Carbohydrate Research 343 (2008) 2393–2400

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Catalytic properties and mode of action of *endo*-(1→3)-β-D-glucanase and β-D-glucosidase from the marine mollusk *Littorina kurila*

Maria S. Pesentseva, Mikhail I. Kusaykin *, Stanislav D. Anastyuk, Victoria V. Sova, Tatyana N. Zvyagintseva

Pacific Institute of Bioorganic Chemistry, Far East Branch of Russian Academy of Sciences, 690022 Vladivostok, 159 Prospect 100 let Vladivostoku, Russia

ARTICLE INFO

Article history:
Received 7 April 2008
Received in revised form 26 June 2008
Accepted 28 June 2008
Available online 6 July 2008

Keywords:
Marine mollusk
(1→3)-β-D-Glucan
(1→3)-β-D-Glucanase
β-Glucosidase
Mode of action

ABSTRACT

A complex of the enzymes from the liver of the marine mollusk *Littorina kurila* that hydrolyzes laminaran was investigated. Two (1→3)-β-D-glucanases (G-I and G-II) were isolated. The molecular mass of G-I as estimated by gel-permeation chromatography and SDS–PAGE analysis was 32 and 40 kDa, respectively. The G-II molecular mass according to SDS–PAGE analysis was about 200 kDa. The pH optimum for both G-I and G-II was pH 5.4. The G-I had narrow substrate specificity and hydrolyzed only the (1→3)-β-D-glucosidic bonds in the mixed (1→3),(1→6)- and (1→3),(1→4)-β-D-glucans down to glucose and glucooligosaccharides. This enzyme acted with retention of the anomeric configuration and catalyzed a transglycosylation reaction. G-I was classified as the glucan *endo*-(1→3)-β-D-glucosidase (EC 3.2.1.39).

G-II exhibited both *exo*-glucanase and β-D-glucoside activities. This enzyme released from the laminaran glucose as a single product, but retained the anomeric center configuration and possessed transglycosylation activity. The hydrolysis rate of glucooligosaccharides by G-I decreased with an increase of the substrate's degree of polymerization. In addition to (1→3)-β-D-glucanase activity, the enzyme had the ability to hydrolyze *p*-nitrophenyl β-D-glucoside and β-D-glucobioses: laminaribiose, gentiobiose, and cellobiose, with the rate ratio of 50:12:1. G-II may correspond to β-D-glucoside glucohydrolase (EC 3.2.1.21).

1. Introduction

Laminarans, the storage polysaccharides of marine brown algae and diatoms, consist of linear mannitol or glucose-ended chains of β-(1→3)-linked glucose residues, with occasional β-(1→6)-linked branches. Laminarans are low-molecular-mass (1→3),(1→6)-β-D-glucans whose heterogeneity depends on differences in molecular masses and on both the localization and content of β-(1→6)-linked glucose residues.[1–3] Thus, laminaran from *Laminaria hyperborea* is a practically linear (1→3)-β-D-glucan, and a laminaran from *Laminaria saccharina* has about two β-(1→6)-branches per molecule.[4] β-(1→6)-Linked glucose residues, however, can be included in the main chain. For example, the *Eisenia bicyclis* laminaran has a block structure: the β-(1→6)-linked glucose residues are included in the chain of β-(1→3)-glucan in a ratio of 1→3:1→6 = 3:1.[5] Laminarans from the *Ishige okamurai* and *Chorda filum* also have a block structure with branches at C-6.[1,6] Finally, the polysaccharide from the calcium alga *Emiliania huxleyi* has a β-(1→6)-linked glucose chain and gentiobiose as branches at C-3 (1→3:1→6 = 2:3).[7] Laminarans possess various pharmacological activities: antitumor, immunomodulating, and radioprotective activity.[8,9]

In this context, (1→3)-β-D-glucanases (laminarinases) of known specificity and mode of action are important tools for study on laminaran and other β-D-glucan structures. Furthermore, enzymatic transformation of laminarans yields a new biologically active (1→3),(1→6)-β-D-glucan.[10] Laminarinases are widespread in bacteria, fungi, plants, and in marine micro- and macroorganisms. Functions of the enzymes are diverse. In bacteria they participate in the pathogenic digestion of cell walls. In higher plants they cleave glucans in seeds and act as inducible defense enzymes. They are involved in autocatalysis of extracellular matrix glucans in fungi and also in yeast cell development. In the animal kingdom, laminarinases are commonly found in marine invertebrates, where they take part in digestion of algal food and play an important role in embryogenesis. The amount of research on marine invertebrate laminarinases is extremely small.

Laminarinases are enzymes that catalyze degradation of β-D-glucan chains by two mechanisms. Exo-action enzymes (EC 3.2.1.58) sequentially split off the terminal bonds, yielding only D-glucose[11] or one of the oligosaccharides, for example, laminaripentaose,[12] as the main hydrolysis product, whereas *endo*-action enzymes (EC 3.2.1.39) hydrolyze the internal linkages in laminaran to release a set of oligosaccharides as the main reaction products. Depending on substrate recognition, two classes of *endo*-(1→3)-β-D-glucanase are known: (1) those that require the presence of at least two adjacent (1→3)-linked β-D-glucosyl residues (EC

* Corresponding author.
E-mail address: mik@piboc.dvo.ru (M. I. Kusaykin).

0008-6215/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.06.025

3.2.1.39),[13] and (2) less-specific enzymes (EC 3.2.1.6) that can also hydrolyze β-(1→4) linkages adjacent to (1→3)-linked β-D-glucosyl residues as in lichenans.[14]

Based on amino acid sequence and folding similarities, *endo*-(1→3)-β-D-glucanases fall into at least five distinct structural families of glucoside hydrolases: 16, 17, 55, 64, and 81.[15] To our knowledge, amino acid sequences were reported for only three metazoan (1→3)-β-D-glucanases: the enzyme from the eggs of the sea urchin *Strongylocentrotus purpuratus*,[16] *endo*-(1→3)-β-D-glucanases from marine bivalve mollusks *Spisula sachalinensis*[17] and *Mizuhopecten yessoensis*.[18]

In this work, we report the purification, properties and mode of action of enzymes from the gastropodean marine mollusk *Littorina kurila* that catalyze the hydrolysis of laminaran.

2. Results and discussion

It has been shown that marine invertebrates, represented by a great number of types and species at various stages of evolution, are rich sources of carbohydrases. Some species, especially mollusks, produce active (1→3)-β-D-glucanases (laminarinases).[19–22]

The enzymes catalyzing the hydrolysis of laminaran were isolated from the liver of the gastropodean marine mollusk *L. kurila*. Two peaks of laminarinase activity (major peak G-I and minor peak G-II) were separated by DEAE-cellulose column chromatography. Each of the enzyme forms was purified to electrophoretic homogeneity, followed by gel filtration on Sephacryl S-100 (Sephacryl S-300) and anion-exchange chromatography with TSK: DEAE-5PW (Tables 1 and 2). As a result of the purification procedure, we obtained a preparation of G-I with specific activity (laminaran as substrate) 0.285 U/mg protein (yield of the enzyme was 3.6%). A purified preparation of G-II had a specific activity of 2.4 U/mg protein (*p*-nitrophenyl β-D-glucopyranoside substrate; yield, 0.9%).

The molecular mass of G-I according to SDS–PAGE analysis was 40 ± 1 kDa (Fig. 1). The SDS electrophoresis showed some minor bands (>3%). Analytical gel filtration G-I on a Superdex 75 HR 10/30 column (FPLC) showed a single symmetrical peak of laminarinase activity, corresponding to 32 ± 5 kDa.

Activity of the laminarinase G-I was observed over a pH range of 4.5–7.5 with a maximum value at pH 5.4 in 0.2 M citrate–phosphate buffer. Note that the optimum pH values for laminarinases from the marine invertebrates are in slightly acidic[19–21] or neutral pH range.[22] Activity of laminarinase G-I was maximal at 40 °C and was inactivated by temperatures over 45 °C.

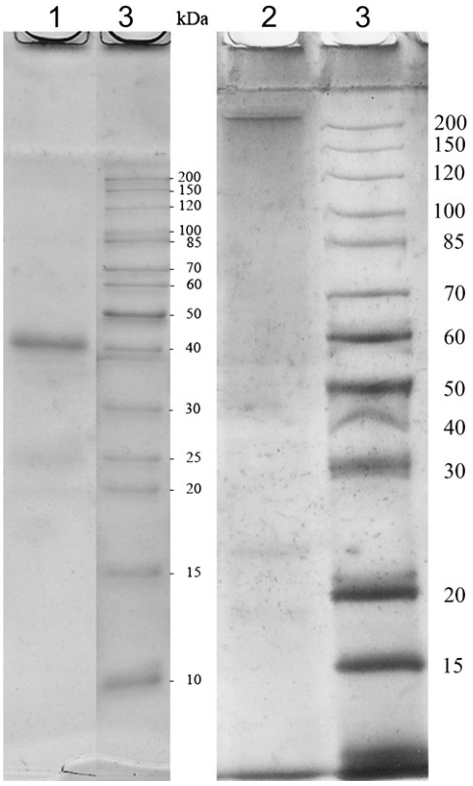

Figure 1. SDS–PAGE of *L. kurila* laminarinases: G-I (1), G-II (2), the molecular mass markers (3).

The K_m values of G-I in the reaction with *L. cichorioides* laminaran as substrate (0.13 mg/mL) were close to those formerly found for other (1→3)-β-D-glucanases.[11,18]

Using a series of glucans with different types of bonds (1→3)-β-D-glucanase G-I was estimated to be specific to β-1→3-bonds in β-D-glucans (Table 3). Glucanase G-I hydrolyzed soluble substrates

Table 1
Purification of a laminarinase G-I

Step	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Purification factor	Yield (%)
Extraction	1174	4.66[b]	0.004	1	100
DEAE-cellulose Batch variation	190	3.14	0.016	4.0	67.4
DEAE-cellulose chromatography	99.6	2.23	0.022	5.5	47.9
Ultrafiltration	38.5	1.56	0.040	10.0	33.5
Sephacryl S-100 chromatography	6.4	0.47	0.073	18.2	10
TSK: DEAE-5PW chromatography	0.6	0.17	0.285	71.3	3.6

[a] Laminaran as substrate.
[b] Summation of the activity of G-I and G-II.

Table 2
Purification of a laminarinase G-II

Step	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Purification factor	Yield (%)
Extraction	1174	55	0.05	1	100
DEAE-cellulose Batch variation	201.6	23	0.11	2.2	41.8
DEAE-cellulose chromatography	62.3	16.5	0.26	5.2	30
Ultrafiltration	32	11	0.34	6.8	20
Sephacryl S-300 chromatography	2.3	1	0.43	8.6	1.8
TSK: DEAE-5PW chromatography	0.2	0.48	2.4	48	0.9

[a] *p*-Nitrophenyl β-D-glucopyranoside as substrate.

Table 3
Relative hydrolysis rates of substrates by G-I and G-II from *L. kurila*

Substrate	Type of bond	M_r (kDa)	Relative hydrolysis rate (%)	
			G-I	G-II
Laminaran	β-1,3; β-1,6 90:10	5–6	100	100
Translam	β-1,3; β-1,6 75:25	8–10	86	80
Periodate-oxidized laminaran	β-1,3; β-1,6 90:10	5–6	95	0
Yeast glucan	β-1,3; β-1,6 90:10	>200	0.24	0
Pachyman	β-1,3; β-1,6 98:2	50–120	0.18	0
CM-pachyman	β-1,3; β-1,6 98:2	>200	0.54	0
Zymosan	β-1,3; β-1,6	>200	0	0
Aubasidan	β-1,3; β-1,6 50:50	500–550	0.17	0
Lichenan	β-1,3; β-1,4 70:30	10–74	0.17	0.06
Xylan	β-1,4	10–20	0	0
Amylopectin	α-1,4	2000	0	0
Pustulan	β-1,6	30	0	0
CM-cellulose	β-1,4	>2000	0	0
p-Nitrophenyl-β-D-glucopyranoside	β	0.301	0	1000

(laminaran and translam) at high rates. The rates of cleavage of high-molecular-mass and partly soluble substrates (yeast glucan, pachyman, CM-pachyman, aubasidan, and lichenan) were low. The enzyme did not digest zymosan, xylan, amylopectin, pustulan, or CM-cellulose. The differences in the rate of hydrolysis of laminaran and translam can be related to structural features of these glucans. Translam, obtained by enzymatic transformation of natural laminaran, contains in comparison with laminaran more β-1→6-bonded glucose residues, which are not only the branches, but are also included in the main chain of the glucan. β-1→6-Bonded glucose residues are concentrated on the nonreducing end of the translam molecule.[10] In laminaran molecules branches are fairly evenly distributed throughout the (1→3)-β-glucan chain.[2] Probably a high concentration of β-(1→6)-bonded glucose residues on the nonreducing end of the translam molecule slows down the rate of its digestion by enzymes in comparison with laminaran. At the same time laminarinase G-I hydrolyzed periodate-oxidized laminaran (modification of the terminal glucose unit of the nonreducing end) at the same rate as natural laminaran that is characteristic of *endo*-enzymes.

The MALDI-TOF mass spectra of the final hydrolysis products of laminaran by G-I revealed the presence of glucose and different oligosaccharides (Fig. 2). They are Glc ($[M+Na]^+$ at $m/z = 203.3$), Glc_2 ($[M+Na]^+$ at $m/z = 365.2$), Glc_3 ($[M+Na]^+$ at $m/z = 527.2$), Glc_4 ($[M+Na]^+$ at $m/z = 689.2$), Glc_5 ($[M+Na]^+$ at $m/z = 851.3$), Glc_6 ($[M+Na]^+$ at $m/z = 1013.5$), Glc_7 ($[M+Na]^+$ at $m/z = 1175.7$), Glc_8 ($[M+Na]^+$ at $m/z = 1338.0$), Glc_9 ($[M+Na]^+$ at $m/z = 1501.0$), Glc_{10} ($[M+Na]^+$ at $m/z = 1663.1$), Glc_{11} ($[M+Na]^+$ at $m/z = 1825.5$), Glc_{12} ($[M+Na]^+$ at $m/z = 1987$). According to HPLC quantitative analysis, glucose and laminaritriose were the major products of the reaction (Fig. 3). Based on the analysis of the reaction products, *endo*-(1→3)-β-D-glucanases that degrade (1→3)-β-D-glucans down to oligosaccharides of high molecular mass are classified as larger-oligosaccharide-producing types of enzymes.[23] (1→3)-β-Glucanases that hydrolyze (1→3)-β-D-glucans in an endo manner yielding glucose, laminaribiose, and other small oligosaccharides as end products are classified as smaller-oligosaccharide-producing types of enzymes.[24] So, G-I apparently catalyzes hydrolysis of laminaran as an *endo*-enzyme like laminarinases from the marine mollusks

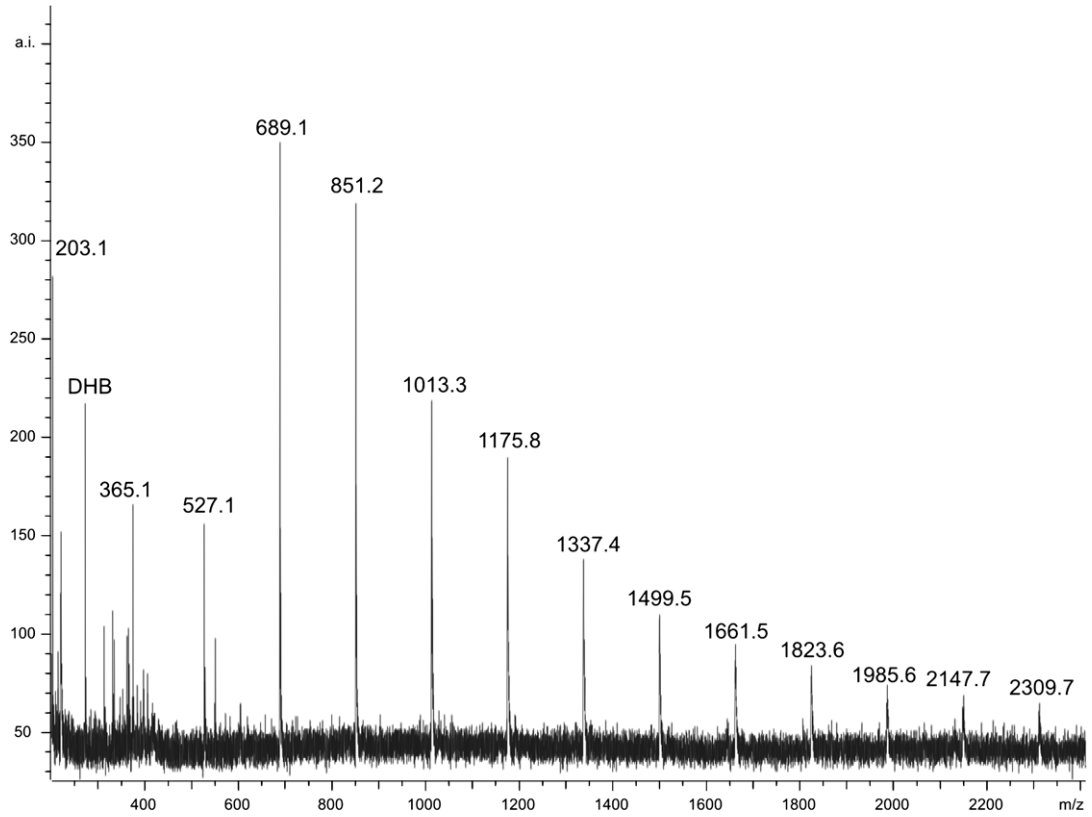

Figure 2. MALDI-TOF mass spectrum of the final products of laminaran hydrolysis by G-I.

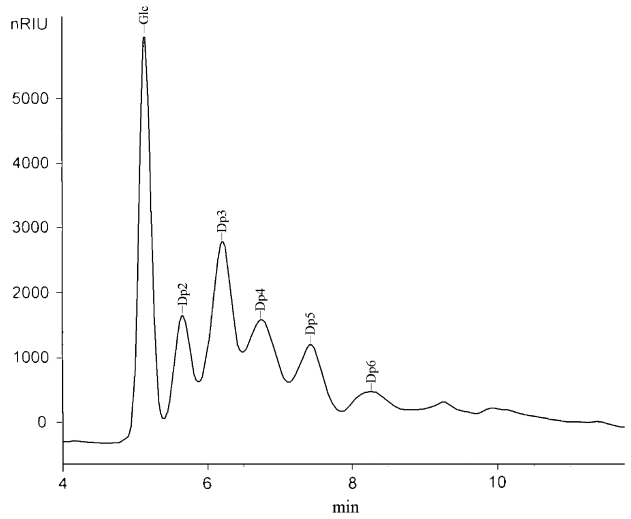

Figure 3. HPLC elution profile of the final products of laminaran hydrolysis by G-I.

H. tuberculata,[22] *S. sachalinensis*,[25] and *Chlamys albidus*[19] and correspond to the smaller-oligosaccharide-producing type of enzyme.

The configuration of products formed during hydrolysis of laminaran was determined by the optical rotation and NMR spectroscopy of the reaction mixture. The optical rotation observed upon addition of G-I did not change practically in course of reaction (from $[\alpha]_D$ −12.7 to −10.6). The negative rotation was maintained after addition of NH_4OH also. It is most likely that β anomers of oligosaccharides were formed in the initial stage of the reaction, and enzymatic cleavage proceeded with retention of configuration. These data are confirmed by NMR experiments (Fig. 4). The very early appearance upon hydrolysis of the β-glucose anomeric signals (97.2 ppm) indicated that enzymatic cleavage proceeded with retention of configuration.

Our previous studies showed that *endo*-(1→3)-β-D-glucanases from sea mollusks, in comparison to other carbohydrases, display high transglycosylation activity.[26] The ability of the enzymes to catalyze a transglycosylation reaction is the typical reaction of retaining glycosyl hydrolyses. Transglycosylating enzymes are traditionally considered to be good tools for the enzymatic synthesis of new substances.[27,28] The purified *endo*-(1→3)-β-D-glucanase G-I possessed transglycosylation activity, that is, it catalyzed the transfer of glyconic parts of the substrate to the hydroxyl-containing acceptors. Laminaran was used as donor while methanol, methyl β-D-glucopyranoside, and methyl β-D-xylopyranoside were employed as acceptors (Fig. 5). The products of hydrolysis (oligosaccharides) and transglycosylation (oligomeric methyl glycosides) were revealed by MALDI-TOFMS (Table 4). G-I catalyzed the transglycosylation reaction more effectively using methyl β-D-glucopyranoside as acceptor (Fig. 5a). The incubation of laminaran with the enzyme in a buffer solution containing 20% of the methanol yielded more reducing oligosaccharides than oligomeric methyl glycosides (Fig. 5b).

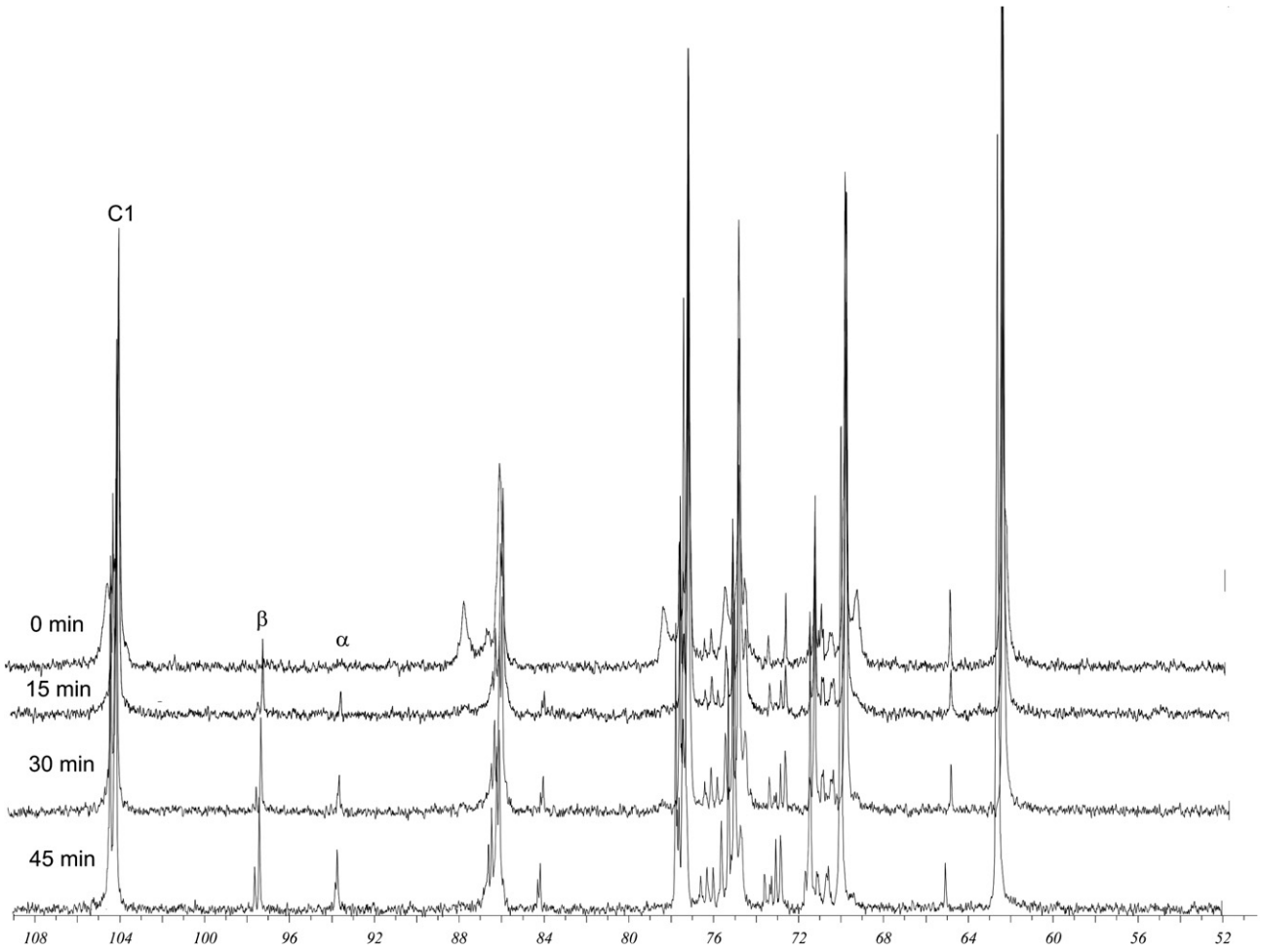

Figure 4. ^{13}C NMR spectra of laminaran recorded after 0–45 min of hydrolysis by G-I.

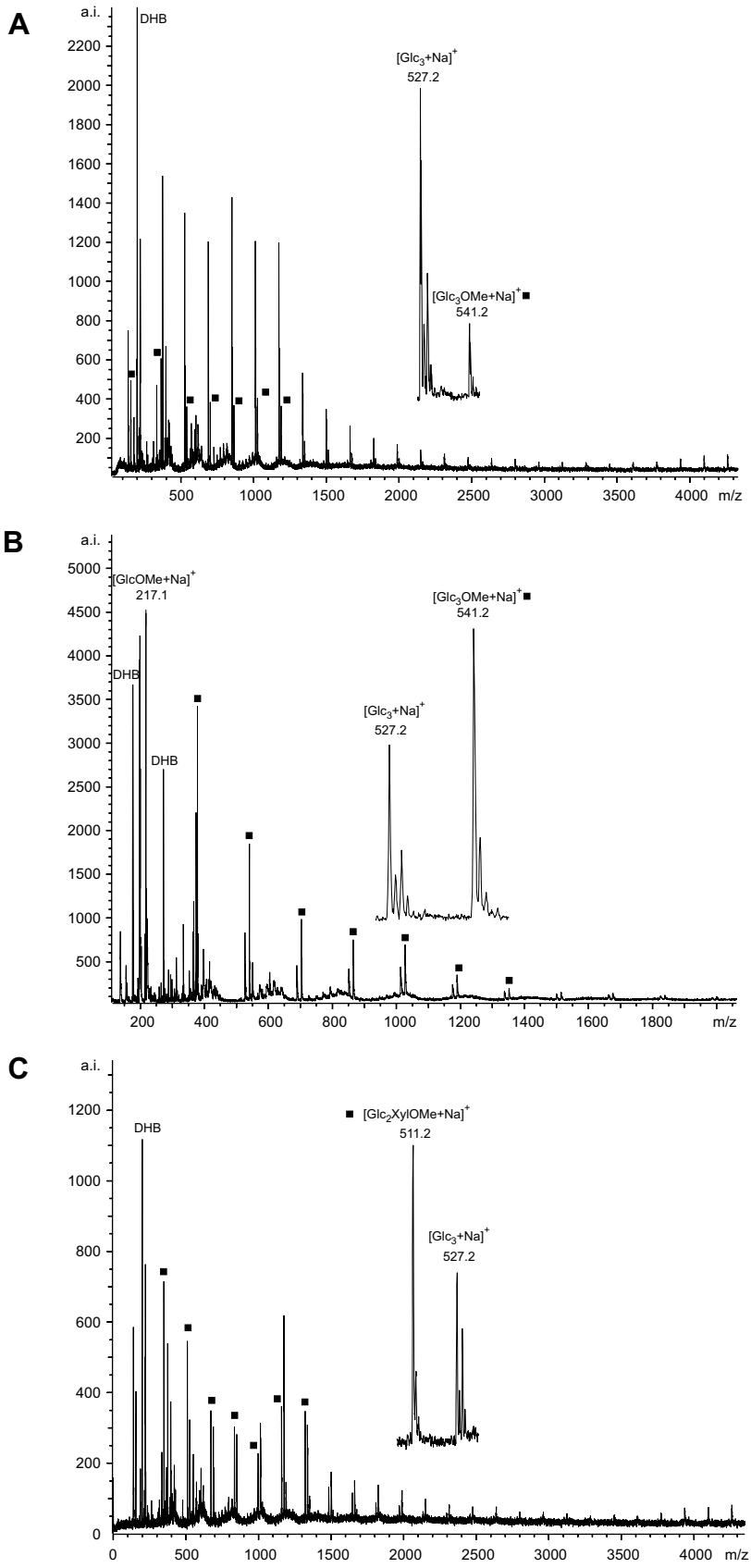

Figure 5. MALDI-TOF mass spectra of the products generated from laminaran with G-I in the presence of various acceptors: (A) methanol, (B) methyl β-D-glucopyranoside, and (C) methyl β-D-xylopyranoside.

Table 4
Products of transglycosylation reaction obtained by the action of G-I on laminaran in the presence of various acceptors

Acceptor	Products, *m/z*						
MeOH	GlcOMe	Glc_2OMe	Glc_3OMe	Glc_4OMe	Glc_5OMe	Glc_6OMe	Glc_7OMe
	217.1	379.1	541.2	703.2	865.3	1027.3	1189.4
Glc-O-Me	Glc_2OMe	Glc_3OMe	Glc_4OMe	Glc_5OMe	Glc_6OMe	Glc_7OMe	Glc_8OMe
	379.1	541.2	703.2	865.3	1027.3	1189.4	1351.4
Xyl-O-Me	GlcXylOMe	$Glc_2XylOMe$	$Glc_3XylOMe$	$Glc_4XylOMe$	$Glc_5XylOMe$	$Glc_6XylOMe$	$Glc_7XylOMe$
	349.1	511.1	673.2	835.3	997.3	1159.4	1321.4

Some of the most important properties of minor laminarinase G-II were examined. The molecular mass of G-II corresponding to a single protein band on SDS 13% polyacrylamide gel was estimated to be about 200 kDa. The activity of the laminarinase G-II was maximal at pH 5.4 and a temperature 35 °C, while the temperature stability of laminarinase G-II was relatively low. The activity of laminarinase G-II was lost very rapidly between 30 °C and 40 °C. The K_m values of G-II in the reaction with laminaran as substrate was 0.34 mg/mL.

MALDI-TOFMS and HPLC analysis of hydrolysis products of laminaran by G-II revealed glucose as the major product at the both initial and final stages of reaction (data not shown). Investigation of the specificity showed that G-II hydrolyzed soluble glucans (laminaran and translam) and *p*-nitrophenyl β-D-glucopyranoside at high rates. The enzyme did not digest zymosan, xylan, amylopectin, pustulan, or CM-cellulose. G-II did not cleave periodate-oxidized laminaran like an *exo*-glucanase. According to the polarimetry data, the optical rotation of the incubation mixture practically did not change during the enzymatic hydrolysis. Addition of NH_4OH also did not influence the optical rotation of the reaction mixture. Thus, the β anomer of glucose appeared in the initial stages of the reaction, and the cleavage of the glucosidic linkages proceeded without inversion of configuration. It is likely that G-II belongs to the class of *exo*-(1→3)-β-D-glucanases or it is a β-D-glucosidase that exhibits a unique ability to catalyze hydrolysis of laminaran. β-D-Glucosidases can be easily distinguished from *exo*-(1→3)-β-D-glucanases by their ability to hydrolyze aryl β-D-glycosides and to decrease in reaction rate with an increase of substrate chain length. It was shown that the hydrolysis rate of glucooligosaccharides by G-II decreased with increase of their degree of polymerization (Fig. 6). G-II also hydrolyzed β-D-glucodisaccharides with various types of bonds. Activity of G-II was observed against laminaribiose (100% hydrolysis), cellobiose (24% hydrolysis), and gentiobiose (2% hydrolysis). An *exo*-(1→3)-β-D-glucanase also exhibiting β-D-glucosidase activity has been only described from the walls of plant cells.[29–31] The other (1→3)-β-D-glucanases isolated from higher plants are *endo*-glucanases with, occasionally, some β-D-glucosidase activity.[32,33] G-II possesses a small transglycosylating activity using laminaran as donor and methyl β-D-glucopyranoside as acceptor. The presence of methyl glucobi- and triosides was revealed with MALDI-TOFMS (data not shown).

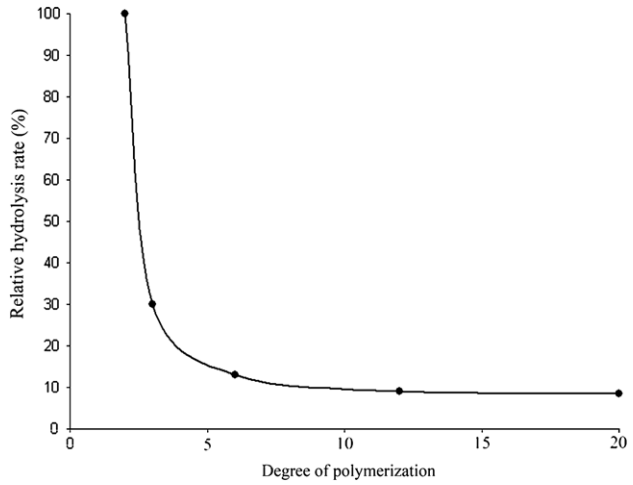

Figure 6. The G-II action on the laminaran and laminarioligosaccharides with various degrees of polymerization.

Thus, G-I from liver *L. kurila* had catalytic properties typical for *endo*-(1→3)-β-D-glucanases from marine invertebrates: high ability to catalyze a transglycosylating reaction and producing a large amount of glucose in the hydrolysis products. According to the rules of enzyme nomenclature, G-I can be classified as glucan *endo*-(1→3)-β-D-glucosidase (EC 3.2.1.39). It is most likely that G-II is β-D-glucoside glucohydrolase (EC 3.2.1.21), with unusual ability effectively to cleavage the laminaran. The purified enzymes G-I and G-II can be used for the structural analysis of laminarans and other (1→3)-β-D-glucans.

3. Experimental

3.1. Substrates

Laminaran from brown alga *Laminaria cichorioides* was obtained as previously described.[34] Periodate-oxidized laminaran was synthesized by a published method.[35] Translam was obtained by the enzymatic transformation of laminaran from *L. cichorioides*.[10]

Aubasidan from *Aureobasidium pullulan* was kindly provided by Professor I. P. Elinov (St. Petersburg, Russia). Pachyman, CM-pachyman, yeast glucan, lichenan, zymosan, xylan, amylopectin, pustulan, CM-cellulose, and laminarioligosaccharides were kindly provided by our colleagues from the Pacific Institute of Bioorganic Chemistry.

Methyl β-D-gluco- and xylopyranosides, *p*-nitrophenyl β-D-glucopyranoside, gentiobiose and cellobiose were purchased from Sigma–Aldrich.

3.2. Analytical methods

Protein concentration was measured by the Lowry procedure using BSA as the standard.[36] The homogeneity and molecular masses of the enzymes were determined by SDS–PAGE according to the method of Laemmli.[37] Analytical gel filtration was carried out by FPLC on an AKTA chromatograph (column: Superose 12 HR 10/30; eluent: 0.025 M acetate buffer, pH 5.4 with 0.15 M NaCl). γ-Globulin (158,000 Da), ovalbumin (44,000 Da), myoglobin (17,000 Da) and vitamin B_{12} were used as standards.

The oligosaccharide composition was analyzed by HPLC on an Agilent 1100 chromatograph (column: Asahipak NH_2P-50 4A; eluent: 6:4 CH_3CN–water). MALDI-TOFMSs of laminaran and products of its transformation were recorded on a Bruker Biflex III MALDI-TOFMS instrument equipped with a nitrogen laser. Sample preparation: a mixture containing 1 μL of sample (1 mg/mL for oligosaccarides or 5 mg/mL for laminaran) and 1 μL of 0.5 M DHB matrix solution in MeOH was introduced onto the sample plate

and air dried. Instrument settings: accelerating voltage, 19 kV; laser power, 40%; number of shots, 30; laser shot rate, 4 Hz. All spectra were recorded in reflector mode.

3.3. Purification of enzymes

Mollusks (*L. kurila*) were collected in Posíeta Bay (northwestern part of the Sea of Japan) in August 2006 near the Marine Experimental Station of the Pacific Institute of Bioorganic Chemistry.

The mollusk liver (150 g) was homogenized and extracted with the 0.2 M acetate buffer, pH 5.4 at a ratio 1:1. Insoluble material was removed by centrifugation (9000*g* for 20 min at 1 °C), and the supernatant was mixed with 0.5 L of DEAE-cellulose (Pharmacia) and loaded on a filter Shott (9 × 12 cm). The proteins were eluted using a stepwise gradient of NaCl (0.1; 0.2; 0.3; 0.4; 0.5 and 1 M) in 0.025 M acetate buffer, pH 5.4 (buffer A). Fractions containing (1→3)-β-D-glucanase activity were pooled, concentrated on a 10 kDa cut-off ultrafiltration membrane (Amicon) and dialyzed against buffer A. The concentrated solution of enzyme was applied to a DEAE-cellulose column (2 × 15 cm), equilibrated with buffer A. The proteins were eluted with a linear NaCl gradient (from 0 to 0.5 M, 200 mL) in buffer A.

Two fractions (G-I or G-II) containing (1→3)-β-D-glucanase activity were further purified by gel-filtration chromatography with a Sephacryl S-300 or Sephacryl S-100 (Pharmacia) column (1.5 × 35 cm) equilibrated with buffer A. Fractions containing the enzyme were loaded onto a TSK DEAE-5PW column (HPLC), equilibrated with buffer A. The proteins were eluted with a linear NaCl gradient (from 0 to 0.4 M, 40 mL) in buffer A.

3.4. Enzyme assay

The activity of the (1→3)-β-D-glucanase was deduced from the increase in the amount of reducing sugars found by the Somogyi–Nelson method.[38] The incubated mixture contained 100 μL of the enzyme and 400 μL of a laminaran solution (1 mg/mL in 0.025 M acetate buffer, pH 5.4). The time of incubation did not exceed that needed to cleave about 10% of the substrate. Glucosidase activity was determined under the same conditions using *p*-nitrophenyl β-D-glucopyranoside as substrate. The amount of the enzyme that catalyzed the formation of 1 μmol of glucose (or *p*-nitrophenol) per minute was accepted as a unit of activity.

3.5. Enzyme characterization

The optimal pH for (1→3)-β-D-glucanases was determined by the standard activity assay at 37 °C using 0.2 M citrate–phosphate buffer in the pH interval of pH 3.5–8.0 The reaction mixture contained 100 μL of the enzyme solution, 200 μL of substrate solution (1 mg/mL), and 200 μL of buffer with various pH values.

The optimal temperature was determined by the standard activity assay at 25, 35, 40, 50, and 55 °C. The concentration of reducing sugars was then measured.

Thermal stability was estimated by measuring of the residual activity after pre-incubation of the enzyme solution at various temperatures (10–70 °C) for 20 min.

The Michaelis–Menten constant was determined from Lineweaver–Burk plots[39] of data obtained by measuring the rate of laminaran hydrolysis by enzyme under the standard assay conditions using a substrate concentration from 0.125 to 2.0 mg/mL.

Substrate specificity was determined with a series of β-D-glucans at a concentration 1 mg/mL. The enzymes were incubated with laminaran and translam for 20 min; the incubation with other substrates was continued for 3–24 h. Relative hydrolysis rate was expressed as a percentage of the rate of *L. cichorioides* laminaran degradation, assumed to be 100%.

3.6. Preparation of products from the hydrolysis of the substrates

Laminaran (2 mg) was dissolved in 1 mL of enzyme solution (5×10^{-2} U). Gentiobiose, cellobiose, and laminarabiose (1 mg) were dissolved in the 0.5 mL of enzyme solution and incubated at 37 °C. Samples of 50 μL were taken in definite intervals and were analyzed by HPLC and MALDI-TOFMS.

3.7. Preparation of transglycosylation products

Laminaran (2 mg) and 2 mg of methyl β-D-glucopyranoside (or methyl β-D-xylopyranoside) were dissolved in 1 mL of enzyme solution (10^{-2} U). The concentration of methanol (as acceptor) in the probe was 20%. Reaction tubes were incubated at 37 °C. Aliquots of 50 μL were taken at 10-min intervals up to 120 min for analysis by MALDI-TOFMS.

3.8. Polarimetric analysis of hydrolysis products

The optical rotations of the enzyme reaction mixtures were measured in quartz cuvettes on a Perkin–Elmer 141 polarimeter (USA). The enzyme solution (0.2 mL, 5×10^{-2} U) prepared in 0.025 M acetate buffer, pH 5.4, was added to solution of laminaran (1.2 mL, 10 mg/mL), and the optical rotation was measured at the time intervals of 5, 10, 15, and 30 min. After 30 min, a drop of concd NH_4OH was added to the polarimeter tube to catalyze the mutarotation of sugars, and the optical rotation was measured again.

3.9. Investigation of the hydrolytic mechanism by NMR spectroscopy

The enzyme solution (0.2 mL, 1 U) in 0.025 M acetate buffer, pH 5.4, was added to solution of laminaran (70 mg in 0.4 mL D_2O), and ^{13}C NMR spectra were recorded at 37 °C at time intervals of 15, 30, and 45 min using a Bruker DRX-500 spectrometer.

Acknowledgments

The work was supported by RFBR (Projects 1 06-04-48540-a), FEB RAS grants and the program 'Molecular and Cellular Biology' for Basic Research of the Presidium of Russian Academy of Science, CRDF (REC-003).

References

1. Maeda, M.; Nisizawa, K. *J. Biochem.* **1968**, *63*, 199–206.
2. Elyakova, L. A.; Zvyagintseva, T. N. *Carbohydr. Res.* **1974**, *34*, 241–248.
3. Read, S. M.; Currie, G.; Bacic, A. *Carbohydr. Res.* **1996**, *281*, 187–201.
4. Nelson, T. E.; Lewis, B. A. *Carbohydr. Res.* **1974**, *33*, 63–74.
5. Usui, T.; Topiyama, T.; Mizuno, T. *Agric. Biol. Chem.* **1979**, *43*, 603–611.
6. Usov, A. I.; Chizhov, A. O. *Bioorg. Khim. (Moscow)* **1989**, *15*, 208–216.
7. Varum, K. M.; Kvam, B. J.; Myklestad, S.; Paulsen, B. S. *Carbohydr. Res.* **1986**, *152*, 243–248.
8. Dalmo, R. A.; Bogwald, J.; Ingebrigtsen, K.; Seljelid, R. *J. Fish. Diseases* **1996**, *19*, 449–457.
9. Goldman, R. *Immunology* **1988**, *63*, 319–324.
10. Zvyagintseva, T. N.; Elyakova, L. A.; Isakov, V. V. *Bioorg. Khim. (Moscow)* **1995**, *21*, 218–225.
11. Burtseva, Yu. V.; Verigina, N. S.; Sova, V. V.; Pivkin, M. V.; Zviagintseva, T. N. *J. Appl. Phycol.* **2006**, *18*, 375–380.
12. Nishimura, T.; Bignon, C.; Allouch, J.; Czjzek, M.; Darbon, H.; Watanabe, T.; Henrissat, B. *FEBS Lett.* **2001**, *499*, 187–190.
13. Suzuki, M.; Horii, T.; Kikuchi, R.; Onishi, T. *Nippon Suisan Gakk.* **1986**, *53*, 311–317.
14. McCarthy, T.; Hanniffy, O.; Savage, A. V.; Tuohy, M. G. *Int. J. Biol. Macromol.* **2003**, *33*, 141–148.
15. Henrissat, B. *Biochem. J.* **1991**, *280*, 309–316.
16. Bachman, E. S.; McClay, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6808–6813.

17. Kozhemyako, V. B.; Rebrikov, D. V.; Lukyanov, S. A.; Bogdanova, E. A.; Marin, A.; Mazur, A. K.; Kovalchuk, S. N.; Agafonova, E. V.; Sova, V. V.; Elyakova, L. A.; Rasskazov, V. A. *Comp. Biochem. Physiol.* **2004**, *137*, 169–178.
18. Kovalchuk, S. N.; Sundukova, E. V.; Kusaykin, M. I.; Guzev, K. V.; Anastiuk, S. D.; Likhatskaya, G. N.; Trifonov, E. V.; Nurminski, E. A.; Kozhemyako, V. B.; Zvyagintseva, T. N.; Rasskazov, V. A. *Comp. Biochem. Physiol.* **2006**, *B143*, 473–485.
19. Privalova, N. M.; Elyakova, L. A. *Comp. Biochem. Physiol. B* **1978**, *60*, 225–228.
20. Sova, V. V.; Elyakova, L. A.; Vaskovsky, V. E. *Biochim. Biophys. Acta* **1970**, *212*, 111–115.
21. Cutler, R. L.; Yellowes, D. *Carbohydr. Res.* **1979**, *75*, 221–229.
22. Lepagnol-Descamps, V.; Richard, C.; Lahaye, M.; Potin, P.; Yvin, J.-C.; Kloareg, B. *Carbohydr. Res.* **1998**, *310*, 283–289.
23. Bacon, J. C.; Gordon, A. H.; Jones, D.; Taylor, I. F.; Webley, D. M. *Biochem. J.* **1970**, *120*, 67–71.
24. de la Cruz, J.; Pintor-Toro, J. A.; Benitez, T.; Llobell, A.; Romero, L. C. *J. Bacteriol.* **1995**, *177*, 6937–6945.
25. Sova, V. V.; Elyakova, L. A. *Biochim. Biophys. Acta* **1972**, *258*, 219–227.
26. Sova, V. V.; Zvyagintseva, T. N.; Svetasheva, T. G.; Burtseva, Y. V.; Elyakova, L. A. *Biochemistry (Moscow)* **1997**, *62*, 1300–1306.
27. Borriss, R.; Krah, M.; Brumer, H.; Kerzhner, M. A.; Ivanen, D. R.; Eneyskaya, E. V.; Elyakova, L. A.; Shishlyannikov, S. M.; Shabalin, K. A.; Neustroev, K. N. *Carbohydr. Res.* **2003**, *338*, 1455–1467.
28. Zvyagintseva, T. N.; Makar'eva, T. N.; Ermakova, S. P.; Elyakova, L. A. *Bioorg. Khim. (Moscow)* **1998**, *24*, 212–223.
29. Cline, K.; Albersheim, P. *Plant. Physiol.* **1981**, *68*, 207–220.
30. Cline, K.; Albersheim, P. *Plant. Physiol.* **1981**, *68*, 221–228.
31. Lienart, Y.; Comtat, J.; Barnoud, F. *Biochim. Biophys. Acta* **1986**, *883*, 353–360.
32. Moore, A. E.; Stone, A. E. *Biochim. Biophys. Acta* **1972**, *258*, 248–264.
33. Wong, Y. S.; Maclachlan, G. A. *Biochim. Biophys. Acta* **1979**, *571*, 244–255.
34. Zvyagintseva, T. N.; Shevchenko, N. M.; Popivnich, I. B.; Isakov, V. V.; Scobun, A. S.; Sundukova, E. V.; Elyakova, L. A. *Carbohydr. Res.* **1999**, *322*, 32–39.
35. Nelson, T. E.; Scarletti, J. V.; Smith, F.; Kirkwood, S. *Can. J. Chem.* **1963**, *245*, 1671–1678.
36. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
37. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
38. Somogyi, M. *J. Biol. Chem.* **1952**, *195*, 19–23.
39. Dixon, M.; Webb, E. C. *Enzymes*, 3rd ed.; Longman: London, 1979; pp 57–60.