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# *SACCORHIZA POLYSCHIDES* (PHAEOPHYCEAE; PHYLLARIACEAE) A NEW SOURCE FOR VANADIUM-DEPENDENT HALOPEROXIDASES

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**Key Word Index**—*Saccorhiza polyschides*; Laminariales; brown algae; vanadium-dependent haloperoxidases; iodoperoxidase; bromoperoxidase.

**Abstract**—Vanadium-dependent iodoperoxidases from the brown seaweed *Saccorhiza polyschides* (Lightfoot) Batters, collected at three different locations along the Portuguese west coast, were extracted, purified and characterized. Several extraction procedures were tested, including two-phase aqueous systems. The purification of the iodoperoxidases was achieved using hydrophobic interaction chromatography followed by chromatofocusing. It was possible to isolate three different isoforms of the enzyme, which show mainly iodoperoxidase activity. The three native enzymes have a relative $M_r$ around 125 kDa, and two subunits of $M_r$ about 64 kDa. Reactivation studies of the apoenzymes with several metal ions revealed that vanadium(V) was essential for enzymatic activity. These enzymes are remarkably thermostable, maintaining their maximum activity up to 50°. The kinetic parameters for the enzyme catalysed iodoperoxidase reaction were obtained at pH 6.1. In the concentration range studied (0.2–8 mM) there was no inhibition by $H_2O_2$ whereas iodide inhibition was already apparent at the top values of the concentration range studied (2–25 mM). © 1998 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

It is currently accepted that haloperoxidases—a subgroup of peroxidases (EC.1.11.1)—have an important role in the production of organic halogenated compounds which are widespread in nature, particularly in marine environments [1, 2]. Haloperoxidases have been extracted and purified from a great variety of organisms, including mammals [3], higher plants [4], sponges [5], algae [6–9], a lichen [10], fungi [11, 12] and bacteria [13–15], and many of them contain the heme as a prosthetic group. All these studies revealed that haloperoxidases from marine organisms show mainly bromoperoxidase activity, whereas in those from terrestrial organisms chloroperoxidase activity is dominant. Iodoperoxidase activity has been found in several seaweeds, in some plants, birds and mammals [16].

In the early eighties, Vilter [17] purified a haloperoxidase from the brown seaweed *Ascophyllum nodosum*. This enzyme lacked the Soret band in the UV-vis spectrum and could be inactivated at low pH in the presence of EDTA. The haloperoxidase activity was only restored when $V^V$ was added to the apoenzyme preparation. This fact called attention to the possible role of vanadium in this enzyme and was subsequently confirmed by Wever *et al.* [18]. Since then, vanadium haloperoxidases have been purified from several brown, red and green seaweeds, from a lichen and from terrestrial fungi [2]. Spectroscopic studies (EXAFS) revealed the presence of $V^V$ in the prosthetic group of these enzymes, bound to oxygen and/or nitrogen donor ligands [19–23]. Recently, the primary and the crystal structures of the vanadium chloroperoxidase from the fungus *Curvularia inaequalis* were determined [24, 25], confirming the former spectroscopic results.

Although the physical properties of vanadium haloperoxidases extracted from several sources are simi-

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lar, more than one isoform may be present in biological material and the reactivity of each can be quite different [26]. Illustrative examples are the algae *A. nodosum*, in which two isoforms of bromoperoxidase have been isolated [27] and *Laminaria digitata* and *Laminaria saccharina* where the presence of several isoforms was also detected [28]. Thus, not only the kind of haloperoxidase activity seems to depend on the *habitat*—marine or terrestrial—of the species considered, but also the reactivity of the isoforms of the same haloperoxidase can vary substantially. It is, therefore, of interest to explore the reasons for the variety, types and properties of haloperoxidases present in different biological sources and relate them to the characteristics and geographical localisation of their habitat.

Several algal species, namely in the brown algal orders Laminariales and Fucales, have their southern limits of geographical distribution along the Portuguese coast, where slightly different variations are observed for some species. This is the case of *A. n. lusitanica*, a Portuguese variety of *A. nodosum* [29].

A screening program on vanadium-dependent haloperoxidases in seaweeds (benthic marine algae) found in the Portuguese coast was therefore undertaken, and we report now the results obtained in the study of *Saccorhiza polyschides* (Lightfoot) Batters, a brown alga of the order Laminariales, family Phyllariaceae. This seaweed is well represented along the western coast; it is found subtidally in both protected and exposed localities and emerges during spring low tides [30].

## RESULTS

### *Extraction and purification of haloperoxidases*

Samples of *S. polyschides* collected at Arrábida, see Fig. 1, were used in pilot experiments to test the ability of four different media for the extraction of haloperoxidases, see Fig. 2. Methods $A_1$ and $A_2$ are based on *tris* extraction of the chopped frozen material, and methods $B_1$ and $B_2$ are two-phase extraction procedures as developed by Jordan and Vilter [28]. In the first cases (methods $A_1$ and $A_2$) the extracts are highly viscous due to the presence of alginates (around 40% of dry weight for the Laminariaceae) which results in incomplete and slow release of the enzyme. In the second case (methods $B_1$ and $B_2$), alkaline solutions are required since large amounts of peroxidases in brown algae are associated with alginates in the cell walls and these are only soluble in alkaline solutions. Therefore, the salts potassium carbonate and hydrogen potassium phosphate were used, together with polyethyleneglycol (PEG). The haloperoxidases are partitioned into the more hydrophobic top phase, rich in PEG. The polymer also absorbs phenolic compounds [28, 31] that under oxidising conditions can bind covalently to native proteins. Most of the polysaccharides remain in the bottom phase, rich in salt. The treatment of the algae in such alkaline medium results in the extraction of the enzyme in the inactive apoform, but reactivation is completely achieved in a few minutes by incubation with excess of vanadate (240 $\mu$M). So, after extraction, $V^V$ was added to the media to reactivate the peroxidases.

The two-phase aqueous system formed by PEG 1550 and $K_2CO_3$–medium ($B_1$) was the most successful for extracting haloperoxidases from *S. polyschides*, as was also found by Jordan and Vilter in studies with *L. digitata* [28].

Partitioning of proteins in an aqueous two-phase system is dependent on the balance between ionic and hydrophobic interactions of the phase system components with proteins, and therefore two-phase aqueous systems varying in the concentration of salt $K_2CO_3$ and the polymer PEG 1500 were tested (see Experimental section for details).

Table 1 summarises the results obtained for *S. polyschides* collected at Viana do Castelo; it is clear that a medium with 15% (w/v) PEG 1500 and 20% (w/v) $K_2CO_3$ is the most efficient and therefore this medium was used for large scale isolations. Identical results were obtained for the algae collected at Porto Côvo. These studies clearly show that, independently from the collection site, all *S. polyschides* specimens exhibit iodoperoxidase activity. This type of activity has already been reported for samples of this algae collected in Brittany, France [32], but this observation was not further explored.

After reactivation by vanadate, the protein extract was loaded directly into a hydrophobic interaction column that removed polysaccharides and other non-peroxidase proteins. Three fractions with iodoperoxidase activity were separated by this procedure (see Fig. 3), for the seaweed collected at all the three locations. These fractions were named, for *S. polyschides* (Sp) collected at Arrábida, $SpA_1$, $SpA_2$, $SpA_3$; for the seaweeds collected at Viana do Castelo, $SpV_1$, $SpV_2$, $SpV_3$ and for those collected at Porto Côvo, $SpP_1$, $SpP_2$, $SpP_3$. After this chromatographic separation, each fraction was subjected to a chromatofocusing step. The purified isoforms differ considerably in their specific activity (Table 2)

### *Characterisation and properties of the enzymes*

The $M_r$s of each native enzyme was ca 125 kDa ($Sp_1$ 113 kDa, $Sp_2$ 129 kDa, $Sp_3$ 132 kDa, respectively) as determined by HPLC gel filtration on a Superdex 200 (see Fig. 4). SDS-PAGE of each enzyme showed only one band when stained for protein (not shown) with an apparent $M_r$ of about 64 kDa. Apparently, the native proteins are dimeric, with two subunits of almost equal $M_r$.

Reactivation of the fully inactivated iodoperoxidases with other metal ions was also performed as shown in Table 3; only vanadium(V) restored the activity completely. Figure 5 shows the time course of the reactivation of the three isoforms by $V^V$. Reac-

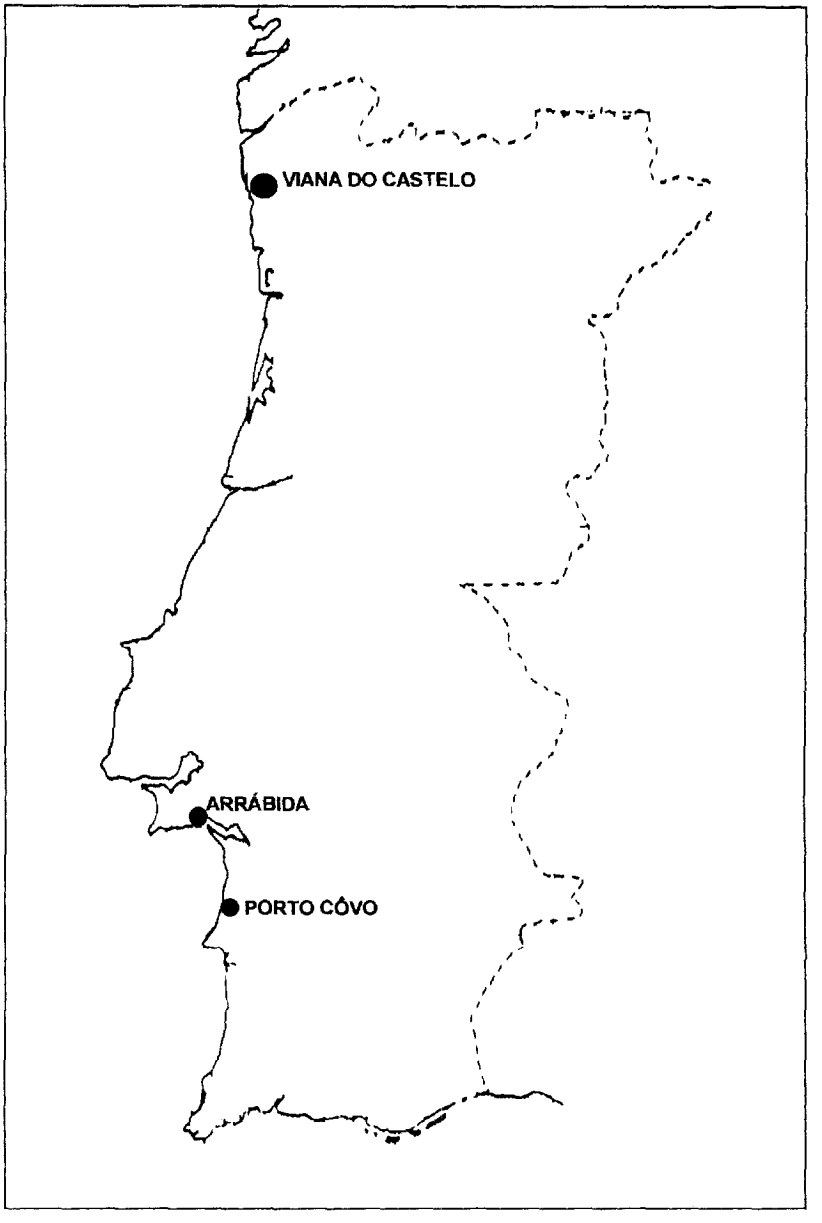


Fig. 1. Collection sites of *Saccorhiza polyschides*.

tivation of $Sp_3$ enzymes was significantly slower than $Sp_1$ or $Sp_2$ and the percentage of reactivation of apo-enzymes with vanadate when compared with initial values, in all the sites, follows the order (Table 3) $Sp_1 > Sp_2 > Sp_3$. These results clearly show that the enzymes are vanadium-dependent iodoperoxidases and that the three isoforms isolated for each seaweed have, indeed, a different reactivation mode.

The variation of activity with increase of temperature was also studied and the results (Fig. 6) clearly show that these enzymes are remarkably thermostable and behave similarly. All of them kept their full activity up to a temperature of 50°, and even after one hour at 70° activity was only reduced to half of its initial activity. This remarkable temperature stability had already been observed for vanadium chloro- and bromoperoxidases [2] and is now also confirmed for iodoperoxidases.

*Steady-state kinetics of the enzymatic reactions*

The steady-state kinetics of the iodoperoxidase catalysed reactions were studied using the three isoforms isolated from *S. polyschides* (Viana do Castelo). Steady state rates of $I_3^-$ formation were investigated as a function of hydrogen peroxide concentration (0.2–8 mM) and iodide concentration (2–25 mM) at pH 6.1.

For the three enzymes, plots of the steady state rates of $I_3^-$ formation vs hydrogen peroxide concentration

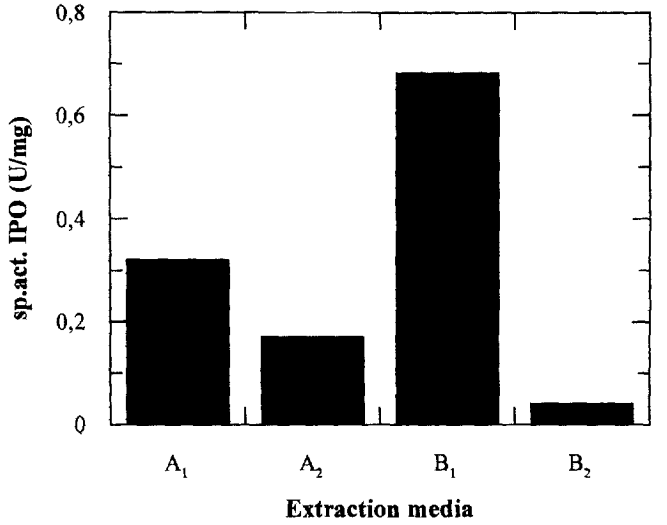


Fig. 2. Efficiency of the extraction of haloperoxidases in four different media: *Medium* $A_1$—0.2 M *tris*-$SO_4$ (pH 8.3); *Medium* $A_2$—0.08 M *tris*; *Medium* $B_1$—1.1 M $K_2CO_3$ (15% (w/v)) and 0.06 M PEG1550 (10% (w/v)); *Medium* $B_2$—1.1 M $K_2HPO_4$ (17.5% (w/v)) and 0.06 M PEG1550 (10% (w/v)). Activity was assayed as described in Experimental (iodoperoxidase).

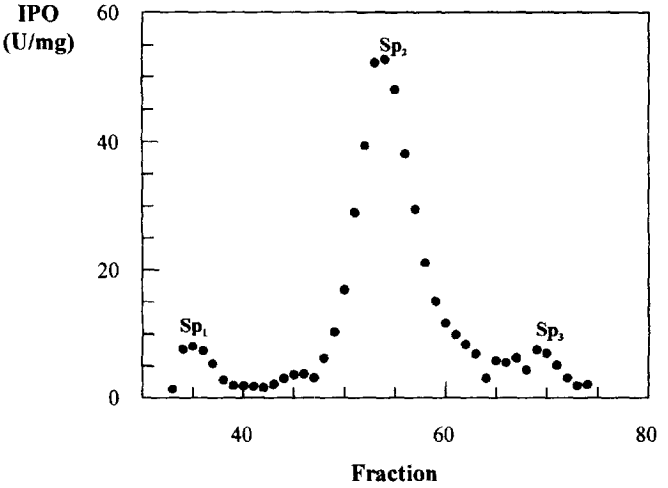


Fig. 3. Separation of the three iodoperoxidase isoenzymes on phenyl-sepharose. Conditions: The enzymes were applied to the column and equilibrated with 1.33 M $(NH_4)_2SO_4$ in 0.05 M *tris* (pH 9.0) and they were eluted with a descending linear gradient of 60% → 0% of the equilibrium solution.

fit a rectangular hyperbolic function satisfying the rate law for a Michaelis-Menten kinetic process. These results are shown in Fig. 7 (Lineweaver-Burk representation) for one of the three enzymes ($SpV_2$). From the slopes and intercepts of these plots, the various Michaelis-Menten constants for hydrogen peroxide ($K_M^{H_2O_2}$) and iodide ($K_M^{I^-}$) and the maximum velocity per mole of enzyme ($k_{cat}$) could be calculated (Table 4).

If we assume that the enzyme contains only one vanadium ion per enzyme molecule, as found in other vanadium haloperoxidases [33], then $k_{cat}$ is identical to the maximum turnover number. The isoforms $SpV_2$ and $SpV_3$ have a higher $k_{cat}$ than isoform $SpV_1$. $SpV_1$ and $SpV_2$ have an identical but slightly smaller $K_M$ for the $I^-$ substrate than $SpV_3$. The larger $K_M$ of $SpV_3$ for $I^-$ may be due to an active site which is more difficult to access. It should be noted that $SpV_3$ was reactivated more slowly than $SpV_1$ and $SpV_2$ (Fig. 5). Iodide is also an inhibitor of the reaction; this inhibition was noticed starting at 10 mM for $SpV_2$ and 15 mM for $SpV_1$ and $SpV_3$. As to $K_M^{H_2O_2}$ the three enzymes also show some differences, but now the $K_M$ of $SpV_3$ for this substrate is considerably smaller than that of $SpV_2$ or $SpV_1$ (which are similar). For the range of hydrogen peroxide concentrations examined (0.2–8 mM) no inhibition was observed.

## DISCUSSION

As described above, three different isoforms of vanadium dependent iodoperoxidases have been iso-

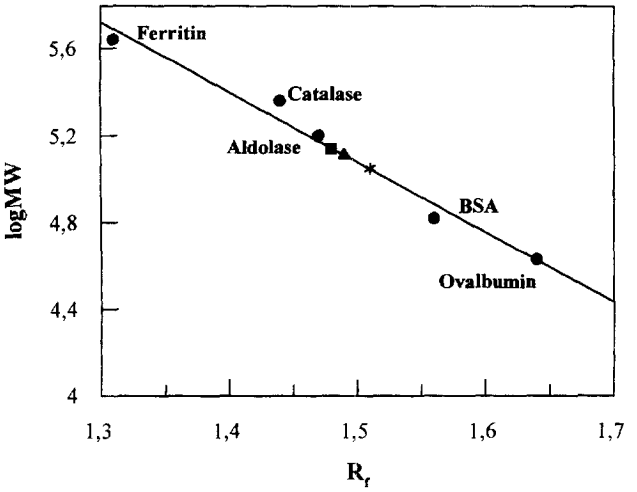


Fig. 4. Molecular mass of the enzymes as determined by gel exclusion HPLC. Conditions: Equilibration and elution solution: 0.3 M NaCl in 0.1 M *tris*-Cl (pH 7.0). ●, Standard proteins; ×, $Sp_1$; ▲, $Sp_2$; ■, $Sp_3$.

Table 1. Efficiency of the aqueous two-phase systems in extracting iodoperoxidase activity from *Saccorhiza polyschides* collected at Viana do Castelo (values as iodoperoxidase specific activity (U/mg) in the top-phase)

| | Station—Viana do Castelo | | | | | |
|---|---|---|---|---|---|---|
| % (w/v) $K_2CO_3$ | % (w/v) PEG 1500 | | | | | |
| | 10.0 | 12.5 | 15.0 | 17.5 | 20.0 | 22.5 |
| 15.0 | * | * | 0.564 | 0.558 | 0.674 | 0.528 |
| 17.5 | * | 0.704 | 0.844 | 1.062 | 1.025 | 0.771 |
| 20.0 | * | 1.297 | 2.167 | 1.621 | 1.524 | † |
| 22.5 | 0.783 | 0.898 | 0.947 | 0.722 | 0.589 | † |
| 25.0 | 0.686 | 0.595 | 0.558 | 0.528 | † | † |

* No formation of two aqueous phases.

† The two aqueous phases were difficult to separate due to the high mucilage content in the interphase.

Table 2. Specific activity of *Saccorhiza polyschides* iodoperoxidases isolated from the three locations

| | Volume (ml) | Total Units (U) | Units/ml | Protein (mg) | sp.act (U/mg)* |
|---|---|---|---|---|---|
| $SpA_1$ | 25 | 16 | 0.655 | 3.00 | 5.5 |
| $SpA_2$ | 75 | 158 | 2.112 | 6.17 | 25.7 |
| $SpA_3$ | 30 | 25 | 0.825 | 6.39 | 3.9 |
| $SpV_1$ | 13 | 25 | 1.907 | 3.14 | 7.9 |
| $SpV_2$ | 38 | 257 | 6.760 | 5.86 | 43.9 |
| $SpV_3$ | 28 | 54 | 1.917 | 9.95 | 5.4 |
| $SpP_1$ | 10 | 12 | 1.146 | 3.56 | 3.2 |
| $SpP_2$ | 45 | 114 | 2.526 | 6.44 | 17.7 |
| $SpP_3$ | 31 | 18 | 0.595 | 14.92 | 1.2 |

* As iodoperoxidase activity.

lated and characterised from *S. polyschides* collected at the different locations along the Portuguese western coast. These isoforms, from each collection site, exhibit differences in $M_r$ of the native enzymes, specific activity values, reactivation mode and kinetic properties. A vanadium-dependent iodoperoxidases was also detected in the brown alga *Phyllariopsis brevipes* collected at the Portuguese southern coast [34] as well as

Table 3. Reactivation of the apoiodoperoxidases with various metal ions (240 $\mu$M). Values are expressed as percentage of IPO specific activity before inactivation

| Enzyme | Fe(III) | Mo(VI) | Mn(II) | V(V) |
|---|---|---|---|---|
| $SpV_1$ | 2.1 | 1.9 | 12.5 | 131.1 |
| $SpV_2$ | 1.3 | 2.4 | 17.6 | 119.3 |
| $SpV_3$ | 2.9 | 3.7 | 10.1 | 116.0 |
| $SpA_1$ | 2.7 | 2.1 | 9.8 | 129.2 |
| $SpA_2$ | 1.7 | 3.1 | 20.1 | 108.6 |
| $SpA_3$ | 3.1 | 4.2 | 9.2 | 105.4 |
| $SpP_1$ | 2.5 | 1.6 | 10.2 | 120.2 |
| $SpP_2$ | 1.9 | 3.5 | 17.6 | 114.3 |
| $SpP_3$ | 2.6 | 4.2 | 9.5 | 109.7 |

in several other algae: *L. saccharina*, *L. hyperborea*, *A. n. lusitanica* and *Pelvetia canaliculata* [29]. For brown algae collected at northern Atlantic locations (France, The Netherlands, Great Britain, Scandinavia coasts), as well as at the Pacific ocean (California and Japan coasts) the presence of mainly vanadium-dependent bromoperoxidase activity was reported [26–28, 33, 35, 36]. These results suggest that bromo- or iodoperoxidase activity may depend on the latitude of the habitat of the algae. However, there may be other factors since California and Portugal have similar latitudes (but the sea water has different salinity [37], for example). It is also curious to note that vanadium-bromoperoxidases and iodoperoxidases are found mainly in organisms living in the marine environment, whereas vanadium-chloroperoxidases are found in

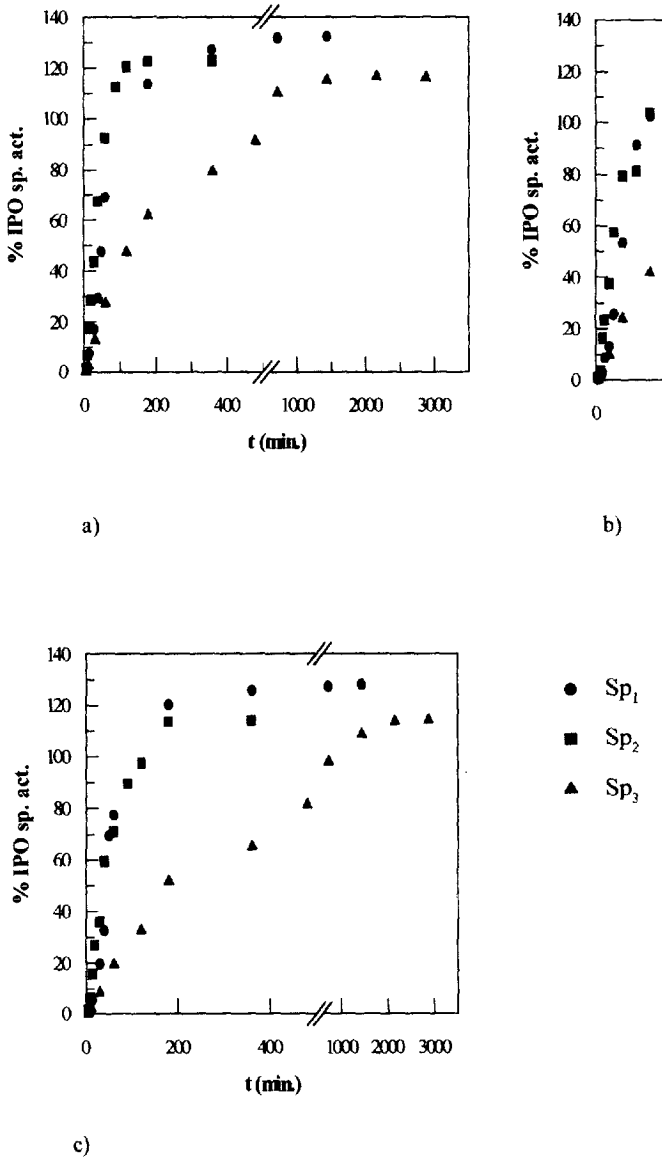


Fig. 5. Reactivation modes of the three enzymes with 240 $\mu$M $V^V$ in 0.05 M *tris*-Cl (pH = 9.0). Specific activity before inactivation was taken as 100%. (a) *Saccorhiza polyschides*—Viana do Castelo; (b) *Saccorhiza polyschides*— Arrábida; (c) *Saccorhiza polyschides*—Porto Côvo.

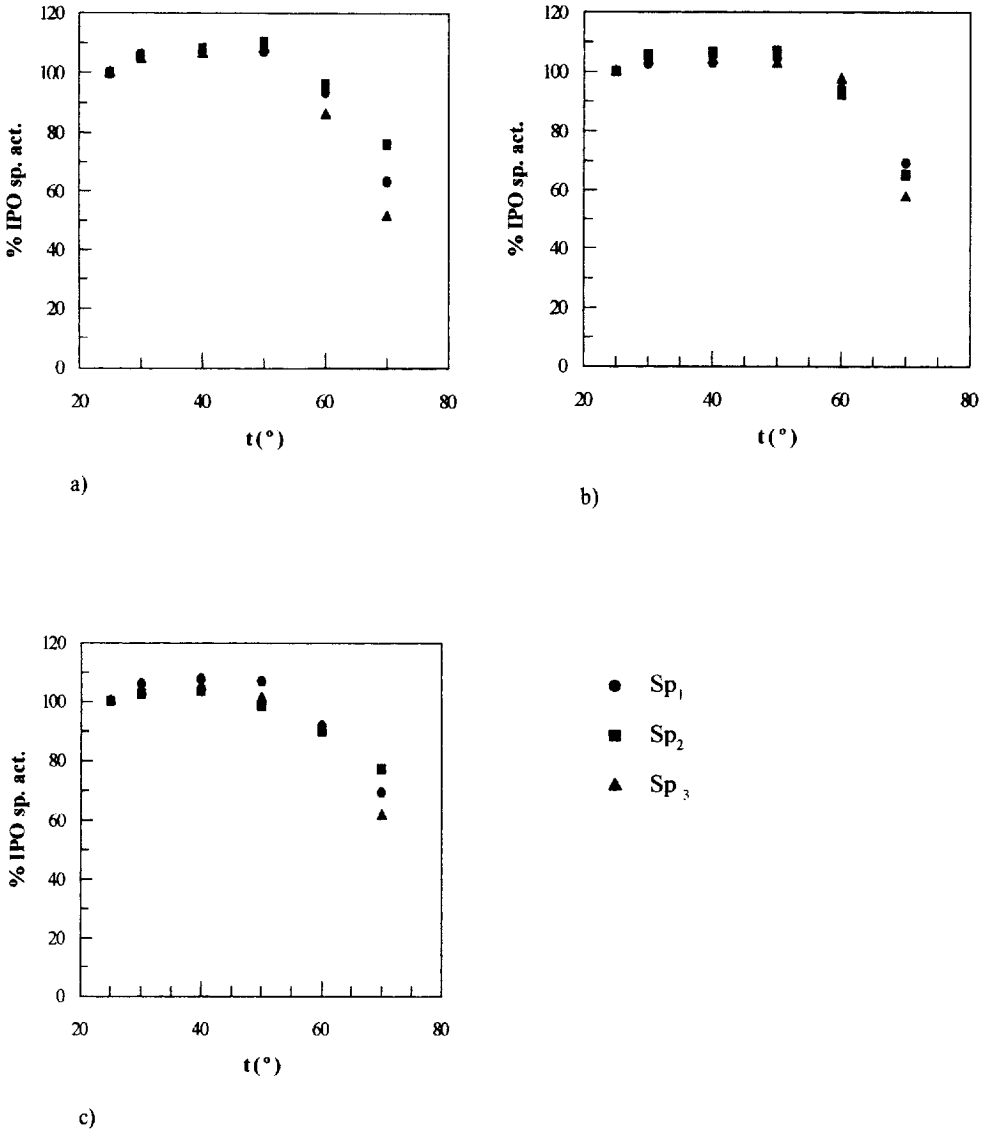


Fig. 6. Thermostability of the *Saccorhiza polyschides* iodoperoxidases. (a) *Saccorhiza polyschides*—Viana do Castelo; (b) *Saccorhiza polyschides*— Arrábida; (c) *Saccorhiza polyschides*—Porto Côvo.

terrestrial organisms (fungi) [2]. The different occurrences of haloperoxidases may be associated with the relative concentration of the halides in the local environment. The high concentration of chloride in the sea water may inhibit chloroperoxidases—this can be the reason for the absence of V-chloroperoxidases in marine organisms. This hypothesis is supported by the fact that high concentrations of bromide inhibit bromoperoxidases [38, 39] and higher concentration of iodide inhibit iodoperoxidases.

The existence of isoenzymes is a common phenomenon in plant peroxidases. These isoenzymes sometimes present several glycosylation patterns or even different amino acid composition which may result in different properties [40]. The observed fractionation of iodoperoxidases may derive from their surface properties, i.e. they may have a different protein architecture or a different glycosylation pattern. The first example of a brown seaweed containing two different vanadium bromoperoxidases was *A. nodosum* [27], although Vilter *et al.* [28] also detected isoforms of V-bromoperoxidase for the brown algae *L. digitata* and *L. saccharina*.

As in the case of *A. nodosum* isoenzymes [27], the three isoforms of *S. polyschides* differ somewhat in their $M_r$s (from 113 to 132 kDa), but the $M_r$ of the subunits is about the same (approx. 64 kDa). These results strongly suggest a dimeric structure for the native enzymes, which is in agreement with previous results obtained with several bromoperoxidases (Br PO) from brown and red algae [26]. However, as already mentionated, the isoform $Sp_3$ differs in several respects from $Sp_1$ and $Sp_2$. Different values of $K_M$ for the substrates and differences in the reactivation

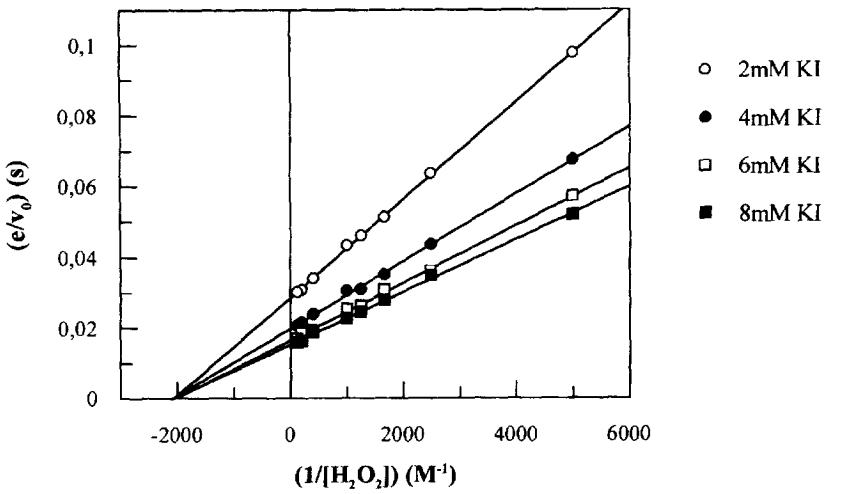


Fig. 7. Primary double-reciprocal plot of the rate of triiodide formation as a function of hydrogen peroxide concentration at fixed potassium iodide concentration for the $SpV_2$ enzyme at pH 6.1.

Table 4. Kinetic parameters for the reaction of $H_2O_2$ and iodide with enzymes at pH 6.1

| | $K_M^{I^-}$ (mM) | $K_M^{H_2O_2}$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) |
|---|---|---|---|
| $SpV_1$ | 3.25 | 478 | 16.2 |
| $SpV_2$ | 3.35 | 494 | 91.0 |
| $SpV_3$ | 5.74 | 84.0 | 91.5 |

process probably reflect an active site which is less accessible. That isoform $Sp_3$ is more strongly attached to the phenyl–sepharose column in the purification process suggests a more hydrophobic nature of the residues and/or a particular structural organisation of the $Sp_3$ isoform perhaps related to a particular action and/or localisation of this isoform in the seaweed.

Different values of haloperoxidase activity have been reported for the two enzymes (BrPO I and BrPO II) of *A. nodosum* (60 U/mg and 51 U/mg), and for the enzymes of *L. saccharina* (134 U/mg) and *Ceramium rubrum* (6.5 U/mg) [27, 41]. However, it should be noted that some of the values refer to the activity of the enzymes as isolated. Higher values are obtained from complete reactivation with vanadate. Substantially higher values (1730 and 1580 U/mg) have been reported for V-bromoperoxidases of the brown alga *Macrocystis pyrifera* and *Fucus distichus* [33], but in the same paper the value of 170 U/mg was reported for BrPO of *A. nodosum*. In the present work we have also observed for the $SpA_2$ a slight but reproducible bromoperoxidase activity (0.1 U/mg); nevertheless this enzyme is essentially an iodoperoxidase.

The specific IPO (iodoperoxidase) activity values for the three isoforms followed the order $Sp_2 \gg Sp_1 > Sp_3$, independently of the site where the seaweeds were collected (see Table 2). It is also interesting to note that, for the three different locations studied, the vanadium-dependent iodoperoxidase activity of the *S. polyschides* enzymes becomes lower as the latitude of the collecting site decreases (see Fig. 1 and Table 2). The reason for this fact is not clear; perhaps it is related to the increase in water temperature.

There is a wealth of information on the kinetic parameters for the reactions catalysed by V-BrPO from different sources [27]. BrPO I and BrPO II from *A. nodosum* have similar $K_M^{H_2O_2}$ (27 $\mu$M) as the BrPO from *L. saccharina*. The red seaweed *Ceramium rubrum* and *Corallina pilulifera* have $K_M$ values which clearly differ, but this may be related to differences in assay conditions. Considerably higher BrPO values have been reported for the lichen *Xanthoria parietina* (870 $\mu$M). Kinetic parameters for the iodoperoxidase catalysed reactions are scarce. The only reported value [42], for the $K_M^{I^-}$ is from *A. nodosum* (0.82 mM). Our results show that the enzymes isolated from *S. polyschides* have a larger $K_M$ for iodide than the enzyme isolated from *A. nodosum*. Also the $K_M$ for hydrogen peroxide is larger when compared with the haloperoxidases from other brown algae, see Table 4.

Presently we do not know which factors determine the specificity of the haloperoxidases in their ability to oxidise chloride, bromide or iodide. The primary structures of a bromoperoxidase and a chloroperoxidase are known and the tertiary structure of a vanadium chloroperoxidase has been determined at 2.1 Å resolution [24].

Sequence comparisons show that the residues involved in binding the vanadate are conserved, with the exception of a lysine residue in the chloroperoxidase which has been substituted by a less basic asparagine residue in the bromoperoxidase. No information is yet available on the primary structure of an iodoperoxidase. A comparison of the sequences may provide some clues to the specificity of the vanadium enzymes but it would not be surprising if the pref-

erence $I^- > Br^- > Cl^-$ (following the so-called Hofmeister lyotropic series of affinity) corresponded to decreasing basicity of residues near the active centre [43].

## EXPERIMENTAL

### *Seaweed collection sites*

The brown alga *S. polyschides* (Lightfoot) Batters was collected along the Portuguese west coast in three different locations: Arrábida, near Setúbal (38° 29'N; 8° 54'W) (20/7/92), Praia do Castelo do Neiva, near Viana do Castelo (41° 41'N; 8° 50'W) (18/9/93) and Porto Côvo, near Sines (37° 57'N; 8° 53'W) (22/9/94), Fig. 1.

### *Sample treatment*

After collection, the seaweeds were transported to the laboratory in cooled containers, extensively washed with distilled water and then stored frozen. *S. polyschides* collected at Arrábida was freeze dried for three days and then reduced to a fine powder using an ultracentrifuge mill. The other seaweed were frozen in liquid $N_2$ and then milled into small particles in a blender device.

### *Extraction and purification procedure*

Several pilot experiments were performed in order to determine the best extraction medium for the vanadium-dependent haloperoxidases. For *S. polyschides* collected in Arrábida, a ratio of 3 g of algal powder/100 ml of extract was used and four extraction media were tested with the following compositions: *Medium* $A_1$—0.2 M *tris*-$SO_4$ (pH 8.3) buffer. *Medium* $A_2$—0.08 M *tris Medium* $B_1$—1.1 M $K_2CO_3$ (15% (w/v)) and 0.06 M PEG 1550 (10% (w/v)). *Medium* $B_2$—1.1 M $K_2HPO_4$ (17.5% (w/v)) and 0.06 M PEG 1550 (10% (w/v)).

For *S. polyschides* collected at Viana do Castelo and Porto Côvo, a ratio of 72 g of fresh seaweed/100 ml of extract was used and only aq. two phase systems were tested with compositions of PEG 1500 varying from 10–22.5% (w/v) and 15–25% (w/v) in $K_2CO_3$.

After mixing the biological material with the extraction medium, the medium was stirred for 1 h. Then, the top phase was separated from the bottom phase and a mixture of 6% (w/v) $(NH_4)_2SO_4$ and three times its vol. of acetone was added to the top phase. The ppt formed was separated from the supernatant by centrifugation for 30 min at 12,000 *g*. The ppt was dissolved in 0.05 M *tris*-Cl (pH 9.0) buffer and dialysed overnight against the same buffer. After dialysis the extracts were subjected to a hydrophobic interaction chromatography step (with phenyl-sepharose CL, Pharmacia) and the haloperoxidases were eluted in the former column by a decreasing linear gradient of $(NH_4)_2SO_4$ from 60 to 0% in 0.05 M *tris*-Cl (pH 9). This was followed by a chromatofocusing step (with polybufferexchanger 94, Pharmacia), and eluted with polybuffer 74-HCl, pH 4, Pharmacia.

### *Haloperoxidase activity tests*

Iodoperoxidase activity was measured by following the conversion at 350 nm of $I^-$ into $I_3^-$ ($\varepsilon_M = 26{,}400$ $cm^{-1}\,M^{-1}$), in the presence of $H_2O_2$ [32]. The measurements were performed against an enzyme-free blank at 25°C and the enzyme activity was determined indirectly by measuring the consumption of $H_2O_2$. Three assays were performed for each determination and their average used in the calculations (where 1 μmol/min. of $H_2O_2$ consumed in the test was defined as 1U). The assay was carried out in buffer pH 6.2 (0.13 M $Na_2HPO_4$ and 0.04 M citric acid) and substrates (0.80 mM $H_2O_2$ and 6.1 mM KI). The vol. of the test sample was 3.3 ml. The bromoperoxidase activity was measured following the conversion of monochlorodimedone ($\varepsilon_M = 20.2$ $cm^{-1}$ $mM^{-1}$) into mono-chlorobromodimedone ($\varepsilon_M = 0.2$ $cm^{-1}$ $mM^{-1}$) at 290 nm [18, 44]. One unit of activity (1U) corresponds to 1 μmol of monochlorodimedone brominated/min. The assay mixture contained buffer pH 6.2 (0.13 M $Na_2HPO_4$ and 0.04 M citric acid, 0.2 M $K_2SO_4$) and substrates (50 μM monochlorodimedone, 0.1 M KBr and 2 mM $H_2O_2$). The vol. of the test sample was 3.3 ml. The solns of $H_2O_2$ were prepared by dilution of a 30% stock solution of Perhydrol (Merck) and their concn was determined at 240 nm ($\varepsilon_M = 43.6$ $cm^{-1}\,M^{-1}$) [45].

### *Protein content determination*

Protein content was determined by the method of Refs [45, 46] and by the method of Refs [47, 48] with BSA as standard, and by *A* readings at 280 and 260 nm [47].

### $M_r$ *determinations*

$M_r$ determinations of the native enzymes were performed in a Superdex 200 HR 10/30 column equilibrated and eluted by 0.3 M NaCl in 0.1 M *tris*-Cl (pH 7.0). A 150 μl aliquot was injected for each sample and the elution performed at 200 μl/min. The standard proteins used were: ferritin ($M_r = 440$ kDa), catalase ($M_r = 232$ kDa), aldolase ($M_r = 158$ kDa), BSA ($M_r = 67$ kDa) and ovalbumin ($M_r = 43$ kDa), all from Pharmacia. The column dead-vol. was determined with dextran blue.

SDS polyacrylamide gel electrophoresis was carried out on 10% gels according to Ref. [49]. Standard proteins used for $M_r$ determination were: phosphorylase b ($M_r = 94$ kDa), BSA ($M_r = 67$ kDa), ovalbumin ($M_r = 43$ kDa), carbonic anhydrase ($M_r = 30$ kDa), trypsin inhibitor ($M_r = 20.1$ kDa) and α-lactoalbumin ($M_r = 14.4$ kDa), all from Pharmacia.

*Reactivation studies*

The haloperoxidases were inactivated at low pH by extensive dialysis for 24 h against citrate-phosphate buffer at pH = 3.8 (0.071 M $Na_2HPO_4$ and 0.064 M citric acid) in the presence of 1 mM EDTA, followed by dialysis against 0.05 M *tris*-Cl buffer (pH 9.0) [17]. The reactivation studies with $Na_3VO_4$ were carried out in 0.05 M *tris*-Cl (pH 9.0). The final vanadium concn during reactivation was 240 $\mu$M. Reactivation studies were also performed with other metal ions, namely Fe(III) ($FeCl_3 \cdot 6H_2O$), Mo(VI) ($(NH_4)_6 Mo_7O_{24}$) and $Mn^{2+}$ ($MnCl_2 \cdot 4H_2O$).

*Thermal stability studies*

The purified enzymes were incubated in a thermostatted vessel for 1 h at temps ranging from 25 to 70°; aliquots were taken and assayed for activity.

*Studies of kinetics*

The steady-state kinetics experiments were done in 0.1 M 2-(*N*-morpholino)ethanesulphonic acid (MES) (pH 6.1) by measuring the initial rate of formation of $I_3^-$ by $H_2O_2$, in the presence of KI, whose concns varied between 2–25 mM. $Na_2SO_4$ was used to maintain a constant ionic strength of 0.2 M when the KI concn was varied. $H_2O_2$ concns varied between 0.2–8 mM. All measurements were performed at 25 ± 0.5°.

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## REFERENCES

1. Neidleman, S. L. and Geigert, J., in *Biohalogenation: Principles, Basic Roles and Applications*. Ellis Horwood Ltd, Chichester, 1986.
2. Vilter, H., in *Metal ions in Biological Systems—Vanadium and its role in life*, Vol. 31, ed. H. Sigel and A. Sigel. Marcel Dekker, New York, 1995, pp. 325–362.
3. Bolscher, B. G. J. M. and Wever, R., *Biochim Biophys. Acta*, 1984, **791**, 75–81.
4. Keilin, D. and Hartree, E. F., *Biochem. J.*, 1951, **49**, 88–104.
5. Baden, D. G. and Corbett, M. D., *Comp. Biochem. Physiol.*, 1979, **64B**, 279–283.
6. Hewson, W. D. and Hager, L. P., *J. Phycol.*, 1980, **16**, 340–345.
7. Theiler, R. F., Siuda, J. S. and Hager, L. P., in *Drugs and Food from the Sea. Myth or Reality*, ed. P. N. Kaul and C. J. Sinderman. The University of Oklahoma, Norman, Oklahoma, 1978, pp. 153–169.
8. Manthey, J. A. and Hager, L. P., *J. Biol. Chem.*, 1981, **256**, 11232–11238.
9. Yamada, H., Itoh, N., Murakami, S. and Izumi, Y., *Agric. Biol. Chem.*, 1985, **49**, 2961–2967.
10. Plat, H., Krenn, B. E. and Wever, R., *Biochem. J.*, 1987, **248**, 277–279.
11. Morris, D. R. and Hager, L. P., *J. Biol. Chem.*, 1996, **241**, 1763–1768.
12. Hunter-Cevera, J. C. and Sotos, L., *Microb. Ecol.*, 1986, **12**, 121–127.
13. Van Pée, K.-H., Lingens, F., *J. Gen. Microbiol.*, 1985, **131**, 1911–1916.
14. Wiesner, W., Van Pée, K.-H., Lingens, F., *Biol. Chem.*, 1985, **366**, 1085–1095.
15. Van Pée, K.-H. and Lingens, F., *J. Bact.*, 1985, **161**, 1171–1175.
16. Kirk, K. L., in *Biochemistry of the Elemental Halogens and Inorganic Halides*. Plenum Press, New York, 1991, 155–189.
17. Vilter, H., *Phytochemistry*, 1984, **23**(7), 1387–1390.
18. Wever, R., Plat, H. and de Boer, E., *Biochim. Biophys. Acta*, 1985, **830**, 181–186.
19. de Boer, E., Boon, K. and Wever, R., *Biochemistry*, 1988, **27**, 1629–1635.
20. Vilter, H. and Rehder, D., *Inorg. Chem. Acta*, 1987, **136**, L7–L10.
21. de Boer, E., Keijzers, C. P., Klaassen, A. A. R., Reijerse, E. J., Collison, D., Garner, C. D. and Wever, R., *FEBS Letters*, 1988, **235**, 93–97.
22. Arber, J. M., de Boer, E., Garner, C. D., Hasnain, S. S. and Wever, R., *Biochemistry*, 1989, **28**, 7968–7973.
23. Hormes, J., Kuetgens, U., Chauvistre, R., Schreiber, U., Anders, N., Vilter, H., Rehder, D. and Weidemann, C., *Biochim Biophys. Acta*, 1988, **956**, 293–299.
24. Simons, L. H., Barnett, P., Vollenbroek, E. G. M., van Schijndel, J. W. P. M., Dekker, H., Muijsers, A. O., Messerschmidt, A. and Wever, R., *Eur. J. Biochem.*, 1995, **229**, 566–574.
25. Messerschmidt, A. and Wever, R., *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 392–396.
26. Wever, R. and Krenn, B. E., in *Vanadium in Biological Systems—Physiology and Biochemistry*, ed. N. D. Chasteen. Kluwer Academic Publishers, 1990, pp. 81–98.
27. Krenn, B., Tromp, M. G. M. and Wever, R., *J. Biol. Chem.*, 1989, **264**, 19207–19292.
28. Jordan, P. and Vilter, H., *Biochim. Biophys. Acta*, 1991, **1073**, 98–106.
29. Almeida, M., Almeida, M. G., Humanes, M., Melo, R., Silva, J. A. and Fraústo da Silva, J. J. R., *Phycologia*, 1997, **36**, 1.
30. Ardré, F., *Portug. Acta Biol. (B)*, 1970, **10**, 137–532.
31. Vilter, H., *Bioseparation*, 1990, **1**, 283–292.
32. Vilter, H., *Bot. Mar.*, 1983, **26**, 331–340.
33. Soedjak, H. S., Butler, A., *Biochemistry*, 1990, **29**, 7974–7981.

34. Almeida, M., Humanes, M., Melo, R., Silva, J. A. L. and Fraústo da Silva, J. J. R., in *Plant Peroxidases—Biochemistry and Physiology*, ed. C. Obinger, U. Burner, R. Ebermann, C. Penel and H. Greppin. University of Geneva, 1996, 146–152.
35. Rush, C., Willets, A., Davies, G., Dauter, Z., Watson, H. and Littlechild, J., *FEBS Letters*, 1995, **359**, 244–246.
36. Wever, R., Tromp, M. G. M., van Schijndel, J. W. P. M., Vollenbroek, Olsen, R. L. and Fogelqvist, E., in *The Biogeochemistry of Global Change: Radiative Trace Gases*, ed. R. S. Oremland. Chapman and Hall, New York, 1992, 811–824.
37. Horne, R. A., in *Marine Chemistry*, Chap. 5. Wiley-Interscience, 1969.
38. Butler, A. and Walker, J. V., *Chem. Rev.*, 1993, **93**, 1937–1944.
39. de Boer, E. and Wever, R., *J. Biol. Chem.*, 1988, **263**, 12326–12332.
40. Gaspar, T., Penel, C., Thorpe, T. and Greppin, H., in *Peroxidases* 1970–1980—*A Survey of Their Biochemical and Physiological Roles in Higher Plants*. Université de Genéve, 1982, pp. 61–70.
41. de Boer, E., Tromp, M. G. M., Plat, H., Krenn, B. E. and Wever, R., *Biochim. Biophys. Acta*, 1986, **872**, 104–115.
42. Vilter, H., *Bot. Mar.*, 1983, **26**, 451–459.
43. Fraústo da Silva, J. J. R. and Williams, R. J. P., *Structure and Bonding*, 1976, **29**, 67–121.
44. Hager, L. P., Morris, D. R., Brown, F. S. and Eberwein, H., *J. Biol. Chem.*, 1966, **241**, 1769–1777.
45. Beers, R. F. and Sizer, I. W., *J. Biol. Chem.*, 1952, **195**, 133–140.
46. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, 1951, **193**, 275.
47. Copeland, R. A., in *Methods for Protein Analysis-Practical Guide to Laboratory Protocols*, Chapman and Hall, 1994.
48. Bradford, M. M., *Anal. Biochem.*, 1976, **72**, 248.
49. Laemmli, U. K., *Nature*, 1970, **227**, 680–685.