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Comparative Biochemistry and Physiology, Part B xx (2006) xxx–xxx
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Purification, cDNA cloning and homology modeling of endo-1,3-β-D-glucanase from scallop *Mizuhopecten yessoensis*

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Received 8 August 2005; received in revised form 29 December 2005; accepted 30 December 2005

Abstract

The retaining endo-1,3-β-D-glucanase (LV) with molecular mass of 36 kDa was purified to homogeneity from the crystalline styles of scallop *Mizuhopecten yessoensis*. The purified enzyme catalyzed hydrolysis of laminaran as endo-enzyme forming glucose, laminaribiose and higher oligosaccharides as products ($K_m \sim 600$ μg/mL). The 1,3-β-D-glucanase effectively catalyzed transglycosylation reaction that is typical of endo-enzymes too. Optima of pH and temperature were at 4.5 and 45 °C, respectively. cDNA encoding the endo-1,3-β-D-glucanase was cloned by PCR-based methods. It contained an open reading frame that encoded 339-amino acids protein. The predicted endo-1,3-β-D-glucanase amino acid sequence included a characteristic domain of the glycosyl hydrolases family 16 and revealed closest homology with 1,3-β-D-glucanases from bivalve *Pseudocardium sachalinensis*, sea urchin *Strongylocentrotus purpuratus* and invertebrates lipopolysaccharide and β-1,3-glucan-binding proteins.

The fold of the LV was more closely related to κ-carrageenase, agarase and 1,3;1,4-β-D-glucanase from glycosyl hydrolases family 16. Homology model of the endo-1,3-β-D-glucanase from *M. yessoensis* was obtained with MOE on the base of the crystal structure of κ-carrageenase from *P. carrageonovora* as template. Putative three-dimensional structures of the LV complexes with substrate laminarihexaose or glucanase inhibitor halistanol sulfate showed that the binding sites of the halistanol sulfate and laminarihexaose are located in the enzyme catalytic site and overlapped.

Keywords: cDNA cloning; Endo-1,3-β-D-glucanase; Specificity; Homology model; Low-resolution docking; Scallop; Predicted structure

1. Introduction

Enzymes that catalyze hydrolysis of *O*-glycosidic bond between glucopyranose residues of 1,3-β-D-glucans and typically referred to as 1,3-β-D-glucanases or laminarinases have been found in bacteria, fungi, plants, marine micro- and macroorganisms. They are involved in various cell functions, such as degradation of seed glucans, control of cell elongation and cell expansion in yeast, fertilization and removal of phloem callus in plants, etc. (Mackay et al., 1985). The marine invertebrates were found to be the richest sources of endo-1,3-β-D-glucanases, but up to date these endo-1,3-β-D-glucanases are poorly explored enzymes. Some laminarinases have been isolated from the crystalline styles of bivalves *Spisula sachalinensis* (*Pseudocardium sachalinensis*) (endo-1,3-β-D-glucanases LIII and LIV) (Sova et al., 1970a,b) and *Chlamys albidus* (L0) (Privalova and Elyakova, 1978), and also from the eggs of the sea urchins *Strongylocentrotus purpuratus* (Bachman and McClay, 1996) and *S. intermedius* (Le) (Sova et al., 1997). Physico-chemical properties of these enzymes as well as their catalytic mechanisms were studied in details (Shevchenko et al., 1986; Bezukladnikov and Elyakova, 1990;

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doi:10.1016/j.cbpb.2005.12.024

Zvyagintseva and Elyakova, 1994; Sova et al., 1997). But there is insufficient information about structure of the invertebrate laminarinases yet. To date only two 1,3-β-D-glucanases from invertebrates have been cloned and their sequences were determined. They are 1,3-β-D-glucanase from *S. purpuratus* (Bachman and McClay, 1996) and endo-1,3-β-D-glucanase (LIV) from *P. sachalinensis* (Kozhemyako et al., 2004). In the paper, we have reported on the isolation, purification, some properties, cDNA cloning and homology modeling of the endo-1,3-β-D-glucanase (LV) from scallop *Mizuhopecten yessoensis*.

2. Materials and methods

2.1. Animals

Scallops *M. yessoensis* were collected in Trinity Bay, Japan Sea.

2.2. Materials and analytical methods

Laminaran (1,3;1,6-β-D-glucan) and fucoidan (sulfated 1,3-α-L-fucan) from *Laminaria cichorioides*, and pustulan (1,6-β-D-glucan) from *Umbellicaria russica* were prepared as described by Zvyagintseva et al. (1999), 1,3-β-D-xylan was provided by Prof. A.I. Usov (IOC RAS, Moscow), lichenan (1,3;1,4-β-D-glucan), pachyman (1,3-β-D-glucan), yeast glucan (1,3;1,6-β-D-glucan) and amylopectin were commercial preparations.

The quantity of total neutral sugar was determined by the phenol/sulfuric acid method with D-glucose as standard (Dubois et al., 1956). The protein quantity was determined by the Lowry method with BSA as standard (Lowry et al., 1951). Polarimetric procedure was made as described earlier (Privalova and Elyakova, 1978).

2.3. Enzyme purification

The crystalline styles (20 g) were homogenized with 60 mL of cooled 0.05 M sodium acetate buffer (pH 5.0) for 60 min at 4 °C. The suspension was then centrifuged for 15 min at 11,000×*g*. The supernatant was stirred with CM-Sephadex C-50 (50 mL), equilibrated with 100 mL 0.05 M sodium acetate buffer (pH 4.5; buffer A) for 1–2 h. The CM-Sephadex C-50 (50 mL) was then washed sequentially with buffer A and 0.1 M sodium chloride in the same buffer. Proteins with 1,3-β-D-glucanase activity were eluted with 0.15 M sodium chloride in buffer A. Fractions containing active enzyme were pooled and loaded onto a Phenyl-Sepharose CL-4B column (1.3×2 cm) equilibrated with 0.05 M sodium acetate buffer (pH 5.1) containing 1 M sodium chloride (buffer B). The column was washed with equilibration buffer to remove material that did not bind, and then 50% ethylene glycol in buffer B was passed through the column. Fractions containing active enzyme were pooled and run on a Sephadex G-75 column (1.5×40 cm) equilibrated with 0.05 M sodium acetate buffer (pH 5.0; buffer C). Elution was carried out with buffer C. Fractions containing 1,3-β-D-glucanase activities were combined and purified further on a CM-Cellulose CM-52 column equilibrated with buffer C.

2.4. Standard activity assay

The standard reaction mixture contained 0.2 mL 0.1% laminaran in 0.05 M sodium acetate buffer (pH 4.5) and 50 μL of the enzyme. The reaction was carried out at 25 °C for 30 min and stopped by heating at 100 °C for 3 min. 1,3-β-D-Glucanase activity was determined by measuring the amount of reducing sugars released from laminaran by the Somogyi–Nelson method (Somogyi, 1952) using glucose as standard. The amount of the enzyme catalyzing the formation of 1 μmol of glucose per minute under above conditions was assumed as 1 unit of enzymatic activity. Specific activity was estimated as units (U) per mg protein.

2.5. Enzyme characterization

The homogeneity and molecular mass of purified 1,3-β-D-glucanase was established by SDS/PAGE (13% w/v acrylamide) according to the Laemmli method (Laemmli, 1970).

The optimal pH was determined by the standard activity assay at 25 °C using 0.1 M sodium acetate buffer in the interval pH 3.5–6.0 and 0.1 M sodium phosphate buffer in the interval pH 5.5–8.0.

The optimal temperature was determined by the standard activity assay at 25°, 35°, 40°, 45°, 50°, and 55 °C. Reaction mixture containing 0.1% laminaran solution, 0.05 M sodium succinate buffer (pH 4.5) with 0.25 M sodium chloride and 0.01 U of the enzyme was incubated at desired temperature for 10 min, and reducing sugars were assayed in samples by standard procedure.

The thermal stability was studied by incubating the enzyme solution in 0.05 M sodium acetate buffer (pH 4.5) with 0.25 M sodium chloride at 25°, 37° and 57 °C and assaying for residual activity by standard procedure at regular intervals.

Michaelis–Menten constant was determined from Lineweaver–Burk plots of data obtained by measuring the rate of laminaran hydrolysis catalyzed by purified laminarinase (10^{-2} U) under the standard assay conditions using a substrate concentration range of 0.01–0.1%. Hydrolysis of other polysaccharides by LV was conducted using a substrate concentration of 0.1%.

2.6. Preparation of products of the enzymatic reaction

2.6.1. Products of the reaction of hydrolysis

Laminaran (5 mg) was dissolved in 1 mL of buffer A, containing 10^{-2} U enzyme. Mixture was incubated at 37 °C for 5, 10 and 30 min, and reaction was terminated by heating at 100 °C for 3 min.

2.6.2. Products of the reaction of transglycosylation

Laminaran (2 mg) and methyl-β-D-glucopyranoside (2 mg) were dissolved in 1 mL of 0.05 M sodium acetate buffer, pH

4.5, containing 10^{-2} U enzyme, and mixture was incubated at 37 °C for 5 min.

2.7. MALDI MS spectra of laminaran and products of the enzymatic reaction

MALDI MS spectra of laminaran and products of the enzymatic reaction were recorded on Bruker Biflex III MALDI-TOF mass spectrometer with nitrogen laser. Sample preparation: mixture containing 1 μl of sample (1 mg/mL for oligosaccharides and 5 mg/mL for laminaran) and 1 μl of 2.5-dihydroxybenzoic acid as matrix was applied onto the sample plate, allowed to dry and then recrystallized with methanol to improve crystal homogeneity. Instrument settings: accelerating voltage—19 kV, laser power—20, number of shots—30, laser shot rate—4 Hz. All spectra were recorded in reflection mode.

2.8. Effect of group-specific reagents

To an inhibitor solution of desired concentration, 0.1 mL of the enzyme solution (0.08 mg/mL) was added. After 30-min incubation at room temperature, the residual activity in samples was assayed by standard procedure. The inhibitors used are listed in Table 2.

2.9. RNA isolation and cDNA synthesis

Total RNA was isolated from 0.2 g of mollusks hepatopancreas by guanidine thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). The cDNA library was synthesized from total RNA and amplified by a SMART PCR cDNA Synthesis Kit (CLONTECH) under the following conditions: 25 cycles at 95 °C (8 s), 63 °C (30 s) and 72 °C (1.5 min).

2.10. Gene cloning and DNA sequencing

A pair of degenerate primers was designed on the basis of sequences GEIDIM and PFDKPFY conservative for endo-1,3-β-D-glucanases. They were, respectively:

Gluc1: 5′-GGCGAGATCGA(C/T)AT(TC)ATG-3′ and
Gluc2: 5′-TAGAACGGCTT(A/G)TCGAA(T/C)GG-3′.

The cDNA library was diluted 50-fold in water and 1 μL of this solution was used for PCR with Gluc1 and Gluc2 primers. PCR reaction was carried out for 37 cycles (95 °C, 10 s; 58 °C, 20 s; 72 °C, 1 min). Approximately 350 bp fragment was obtained. PCR product was cloned by InsT/Aclone™ PCR Product Cloning Kit (Fermentas) and sequenced using an ABI 310 automated sequencer with Big Dye™ (ABI Prism) dideoxyterminators.

The 3′- and 5′-terminal regions of the cDNA encoding LV were amplified by the method of 3′- and 5′-RACE with specific primers designed on the determined sequence and adapter-specific primer as described (Matz et al., 1999). Gene-specific primers for 5′- and 3′-RACEs were Gluc5: 5′-GTGTAGAAGCAACATAGTTG-3′ and Gluc3: 5′-CGGAAA-CAACATATGGGC-3′, respectively. The PCR profile was 95 °C, 10 s; 57 °C, 20 s; 72 °C, 1 min. Two obtained fragments with lengths 600 bp and 320 bp were cloned and sequenced as described above.

2.11. Sequence analysis and comparisons

Sequences were analyzed using the BLAST website. Domain organization was verified by the web-based SMART tool (http://smart.embl-heidelberg.de). Multiple sequence alignment was performed with the Clustal W version 1.8 software (Higgins et al., 1994) with subsequent manual optimization.

2.12. Molecular modeling

The tertiary structure of the endo-1,3-β-D-glucanase from the scallop *M. yessoensis* was predicted by the homology modeling approach (Sanchez and Sali, 1997; Baker and Sali, 2001). The three-dimensional structure of LV was built up based on the analysis of its primary structure with the help of servers 3D-PSSM (Kelley et al., 2000; www.sbg.bio.ic.ac.uk/~3dpssm/), FUGUE(Shi et al., 2001; www.cryst.bioc.cam.ac.uk/~fugue/) and CPHmodel (Lund et al., 2002; www.cbs.dtu.dk/services/CPHmodel/). Homology model of the endo-1,3-β-D-glucanase from *M. yessoensis* was generated using the molecular graphics package Molecular Operating Environment (MOE; Chemical Computing Group; www.chemcomp.com/) on the base of the crystal structure of κ-carrageenase from *P. carrageonovora* (PDB codes 1DYP) as template (Michel et al., 2001). The alignment of LV and κ-carrageenase was obtained with Clustal (matrix: BLOSUM62) in the package MOE. The evaluation of structural parameters and the prediction quality of the modeled structure was done using the programs MOLMOL (Koradi et al., 1996), SPDBV (Guex and Peitsch, 1997) and MOE. The superposition of the coordinates of Cα-atoms of the theoretical model with template was carried out by MOE Homology module and by DALI server (Holm and Sander, 1993; www.ebi.ac.uk/dali/fssp/). The disulfide bond in the endo-1,3-β-D-glucanase model was built with MOE molecular building module. The content of the secondary structural elements of the endo-1,3-β-D-glucanase model was obtained with MOLMOL (Koradi et al., 1996). Energy minimization of LV model was carried out by GROMACS 3.2.1 (Lindahl et al., 2001; www.gromacs.org/) using a steepest descent method and energy minimization options: force tolerance (emtol) equals 100.0 kJ/mol and initial step-size (emstep) equals 0.01. The atomic coordinates of the kappa-carrageenase and laminarihexaose were taken from the Brookhaven Protein Data Bank (PDB, Berman et al., 2000; www.rcsb.org/pdb/) with the PDB codes 1DYP and 1WYW, respectively. The 3D-structure of halistanol sulfate (Fusetani et al., 1981) was modeled using the program MOE from the structure of cholesterol sulfate obtained from the Cambridge Structural Database (CSD code CHOLSF10, http://www.ccdc.cam.ac.uk/). The programs SPDBV 3.7 (Guex and Peitsch, 1997), MOLMOL, RASMOL (Sayle and Milner-

White, 1995) and MOE were used for visualization and analysis of the molecular structures. Molecular dockings of the scallop endo-1,3-β-D glucanase with its substrate laminarihexaose or glucanase inhibitor halistanol sulfate were carried out by GRAMM release 1.03 (Katchalski-Katzir et al., 1992; Vakser et al., 1999) implemented on MVS-1000/16 of FEB RAS supercomputing center.

3. Results

3.1. Enzyme purification

The 1,3-β-D-glucanase from *M. yessoensis* (LV) was purified 65-fold with a 14% recovery yield from the 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. The results of the purification are summarized in Table 1.

3.2. Enzyme characterization and specificity

The purified enzyme was subjected to SDS-PAGE and showed a single protein band of about 36 kDa (Fig. 1). It displayed the hydrolytic activity with laminaran as substrate within a relatively broad pH optimum between 4.0 and 5.0. The optimal temperature for the reaction under the conditions used was 30–45 °C whereas rapid denaturation of the enzyme occurred above 45 °C. LV was fairly stable keeping the initial activity at 25 °C for 1 month, but the activity was completely lost after incubation at 37 °C for 2 h. The activity of the 1,3-β-D-glucanase increased in the presence of NaCl, being at the highest at NaCl concentration of 0.5 M. The effect of substrate concentration (laminaran from *L. cichorioides*) on enzyme activity was estimated and an apparent K_m value of 600 μg/mL was obtained. LV did not act on the other tried substrates, including insoluble or high branchy 1,3-β-D-glucans, such as pachyman, lichenan and yeast glucan, and also 1,3-β-D-xylan, pustulan, fucoidan and amylopectin.

Table 1
Purification of the endo-1,3-β-D-glucanase from *M. yessoensis*

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Recovery yield (%)
Extraction	2810	267	0.095	1	100
Ion-exchange chromatography on CM-Sephadex C-50	210	224	1.07	11	84
Hydrophobic chromatography on Phenyl-Sepharose followed by gel-filtration on Sephadex G-75	15	66.7	4.2	44	25
Ion-exchange chromatography on CM-cellulose CM-52	6.3	37.8	6.0	65	14

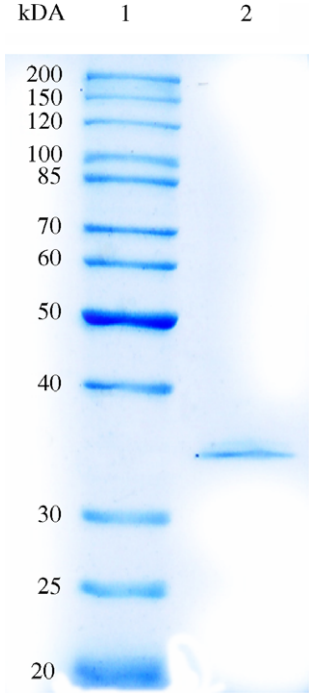

Fig. 1. SDS-PAGE of *M. yessoensis* endo-1,3-β-D-glucanase: (1) standard molecular mass markers (kDA); (2) purified LV under reducing conditions.

The MALDI-TOF mass spectra of the laminaran hydrolysis products revealed the presence of glucose and different oligosaccharides (Fig. 2). They are (*m/z* of $[M+Na]^+$): Glc_2 (365.5), Glc_3 (527.2), Glc_4 (689.1). Glc_5 (851.3), Glc_6 (1013.4), Glc_7 (1175.5), Glc_8 (1337.6), Glc_9 (1500.1), Glc_{10} (1662.5). The method reveals glucose weekly. Therefore, the amount of glucose in products of laminaran hydrolysis was determined by glucose oxidase method (Keston, 1956) and reached 40% at the initial stages of reaction (the degree of laminaran hydrolysis did not exceed 10%).

The same oligosaccharides were revealed after exhaustive hydrolyses of the laminaran. So, LV apparently catalyzed hydrolysis of laminaran as endo-enzyme.

The ability of the endo-1,3-β-D-glucanase from *M. yessoensis* to catalyze transglycosylation reaction, known as the typical reaction of retaining glycosyl hydrolyses, was examined. LV effectively catalyzed transglycosylation reaction using laminaran as donor and methyl-β-D-glucopyranoside as acceptor. The products of this reaction were identified by MALDI-TOF MS as methyl-glucooligosides (*m/z* of $[M+Na]^+$) as follows: Glc_2–CH_3 (379.5), Glc_3–CH_3 (541.17), Glc_4–CH_3 (703.15), Glc_5–CH_3 (865.3, Glc_6–CH_3 (1027.5), Glc_7–CH_3 (1189.7), Glc_8–CH_3 (1351.7), Glc_9–CH_3 (1514.2), Glc_{10}–CH_3 (1676.4) (Fig. 3).

According to the data of polarimetry, the optical rotation of the incubation mixture did not change practically in course of reaction (from −31.3 to −27.5). The negative rotation kept after the ammonia addition also. It is most likely that the β-anomers of oligosaccharides are formed in the initial stage of the reaction.

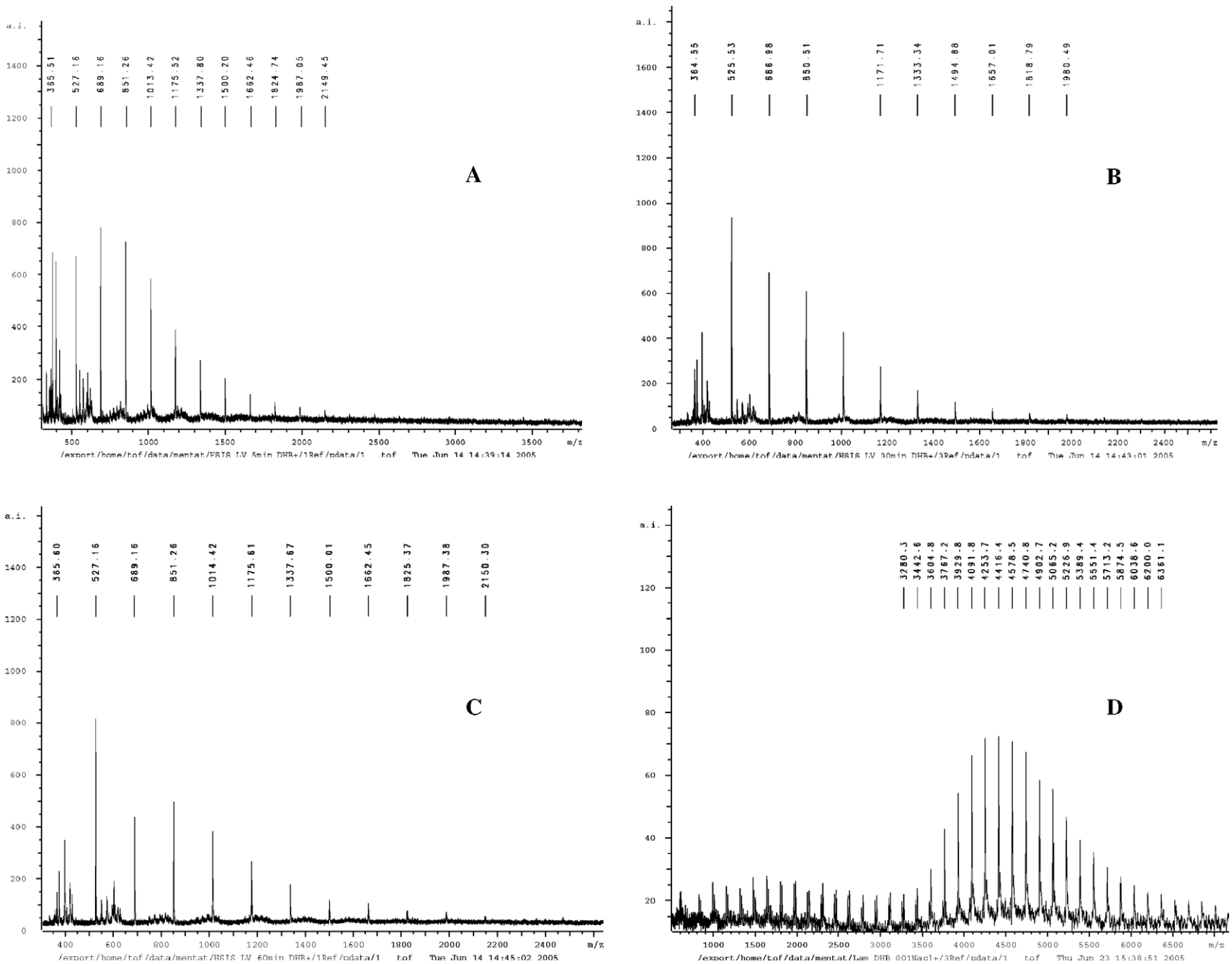

Fig. 2. MALDI-TOF MS of the products of laminaran hydrolysis by LV: (A) 5 min of reaction, (B) 10 min, (C) 30 min, (D) laminaran.

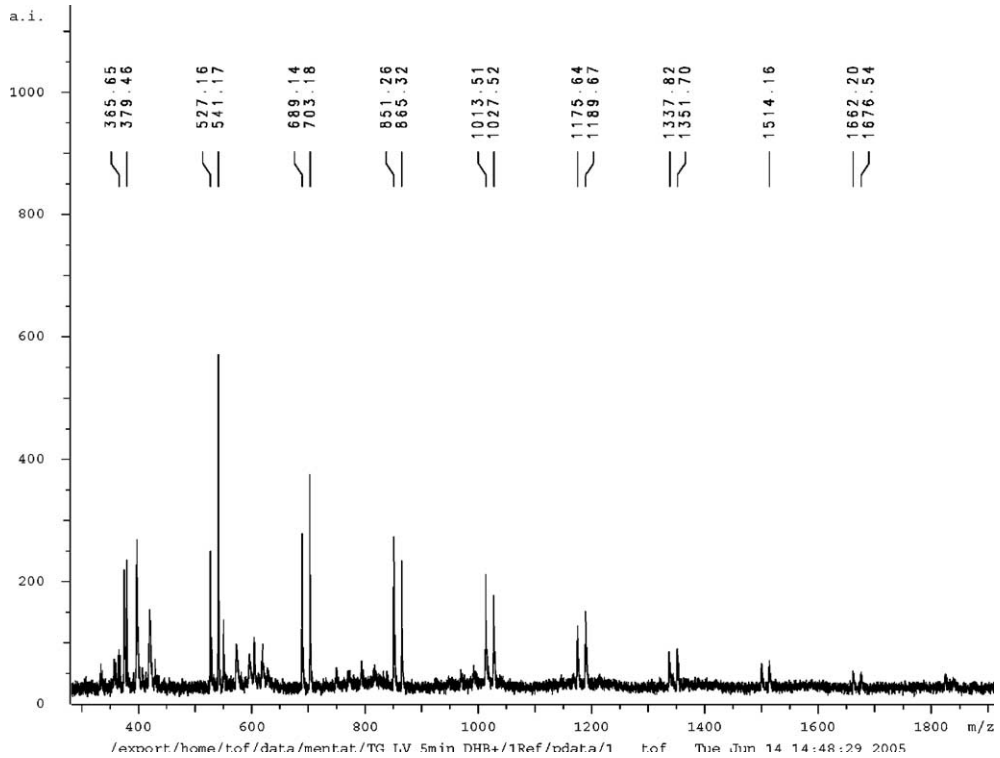

Fig. 3. MALDI-TOF MS of products of the transglycosylation reaction catalyzed by LV.

3.3. Effect of group-specific reagents

To elucidate a role of some functional groups in catalytic activity of LV, an inhibitor analysis was done (Table 2). Reagents modifying tyrosine and lysine residues and SH-groups had no effect on the enzyme activity. However, the enzyme activity decreased after treatment of LV with propylene oxide (up to 85%) and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) (up to 15%). Inhibition of enzyme activity (up to 70%) was observed at the joint action of EDC and *o*-dianisidine (nucleophilic agent). These data suggest the significant role of carboxylic groups of dicarbonic amino acids in the enzyme catalysis. Complete loss of the enzyme activity was observed after treatment of the enzyme with *N*-bromosuccinimide, which specifically oxidizes tryptophan residues at weakly acidic pH. The important role of tryptophan residues for substrate binding by glucanase has been shown by Din et al. (1994).

Table 2
Effect of group-specific reagents on endo-1,3-β-D-glucanase from *M. yessoensis*

Reagent	Residues or group modified	Reagent concentration (M)	pH	Residual activity (%)
Ethylendiaminetetraacetic acid	Metal	10^{-2}	5.2	100
p-Chloromercuribenzoate	SH-group	$5 \cdot 10^{-4}$		100
Iodoacetamide		10^{-2}		100
N-Ethylmaleimide		10^{-2}		100
Ellman's reagent		10^{-3}		100
N-Acetylimidazole	Tyrosine	$5 \cdot 10^{-2}$	5.2	100
Tetranitromethane		$2.5 \cdot 10^{-2}$	7.0	100
2,4-Pentanedione	Lysine	1.0	7.0	100
Trinitrobenzolsulfonic acid		$2 \cdot 10^{-3}$		100
N-Bromosuccinimide	Tryptophan	10^{-4}	4.0	0
Diethyl pyrocarbonate	Histidine	$1.2 \cdot 10^{-2}$	6.0	70
		$2.4 \cdot 10^{-2}$		10
		$3.6 \cdot 10^{-2}$		0
Propylene oxide	COOH	1.5	4.0	93
		3.0		83
		4.5		64
		6.0		15
EDC		10^{-2}	5.0	85
EDC and *o*-dianisidine		10^{-2} and $4 \cdot 10^{-3}$	5.0	30

3.4. Primary structure of endo-1,3-β-D-glucanase from *M. yessoensis*

The nucleotide sequence of full cDNA encoding LV was estimated by RT-PCR, 5′-, 3′-RACE methods and technique based on selective suppression of polymerase chain reaction (Lukyanov et al., 1995; Siebert et al., 1995). Its GenBank accession number is AY848857.

The nucleotide and deduced amino acid sequences of the endo-1,3-β-D-glucanase from *M. yessoensis* are shown in Fig. 4. The cDNA was composed of 1115 bp. An open reading frame of 1017 bp (from 17 to 1036 nucleotides) encoded a polypeptide consisting of 339 amino acid residues. The start methionine is followed by hydrophobic stretch of 15 amino acid residues being probably a signal sequence. The mature enzyme has 324 amino acids with a calculated molecular mass of 36.9 kDa that is in close agreement with the experimental results for the purified enzyme.

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acgcattggtaacaccatggatcccctcctttgtcttgtactgttgccgttggtggctggc  61
                M  D  P  L  L  C  L  V  L  L  P  L  V  A  G   15
gcaggcttccgtgacgatttcacaacatgggacccgagtgattaccagattgaagtctcg  121
 A  G  F  R  D  D  F  T  T  W  D  P  S  D  Y  Q  I  E  V  S    35
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 A  W  G  G  G  N  H  E  F  Q  V  F  T  P  E  P  S  N  L  F    55
gtcagaaatggtaacctgtacatcaaaccgacgttcacgagggacagtgcccactttaac  241
 V  R  N  G  N  L  Y  I  K  P  T  F  T  R  D  S  A  H  F  N    75
gacgggagcctgtattacgggaccatggacgttaactccctgtggcataggtgcacccag  301
 D  G  S  L  Y  Y  G  T  M  D  V  N  S  L  W  H  R  C  T  Q    95
catgacaataatggttgtcacaaacaatcttacggtggcgacagtgagatcctgcccccc  361
 H  D  N  N  G  C  H  K  Q  S  Y  G  G  D  S  E  I  L  P  P   115
gtcatgtccggaaagattaccaccaactttgcgatgacatacggacgtgttaatgtacgc  421
 V  M  S  G  K  I  T  T  N  F  A  M  T  Y  G  R  V  N  V  R   135
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 Y  D  V  P  K  P  W  G  N  H  N  P  M  R  S  F  W  E  A  R   315
cactcctgggaacacacctggcaaggagatgaggttgctttggtaatagattacattgag 1021
 H  S  W  E  H  T  W  Q  G  D  E  V  A  L  V  I  D  Y  I  E   335
atgattcctcactaatttaatcaaattcatgatggctgtcagtgaacatgaaataaaatg 1081
 M  I  P  H  #                                                 339
catgttgtatataccaaaaaaaaaaaaaaaaaaa                            1115
```

Fig. 4. Nucleotide sequence of LV cDNA and deduced amino acid sequence. Amino acids are numbered from the initial methionine. Predicted signal peptide sequence is given in italic. Glucanase-like domain is given in shaded box.

3.5. Sequence analysis and comparisons

The deduced endo-1,3-β-D-glucanase amino acid sequence was revealed by SMART analysis (Schultz et al., 2000) to contain a characteristic domain of the glycosyl hydrolases family 16 starting at position 80 aa and ending at position 304 aa (Fig. 4).

GenBank databases searching by the BLAST software disclosed a significant sequence homology of the scallop endo-1,3-β-D-glucanase with the invertebrates glucanases as well as with the lipopolysaccharide- and β-1,3-glucan-binding proteins. Comparison of the LV with sequences in protein database revealed its closest homology with *P. sachalinensis* β-1,3-glucanase (GenBank accession no. AAP74223) (Kozhemyako et al., 2004), 48% identity and 60% similarity; lipopolysaccharide and β-1,3-glucan-binding proteins from *Pacifastacus leniusculus* (LGBP) (GenBank accession no. JC6141) (Lee et al., 2000), 42% identity and 58% similarity; LGBP from *Litopenaeus stylirostris* (GenBank accession no. AAM73871) (Roux et al., 2002), 42% identity and 56% similarity; as well as with the coelomic cytolytic factors from *Eisenia fetida* (GenBank accession no. AAC35887) (Beschin et al., 1998), 40% identity and 58% similarity, and from *Lumbricus terrestris* (GenBank accession no. AAL09587) (Bilej et al., 2001), 39% identity and 56% similarity. The scallop endo-1,3-β-D-glucanase also shows 39% identity and 58% similarity with β-1,3-glucanase from the sea urchin *S. purpuratus* (GenBank accession no. AAC47235) (Bachman and McClay, 1996). A somewhat lower degree of similarity (about 29% identity and 40% similarity) is observed with bacterial beta-1,3-glucanases from *Bacillus circulans* (GenBank accession no. AAC60453 (Yamamoto et al., 1993), *Pyrococcus furiosus* (GenBank accession no. AAC25554) (Gueguen et al., 1997); *Rhodothermus marinus* (GenBank accession no. AAC69707) (Krah et al., 1998) and *Thermotoga neapolitana* (GenBank accession no. CAA88008 (Dakhova et al., 1993).

The multiple alignment of glycosyl hydrolases family 16 domain of LV with those of similar invertebrates and bacterial 1,3-glucanases is shown in Fig. 5.

3.6. Homology model of the endo-1,3-β-D-glucanase from *M. yessoensis*

Analysis of the amino acid sequence of the *M. yessoensis* endo-1,3-β-D-glucanase with 3D-PSSM, FUGUE and CPHmodel servers displays that fold of LV coincides with folds of enzymes belonging to family GH-16. The identity of amino acid sequence of LV and enzymes of the family GH-16 with determined three-dimensional structure is poor and equal to 16–20%. However, according to the results of 3D-PSSM server studies the fold of the scallop endo-1,3-β-D-glucanase coincides with probability 95% with folds of bacterial β-1,3-1,4-glucanase (PDB code 2AYH) and β-agarase (PDB code 1O4Z) and coincides with probability 80% with fold of κ-carrageenase (PDB code 1DYP). According to the FUGUE server results, the fold of LV coincides with probability 99% with folds of enzymes belonging to family GH-16. The values ZSCORE obtained by FUGUE are equal to 28.11 for κ-carrageenase, 22.37 for β-agarase and 19.70 for bacterial β-1,3-1,4-glucanase. The structure of κ-carrageenase was selected as the best template for *M. yessoensis* endo-1,3-β-D-glucanase with the CPHmodel server. Thus homology model of LV three-dimensional structure was built on the base of the crystal structure of κ-carrageenase from *P. carrageonovora* as template by MOE (Fig. 6B,C). Sequence alignment of the 16–290 residues fragment of *M. yessoensis* endo-β-1,3-D-glucanase and 27–290 residues fragment of κ-carrageenase *P. carrageenovora* (Fig. 6A) displayed their identity 21.8% and similarity 36%. The quality of LV homology model was checked with MOE. Energy minimization of the endo-1,3-β-D-glucanase model was carried out by GROMACS 3.2.1 (Lindahl et al., 2001; www.gromacs.org/) using steepest descent method and after energy minimization in vacua the energy value was −16,045 kJ/mol. The LV displays antiparallel β-strand jellyroll architecture. It consists of two antiparallel β-sheets of six and seven strands creating a deep cavity (Fig. 6B,C). The nuceophile (Glu163) and acid/base (Glu168) catalytic residues of *P. carrageonovora* κ-carrageenase correspond to residues Glu165 (nuceophile) and Glu170 (acid/base) of *M. yessoensis* endo-1,3-β-D-glucanase.

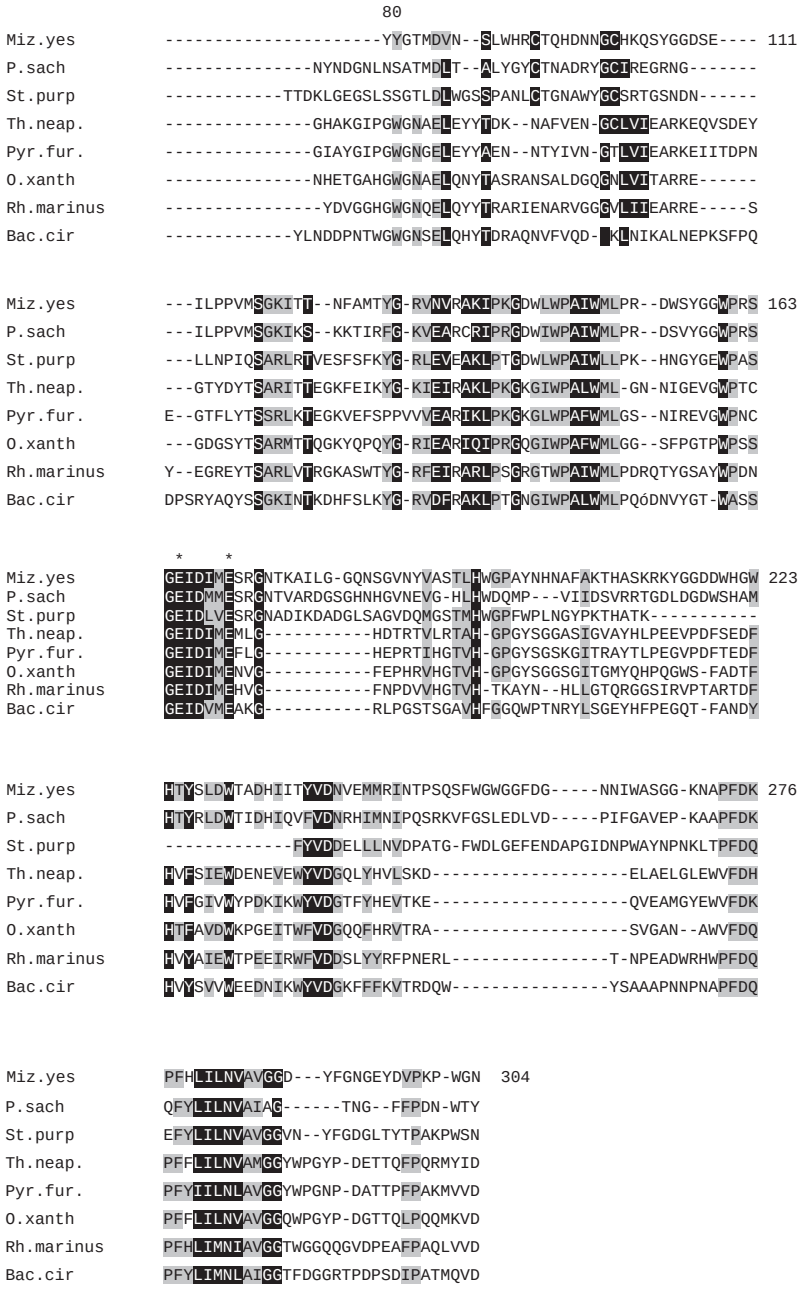

Fig. 5. Alignment of glycosyl hydrolases family 16 domain of LV and those of other similar invertebrates and bacterial glucanases. The numbering of LV is indicated. Conserved residues of the sequences are highlighted in black (strictly conserved) or in gray. The asterisks above the sequences indicate catalytic residues. Miz.yes—*M. yessoensis* endo-1,3-β-D-glucanase (GeneBank accession no. AY848857); P.sach—*Pseudocardium sachalinensis* β-1,3-glucanase (GenBank accession no. AAP74223); St.purp—*Strongylocentrotus purpuratus* β-1,3-glucanase (GenBank accession no. AAC47235); Th.neap.—*Thermotoga neapolitana* laminarinase (GenBank accession no. CAA88008); Pyr.fur—*Pyrococcus furiosus* endo-β-1,3-glucanase (GenBank accession no. AAC25554); O.xanth—*Oerskovia xanthineolytica* β-1,3-glucanase (GenBank accession no. AAC38290); Rh.marinus—*Rhodothermus marinus* laminarinase (GenBank accession no. AAC69707); Bac.cir—*Bacillus circulans* β-1,3-glucanase (GenBank accession no. AAC60453).

The active site of this enzyme is located in the cavity, where three residues Glu165, Asp167 and Glu170 form an electronegative area. A possible disulfide bond between Cys93 and Cys101 connects different sides of the loop between β3- and β4-strands. The secondary structure of LV calculated with MOLMOL (Koradi et al., 1996) consists of 2.4% of α-helices, 32.8% of β-strands, 6.2% turns and 58.6% of loops and others. It is in agreement with prediction of the *M. yessoensis* endo-1,3-β-D-glucanase's secondary structure by 3D-PSSM and PSIPRED (McGuffin et al., 2000) servers: the model of the mature protein fragment (residues 16–305) contains 2 α-helices and 15 β-strands. The structure's comparison of the LV model and κ-carrageenase was made by superposition of the coordinates of Cα-atoms using the MOE Homology models module. The value of root mean square deviation (RMSD) for Cα-atoms of the endo-1,3-β-D-glucanase and κ-carrageenase was 1.66 Å. For 6 Cα-atoms in the active site region (Glu165–Glu170), the value of RMSD was 0.36 Å. Thus, we obtained the

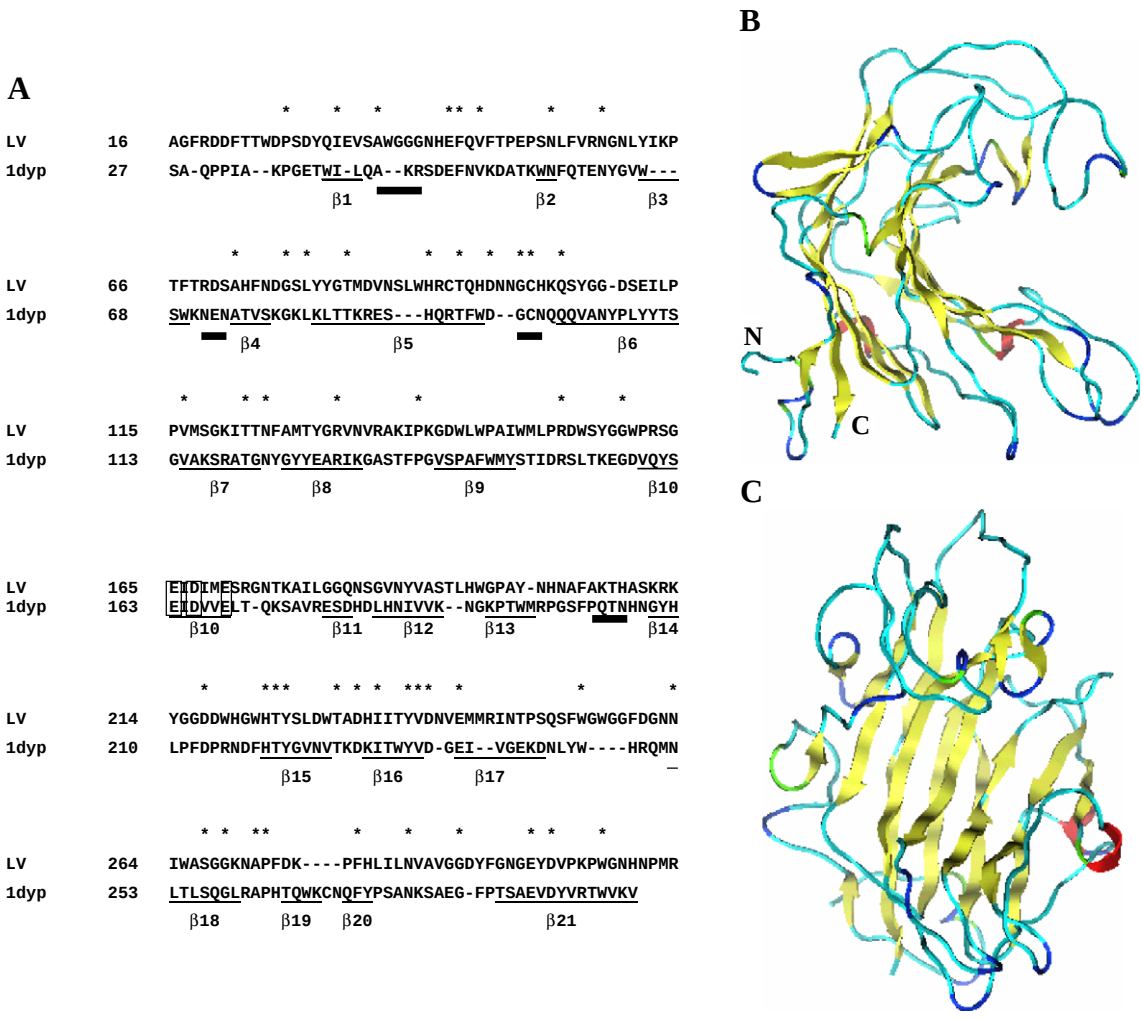

Fig. 6. Homology model of the 16–290 residues fragment of LV build base on the crystal structure of κ-carrageenase *P. carrageenovora* (PDB code 1DYP) as template. Sequence alignment of the 16–290 residue fragment of *M. yessoensis* endo-β-1,3-D-glucanase and 27–290 residues fragment of κ-carrageenase *P. carrageenovora* (A). The secondary structure elements of κ-carrageenase are shown as arrows (β-sheet) and bold lines (α-helix), catalytic residues are shown in the frame and identical residues are marked with asterisks. The front view (B) and side-face view (C) of the ribbon representation of the LV model are shown. The N terminus and C terminus are labeled. Figure is prepared with MOE (www.chemcomp.com/).

endo-1,3-β-D-glucanase model with correct fold for family GH-16 and high accuracy in the region of active site.

Comparative analysis of the protein contacts for the scallop endo-1,3-β-D-glucanase model, κ-carrageenase and β-agarase was carried out with MOE. It was found that LV model contains 65 H-bonds, 45 hydrophobic contacts, 11 ion bonds and 1 disulfide bond, whereas κ-carrageenase contains 84 H-bonds, 61 hydrophobic contacts, 17 ion bonds and 1 disulfide bond, and β-agarase contains 69 H-bonds, 86 hydrophobic contacts, 16 ion bonds and no disulfide bond. In the region of active site of LV, the nucleophile Glu165 (Glu163 for κ-carrageenase) is ion bonded with His201 (His183 for κ-carrageenase). The acid/base Glu170 (Glu168 for κ-carrageenase) catalytic residue of *M. yessoensis* β-1,3-glucanase is hydrogen bonded to Gln182 (forms ion bond with Arg260 of κ-carrageenase). The catalytic residues of β-agarase Glu147 and Glu152 interact with His172 and His168. The Asp167 of *M. yessoensis* endo-1,3-β-D-glucanase interacts with Ser184. Similarly, the Asp165 of the active site of *P. carrageonovora* κ-carrageenase interacts with His183, whereas Asp149 of *Z. galactanivorans* β-agarase interacts with His172. Thus, contacts within the active sites of these enzymes are different. The replacement of Val167 of κ-carrageenase on to Met169 of *M. yessoensis* endo-1,3-β-D-glucanase suggests to promote increasing of the hydrophobic contacts in the region of the residue Glu170. It should be pointed out that the structural positions of the catalytic residues within the enzymes compared have been found to be conserved, apparently, that suggests that the different substrate specificity of family GH-16 enzymes is a result on some differences of the residue contacts within the catalytic site.

3.7. Molecular docking of M. yessoensis endo-1,3-β-D-glucanase model with substrate and inhibitor

The first structure of family GH-16 enzyme β-agarase A in complex with agaro-octaose has been solved recently (Allouch et al., 2004). It was shown that no enzyme conformational changes were observed upon binding of

oligosaccharide (Allouch et al., 2004). It is allowed to use the "rigid-body" docking for prediction the structure of the family GH-16 enzymes with substrates. The homology model of *M. yessoensis* endo-1,3-β-D-glucanase was used for theoretical prediction of the structures of the enzyme LV complexes with substrate or inhibitor. The "rigid-body" low-resolution molecular docking (Vakser et al., 1999) was carried out for prediction of the putative binding sites of laminarihexaose and glucanase inhibitor halistanol sulfate (Fig. 7) with the *M. yessoensis* endo-1,3-β-D-glucanase. It was found that laminarihexaose interacts with the Glu165 in the catalytic cavity of LV by the reducing end (Fig. 8A,B). The O6 of the second β-D-glucose of laminarihexaose is hydrogen bonded with Glu165 OE1. The second carbohydrate-binding site of the endo-1,3-β-D-glucanase is on the opposite side of its surface.

The sulfated steroid halistanol sulfate isolated from *Halichondriidae* sp. was shown to be natural irreversible inhibitor of the mollusk endo-1,3-β-D-glucanase with IC_{70} 1 μM (Zvyagintseva et al., 1986). The putative structure of the complex of LV with halistanol sulfate was first predicted (Fig. 8C and D). It was found that halistanol sulfate binds with the catalytic cavity of *M. yessoensis* endo-1,3-β-D-glucanase.

4. Discussion

In the paper we report on the isolation, some biochemical properties and predicted structure of the 1,3-β-D-glucanase from the scallop *M. yessoensis,* designated here as LV. As follows from results of biochemical research the enzyme represents typical retaining endo-1,3-β-D-glucanase.

Earlier we have reported cDNA cloning of the endo-1,3-β-D-glucanase from *P. sachalinensis* and estimated its primary structure (Kozhemyako et al., 2004). The similar information about primary structure of the 1,3-β-D-glucanase from *S. purpuratus* was obtained by Bachman and McClay (1996). The deduced amino acid sequence of the LV was up to 48% identical (60% similarity) to the 1,3-β-D-glucanase from *P. sachalinensis*, which correlated with the data on similarity of biochemical features and mode of action of these two enzymes and reflected the phylogenetic relationships of both species belonging to Bivalves. LV effectively catalyzes transglycosylation reaction as endo-1,3-β-D-glucanase *P. sachalinensis* does (Zvyagintseva and Elyakova, 1994; Sova et al., 1997). Studies of the oligomer set formed during the enzyme hydrolysis of laminaran suggest that LV splits substrate by so-called "multiple attack mechanism" like 1,3-β-D-glucanase from *P. sachalinensis* (Zvyagintseva and Elyakova, 1994; Bezukladnikov and Elyakova, 1990) (Fig. 2). So both enzymes from sea mollusks are retaining endo-1,3-β-D-glucanases and are distinguished, on the one hand, by high ability to catalyze the reactions of transglycosylation, and on the other hand, by high degree of multiple attack, characteristic for exo-enzymes.

The endo-1,3-β-D-glucanases from *M. yessoensis* and *P. sachalinensis* had less homology with the sea urchin enzyme. The differences in mollusks and urchin 1,3-β-D-glucanases primary structures agreed with the data on dissimilarity of the biochemical properties of these enzymes (Bachman and McClay, 1996).

The scallop endo-1,3-β-D-glucanase was shown by sequence analysis to belong to glycosyl hydrolases of family 16 similarly to 1,3-β-D-glucanases from *S. purpuratus* and *P. sachalinensis*. The multiple alignment of LV amino acid sequence with the invertebrate 1,3-β-D-glucanases and bacterial laminarinases revealed that the sequences with the highest homology matched the amino acids around the catalytic residues (Glu165–Glu170) (Fig. 4). Glycine residues at positions 100, 130, 141, 164, 173, 287 and 288 are revealed

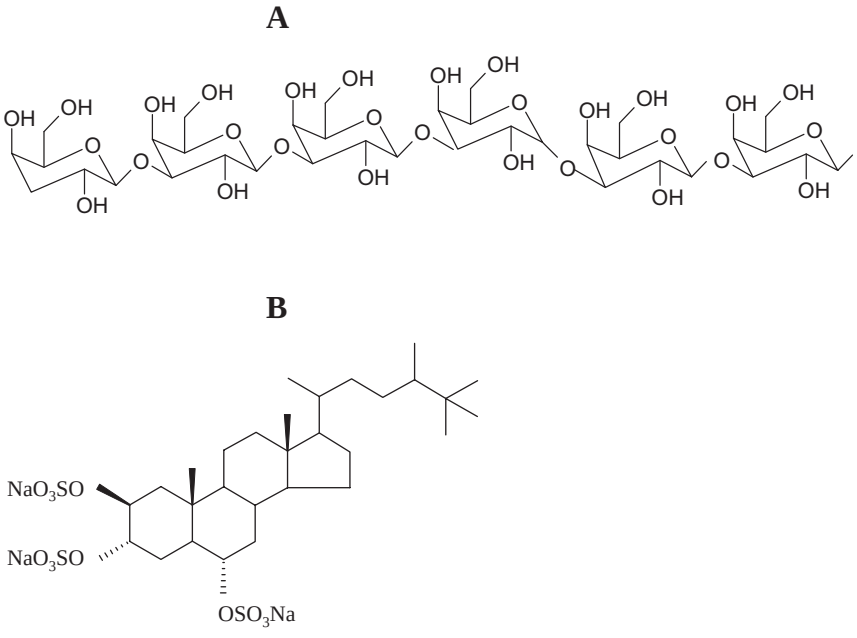

Fig. 7. Chemical structures of laminarihexaose (A) and halistanol sulfate (B).

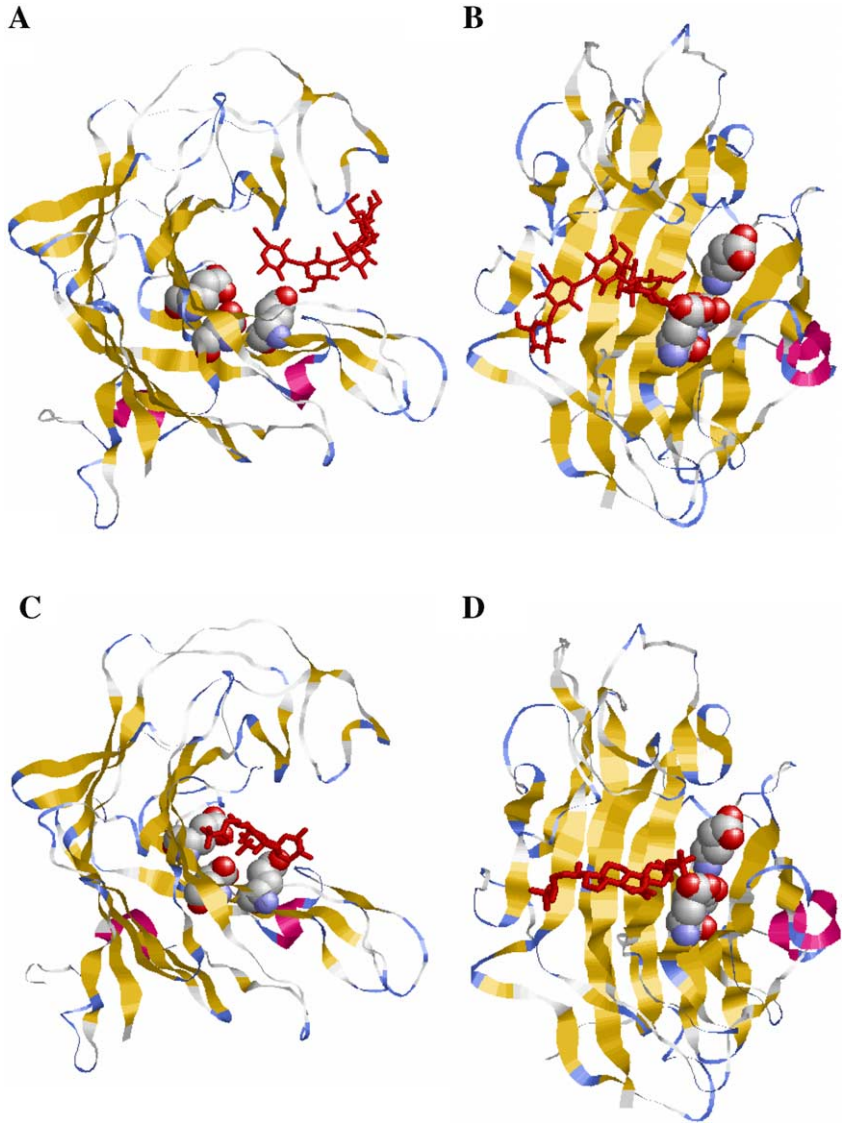

Fig. 8. Views of the endo-β-1,3-D-glucanase complexes with substrate laminarihexaose (A, B) or with glucanase inhibitor halistanol sulfate (C, D) in front (A, C) and side-face (B, D). Glucanase is shown by ribbon diagram, catalytic residues Glu165, Asp167, Glu170 are shown as spacefill and ligands are shown by stick. Figure is prepared with Rasmol (Sayle and Milner-White, 1995).

to have a high conservatism. Glycine, owing to its structural feature, was suggested to play an important role in protein folding and conformational mobility of active site (Varfolomeev et al., 2002). LV was shown to have no free SH-groups. As the predicted protein contained only two cysteine residues, namely Cys93 and Cys101, they likely formed disulfide bond. These cysteine residues are conserved for all invertebrate laminarinases studied.

The three-dimensional structures of lichenase, carrageenase and agarase from family GH-16 are available at present. The sequence and fold similarities between enzymes of family GH-16 have allowed to generate the endo-1,3-β-D-glucanase homology model. The *M. yessoensis* endo-1,3-β-D-glucanase model with correct fold for family GH-16 and high accuracy in the region of active site was generated (Fig. 5B,C). Superposition of the Cα-atoms of LV model with another members of family GH-16 by DALI server (Holm and Sander, 1993) displays RMSD: 1.4 Å for 261 atoms of *P. carrageonovora* κ-carrageenase (1DYP) before disulfide bond building and 1.9 Å for 252 atoms of *P. carrageonovora* κ-carrageenase after disulfide bond building, 2.9 Å for 212 atoms of *Z. galactanivorans* β-agarase (1O4Y), 2.7 Å for 174 atoms of xyloglucan endotransglycosylase (1UMZ). In the region of active site, the superposition of the 6 Cα-atoms (Glu165–Glu170) of *M. yessoensis* endo-1,3-β-D-glucanase with corresponding κ-carrageenase atoms displays the value of RMSD 0.36 Å. Similar values of RMSD were observed in the compared structures of β-agarase A and κ-carrageenase (Jam et al., 2005). The content of the secondary structure elements in the *M. yessoensis* endo-1,3-β-D-glucanase model is in agreement with the secondary structure of *P. furiosus* endo-β-1,3-glucanase model (2.6% α-helices, 42.4% β-strands, 12.4% turns and 42% loops) which was consistent with spectroscopy data (Chiaraluce et al., 2004).

To date, no 3D-structure of any β-1,3-glucanase or its complex with substrate or inhibitor has been reported. The theoretical predictions of the structures of LV complexes with substrate or glucanase inhibitor were carried out with low-resolution molecular docking (Fig. 7). It was found that laminarihexaose has putative catalytic and non-catalytic binding sites. The first site is located within the catalytic cavity whereas the second is outside the catalytic cavity. The structure of mutant β-agarase A E147S in the complex with agaro-octaose has been solved recently (Allouch et al., 2004). It was shown that two oligosaccharide chains are bound to the protein. The first one resides in the active site channel, and the second oligosaccharide-binding site is revealed to be outside of the catalytic cavity of the protein. The β-agarase was found to have non-catalytic carbohydrate-binding site, which is involved in the adhesion of the enzyme with its natural substrate or is involved in the substrate dissociation (Allouch et al., 2004; Jam et al., 2005). The laminarihexaose interacts in the catalytic cavity with the endo-1,3-β-D-glucanase's Glu165 by the oligosaccharide-reducing end (Fig. 8A). Predicted first substrate-binding site in the catalytic site of the LV is similar to the same of the β-agarase (Allouch et al., 2004). Hydrolysis of glycosidic bonds in laminaran by the *M. yessoensis* endo-1,3-β-D-glucanase results in the appearance of monosaccharides as the predominant product. The predicted structure of the β-1,3-glucanase-laminarihexaose complex displays possible hydrolysis of bond near the reducing end of polysaccharide and monosaccharide formation. The second carbohydrate-binding site of LV is outside of the catalytic cavity of and does not coincide with the β-agarase second site. It seems likely that surface carbohydrate-binding sites of the endo-1,3-β-D-glucanase and β-agarase are different.

The halistanol sulfate isolated from *Halichondriidae* sp. is a natural, irreversible inhibitor of the *M. yessoensis* endo-1,3-β-D-glucanase with IC_{70} equal to 1 μM (Zvyagintseva et al., 1986). The putative structure of the LV complex with halistanol sulfate was first predicted (Fig. 8C,D). It was found that halistanol sulfate binds with LV catalytic cavity. It is a possible electrostatic interaction of two sulfate groups of halistanol sulfate with Arg57 and Arg153 of the scallop 1,3-β-D-glucanase near the catalytic site. It was theoretically predicted that the binding sites of the halistanol sulfate and laminarihexaose are located in the enzyme catalytic site and overlapped. This suggestion was confirmed by experimental data on protection of LV from halistanol sulfate inactivation with glucose which is competitive inhibitor for the endo-1,3-β-D-glucanase (Zvyagintseva and Elyakova, 1994; Zvyagintseva et al., 1986).

Acknowledgments

This work was supported by the grants of the Russian Foundation for Basic Research 05-04-49881 and 03-04-49534 and the grant of the Russian Academy of Sciences on Program of Fundamental Research "Physico-chemical biology" 03-1-0-05-002.

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