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Endo-(1→3)-β-D-glucanase GI from marine mollusk *Littorina sitkana*: Amino acid sequence and ESIMS/MS-estimated features of transglycosylation and hydrolysis reactions in comparison to analogous enzyme LIV from *Pseudocardium sachalinensis*

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ABSTRACT

The cDNA encoding the glucanase GI from *Littorina sitkana* (formerly named *Littorina kurila*) was cloned and sequenced, and the enzyme was assigned to glycoside hydrolases family 16 (GHF16) on the basis of amino acid sequence similarity. Structural features of the products of transglycosylation reaction using 6-O-methyl-β-D-glucuronic acid as an acceptor were established. Using colisionally induced dissociation (CID) tandem electrospray ionization mass spectrometry (ESIMS/MS) it was shown that GI transfers the residues of glyconic parts of the substrate mainly at C3 and C4 positions of 6-O-methyl-β-D-glucuronic acid and strictly at C3 position of glucose residue. The semi-quantitative characteristics of simultaneously passing hydrolysis and transglycosylation reactions catalyzed by retaining endo-(1→3)-β-D-glucanases GI from *L. sitkana* and LIV from *Pseudocardium sachalinensis* (formerly named *Spisula sachalinensis*) have been obtained by ESIMS. Laminaran was used as a donor, while glycerol was employed as an acceptor. The significant distinctions of catalytic properties of LIV and GI were revealed.

1. Introduction

Laminarinases or (1→3)-β-D-glucanases belong to O-glycoside hydrolases, key enzymes of carbohydrate metabolism. These enzymes are widely distributed in various organisms such as archaea, bacteria, fungi, yeasts, plants, insects, fishes, invertebrates, and are involved in many physiological processes [1]. Complex structure of substrates (mixed (1→3);(1→6)- or (1→3);(1→4)-β-D-glucans), as well as considerable differences in their molecular masses, conformations and other characteristics determine the diversity of catalytic properties of (1→3)-β-D-glucanases and their functions in living organisms. These enzymes were studied in many respects, but our knowledge of their structure and mechanism of action is still incomplete, especially for enzymes from marine organisms. The marine invertebrates were found to be the rich sources of (1→3)-β-D-glucanases. They take part in digestion of dietary polysaccharides and play an important role in embryogenesis. Some (1→3)-β-D-glucanases have been isolated from the eggs of the sea urchin *Strongylocentrotus purpuratus* [2], marine mollusks *Pseudocardium sachalinensis* [3], *Mizuhopecten yessoensis* [4], *Chlamys albidus* [5], *Haliotis discus hannai* [6], *Perna viridis* [7]. Physico-chemical and catalytic properties of enzymes from these animals were studied in details and amino-acid sequences have been reported. All (1→3)-β-D-glucanases of marine invertebrates were classified as retaining endo-enzymes and according to amino-acid sequence analysis were assigned to the 16 family of glycoside hydrolases. A distinguishing feature of the catalytic action of (1→3)-β-D-glucanases from sea mollusks is their high transglycosylating activity, which is successfully used in the enzymatic synthesis of novel compounds and the transformation of natural glucans with the aim of increasing their biological activity [8,9]. Previously we isolated endo-(1→3)-β-D-glucanase from the gastropodian mollusk *Littorina sitkana* and investigated its basic catalytic properties [10]. In present work we report amino acid sequence of endo-(1→3)-β-D-glucanase from *L. sitkana* and comparative investigation of transglycosylating properties of endo-(1→3)-β-D-glucanases from *L. sitkana* and *P. sachalinensis* by the means of mass spectrometry. The comparative investigation of

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catalytic properties of enzymes is aimed for better understanding of structure–function relationship and for finding the sources with advanced biotechnical potential.

2. Experimental

2.1. Materials

Marine mollusks *L. sitkana* and *P. sachalinensis* were collected in Posíeta Bay (northwestern part of the Sea of Japan) in August 2010 near the Marine Experimental Station of the Pacific Institute of Bioorganic Chemistry. Digestive organs: liver of *L. sitkana* and crystalline styles of *P. sachalinensis* were isolated and frozen at −70 °C. Laminaran from brown alga *Laminaria cichorioides* was isolated as described [11]. Glycerol was purchased from Sigma–Aldrich, 6-O-methyl-β-D-glucuronic acid was synthesized as described [12].

2.2. Analytical methods

Protein concentration in solution was determined by the method of Bradford [13] using BSA as a standard. The homogeneity and molecular masses of the enzymes were determined by SDS–PAGE according to the method of Laemmli [14] and MALDI mass spectrometry. The oligosaccharide composition was analyzed by high performance liquid chromatography (HPLC) on an Agilent 1100 chromatograph under following condition: Asahipak NH_2P-50 4A column, acetonitrile and filtered distilled water (6:4) as the mobile phase, flow rate of 0.5 mL/min and refractive index detector. The retention times of products were determinated using glucose (Sigma–Aldrich) and collection of laminarioligosaccharides (kindly provided by our collaborators from PIBOC) as standards.

2.3. Purification of enzymes

Endo-1,3-β-D-glucanase from the liver of *L. sitkana* was purified as described previously [10]. Endo-1,3-β-D-glucanase from the crystalline styles of *P. sachalinensis* was purified reported in [15]. Homogeneity of 1,3-β-glucanases was established by SDS electrophoresis in 15% PAGE under reducing conditions [14].

2.4. Determination of enzyme activity

A standard reaction mixture contained a 0.1% (w/v) substrate solution (200 μL) and an enzyme solution (50 μL) in 0.025 M sodium acetate buffer, pH 5.2. The incubation was carried out for 20 min at 37 °C. The activity was determined from an increase in the amount of reducing sugars by the method of Nelson using glucose as a standard [16]. A unit of enzyme activity was defined as that amount of the enzyme required to catalyze the formation of 1 μmol of glucose per minute under standard conditions.

2.5. Preparation of hydrolysis and transglycosylation products

An enzyme solution (50 μL; 10^{-2} units) was added to 250 μL of laminaran solution (5 mg/mL), and the mixture was incubated at 37 °C. At definite intervals of 1, 5, 10, 20, 40 and 24 h, samples (50 μL) were taken. The reaction was terminated by adding of 50 μL NH_4OH (2.5%). The hydrolysis products were analyzed by ESIMS.

Transglycosylation products for ESIMS analyses were obtained by adding the enzyme solution (50 μL; 10^{-2} units) in buffer to 250 μL of a solution containing laminaran solution (5 mg/mL) and acceptors: glycerol (1%, 5%, and 10%) or 6-O-methyl-β-D-glucuronic acid (1 mg/mL). The incubation was performed at 37 °C. At definite intervals of 1, 5, 10, 20, 40 and 24 h, aliquots of 50 μL were taken, and NH_4OH (2.5%, 50 μL) was added to the aliquots to terminate the reaction. The samples were analyzed by ESIMS.

2.6. Mass spectrometric analysis

MALDI-TOFMS spectra for determination of the molecular mass of the enzymes were recorded with an ULTRAFLEX III MALDI-TOF/TOF (Bruker, Germany) mass spectrometer with delayed ion extraction and reflector modes, equipped with nitrogen laser (337 nm), with accelerating voltage of 25 kV. Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic) was used as the MALDI matrix in the positive-ion mode at a concentration of 10 mg/mL in 1:1 acetonitrile–water. Sample preparation: 1 μL of the matrix solution was applied to a stainless steel plate and air dried, then 1 μL of a sample solution in water (∼0.1 mg/mL) was applied as a second layer. The mixture was air dried and then introduced into the mass spectrometer.

ESIMS spectra were recorded with a 6510 LC Q-TOF (Agilent, USA) mass spectrometer with a dual electrospray-ionization source. Spectra were acquired in the positive-ion mode, with pre-calibration with a standard "HP-mix" for positive-ion mode. Capillary voltage was set to 3500 V, and the drying gas temperature was 325 °C. The fragmentor voltage was set to 215 V. Isolation window for MS/MS experiments was set to 1.3 mass units. Collision energy was optimized between 10 and 45 V by fragmentation abundance. The dried sample was dissolved in 1:1 acetonitrile/water (concentration of the sample ranged from approx. 0.001 to 0.5 mg/mL) and introduced into the mass spectrometer at flow rate of 5 μL/min using a syringe pump, manufactured by KD Scientific (USA).

2.7. RNA isolation and cDNA sequencing

Total RNA was isolated from whole *L. sitkana* by the guanidine thiocyanate/phenol/chloroform method [17]. cDNA was synthesized from total RNA and subjected to 22 cycles of amplification (10 s at 95 °C, 20 s at 63 °C and 90 s at 72 °C) with a SMART cDNA Amplification Kit (Clontech, USA) in accordance with the manufacturer's instructions. The cDNA was diluted 20-fold and 1 μL of this solution was used for PCR with Gluc1: 5′-TGGCC(A/T)GC(C/T)AT(T/C)TGGATG-3′ and Gluc2: 5′-CCATT(C/T)GA(T/C) AA(A/G)CCATT-3′ primers designed on the basis of the peptide sequences WPAIWM and PFDKPF, conserved for all endo-(1→3)-β-D-glucanases GHF16. PCR was set up using Encyclo PCR Kit (Evrogen, Russia) and amplification was carried out for 35 cycles (10 s at 95 °C, 15 s at 58 °C, 1 min at 72 °C). Obtained PCR product of 380 bp was cloned with InsTAclone PCR Cloning Kit (Fermentas, Lithuania) and sequenced using ABI Prism Big Dye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, USA) on ABI Prism 310 Genetic Analyzer.

The 3′- and 5′-terminal regions of the cDNA encoding GI were amplified by PCR with specific primers designed on the basis of 380 bp cDNA fragment sequence and adapter-specific primer.[36] Gene specific primers for 5′- and 3′-RACE were Gluc5: 5′-GTGGTTGTCGCCTGCGGAGGTGCC-3′ and Gluc3: 5′-GGCCACACCTCAAACATCTGG-3′, respectively. Two obtained cDNA fragments of 1080 bp and 400 bp were cloned and sequenced as described above.

2.8. Sequence analysis and comparisons

Sequence analysis was carried out using the NCBI-BLAST2 website (http://www.ebi.ac.uk/blastall/). Domain architectures were identified by SMART tool (http://smart.embl-heidelberg.de).

Multiple sequence alignment was performed with the ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

3. Results and discussion

Previously we isolated (1→3)-β-D-glucanase GI from the liver of the marine gastropodean mollusk *L. sitkana* and its general properties were investigated [10]. Glucanase GI had characteristic features of (1→3)-β-D-glucanases from marine mollusks. Molecular mass of GI as estimated by gel-permeation chromatography and SDS–PAGE analysis was 32 and 40 kDa, respectively and 39.3 kDa (data not shown) according to MALDI-TOFMS analysis. It exhibited a maximum activity at pH 5.4 and 40 °C. The Km value for laminaran hydrolysis was 0.13 mg/mL. The (1→3)-β-D-glucanase GI had narrow substrate specificity and hydrolyzed only (1→3)-β-D-glycosidic bonds in 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 using laminaran as donor and p-nitrophenyl-β-D-glucoside as acceptor. GI was classified as the glucan endo-(1→3)-β-D-glucosidase (EC 3.2.1.39) [10].

3.1. cDNA cloning and GI primary structure

The nucleotide sequence of full cDNA encoding GI was estimated by RT-PCR, 5′-, 3′-RACE methods. The obtained cDNA sequence of 1636 bp contains an open reading frame of 1017 bp encoding a polypeptide of 442 amino acid residues (GeneBank accession number FJ641204). Analysis of the *L. sitkana* endo-(1→3)-β-D-glucanase deduced amino acid sequence by SMART tool identified the domain of glycoside hydrolase family 16 (pfam00722 Glyco_hydro-16, E = 9.10e−09) located from Met149 to Lys374. The predicted mature enzyme GI with calculated molecular mass of 36.7 kDa consists of 322 amino acids residues. Molecular mass GI corrected by MALDI mass spectrometry was 39.3 kDa. Probably, the molecule of enzyme was highly derivatized due to post-translational modifications.

Sequence comparisons of GI deduced amino acids showed similarity of 67%, 65%, 64%, 65%, 63%, 61% and 59% to endo-(1→3)-β-D-glucanases of surf clam *P. sachalinensis* (GenBank AAP74223), scallops *M. yessoensis* (GenBank AY848857) and *Ch. albidus* (GenBank DQ093347), the mussel *P. viridis* (GenBank ACM68926), Pacific abalone *Haliotis discus hannai* (GenBank BAH84971), the cotton bollworm *Helicoverpa armigera* (GenBank ABU9862) and the yellow mealworm *Tenebrio molitor* (GenBank ACS36221), respectively. A somewhat lower degree of similarity is observed with endo-(1→3)-β-D-glucanases from the Antarctic springtail *Cryptopygus antarcticus* (GenBank ACD93221) and the pinewood nematode *Bursaphelenchus xylophilus* (GenBank BAE48357) – 52% and 54%, respectively. The multiple alignment of the endo-(1→3)-β-D-glucanase from *L. sitkana* with those of other invertebrate is shown in Fig. 1.

Analysis of the GI primary structure revealed that the enzyme belongs to glycoside hydrolase family 16. GI shares high (52–67%) sequence similarity with known invertebrates' endo-(1→3)-β-D-glucanases and contains two conserved motives, which are characteristic for endo-(1→3)-β-D-glucanases GHF16 (Fig. 1). One of them, catalytic EIDIME motif, includes two conserved glutamic acid residues, which catalytic importance has been proved by mutational analysis of laminarinase from *Rhodothermus marinus* [18] and by the crystal structure and mutational analysis of the endo-(1→3)-β-glucanase GHF16 from *Nocardiopsis* sp. [19,20]. Also the crucial role of these Glu residues has been suggested by the crystal structure and mutational analyses of a *Bacillus* 1→3;1→4-β-D-glucanase [21–23] and by analysis of crystal structure of κ-carrageenase from *Pseudoalteromonas carrageenovora* [24] and agarase from *Zobellia galactanivorans* [25]. All above mentioned enzymes belong to glycoside hydrolase family 16 (http://www.cazy.org/GH16.html), possess common enzyme mechanism and have similar all-antiparallel β-sheet jellyroll architecture. The second conserved motif, WLWPAIW, have been suggest to be substrate binding site [26]. The role of the last tryptophan residues in the WPAIW motif have been predicted to provide aromatic stacking interaction with glucose residue on subsite – 1 in the endo-(1→3)-β-D-glucanase BglF from *Nocardiopsis* sp. [20]. Another conserved tryptophan residues (Trp118 in BglF), which interacts with glucose residue on subsite – 2, is observed in GI at Trp146. It is worth mentioning that endo-(1→3)-β-D-glucanases of GHF16 are fairly rich in aromatic residues. Besides two above-mentioned tryptophan residues, analysis of the multiple alignment revealed Trp129, Trp131, Trp209, Trp283, Trp295, Trp301, Trp305, Phe9, Phe252, Phe256, Phe271 and Phe294 (residues numbering of GI), conserved for the majority of endo-(1→3)-β-D-glucanase GHF16, and five sites (Phe5, Trp19 Trp177, Trp205, and Phe270) with homologous substitutions (Fig. 1). It could be speculated that these aromatic residues (or some of them) participate in laminaran binding beyond the active-site cleft. The important role of tryptophan, phenylalanine and tyrosine residues for binding of glucose residues of polysaccharides was shown [21,24,25,27,28].

The multiple alignments revealed that two histidine residues, His176 and His203, are conserved for the endo-(1→3)-β-D-glucanases (Fig. 1). The importance of histidine residues for catalysis was shown by its chemical modifications in endo-(1→3)-β-D-glucanases from *M. yessoensis* and *T. molitor* [4,29]. Localization of His142 of the endo-(1→3)-β-D-glucanase from *Nocardiopsis* sp. in active site was demonstrated by Fibriansah [20]. Moreover, hypothesis that the histidine and aspartic acid residues, conserved in κ-carrageenases, β-agarases and laminarinases of GHF16 and located in catalytic cleft, participate in proton trafficking during the deglycosylation step of the catalytic cycle, was suggested by a close inspection of the three-dimensional structure of the κ-carrageenase from *P. carrageenovora* [24]. Thus, His176 of GI together with Glu 151, Asp153 and Glu156 seems to be involved in catalytic cycle.

GI contains two cysteine residues, Cys82 and Cys90, which are conserved for the majority invertebrate' endo-(1→3)-β-D-glucanase and formed a disulfide bond connecting different sides of the loop between β3- and β4-strands [4]. The exceptions are endo-(1→3)-β-D-glucanase from Antarctic springtail *C. antarcticus* and from pinewood nematode *B. xylophilus*. They lack these cysteine residues because of deletion of about 40 amino acid residues in the corresponding region as well as of about 20 amino acids at C-end of their molecules. Phylogenetic analysis showed that this endo-(1→3)-β-D-glucanases are clustered together with glucanases from bacteria with high bootstrap values indicating that endo-(1→3)-β-D-glucanases from *B. xylophilus* and *C. antarcticus* are more closely related to those from bacteria than from eukaryotes. This suggests that the enzymes were acquired by horizontal gene transfer from bacteria [30,31].

The primary structure of GI enzyme from marine invertebrates is one of the few examples of that. We believe that it is a valuable contribution for the future investigations. Though finding relationship between the primary structure and exact features of enzyme is a complex task and requires separate investigations, the data, presented below could partly enlighten this relationship.

3.2. Hydrolysis and transglycosylation

It has been known that endo-(1→3)-β-D-glucanases from sea mollusks, in comparison to other O-glycoside hydrolases

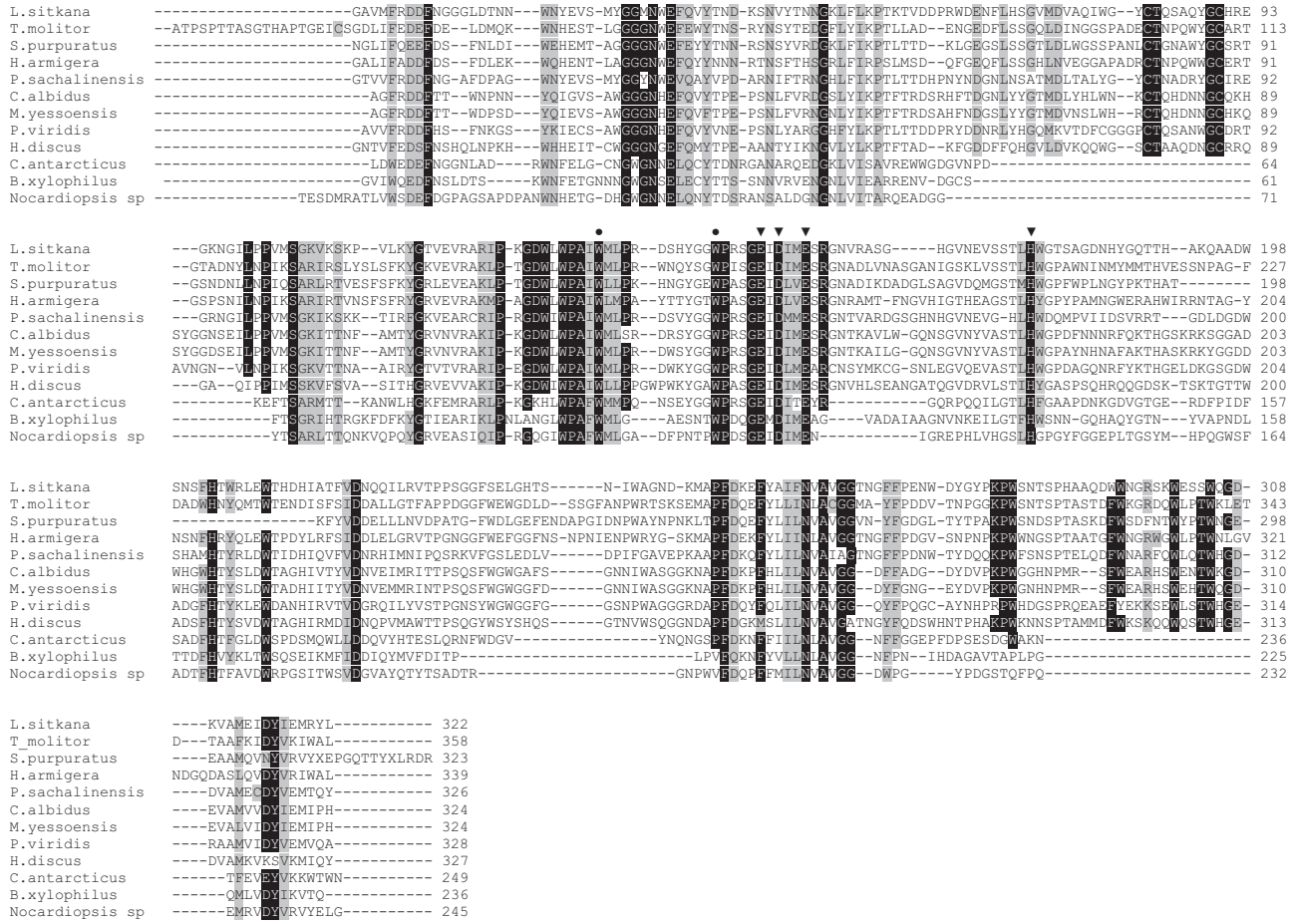

Fig. 1. Alignment of invertebrates' and bacterial endo-(1→3)-β-D-glucanases sequences. Conserved residues are highlighted in black (strictly conserved) or in grey (homologous replacements). Triangles above the sequences indicate catalytic residues; circlets indicate substrate-binding tryptophan residues of the enzymes active site.

display high transglycosylation activity [7,32,33]. Transglycosylating enzymes are traditionally considered to be good tools for the enzymatic synthesis of new biologically active carbohydrates [9]. In general, enzyme-catalyzed synthesis of oligosaccharides is a useful method because it allows the formation of well-defined oligosaccharides selectively in the absence of any protecting groups. Previous study has been shown that the purified endo-(1→3)-β-D-glucanase GI possessed transglycosylating activity [10]. Transglycosylating properties were studied also for glucanases LIV (from *P. sachalinensis*), L0 (from *Ch. albidus*), LV (from *M. yessoensis*) and glucanase from *P. viridis* with some aryl glycosides, glycerol and D-glucose as acceptor [32,33]. Early studies have been shown that glycerol is a better acceptor than water and other alcohols but it is less effective than glucose, p-nitrophenyl- and methylglycosides for endo-(1→3)-β-D-glucanases (LIV, L0, LV and glucanase from *P. viridis*) [7,32]. It is interesting that glycerol as acceptor has different action upon enzymatic reactions: it increases the rate of reaction and changes the ratio of hydrolysis products for LIV. Probably, these characteristics might be connected to the structure of active center of the enzyme.

The features of transglycosylation reaction catalyzed by GI were established by the means of mass spectrometry. Two acceptors were chosen: 6-O-methyl-β-D-glucuronic acid (GlcAOMe) for structural analysis, glycerol for semi-quantitative analysis and laminaran as a donor. As GI was unable to transfer glucose residues to GlcA directly, GlcAOMe compound was chosen as an acceptor in order to obtain GlcA-labeled products (by removing methyl group), since structural features of these compounds could be elucidated with a negative-ion CID ESIMS/MS [34]. Transglycosylation products were detected by ESIMS for both acceptors. It should be noted, that neutral substances (products of hydrolysis, built up of glucose and transglycosylation products, built up of glucose and glycerol) showed fragment ions from the glycosidic bonds cleavage only. Thus, identification of glycosylation sites could only be possible by CID ESIMS/MS analysis of GlcA-labeled products, which were prepared by removing methyl groups from GlcAOMe-products by treatment with aqueous ammonia as described for deacetylation procedure [35]. Surprisingly, intensities of the ions of GlcA-containing products were higher in the positive-ion mode. However, fragment ions from cross-ring cleavages were observed, providing information on the linkage types (Fig. 2). In this way, by analyzing oligosaccharides with longer DP by ESIMS/MS it has been shown that GI transfers residues of glyconic parts of the substrate mainly at C3 and C4 positions of the GlcA and strictly at C3 of Glc residues. Thus, disaccharides Glc-(1→3)-GlcA; Glc-(1→4)-GlcA and trisaccharides Glc-(1→3)-Glc(1→3)-GlcA; Glc-(1→3)-Glc(1→4)-GlcA were found and characterized in the products of reaction.

The further study of the features of transglycosylation reaction catalyzed by (1→3)-β-D-glucanase GI from the liver of marine gastropodean mollusk *L. sitkana* using glycerol as acceptor was performed in comparison with (1→3)-β-D-glucanase LIV from the crystalline styles of marine bivalve mollusk *P. sachalinensis*. LIV is one of the first enzymes isolated from marine mollusks and its kinetics and catalytic mechanism were studied by various

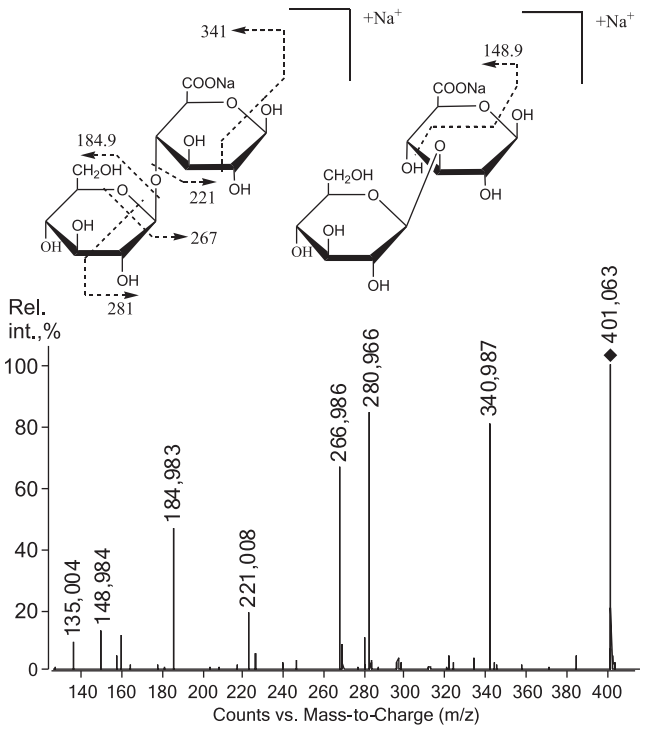

Fig. 2. A positive-ion mode CID ESIMS/MS of transglycosylation products, obtained under action of endo-1,3-β-glucanases GI (10^{-2} U) on laminaran (5 mg/mL) as a donor and GlcAOMe (1 mg/mL) as acceptor: square indicates ion [GlcGlcA-H+2Na]$^+$ at *m/z* 401.063.

methods in detail [32,36]. Amino acid sequence of LIV is a second such example of (1→3)-β-D-glucanase from animal source [3].

Products of laminaran hydrolysis by GI and LIV were analyzed by ESI mass spectrometry. These methods provide wide dynamic range, high precision and ultimate sensitivity with some restrictions. There is a problem of detection of the monosaccharide ions, such as [Glc+Na]$^+$ and ionization ability of ESIMS seems to fall with increasing DP [37,38]. Though the ESIMS method does not allow carrying out accurate quantitative measurements, if the substances are close on molecular weights and have a similar structure, it is possible to measure a ratio between the intensities of the ions and thus calculate kinetic parameters. Furthermore, use of HPLC combined with mass spectrometry allows having total information about hydrolysis products as well as transglycosylation products.

Glucose (main component due to HPLC, but its intensity is lowered in MS due to the restrictions, mentioned above) and glucooligosaccharides with different degree of polymerization (DP) were found in the reaction products (20% of laminaran hydrolysis) of both GI and LIV enzymes (Fig. 3A, 3B). Mass spectra showed the peaks of the following ions: [Glc+Na]$^+$ at *m/z* 203, [Glc_2+Na]$^+$ at *m/z* 365, [Glc_3+Na]$^+$ at *m/z* 527, [Glc_4+Na]$^+$ at *m/z* 689, [Glc_5+Na]$^+$ at *m/z* 851, [Glc_6+Na]$^+$ at *m/z* 1013, [Glc_7+Na]$^+$ at *m/z* 1175, [Glc_8+Na]$^+$ at *m/z* 1337.0. An analysis of the composition of the products at different stages of the reaction showed that the type of action of LIV and GI is characteristic for endo-enzymes from marine sources [39]. From ESIMS one can directly deduce the products of primary and secondary hydrolysis [7], since laminaran from *L. cichorioides* is known to contain large percent of mannitol residues, located at the reducing end [11]. Thus, by observing "+2 Da" satellite signals it was confirmed that GI (Fig. 3A) accumulated Glc_3, Glc_4, Glc_5 and LIV (Fig. 3B) accumulated Glc_3, Glc_4, Glc_5, Glc_7 as products of primary attack. Probably, LIV has more extensive substrate-binding center. Both enzymes also produced glucose from the first minutes of hydrolysis (data not shown). Hence, probably, GI and LIV attacked mannitol-free reducing ends of polysaccharide first. The tendency of LIV to attack substrate near the reducing end was shown earlier [39]. ESIMS data was confirmed by HPLC (Table 1). Both (1→3)-β-D-glucanases are classified as endo-enzyme of smaller-oligosaccharide-producing type according to previous study [40].

Table 1
Yield (%) of the products of laminaran hydrolysis by endo-(1→3)-β-D-glucanases GI and LIV detected by HPLC. The degree of laminaran hydrolysis is about 20%.

Enzymes	Products[a] (%)				
	Glc	Glc_2	Glc_3	Glc_4	Glc_5
G-I	44	17.2	22.2	9.0	7.6
L-IV	27.2	26.8	26.0	10.8	9.2

[a] Glc, Glc_2, Glc_3, Glc_4, Glc_5 – glucose and glucobi-, tri-, tetra- and pentaose, respectively.

To calculate kinetic parameters of transglycosylation reaction for both LIV and GI enzymes, laminaran was used as a donor, while glycerol was employed as an acceptor. The optimal concentration of laminaran was found to be 5 mg/mL. The enzymatic activities at various concentrations using 0.1, 0.5 and 1.0 M glycerol as the acceptor were investigated by ESIMS [4, 7, 10, and 33]. ESIMS showed the ions of hydrolysis (see above) and transglycosylation reactions products: [GlyGlc+Na]$^+$ at *m/z* 277.1, [$GlyGlc_2$+Na]$^+$ at *m/z* 439.4, [$GlyGlc_3$+Na]$^+$ at *m/z* 601.6, [$GlyGlc_4$+Na]$^+$ at *m/z* 763.8, [$GlyGlc_5$+Na]$^+$ at *m/z* 925.4, [$GlyGlc_6$+Na]$^+$ at *m/z* 1088.1, [$GlyGlc_7$+Na]$^+$ at *m/z* 1250.3, [$GlyGlc_8$+Na]$^+$ at *m/z* 1412.4 (Fig. 4). The results of investigation for LIV enzyme have shown that the rate of hydrolysis is higher that the rate of transglycosylation reaction (Fig. 4A). Contrary results were obtained for GI enzyme (Fig. 4B), when the speed of hydrolysis reaction was higher only at low concentration of the glycerol.

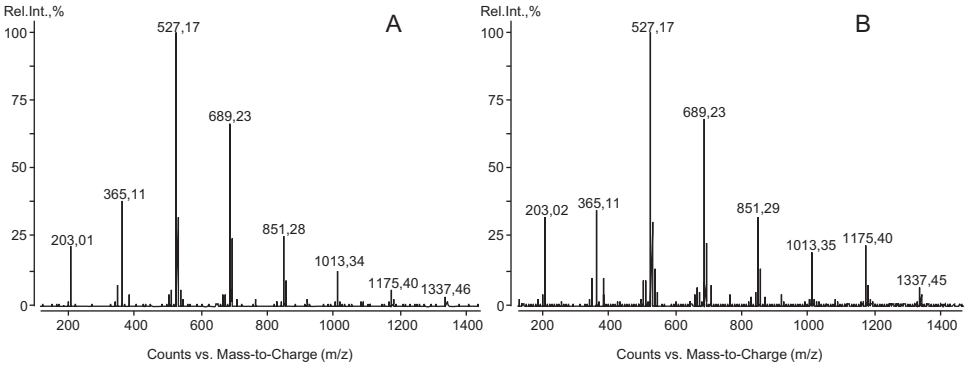

Fig. 3. ESIMS of the products of laminaran hydrolysis by (1→3)-β-D-glucanases GI (A) and LIV (B). The degree of substrate hydrolysis is about 20%.

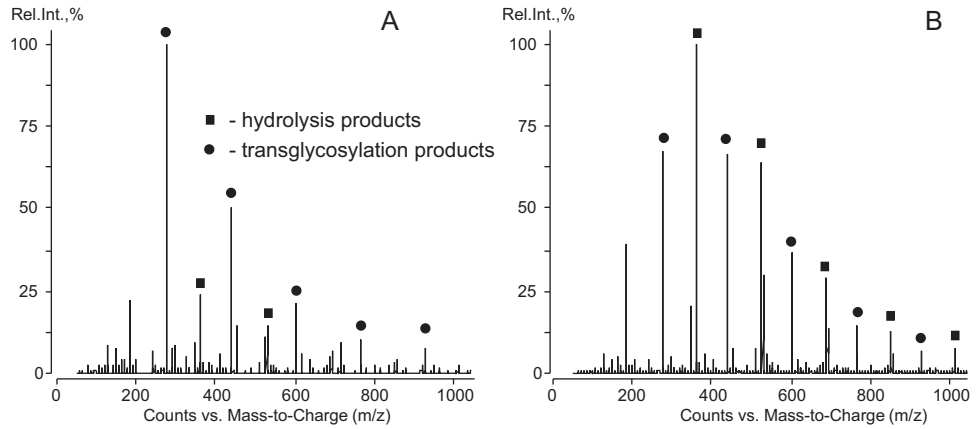

Fig. 4. ESIMS of transglycosylation and hydrolysis products, obtained under action of endo-1,3-β-glucanases GI (A) and LIV (B) (10^{-2} U) on laminaran (5 mg/mL) as a donor and glycerol (1.0 M for GI and 0.5 M for LIV) as acceptor.

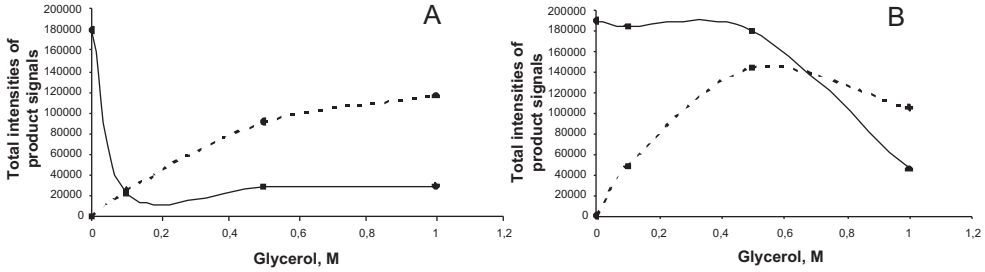

Fig. 5. The products of transglycosylation (dotted line) and hydrolysis (line) reactions catalyzed by endo-1,3-β-glucanases GI (A) and LIV (B) (10^{-2} U) at various concentrations of glycerol (0.1, 0.5 and 1.0 M). These plots were calculated from ESIMS spectra as total intensity basis products of reactions (glucose and oligosaccharides with DP 2–5) in initial stage of reaction.

This observation was investigated more deeply. The plot (Fig. 5) is based on the ESIMS spectral data of transglycosylation reaction products (glucose and oligosaccharides with DP 2–5), which were recorded at various concentrations of glycerol at the initial stage of reaction (first minutes). Total intensity was calculated as a sum of the intensities of the ions of glucose and oligosaccharides. It was found that total amount of transglycosylation reaction products increased with increasing of glycerol concentration. For GI enzyme reaction still ran at 1.0 M of glycerol concentration. For LIV enzyme it was found that amount of transglycosylation products increased up to 0.5 M of glycerol concentration but decreased when the glycerol concentration was higher. According to the dynamics of transglycosylation and hydrolysis products accumulation for LIV we concluded that enzyme partly inactivated with increasing of glycerol concentration. Probably, the affinity of the glycerol to the catalytic center of LIV was higher. To confirm the influence of glycerol concentration on the hydrolysis reaction we used independent method (Nelson) for registration of the yield of reaction products (Fig. 6). The data shows that glycerol suppresses hydrolysis of laminaran by GI enzyme while the transglycosylation products are accumulated when the rate of hydrolysis by LIV has a linear decrease. The ratio of $K_{tr,gly}$ (transfer to glycerol) to K_{hydr} (transfer to water) can be estimated from the experiment with glycerol as

$$\alpha = \frac{K_{tr,gly}}{K_{hydr}} = \frac{I_{tr,gly}}{I_{hydr}} \cdot \frac{[H_2O]}{[Glycerol]}$$

were $I_{tr,gly}$ and I_{hydr} are the maxima of total intensity of transglycosylation and hydrolysis products, respectively. The coefficients were calculated: at 0.5 M of glycerol for LIV α was 80 and for 1 M glycerol for GI α was 225. It must be noted that the value α for LIV identified by ESIMS is close to the value $\alpha = 50$ calculated in a former study [33].

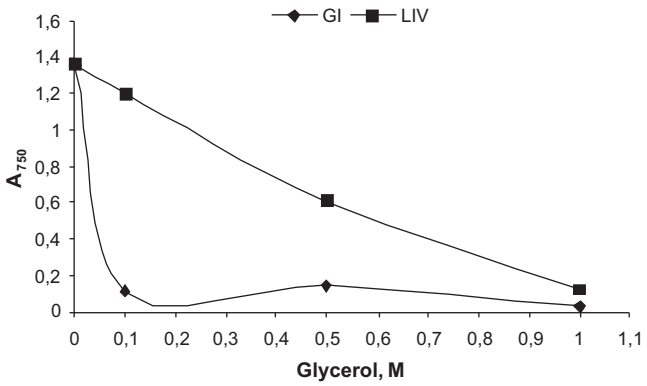

Fig. 6. The effect of glycerol on hydrolysis of laminaran (1 mg/mL) by 10^{-2} U of endo-1,3-β-glucanases GI and LIV (registration of products by Nelson method).

4. Conclusion

Both (1→3)-β-D-glucanases GI and LIV were assigned to 16 family of glycoside hydrolases. They showed high sequence similarity, they hydrolyzed substrate according to endo-type with retention of the anomeric configuration. Despite the same specificity, both enzymes have significant differences in hydrolysis products and the transglycosylation and hydrolysis reactions, passing at the same time. The use of mass spectrometry allowed us to simultaneously study the dynamics of hydrolysis and transglycosylation reactions and to elucidate the structure of oligosaccharides and oligosides, synthesized by the enzymes. Unfortunately, some limitations of the MS technique do not allow identifying which hydroxyl groups are

glycosylated during transglycosylation in the case with glycerol as acceptor.

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