protein was precipitated by salting-out with 80% ammonium sulphate. After centrifugation (14,000 × *g*, 45 min), the precipitate was resuspended in 60% (v/v) ethanol in 20 mM Tris–SO_4 (pH 8.3), and again centrifuged (14,000 × *g*, 20 min). The upper phase was dialysed overnight against 20 mM Tris–HCl (pH 7.3) and this extract was applied to a ConA column (1.7 × 11 cm) eluted with 1 mM $CaCl_2{\cdot}2H_2O$, 1 mM Na_2SO_4, 500 mM NaCl, 500 mM methylmannopyranoside in 20 mM Tris–HCl (pH 7.3). All the active fractions were pooled and submitted to a second chromatographic separation on a HPLC system with a gel filtration Superdex 200HR 10/30 resin (Pharmacia). The elution was carried out with a solution of 100 mM NaCl in 50 mM Tris–HCl (pH 9.0).

Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin as standard (Sigma). The relative molecular mass was determined by HPLC/gel filtration chromatography (Superdex 200HR 10/30 column on a Shimadzu system) with a mobile phase of 100 mM NaCl in 50 mM Tris–HCl (pH 9.0). Standard proteins from Pharmacia and Sigma were used for column calibration.

SDS–PAGE was carried out according to Laemmli (1970) using a Hoefer SE600 unit. The standard proteins used for molecular mass determination were from Bio-Rad (broad range kit). Native gel electrophoresis was carried out under the same conditions but without SDS. Generally, the gels were silver stained, according to the method of Blum et al. (1987).

The detection of glycoproteins was carried out by the Concanavalin A-peroxidase method of Faye and Chrispeels (1985). The enzymes were previously separated by SDS–PAGE on a 12.5% acrylamide mini-gel and electrophoretic-blotted to a PVDF membrane (ProBlott™, Bio-Rad), using 192 mM glycine in 25 mM Tris (pH 8.3) buffer, 10% (v/v) methanol, as transfer buffer and the Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad).

Isoelectric focussing was carried out in a Multiphor II electrophoresis system (Pharmacia) with a 5% acrylamide and 3% bisacrylamide gel. The ampholites, as well as the pI markers, were from Pharmacia (Ampholine® pH 3.5–10).

Activity as iodoperoxidase (IPO) was measured by the conversion at 350 nm of I^- into I_3^- (ε_M 26,400 cm^{-1} M^{-1}) using H_2O_2 as the electron acceptor (Björkstén, 1968). The H_2O_2 solutions were prepared by dilution of 30% stock solution of Perhydrol (Merck) and their concentration was determined spectrophotometrically at 240 nm (ε_M 43.6 cm^{-1} M^{-1}) (Beers and Sizer, 1952). Bromination activity was measured spectrophotometrically at 290 nm, using the molecular absorbance of monochlorodimedone (ε_M 19.9 mM^{-1} $.cm^{-1}$) in an assay system which contained 50 μM monochlorodimedone, 2 mM H_2O_2, 100 mM KBr and 200 mM Na_2SO_4, in 100 mM phosphate (pH 6.5) buffer (Murphy and O'hEocha, 1973).

Enzymes were inactivated by extensive diafiltration using a Centricon-30 (Amicon) device against 100 mM citrate–phosphate pH 3.8 buffer in the presence of 1 mM EDTA, followed by a second diafiltration with 50 mM Tris–HCl (pH 9.0). Reactivation studies were carried out with ferric citrate, manganese sulphate,

ammonium molybdate and sodium vanadate in the later buffer.

The thermostability of the enzyme was tested by heating the extract at the required temperature for 1 h and checking the activity (iodide assay) immediately after. The solutions used in this enzyme assay were incubated at each studied temperature.

The steady-state kinetics experiments were done by measuring the iodination of I^- to I_3^- by H_2O_2. The solutions were buffered at pH 6.2, using a citrate–phosphate buffer, at an ionic strength of 0.1 M (Na_2SO_4). The experimental data were fitted to a rectangular hyperbolae equation (Michaelis–Menten form) by means of a non-linear least-squares treatment (Roberts, 1977). For the determination of the optimum pH, the solutions were buffered at the desired pH using acetate buffer (pH 4.0–5.6) and phosphate buffer (pH 5.7–7.8), at an ionic strength of 0.1 M. All measurements were carried out at 25°C.

Preliminary histochemical studies were made in transversal cuts of the blade soaking them in *o*-dianisidine dye prepared according to Vilter (1983).

All chemicals used were of analytical grade except potassium phosphate and ammonium sulphate, used in the first steps of the purification processes, which were of technical grade.

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References

Almeida, M., Almeida, M.G., Humanes, M., Melo, R.A., Silva, J.A., Fraústo da Silva, J.J.R., 1997. Novel vanadium-dependent haloperoxidases in brown algae (Fucacae and Laminareacae) from Portugal. Phycologia 36 (4), 1.

Almeida, M., Humanes, M., Melo, R.A., Silva, J.A., Fraústo da Silva, J.J.R., Vilter, H., Wever, R., 1998. *Sacchoriza polyschides*: a new source for vanadium-dependent iodoperoxidases. Phytochemistry 48 (2), 229–239.

Ardré, F., 1969. Contribution a l'étude des algues marines du Portugal. Portug. Acta Biol. (B) X, 137–532.

Beers, R.F., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133–140.

Björkstén, F., 1968. A kinetic study of the horse-radish peroxidase-catalysed oxidation of iodide. Eur. J. Biochem. 5, 133–184.

Blum, H., Beier, H., Gross, H.J., 1987. Improved silver staining of plant proteins RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93–99.

de Boer, E., Tromp, M.G.M., Plat, H., Kreen, B.E., Wever, R., 1986. Vanadium (V) as an essential element for haloperoxidase activity in marine brown algae: purification and characterisation of a vanadium (V)-containing bromoperoxidase from *Laminaria saccharina*. Biochim. Biophys. Acta 872, 104–115.

Faye, L., Chrispeels, M.J., 1985. Characterization of N-linked oligosaccharides by affinoblotting with concanavalin-A peroxidase and treatment of the blots with glycosidases. Anal. Biochem. 149, 218–224.

Itoh, N., Sasaki, H., Ohsawa, N., Shibata, M.S., Miura, J., 1996. Phytochemistry 42 (2), 277–281.

Jackson, P., Paulo, S., Ricardo, C.P., 1996. B2, the major soluble peroxidase of lupins, in vegetative development and the plant response to pathogenic agents. In: Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H. (Eds.), Plant peroxidases — biochemistry and physiology, IV International Symposium. University of Geneva, Geneva, pp. 247–254.

Kirk, K.L., 1991. Biochemistry of the elemental halogens and inorganic halides. Plenum Press, New York.

Kohlmeyer, J., Kohlmeyer, E., 1972. Is *Ascophyllum* lichenized? Bot. Mar. 15, 109–112.

Krenn, B.E., Izumi, Y., Yamada, H., Wever, R., 1989a. A comparison of different (vanadium) peroxidases; the bromoperoxidase from *Corallina pilulifera* is also a vanadium enzyme. Biochim. Biophys. Acta 998, 63–68.

Krenn, B.E., Tromp, M.G.M., Wever, R., 1989b. The brown algae *Ascophyllum nodosum* contains two different vanadium bromoperoxidases. J. Biol. Chem. 264, 19287–19292.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage. Nature 227, 680–685.

Littlechild, J., 1999. Haloperoxidases and their role in biotransformation reactions. Curr. Opin. Chem. Biol. 3, 28–34.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Lüning, K., 1990. Seaweed their environment, biogeography, and ecophysiology. Wiley, New York.

Messerschmidt, A., Wever, R., 1995. X-ray structure of a vanadium-containing enzyme: chloroperoxidase from the fungus *Curvularia inaequalis*. Proc. Nat. Acad. Sci. 93, 392–396.

Murphy, M.J., O'hEocha, C., 1973. Peroxidase activity in the brown algae *Laminaria digitata*. Phytochemistry 12, 2645–2648.

Neidleman, S.L., Geigert, J., 1986. Biohalogenation: principles, basic roles and applications. Ellis Horwood, Chichester.

Roberts, D.V., 1977. Enzyme kinetics. Cambridge University Press, London.

Sheffield, D.J., Harry, T., Smith, A.J., Rogers, L.J., 1993. Purification and characterisation of the vanadium bromoperoxidase from the macroalga *Corallina officinalis*. Phytochemistry 32, 21–26.

Siegel, H., Siegel, A. (Eds.), 1995. Metal ions in biological systems, Vol. 31. Marcel Dekker, New York.

Vilter, H., 1983. Peroxidases from Phaeophyceae. Part IV: Fractionation and location of peroxidase enzymes. Bot. Mar. XXVI, 451–455.

Vilter, H., 1995. Vanadium-dependent haloperoxidases. In: Siegel, H., Siegel., A. (Eds.), Metal ions in biological system. Marcel Dekker and references there in, New York, 31, 325–362.

Vilter, H., Glombitza, K.-W., 1983. Peroxidases from Phaeophyceae. Part II: Analytical disc-electrophoresis and isoelectric focusing of peroxidases from Phaeophyceae. Bot. Mar. XXVI, 341–344.

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

Wever, R., Tromp, M.G.M., Krenn, B.E., Marjani, A., Tol, V.,

1991. Brominating activity of the seaweed *Ascophyllum nodosum*: impact on the biosphere. Envirom. Sci. Tecnol. 25, 446–449.

Weyand, M., Hetch, H., Kies, M., Liaud, M., Vilter, H., Schomburg, D., 1999. X-ray structure determination of a vanadium-dependent haloperoxidase from *Ascophyllum nodosum* at 2.0A resolution. J. Mol. Biol. 293 (3), 595–611.