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Vanadium haloperoxidases from brown algae of the Laminariaceae family

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Abstract

Vanadium haloperoxidases were extracted, purified and characterized from three different species of Laminariaceae — *Laminaria saccharina* (Linné) Lamouroux, *Laminaria hyperborea* (Gunner) Foslie and *Laminaria ochroleuca* de la Pylaie. Two different forms of the vanadium haloperoxidases were purified from *L. saccharina* and *L. hyperborea* and one form from *L. ochroleuca* species. Reconstitution experiments in the presence of several metal ions showed that only vanadium(V) completely restored the enzymes activity. The stability of some enzymes in mixtures of buffer solution and several organic solvents such as acetone, ethanol, methanol and 1-propanol was noteworthy; for instance, after 30 days at least 40% of the initial activity for some isoforms remained in mixtures of 3:1 buffer solution/organic solvent. The enzymes were also moderately thermostable, keeping full activity up to 40°C. Some preliminary steady-state kinetic studies were performed and apparent Michaelis–Menten kinetic parameters were determined for the substrates iodide and hydrogen peroxide. Histochemical studies were also performed in fresh tissue sections from stipe and blade of *L. hyperborea* and *L. saccharina*, showing that haloperoxidase activity was concentrated in the external cortex near the cuticle, although some activity was also observed in the inner cortical region.

Keywords: *Laminaria saccharina*; *Laminaria hyperborea*; *Laminaria ochroleuca*; Laminariaceae; Vanadium in biology; Vanadium-dependent haloperoxidases

1. Introduction

The bioinorganic chemistry of vanadium has aroused much interest in recent years due to the curious roles and states of this metal in various lower organisms (see reviews by Chasteen, 1990; Wever and Kustin, 1990; Butler and Carrano, 1991; Redher, 1991; Sigel and Sigel, 1995; Tracey and Crans, 1998). One case which received more attention in the last few years is that of the vanadium haloperoxidases, a new class of enzymes that contain vanadium(V), as vanadate, in the active site and catalyse halogenation reactions of several substrates. These enzymes are found in many brown, in some red and in one green marine alga (Butler and Walker, 1993) and in the lichen *Xantoria parietina* (Plat et al., 1987). Recently, vanadium dependent haloperoxidases were also found in some fungi (van Schijndel et al., 1993; Barnett et al., 1998).

All the vanadium haloperoxidases isolated to date share some common features: they are composed of one or more subunits of relative molecular mass around 67 kDa; they can be inactivated by dialysis against EDTA at low pH; their activity is only restored by addition of vanadium (as vanadate) and they seem to have similar coordination of vanadium in the active site (Messerschmidt and Wever, 1996; Messerschmidt et al., 1997; Weyand et al., 1999).

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Vanadium haloperoxidases have been traditionally classified as chloro, bromo and iodoperoxidases. Vanadium chloroperoxidases have only been detected in terrestrial organisms, whereas bromo and iodoperoxidases are dominant in the marine environment.

Marine organisms, particularly seaweeds, produce large quantities of halometabolites, which are held within algal membrane bound vesicles and are thought to result from the catalytic activity of haloperoxidases. These halometabolites probably act as hormones or as repellents in biological defence mechanisms (Jordan and Vilter, 1991). Interestingly in this respect, thirty years ago, Siegel and Siegel (1970) proposed that an anomalous substrate specificity of haloperoxidases might be the reason why seaweeds are not lignificated. More recently, Küpper et al. (1998) reported that haloperoxidases might be involved in the uptake of iodide from seawater, a process that, according to these authors, may be related to conditions of oxidative stress. In the same year, ten Brink et al. (1998) reported that marine vanadium haloperoxidases were able to catalyse enantioselective sulfoxidation reactions. Despite these many studies, the reason for the selection of vanadium for these enzymes remains elusive since other more common biological metals, e.g. iron (heme) or manganese could also have been used (Fraústo da Silva and Williams, 1991).

The isolation of more than one form of vanadium haloperoxidase has been reported for *Ascophyllum nodosum* (Krenn et al., 1989). Recently, two papers from our laboratory were published showing that it is also possible to purify more than one form of the enzyme (Almeida et al., 1998; Almeida et al., 2000) from the brown seaweeds *Sacchoriza polyschides* and *Pelvetia canaliculata*. Histochemical tests are a very important control tool in this kind of studies, since contamination of the collected seaweeds with symbiont organisms living in and on the seaweeds is very common (Pedérsen and Fries, 1977). These controls were performed on *A. nodosum* and indicated that the enzyme activity was located on the surface of the cell wall of the thallus and inside the alga between the cortex and the medulla and especially around the conceptacles (Krenn et al., 1989). Similar experiments in *P. canaliculata* revealed that enzyme activity was located, on the surface cuticle of the thallus (Almeida et al., 2000).

Strangely, the seaweeds collected along the Portuguese coast, which were previously studied, exhibited lower haloperoxidase activities than most of the algae from northern regions of the Atlantic Ocean (Almeida et al., 1998; Almeida et al., 2000). To confirm if this is a general trend, which would suggest an environment dependence of the activity, we decided to carry out a comparative study of the properties of the haloperoxidases extracted from *L. saccharina, L. hyperborea* and *L. ochroleuca* since the Portuguese coast is the southern European habitat limit for these species.

2. Results and discussion

2.1. Extraction of the enzymes

The algae from the Laminariaceae family are extremely rich in alginates and polyphenolic compounds that complicate the extraction and purification of these proteins. An aqueous salt/polymer two-phase system was employed for the extraction. We have used polyethyleneglycol (PEG) and potassium carbonate in this system, since previous experience has shown that this salt is the most adequate for the extraction of this particular type of seaweed (Jordan and Vilter, 1991). From several compositions tested, the medium with 22.5% (w/v) K_2CO_3 and 15% (w/v) PEG 1500 in water was found to be the most efficient for the extraction of the haloperoxidases from these algae. The vanadium haloperoxidases (V-HPOs) were mainly found in the top-phase, bounded to the polymer (PEG); the bottom aqueous phase, rich in salts, accumulated most of the alginates and hydrophilic compounds. The separation of the enzyme from the polymer was easily achieved by the addition of acetone. After centrifugation the pellet thus obtained was dissolved in the minimum amount of 50 mM Tris–HCl (pH 9.0). Since the pH is quite high, a partial loss of the prosthetic group occurred; activity was fully restored by addition of vanadate (20 mM).

2.2. Purification of the enzyme — isoforms

The extracts from the three seaweeds were subjected to several chromatographic steps to purify the enzymes. The first stage was a hydrophobic interaction step with phenyl-sepharose. For *L. saccharina* and *L. ochroleuca* two sets of fractions with activity (named Ls1 and Ls2; Lo1 and Lo2, respectively) were obtained, whereas for the *L. hyperborea* extract only one set of active fractions was obtained. When subjected to electrophoresis under non-denaturing conditions all these fractions revealed several bands (results not shown); hence, a second chromatographic step was necessary.

Two different approaches were used for the second purification step; a further hydrophobic interaction chromatography with butyl-sepharose, for *L. saccharina* and *L. hyperborea,* and an ion exchange chromatography with DEAE-Sephacel for *L. ochroleuca*.

The active fraction from *L. hyperborea* yielded, after this second chromatographic step two active bands (named Lh1 and Lh2), while for *L. saccharina* this second step provided separation from most of the contaminants. A chromofocusing chromatography was used as the final purification step and yielded the two forms of the vanadium-haloperoxidases (V-HPO) from *L. saccharina* and *L. hyperborea* species. For *L. ochroleuca* the ion exchange chromatographic step with DEAE-Sephacel resolved the Lo2 fraction into a major fraction

(named α) and a minor fraction (named β). The Lo1 fraction lost its activity very rapidly and could not be reactivated.

Table 1 shows the specific activities of the purified forms from the three Laminariaceae studied. It is clear that the *L. saccharina* isoforms exhibit considerably higher iodoperoxidase (IPO) and bromoperoxidase (BrPO) activities than the isoforms from *L. hyperborea*. It is also interesting to notice that the two forms of the enzyme purified from this seaweed show different types of activity (Lh1 is a IPO while Lh2 is a BrPO). The *L. ochroleuca* fractions exhibited very low IPO activity only, so that we limited the study of the corresponding enzyme to the activity tests, molecular mass determination and reactivation with vanadium(V).

The IPO activity values found for the *L. saccharina* two isoforms of V-HPO (1067 and 320 U/mg) are comparable to the values obtained by Jordan and Vilter (1991) for the two V-HPO isoforms from *L. digitata*, a species commonly related to *L. saccharina* and *L. hyperborea* at higher latitudes (1140 and 797 U/mg, respectively). In a previous work, de Boer et al. (1986), purified only one enzyme form from *L. saccharina* using a classical extraction method. This enzyme had a specific activity in the bromination reaction of 608 U/mg after reactivation with vanadate, which is also much higher than the values obtained in the present study for the same kind of activity. This had already been observed for the haloperoxidase extracted from *P. canaliculata* and *S. polyschides* (Almeida et al., 1998; Almeida et al., 2000) and suggests a decreasing trend in enzymatic power of the vanadium haloperoxidases as one goes south in the habitat of the algae. If real, this trend may derive from specific conditions along the Portuguese coast, perhaps the higher temperature of the seawater, but this requires confirmation since the effect of this variable is not obvious. Another important observation is that the extraction procedure may determine the number of forms of the enzyme purified from these seaweeds. Using aqueous two-phase systems, which involve a delicate balance between hydrophobic and hydrophilic interactions, several isoforms can be separated. The classical extraction procedure used by de Boer et al. (1986) is not so selective; thus the fact that we have used the two-phase aqueous system may explain why we found two isoforms in *L. saccharina*. The reason for the existence of different forms of the enzyme in the same seaweed is not yet understood. It is possible that they have different functions and distribute differently, see below the histochemical tests. A more extensive study is required to clarify both of these aspects. It can not be excluded, however, that the life cycle of the seaweeds determines the production of isoforms since we have observed that for other specimens of *L. saccharina* collected at the same sites but in a different period of the year only one isoform was isolated although we have used the same extraction and purification procedures described in this work.

Table 1
IPO and BrPO specific activity values for the isoforms of V-HPO separated from *Laminara saccharina*, *Laminaria hyperborea* and *Laminaria ochroleuca*

Isoforms	IPO (U/mg)	BrPO (U/mg)
Ls_1	320	12
Ls_2	1067	32
Lh_1	131	–[a]
Lh_2	300	16
Lo1	(0.3)	–[a]
Lo2 (α)	16	–[a]
Lo2 (β)	9	–[a]

[a] The fractions did not show BrPO activity under the test conditions.

2.3. Determination of molecular mass

Each form of V-HPO purified from *L. saccharina* and *L. hyperborea* yielded only one major band on polyacrylamide gels under non-denaturating conditions; these bands stained both for protein and for HPO activity. Compared to standard proteins, these enzymes hardly migrate, indicating a high relative molecular mass, probably due to aggregation of the enzyme. Determination of the relative molecular mass for the enzymes from *L. saccharina* and *L. hyperborea* was difficult at first since in the presence of 0.1% SDS, chosen for this purpose, they were still very strongly aggregated. Subsequently, gel filtration on Sephacryl S-300R was successful, giving two bands, corresponding to relative molecular masses of 169 and 58 kDa (results not shown). The first band probably corresponds to an aggregated form of the enzyme (all peroxidases extracted from seaweeds aggregate easily) while the second band corresponds to the molecular mass of the subunit. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), the purified enzymes showed only one major band, with M_r around 67 kDa (Fig. 1). This is in agreement with the reported values for *L. saccharina* (64 and 66 kDa) subunits (de Boer et al., 1986). Recently, the molecular mass of the *A. nodosum* bromoperoxidase monomer was determined as 60 kDa (Weyand et al., 1999). For *L. ochroleuca* the relative molecular mass of fraction α was determined as 133 kDa on a non-denaturating polyacrylamide gel electrophoresis (Fig. 1) and 65 kDa on a SDS-denaturating polyacrylamide gel electrophoresis, which seem to indicate the presence of two subunits of identical molecular masses.

2.4. Inactivation and reactivation

By extensive dialysis with citrate–phosphate buffer in the presence of EDTA the enzymes were inactivated due to removal of the prosthetic group (Table 2). Some enzymes were easily deactivated, while for others deactivation was incomplete. It is also interesting to

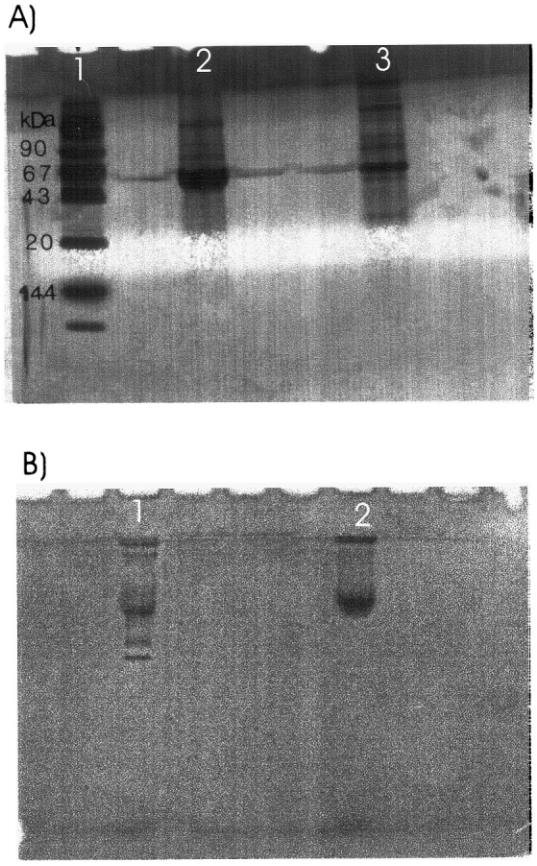


Fig. 1. SDS–PAGE 12.5% as laemmli of V-HPO: (A) V-HPO from *Laminaria saccharina* and *Laminaria hyperborea*: lane 1, standard proteins; lane 2, Ls1 enzyme; lane 3, Lh2 enzyme; (B) V-HPO from *Laminaria ochroleuca*: lane 1, standard proteins; lane 2, Lo2 (α) enzyme.

Table 2
Percentage of IPO specific activity of the isoforms of V-HPO, remaining after deactivation (specific IPO activity before the deactivation process was taken as 100%)[a]

Enzyme	% remaining IPO specific activity
Ls_1	1
Ls_2	9
Lh_1	5
Lh_2	3.5
Lo2 (α)	10
Phyllariopsis brevipes[b]	19

[a] For details see Experimental section.
[b] Almeida et al. (1996).

note the differences in deactivation between the two forms of the enzyme. For instance, Ls2 showed a higher resistance to deactivation than Ls1, whereas Lh1 is more resistant than Lh2. A similar behaviour has also been observed for the enzyme extracted from *P. canaliculata*, for which one of the forms showed a higher resistance to deactivation (Almeida et al., 2000). As Fig. 2 illustrates, the time required for complete reactivation of the enzymes was again different for each form of the enzyme. Since vanadium uptake (as vanadate) depends on the polypeptide chain and on the bonding of this species to the active site, the difference may be due to different conformations of the protein near the active site, which may alter the binding of vanadate. We note also, that the values of specific haloperoxidase activity were usually higher after reactivation with vanadate, which means that the vanadate group is not tightly bound at the active site and partial losses during the processes of purification may have occurred.

The effect of other metal ions, such as Mo(VI) (as molybdate), Mn(II) and Fe(III) on the reactivation of the apo-haloperoxidases was also tested; reactivation was always found to be less then 15% of that observed with vanadate.

2.5. Effect of organic solvents

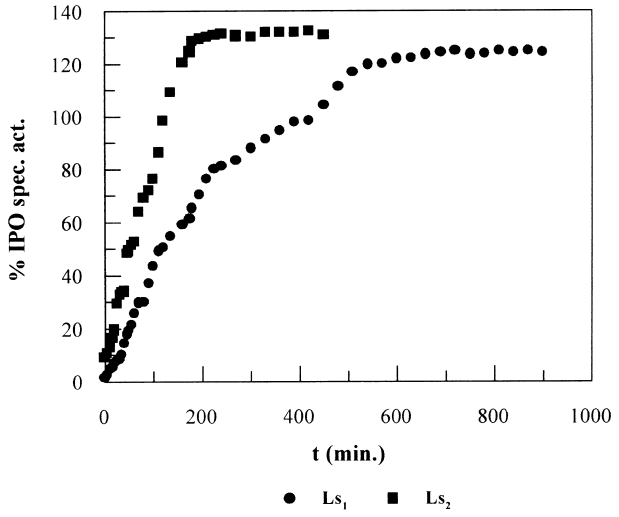
The long term effect of organic solvents on haloperoxidase activity is shown in Fig. 3. After 1 month incubation, the Ls1 form showed the highest activity in the mixtures with 25% of organic solvent (Fig. 3A), especially in the case of acetone when the activity was actually higher than in aqueous media; a possible explanation may be that the enzyme is aggregated in the buffer media and the presence of acetone could contribute to its desaggregation. For the other two forms (Ls2 and Lh2) appreciable activity was still observed after 1 month. For mixtures with 50% of organic solvent (Fig. 3B) the behaviour was quite different. The stability of Ls1 decreased significantly, whereas for Ls2 and Lh2 the decrease was not so pronounced.

It is also curious to notice that for mixtures with 50% organic solvent the forms Ls2 and Lh2 were more stable than Ls1, in clear contrast to what was observed in the mixtures with 25% organic solvent. Clearly, further work is needed to clarify these features of the vanadium haloperoxidases.

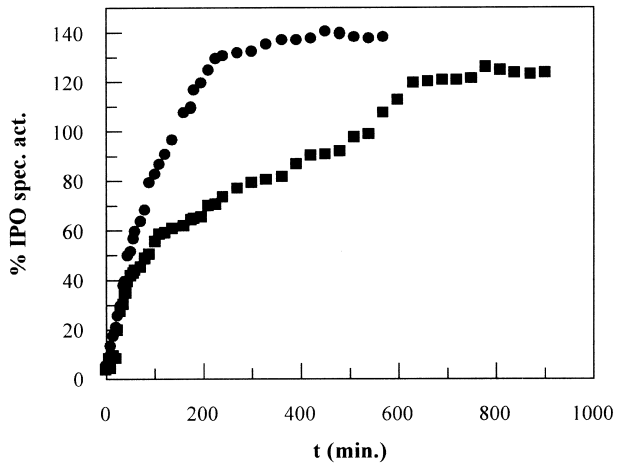
2.6. Thermal stability

The thermal stability of *L. saccharina* enzymes was also investigated. For temperatures in the range 25–50°C the enzymes studied were quite stable, catalysing with considerable efficiency the formation of triiodide (Fig. 4). An enhanced activity was shown by the Ls1 enzyme in the range 30–50°C, with a maximum at 40°C, contrasting the behaviour of Ls2 (Fig. 4). Above 50°C, the catalytic activity of both the Ls1 and Ls2 isoforms is drastically reduced. The thermal stability has also been studied for the haloperoxidases from the red algae *Corallina officinalis* (Sheffield et al., 1993), *Ceramium rubrum* (Krenn et al., 1987), the brown algae *Phyllariopsis brevipes* (Almeida et al., 1996) and *A. nodosum* (Tromp et al., 1989), and the lichen *Xantoria parietina* (Plat et al., 1987). These studies indicate that the thermal stability

A) *L. saccharina*



B) *L. hyperborea*



C) *L. ochroleuca* (Lo2(α))

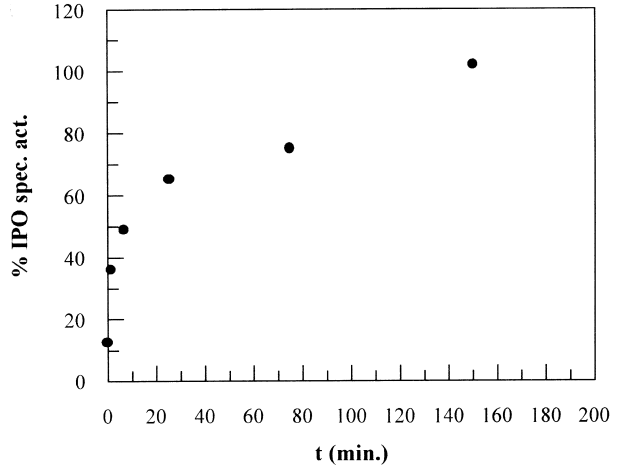


Fig. 2. Reactivaction with V(V) of the enzyme forms purified from *Laminaria saccharina, Laminaria hyperborea* and *Laminaria ochroleuca*. The values of IPO specific activity values the deactivaction/reactivaction process were taken as 100%. The values presented (% IPO specific activity) correspond to a percentage of those initial values.

of V-HPO is generally higher for the lichen comparatively to the seaweeds. This fact is probably related to the nature of the habitat, since *Xanthoria parietina* lives in the terrestrial environment, subject to larger temperature variation while seaweeds live in a more stable marine environment.

2.7. Steady-state kinetics

Some preliminary steady-state kinetic studies were performed by measuring the initial rate of I_3^- formation, catalysed by these enzymes, following the experimental conditions described in the Experimental section for the two substrates used (iodide and hydrogen peroxide) and for three pH values. Values of the apparent Michaelis–Menten constants for the two substrates and the values of maximum rate (V_{max}) were obtained and are presented in Table 3.

This table shows that there are no significant differences between kinetic parameters for the enzymes studied in this work. All the enzyme forms show a V_{max} at pH 6.1.

The apparent Michaelis–Menten constant for the substrate iodide ($K_m^{app\ I-}$) decreased slightly with the decrease of pH, showing that the affinity for iodide decreases when the pH increases. Previous studies on the bromoperoxidases from other seaweeds (de Boer and Wever, 1988; Everett and Butler, 1989; Soedjack and Butler, 1991) gave analogous results. It was not possible to increase the pH further since the non-catalysed reaction between H_2O_2 and I^- interferes strongly with the assay (Vilter, 1995).

The binding of hydrogen peroxide is affected in a different way; since the values of the apparent Michaelis–Menten constant for the substrate hydrogen peroxide ($K_m^{app\ H_2O_2}$) decreased with the increase in pH, this implies that the affinity of hydrogen peroxide for the enzyme increases with the increase of pH. Probably, protonation of a group at lower pH values, possibly a histidine side chain, prevents the reaction with peroxide. Studies carried out with the bromoperoxidase extracted from *A. nodosum* (de Boer and Wever, 1988) did indeed indicate the presence of a group, probably a histidine residue, with a pK_a of 5.7, which may be responsible for the pH dependence of the catalytical process. In fact, it was found for other peroxidases that a histidine with a pK_a between 4 and 6 could control the binding of peroxide to the active center, reflecting the importance of the polypeptide chain on activity (de Boer and Wever, 1988). Recently, the detailed role of a particular histidine residue (His 496) in the catalytic behaviour of haloperoxidases was discussed (Renirie et al., 2000).

2.8. Histochemical tests

From the observation of *L. saccharina* and *L. hyperborea* stained sections, it is possible to confirm that the

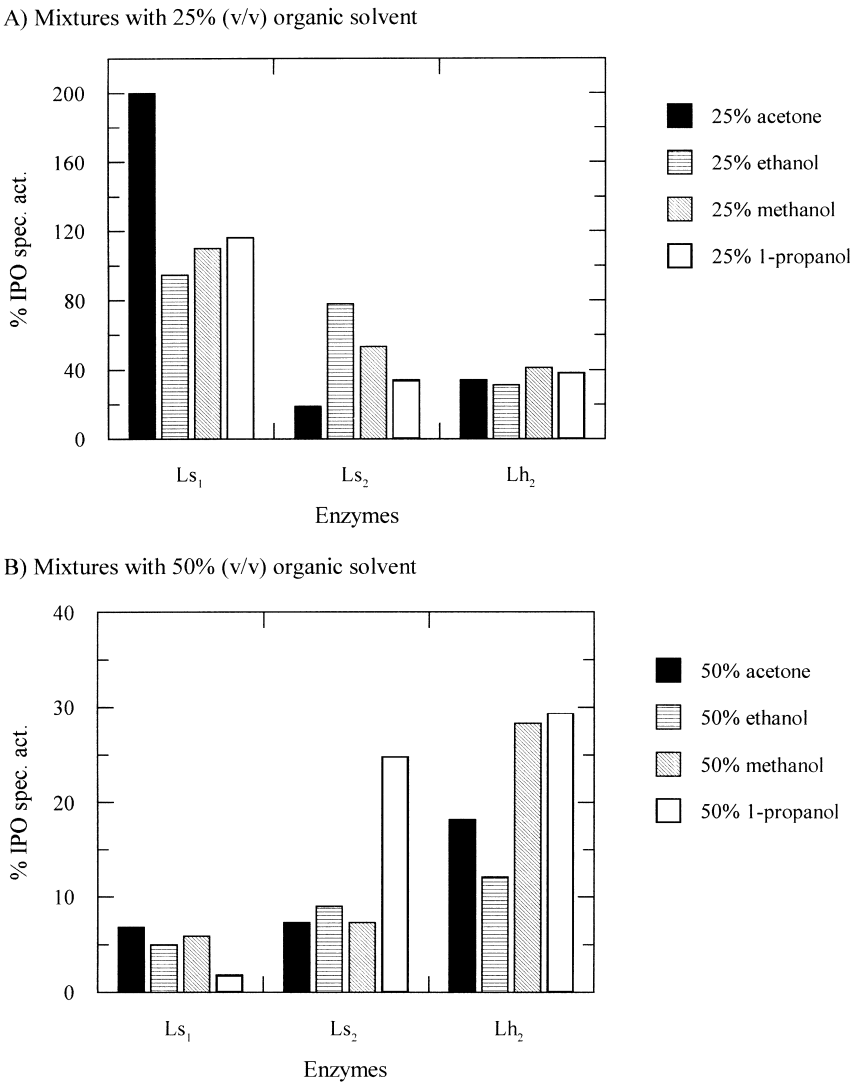


Fig. 3. Effect of buffer solution/organic solvent mixtures on IPO activity of the enzymes after 30 days incubation. IPO specific activity before mixture with organic solvent was taken as 100% (see Experimental section for details).

Table 3
Apparent kinetic parameters ($K_m^{app\ I-}$ and $K_m^{app\ H_2O_2}$) of the vanadium haloperoxidases from *Laminaria saccharina* and *Laminaria hyperborea*

pH	Enzyme	$K_m^{app\ I-}$ (mM)	$K_m^{app\ H_2O_2}$ (μM)	$V_{max}\times10^{-7}$ (M s^{-1})
5.5	Ls_1	1.3	376	7.6
	Ls_2	1.9	333	11.2
	Lh_1	1.9	334	9.8
	Lh_2	2.3	285	12.1
6.1	Ls_1	3.4	243	9.1
	Ls_2	2.7	273	12.1
	Lh_1	3.4	275	16.0
	Lh_2	3.8	217	17.5
6.7	Ls_1	3.7	120	8.3
	Ls_2	4.3	137	7.1
	Lh_1	3.5	166	9.4
	Lh_2	3.8	173	10.8

haloperoxidases are located, near the cuticle as well as in the external cortex region of the thallus (Fig. 5). However, the *L. hyperborea* sections exhibited also strong haloperoxidase activity around mucilaginous channels. The inner cortex region shows the presence of haloperoxidase activity for *L. saccharina*, absent in *L. hyperborea*. Some residual haloperoxidase activity was also observed in the medullar region of *L. hyperborea*. These results are in agreement with those of Krenn et al. (1989) and Almeida et al. (2000).

3. Experimental

3.1. Collection of algae

All the seaweeds were collected at low tide from the Portuguese west coast, in the northern part of the

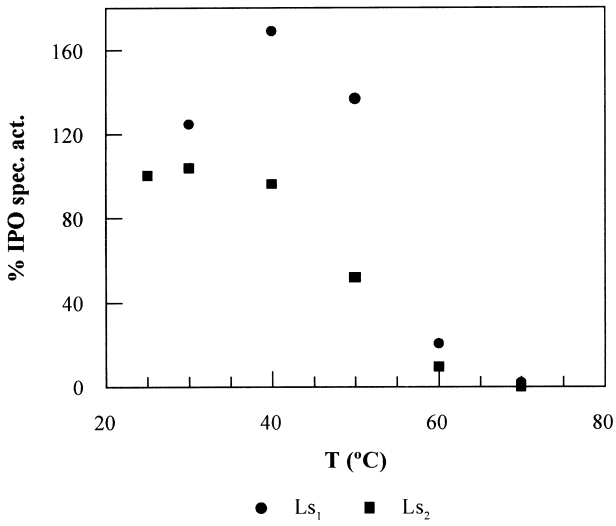


Fig. 4. The effect of temperature on the IPO activity for the two isoforms of V-HPO isolated from *Laminaria saccharina*. IPO specific activity before thermal treatment was taken as 100%.

country, near Viana do Castelo, at the end of summer (*L. hyperborea* and *L. saccharina*) and in spring (*L. saccharina* and *L. ochroleuca*). After collection, the algae were transported to the laboratory, thoroughly washed with distilled water, chopped and stored frozen until required.

3.2. Extraction and purification

For the extraction of HPO, two-phase aqueous systems, formed with an aqueous solution of PEG 1500 and an aqueous solution of potassium carbonate, were used. For each species, the process was optimised in order to select the most effective extraction media. In all cases, the media with 22.5% (w/v) K_2CO_3 and 15% (w/v) PEG 1500 proved to be the best.

After centrifugation for 30 min at 5000 *g* the upper phase was collected and the proteins precipitated by adding an equal volume of acetone. After a second centrifugation (30 min at 10,000 *g*) the resulting pellet was collected and resuspended in 50 mM Tris–HCl (pH

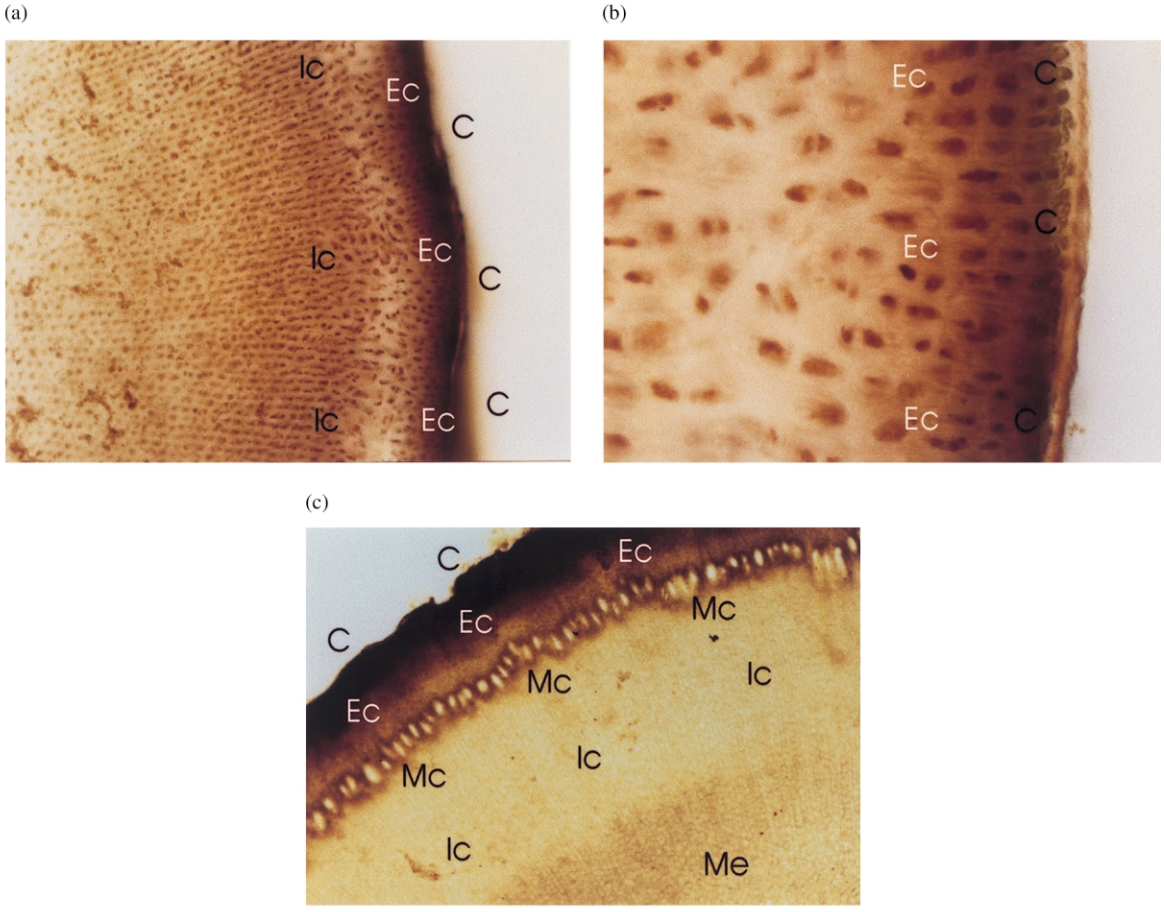


Fig. 5. Histochemical staining of fresh tissue samples of *Laminaria saccharina* and *Laminaria hyperborea*: (a) *Laminaria saccharina* (amplification: 10×); (b) *Laminaria saccharina* (amplification: 50×); (c) *L. hyperborea* (amplification: 10×); (C, cuticle; Ec, external cortex; Mc, mucilaginous channels; Ic, internal cortex; Me, medulla).

9.0) buffer. In order to reactivate the HPO, the extract was dialysed overnight against 20 mM $NaVO_3$ in 50 mM Tris–HCl (pH 9.0).

The sample was then loaded onto a hydrophobic interaction column (phenyl-sepharose from Pharmacia) and eluted with a decreasing linear gradient (1.3–0 M) of $(NH_4)_2SO_4$ in 50 mM Tris–HCl (pH 9.0). Resultant samples were then applied to another hydrophobic interaction column (butyl-sepharose from Pharmacia) and eluted also with a decreasing linear gradient (1.3–0 M) of $(NH_4)_2SO_4$ in 50 mM Tris–HCl (pH 9.0) or alternatively (*L. ochroleuca*) applied to an anionic exchange column (DEAE-Sephacel, from Pharmacia) and eluted with an increasing linear gradient of 0–1.3 M of $(NH_4)_2SO_4$ in 0.2 M Tris–Cl (pH 9.0). For the chromofocusing chromatography step (polybuffer exchanger-PBE 94, from Pharmacia) the samples were eluted with Polybuffer 74-imidazole (pH 4.0) also from Pharmacia.

3.3. Determination of protein content

Protein was determined by Bradford's method (Bradford, 1976), using bovine serum albumin as standard (Sigma).

3.4. Molecular mass determination and electrophoresis

The relative molecular mass was determined by FPLC/gel filtration chromatography on Sephacryl S-300R (Pharmacia) with a mobile phase of 0.5 M NaCl in 50 mM Tris–HCl (pH 9.0). Standard proteins from Pharmacia (ribonuclease A, M_r 13.7 kDa; chymotrypsinogen, M_r 25 kDa; ovalbumin, M_r 43 kDa; aldolase, M_r 158 kDa; and catalase, M_r 232 kDa) were used for the calibration of the column. SDS polyacrylamide gel electrophoresis was carried out on 12.5% gels according to Laemmli (1970). Standard proteins used for molecular mass determination were: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactoalbumin (14.4 kDa), all from Pharmacia.

3.5. Reactivation studies

The HPO were inactivated at low pH by extensive dialysis for 24–72 h against a citrate–phosphate buffer at pH 3.8, in the presence of 1 mM EDTA, followed by dialysis against 50 mM Tris–HCl (pH 9.0). Reactivation studies with Na_3VO_4 were carried out in 50 mM Tris–HCl (pH 9.0). The final vanadium concentration for reactivation was 240 μM. The same conditions were used in the reactivation studies with other inorganic salts (ferric chloride, ammonium molybdate and manganese chloride).

3.6. Operational stability in organic solvents

The effect of organic solvents upon the haloperoxidase activity was determined by incubation of the enzyme with 25 and 50% (v/v) acetone, ethanol, methanol and 1-propanol mixed with 50 mM Tris–Cl pH 9.0, for 30 days at room temperature. The vials were tightly capped and kept in the dark. Each incubation was carried out in duplicate.

3.7. Thermal stability studies

The purified enzymes were incubated in a thermostated vessel for one hour at temperatures ranging from 25 to 70°C. Activity was determined after subsequent equilibration at room temperature.

3.8. Activity determinations

Activity as iodoperoxidase (IPO) was measured by following the conversion of I^- to I_3^- at 350 nm ($\varepsilon_M = 26400\ M^{-1}\ cm^{-1}$) using H_2O_2 as the electron acceptor (Björkstén, 1968). The H_2O_2 solutions were prepared by dilution of a 30% stock solution of Perhydrol (Merck) and their concentration was determined spectrophotometrically at 240 nm ($\varepsilon_M = 43.6\ M^{-1}\ cm^{-1}$). Bromination activity was measured spectrophotometrically at 290 nm in an assay system containing 50 μM monochlorodimedone ($\varepsilon_M = 20.2\ mM^{-1}\ cm^{-1}$), 2 mM H_2O_2, 100 mM KBr, in 100 mM phosphate buffer (pH 6.5) (Wever et al., 1985). Activity in native electrophoresis gels was detected by incubation of the gel in *o*-dianisidine, iodide and hydrogen peroxide (Vilter, 1981).

3.9. Steady-state kinetic experiments

These experiments were carried out in 0.1 M 2-(morpholino)ethanesulphonic acid (MES) (pH 5.5, 6.1 and 6.7) by measuring the oxidation of I^- by H_2O_2 to I_3^-. For each pH value the initial rate of formation of I_3^- was determined at a constant concentration of hydrogen peroxide (0.8 mM) and the potassium iodide concentration was varied between 0.2 and 10 mM. Alternatively, a constant concentration of potassium iodide (6 mM) was used and the hydrogen peroxide concentration was varied between 0.2 and 1.2 mM. Sodium sulphate was added to keep a constant ionic strength of 0.2 M when the potassium iodide concentration was varied. All measurements were performed at 25.0±0.5°C.

3.10. Steady-state kinetic analysis

The initial rates, v_0 plotted as a function of hydrogen peroxide or iodide concentration were fit to a Michaelis–Menten equation. The apparent kinetic parameters $K_m^{app\ I-}$ and $K_m^{app\ H_2O_2}$ as well as V_{max} were obtained from primary double reciprocal plots.

3.11. Histochemical studies

Tissue samples of fresh seaweeds cut with a blade razor, were immersed in filtered natural seawater followed by incubation with the haloperoxidase activity test solution, containing of 0.19 mM *o*-dianisidine, 6.06 mM KI and 0.8 mM H_2O_2 in distilled water.

After incubation, the tissue samples were washed with distilled water and observed in the light microscope.

The presence of haloperoxidase activity was ascertained from the development of a dark reddish brown colour due to *o*-dianisidine oxidation assisted by iodide. Control tests were performed for each tested tissue sample.

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