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The Brown Algal Kelp *Laminaria digitata* Features Distinct Bromoperoxidase and Iodoperoxidase Activities*

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Different haloperoxidases, one specific for the oxidation of iodide and another that can oxidize both iodide and bromide, were separated from the sporophytes of the brown alga *Laminaria digitata* and purified to electrophoretic homogeneity. The iodoperoxidase activity was approximately seven times more efficient than the bromoperoxidase fraction in the oxidation of iodide. The two enzymes were markedly different in their molecular masses, trypsin digestion profiles, and immunological characteristics. Also, in contrast to the iodoperoxidase, bromoperoxidases were present in the form of multimeric aggregates of near-identical proteins. Two full-length haloperoxidase cDNAs were isolated from *L. digitata*, using haloperoxidase partial cDNAs that had been identified previously in an Expressed Sequence Tag analysis of the life cycle of this species (1). Sequence comparisons, mass spectrometry, and immunological analyses of the purified bromoperoxidase, as well as the activity of the protein expressed in *Escherichia coli*, all indicate that these almost identical cDNAs encode bromoperoxidases. Haloperoxidases form a large multigenic family in *L. digitata*, and the potential functions of haloperoxidases in this kelp are discussed.

Brown algae from the order Laminariales (kelps) are characterized by a heteromorphic haplodiplophasic life cycle alternating between a microscopic filamentous gametophyte and a macroscopic sporophyte, which can reach several meters in length depending of the species. Kelps' sporophytes accumulate iodine to more than 30,000 times the concentration of this element in seawater, up to levels as high as 1% of dry weight (*e.g.* see Refs. 2 and 3). Not much is known, however, on the iodine-concentrating mechanisms and on the biological functions of iodine in these kelps and other marine plants. Only one aspect of halogen metabolism, the production of volatile halocarbons, has attracted attention, because these compounds, and in particular the iodinated forms, have a significant impact on the chemistry of atmosphere (4, 5). In the biology of marine algae, volatile halocarbons are viewed as defense metabolites, *i.e.* products of the scavenging of activated oxygen species and/or potent biocides (6–10).

Halogen uptake (3) and the production of halo-organic compounds (6, 11) by marine algae are thought to involve vanadium-dependent haloperoxidases. Haloperoxidases catalyze the oxidation of halides, and they are named according to the most electronegative halide that they can oxidize; chloroperoxidases can catalyze the oxidation of chloride, as well as of bromide and iodide, bromoperoxidases (BPOs)[1] react with bromide and iodide, whereas iodoperoxidases (IPOs) are specific of iodide. The ability of vanadium-dependent haloperoxidases to halogenate a broad range of organic compounds of both commercial and pharmaceutical interest, as well as their high stability toward high temperatures, oxidative conditions, and in the presence of organic solvents, makes them good candidates for use in industrial biotransformations (12, 13). These properties have elicited detailed structural and mechanistic studies on several vanadium-dependent haloperoxidases, namely the chloroperoxidase from the fungus *Curvularia inaequalis* (14) and the bromoperoxidases from the red algae *Corallina pilulifera* (15, 16) and *Corallina officinalis* (17, 18) or from the fucalean brown algae *Fucus distichus* (19) and *Ascophyllum nodosum* (20).

Various vanadium-dependent haloperoxidase isoforms have been described in Laminariales (21–28), but nothing is known on the structure of these haloperoxidases. To further understand the biochemical and biological functions of haloperoxidases in Laminariales, we now have undertaken the purification and molecular characterization of these enzymes. We show here that *Laminaria digitata* sporophytes feature distinct iodoperoxidase and bromoperoxidase activities and that the bromoperoxidases consist of near-identical proteins on the form of multimeric aggregates. From partial cDNAs displaying homologies with the *F. distichus* vBPO, which were identified in an Expressed Sequence Tag analysis of the life cycle of *L. digitata* (1), we have isolated two full-length haloperoxidase cDNAs from the sporophyte of this species. Sequence comparisons, mass spectrometry, and immunological analyses of the purified bromoperoxidase, as well as the activity of the protein expressed in *Escherichia coli*, all indicate that these almost identical cDNAs encode bromoperoxidases. In addition peptide sequencing and Western blotting show that the primary structure of iodoperoxidase markedly departs from that of bromoperoxidase.

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The nucleotide sequence(s) reported in this paper has been submitted to the EBI Data Bank with accession number(s) AJ491786 and AJ491787.

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[1] The abbreviations used are: BPO, bromoperoxidase; vBPO, vanadium-dependent bromoperoxidase; IPO, iodoperoxidase; MS, mass spectrometry; MS/MS, tandem MS; LC, liquid chromatography; EST, expressed sequence tag; UTR, untranslated region.

EXPERIMENTAL PROCEDURES

Plant Material—L. digitata young sporophytes (blade length less than 25 cm) were collected from the shore in the vicinity of Roscoff (Brittany, France) and maintained under a 12:12 light-dark cycle in running seawater at 15 °C or immediately frozen in liquid nitrogen and kept at −80 °C. Gametophyte cultures were established and maintained as described previously (29).

Purification of Vanadium-dependent Haloperoxidases—As the algae from the Laminariaceae family are extremely rich in alginates and polyphenolic compounds, an aqueous salt/polymer two-phase system developed by Vilter (30) was used for the extraction of haloperoxidases. Briefly, 120 g of *L. digitata* sporophytes were powdered in liquid nitrogen and extracted using 20% (w/v) K_2CO_3 (100 ml per aliquots of 20-g fresh weight) and 13% (w/v) polyethylene glycol (1550 Da). The two phases were separated by centrifugation at 5,000 × *g* for 15 min, and 6% (w/v) $(NH_4)_2SO_4$ and 3 volumes of acetone were added to the top phase. After 1 h at −20 °C, the protein extract was pelleted by centrifugation at 10,000 × *g* for 30 min, dissolved in 50 mM Tris-HCl, pH 9.0, buffer, and dialyzed overnight against the same buffer. The extract was then loaded on a phenyl-Sepharose CL4B hydrophobic interaction column, equilibrated with 30% $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 9.0. Proteins were eluted by a decreasing linear gradient down to salt-free Tris buffer. The haloperoxidase active fractions were pooled, dialyzed against 50 mM Tris-HCl, pH 9.0, and concentrated by filtration (YM-10; Millipore) on stirred ultracentrifugation cells (model 8200; Millipore). For the final purification of native enzymes, semi-preparative electrophoresis (model 491 Prep Cell; Bio-Rad) or electroelution (model 422 Electro-eluter; Bio-Rad) was used according to the manufacturer's recommendations.

Proteins of *L. digitata* gametophytes were extracted in 50 mM Tris-HCl, pH 9.0, 15 mM $MgCl_2$, 2 mM dithiothreitol buffer, containing 0.5% polyvinylpolypyrolidone and 0.5% Igepal CA-630 (Sigma-Aldrich). After sonication (three times, 30 s), the pellet was discarded by centrifugation at 10,000 × *g* for 10 min, and the protein extract was recovered from the supernatant.

Protein concentrations were determined using the Bio-Rad Coomassie protein assay (31) with bovine serum albumin as standard (Sigma-Aldrich), and the holoenzymes were re-constituted by adding 2 mM $NaVO_3$. Gel electrophoresis was carried out using 9% polyacrylamide slab gels according to Laemmli (32) and using protein molecular mass standards (Bio-Rad). SDS samples were denatured by boiling at 100 °C for 10 min.

Enzyme Activity Assays—Haloperoxidase activities were detected on non-denaturing gels, soaked with 100 mM potassium phosphate buffer, pH 7.4, in the presence of 0.1 mM *o*-dianisidine, 0.45 mM H_2O_2, and 10 mM potassium iodide, potassium bromide, or potassium chloride for revealing iodoperoxidase, bromoperoxidase, or chloroperoxidase activities, respectively. To detect haloperoxidase activity on denaturating gels, SDS was removed by washing the gel in Tris-glycine buffer containing 0.1% Igepal CA-630 (Sigma-Aldrich).

Bromoperoxidase activity was measured spectrophotometrically by monitoring at 290 nm the conversion of monochlorodimedone (20.0/cm/mM) into monochlorobromodimedone (0.2/cm/mM) at pH 6.1 (33). Specific activities are expressed in units per milligram of protein, where one unit of bromoperoxidase activity is defined as the amount required for brominating 1 μmol of monochlorodimedone per min. Iodoperoxidase activity was recorded at pH 6.2 following the conversion of iodide into triiodide (26.4/cm/mM) at 350 nm (34). The differences between the non-enzymatic and the non-enzymatic-plus-enzyme reactions were calculated for each sample. The specific activity is expressed in units per milligram of protein, where one unit of iodoperoxidase activity is defined as the amount required for consuming 1 μmol of H_2O_2 per min. For optimal pH determination, iodoperoxidase specific activities were measured in the same conditions at 20 °C, using the following buffers: 0.1 M sodium acetate, pH 4.0–5.5, or 0.1 M MES, pH 5.5–6.8. For thermostability studies, proteins were maintained at the appropriate temperature for 10 min before the iodoperoxidase assay.

Molecular Weight Determination of the Native Enzymes—Using non-denaturating gel electrophoresis, protein samples were run under a range of acrylamide concentrations from 4 to 14% (35). A molecular size standard curve was established using standard proteins (albumin, aldolase, catalase, ferritin, and urease from Amersham Biosciences), which were electrophoresed under the same conditions. The relative molecular mass of native proteins was also determined by fast protein liquid chromatography/gel filtration chromatography (Superdex 200 HR 10/30 column; Amersham Biosciences) with a mobile phase consisting of 100 mM NaCl in 50 mM Tris-HCl, pH 9.0. Standard proteins (Amersham Biosciences) were used for column calibration.

Mass Spectrometry Analyses—Phenyl-sepharose haloperoxidase fractions were separated by fast protein liquid chromatography/gel filtration chromatography (Superdex 200 HR) followed by a 9% SDS-PAGE. The in-gel digestion by trypsin was performed on excised activity bands as described by Rabilloud *et al.* (36).

Nanoscale capillary liquid chromatography-tandem mass spectrometric (nano-LC-MS/MS) analyses of the digested proteins were performed using a CapLC capillary LC system (Micromass, Manchester, United Kingdom) coupled to an hybrid quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Q-TOF II; Micromass, Manchester, United Kingdom). Chromatographic separations were conducted on a reversed-phase capillary column (Pepmap C18; 75-μm internal diameter, 15-cm length; LC Packings) with a 200 nl/min flow. LC analyses were performed using a linear gradient from 95% A (H_2O/0.05% HCOOH) to 45% B (acetonitrile/0.05% HCOOH) in 35 min, followed by a linear gradient to 95% B in 1 min, and finally followed by an isocratic step at 95% B during 4 min. Mass data acquisitions were performed using automatic switching between the MS and MS/MS modes. Fragmentation of precursor was performed using collision with argon gas. The collision energy was selected automatically for each precursor ion depending on precursor ion mass (between 20 and 100 eV). The m/z scale was calibrated by using the synthetic polyalanine ions.

Micro-LC-MS/MS experiments were performed using an ion trap mass spectrometer Esquire 3000+ operating with an electrospray source in positive mode (Bruker-Daltonik GmbH, Bremen, Germany) coupled to an Agilent 1100 Series capillary LC system (Agilent Technologies, Palo Alto, CA) as described (37). The samples were loaded onto a 0.3 × 35-mm ZORBAX SB-C18 enrichment column at a flow rate of 50 μl/min with H_2O/0.1% HCOOH for 5 min. The trapped tryptic peptides were back-flushed onto the analytical column (0.3 × 150-mm; ZORBAX 300 SB-C18) at a flow rate of 4 μl/min using a linear gradient from 95% A (H_2O/0.1% HCOOH) to 60% B (acetonitrile/0.1% HCOOH) in 60 min followed by a linear gradient to 80% B in 5 min and finally followed by an isocratic step at 80% B for 5 min. For MS experiments (in auto-MS/MS mode) the two most important ions of each MS spectrum were fragmented by applying a resonance frequency on the end-cap electrodes (peak-to-peak amplitude from 0.3 to 2.0 V) matching the frequency of the selected ions. Fragmentation of the precursor ions occurred in the ion trap because of collisions with Helium buffer gas (pressure 5.10^{-3} millibar). Calibration of the ion trap analyzer was performed by using multiply charged ions mixture made with the following peptides: Arg-Leu-enkephalin, angiotensin, Substance P, bombesin, and ACTH.

Mass data collected during the nano- and micro-LC-MS/MS analyses were processed and converted into peak list and MGF (Mascot Generic File) files, respectively, and then submitted to the search software MASCOT (Matrix Science, London, United Kingdom), with a tolerance on mass measurement of 0.5 Da. The peptide sequences were obtained by mass spectrometry *de novo* sequencing. Data from the nano-LC-MS/MS analysis (Q-TOF II) were deconvoluted with the algorithm Maxent3 (Micromass, Manchester, United Kingdom).

cDNA Isolation, Cloning, and Sequencing—The longest of the vBPO EST, LamdiSest169est (NCBI accession number AW400475), identified previously in *L. digitata* sporophytes (1), was subcloned in *Sac*I pBluescript SK vector (Stratagene, La Jolla, CA). Plasmid DNA was sequenced on both strands using the Vistra Thermosequenase core sequencing kit on a Vistra automated DNA sequencer (Amersham Biosciences). The *L. digitata* sporophyte cDNA λZAP II library (1) was used as a template in polymerase chain reaction to amplify the 5′end of the BPO cDNA using pBluescript universal primer (forward) and a 5′end-specific primer from the LamdiSest169est (reverse; 5′-CTGCAGGTTCTCTGCGGCGA-3′). The 1000-bp PCR fragment obtained was cloned in the pCR 2.1-TOPO vector (Invitrogen) and labeled with [α-^{32}P]dCTP using the Megaprime labeling kit (Amersham Biosciences) to screen the *L. digitata* sporophyte cDNA library. Positive cDNA clones were subcloned by PCR using insert-specific oligonucleotides in the pCR 2.1-TOPO vector (Invitrogen) and were sequenced on both strands.

Sequence and Structural Analyses—Primary sequence analysis and translation were carried out using the DNAMAN program version 4.15. Prediction of protein sorting signal and of signal peptide cleavage site were performed using the TargetP, SignalP, and PSORT programs, respectively; *in silico* tryptic digestion of protein sequences was performed using the PeptideCutter program (programs available on the www.expasy.org server and the www.cbs.dtu.dk/services site). The vBPO protein sequences were aligned using DNAMAN and displayed using GENEDOC (free access on www.psc.edu/biomed/genedoc). The

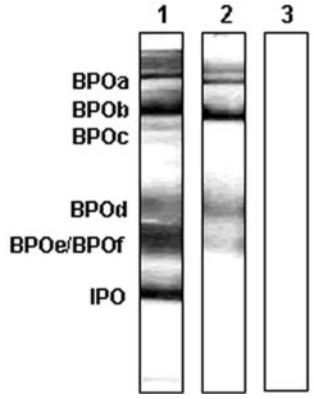

FIG. 1. **In-gel haloperoxidase assay of protein extracts from *L. digitata* sporophytes under non-denaturing conditions.** Polyacrylamide gels were loaded with haloperoxidase fractions (1.6 μg of proteins) and stained for iodoperoxidase activity (*lane 1*), bromoperoxidase activity (*lane 2*), and chloroperoxidase activity (*lane 3*). Bromoperoxidase activity bands are referred to as BPOa–f, and the iodoperoxidase band is indicated as IPO.

protein structure from *L. digitata* vBPO was predicted using the SWISS-MODEL program based on the three-dimensional structure of *A. nodosum* vBPO (NCBI accession number 1QI9A). The three-dimensional representations were visualized by the Swiss-PdbViewer software (free access on www.expasy.org server).

Southern Analyses—Genomic DNA was isolated from frozen *L. digitata* sporophytes as described by Apt *et al.* (38). Digested DNA (5 μg/*lane*) was electrophoresed on a 0.8% agarose gel and blotted onto nylon filters. The vBPO intragenic probe was prepared from an 800-bp *Eco*RI-*Sac*I fragment of LamdiSest169est (corresponding to Leu-182-Leu-412 in vBPO1; see Fig. 5). Southern blot hybridization, probe labeling, and detection were performed using the Gene Images random prime labeling module kit according to the manufacturer's recommendations (Amersham Biosciences).

Immunoblotting—Denaturated proteins were resolved on a 9% SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). Immunoblotting and ECL detection were performed using the ECL Western blotting System (Amersham Biosciences). The polyclonal antibodies against the vBPO isoenzyme-I from *A. nodosum* were kindly supplied by H. Vilter (34) and used at a 1:2,500 dilution. A polyclonal antipeptide to vBPO of *L. digitata* was raised in rabbits against a synthetic peptide (see Fig. 5) by Eurogentec and used at 1:1,700 dilution.

Expression of L. digitata vBPO in E. coli—An 1836-bp fragment from the putative mature protein v-BPO1 was amplified using the primers 5′-GGGGACAAGTTTGTACAAAAAGCAGGCTTCTACGAGGAGC-CTCCCTCCCGAGCC-3′ (forward) and 5′-GGGGACCACTTTGTACAA-GAAAGCTGGGTCCTAGAGCTCGGTGTGTCCGTGGAG-3′ (reverse; *Att*P1 and *Att*P2 recombination sites of the Gateway system are underlined; Invitrogen). The PCR product was inserted into the pDEST17 expression vector and amplified in DH5α *E. coli* strain for DNA replication and sequence verifications and in *E. coli* BL21-SI (Novagen, Darmstadt, Germany) for expression studies. A 2-ml overnight preculture of the transformed bacteria was used to inoculate a 250-ml culture of LBON medium (LB medium without salts) containing 100 μg/ml of ampicillin. Recombinant bacteria were grown at 37 °C until 0.5 A_{600} and then with 150 mM NaCl for 16 h at 30 °C in the presence or in the absence of 3% ethanol (39). Cytoplasmic and membrane proteins, hereafter referred to as the cytoplasmic fraction, were extracted from the bacterial pellet by solubilization in B-PER detergent (Pierce), and the extract was supplemented with 1 mM phenylmethylsulfonyl fluoride (anti-protease protection) and 2 mM $NaVO_3$. Aggregated proteins from inclusion bodies were solubilized by 6 M urea in 50 mM Tris, pH 8.0.

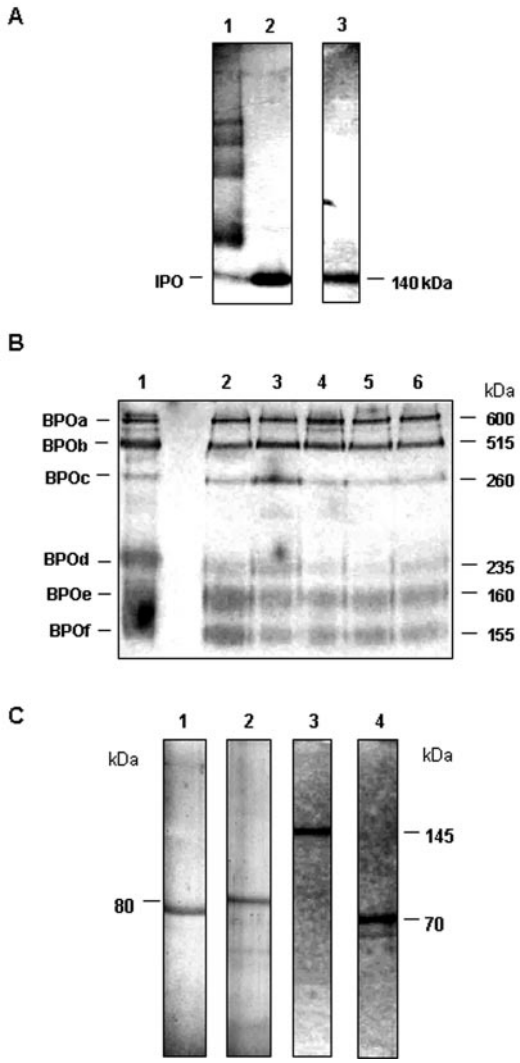

FIG. 2. **Purification of iodoperoxidase and bromoperoxidase from *L. digitata* followed by activity staining.** *A*, polyacrylamide gel electrophoresis of the iodoperoxidase fraction under non-denaturing conditions. *Lane 1*, aqueous salt/polymer two-phase system extract (130 ng) stained for iodoperoxidase activity. *Lanes 2* and *3*, IPO fraction purified by semi-preparative electrophoresis (130 ng), stained for iodoperoxidase activity and with silver nitrate, respectively. *B*, polyacrylamide gel electrophoresis under non-denaturing conditions of the bromoperoxidase fractions. *Lane 1*, phenyl-Sepharose partially purified extract, from which five distinct bromoperoxidase activity bands, annotated in the *left* of the gel, were extracted by electroelution from another gel run under identical conditions. *Lanes 2-6*, PAGE patterns of the proteins electroeluted from the BPOa, BPOb, BPOc, BPOd, and BPOe-f bands, respectively. The gel was loaded with 200 ng of proteins and stained for bromoperoxidase activity. The relative molecular masses of native enzymes indicated on the *right* were established as described under "Experimental Procedures." *C*, SDS-PAGE analyses of the purified IPO (*lanes 1* and *2*) and BPO (*lanes 3* and *4*) from *L. digitata*. Proteins were denatured in the absence (*lanes 1* and *3*) or in the presence of β-mercaptoethanol (*lanes 2* and *4*). Each *lane* was loaded with 30 ng of IPO and 100 ng of BPO and stained with silver nitrate. The relative molecular masses indicated on the *right* were established using a standard protein kit.

RESULTS

Purification of Haloperoxidases from L. digitata Sporophytes—Haloperoxidases from *L. digitata* were extracted using an aqueous salt/polymer two-phase system (30) and analyzed by non-denaturing electrophoresis followed by an in-gel activity assay (Fig. 1). Haloperoxidase activities were apparent only after adding orthovanadate to the protein extract, indicating that these enzymes are vanadium-dependent. Whereas no chloroperoxidase activity was detected, at least six major bands (referred to as BPOa–f) with bromoperoxidase activity were revealed in the presence of either bromide or iodide. A single enzyme activity specific for the oxidation of iodide (referred to as IPO) was apparent (Fig. 1).

Haloperoxidases were further fractionated by hydrophobic interactions on a phenyl-Sepharose CL4B column. The IPO activity was then purified to homogeneity by semi-preparative electrophoresis (Fig. 2*A*). The bands with BPO activity were excised from the non-denaturing electrophoresis gel and elec-

TABLE I
Bromoperoxidase and iodoperoxidase activities from L. digitata

IPO and BPO were purified on a phenyl-Sepharose column followed by semi-preparative electrophoresis for IPO and by electroelution for BPO. Specific activities are expressed in units per milligram of protein. One unit of bromoperoxidase activity is defined as the amount required for brominating 1 μmol of monochlorodimedone per min (33). One unit of iodoperoxidase activity is defined as the amount required for consuming 1 μmol of H_2O_2 per min (34).

Sample	Specific activity	
	Bromoperoxidase	Iodoperoxidase
	units/mg	
Gametophyte crude extract	0.7	0.
Sporophyte extract	2.6	10.4
Pure sporophyte BPO	42.	62.
Pure sporophyte IPO	0.	310.

troeluted separately. When re-analyzed by native gel electrophoresis, however, every band again showed a same, complex pattern (Fig. 2*B*). Under denaturing conditions (SDS-PAGE), IPO ran as a single protein with an apparent molecular mass of 75 kDa and as a protein of ~80 kDa in the presence of β-mercaptoethanol (Fig. 2*C*, *lanes 1* and *2*). Upon SDS-PAGE analysis, all of the BPOs were also resolved as one single band, each with relative molecular mass of 145 kDa. In the presence of β-mercaptoethanol they again appeared as a single band, yet with a molecular mass of ~70 kDa (Fig. 2*C*, *lanes 3* and *4*).

Biochemical Properties of Haloperoxidases from L. digitata—The specific activities of *L. digitata* haloperoxidases are summarized in Table I. In contrast to the sporophytes, a weak bromoperoxidase activity and no iodoperoxidase activity was detected in the crude protein extracts from the gametophytes of *L. digitata*. The IPO purified from the sporophytes displayed a specific activity of 310 units/mg toward iodide at pH 6.2. The purified BPO fraction had a lower efficiency toward iodide, in the iodoperoxidase assay. The purified haloperoxidases displayed the same pH optimum, at around 5.5 (Fig. 3). At this pH, iodoperoxidase-specific activities were 1200 units/mg for IPO and 180 units/mg for BPOs. Upon heating for 10 min, the purified IPO and BPOs remained fully active up to 60 °C (Fig. 3), with the high molecular mass BPOs retaining the highest activity up to 80 °C (data not shown).

Mass Spectrometry Analyses of Excised BPOs and IPO—Mass spectrometry analyses were carried out for three of the bromoperoxidase bands, BPOb–d, and for the iodoperoxidase. As the N-terminal sequences of these proteins were blocked, they were digested by trypsin and analyzed by nano- or micro-LC-MS/MS. Very similar MS data were obtained for the three BPOs, and among those data, an identical set of eleven peptides was identified in BPOb–d tryptic digests (Table II). As illustrated in Fig. 4, four peptides were fully sequenced, resulting in identical sequences for all of the three BPO bands. None of the eleven peptide masses observed in BPO tryptic digests was found in the IPO tryptic digest, which markedly differed from the BPOs by its mass spectra (Table II) and by the partial sequences obtained by *de novo* sequencing (AVNVA, SAPG(I/L)NG, (I/L)VAADTVNTEAYR).

Molecular and Immunological Characterization of L. digitata Bromoperoxidases—In an EST analysis of the life cycle stages of *L. digitata*, one contig from 12 EST reconstructed a partial cDNA showing high homology with the vBPO of *F. distichus* (1). The cDNA library from *L. digitata* sporophytes was thus screened for full-length vBPO cDNAs using a PCR-amplified probe designed from the 5′end of the partial cDNA, yielding several positive clones, which were mapped by restriction analyses. The two longest inserts, referred to as vBPO1 (3379 bp) and vBPO2 (3420 bp), were fully sequenced. They

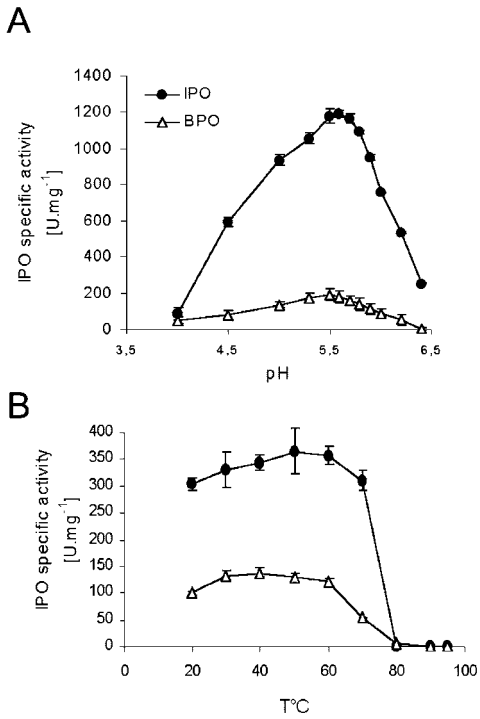

FIG. 3. **Iodoperoxidase specific activity of the purified IPO and BPO as a function of pH at 20 °C (*A*) and as a function of temperature at pH 6.2 (*B*).** *Error bars* indicate standard deviations (n = 3).

encoded two distinct full-length cDNA, with short 5′UTRs (104 and 36 bp, respectively) and with large 3′UTRs (1337 and 1338 bp, respectively).

The two cDNAs had 99.2 and 99.5% identity at the nucleotide and protein levels, respectively. They mainly differed in their 5′ends, vBPO1 presenting a putative 20-amino acid signal peptide with the cleavage site after Gly-20 whereas vBPO2 harbored a longer one (56 amino acids), with the cleavage site located at Gly-56. The putative mature proteins had molecular masses of 68,953 Da (vBPO1) and 68,957 Da (vBPO2), differing by only three amino acids, Ile-75/Val-111, Gly-205/Ser-241, Val-365/Ser-401 (Fig. 5). The vBPO protein sequences from *L. digitata* presented 38 and 40% identity with those from *F. distichus* and *A. nodosum*, respectively (Fig. 5). In particular, *L. digitata* vBPOs differed from the other brown algal bromoperoxidases by longer C-terminal ends, including 17-amino acid-long insertions, between Gly-494 and Gly-511 (numbering of vBPO1). They featured, however, the two catalytic histidine residues, as well as all of the residues known to be involved in the fixation of vanadium (20).

The eleven peptides characterized by LC-MS on the purified bromoperoxidases corresponded with a good accuracy to those identified by *in silico* tryptic digestion of vBPO protein sequences (Table II), and they homogeneously covered 22% of the vBPO mature proteins (Fig. 5). Three of the four peptides fully sequenced by LC-MS/MS were present in both vBPO sequences. The fourth peptide (Fig. 4) was only present in vBPO1. In this sequence vBPO2 departed from vBPO1 by replacement of glycine by serine (Fig. 5). In a Southern blot analysis with the vBPO intragenic probe a large number of hybridization bands was detected in *L. digitata* (Fig. 6). A synthetic peptide (see *box* in Fig. 5) was used to produce a polyclonal antibody against *L. digitata* vBPOs. This antibody cross-reacted with the purified BPOs but not with the purified IPO. This latter enzyme, however, was specifically recognized by antibodies against the vBPO from *A. nodosum* (Fig. 7).

TABLE II

LC-MS-MS analyses of purified IPO and BPO tryptic digests and comparison with the theoretical tryptic digest of vBPO1 from L. digitata

The native electrophoresed bands corresponding to IPO and BPOb-d were excised and submitted to in-gel digestion by trypsin. The first two columns refer to the main precursor ions observed by LC-MS for IPO (1st column) and for the BPO bands (2nd column). The observed monoisotopic masses of BPO peptides (2nd column) were compared with the calculated vBPO1 tryptic digest masses (3rd column), and accuracies are reported in the 5th column. The peptides, partially or fully sequenced by LC-MS/MS, are shown in bold.

IPO, MH^+ (obs.)	BPOb-d, MH^+ (obs.)	vBPO1		
		MH^+ (calc.)	Tryptic peptide residues	ΔM
Da	*Da*	*Da*		*ppm*
1197.78	1856.96	1856.84	110-124	65
1423.78	3326.80	3326.65	137-168	45
983.58	**1371.86**	1371.76	198-210	73
1453.82	**1350.60**	1350.62	287-297	15
875.54	**1142.80**	1142.55	312-320	219
1646.88	1338.82	1338.76	396-407	45
1524.84	**1083.80**	1083.55	463-472	231
946.58	**1563.80**	1563.71	473-486	57
1151.62	**1464.80**	1464.76	508-520	27
	942.64	942.57	521-529	74
	1350.80	1350.74	555-566	44

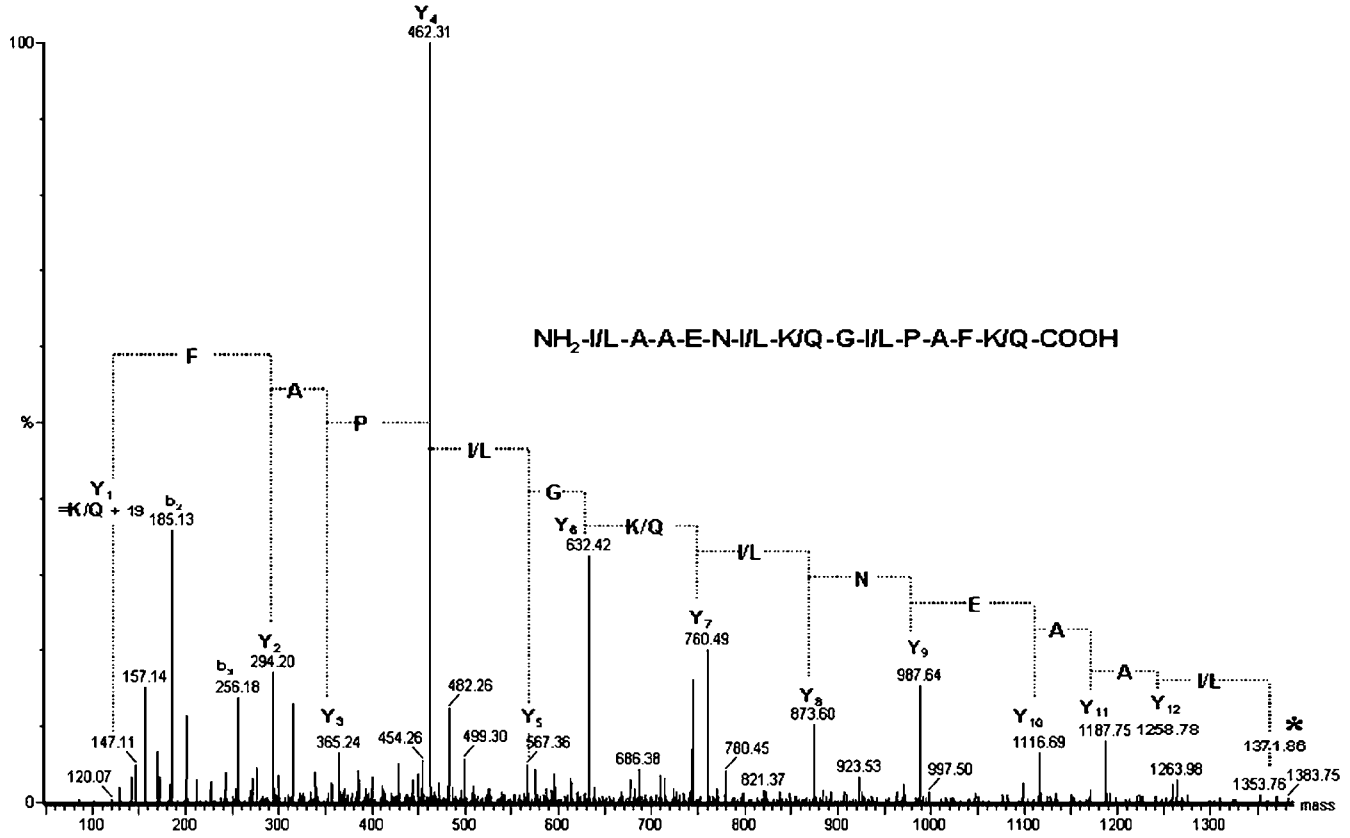

FIG. 4. **MS/MS spectrum and deduced amino acids of the 1371.86-Da tryptic peptide from purified BPO.** Mass data are the average of seven MS/MS analyses. An *asterisk* (*) on the *right* of the spectrum indicates the precursor ion.

Three-dimensional Structure of L. digitata Bromoperoxidases—The three-dimensional structure of *L. digitata* vBPO1 (Fig. 8) was modelized from the homo-dimeric structure of *A. nodosum* vBPO (20). The two tertiary structures were highly conserved in their overall folding. The six cysteine residues known to be involved in intramolecular disulphide bridges within the *A. nodosum* monomer (20) were present at similar positions in *L. digitata* bromoperoxidases. The two cysteine residues involved in the dimer interface of the *A. nodosum* enzyme are also conserved in *L. digitata* vBPOs (Cys-3 and Cys-41; numbering of *A. nodosum* vBPO). The 17-amino acid-long insertion in the C-terminal part of *L. digitata* bromoper-oxidases appeared as an α-helix in the three-dimensional structure model (Fig. 8).

Expression of v-BPO1 cDNA in E. coli—Following overexpression of vBPO1 in *E. coli*, a protein with the expected size of 70 kDa was produced in both the inclusion bodies and cytoplasmic protein fractions. Addition of 3% ethanol increased the proportion of overexpressed protein in the cytoplasmic fraction (Fig. 9*A*). Consistently, bromoperoxidase activity was higher in the latter fraction than in cytoplasmic protein fractions from cultures induced with 150 mM NaCl only (Fig. 9*B*). The recombinant bromoperoxidase activity consisted of one split band only, with an apparent molecular mass similar to that of the native protein band referred to as BPOd (Fig. 9*B*).

DISCUSSION

L. digitata Sporophytes Feature Distinct Bromo- and Iodoperoxidases—We here have separated from the sporophytes of

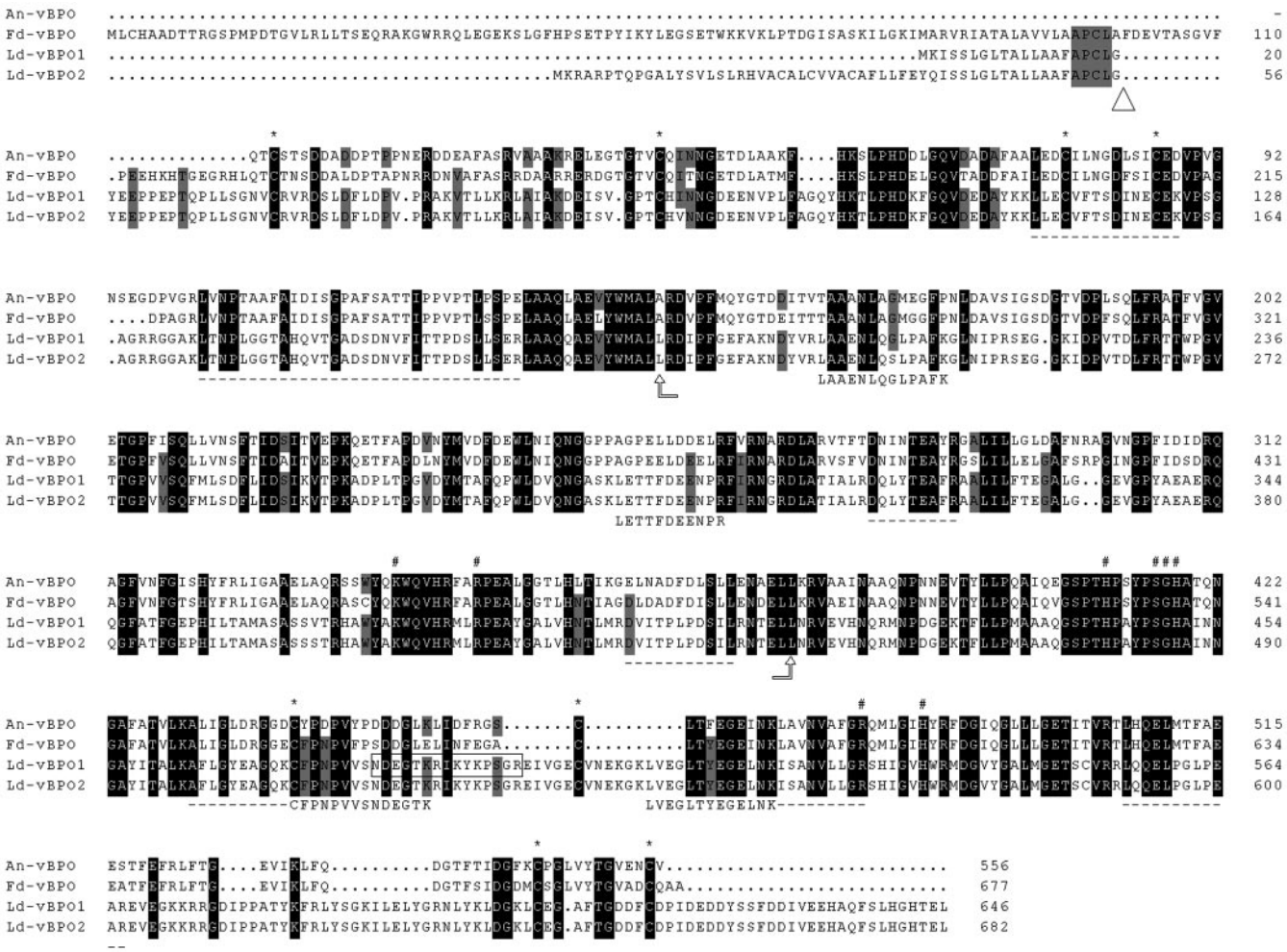

FIG. 5. **Multiple amino acid sequence alignment of known vanadium bromoperoxidases from brown algae.** *An-vBPO*, *A. nodosum* vBPO (Swiss-Prot accession number P81701); *Fd-vBPO*, *F. distichus* vBPO (NCBI accession number AAC35279); *Ld-vBPO1* and *Ld-vBPO2*, *L. digitata* vBPO1 and vBPO2 (this study) (EBI accession numbers AJ491786 and AJ491787, respectively). The conserved residues in all of the four sequences are shown in *white capitals* on a *black background*, and the residues that occur in at least three of the sequences are in *black capitals* on a *gray background*. The amino acid residues associated with the active site vanadium cofactor are indicated by the number symbol (#), and the cysteine residues involved in disulphide bridges according to the *An-vBPO* structure (20) are marked with an *asterisk* (*) *above* the alignment. The putative cleavage site of signal peptides in *Ld-vBPO* is referred to by a *triangle*. The seven peptides identified by LC-MS from the purified bromoperoxidases of *L. digitata* are *underlined*, with the four peptides sequenced by LC-MS/MS out in full *below* the alignment. The peptide used to produce an antipeptide to the vBPO from *L. digitata* is *boxed*. *Arrows* point out the intragenic probe used for Southern blot.

the brown alga *L. digitata* different haloperoxidase fractions, one specific for the oxidation of iodide and another that can oxidize both iodide and bromide (Table I). The iodoperoxidase and bromoperoxidase activities were purified to electrophoretic homogeneity (Fig. 2). They markedly differed in their molecular masses (Fig. 2), trypsin digestion profiles (Table II), and immunological characteristics (Fig. 7). Altogether, it appears that *L. digitata* features distinct iodoperoxidase and bromoperoxidase activities, *i.e.* which are borne by different proteins. At the optimal pH (5.5) the iodoperoxidase from *L. digitata* was completely inactive in the presence of bromide whereas it was 6.7 times more efficient than the bromoperoxidase fraction in the oxidation of iodide (Fig. 3). Although specific iodoperoxidase activities have already been reported in two Laminariaceae, *L. hyperborea* and *L. ochroleuca* (28), this is, to our knowledge, the first complete isolation of a genuine iodoperoxidase. At pH 6.2 (classically used in iodoperoxidase assays), its specific activity was about 3–20 times higher than the other kelp IPOs (28). Based on both immunological relatedness (Fig. 7) and LC-MS/MS peptide sequences, the IPO from *L. digitata* appears to be more closely related to the vBPO from *A. nodosum* than to the vBPOs from *L. digitata*.

The BPOs of L. digitata Are Multimeric Proteins—Given that the IPO was resolved as one single band with an apparent molecular mass of 80 kDa in the presence of SDS and β-mercaptoethanol (Fig. 2*C*), down from one 140-kDa band in native conditions (Fig. 2*A*), it is likely that this enzyme is a dimeric protein. Like *A. nodosum* vBPO (20), the bromoperoxidases from *L. digitata* also appear as dimeric proteins, with apparent molecular masses of 70 kDa in the complete denaturing conditions and of 145 kDa in the presence of SDS only (Fig. 2*C*). However, under non-denaturing conditions, purified bromoperoxidases consisted of six major bands, ranging from 155–600 kDa in sizes (Fig. 2*B*), indicating that they further reassociate into multimeric proteins. This observation is supported by mass spectrometry analyses (Table II), which show that the bromoperoxidase bands vBPOb–d actually consist of the same protein or of structurally very close proteins that cannot be separated by one-dimensional electrophoresis.

The crystal structure analysis of the bromoperoxidase from the red alga *C. officinalis* shows that this protein is organized as an assembly of six homodimers (17). It was suggested recently (28) that brown algal haloperoxidases also tend to aggregate into forms with a high molecular mass. We here show

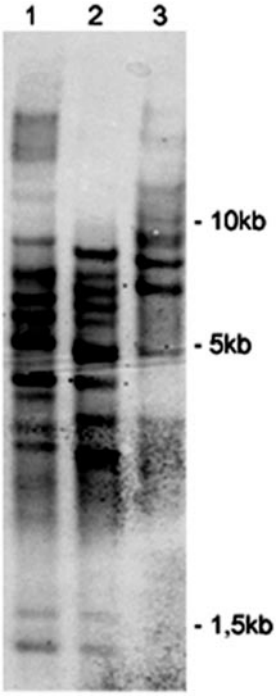

FIG. 6. **Southern blot hybridization of *L. digitata* genomic DNA with an intragenic probe from *L. digitata* vBPO cDNA.** *Lanes 1-3* contain *L. digitata* genomic DNA digested with *Sal*I, *Sal*I/*Xho*I, and *Xho*I, respectively. The intragenic probe is shown in Fig. 5.

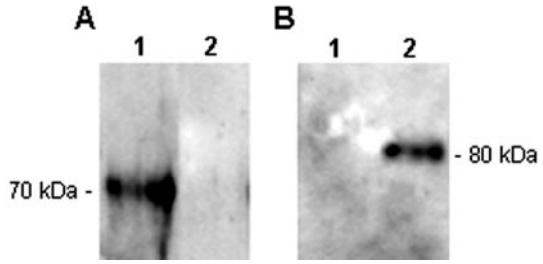

FIG. 7. **Western blot hybridization of the purified bromo- (*lane 1*) and iodoperoxidase (*lane 2*).** Each *lane* contains 200 ng of protein, treated with SDS and β-mercaptoethanol. Blots were stained with antibodies directed against *L. digitata* vBPO (*A*) or against *A. nodosum* vBPO (*B*).

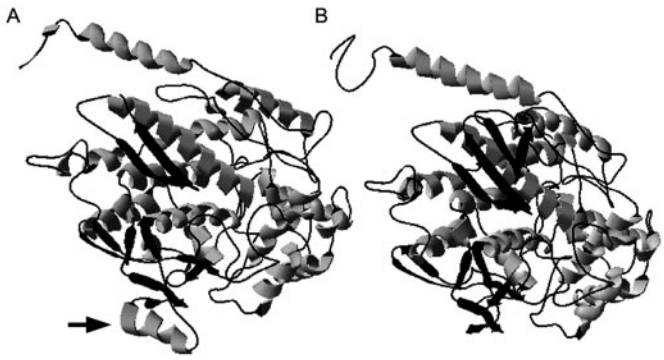

FIG. 8. **Structure model of the vBPO1 monomer from L. digitata (*A*) compared with the ribbon-type representation of the vBPO monomer from *A. nodosum* (*B*).** Secondary structure assignments: α-helices are shown in *gray*, and β-strands and coils are shown in *black*. The N- and C-terminal ends of *L. digitata* vBPO1 protein (amino acid residues 1–42 and 583–646, respectively) were not modelized because of the lack of homology with the vBPO protein of *A. nodosum*. The additional α-helix in *L. digitata* vBPO1 is marked by an *arrow*.

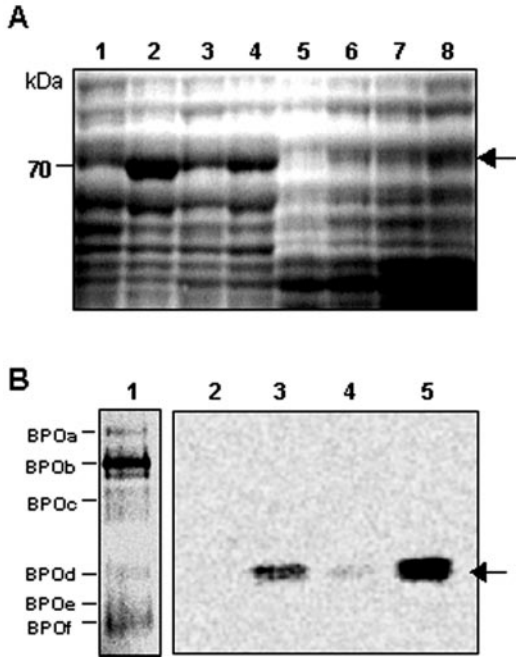

FIG. 9. **Expression of *L. digitata* vBPO1 in *E. coli*.** *A*, denaturing SDS-PAGE analysis (8% polyacrylamide) of *E. coli* protein extracts (25 μg), stained with Coomassie Blue, from inclusion bodies fractions (*lanes 1-4*) and from cytoplasmic fractions (*lanes 5-8*), under the following culture conditions: *lanes 1* and *5*, uninduced culture; *lanes 2* and *6*, culture induced with 150 mM NaCl; *lanes 3* and *7*, uninduced culture supplemented with 3% EtOH; *lanes 4* and *8*, culture induced with 150 mM NaCl and supplemented with 3% EtOH. *B*, in-gel iodoperoxidase assay of the purified BPO extract (100 ng) from *L. digitata* (*lane 1*) and of the recombinant protein extracts (50 μg) from the cytoplasmic fractions, under the following culture conditions: *lane 2*, uninduced culture; *lane 3*, culture induced with 150 mM NaCl; *lane 4*, uninduced culture supplemented with 3% (v/v) EtOH; *lane 5*, culture induced with 150 mM NaCl and supplemented with 3% (v/v) EtOH. Note that, in the original gels, the bromoperoxidase activity did appear as one double band. *Arrows* indicate the bromoperoxidase bands.

unequivocally that *L. digitata* vBPOs consist of dimers that, in solution, spontaneously self-associate into higher molecular mass oligomers. It follows that so-called bromoperoxidase isoforms (23, 24) may actually be different reassociation states of the same protein or of closely related proteins. It is worth noting here, however, that under non-denaturing conditions the recombinant bromoperoxidase vBPO1 ran as one double band with an apparent molecular mass similar to that of the BPOd native band, ~235 kDa. This observation suggests that the recombinant protein lacks some post-translational modifications, which are not essential for activity but which are important for reaggregation into a multimeric protein.

Bromoperoxidases Form a Multigenic Family in L. digitata—From sequence tags with homology to brown algal bromoperoxidases, we here have characterized two full-length cDNAs from *L. digitata*, vBPO1 and vBPO2. A body of convergent evidence shows that vBPO1 and vBPO2 encode bromoperoxidases identical or very similar to the proteins referred above to as vBPOa–f. (i) The mature proteins feature all of the residues known to be involved in the bromoperoxidase active site (Fig. 5), including the additional histidine residue essential for bromoperoxidase activity (18, 20). (ii) They are immunologically related to the purified BPOs (Fig. 7). (iii) They harbor the very peptides that were found by LC-MS/MS in the purified fractions (Fig. 5). (iv) The protein produced from vBPO1 in *E. coli* featured bromoperoxidase activity (Fig. 9).

Although the bromoperoxidases from *L. digitata* and those of fucalean algae markedly diverge in their primary sequences (Fig. 5), they are superimposable in their monomeric three-

dimensional structure (Fig. 8). It is, therefore, likely that the conserved cysteine residues in *L. digitata* sequences are involved with the enzyme dimerization (20) and that, as in the case of other bromoperoxidases, these fold into multimeric, extremely robust proteins. As proposed for the other bromoperoxidases (17, 20), protein multimerization may result in a higher thermostability in terms of activity (Fig. 3).

In contrast to the fungi *C. inaequalis* and *Embellisia didymospora*, where only one copy of a vanadium-dependent chloroperoxidase gene was identified (40, 41), Southern blot analysis (Fig. 6) indicated the presence of a high copy number of BPO genes in *L. digitata*. vBPO1 and vBPO2 are very similar in their 3′UTR and 5′UTR regions, suggesting that they have arisen by a recent gene duplication. Minor differences were found indeed among the various purified BPOs upon trypsin degradation and LC-MS analyses (data not shown), indicating that these proteins present subtle differences in their primary sequences or post-translational modifications. In particular, as reported earlier in *L. digitata* (42), only one of the bromoperoxidase fractions (BPOb) was found to bind to ConA (data not shown). Altogether, it appears that bromoperoxidases form a large multigenic family in *L. digitata*.

Potential Functions of Haloperoxidases in L. digitata—Several lines of indirect evidence suggest that haloperoxidases are involved in the uptake of iodine in kelps. (i) The uptake of iodine by *L. digitata* gametophytes was enhanced in the presence of an *A. nodosum* vBPO (3). (ii) Both the vBPO expression (as seen from EST analysis (1)) and activity (this study) are high in *L. digitata* sporophytes, which accumulate high levels of iodine, compared with the gametophytes, which do not. We, therefore, have proposed that this protein is involved with iodine uptake in *L. digitata* (1). However, we here show that *L. digitata* sporophytes also feature a iodoperoxidase, specialized in the oxidation of iodide and with no activity toward bromide (Table I). Because the concentration of bromide (3 mM) is in seawater several orders of magnitude higher than those of iodide (0.25 μM), the iodoperoxidase is more likely than the bromoperoxidase to account for the uptake of iodide from seawater.

It remains that *L. digitata* features a high number of vBPO genes (Fig. 6), which, based on their high proportion in the cDNA library (2% of transcripts (1)) and the occurrence of corresponding products in the sporophytes (see Fig. 1 and Table I), are constitutively expressed. These findings raise the question of the functions of bromoperoxidases in this alga. Because Laminariales feature bromine concentrations in the millimolar range (2), they may be involved in the metabolism of bromide. They nevertheless may also be involved in the metabolism of iodide, when iodide concentrations are high compared with those of bromide, *i.e.* in intracellular compartments. In this respect, it is worth noting here that vBPO1 and vBPO2 have different signal peptides, indicating that these two bromoperoxidases probably have different subcellular locations. Further information on the respective biological functions of halogen-oxidizing enzymes in kelps must now, however, await the complete characterization of iodoperoxidase(s), as well a detailed investigation of the localization and expression of haloperoxidases in these algae.

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