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An *endo*-(1→3)-β-D-glucanase from the scallop *Chlamys albidus:* catalytic properties, cDNA cloning and secondary-structure characterization

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ABSTRACT

An *endo*-(1→3)-β-D-glucanase (L_0) with molecular mass of 37 kDa was purified to homogeneity from the crystalline style of the scallop *Chlamys albidus*. The *endo*-(1→3)-β-D-glucanase was extremely thermolabile with a half-life of 10 min at 37 °C. L_0 hydrolyzed laminaran with $K_m \sim 0.75$ mg/mL, and catalyzed effectively transglycosylation reactions with laminaran as donor and *p*-nitrophenyl β D-glucoside as acceptor ($K_m \sim 2$ mg/mL for laminaran) and laminaran as donor and as acceptor ($K_m \sim 5$ mg/mL) yielding *p*-nitrophenyl β D-glucooligosaccharides ($n = 2$–6) and high-molecular branching (1→3),(1→6)-β-D-glucans, respectively. Efficiency of hydrolysis and transglycosylation processes depended on the substrate structure and decreased appreciably with the increase of the percentage of β-(1→6)-glycosidic bonds, and laminaran with 10% of β-(1→6)-glycosidic bonds was the optimal substrate for both reactions. The CD spectrum of L_0 was characteristic for a protein with prevailing β secondary-structural elements. Binding L_0 with D-glucose as the best acceptor for transglycosylation was investigated by the methods of intrinsic tryptophan fluorescence and CD. Glucose in concentration sufficient to saturate the enzyme binding sites resulted in a red shift in the maximum of fluorescence emission of 1–1.5 nm and quenching the Trp fluorescence up to 50%. An apparent association constant of L_0 with glucose ($K_a = 7.4 \times 10^5 \pm 1.1 \times 10^5$ M^{-1}) and stoichiometry ($n = 13.3 \pm 0.7$) was calculated. The cDNA encoding L_0 was sequenced, and the enzyme was classified in glycoside hydrolases family 16 on the basis of the amino acid sequence similarity.

1. Introduction

(1→3)-β-D-Glucanases, the enzymes hydrolyzing (1→3)-β-D-glucosidic linkages in (1→3)-β-D-glucans, are distributed widely in bacteria, fungi, higher plants and invertebrates. Terrestrial microorganisms and plants possess a set of (1→3)-β-D-glucanases including both *exo*- and *endo*-(1→3)-β-D-glucanases, which split β-(1→3)-glycosidic linkages in (1→3), (1→3),(1→6)- or (1→3),(1→4)-β-D-glucans as typical hydrolases.[1–3] Marine invertebrates contain exclusively *endo*-(1→3)-β-D-glucanases,[4,5] which are able to equally catalyze transglycosylation and hydrolysis reactions.[6–8] To date many bacterial, fungal and plant *endo*-(1→3)-β-D-glucanases have been studied in detail, but there are few reports on the isolation, biochemical and structural characterization of *endo*-(1→3)-β-D-glucanases from marine invertebrates.[9–12]

Earlier we reported the isolation and some catalytic properties of an *endo*-(1→3)-β-D-glucanase from crystalline styles of the scallop *Chlamys albidus*.[13–15] This enzyme was shown to catalyze two reactions depending on the acceptors used: (a) hydrolysis (transfer of substrate glycosyl residues to water) and (b) transglycosylation (transfer of substrate glycosyl residues to glycosides, alcohols, other hydroxyl-containing compounds, a substrate and gluco-oligosaccharides). A new (1→3),(1→6)-β-D-glucan (translam) with potent immunomodulating, radioprotective and antitumour activities was isolated from the end products of laminaran transformation catalyzed by L_0.[15–18] Translam had higher molecular weight than the initial laminaran (8–10 kDa vs 3–5 kDa) and more β-(1→6)-glycosidic bonds (25% vs 10%). Oligosaccharides with polymerization degrees of 2–6 or 2–7, respectively, and either glycerol or ^{14}C-labelling at the pseudo-reducing or reducing end were obtained by the action of L_0 on laminaran in the presence of glycerol or [1-^{14}C]-D-glucose as acceptor.[6,15] Besides, L_0 was shown to catalyze the transfer of the glyconic part of a substrate to methanol, ethanol, methyl glycosides and *p*-nitrophenyl β-D-glucosides with production of alkyl- or aryl derivative of (1→3),(1→6)-β-D-gluco-oligosaccharides with polymerization degree of 2–6.[6–8,15] Earlier on it was shown that one of the products of transglycosylation catalyzed by L_0 with methyl and *p*-nitrophenyl β-D-glucosides as

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acceptors were methyl and *p*-nitrophenyl β-D-cellobiosides, respectively. Thus, L_0 has the ability to synthesize not only β-(1→3)-, but also β-(1→4)-glycosidic bonds.[7,15] and can be used for polysaccharide depolymerization, but as well for the direct synthesis of new β-D-glucans and gluco-oligosaccharides and their glycosides of medicinal and synthetic interest.

We now report on some catalytic properties and structural data of the *endo*-(1→3)-β-D-glucanase from the scallop *C. albidus* in comparison with those of *endo*-(1→3)-β-D-glucanases from the marine molluscs *Mizuhopecten yessoensis* and *Spisula* (*Pseudocardium*) *sachalinensis*, studied previously.[4–6,10,11]

2. Experimental

2.1. Materials

Molluscs *C. albidus* and *Spisula sachalinensis* were collected in the Okhotsk Sea (near the Onekotan Island) and in Trinity Bay (north-western part of the Japan Sea), respectively. Laminarans from *Laminaria gurjanovae*, *L. cichorioides* and *Fucus evanescens* and translam ((1→3),(1→6)-β-D-glucans containing 2%, 10%, 35% and 25% of β-(1→6)-bonds, respectively), fucoidan (sulfated (1→3)-α-L-fucan) from *L. cichorioides* and pustulan ((1→6)-β-D-glucan) from *Umbellicaria russica* were prepared as described.[18] (1→3)-β-D-Xylan was provided by Professor A.I. Usov (Institute of Organic Chemistry of the Russian Academy of Sciences, Moscow). Lichenan ((1→3),(1→4)-β-D-glucan), pachiman ((1→3)-β-D-glucan), carboxymethyl pachiman, a glucan from *Sclerotium rolfsii* ((1→3),(1→6)-β-D-glucan with about 50% of β-(1→6)-bonds), amylopectin and *p*-nitrophenyl β-D-glucoside were commercial preparations. The SMART cDNA Amplification Kit was purchased from Clontech (USA); the InsT/Aclone PCR Product Cloning Kit, from Fermentas (Lithuania) and the ABI Prism Big Dye terminator cycle sequencing ready reaction kit, from Applied Biosystems (USA).

2.2. Enzymes purification

The *endo*-(1→3)-β-D-glucanases from *C. albidus* and *S. sachalinensis* were purified as described previously.[11]

2.3. Standard activity assay

The standard reaction mixture contained 0.05 mL of the enzyme (10^{-2} U) and 0.2 mL of 0.1% substrate in 0.05 M sodium acetate buffer (pH 4.5) with 0.25 M NaCl. The reaction was carried out at 20 °C for 10 min and stopped by heating at 100 °C for 3 min or by adding the Somogyi–Nelson reagent. (1→3)-β-D-Glucanase activity was determined by measuring the amount of reducing sugars released from substrates by the Somogyi–Nelson method[19] using glucose as standard, or by measuring viscosity as described by Hultin and Wanntorp[20] using carboxymethyl pachiman as substrate. The amount of enzyme catalyzing the formation of 1 μmol of glucose min^{-1} or decreasing the relative viscosity of the substrate solution by 1 mpoise s^{-1} under the above conditions was assumed to be one unit of hydrolytic activity or of viscosity measurement, respectively. The specific activity was estimated as units (U) per mg protein.

Hydrolysis of carboxymethyl pachiman (0.2% substrate in 0.05 M sodium acetate buffer with 0.25 M NaCl, pH 4.5) catalyzed by L_0 (5–20 μL, 10^{-2} U), both in the presence of glucose (final concentrations of 5%, 15% and 30%) and in its absence (as control), was measured by the Hultin and Wanntorp method.[20]

The amount of total neutral sugar was determined by the phenol/sulfuric acid method with D-glucose as standard.[21] The protein was determined by the Lowry method with BSA as standard.[22]

2.4. Peptide sequencing

The N-terminal amino-acid sequence of L_0 was performed on a Beckman 890C sequencer according to standard procedures.[23]

2.5. Enzyme characterization

The homogeneity and molecular mass of the purified *endo*-(1→3)-β-D-glucanase were established by SDS/PAGE using 13% (w/v) polyacrylamide gel according to the method of Laemmli.[24]

The thermal stability was studied by incubating the enzyme solution in 0.05 M sodium acetate buffer with 0.25 M sodium chloride, pH 4.5, at 15 °C, 25 °C, 37 °C then assaying for *endo*-(1→3)-β-D-glucanase activity as described in Section 2.3 at time intervals from 10 min to 2 h.

The Michaelis–Menten constant (K_m) for the hydrolysis reaction was determined from Lineweaver–Burk plots of data obtained by measuring the rate of laminaran hydrolysis catalyzed by the purified enzyme (10^{-2} U) under the standard assay conditions using a substrate concentration range of 0.1–1 mg/mL. The K_m value for transglycosylation reactions was determined in the same conditions using laminaran from *L. cichorioides* as substrate at the concentration range of 1–10 mg/mL. Transglycosylation reaction products using *p*-nitrophenyl β-D-glucoside (5 mg/mL) as acceptor were measured at 280 nm by HPLC of the appearing nitrophenyl gluco-oligosaccharides as described previously.[8] High-molecular weight products of enzymatic transformation of laminaran from *L. cichorioides* were isolated using the hydrophobic resin Polychrome-1 (polytetrafluoroethylene, Russia) and were determined as described previously.[16] The initial rates of hydrolysis (v_h) and transglycosylation (v_t) were measured at substrate conversion levels below 10%. To study the influence of substrate structure on the above-mentioned reactions, laminarans from *L. gurjanovae*, *L. cichorioides* and *F. evanescens*, translam, glucan from *S. rolfsii*, which are (1→3),(1→6)-β-D-glucans containing 2%, 10%, 25%, 35% and 50% of β-(1→6)-glycosidic bonds, respectively, were used. The degree of hydrolysis (% hydrolyzed glycosidic linkage of substrate) was determined after exhaustive hydrolysis of substrates, when the amount of product did not change after adding new portions of the enzyme.

2.6. Ultraviolet absorption spectrum

The UV absorption spectrum of L_0 was recorded with a Specord M-40 spectrophotometer (Carl Zeiss, Germany). The protein concentration was evaluated spectrophotometrically using $\varepsilon = 125\,485\ M^{-1}\,cm^{-1}$ at 280 nm calculated from the primary structure of L_0 by the ExPASy server (http://cn.expasy.org/tools/protparam.html).

2.7. Circular dichroism (CD) and secondary structure analysis

The secondary structure elements of the *endo*-(1→3)-β-D-glucanase from *C. albidus* were determined by CD spectroscopy at 20 °C. The CD spectrum was measured with a Jasco-500 A spectropolarimeter (Jasco Instruments S.A.) at 200–350 nm using 0.1 and1 cm path length cells and a protein concentration of 0.18 mg/mL. Results were expressed as molar ellipticity $[\theta]_\lambda$ (deg cm^2 $dmol^{-1}$), calculated as described by Adler et al.[25]

$$[\theta]_\lambda = (\theta_{obs} \cdot MRW/10 \cdot d \cdot c),$$

where θ_{obs} is the observed ellipticity in degrees; MRW is a mean amino acid residue weight of 114 Da calculated from the L_0 primary structure; d is a path length (cm); c is the protein concentration (g/mL).

The content of secondary-structure elements was determined using the CDSSTR algorithm.[26] The tertiary arrangement of the protein was determined by the CLUSTER algorithm.[27]

2.8. Fluorescence measurements

Fluorescence spectra were recorded at room temperature with a laboratory-made spectrofluorimeter, described earlier.[28] The monochromator slit did not exceed 2 nm. Spectra were measured at 300–400 nm and processed with a 5-nm step. All spectra were corrected for wavelength-dependent instrumental response characteristics. Intensities in the corrected spectra are proportional to the number of photons emitted per unit wavelength interval.

The protein fluorescence quantum yield was evaluated by comparing areas of corrected fluorescence spectra of a protein sample with that of aqueous tryptophan (0.23 at 20 °C) with the same absorbance at the excitation wavelength of 296.7 nm. The equilibrium binding constant $K_a = 1/K_d$ (K_d is the dissociation constant) and stoichiometries n for the protein–glucose complex were determined by plotting the relative quantum yield versus the molar ratio of total glucose G_t to the total L_0 concentration P_t and fitting to a function describing the binding of glucose to n identical sites in the protein.[29]

$$q = q_o - (q_o - q_\infty) \cdot \{(n + G_t/P_t + K_d/P_t) - [(n + G_t/P_t + K_d/P_t)^2 - 4 \cdot n \cdot G_t/P_t)]^{1/2}\}/2 \cdot n,$$

where q is the quantum yield in the presence of glucose, q_o is the quantum yield in the absence of glucose; q_∞ is the quantum yield at complete saturation with glucose.

2.9. cDNA cloning

Total RNA was isolated from 0.2 g of scallops hepatopancreas by the guanidine thiocyanate/phenol/chloroform method.[30] cDNA was synthesized from total RNA and subjected to 25 cycles of amplification (10 s at 95 °C, 20 s 63 °C and 90 s min at 72 °C) with a SMART cDNA Amplification Kit (Clontech, USA).

A pair of degenerate primers Gluc1: 5′-GGCGAGATCGA(C/T)AT(T/C)ATG-3′ and Gluc2: 5′-TAGAACGGCTT(A/G)TCGAA(T/C)GG-3′ was designed on the basis of the peptide sequences GEIDIM and PFDKPFY that are conserved for all *endo*-(1→3)-β-D-glucanases GHF16. The cDNA was diluted 20-fold and 1 μL of this solution was used for PCR with Gluc1 and Gluc2 primers. PCR was carried out for 37 cycles (10 s at 95 °C, 20 s at 58 °C, 1 min at 72 °C), and the 350 bp PCR product was cloned with InsT/AclonePCR Product Cloning Kit and sequenced with ABI Prism Big Dye terminator cycle sequencing ready reaction kit on ABI Prism 310 Genetic Analyzer.

The 3′- and 5′-terminal regions of the cDNA encoding L_0 were amplified by PCR with specific primers designated on the basis of the 350 bp cDNA fragment sequence and adapter-specific primer.[36] Gene specific primers for 5′- and 3-RACE were Gluc5: 5′-GTGTAGAAGCAACATAGTTG-3′ and Gluc3: 5′-CGGAAACAACATATGGGC-3′, respectively. Two obtained cDNA fragments with lengths of 600 bp and 320 bp were cloned and sequenced as described above.

2.10. Sequence analysis and comparisons

Sequences were analyzed using the NCBI-BLAST2 website (http://www.ebi.ac.uk/blastall/). Domain architectures were identified by SMART tool (http://smart.embl-heidelberg.de). The presence and location of the signal peptide cleavage site within the amino acid sequence was predicted by SignalP (http://www.cbs.dtu.dk/services/SignalP/). Multiple sequence alignment was performed with the CLUSTALW version 1.8 software (http://www.ebi.ac.uk/clustalw/index.html) using Blosum matrix. The secondary-structure elements were predicted with SSPRO server (http://scratch.proteomics.ics.uci.edu/) and SOPMA program (http://searchlauncher.bcm.tmc.edu/seq-search/struc-predict.html).

3. Results

3.1. *endo*-(1→3)-β-D-Glucanase characterization

L_0 was purified from scallops crystalline styles by combining ion-exchange chromatography on CM-Sephadex C-50, hydrophobic chromatography on Phenyl-Sepharose CL-4B, gel-filtration on Sephadex G-75 and ion-exchange chromatography on CM-Cellulose CM-52 as described previously.[11] The purified enzyme was subjected to SDS–PAGE and showed a single protein band of 37 kDa (Fig. 1). The N-terminus sequence of L_0 was revealed to be AGFRDDF.

The specific enzyme activities of purified L_0 with various substrates were determined, and the results indicated that L_0 had the highest specificity for the laminaran with a degree of hydrolysis of the laminaran glycosidic linkages of about 60%. L_0 acted weakly on the insoluble β-D-glucans such as pachiman and lichenan (hydrolysis degree about 8% and 4%, respectively), and did not act on the other assayed substrates, including a yeast (1→3),(1→6)-β-D-glucan, (1→3)-β-D-xylan, pustulan, fucoidan and amylopectin.

The thermal stability of the *endo*-(1→3)-β-D-glucanases from *C. albidus* and *S. sachalinensis* (L_{IV}) were investigated by incubating the purified enzymes at different temperatures (Fig. 2). The half-life for L_0 was determined at 15, 25 and 37 °C. The scallop enzyme showed a half-life of 30 min at 25 °C, and was rapidly inactivated at 37 °C with a half-life of 10 min. At 50 °C, L_0 was inactivated instantly. The hydrolytic activity of the *endo*-(1→3)-β-D-glucanase from *S. sachalinensis* did not change under the same conditions (Fig. 2).

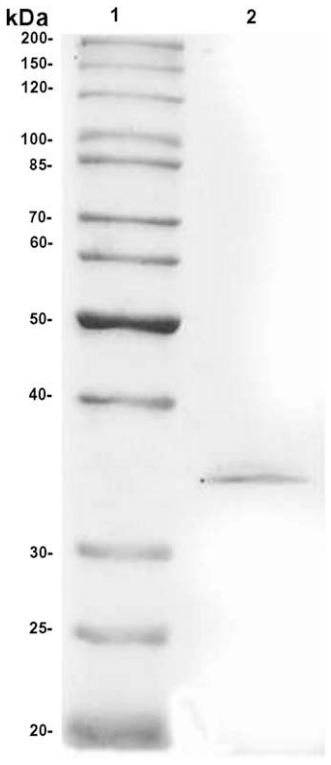

Figure 1. SDS–PAGE of the *endo*-(1→3)-β-D-glucanase from *C. albidus*: (1) standard molecular mass markers (kDa); (2) purified L_0 under reducing conditions.

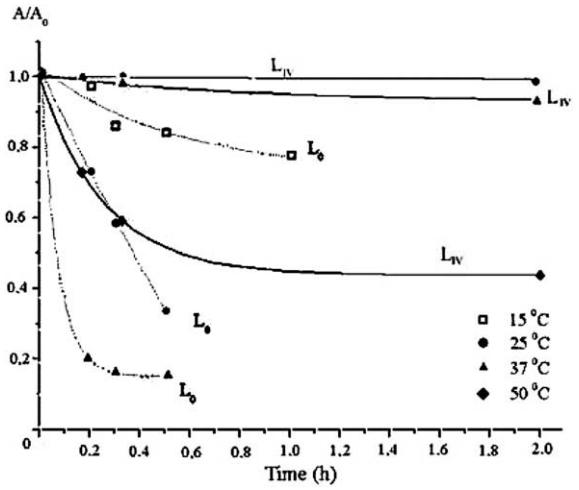

Figure 2. Thermal stability of the *endo*-(1→3)-β-D-glucanase from *C. albidus* (L_0) and *S. sachalinensis* (L_{IV}).

The effect of laminaran concentration on hydrolytic and transglycosylation (*p*-nitrophenyl β-D-glucoside and laminaran as acceptors) activities of L_0 were estimated and apparent K_m values of these reactions were 0.75 mg/mL, 2 mg/mL and 5 mg/mL, respectively.

The influence of substrate structure on initial velocities of hydrolysis (v_h) and transglycosylation (v_t) was studied. Velocity ratios v_h and v_t for (1→3),(1→6)-β-D-glucans with a different proportion of β-(1→6)-glycosidic bonds, namely laminarans from *L. gurjanovae* (1) and *L. cichorioides* (2), translam (3), laminaran from *F. evanescens* (4) and glucan from *S. rolfsii* (5), containing 2%, 10%, 25%, 35% and 50% of β-(1→6)-glycosidic bonds, respectively, were as follows: $v_h(1):v_h(2):v_h(3):v_h(4):v_h(5) = 80:100:70:50:0$ and $v_t(1):v_t(2):v_t(3):v_t(4):v_t(5) = 80:100:50:20:0$.

Some physicochemical and catalytic properties of L_0 in comparison with those of the *endo*-(1→3)-β-D-glucanases from *S. sachalinensis* (L_{IV}) and *M. yessoensis* (L_V) are summarized in Table 1.

3.2. CD measurement

CD measurements in the far UV-region (200–250 nm) revealed that L_0 contained about 8.7% α-helices, 30% β-sheet, 22% β-turn and 40% unordered structure.

3.3. Equilibrium binding of L_0 with D-glucose

The tryptophane fluorescence spectrum of L_0 showed an emission maximum at 334 nm. Addition of glucose up to a concentration sufficient for saturation of the enzyme's binding sites resulted in Trp fluorescence quenching of about 50% as well as a red shift in the maximum of fluorescence emission of 1–1.5 nm. The titration curve series under the defined experimental conditions is presented in Figure 3. The decrease in the fluorescence quantum yield was plotted against the mole ratio of total glucose to total L_0 concentration. An apparent association constant of glucose to the enzyme of $K_a = 7.4 \times 10^5 \pm 1.1 \times 10^5\ M^{-1}$ and stoichiometry of $n = 13.3 \pm 0.7$ were calculated.

3.4. Primary structure of L_0 and comparisons

The primary structure of L_0 was determined by cDNA sequencing. The GeneBank accession number of L_0 is DQ093347. The cDNA of 1119 bp contains an open reading frame of 1017 bp encoding a polypeptide consisting of 339 amino acid residues. The start methi-

Table 1
Characteristics of the *endo*-(1→3)-β-D-glucanases from marine molluscs[6–8,11,14–16,32]

Enzyme, source	*Mr* (kDa)	pI	Amino acid residues significant functionally	Optimal conditions			Hydrolysis reaction				Transglycosylation reaction			
				pH	*T* (°C)	NaCl (M)	Glycosidic bond hydrolyzed	K_m (laminaran, mg/mL)	Degree of laminaran hydrolysis (%)	Amount of Glc* in final product (%)	Synthesized glucosidic bonds	Inclusion of Np-Glc** (%/h)	K_m (laminaran, mg/mL)	Yield of HMF*** (%)	K_m (laminaran, mg/mL)
L_0, *C. albidus*	37	7.5	Glu(Asp), Trp, His	4.4	20	0.1–0.3	β-(1→3)	0.75	60	30	β-(1→3) β-(1→4) β-(1→6)	13	2	20	5
L_{IV}, *S. sachalinensis*	38	7.6	Glu(Asp), Trp, His	5.8	45	0.01–0.3	β-(1→3)	0.25	70	40	β-(1→3)	11	1	4	nd
L_V, *M. yessoensis*	36	nd	Glu(Asp), Trp, His	4.5	45	0.2–0.5	β-(1→3)	0.6	60	30	β-(1→3)	12	nd	nd	nd

nd—Not detected.
* Glc—glucose.
** Np-Glc—*p*-nitrophenyl-β-D-glucoside.
*** HMF—high-molecular weight fraction.

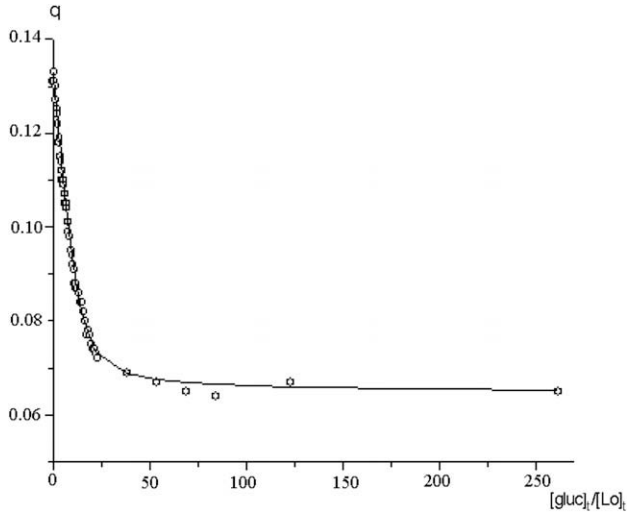

Figure 3. Binding of D-glucose with the *endo*-(1→3)-β-D-glucanase from *C. albidus*. The fluorescence quantum yield (points) is plot versus the mole ratio of total glucose and total L_0. The enzyme concentration was 0.81 μM in 0.05 M acetate buffer; pH 5.15. The solid line represents the best fit of the data to Eq. 1.

onine is followed by a hydrophobic stretch of 15 amino acid residues, which has been predicted to be a signal sequence by SIGNALP (http://www.cbs.dtu.dk/services/SignalP/). The predicted mature enzyme consists of 324 amino acid residues and has a calculated molecular mass of 36.9 kDa, agreeing with the experimental result for the purified enzyme. The N-terminal region of the deduced amino acid sequence coincides completely with the N-terminus moiety of L_0 determined by the Edman method.

Analysis of the *C. albidus endo*-(1→3)-β-D-glucanase deduced amino acid sequence by SMART tool revealed that the sequence from Tyr-80 to Gly-304 is a characteristic domain of the glycoside hydrolase family 16. Thus L_0 is a novel member of the *endo*-(1→3)-β-D-glucanase (laminarinase) subfamily of glycoside hydrolase family 16 (GHF16).

Results of BLAST2 search elicited the highest homology of L_0 with *endo*-(1→3)-β-D-glucanases from the scallop *M. yessoensis* (GeneBank accession no. AY848857) and from the bivalve *S. sachalinensis* (GeneBank accession no. AAP74223) (86% and 45% identity, 93% and 60% similarity, respectively). Significant sequence homology (about 40% identity and 55% similarity) was also found with the *endo*-(1→3)-β-D-glucanase from sea urchin *S. purpuratus* (GeneBank accession no. AAC47235) and the invertebrates lipopolysaccharide- and β-(1→3)-glucan binding proteins activating the prophenoloxidase and clotting system. The primary structure similarity between L_0 and GHF16 *endo*-(1→3)-β-D-glucanases from marine bacteria *Rhodothermus marinus* (GeneBank accession no. AAC69707), *Pyrococcus furiosus DSM* (GeneBank accession no. AAC25554), *Thermotoga neapolitana* (GeneBank accession no. CAA88008) and the *endo*-(1→3)-β-D-glucanase from nematode *Bursaphelenchus xylophilus* (GeneBank accession no. BAE02683) was 28%, 27%, 22% and 25%, respectively.

4. Discussion

Our earlier studies have shown that marine invertebrates were important sources of *endo*-(1→3)-β-D-glucanases, and the highest *endo*-(1→3)-β-D-glucanase activities have been found within crystalline styles of bivalves.[4,5] *endo*-(1→3)-β-D-Glucanases from crystalline styles of *Spisula sachalinensis*, *Mezuhopecten yessoensis* and *C. albidus* have been studied in greater detail.[4–8,10,13–17,31,32] The molluscs enzymes have similar molecular mass and isoelectric point (pI), but differ in thermal stability and dependence of activity on NaCl concentration (Table 1). The *endo*-(1→3)-β-D-glucanase from *C. albidus* was more thermolabile and required higher ionic strength, than L_{IV} and $L_{V,}$ whose catalytic activities did not change over a wide range of ionic strength. The scallop *C. albidus* inhabits the depths of 60–120 m with summer temperature near the bottom of 1–10 °C, whereas *S. sachalinensis* and *M. yessoensis* inhabit depths with higher summer temperatures (up to 20 °C). The extreme thermolability of L_0 is probably a result of the enzyme adaptation to a low temperature environment.

L_0 as well as L_V and L_{IV} hydrolyze (1→3)-β-D-glucans randomly with glucose, laminaribiose, laminaritriose and higher mixed (1→3),(1→6)-β-D-glucooligosaccharides as final products, weakly act on insoluble β-D-glucans, such as pachiman and lichenan, and do not act on the highly branched yeast (1→3),(1→6)-β-D-glucan and (1→3)-β-D-xylan, pustulan, fucoidan and amylopectin. The enzymes catalyze hydrolysis of the β-(1→3)-glycosidic bonds with retention of the anomeric configuration at the cleavage site.[13] These results confirm that L_0 as well as L_V and L_{IV} are *endo*-(1→3)-β-D-glucanases (laminarinase; EC 3.2.1.39).

One of the characteristics of laminaran hydrolysis by L_0 and other marine molluscs *endo*-(1→3)-β-D-glucanases is the large amount of glucose formation, namely about 30% for the *endo*-(1→3)-β-D-glucanases from *C. albidus* and *M. yessoensis* and 40% for the *endo*-(1→3)-β-D-glucanase from *S. sachalinensis*[32] (Table 1). This is unusual for *endo*-(1→3)-β-D-glucanases. It was shown earlier that marine invertebrates contained only *endo*-(1→3)-β-D-glucanases[4,5,15] unlike terrestrial microorganisms and plants which possess a set of *exo*- and *endo*-(1→3)-β-D-glucanases.[1–3] Probably, this mode of action of *endo*-(1→3)-β-D-glucanases is a compensation for the lack of *exo*-(1→3)-β-D-glucanases in marine molluscs, and multiple attack model of action was proposed previously.[6,31]

Another characteristic of the marine molluscs *endo*-(1→3)-β-D-glucanases is their ability to catalyze transglycosylation reactions. It was shown that L_0 performed transglycosylation to generate both β-(1→3)- and β-(1→4)-glycosidic linkages in the final products, whereas the *endo*-(1→3)-β-D-glucanases from *M. yessoensis* and *S. sachalinensis* formed only β-(1→3)-glycosidic bonds (Table 1).[6–8] Efficiency of hydrolysis and transglycosylation catalyzed by L_0 depended substantially on the substrate structure and decreased appreciably with the increase of β-(1→6)-glycosidic linkage content. The transglycosylation activity to *p*-nitrophenyl β-D-glucoside as acceptor was more sensitive to substrate structure than was hydrolysis. Laminaran from *L. cichorioides* with 10% of β-(1→6)-glycosidic bonds had the most optimal structure for both reactions.

D-Glucose was shown to be the best acceptor for transglycosylation.[6,15] To investigate the binding of glucose with L_0, the methods of intrinsic tryptophan (Trp) fluorescence and CD were used. The fluorescence of L_0 was more sensitive to the changes of Trp residues' microenvironment under glucose binding as seen from variations in the near-UV CD band at 270–300 nm. The shift of fluorescence maximum and the decrease of quantum yield indicated that the environment of some of the 19 Trp residues found in L_0 became less hydrophobic. Highly similar far- and near-UV CD-spectra were observed for L_0 unbound and bound with glucose (data not shown), indicating that the binding caused no major changes in the secondary and tertiary structure of the protein. It was shown that D-glucose with concentration of up to 30% did not inhibit hydrolysis of carboxymethyl pachiman, and formed complexes with L_0 ($K_d \sim 10^{-6}$ M).[7,8] The K_m ($\sim K_d$ of enzyme-substrate complexes) determined with laminaran from *L. cichorioides* (molecular mass of about 5 kDa) was 0.75 mg/mL, or about 15×10^{-5} M. Hence, both laminaran and D-glucose were bound by the enzyme effectively, but did not compete with each other,

possibly because D-glucose binds in an acceptor site beyond the catalytic or substrate binding region.

It was shown that L_0, unlike other molluscs *endo*-(1→3)-β-D-glucanases, possessed ability to transform laminaran into more branched and high-molecular weight (1→3),(1→6)-β-D-glucans due to synthesis of not only β-(1→3)- but also β-(1→6)-glycosidic linkages (Table 1).[16] Two fractions of such β-D-glucans designated as 'translam' and 'antivir' exhibited immunostimulating activity.[17]

Analysis of the L_0 primary structure revealed that the enzyme, as well as *endo*-(1→3)-β-D-glucanases from *S. sachalinensis*, *M. yessoensis*, sea urchin *S. purpuratus*, the nematode *B. xylophilus*[12] and from marine bacteria, belongs to glycoside hydrolase family 16. Despite high sequence homology between L_0, L_V and L_{IV} (93% and 60%, respectively), the *endo*-(1→3)-β-D-glucanase from *C. albidus* showed significant difference in physicochemical and catalytic properties. Identification of the amino acid residues responsible for the unique enzymatic properties of L_0 is of great interest, and will be the subject of further investigation.

The multiple alignment of GHF16 domains of the bacterial and invertebrates' *endo*-(1→3)-β-D-glucanases (Fig. 4) showed that they contained the conserved site Gly149-Glu150-Xxx151-Asp152-Xxx153-Met/Val154-Glu155 (numbering of the L_0, Xxx—hydrophobic amino acid residue) including the two catalytic residues Glu150 and Glu155,[33] and the substrate binding site Trp130-Pro131-Ala132-Xxx133-Trp134-Met135-Leu136.[34] The significance of tryptophan residues for the enzymatic activity of molluscs *endo*-(1→3)-β-D-glucanases was determined by the chemical modification methods previously.[11] The important role of tryptophan residues of endoglucanase A for substrate binding was shown by Din et al.[35] Multiple alignments revealed that two histidine residues, His179 and His208, are invariant for all known *endo*-(1→3)-β-D-glucanases (Fig. 4). Studies of chemical modifications of histidine residues in molluscs *endo*-(1→3)-β-D-glucanases demonstrated their importance for catalysis.[11] Moreover, hypothesis about participation of the histidine and aspartic acid residues, conserved in κ-carrageenases, β-agarases and laminarinases of GHF16, 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 *Pseudoalteromonas carrageenovora*.[36]

The CD measurements in the far UV-region as well as a theoretical prediction of a secondary structure of L_0 performed on the basis of the amino acid sequence by SOPM program and SSpro8 server show that the *endo*-(1→3)-β-D-glucanase belongs to the β class of protein and has a jelly-roll structure, as the other glycoside hydrolases family 16.[36–38]

5. Conclusion

Our studies of the biochemical properties of the *endo*-(1→3)-β-D-glucanase from the scallop *C. albidus* reveals significant differences from other marine molluscs *endo*-(1→3)-β-D-glucanases, despite their high structural homology. The unique ability of L_0 to catalyze the transfer of glycosyl residues to laminaran and gluco-oligosaccharides forming (1→3),(1→6)-β-D-glucans is of

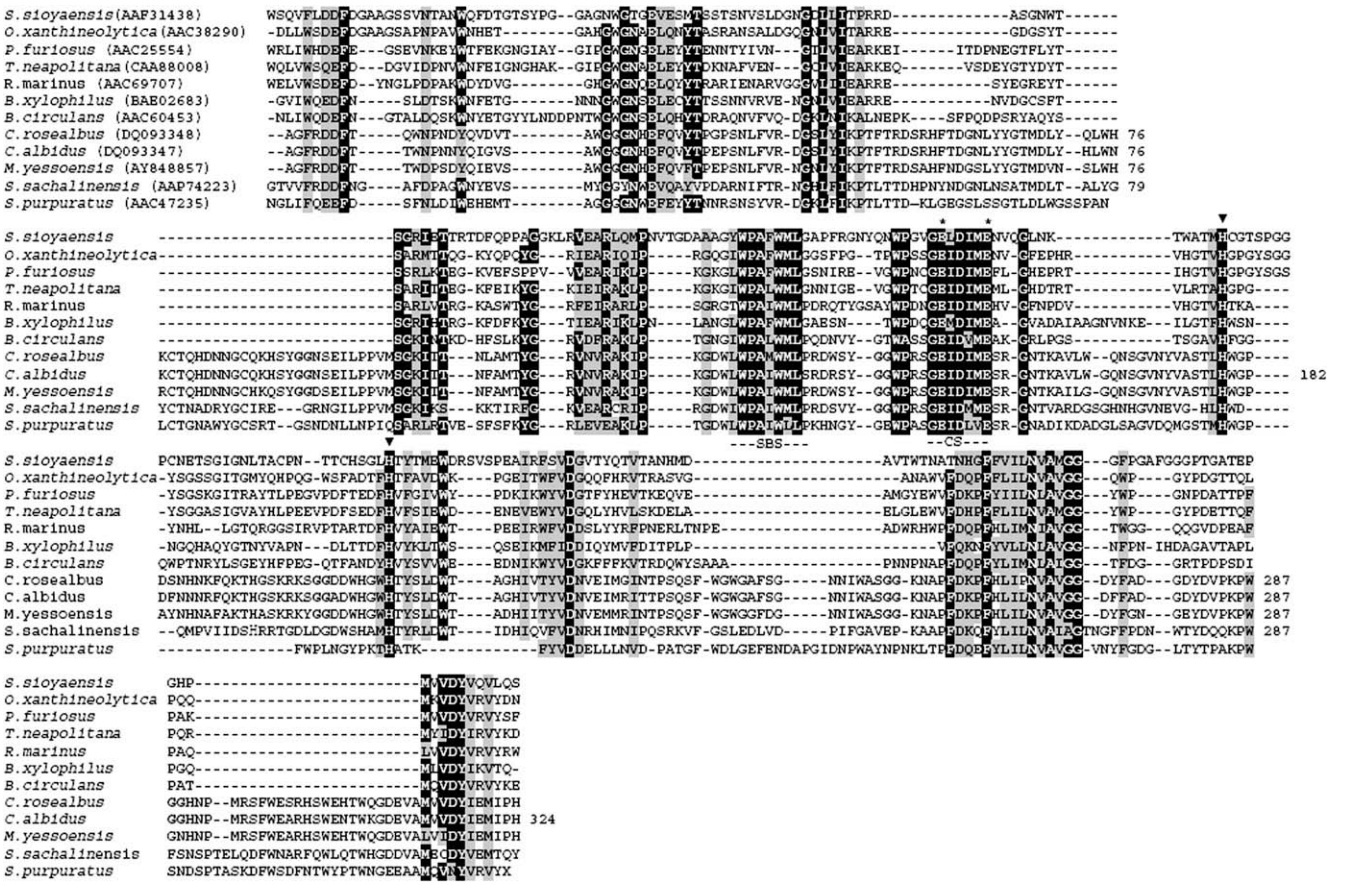

Figure 4. Alignment of GHF16 domain sequences of invertebrates' and bacterial *endo*-(1→3)-β-D-glucanases (GeneBank accession numbers are in brackets). Numbering corresponds to the mature L_0 sequence (without signal peptide). Conserved residues of the sequences are highlighted in black (strictly conserved) or in grey (homologous replacements). Substrate binding site (SBS) and catalytic site (CS) are dot-lined. The asterisks above the sequences indicate catalytic residues. The arrows indicate the conserved histidine residues.

peculiar interest for further investigation of the enzyme structure and function as well as for application in the direct synthesis of biologically active β-D-glucans.

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References

1. Kobayashi, Y.; Tanaka, H.; Ogasawara, N. *Agric. Biol. Chem.* **1974**, *38*, 959–965.
2. Jeffries, T. W.; MacMillan, J. D. *Carbohydr. Res.* **1981**, *95*, 87–100.
3. Bulgakov, V. P.; Kusajkin, M. G.; Tchernoded, G. K.; Zvyagintseva, T. N.; Zhuravlev, Y. N. *Fitoterapia* **2002**, *73*, 638–643.
4. Sova, V. V.; Elyakova, L. A. *Biochim. Biophys. Acta* **1972**, *258*, 219–227.
5. Elyakova, L. A.; Shilova, T. G. *Comp. Biochem. Physiol.* **1979**, *64*, 245–248.
6. Bezukladnikov, P. W.; Elyakova, L. A. *Carbohydr. Res.* **1990**, *203*, 119–127.
7. Zvyagintseva, T. N.; Evtushenko, E. V.; Elyakova, L. A. *Bioorg. Khim.* **1989**, *15*, 1206–1214.
8. Zvyagintseva, T. N.; Makar'eva, T. N.; Ermakova, S. P.; Elyakova, L. A. *Bioorg. Khim.* **1998**, *24*, 219–223.
9. Bachman, E. S.; McClay, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6808–6813.
10. 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.
11. Kovalchuk, S. N.; Sundukova, E. V.; Kusaykin, M. I.; Guzev, K. V.; Anastyuk, S. D.; Likhatskaya, G. N.; Trifonov, E. V.; Nurminski, E. A.; Kozhemyako, V. B.; Zvyagintseva, T. N.; Rasskazov, V. A. *Comp. Biochem. Physiol.* **2006**, *143*, 473–485.
12. Kikuchi, T.; Shibuya, H.; Jones, J. T. *Biochem. J.* **2005**, *389*, 117–125.
13. Privalova, N. M.; Elyakova, L. A. *Comp. Biochem. Physiol.* **1978**, *60*, 225–228.
14. Lakisova, I. Yu.; Elyakova, L. A.; Emelyanenko, V. I.; Permyakov, E. A.; Burstein, E. A. *Biophysika (Russia)* **1988**, *23*, 31–35.
15. Zvyagintseva, T. N.; Elyakova, L. A. *Bioorg. Khim. (Moscow)* **1994**, *20*, 453–474.
16. Zvyagintseva, T. N.; Elyakova, L. A.; Isakov, V. V. *Bioorg. Khim. (Moscow)* **1995**, *21*, 218–225.
17. Solov'eva, T. F.; Elyakova, L. A.; Zvyagintseva, T. N.; Ermak, I. M. *MTSJ (U.S.A.)* **1996**, *30*, 35–39.
18. Zvyagintseva, T. N.; Shevchenko, N. M.; Popivnich, I. B.; Isakov, V. V.; Scobun, A. S.; Sundukova, Y. V.; Elyakova, L. A. *Carbohydr. Res.* **1999**, *322*, 32–39.
19. Somogyi, M. *J. Biol. Chem.* **1952**, *195*, 19–23.
20. Hultin, E.; Wanntorp, I. *Acta Chem. Scand.* **1966**, *3*, 2667–2677.
21. Dubois, M.; Gilles, K. A.; Hamilton, J.; Rebers, P. A.; Smith, F. *Nature* **1951**, *168*, 167.
22. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
23. Edman, P.; Begg, G. *Eur. J. Biochem.* **1967**, *1*, 80–91.
24. Laemmli, U. K. *Nature* **1970**, *227*, 680–685.
25. Adler, A. J.; Greenfield, N. J.; Fasman, G. D. *Methods Enzymol.* **1971**, *27*, 675–735.
26. Provencher, S. W.; Glöckner, *J. Biochem.* **1981**, *20*, 33–37.
27. Venyaminov, S. Yu.; Vassilenko, K. S. *Anal. Biochem.* **1994**, *222*, 176–184.
28. Permyakov, E. A.; Burstein, E. A.; Sawada, V.; Yamazaki, I. *Biochim. Biophys. Acta* **1977**, *491*, 149–154.
29. Tanford, C. *Physical Chemistry of Macromolecules*; Wiley: New York, 1961. p 710.
30. Chomczynski, P.; Sacchi, N. *Anal. Biochem.* **1987**, *162*, 156–159.
31. Mazur, A. K. *Biopolymers* **1984**, *23*, 859–876.
32. Shevchenko, N. M.; Zvyagintseva, T. N.; Elyakova, L. A. *Carbohydr. Res.* **1986**, *148*, 57–62.
33. Krah, M.; Misselwitz, R.; Politz, O.; Thomsen, K. K.; Welfle, H.; Borriss, R. *Eur. J. Biochem.* **1998**, *257*, 101–111.
34. Ferrer, P.; Halkier, T.; Hedegaard, L.; Savva, D.; Diers, I.; Asenjo, J. A. *J. Bacteriol.* **1996**, *178*, 4751–4757.
35. Din, N.; Forsythe, I. J.; Burtnick, L. D.; Gilkes, N. R.; Miller, R. C., Jr.; Warren, R. A.; Kilburn, D. G. *Mol. Microbiol.* **1994**, *11*, 747–755.
36. Michel, G.; Chantalat, L.; Duee, E.; Barbeyron, T.; Henrissat, B.; Kloareg, B.; Dideberg, O. *Structure (Cambridge)* **2001**, *9*, 513–525.
37. Keitel, T.; Simon, O.; Borriss, R.; Heinemann, U. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5287–5291.
38. Allouch, J.; Jam, M.; Helbert, W.; Barbeyron, T.; Kloareg, B.; Henrissat, B.; Czjzek, M. *J. Biol. Chem.* **2003**, *8*, 47171–47180.