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Note

Purification and determination of the action pattern of *Haliotis tuberculata* laminarinase

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Abstract

The major laminarinase activity (EC 3.2.1.39) from the gastropodean marine mollusc *Haliotis tuberculata* was purified to homogeneity by cation exchange chromatography and its action pattern was investigated by HPAEC-PAD analysis of the degradation of various laminarin samples. It consists of a 60 kDa protein capable of depolymerizing the unbranched portions of the β-(1→3), β-(1→6)-glucan, down to laminaritriose. The enzyme operates via a molecular mechanism retaining the anomeric configuration. As the purified protein does not cleave the β-(1→6) linkages, it can be used for the structural analysis of laminarins. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: β-(1→3)-endoglucanase; *Haliotis tuberculata*; HPAEC-PAD; Laminarin; oligosaccharides

Laminarin, the storage polysaccharide of marine brown algae and diatoms, consists of linear, mannitol- or glucose-ended chains of β-(1→3) linked glucose residues, with occasional β-(1→6)-linked branches [1–4]. The main chain length and the overal degree of polymerisation (DP) of laminarin range from 7 to 19 and 16 to 31, respectively, indicating a structural polydispersity, from essentially linear β-(1→3)-glucans to branched β-(1→3), β-(1→6)-glucans with an average of three ramifications per molecule. Solubility in cold water depends on the branching characteristics, with increasing linearity resulting in a greater ability to form intermolecular hydrogen bonds and insoluble aggregates [5]. Fungal or brown algal β-(1→3), β-(1→6)-glucans are known to elicit defence responses in both plants [6–12] and animals [13–16], and thus have potential applications in crop protection or as immunomodulatory drugs in aquaculture and biomedicine.

In this context, β-(1→3)-endoglucanases of known action pattern would be useful for the routine analysis of laminarin structure from different sources. Laminarinases are widespread among living organisms, throughout archea, eubacteria and several eukaryotic lineages. Depending on substrate recognition two classes of laminarinases are

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known, those that require the presence of at least two adjacent (1→3)-linked β-D-glucosyl residues (EC 3.2.1.39), and less specific laminarinases (EC 3.2.1.6) which can also hydrolyse such β-(1→4) linkages adjacent to (1→3)-linked β-D-glucosyl residues as in lichenans. Based on aminoacid sequence and folding similarities, endo-β-(1→3)-glucanases fall into at least three distinct structural families, the family 16 [17], the family 17 [18] and the family 64 [19] of glycoside hydrolases.

In metazoa, laminarinase activity was reported from the digestive track and the hepatopancreas of a number of marine organisms [20,21]. Yet, so far only two endo-β-(1→3)-glucanases have been purified to electrophoretic homogeneity, from the crystalline style of the bivalvean mollusc *Spisula sachalinensis* [22] and from the cephalothorax of the krill crustacean species *Euphausia superba* [23]. Similarly, only two metazoan laminarinase sequences are known, that of the enzyme from the eggs of the sea urchin *Strongylocentrotus purpuratus* [24] and that of the clotting factor from the horseshoe crab *Tachypleus tridentatus* [25]. We report here the purification and characterization of an endo-β-(1→3)-glucanase from the ormer *Haliotis tuberculata* (Gastropoda, Mollusca) as well as a detailed analysis of its mode of action on various laminarin samples.

1. Results and discussion

Purification of the laminarinase and alginate lyase from H. tuberculata.—Based on the hydrolysis of pachyman (Megazyme test) and of laminarin, the hepatopancreas extracts of *H. tuberculata* contained laminarinase activity. The laminarinase was purified to electrophoretic homogeneity by ammonium sulfate precipitation and CM-Sephadex 50 (CMC50) cation-exchange chromatography, followed by gel filtration on Sephacryl HR 300 and cation-exchange chromatography with a MonoS column. Upon elution with a sodium chloride gradient, proteins were resolved from the MonoS column into one major and several minor peaks. Only the major peak, which eluted at 0.35 M NaCl, displayed laminarinase activity in the reducing sugars assay (Fig. 1). It contained a single protein band, with a molecular weight of approximatively 60 kDa (Fig. 2) and a pI of 8.50 (data not shown). The procedure resulted in a 32.9-fold purification of the laminarinase activity,

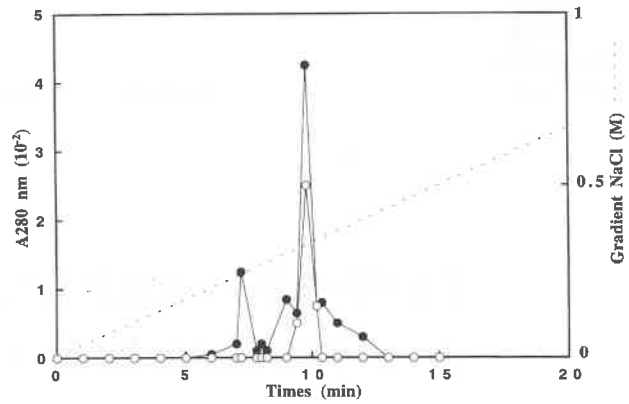

Fig. 1. Chromatography on MonoS column of the Sephacryl fraction of *H. tuberculata* laminarinase. The enzyme was eluted from the column with a linear gradient of 5 mM–1 M NaCl and the eluent was monitored for proteins (–○–) by absorbance at 280 nm and for laminarinase activity (–●–) by the reducing sugars assay.

with a final recovery of 3.5%. Most of the loss occurred prior to gel filtration, upon concentration and dialysis with the Amicon ultrafiltration cell (Table 1). The yield decrease at this step, however, is also partly accountable to the removal of exo-β-(1→3)-glucanase activity as well as of debranching activity (see below). Alginate lyase activity was separated from laminarinase during the Sephacryl HR 300 step. It was further purified[1] with the MonoS column, yielding a major, 35 kDa protein band upon SDS-PAGE analysis (Fig. 2).

Based on the pachyman test and as indicated by the generation from laminarin of a mixture of oligosaccharides in addition to glucose (see below), *H. tuberculata* laminarinase is an endo-β-(1→3)-glucanase (EC 3.2.1.6.). No exoglucanase activity was detected using p-nitrophenyl- or methylumbelliferyl β-D-glucopyranoside as substrates. Laminarinase activity was observed over pH 5.0–8.0, with a maximum value at pH 7.0 in 20 mM phosphate buffer. Activity was maximal at 37 °C and the enzyme was inactivated by temperatures over 45 °C. The ormer laminarinase ressembles the β-(1→3)-glucanase from the crustacean *E. superba* in its molecular mass, ca. 60 kDa, but differs by its optimal pH, 7.0 instead of 4.3–5.0 and its optimal temperature, 37 °C, intermediate between those of *S. sachalinensis* (25 °C) and of *E. superba* (65 °C) endo-β-(1→3)-glucanases.

[1] Analysis of the substrate specificity of the purified alginate lyase will be reported elsewhere.

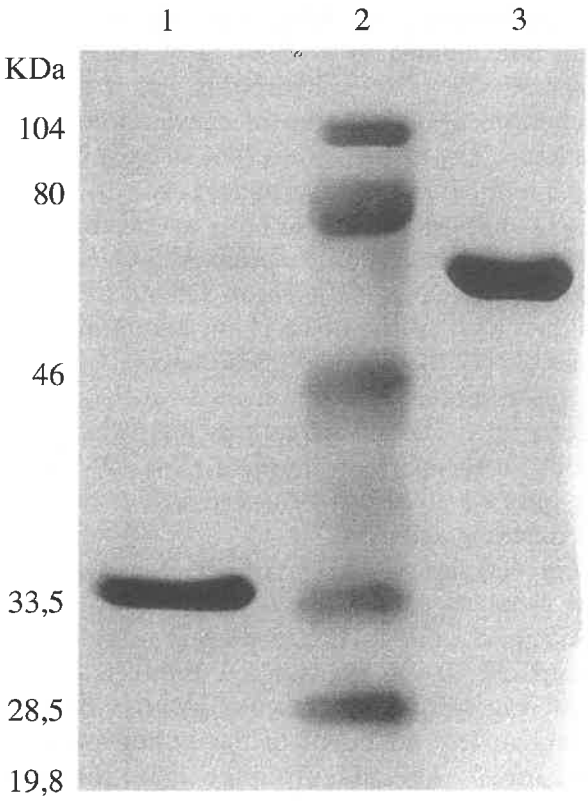

Fig. 2. SDS-PAGE analysis of the β-(1→3)-endo-glucanase (lane 3) and the alginate lyase (lane 1) from *H. tuberculata*. The fractions from the MonoS column were submitted to electrophoresis on a SDS 12.5% polyacrylamide gel and stained with Coomassie blue. The molecular weight marker (lane 2) consisted of lysozyme, soybean trypsin, carbonic anhydrase, ovalbumin, serumalbumin and phosphorylase B (Biorad).

HPAEC-PAD chromatographic analysis of the hydrolysis of linear β-*(1→3)-glucooligosaccharides by* H. tuberculata *laminarinase.*—A standard mixture of linear homo-oligomers of laminarin (Seikagaku) was analysed by High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD), using a CarboPac PA 100 column and ionization with 150 mM NaOH. Optimal separation of glucose, laminaribiose, laminaritriose, laminaritetraose, laminaripentaose, laminarihexaose and laminariheptaose (DP 1–7) was achieved in a single run within 25 min, using a four-step 150–500 mM sodium acetate gradient [Fig. 3(b)]. These chromatographic conditions also allowed for the detection of laminarin, which eluted as a wide peak at the end of the gradient, corresponding to an average DP of 25–35 [Fig. 3(a)]. As reported recently with a PA 1 column by Fontaine et al. [26], HPAEC-PAD can therefore be regarded as a well suited new method for analyzing the catalytic properties of endo-β-(1→3)-glucanases.

The end-products of the hydrolysis of laminarin standard oligomers by the purified laminarinase from *H. tuberculata* are shown in Fig. 3(c). After 2 h hydrolysis, D-glucose and oligomers up to laminariheptaose were detected, but the larger DP 5–7 oligosaccharides were less abundant than in the control (not shown). When each oligomer of DP 1–7 was incubated independently in the presence of purified laminarinase, the larger oligomers (DP 6 and DP 7) were degraded more rapidly whereas no hydrolysis was observed for oligomers smaller than DP 4 (Table 2), indicating that this enzyme requires a minimum of four contiguous β-(1→3)-linked residues for its hydrolytic activity. Consistently, at the end of hydrolysis, DP 4–7 oligosaccharides were no longer detected whereas the proportions of DP 1 and DP 2 had markedly increased [Fig. 3(c)].

*Degradation of laminarin fractions by the endo-*β-*(1→3)-glucanase of* H. tuberculata.—The degradation of laminarin either with the *H. tuberculata* CMC-50 fraction or with the purified enzyme was then monitored by HPAEC-PAD chromatography. A variety of laminarin oligomers were detected on the chromatograms, including β-(1→3)-glucooligosaccharides ranging from DP 1–7. In the presence of unpurified laminarinase (CMC-50 fraction), laminarin was extensively degraded within 24 h, yielding glucose, laminaribiose and laminaritriose as the major linear β-(1→3)-oligosaccharide products [Fig. 4(a)]. In contrast, when using the MonoS purified fraction, degradation of laminarin was uncomplete after

Table 1
Purification of the laminarinase from the hepatopancreas of *H. tuberculata*

Step	U/mL	Recovery (%)	Proteins (mg/mL)	Specific activity (U/mg)	Purification (fold)
Crude extract	27.3	100	10.4	2.63	1
$(NH_4)_2SO_4$ precipitation	74.3	46	17.4	4.27	1.6
CMC-50	324.6	40	27.9	11.63	4.4
Sephacryl	17.6	5.9	0.22	80.00	30.4
MonoS	2.6	3.5	0.03	86.66	32.9

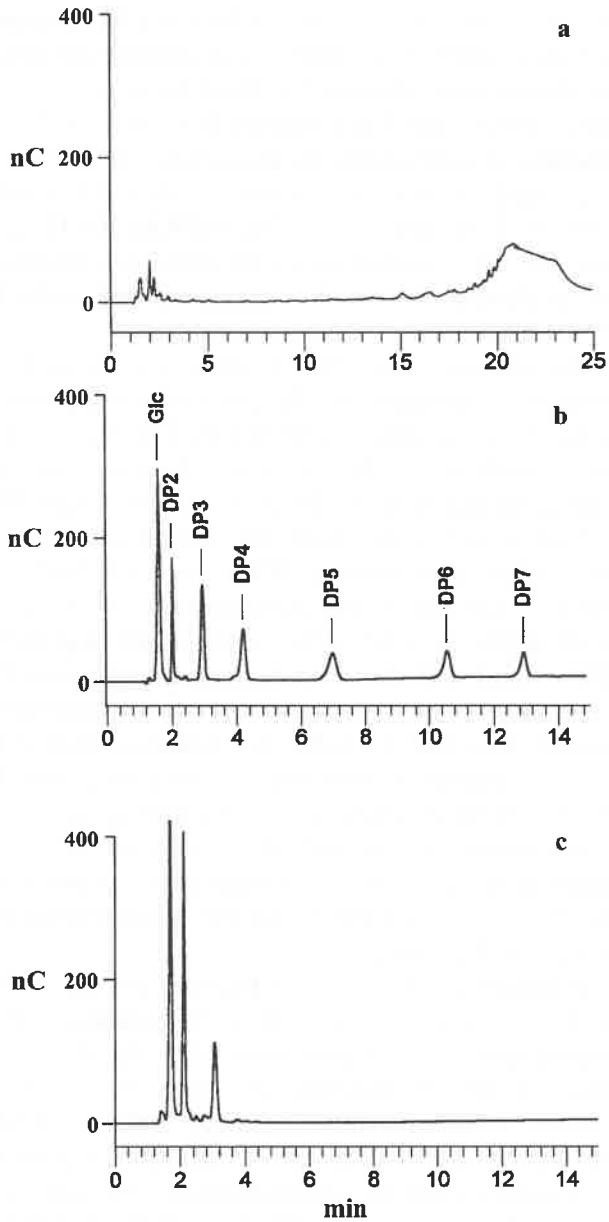

Fig. 3. HPAEC-PAD analysis of (a) Sigma laminarin, of (b) the standard mixture of laminarin linear homo-oligomers and of (c) the standard mixture after degradation to completion by the purified laminarinase.

Table 2
Action pattern of *H. tuberculata* laminarinase towards various homo-oligolaminarins. Linear laminarin standards with DP ranging from 2 to 7 were submitted to hydrolysis with the purified laminarinase, then analysed by HPAEC-PAD

Substrate	6 h Hydrolysis	24 h Hydrolysis
Laminaribiose	DP 2	DP 2
Laminaritriose	DP 3	DP 3
Laminaritetraose	DP 3 + DP 1	DP 3 + DP 1
Laminaripentaose	DP 3 + DP 2	DP 3 + DP 2
Laminarihexaose	DP 3 + DP 3	DP 3 + DP 3
Laminariheptaose	DP 4 + DP 3	DP 3 + DP 1 + DP 3

24 h hydrolysis [Fig. 4(b)]. The digestion pattern was not significantly different from the one observed after 25 min hydrolysis and it remained unchanged upon addition of enzyme followed by one more day of hydrolysis (not shown). It exhibited a number of products unassignable to linear β-(1→3)-oligosaccharides, notably at retention times 8.73, 11.50, and 13.60, respectively. As *Laminaria digitata* laminarin has a mean DP of 25 glucosyl residues with up to three β-(1→6) ramifications per molecule [4], these unidentified oligomers are likely to correspond to 6-branched β-(1→3)-oligosaccharides. These observations suggest that, contrarily to the partially purified CMC-50 fraction, the purified endo-β-(1→3)-glucanase is devoid of debranching activity.

Fig. 4(c) shows the HPAEC-PAD analysis of the end products of the Phycarine® fraction with the purified *H. tuberculata* endo-β-(1→3)-glucanase. Compared to the degradation products of laminarin [Fig. 4(b)], unidentified oligosaccharides were absent or less abundant in the elution pattern of Phycarine®. Assuming from the above results that the purified endo-β-(1→3)-glucanase cannot cleave the β-(1→6) ramifications of the β-(1→3)-glucan backbone, Phycarine® is therefore probably a β-(1→3)-glucan more linear than the laminarin provided by Sigma. Such structural differences may represent seasonal or geographical variability in *L. digitata* or differences in the extraction process, or both.

Stereochemical course of the hydrolysis.—As already reported by others [27], laminarin, which is not fully solubilized at 30 °C, exhibited a ^{13}C NMR spectrum with 6 large major carbon signals assigned to the six carbon resonances of glucose (C-1: 103.3; C-2: 73.9; C-3: 85.0; C-4: 68.9; C-5: 76.3; C-6: 61.5 ppm, Fig. 5). As the enzymatic hydrolysis progressed, the signals became thinner and new series of resonances were observed. Among those, the signals in the 96.0 and 92.5 ppm regions were attributed to the C-1 of reducing β- and α-glucose residues. The various signals in this region (96.3–96.1 ppm and 92.5–92.2–92.4 ppm), the intensity of which increased differently over hydrolysis, may reflect different chemical environments such as the presence of a β-(1→6) branched glucosyl residue on the glucose next to the reducing end and/or different conformational arrangements of the glucan chains. The very early appearance upon hydrolysis of the β-glucose anomer signals indicated that enzymic cleavage proceeded by

retention of configuration. Exact values of the ratio of the reducing-end glucose anomeric signal integrals were not obtained because of the multiplicity of signals in the anomeric regions. However, the progressive increase of the α-anomer population with time also supported a mechanism retaining the anomeric configuration.

Peptide sequences were obtained from the purified laminarinase by Edman degradation. The N-terminal sequence was DTTVTIYNEWLSQGG, and two internal fragments were determined, ILDSTTTIAFHK and TFDYYMQQVHAYGDG. No significant homology was found between any of the above peptide sequences and the various protein sequences available for laminarinases [17–19]. In particular, no information as to the appartenance of the laminarinase of *H. tuberculata* to either family 16 or family 17 could be deduced from the molecular mechanism investigated above since these glycoside hydrolases were both shown to proceed with overall retention of the anomeric configuration [17,18].

In conclusion, we have purified to homogeneity the major laminarinase activity from the gastropodean marine mollusc *H. tuberculata*. It consists of a 60 kDa protein capable of depolymerizing the unbranched portions of the β-(1→3), β-(1→6)-glucan, down to laminaritriose. As the purified protein does not cleave the β-(1→6) linkages, it can be used for the structural analysis of the various laminarins from brown algae and diatoms. It may also be helpful for the preparation of specific β-(1→3)-(1→6)-glucoligosaccharides from laminarin or from fungal cell walls.

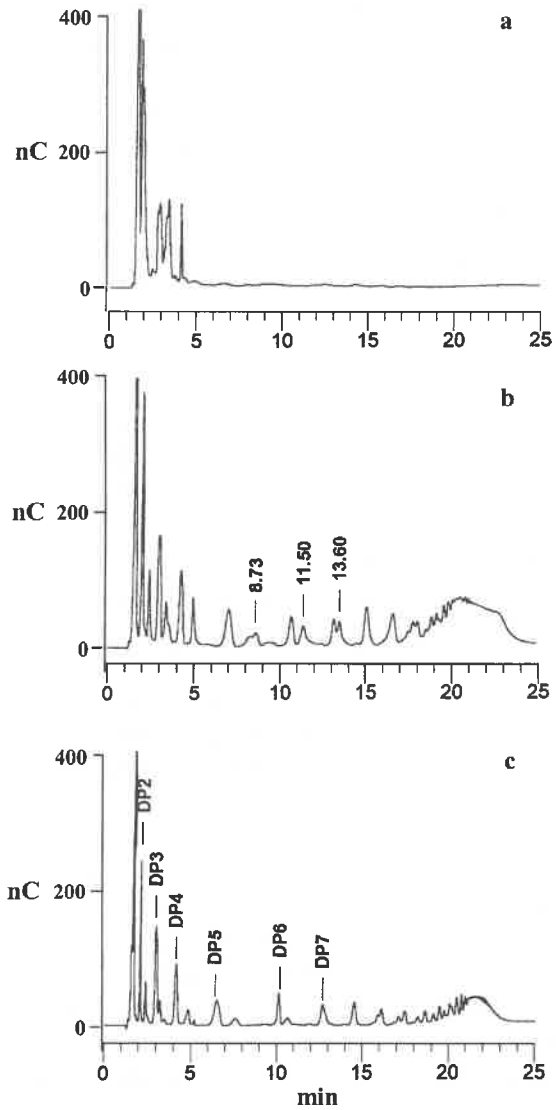

Fig. 4. HPAEC-PAD elution profiles of (a) the end products of laminarin, respectively with the CMC-50 laminarinase fraction and (b) the purified MonoS fraction and of (c) Phycarine® by the MonoS fraction. Hydrolysis was performed for 24 h with an enzyme:substrate ratio of 0.08 U mg^{-1}.

2. Experimental

Materials and methods.—Enzyme and protein assays. Proteins concentrations were measured according to Bradford [28], using the Bio-Rad

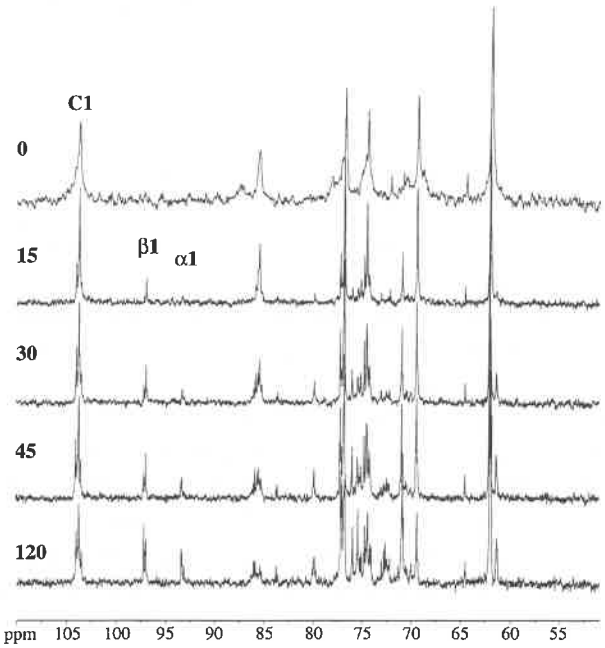

Fig. 5. ^{13}C NMR spectra of laminarin recorded after 0–120 min of hydrolysis by the purified glucanase. C1, β1 and α1 refer to the C-1 of internal glucose residues and of β- and α-glucose residues at the reducing end of laminarin, respectively.

Protein Assay Reagent and bovine serum albumin (Sigma) as standard. Alginate lyase activity was measured with 1–100 μL aliquots by monitoring at 20 °C the absorbance at 235 nm [29] of a 2.0 mL reaction mixture consisting of 0.25% (w/v) sodium alginate from *Macrocystis pyrifera* (Sigma) in 10 mM Tris-MES buffer pH 7.5 supplemented with 100 mM NaCl and 20 mM $MgCl_2$.

Laminarinase activity was determined by the reducing sugars method of Kidby and Davidson [30]. Aliquots (1–100 μL) were incubated at 37 °C for 15 min in a 1.0 mL of 20 mM sodium hydrogen phosphate buffer, pH 7.0, containing 1.0 mg of Phycarine® (Goëmar, France). One unit of enzyme activity (U) is defined as the amount of enzyme which results in an increase of the optical density at 237 nm of 0.1 U min^{-1}. β-(1→3)-Endoglucanase activity was monitored with pachyman, using the β-(1→3)-Glucazyme tablet from Megazyme (Bray, Ireland) while β-glucosidase activity was assayed with *p*-nitrophenyl- or 4-methylumbelliferyl β-D-glucopyranoside from Sigma. The effect of pH on laminarinase activity was investigated with 0.02 M citrate–phosphate buffer (pH 3.0–6.0), 0.02 M MES (pH 5.0, 6.5), 0.02 M phosphate (pH 6.0–8.0) and 0.02 M Tris-HCl (pH 7.0–8.0) and the temperature dependence was determined from 30 to 60 °C at pH 7.0 in 0.02 M phosphate buffer.

Purification of laminarinase and alginate lyase from H. tuberculata.—Ormers (*H. tuberculata*) were collected at Roscoff, Brittany, France and their hepatopancreas were fractionated as described previously by Boyen et al. [31]. Briefly, about 40–50 hepatopancreas were lacerated and homogenized for 1 h at 4 °C in 3 vol of 10 mM sodium hydrogen phosphate buffer pH 6.0, containing 10 mM EDTA (buffer I). Insoluble material was removed by centrifugation (10,000×*g* for 15 min) and the supernatant was fractionated with ammonium sulfate (Merck). The precipitate between 40 and 60% ammonium sulfate saturation was resuspended with 20 vol of buffer I and stirred for 2 h in the presence of 50 mL of CM-Sephadex C-50 beads (Pharmacia). Beads were then extensively washed with buffer I, packed in a 5×30 cm column and eluted with 100 mM sodium hydrogen phosphate buffer (pH 8.0) containing 5 mM EDTA. Fractions were monitored for protein (280 nm) and assayed for laminarinase and alginate lyase activity, which co-eluted under these chromatographic conditions. The active fractions were pooled, concentrated ten times using a 10,000 Da cut-off ultrafiltration membrane (Amicon) and dialysed against buffer I. An aliquot (1 mL) was eluted with buffer I through a Sephacryl HR 300 (Pharmacia) column (1.6×100 cm). The laminarinase and alginate lyase fractions (250 μL) were then diluted four times in 10 mM MES buffer pH 6.0, 5 mM NaCl and chromatographed through a cation exchange column (MonoS, Pharmacia), using a 5 mM–1 M NaCl gradient in 10 mM MES buffer (pH 6.0).

The active fractions were concentrated by ultrafiltration (Microcon, 10,000 Da, Millipore) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) at 30 mA in a 0.75 mm slab gel consisting of a 6% (w/v) bis-acrylamide stacking and a 12.5% running gel. Proteins were stained with Coomassie Blue (Pharmacia) and the molecular mass was determined using the SDS-PAGE low range standard from Bio-Rad. Isoelectric point was measured with a Phast System using a 3–9 IEF Phastgel and a 3–10 IEF calibration kit (Pharmacia). Microsequences from the purified protein were determined by Edman degradation at the Institut Pasteur (Paris, France).

HPAEC of laminarin and oligolaminarins.—Standard *L. digitata* laminarin was obtained from Sigma. Alternatively, laminarin referred to as Phycarine® was prepared as follows. Freshly harvested *L. digitata* sporophytes were extracted with 0.1 M sulfuric acid at 70 °C for 3 h; the supernatant was ultrafiltrated at 300 kDa then concentrated by diafiltration at 3 kDa with deionized water and lyophilized. Oligolaminarin standards, consisting of mixed or separated oligomers DP 1–7, were purchased from Seikagaku, Japan. They were prepared as stock solutions of 0.5 mg mL^{-1} in water then diluted 10 times in 150 mM NaOH before chromatography.

Laminarin and related oligomers were analysed by High Performance Anion Exchange Chromatography and Pulsed Amperometric Detection (HPAEC-PAD), using a Dionex chromatograph in the configuration DX500 (Dionex, Sunnyvale, CA), a 50 μL injection loop, a CarboPac PA100 column (4×250 mm) and an electrochemical detector with a gold working electrode (E1 = 0.05 V, 0.4 s; E2 = 0.75 V, 0.2 s; E3 = −0.15 V, 0.4 s). Elution was performed at 1 mL min^{-1} first isocratically with a 7:3 ratio of 150 mM NaOH (eluent A) and 150 mM NaOH, 500 mM NaOAc (eluent B) for 5 min, followed for 11 min by a linear

gradient up to 60% of eluent B then by a 4 min gradient to reach 100% of eluent B. Laminarinase products were preserved before injection by dilution with 1 vol of 150 mM NaOH.

NMR investigation of the hydrolytic mechanism.—Laminarin (Phycarine®, 75 mg in 200 μL D_2O and 300 μL H_2O) was hydrolyzed by 200 μL of purified laminarinase (40 U) in a 5 mm OD NMR tube at 30 °C. ^{13}C NMR spectra were recorded at 30 °C over periods of acquisition lasting about 15 min each. Recording conditions were: 8 K data points, 80° pulse angle, 0.45 s of recycling time, 1000 scans. Chemical shifts are expressed in ppm from external aceton, set to 31.4 ppm.

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