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Carbohydrate Research 322 (1999) 291–297

CARBOHYDRATE RESEARCH

Note

Degradation of algal (*Ascophyllum nodosum*) fucoidan by an enzymatic activity contained in digestive glands of the marine mollusc *Pecten maximus*

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Received 6 February 1999; revised 3 August 1999; accepted 12 August 1999

Abstract

A protein extract from digestive glands of the marine mollusc *Pecten maximus* was shown to possess fucoidan-degrading activity. This activity was able to release L-fucose from fucoidan derived from the brown algae *Ascophyllum nodosum*, and markedly reduce the molecular size of the polysaccharide. An enzymatically degraded fucoidan was produced and analysed by NMR spectroscopy. The 2D ^{1}H NMR data obtained for the first time on low-molecular-weight fractions of algal fucoidan provided new insight into the structure of the polysaccharide. The latter has a randomly organised structure, involving (1→3)- and (1→4)-linked unsulfated and 2-sulfated-α-L-fucose residues.

Keywords: Fucoidan; Fucosidase; Enzymatic depolymerisation; Fucanolytic activity

The sulfated polysaccharide fucoidan from brown algae exhibits biological activities in mammalian systems very promising for human therapeutics [1–4]. Algal fucoidan is mainly composed of sulfated fucose, but its molecular structure remains to be established, as the type of glycosidic bond and the distribution of the sulfate groups are still debated [5–8]. The polydisperse and high molecular weight of the polysaccharide as well as its molecular heterogeneity limited the structural studies. Low-molecular-weight fucoidan, which is easier to characterise, will be of great value for structural investigations. Enzymatic degradation of polysaccharides is known to produce oligosaccharides under mild conditions that do not alter the overall structure [9], but, to date, no fucoidan-specific degrading enzymes are available. Our purpose was to find a source of depolymerising enzymatic activities, which could be used without further extensive purification for the degradation of algal fucoidan. We report data showing that digestive glands of the common marine mollusc *Pecten maximus* are an effective and facile source of such activities. We show that the resulting enzyme extract is active on algal fucoidan, and we describe its use to signifi-

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0008-6215/99/$ - see front matter
PII: S0008-6215(99)00223-2

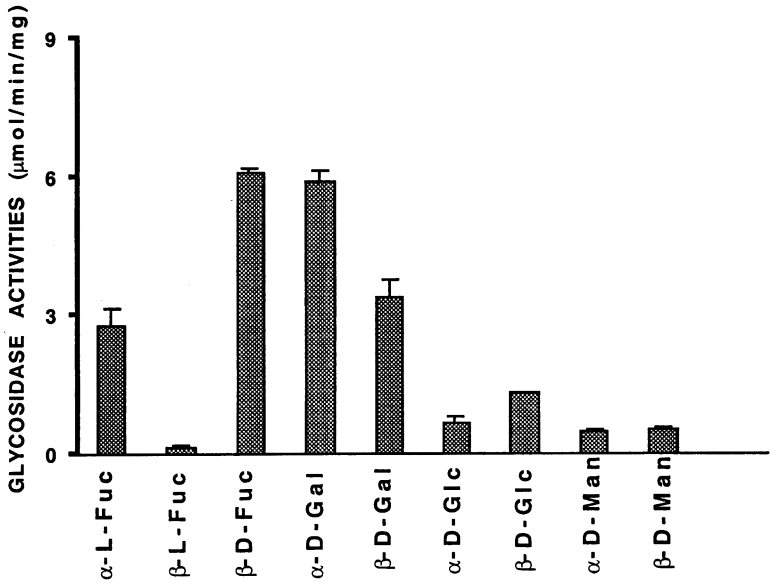

Fig. 1. Glycosidase activities contained in protein extract from *P. maximus* digestive glands. The following *p*-nitrophenyl (PNP) substrates were used: PNP-α-L-fucose (α-L-Fuc), PNP-β-L-fucose (β-L-Fuc), PNP-β-D-fucose (β-D-Fuc), PNP-α-D-galactose (α-D-Gal), PNP-β-D-galactose (β-D-Gal), PNP-α-D-glucose (α-D-Glc), PNP-β-D-glucose (β-D-Glc), PNP-α-D-mannose (α-D-Man), PNP-β-D-mannose (β-D-Man). Activity was determined from the OD at 405 nm of the released PNP (average values of five determinations per PNP substrate) as described in Section 1.

cantly reduce the size of the polysaccharide, providing low-molecular-weight fractions that we submitted to a first structural study.

Protein extract from digestive glands of *P. maximus* contained high levels of glycosidases, which were tested using several synthetic *p*-nitrophenyl substrates (Fig. 1). The fucosidase and galactosidase were the two major glycosidase activities present. L-Fucosidase activity exhibited specificity toward the α-glycosidic bond, while D-galactosidase activity was observed for both α- or β-D-galactose. An optimal pH of 5.5 and an optimal temperature of 50 °C were determined for the α-L-fucosidase activity; no loss of activity was observed when the enzyme extract was incubated for several days at 30 °C. The fucosidase activity is thus highly stable, as described in the literature for others glycosidases [10].

As the digestive glands of the marine mollusc *P. maximus* are an effective source of fucosidase, the enzyme extract was then incubated with fucoidan from *A. nodosum* in order to test its ability to hydrolyse the sulfated polysaccharide. Analysis of neutral monosaccharides by HPLC on an amino column with pulsed amperometric detection showed that only fucose was released during the incubation. The formation of free L-fucose during the incubation was confirmed by the L-fucose dehydrogenase (FDH) spectrophotometric assay (Fig. 2). The enzyme extract thus contained a fucosidase active on algal fucoidan. Monosaccharide was not detected by either method in incubation medium containing either fucoidan alone or fucoidan plus heat-de-

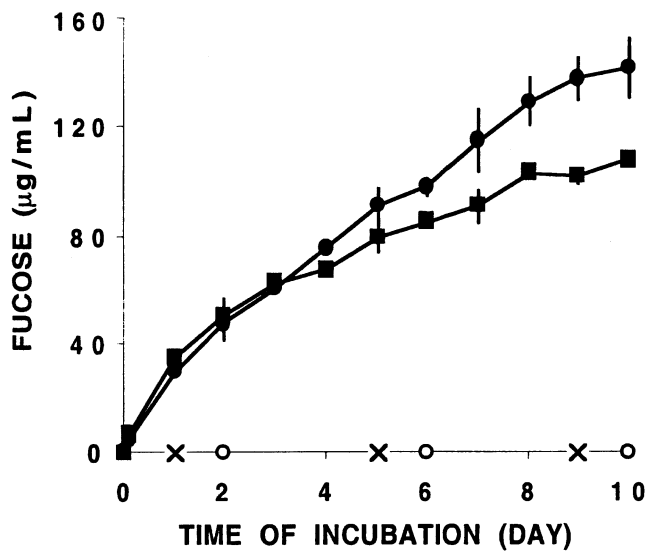

Fig. 2. Measurement of fucose by HPLC (●) and spectrophotometric (■) methods during incubation of fucoidan (1 mg/mL) with protein extract (5% v/v) from *P. maximus* digestive glands. Controls were fucoidan incubated alone (○) and fucoidan incubated with heat-denatured protein extract (×).

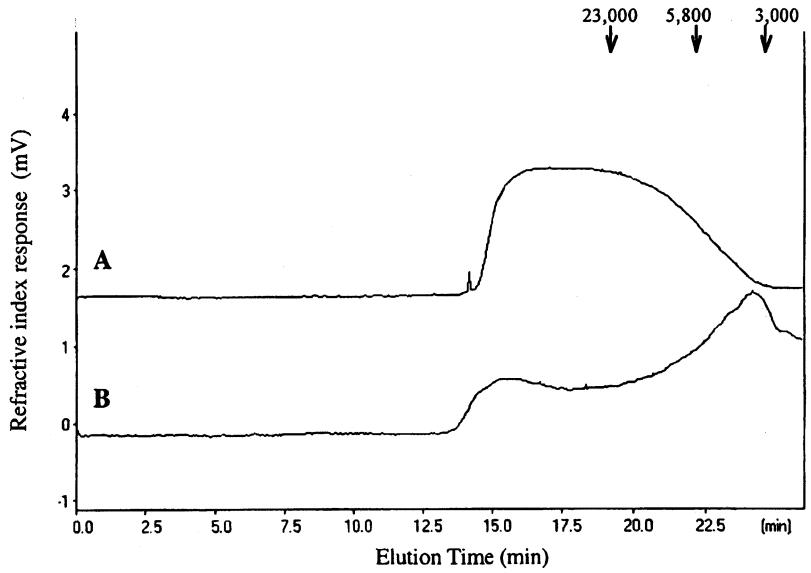

Fig. 3. High-performance size-exclusion chromatograms (TSK G2000 SWXL column, flow rate 0.5 ml/min) of fucoidan (A) before and (B) after 7 days of incubation with protein extract from *P. maximus* digestive glands. Arrows indicate the positions of elution of molecular-weight standards (pullulans).

natured enzyme extract. α-L-Fucosidase activity on *p*-nitrophenyl α-L-fucose, measured on aliquots from the reaction medium, was remarkably stable throughout the incubation in agreement with the aforementioned glycosidase stability. The extent of the enzymatic degradation of fucoidan was then analysed by high-performance size-exclusion chromatography (HPSEC). A dramatic decrease of the apparent molecular weight of the polysaccharide was observed upon incubation, from 25,000 for the starting fucoidan to about 3000 for the degraded fucoidan (Fig. 3). No degradation was observed for the controls (fucoidan alone and fucoidan plus heat-denatured extract) under the same conditions. Therefore, an efficient enzymatic degradation of fucoidan occurred, which was also demonstrated by electrophoresis analysis of the sulfated polysaccharide on polyacrylamide gel (data not shown). The amount of produced reducing ends measured as equivalent fucose was 10% of the total fucose contained in fucoidan after 5 days of incubation. Half of this amount resulted from free fucose formed during the enzymatic degradation as determined by HPLC and by the FDH assay. Therefore in addition to the fucosidase activity, a depolymerising enzymatic activity acting by endolytic cleavage of the macromolecular chain must be inferred to explain the important decrease of molecular weight associated with the release of a small amount of reducing sugar.

The enzymatically degraded fucoidan of $M_r \approx 3000$ were then prepared by gel-filtration chromatography and analysed by ^{1}H NMR. The 500 MHz ^{1}H spectrum (not shown) of the starting fucoidan exhibited broad, complex signals as expected for a such high-molecular-weight and heterogeneous polymer. Still two distinct parts of the spectrum characteristic of the fucoidan were easily located, i.e., the anomeric proton signals at 5.0–5.6 ppm and the methyl signals at 1.2–1.4 ppm. These signals were consistent with the presence of α-L-fucose as the constitutive unit of the polysaccharide. The spectra obtained for the enzymatically degraded fucoidan were still complex but much more resolved, allowing structural investigation through 2D ^{1}H–^{1}H correlated spectroscopy. The DQF-COSY spectra (Fig. 4) and the TOCSY spectra (not shown) illustrated the heterogeneity of the algal fucoidan regarding the position of the glycosidic linkage and the sites of sulfation on the fucose unit. For instance, at least 16 different fucose H-1 signals and 16 different H-5–H-6 pairs were distinguishable on the spectra. Anomeric proton signals were spread from 5.0 to 5.6 ppm, including signals at 5.5 and 5.6 ppm that have no counterpart in any of the

echinoderm fucoidans described previously [11]. A vicinal constant of 3.6 Hz characteristic of an α-fucopyranosyl structure was determined for all of these anomeric signals.

The ^{1}H chemical shifts are characteristic of the position of the sulfate substitution. The chemical shifts of protons at sulfated positions are typically 0.6–0.7 ppm downfield of the equivalent proton at an unsulfated position [11]. Cross-peaks from the anomeric α-H-1 were observed in COSY spectra at 5.0–5.2 ppm with correlated H-2 at 3.7–4.0 ppm, and at 5.3–5.5 ppm with correlated H-2 at 4.45–4.7 ppm (Fig. 4). The latter anomeric signals shifted downfield could then be assigned to α-L-fucose residue sulfated at C-2, and the former to α-L-fucose residue unsulfated at C-2.

TOCSY spectra gave connectivities for H-1 through to H-4 and for H-5–H-6. The coupling constant between H-4 and H-5 is too small in the case of fucose residue, so that H-4–H-5 cross-peaks could not be seen. H-3 chemical shifts were observed at 4.15–4.35 ppm, as expected for α-L-fucose residues. However, these H-3 chemical shifts could not be considered as indicative of the sulfation status of the carbons C-2 and C-4 nor of the glycosidic linkage. H-4 chemical shifts were observed at 4.0–4.15 ppm corresponding to a C-4 position, which is not sulfated. H-4 signals were described at 4.7–4.8 ppm when position 4 is sulfated [11,12]. A sulfated C-4 position is not excluded here as HOD presaturation suppressed the signal at the corresponding chemical shift region.

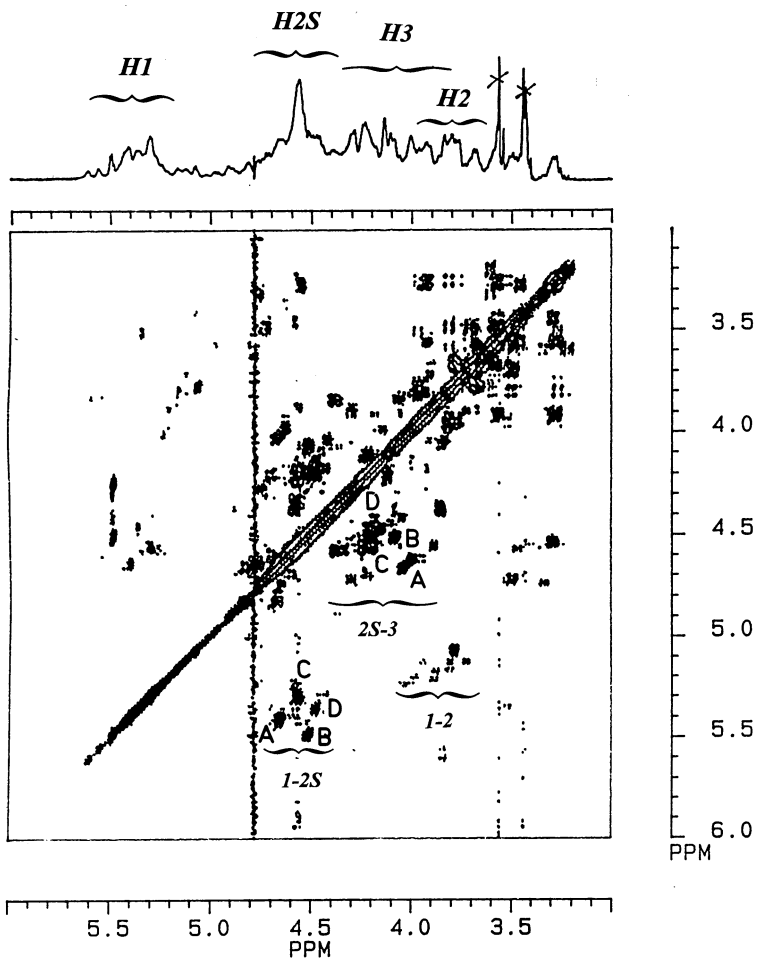

Fig. 4. Expansion of the 6.0–3.0 ppm region of the DQF-COSY spectrum at 500 MHz of the enzymatically degraded fucoidan. The spectrum was recorded at 25 °C in D_2O. The HOD signal (4.8 ppm) was suppressed by presaturation. Signals marked × arise from contaminants. Four distinct anomeric H-1 groups (A, B, C and D) and some cross-peaks between the vicinal protons H-1–H-2 and H-2–H-3 are indicated (signals designated by 2S refer to a sulfated C-2 of the fucose unit).

H-6 (methyl) signals were observed as two groups of equal proportions by integration, respectively located at 1.2–1.25 ppm and at 1.3–1.39 ppm. Correlated signals for protons H-5 were observed at 4.2–4.7 ppm for the two groups. H-6 chemical shifts at 1.2–1.25 ppm are consistent with α-L-fucose residues joined by a (1→3) linkage, while those at 1.3–1.4 ppm are similar to H-6 of (1→4)-linked α-L-fucose residues as recently reported [13]. Cross-peaks were noticed in NOESY spectra (not shown) from H-1 (5.3 ppm) to H-6 (1.38 ppm). This NOE pattern is indicative of (1→4) linkages rather than (1→3) linkages for those residues [11,13]. Consequently, all these structural data indicated that the algal fucoidan from *A. nodosum* contained sequences of α-(1→3)- and α-(1→4)-L-fucose. Both unsulfated and sulfated fucose units occurred with sulfate groups on C-2 and possibly C-4.

In conclusion, an efficient fucanolytic activity was evidenced in this study through chromatographic analysis and determination of the reducing power. This fucoidan-degrading activity contained in the digestive glands of *P. maximus* was well suited for the production of low-molecular-weight fractions of fucoidan. Previously reported fucoidan-degrading enzymes were fucosidase, which is useless for production of oligosaccharides [14], or only a low level of fucanolytic activity leading to a very limited decrease of the molecular weight of the sulfated polysaccharide [15]. Consequently, these fucoidan-degrading enzymes were not suitable for structural studies. Here the enzymatic degradation of fucoidan led to a fraction of low molecular weight, i.e., $\leq$ 5000. These low-molecular-weight fractions were then purified for structural studies.

This is the first report of a NMR investigation on oligosaccharides from algal fucoidan. A structural model for fucoidan extracted from the brown algae *Fucus vesiculosus* has been proposed by Patankar et al. based on methylation analysis [8]. This model consisted of α-(1→3)-linked L-fucose backbone with α-(1→4)-linked L-fucose branches and sulfate group mainly on C-4. Furthermore, the isolation of 2-sulfo-α-L-fucopyranose from *F. vesiculosus* fucoidan [16] has been reported recently. Our results from NMR spectroscopy analysis of *A. nodosum* fucoidan are in agreement with this model as regards the glycosidic linkages, since we propose a structure with α-(1→3)/α-(1→4)-L-fucose. Our data unambiguously indicated sulfate group on C-2; a sulfation on C-4 is not excluded. Finally, a structure could be drawn consisting of 2- or 4-sulfated-α-L-fucose and of some unsulfated (1→3)- and (1→4)-linked α-L-fucose residues. This is an average structure, as it appears from our data that algal fucoidan is randomly organised unlike echinoderm fucoidans [11].

1. Experimental

Chemicals.—*p*-Nitrophenyl glycosides and fucose dehydrogenase were purchased from Sigma (France). Fucoidan (M_r 25,000, fucose 47 g/100 g, sulfate 30 g/100 g, uronic acid 6 g/100 g) used in this study was extracted from the brown algae *Ascophyllum nodosum* and purified as previously published [17,18]. HPLC grade MeCN was from Carlo Erba (France). Sodium hydroxide (99.99% semiconductor grade) used for pulsed amperometric detection was obtained from Aldrich (France). Standard polysaccharides, pullulans and dextrans, were from Interchim (France). Others chemicals and reagents were obtained from commercial sources at the highest level of purity available.

Preparation of enzyme extract.—The marine molluscs *P. maximus* were provided by IFREMER (Brest, France). Digestive glands of *P. maximus* were homogenised, and proteins were extracted by fractionated ammonium sulfate precipitation according to a previously described procedure [19]. Lipids were removed from the resulting protein solution by a washing step with 30% (v/v) EtOAc. The aq phase, which contained all the enzymatic activities of interest, was recovered by centrifugation and dialysed against 0.1 M acetate buffer, pH 5.5. The dialysed protein solution constituted the enzyme extract used in this study for the degradation of fucoidan. A yield of 3 mg protein per g of fresh digestive glands was usually obtained, with a specific activity ranging from 15 to 20 nmol of reducing sugars equivalent fucose per min and mg

of protein. Protein concentrations were determined by the Bio-Rad dye-binding method with BSA as the standard [20].

Enzymatic degradation of fucoidan.—The enzyme extract (5% v/v) was incubated at 30 °C in 0.1 M acetate buffer, pH 5.5, containing 1 mg/mL fucoidan. The reaction mixture was slightly stirred during the incubation (up to 10 days). Monosaccharides formed upon the enzymatic degradation of fucoidan were analysed by HPLC with a pulsed amperometric detection, using an Astec polymeric amino column (25×0.46 cm) fitted with a guard column (1×0.46 cm) (Interchim, France). Elution was performed at 0.7 mL/min with 0.1 μm filtered and degassed 3:2 MeCN:water mobile phase. A pneumatic post-column delivery system (Dionex, France) added 0.3 M NaOH (at 50 psi with helium to pressurise) to the column effluent before an electrochemical detection cell (ESA 5040, Eurosep, France). The following pulse potentials were applied to the working gold electrode by a Coulochem 2 module (Eurosep, France): $E_1 = 0.16$ V (t_1 700 ms, acquisition delay = 650 ms); $E_2 = +0.7$ V (t_2 100 ms); $E_3 = -0.6$ V (t_3 100 ms). The amount of fucose released from fucoidan during the incubation was determined by the L-fucose dehydrogenase spectrophotometric method [21] as follows: 200 μL aliquots were removed from the reaction mixture and mixed with 800 μL 0.1 M borax buffer, pH 9.5, containing 725 μM NADP and 0.2 unit of fucose dehydrogenase. The optical density was recorded at 340 nm and the concentration of fucose was deduced from the final absorbance. The extent of the enzymatic degradation was analysed by the measure of the reducing sugars using the method of Kidby and Davidson [22] and by HPSEC. The reducing ends were determined from 80 μL of the reaction mixture diluted to 1 mL with a 7.6 mM potassium ferricyanide solution. The increase of the optical density was measured at 237 nm and the amount of the reducing sugars was expressed as fucose equivalent. HPSEC was performed as previously described [23] using a TSK G2000 SWXL column (30×0.78 cm) equipped with a guard column, and connected to a Gilson 132 RI refractometer. The column was calibrated with standard polysaccharides, pullulans and dextrans ($M_r = 1500$–$853{,}000$), and eluted with 0.05 M phosphate buffer, 0.15 M NaCl, pH 7.3, at a flow rate of 0.5 mL/min.

Fucosidase assays.—Fucosidase activity was routinely assayed with *p*-nitrophenyl-α-L-fucose as substrate using the spectrophotometric determination of *p*-nitrophenol at 405 nm [24]. The reaction was initiated by the addition of enzyme extract (50 μL) in 1 mL of 0.1 M acetate buffer, pH 5.5, containing 3.5 mM substrate. The reaction was carried out at 50 °C for 10 min, then was stopped by one tenth dilution of reaction mixture in 0.1 M borate buffer, pH 9.5, and absorbance at 405 nm was measured. The other *p*-nitrophenyl glycosides were assayed in the same conditions. Enzyme-specific activity was defined as the number of nanomoles of *p*-nitrophenol released per minute per milligram of protein. The effect of pH on fucosidase activity was investigated with 0.1 M acetate buffer (pH 4.5–5.5), 0.1 M phosphate (pH 5.5–7.5), 0.1 M Tris–HCl (pH 7–8.5), and the temperature dependence was determined from 20 to 70 °C at pH 5.5 in 0.1 M acetate buffer.

Gel-filtration chromatography.—Degraded fucoidan was purified from the hydrolysis mixture by gel-filtration chromatography on a Sephacryl S-100 (Pharmacia, France) column (21×5 cm) equilibrated and eluted with 0.2 M ammonium acetate buffer, pH 5.5 (flow rate 0.5 mL/min). The fucoidan fractions were pooled according to their molecular weight and freeze dried. The low-molecular-weight fractions from the purified degraded fucoidan were prepared by gel-filtration chromatography on a Bio-gel P2 (Bio-Rad, France) column (90×1.6 cm) equilibrated and eluted with the same ammonium acetate buffer (flow rate 6.6 mL/h).

NMR spectroscopy experiments.—^{1}H NMR spectra were recorded at 500 MHz using a 500 AM Bruker spectrometer. The sulfated polysaccharide samples (10–20 mg) were exchanged twice with 99.8% D_2O (Sigma, France) with intermediate lyophilisation and dissolved in 0.6 mL of 100% D_2O (Sigma, France). Spectra were recorded at 25 °C with suppression of the HOD signal by presaturation. Chemical shifts are reported in ppm us-

ing sodium 3-trimethylsilylpropanoate (δ_H: 0.00) as internal reference.

$^1H-^1H$ COSY, $^1H-^1H$-relayed COSY spectra were performed using standard pulse sequences and data were recorded in magnitude mode. Two-dimensional double-quantum filtered COSY (DQF-COSY), TOCSY and NOESY experiments were recorded in the phase-sensitive mode. TOCSY spectra were run with a spin-lock field of about 8 kHz and a mixing time of 230 ms; NOESY spectra were run with a mixing time of 300 or 600 ms.

Acknowledgements

We are indebted to Dr P. Roy (IFREMER, France) for technical assistance in providing the digestive glands from *P. maximus*. We thank Dr B. Mulloy (NIBSC, UK) for useful discussions and Dr D. Letourneur (CNRS, France) for critical reading of the manuscript.

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