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Catalytic Properties and Amino Acid Sequence of Endo-1→3-β-D-glucanase from the Marine Mollusk *Tapes literata*

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Abstract—A specific 1→3-β-D-glucanase with molecular mass 37 kDa was isolated in homogeneous state from crystalline style of the commercial marine mollusk *Tapes literata*. It exhibits maximal activity within the pH range from 4.5 to 7.5 at 45°C. The 1→3-β-D-glucanase catalyzes hydrolysis of β-1→3 bonds in glucans as an endoenzyme with retention of bond configuration, and it has transglycosylating activity. The K_m for hydrolysis of laminaran is 0.25 mg/ml. The enzyme is classified as a glucan endo-(1→3)-β-D-glucosidase (EC 3.2.1.39). The cDNA encoding this 1→3-β-D-glucanase from *T. literata* was sequenced, and the amino acid sequence of the enzyme was determined. The endo-1→3-β-D-glucanase from *T. literata* was assigned to the 16th structural family (GHF 16) of O-glycoside hydrolases.

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Laminarinases (1→3-β-D-glucanases) are O-glycoside hydrolases, which are key enzymes of carbohydrate metabolism. They catalyze hydrolysis of O-glycoside bonds in 1→3-β-D-glucans and are widespread among various organisms such as archae, bacteria, fungi, yeast, plants, fishes, and invertebrates. The 1→3-β-D-glucanases participate in many physiological processes such as biosynthesis of glucans in cell walls of microorganisms, plant protection from pathogens, digestion and fertilization of invertebrates, etc. [1]. Animal 1→3-β-D-glucanases are much less studied than the plant and bacterial enzymes [2, 3].

Marine invertebrates are rich and relatively available source of various O-glycoside hydrolases. Some species of marine invertebrates are important in mariculture (e.g. scallop, mussel, and trepang). Usually O-glycoside hydrolases are obtained from digestive organs of marine invertebrates, which are removed as wastes during their industrial processing.

Earlier we showed that 1→3-β-D-glucanases are widespread in marine invertebrates inhabiting various regions of the World Ocean. The highest level of activity of 1→3-β-D-glucanases was found in crystalline styles of ark shell mollusks – unique organs concentrating enzymes for extracellular digestion of carbohydrate food [4]. We have isolated homogeneous 1→3-β-D-glucanases from crystalline styles of the several shell mollusks (*Spisula* (*Pseudocardium*) *sachalinensis* [5], *Chlamys albidus* [6], *Mizuhopecten yessoensis* [7], and *Perna viridis* [8]), studied their physicochemical properties and catalytic mechanism, and determined their amino acid sequences. Comparison of the established amino acid sequences of eleven 1→3-β-D-glucanases from marine invertebrates (http://www.ncbi.nlm.nih.gov/protein/) allowed them to be assigned to the 16th structural family of O-glycoside hydrolases. The 1→3-β-D-glucanases of marine invertebrates are available and stable enzymes that can be easily isolated; they are convenient models for study of structural and functional features of O-glycoside hydrolases. They are successfully used for carbohydrate structure determination, enzymatic synthesis, and trans-

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formation of natural glucans to increase their biological activity [9, 10].

The goal of the present work was to characterize the catalytic properties and determine the amino acid sequence of a novel 1→3-β-D-glucanase from crystalline styles of the commercial ark shell mollusk *Tapes literata*.

MATERIALS AND METHODS

Biological material. Specimens of the marine mollusk *Tapes literata* were collected in the South China Sea in the coastal zone of the Socialist Republic of Vietnam in August 2009. Crystalline styles were immediately isolated and frozen.

Reagents. Laminaran was isolated from the brown alga *Laminaria cichorioides* according to [11], periodate-oxidized laminaran was synthesized as described earlier [12], and pustulan from the lichen *Umbilicaria russica*, yeast glucan from cell walls of baker's yeast, pachyman from *Poria cocos*, and aubasidan from *Aureobasidium pullulans* were kindly donated by researchers of the Pacific Institute of Bioorganic Chemistry (Far East Branch, Russian Academy of Sciences). Other reagents used in this work – carriers for chromatography, CM cellulose, amylopectin, methyl-β-D-glucopyranoside, methyl-β-D-xylopyranoside, glycerol, methanol, group-specific reagents, and inorganic salts – were from Sigma (USA).

Isolation and purification of 1→3-β-D-glucanase from *T. literata*. Crystalline styles (9.1 g) were homogenized with 90 ml of cooled 0.025 M sodium acetate buffer, pH 5.4 (working buffer). The homogenate was incubated for 3 h at 4°C and then centrifuged at 9000*g* for 15 min. The supernatant was applied on a column with CM-MacroPrep pre-equilibrated with the working buffer. The column was eluted in a gradient of NaCl concentration in buffer (0-1.5 M, 200 ml portions) at the rate 0.5 ml/min, while fractions 5 ml in volume were collected. Fractions exhibiting 1→3-β-D-glucanase activity were pooled and concentrated to 3.5 ml by ultrafiltration on a PM-10 membrane from Amicon (The Netherlands). The enzyme preparation was applied on a column (1.0 × 80 cm) with P-30 Biogel equilibrated with working buffer. The enzyme was eluted with buffer with rate 0.3 ml/min and fraction volume 1 ml. Fractions exhibiting 1→3-β-D-glucanase activity were pooled, concentrated by ultrafiltration, and applied on a column (1.5 × 110 cm) with Sephadex G-50. Conditions for separation were analogous to those for ultrafiltration on P-30 Biogel.

Estimation of 1→3-β-D-glucanase activity. The standard reaction mixture contained 200 μl of laminaran solution (1 mg/ml) in the working buffer and 50 μl of enzyme solution. Incubation time was 20 min at 37°C. Activity was estimated via increase in concentration of reducing saccharides in the reaction mixture according to Nelson [13]. The amount of enzyme catalyzing formation of 1 μmol of glucose per minute was taken as the activity unit. The specific activity is expressed in U/mg protein.

Protein concentration in solutions was determined according to Bradford [14], and that during chromatography via optical absorption at 280 nm.

Electrophoresis of proteins was performed under denaturing conditions in 12% polyacrylamide gel [15]. A mixture of proteins from BioRad (USA) with molecular masses from 15 to 250 kDa was used as the standard.

Determination of optimal pH. The reaction mixture containing 20 μl of enzyme solution, 100 μl of 0.2 M citrate-phosphate buffer with various pH values (from 3.4 to 7.4), and 400 μl of aqueous laminaran solution (1 mg/ml) was incubated for 20 min at 37°C, and then the samples were tested for reducing ability [13].

Determination of pH stability. Enzyme solution (50 μl) in 0.05 M citrate-phosphate buffer of the required pH value was incubated for 20 min at room temperature, then substrate solution (200 μl) in 0.2 M working buffer was added, and the remaining enzyme activity was assayed.

Determination of temperature optimum. A standard mixture of enzyme and laminaran was incubated for 20 min at various temperature values (from 4 to 60°C), and then samples were tested for reducing ability [13].

Determination of thermal stability. Enzyme solution in working buffer (500 μl) was incubated at various temperatures (from 20 to 70°C). Aliquots (20 μl) of enzyme solution were taken after certain time periods. Then 200 μl of laminaran solution (1 mg/ml) was added to the cooled samples, and the remaining enzyme activity was assayed after incubation for 20 min at 37°C.

Effect on various substrates. The reaction mixture contained 50 μl of enzyme solution in working buffer (10^{-2} units) and 200 μl of substrate solution (1 mg/ml). Laminaran and periodate-oxidized laminaran were incubated for 20 min, and other substrates were incubated from 3 to 24 h. The rates of hydrolysis of various substrates were expressed in percent of the rate of laminaran cleavage.

The Michaelis constant (K_m) for hydrolysis of laminaran was calculated according to the Lineweaver–Burk procedure. The initial rates of laminaran (concentrations 0.01-0.1%) hydrolysis by the purified 1→3-β-D-glucanase (10^{-2} units) under standard conditions were measured.

Products of hydrolysis and transglycosylation. Enzyme solution ($2\cdot 10^{-2}$ units, 100 μl) was added to that of laminaran (1 mg/ml, 400 μl), and the mixture was incubated at 37°C. Aliquots (50 μl) were taken after certain time periods, and the reaction was terminated by boiling. Products were analyzed by MALDI-TOF mass spectroscopy.

To obtain transglycosylation products, enzyme solution in buffer (10^{-2} units, 50 μl) was added to 1 ml of solution containing 2 mg of laminaran and 2 mg of an acceptor (methyl-β-D-glucopyranoside, methyl-β-D-xylopy-

ranoside, glycerol, and methanol). The mixture was incubated at 37°C. Aliquots (50 µl) were taken after certain time periods, and the reaction was terminated by boiling. Products were analyzed by MALDI-TOF mass spectroscopy.

Mass spectroscopy. Mass spectra were recorded using an ULTRAFLEX III MALDI-TOF/TOF mass spectrometer from Bruker (Germany). Sample (1 µl) was twice applied on DHB (2,5-dihydroxybenzoic acid) matrix and dried.

NMR spectroscopy. ^{13}C-NMR spectra were recorded at 37°C using a DRX spectrometer from Bruker (1000 scans, relaxation time 1 sec).

Laminaran was several times evaporated with D_2O, dissolved in D_2O (0.5 ml, 10 mg/ml), and then its spectrum was recorded. Enzyme (1 unit, 0.1 ml) in 0.025 M sodium acetate buffer, pH 5.4, was added to the laminaran solution, and spectra of the products were recorded after each 10 min for 2 h.

Isolation of RNA and plasmid DNA. RNA from *T. literata* hepatopancreas and plasmid DNA were isolated using kits from Fermentas (Lithuania) according to the producer's recommendations.

Synthesis and amplification of cDNA. cDNA from *T. literata* hepatopancreas was obtained using a SMART PCR cDNA Synthesis Kit from Clontech Laboratories (USA) according to the producer's recommendations.

Polymerase chain reaction (PCR). cDNA from *T. literata* hepatopancreas was used for amplification of cDNA encoding endo-1→3-β-D-glucanase from *T. literata*.

Cloning and sequencing of cDNA of endo-1→3-β-D-glucanase from *T. literata*. To clone PCR products, an InsT/Aclone™ PCR Product Cloning Kit (Fermentas) was used according to the producer's recommendations. Bacterial colonies containing plasmids with the desired insertions were screened by PCR using M13 universal primers. Nucleotide sequences were determined according to Sanger using an ABI PRISM 310 Genetic Analyzer automatic sequencer and Big Dye 3.1 Kit (Applied Biosystems, USA) according to the producer's recommendations.

Nucleotide and amino acid sequences were analyzed using the BLAST2 (http://www.ebi.ac.uk/blastall/), ClustalW 1.8 (http://www.ebi.ac.uk/clustalw/index.html) (Higgins et al., 1994), and SignalP (http://www.cbs.dtu.dk/services/SignalP) packages. Domain organization of proteins was determined using the SMART server (http://smart.embl-heidelberg.de). Amino acid sequence was determined on the basis of cDNA nucleotide sequence using the EXPASY server (http://expasy.org/tools/dme.html).

RESULTS AND DISCUSSION

Tapes literata is a commercial ark shell mollusk inhabiting the coastal zone of the Socialist Republic of Vietnam. Analysis of composition of O-glycoside hydrolases in the extract from its crystalline styles showed that this 1→3-β-D-glucanase is the main digestive enzyme. The activity of other glucanases catalyzing hydrolysis of amylopectin, CM cellulose, and pustulan was negligible.

The purification of the enzyme included the following stages: extraction, centrifugation, ion-exchange chromatography on CM-MacroPrep, ultrafiltration, and gel filtration on P-30 Biogel and Sephadex G-50 (Table 1). High concentration of 1→3-β-D-glucanase in the initial extract yielded the enzyme with molecular mass 36 kDa in the homogeneous state according to SDS-PAGE data, although the degree of purification was not high (8.24 times) (Fig. 1). The yield of 1→3-β-D-glucanase exceeded the known yields of such enzymes from mollusks and was 52.3% enzyme content (activity units) in the initial extract from the crystalline style of the mollusk.

The 1→3-β-D-glucanase exhibited maximal activity over a broad pH range (from 4.5 to 7.5, Fig. 2) at temperature 45°C (Fig. 3). The enzyme appeared to be sufficiently stable to the action of temperature and NaCl, which is typical of enzymes from marine organisms. Half-inactivation time of the 1→3-β-D-glucanase was 18 min at 50°C (Fig. 4). The enzyme activity did not change in the range of NaCl concentrations from 0.1 to 1.5 M.

Table 1. Purification of 1→3-β-D-glucanase from crystalline style of *T. literata*

Stage of purification	Total protein, mg	Total activity, units	Specific activity, units/mg protein	Degree of purification, times	Yield, % (via activity units)
Extraction	91.3	15.5	0.17	1	100
CM-MacroPrep	20.7	13.2	0.6	3.8	85.4
Gel filtration on P-30 Biogel	8.4	9.2	1.1	6.5	59.5
Gel filtration on Sephadex G-50	5.8	8.1	1.4	8.2	52.3

Fig. 1. SDS-PAGE of protein preparations from crystalline style of *T. literata* at different stages of purification: a) extract; b) after chromatography on CM-MacroPrep; c) after chromatography on Sephadex G-50. *1*) Protein preparation; *2*) standards.

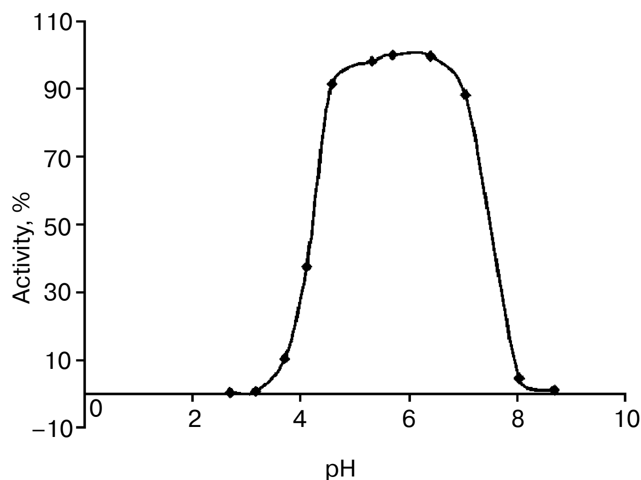

Fig. 2. The effect of pH on activity of the 1→3-β-D-glucanase from crystalline style of *T. literata*.

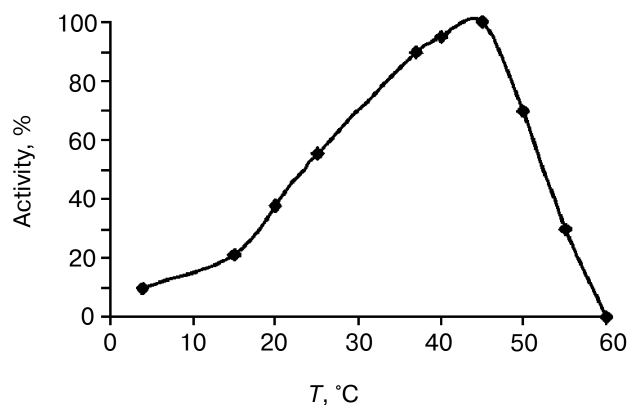

Fig. 3. Temperature optimum of the 1→3-β-D-glucanase from crystalline style of *T. literata*.

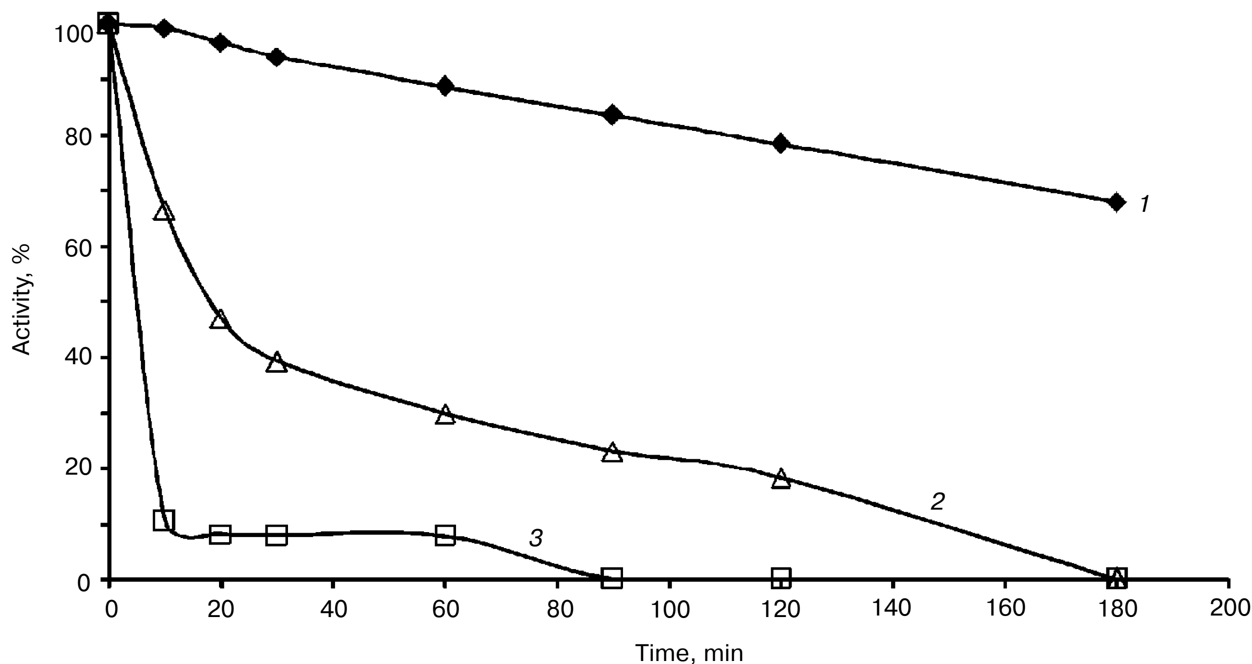

Fig. 4. Thermal stability of the 1→3-β-D-glucanase from crystalline style of *T. literata*. Temperature (°C): *1*) 37; *2*) 50; *3*) 55.

The specificity of the 1→3-β-D-glucanase to β-1→3 bonds was demonstrated for a series of substrates. The enzyme was shown to hydrolyze laminaran and periodate-oxidized laminaran efficiently, whereas high molecular weight and poorly soluble 1→3;1→6-β-D-glucans (pachyman, yeast glucan, and aubasidan) were hydrolyzed to only slightly. Glucans with another bond type (pustulan, amylopectin, and CM cellulose) were not hydrolyzed at all (Table 2). Modification of the terminal glucose residues in the laminaran molecule (periodate-oxidized laminaran) did not change the rate of its hydrolysis by the 1→3-β-D-glucanase from *T. literata* (Table 2), which is typical of endo-enzymes cleaving internal bonds in glucan molecule. It is known that exo-1→3-β-D-glucanases successively splitting out glucose (or oligosaccharides) from the non-reducing end of glucan do not hydrolyze periodate-oxidized laminaran [12].

The Michaelis constant (K_m) for hydrolysis of laminaran by 1→3-β-D-glucanase from *T. literata* was calculated according to Lineweaver–Burk and was 0.25 mg/ml (Fig. 5); this value is typical of 1→3-β-D-glucanases from various sources: marine fungi, mollusks, and others.

Table 2. Specificity of 1→3-β-D-glucanase from *T. literata*

Substrate	Bond type, ratio	Molecular mass (kDa), solubility	Activity, %
Laminaran from *L. cichorioides*	β-1→3; -1→6 90:10	5-6, soluble	100
Periodate-oxidized laminaran	β-1→3	5-6, soluble	100
Yeast glucan	β-1→3; β-1→6 90:10	>200, poorly soluble	2
Pachyman	β-1→3; β-1→6 98:2	50-120, poorly soluble	1
Aubasidan	β-1→3; -1→6 50:50	500-550, poorly soluble	0.5
Pustulan	β-1→6	30, soluble	0
Amylopectin	α-1→4	2000, soluble	0
CM cellulose	β-1→4	>2000, soluble	0

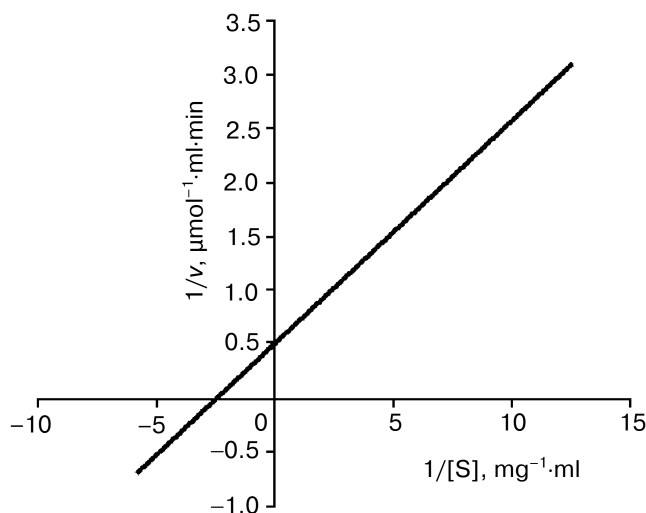

Fig. 5. Lineweaver–Burk plot for determination of the Michaelis constant of 1→3-β-D-glucanase from crystalline styles of *T. literata*. The substrate was laminaran from *L. cichorioides*.

Endo-1→3-β-D-glucanases can be conventionally divided into two groups by composition of the products formed by the action of these enzymes on 1→3-β-D-glucans [8]. Enzymes catalyzing formation of high molecular weight compounds (oligosaccharides with high degree of polymerization) belong to the first group. Enzymes that hydrolyze substrate thus forming low molecular weight products (mono- and oligosaccharides with low degree of polymerization) belong to the second group. Earlier we showed that endo-1→3-β-D-glucanases of marine invertebrates belong to the second group. High concentration of glucose (up to 40%) in the products of laminaran hydrolysis is their specific feature [4].

The products of laminaran hydrolysis by 1→3-β-D-glucanase from *T. literata* were analyzed by MALDI-TOF mass spectroscopy (Fig. 6). The composition of the products supported the endo-type of enzyme action. Glucose and oligosaccharides with various degree of polymerization were detected among the products of hydrolysis when the substrate was ~5-10% hydrolyzed. The following main peaks with *m/z* $[M+Na]^+$ were observed in the spectra: Glc_1 (203.3), Glc_2 (365.2), Glc_3 (527.2), Glc_4 (689.2), Glc_5 (851.3), Glc_6 (1013.3), Glc_7 (1175.4), Glc_8 (1337.4), Glc_9 (1499.4), Glc_{10} (1661.5), Glc_{11} (1823.5), Glc_{12} (1987.6), Glc_{13} (2149.7), Glc_{14} (2311.8), Glc_{15} (2473.9), Glc_{16} (2636.0), Glc_{17} (2798.2). So, the 1→3-β-D-glucanase from *T. literata* can be classified as endo-1→3-β-D-glucanase of the second group from the composition of the products of hydrolysis.

The kinetics of hydrolysis of laminaran by the 1→3-β-D-glucanase from *T. literata* were analyzed by ^{13}C-NMR spectroscopy. The spectra were completely identical to those for endo-1→3-β-D-glucanase from the marine mollusk *P. viridis* obtained by us earlier [8]. New signals in the area 97.2 ppm corresponding with C1 of the reducing glycoside residue with β-configuration of glycoside bond appeared in the spectrum of laminaran from the very beginning of the reaction. Signal in the area 93.7 ppm corresponding to C1 of a reducing glycoside residue with α-configuration of the glycoside bond appeared significantly later as a consequence of mutarotation. So, the 1→3-β-D-glucanase from *T. literata* hydrolyzes the bond retaining configuration of the anomeric monosaccharide center, which is typical of endo-type enzymes.

Glucanases retaining configuration of the cleaved bond are known to be able to catalyze transglycosylation, i.e. for reactions of transfer of the glycon part of the substrate not only on water (hydrolysis) but also onto various hydroxyl-bearing compounds. Being typical hydrolases, endo-1→3-β-D-glucanases from marine mollusks having enhanced transglycosylating activity compared with that of enzymes from terrestrial sources [4].

The transglycosylating ability of the 1→3-β-D-glucanase from *T. literata* was studied by MALDI-TOF mass spectroscopy. This method allows simultaneous registration of the products of hydrolysis and transglycosylation and evaluation of dynamics of substrate consumption. Laminaran was used as a donor of the carbohydrate component, whereas methyl-β-D-glucopyranoside (β-D-Glc*p*-O-Me), methyl-β-D-xylopyranoside (β-D-Xyl*p*-O-Me), glycerol, and methanol were used as acceptors. Samples were taken 1, 5, and 15 min and 24 h after the beginning of the reaction. Mass spectra of the products of transglycosylation and hydrolysis during 5 min action of the enzyme are presented in Fig. 6. Analysis of mass spectra indicated that the enzyme actively transferred to all the studied acceptors except methanol. Products of transfer to methanol were not detected. The intensity of ions of the products of transfer to β-D-Glc*p*-O-Me acceptor was the maximal for this experiment, beginning from the very first minutes of reaction (Fig. 6a). The efficiency of β-D-Xyl*p*-O-Me as an acceptor was less than that of β-D-Glc*p*-O-Me, the intensity of ions of low molecular weight products of transfer being lower, but intensive signals of high molecular weight products of transfer were observed (Fig. 6b). Intensity of signals of the products of hydrolysis was also significantly higher.

Earlier it was shown that for the 1→3-β-D-glucanase from the ark shell mollusk *Perna viridis*, glycerol was the optimal acceptor in the transglycosylation reaction [8]. Analysis of mass spectra of the 1→3-β-D-glucanase from *T. literata* recorded under analogous conditions did not indicate such a tendency. As can be seen in Fig. 6c, after 5 min of reaction with glycerol as acceptor almost unhydrolyzed laminaran was present in the reaction mixture along with low-intensity signals of high molecular weight products of transfer. Figure 6d demonstrates that in the control the laminaran was cleaved into oligosaccharides within 5 min. Decreased rate of sub-

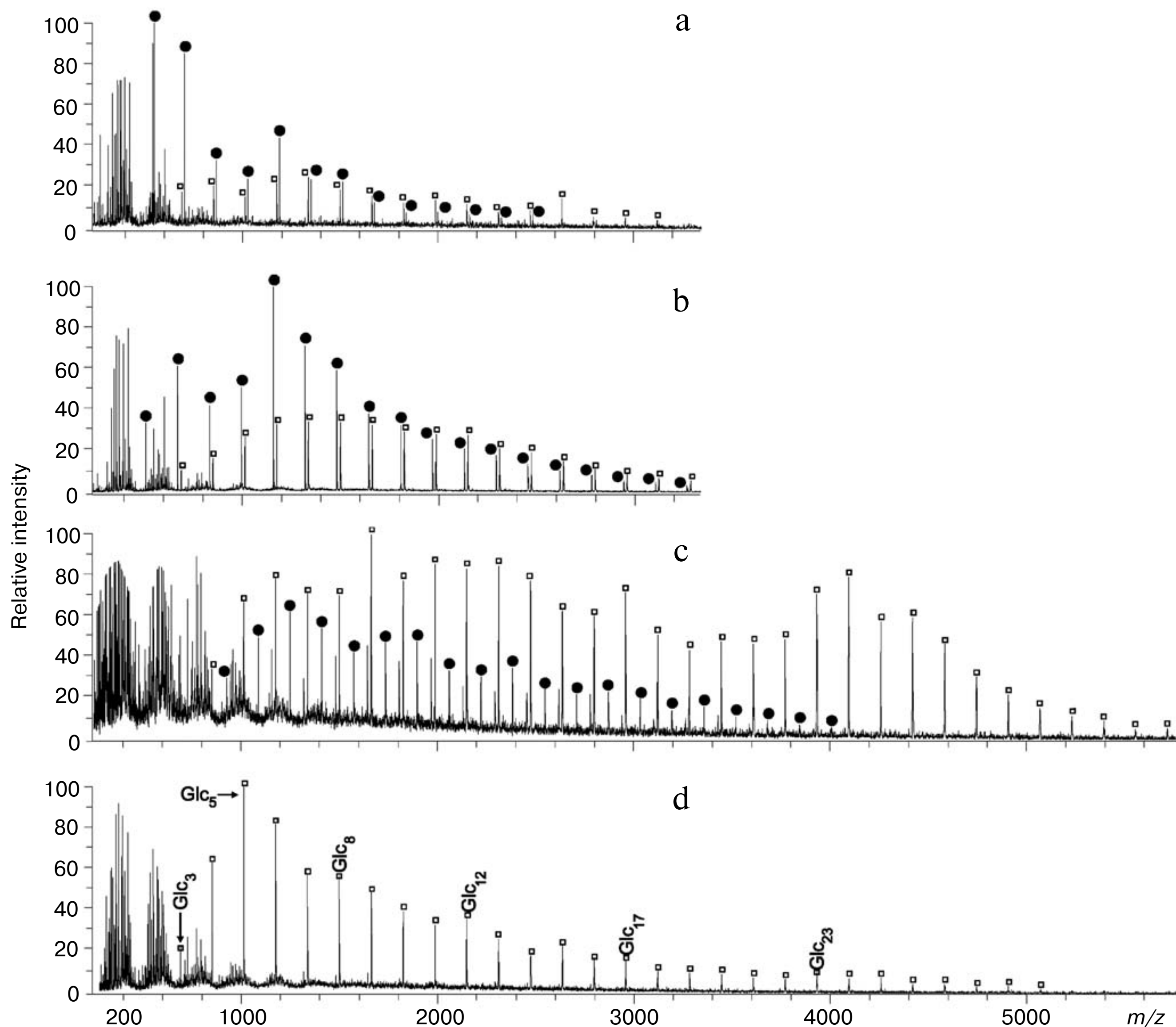

Fig. 6. MALDI-TOF mass spectra of the products of transglycosylation obtained by the action of 1→3-β-D-glucanase from *T. literata* on laminaran for 5 min at the presence of acceptors: β-D-Glc*p*-O-Me (a), β-D-Xyl*p*-O-Me (b), glycerol (c), water (hydrolysis) (d). Products of hydrolysis (open square); products of transglycosylation (closed circle).

strate consumption can be caused by inhibitory action of glycerol. This also follows from analysis of mass spectra of the products of the transglycosylation reaction catalyzed by the 1→3-β-D-glucanase from *Littorina sitkana* [16].

The role of certain functional groups for catalytic activity of the 1→3-β-D-glucanase was evaluated by inhibitor analysis (Table 3). N-Bromosuccinimide selectively oxidizing tryptophan residues in weakly acidic medium almost completely inactivated the enzyme. These data suggest that tryptophan residues are very important for catalytic activity of the 1→3-β-D-glucanase. It was shown that in some O-glycoside hydrolases tryptophan residues are located in the binding site of the substrate and participate in binding of monosaccharide residues [17, 18]. Participation of carboxyl groups in catalysis cannot be excluded. Carbodiimide did not decrease the enzyme activity, but its action in the presence of a nucleophilic agent (butylamine) resulted in 34% loss of activity. The enzyme activity decreased by 22% in response to histidine-modifying reagents – both the highly specific diethylpyrocarbonate and the less specific acetylimidazole.

The amino acid sequence of the endo 1→3-β-D-glucanase from *T. literata* was determined by molecular-biological methods. Total RNA isolated from *T. literata* hepatopancreas was used for synthesis of the first strand

Table 3. Effect of group-specific reagents and metal ions on the activity of the 1→3-β-D-glucanase from crystalline styles of *T. literata*

Reagent	Group	Concentration, M	Residual activity, %*
Acetylimidazole	Tyr, His	10^{-2}	88
Diethylpyrocarbonate	His	10^{-2}	88
N-Bromosuccinimide	Trp	10^{-2}	4
N-Ethylmaleimide	-SH	10^{-2}	93
Sodium *p*-chloromercury benzoate	-SH	10^{-2}	97
p-(3-dimethylaminopropyl)-*p*′-ethylcarbodiimide hydrochloride	-COOH	10^{-2}	95
p-(3-dimethylaminopropyl)-*p*′-ethylcarbodiimide hydrochloride and butylamine	-COOH	10^{-2}	66
Butylamine (control)		10^{-2}	94
EDTA	Me	10^{-2}	90
Mg^{2+}	–	5×10^{-3}	97
Co^{2+}	–	5×10^{-3}	96
Sn^{2+}	–	5×10^{-3}	97
Cu^{2+}	–	5×10^{-3}	94
Mn^{2+}	–	5×10^{-3}	89

* Enzyme activity in a sample without addition of reagent is taken as 100%.

of cDNA, which was then amplified by PCR. For amplification of cDNA fragments encoding the endo-1→3-β-D-glucanase, we used degenerated oligonucleotide primers Glu1 and Glu2 synthesized on conservative sites WPAIWM and PFDKPF of amino acid sequences of endo-1→3-β-D-glucanases. PCR gave a cDNA fragment ~400 bp in length, which was cloned in plasmid vector pTZ57R/T and sequenced. Analysis of the determined nucleotide sequence of this cDNA fragment using BLAST2 indicates that cDNA of endo-1→3-β-D-glucanases of mollusks *M. yessoensis*, *Ch. albidus*, *P. sachalinensis*, and *P. viridis* are its nearest homologs; the structures of these cDNA were determined by us earlier [5-8]. Oligonucleotide primers for amplification of terminal cDNA sites of the endo-1→3-β-D-glucanase from *T. literata* were constructed on the basis of nucleotide sequence of cDNA fragment. Amplification was performed by the modified RACE method [19]. PCR gave cDNA fragments 380 and 1100 bp in length, which were cloned in plasmid vector pTZ57R/T and sequenced. Thus, we established nucleotide sequence of three overlapping fragments of cDNA of endo-1→3-β-D-glucanase from *T. literata*. Their mutual correlation allowed reconstruction of the complete nucleotide sequence of cDNA encoding the endo-1→3-β-D-glucanase with the length of 1481 bp.

The amino acid sequence of the endo-1→3-β-D-glucanase was established on the basis of the nucleotide sequence of the cDNA encoding this protein. Analysis of the cDNA sequence indicates that it includes one prolonged open reading frame 1329 bp in length, which encodes a polypeptide of 442 amino acid residues (a.a.). The N-terminal sequence of the mature protein possibly begins with 121 a.a. (SVFRDDFNGA). The first 120 a.a. are probably cleaved during posttranscriptional modification of a precursor of the endo-1→3-β-D-glucanase. The mature protein consists of 322 a.a. Its calculated molecular mass is 36.8 kDa, which is in accord with experimental data obtained by mass spectroscopy (37.0 kDa). The isoelectric point of the protein is 6.32.

Comparison of the amino acid sequence of the endo-1→3-β-D-glucanase from *T. literata* with those of endo-1→3-β-D-glucanases from other mollusks revealed the maximal degree of homology with the protein from *P. sachalinensis* (68% identity and 77% homology). The degree of structural homology with the other endo-1→3-β-D-glucanases from mollusks is also high: 62-71% (identity 51-58%) (Table 4). Structural homology with

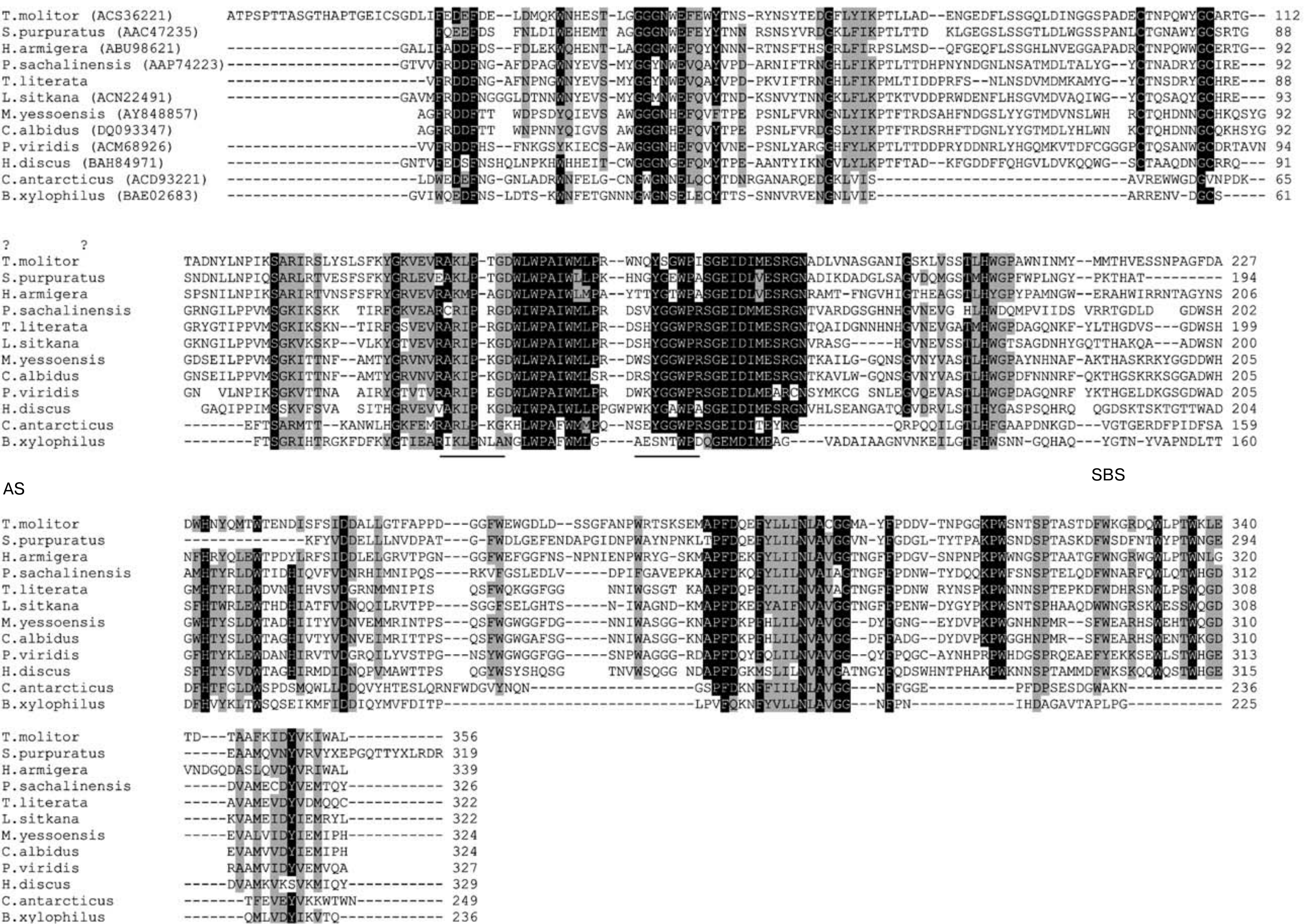

Fig. 7. Multiple alignment of amino acid sequences of endo-1→3-β-D-glucanases from invertebrates. Numbers of sequences in the GenBank database are given in parentheses. Identical amino acid residues are colored black and homologous amino acid residues are in gray. Active site (AS) and substrate binding site (SBS) are marked. ?, catalytic amino acid residues.

Table 4. Degree of identity/homology of endo-1→3-β-D-glucanases from mollusks (%)

Species	*T. literata*	*Ch. albidus*	*P. viridis*	*M. yessoensis*	*P. sachalinensis*
T. literata	100	51/62	51/66	52/65	68/77
Ch. albidus		100	53/66	86/93	48/58
P. viridis			100	53/68	44/57
M. yessoensis				100	48/60
P. sachalinensis					100

endo-1→3-β-D-glucanases from other invertebrates is somewhat lower: 44-58% [20]. Analysis of sequence of endo-1→3-β-D-glucanase from *T. literata* using the SMART server indicates that it contains a catalytic domain of the 16th family of glycoside hydrolases 199 a.a. in length (Asn199-Tyr397). So, according to structural classification the endo-1→3-β-D-glucanase from *T. literata* is a new representative of the 16th family (GHF 16) of O-glycoside hydrolases.

Multiple alignment of the amino acid sequences of endo-1→3-β-D-glucanases from invertebrates is presented in Fig. 7. Their comparison demonstrated that the fragment from 145 to 151 a.a. Gly-Gly-Ile/Met-Asp-Xxx-Xxx-Glu, which is the active site of glycoside hydrolases of the 16th family, is the most conservative (hereafter numeration is according to the endo-1→3-β-D-glucanase from *T. literata*). As shown earlier for 1→3;1→4-β-D-glucanases belonging to the 16th family of O-glycoside hydrolases [21, 22], two glutamic acid residues (Glu146 and Glu151) directly participate in catalysis. The sequence Trp-Pro-Ala-Xxx-Trp-Met-Leu (Xxx – hydrophobic amino acid residue), the binding site of substrate, is also conservative [23]. Tryptophan residues Trp126, Trp130, and Trp141 are invariant for the endo-1→3-β-D-glucanases. Multiple alignment of amino acid sequences of endo-1→3-β-D-glucanases also revealed the presence of two conservative histidine residues, His176 and His202 (Fig. 7); this suggests their important role in the activity of these enzymes. The results of chemical modification of the endo-1→3-β-D-glucanases, which is an indirect method for identification of functional groups of the active site, are consistent with these data [7, 24].

In spite of the relatively high degree of homology, the endo-1→3-β-D-glucanases from mollusks vary in physicochemical properties, in particular, in thermal stability. Earlier it was reported [5-8] that the thermal stability of endo-1→3-β-D-glucanases from mollusks increases in the following series (biological sources of enzymes are given): *Ch. albidus*, *M. yessoensis*, *T. literata*, *P. sachalinensis*, *P. viridis*.

It is known that the thermal stability of enzymes in many cases is defined by the degree of their conformational mobility: the higher the conformational mobility of a protein molecule, the less their thermal stability usually is [25]. The presence of disulfide bonds in the macrostructure of protein molecule is one of the factors decreasing conformational mobility. Endo-1→3-β-D-glucanases from marine mollusks vary in content of cysteine residues: enzymes from scallops *Ch. albidus* and *M. yessoensis* have two cysteine residues, the enzyme from *T. literata* has three cysteine residues, that from *P. sachalinensis* four, and from *P. viridis* seven. This suggests the following regularity: the higher the number of cysteine residues (and probably, the number of disulfide bonds) in an endo-1→3-β-D-glucanase molecule, the higher is its thermal stability. Analysis of amino acid residues of endo-1→3-β-D-glucanases demonstrated that two cysteine residues (Cys77 and Cys85) are conservative for all endo-1→3-β-D-glucanases from mollusks and also for endo-1→3-β-D-glucanases from other sources (*T. molitor*, *S. purpuratus*, *H. armigera*, and *H. discus*) (Fig. 7). These residues possibly form a disulfide bond, which is very important for stabilization of molecules.

So, a new 1→3-β-D-glucanase was isolated to homogeneity from crystalline style of the ark shell mollusk *T. literata*. This enzyme catalyzes hydrolysis of β-1,3-bonds in glucans, retaining configuration of the cleaved bond, is catalyzes transglycosylation, and is classified as a glucan-endo-1→3-β-D-glucosidase (EC 3.2.1.39). Analysis of the amino acid sequence allowed assignment of the endo-1→3-β-D-glucanase to the 16th structural family of O-glycoside hydrolases. Comparison of the structures of endo-1→3-β-D-glucanases of marine origin revealed conservative molecular fragments important for catalytic activity and demonstrated that thermal stability of the enzymes depends on the number of cysteine residues.

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