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Enzymatic properties and the primary structure of a β -1,3-glucanase from the digestive fluid of the Pacific abalone *Haliotis discus hannai*

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ABSTRACT

A β -1,3-glucanase (EC 3.2.1.6) with a molecular mass of 33 kDa was isolated from the digestive fluid of the Pacific abalone *Haliotis discus hannai* by ammonium sulfate fractionation followed by conventional column chromatography. This enzyme, named HdLam33 in the present study, degraded laminarin and laminarioligosaccharides to laminaribiose and glucose with the optimal temperature and pH at 50°C and 6.0, respectively. HdLam33 possessed transglycosylation activity, a characteristic property of glucan hydrolases that split glycoside linkage with a retaining manner. By the transglycosylation reaction of HdLam33, the laminaribiose unit in the non-reducing terminus of laminaritriose (donor substrate) was transferred to a free laminaribiose (acceptor substrate) resulting laminaritetraose and glucose. The resulted laminaritetraose was subsequently hydrolyzed by HdLam33 into two moles of glucose and one mole of laminaribiose. The primary structure of HdLam33 was analyzed by the cDNA method. The deduced amino-acid sequence of 329 residues corresponding to the catalytic domain of HdLam33 showed 56-61% amino-acid identity with those of other molluscan β -1,3-glucanases which have been identified as glycoside hydrolase family 16 enzymes.

Keywords: abalone, mollusks, gastropod, β -1,3-glucanase, laminarinase, laminarin, laminarioligosaccharide, transglycosylation.

1. Introduction

It has been generally known that herbivorous marine invertebrates such as abalone and scallop possess various kinds of polysaccharide-degrading enzymes in their digestive fluid (Mai et al., 1995; Takami et al., 1998; Johnston et al., 2005). These enzymes depolymerize dietary polysaccharides to oligo- and monosaccharides providing carbon and energy sources of the animals. The composition of enzymes in the digestive fluid is closely related to the feeding habits of the animals. For example, digestive fluid of marine gastropods such as abalone and sea hare contains alginate lyase, amylase, cellulase, and mannanase, which can depolymerize seaweeds' alginic acid, dextran, cellulose, and β -mannan, respectively (Shimizu et al., 2003; Suzuki et al., 2003; Andreotti et al., 2005 and 2006; Suzuki et al., 2006; Ootsuka et al., 2006; Nikapitiya et al., 2009). The oligosaccharides and monosaccharides produced by these enzymes are considered to be directly assimilated by the animals themselves or indirectly through fermentation by intestinal bacteria (Erasmus et al., 1997; Sawabe et al., 2003).

Abalone feeds mainly on brown seaweeds and degrades alginic acid, cellulose, and laminarin contained in the frond with alginate lyase, cellulase, and laminarinase in the digestive fluid. Among these seaweeds' polysaccharides, laminarin seems to be the most potential glucose source for abalone since laminarin is a major storage polysaccharide consisting of β -1,3-linked glucose main chains possessing occasional β -1-6-linked glucose branches (Maeda and Nisizawa, 1967; Pang et al., 2005). Previously, a laminarin-degrading enzyme, i.e., endo- β -1,3-glucanase that hydrolyzes β -1,3-glucoside linkages of laminarin, was isolated from hepatopancreas of the ormer *Haliotis tuberculata* (Lépagnot-Descamps et al., 1998). On the other hand, laminarin content in brown seaweeds has been shown to come up to approximately 20% (w/w, in dried materials). Thus, the endo- β -1,3-glucanase seems to be a key factor for understanding the abalone's strategy for acquiring energy and carbon sources from brown seaweeds' laminarin.

Endo- β -1,3-glucanases have distributed over various organisms such as fungi, yeasts, bacteria, plants, and marine invertebrates (Sova et al., 1970a; Erfle et al., 1988; Tangarone et al., 1989; Hrmova and Fincher, 1993; Mrsa et al., 1993; Miyanishi et al., 2003). Currently, three different types of endo- β -1,3-glucanases are known, i.e., 1) endo-1,3;4- β -glucanase (EC 3.2.1.6) which degrades not only β -1,3-linkage but also β -1,4-linkage adjacent to the reducing terminal side of the β -1,3-linkage in β -1,3;1,4-glucan, 2) lichenase (EC 3.2.1.73) which degrades only β -1,4-linkage adjacent to the reducing terminal side of the β -1,3-linkage in

β -1,3;1,4-glucan, and 3) glucan endo-1,3- β -D-glucosidase (EC 3.2.1.39) which requires the presence of at least two adjacent β -1,3-linkages of β -1,3-glucan and degrades only β -1,3-linkage. β -1,3-Glucanases from surf clam (*Spisula sachalinensis*), scallop (*Chlamys albidus*, *Patinopecten yessoensis*), and the ormer (*H. tuberculata*) have been classified to the first type enzyme (EC 3.2.1.6). These marine molluscan enzymes are also called laminarinase since they can efficiently hydrolyze the β -1,3-linkage of laminarin from brown seaweeds (Sova et al., 1970b; Privalova and Elyakova, 1978; L  pagnol-Descamps et al., 1998; Kumagai et al., 2008). Primary structures of molluscan β -1,3-glucanases have been reported in the surf clam and scallop enzymes (Kozhemyako et al., 2004; Kovalchuk et al., 2006; Kovalchuk et al., 2009). According to the hydrophobic cluster analysis of the primary structure (CAZy <http://www.cazy.org/>), these bivalve β -1,3-glucanases have been classified under the glycosyl hydrolase family16 (GHF16).

Previously, L  pagnol-Descamps et al. (1998) isolated a β -1,3-glucanase from hepatopancreas of the ormer *H. tuberculata* (a gastropod), and investigated its basic properties. The end-products of laminarioligosaccharides by the ormer enzyme were laminaritriose, laminaribiose, and glucose. The ormer enzyme could degrade oligosaccharides larger than laminaritriose but not laminaritriose and laminaribiose. On the other hand, the β -1,3-glucanase isolated from the digestive fluid of scallop produced laminaribiose and glucose as end-products (Kumagai et al, 2008). The molecular mass of the ormer enzyme was estimated to be 60 kDa; however, those of bivalve enzymes were around 35-38 kDa. The bivalve enzymes have been classified to GHF16 on the basis of their primary structure; however, the ormer enzyme has not been classified to any GHFs since the complete amino-acid sequence has not been determined yet. Thus, information about the gastropod β -1,3-glucanase is still poor compared with that for bivalve enzymes.

Therefore, in the present study, we isolated a β -1,3-glucanase from the digestive fluid of the Pacific abalone *H. discus hannai*, a related species of the ormer *H. tuberculata*, and investigated its general properties. The molecular mass of the abalone β -1,3-glucanase was approximately 33 kDa which was similar to bivalve β -1,3-glucanases but considerably different from that of the ormer enzyme. Further, we analyzed the primary structure of the abalone enzyme by the cDNA method and found that the abalone enzyme is also classified to GHF 16 as in the cases of bivalve β -1,3-glucanases.

2. Materials and methods

2.1. Materials

The Pacific abalone, *H. discus hannai*, was commercially obtained from a local market in Hakodate, Hokkaido Prefecture, Japan. Laminarin from *Laminaria digitata* and lichenan from *Cetraria islandica* were purchased from Sigma-Aldrich (St. Louis, MO, USA), laminarioligosaccharides (laminaribiose – laminariheptaose, abbreviated to L2-L7) from Seikagaku Kogyo (Tokyo, Japan), TOYOPEARL Phenyl-650M and TOYOPEARL CM-650M from Toyo Soda Mfg. Co. (Tokyo, Japan), Superdex 75 10/300GL from GE Healthcare UK Ltd. (Little Chalfont, Buckinghamshire, England), Oligotex-dT(30), TaKaRa Premix *Taq* DNA polymerase, 5'-Full RACE and 3'-Full RACE kits, restriction endonucleases from TaKaRa (Tokyo, Japan), and pCR TOPO 2.1 TA cloning kit from Invitrogen (Carlsbad, CA, USA). Other reagents were purchased from Wako Pure Chemicals Industries Ltd (Osaka, Japan).

2.2. Assay for β -1,3-glucanase activity

β -1,3-Glucanase activity was assayed at 30°C in 1 mL of reaction mixture containing 0.01-0.05 units of enzyme, 0.2% (w/v) laminarin, and 10 mM sodium phosphate buffer (pH 6.0). The reducing sugars liberated by the enzyme reaction were determined by the method of Park and Johnson (1949). One unit of β -1,3-glucanase was defined as the amount of enzyme that liberates reducing sugars equivalent to 1.0 μ mol glucose per min under the standard conditions. pH dependence of the enzyme was measured at 30°C using reaction mixtures containing 50 mM sodium citrate buffer (pH 3.5-6.0) and 50 mM sodium phosphate buffer (pH 6.0-9.5). Temperature dependence of the enzyme was measured at 4-70°C using the reaction mixture adjusted to pH 6.0 with 10 mM sodium phosphate buffer. Thermal stability was determined by measuring the activity remaining after the heat treatment of enzyme at 4-60°C and pH 6.0 for 15 min.

2.3. Purification of β -1,3-glucanase from abalone

A β -1,3-glucanase was purified from digestive fluid of abalone as follows: the abalone (shell size, 10 x 6 cm) was dissected with a scalpel and the adductor muscle was removed. The digestive fluid was then collected from the stomach lumen by using Pasteur's pipette. Approximately 30 mL of the digestive fluid was obtained from 15 abalones. The digestive fluid was dialyzed against 10 mM sodium phosphate buffer (pH 6.0) for 12 h with occasional buffer changing. After the dialysis, the digestive fluid was centrifuged at 10,000 x g for 10

min and the supernatant (crude enzyme) was subjected to ammonium sulfate fractionation. The precipitates formed between 20-60% saturation of ammonium sulfate were collected by centrifugation at 10,000 x g for 10 min. The precipitates were dissolved in 40% saturated ammonium sulfate containing 10 mM sodium phosphate buffer (pH 6.0) and applied to a TOYOPEARL Phenyl-650M column (2 x 30 cm) pre-equilibrated with the same solution. The adsorbed proteins were eluted by a stepwise elution system with 40%, 30%, 20%, and 0% saturated ammonium sulfate in 10 mM sodium phosphate buffer (pH 6.0). β -1,3-Glucanase was eluted by the buffer containing 20% saturated ammonium sulfate (Fig. 1A). The active fractions (fraction numbers 102-107) were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0), and applied to a TOYOPEARL CM-650M column (2 x 45 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0-300 mM NaCl (Fig. 1B). In this chromatography, β -1,3-glucanase was eluted at around 200 mM NaCl (fraction numbers 67-70). These fractions were pooled and concentrated by lyophilization to less than 5 ml and applied to an AKTA FPLC (Amersham Biosciences, Uppsala, Sweden) equipped by a Superdex 75 10/300 GL column pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0) containing 300 mM NaCl (Fig. 1C). β -1,3-Glucanase was eluted in fractions 32 and 33 and these fractions were combined to use as the abalone β -1,3-glucanase. According to SDS-PAGE, the abalone β -1,3-glucanase was consisting of a single protein band with a molecular mass of approximately 33 kDa (Fig. 2). By the above purification procedure, the abalone β -1,3-glucanase was purified 340-fold at a yield of 17% and the specific activity 68 U/mg (Table 1). In the present study, we named this β -1,3-glucanase HdLam33 after the scientific name of the abalone *H. discus hannai* and the molecular mass of 33 kDa.

2.4. Analysis of laminarioligosaccharides by TLC

Laminarioligosaccharides produced by HdLam33 were analyzed by thin-layer chromatography (TLC). The oligosaccharides were developed on a TLC-60 plate (Merck, Darmstadt, Germany) with a solvent consisting of ethyl acetate, acetic acid, and water (2:2:1 (v:v:v)) and detected by spraying 10% (v/v) sulfuric acid in ethanol followed by heating at 130°C for 10 min.

2.5. Analysis of transglycosylation activity

Transglycosylation activity of β -1,3-glucanase was assayed at 30°C in a reaction

mixture containing 10 mM laminaritriose (a donor substrate), 30 mM laminaribiose (an acceptor substrate), 0.01 U/ml HdLam33, and 10 mM sodium phosphate buffer (pH 6.0). The transglycosylation reaction was performed at 30°C for up to 24 h and terminated by the addition of an equal amount of 10% trichloroacetic acid. The sugar composition of the reaction products were analyzed by TLC, while the amount of glucose released by the reaction was determined with a Glucose CII test kit Wako (Wako Pure Chemicals Industries Ltd.).

2.6. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 0.1% (w/v) SDS-10% (w/v) polyacrylamide gel according to the method of Porzio and Pearson (1977). After the electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol-10% (v/v) acetic acid, and destained with 5% (v/v) methanol-7% (v/v) acetic acid. Protein Marker, Broad Range (New England BioLabs, Ipswich, MA, USA) was used as a molecular mass marker.

2.7. Determination of partial amino-acid sequence

The N-terminal amino-acid sequence of HdLam33 was determined with an ABI Procise 492 sequencer (Applied Biosystems). For the determination of internal amino-acid sequences of HdLam33, it was digested with 0.5% lysylendopeptidase at 37°C for 2 h, and blotted to a polyvinylidene difluoride membrane after SDS-PAGE. Several fragments well separated on the membrane were subjected to the sequencer.

2.8. Mass spectrometry of HdLam33.

Molecular mass of HdLam33 was determined by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) by using an ABI Proteomics Analyzer 4700 (Applied Biosystems, Foster City, CA, USA). The purified HdLam33 was mixed with an equal volume of 10 mg/mL sinapinic acid (Sigma-Aldrich) and subjected to the spectrometer.

2.9. Determination of protein concentration

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin fraction V as a standard protein.

2.10. cDNA cloning of HdLam33

Total RNA was extracted from a hepatopancreas of the abalone by the guanidinium thiocyanate-phenol method (Chomczynski and Sacchi, 1987). mRNA was selected from the total RNA with an Oligo-dT(30) kit (TaKaRa) according to the manufacturers' protocol. cDNA was synthesized from the mRNA with a cDNA synthesis kit (TaKaRa) employing random oligonucleotide primers. cDNAs encoding HdLam33 were amplified by the PCR with degenerated primers synthesized on the basis of partial amino-acid sequences of HdLam33. For the amplification of cDNAs, a successive reaction at 95°C for 30s, 45°C for 30s, and 72°C for 90s was repeated 30 cycles. The amplified cDNAs were cloned with pCR 2.1 TOPO vector and the TA cloning kit (Invitrogen) and sequenced with an ABI 310 Genetic Analyzer (Applied Biosystems). Homology search for deduced amino-acid sequences was performed using the BLAST tool provided by National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

3. Results

3.1. General properties of HdLam33

In the present study, a β -1,3-glucanase HdLam33 was purified to electrophoretic homogeneity from the digestive fluid of the Pacific abalone (Fig. 1, 2 and Table 1). The molecular mass of this enzyme was estimated to be 33 kDa by SDS-PAGE (Fig. 2). Optimal temperature and pH of HdLam33 were 50°C and 6.0, respectively, and the temperature that caused a half inactivation during 15-min incubation was approximately 50°C (Fig. 3A-C). HdLam33 degraded laminarin (β -1,3;1,6-glucan) and lichenan (β -1,3;4-glucan); however, it could not degrade CMC, xylan, mannan, and agar (Fig. 3D). Accordingly, HdLam33 was regarded as a typical β -1,3-glucanase with a similar substrate preference to bivalve β -1,3-glucanase like PyLam38 (Kumagai et al., 2008).

Degradation products of laminarioligosaccharides and laminarin by HdLam33 were analyzed by TLC (Fig. 4A-F). HdLam33 degraded oligosaccharides larger than laminaribiose producing laminaribiose and glucose but it could not degrade laminaribiose. Laminarin was also degraded by HdLam33 into laminaribiose and glucose. These results indicate that the major end-products of HdLam33 are disaccharide and monosaccharide. However, the small amount of tetrasaccharide was also produced from laminarin by HdLam33 (Fig. 4G). This is

considered to be 6-*O*-glycosyl-laminaritriose that has been derived from the β -1,6-linked branching region of laminarin since endo- β -1,3-glucanases are generally known to be incapable of degrading the glycoside linkages neighboring β -1,6-linkage.

It is noteworthy that small amounts of oligosaccharides with the size larger than the original substrates were generated along with the degradation of laminarioligosaccharides. For example, tetrasaccharide was detected in 1 h-reaction products of laminaritriose (Fig. 4B). The generation of oligosaccharides larger than the original substrates indicated that HdLam33 catalyzed the transglycosylation which is known as a reaction caused by the enzymes that hydrolyzes glycosyl linkages retaining the anomeric configuration.

3.2. Degradation of laminaribiose by HdLam33 through the transglycosylation with laminaritriose

As described above, HdLam33 was considered to possess transglycosylation activity. Previously, we reported that the scallop β -1,3-glucanase, PyLam38, catalyzes the transglycosylation reaction and can produce various kinds of heterooligosaccharides consisting of laminarioligosaccharides and various compounds possessing hydroxyl groups (Kumagai et al., 2008). Similar transglycosylation activity was also found in HdLam33. Namely, HdLam33 cleaved the glycosyl linkage in the reducing terminus of laminaritetraose and transferred the laminaritriose unit from the non-reducing terminus of the cleavage site to the acceptor substrates such as β -D-methyl glucoside and xylooligosaccharides as did PyLam38 (Kumagai et al, 2008). Therefore, in the present study, we further investigated whether or not HdLam33 can degrade laminaribiose, which was not degraded by HdLam33 as a sole substrate (Fig. 4A), through the transglycosylation reaction. At first, we determined the amount of glucose released from laminaritriose and laminaribiose by the HdLam33 digestion. As shown in Fig. 5A, practically no glucose was released from 30 mM laminaribiose by HdLam33; however, approximately 15 mM of glucose was released from 10 mM of laminaritriose. Thus, the amount of glucose released from laminaritriose was 1.5 times larger than the expected value, i.e., 10 mM. These results led us to consider that some parts of laminaribiose derived from laminaritriose were further degraded by HdLam33; provably through the transglycosylation. Then, the mixture of laminaribiose and laminaritriose was subjected to the degradation by HdLam33. In this case, the amount of glucose released was greatly increased, i.e., it reached 45 mM in 10 h reaction (Fig. 5A). This was three times larger than that produced in the reaction mixture containing laminaritriose alone (15 mM). Since the concentrations for laminaritriose and laminaribiose in the reaction mixture were 10

mM and 30 mM, respectively, the total glucose concentration was theoretically 90 mM (30 mM plus 60 mM). Accordingly, the results in Fig. 5A indicated that approximately a half of the total glucose was released from the mixture of laminaritriose and laminaribiose by HdLam33. In order to interpret this high glucose yield, we have to consider that at least a certain part of laminaribiose along with laminaritriose was degraded by HdLam33. Actually, the moderate decrease in laminaribiose is seen in the TLC analysis, i.e, the amount of laminaribiose was decreased to almost a half of the original value along with the decrease in laminaritriose (Fig. 5B). Based on these results, we consider that the high yield of glucose from the mixture of laminaritriose and laminaribiose is due to the transglycosylation reaction between laminaritriose and laminaribiose. The mechanism can be explained as follows. At the first step of the reaction, the laminaribiose unit of laminaritriose is transferred to a laminaribiose producing laminaritetraose and glucose by the transglycosylation activity of HdLam33 (Fig. 6). At the second step, the laminaritetraose is hydrolyzed to laminaritriose and glucose. A part of laminaritriose is introduced to the first step and reacts with laminaribiose. Another part of laminaritriose is degraded to laminaribiose and glucose at the third step. The thus produced laminaribiose is introduced to the first step and react with laminaritriose. Repeating these steps, HdLam33 efficiently degrades laminaribiose along with laminaritriose.

3.3. Primary structure of HdLam33

The N-terminal amino-acid sequence of 17 residues of HdLam33 was determined as GNTVFEDSFNSHQLNPK. This sequence showed no similarity to any protein sequences currently deposited in the data bases. On the other hand, the amino-acid sequence of a lysylendopeptidyl fragment of HdLam33, VFSVASITHGRVEVVAKIPKGDWIWP, showed 73-85% similarity to the internal amino-acid sequences of β -1,3-glucanases from scallop, surf clam, and sea urchin. We then synthesized the forward primers F1 and F2 on the basis of the N-terminal sequence, and the reverse primers R1 and R2 on the basis of the internal sequences (Table 2). cDNAs encoding HdLam33 were amplified by the PCR using these primers from the abalone hepatopancreas cDNA library. As a result, a cDNA consisting of approximately 350 bp (Hd1-cDNA) was successfully amplified by the PCR using a primer set of F2 and R2. Hd1-cDNA was consisting of 345 bp that encodes an amino-acid sequence of 115 residues. Then, we synthesized a series of specific primers on the basis of the nucleotide sequence of Hd1-cDNA (Table 2), and amplified Hd3RACE-cDNA (808 bp) covering the 3'-terminal region and Hd5RACE-cDNA (459 bp) covering the 5'-terminal region by 3'-RACE and 5'-RACE, respectively. By combining the nucleotide sequences of

Hd5RACE-cDNA, Hd1-cDNA, and Hd3RACE-cDNA in this order, we determined a nucleotide sequence of 1180 bp encoding the amino-acid sequence of 366 residues of HdLam33. The reliability of this sequence was confirmed with HdFull-cDNA, which we newly amplified by PCR with a specific primer pair, 5'fullF and 3'fullR (Table 2). These sequence data are available from DNA Data Bank of Japan (<http://www.ddbj.nig.ac.jp/>) with an accession number AB488493. The N-terminal sequence of 36 residues except for the initiation methionine in the deduced sequence was regarded as the signal peptide region of HdLam33 because this region was absent in the N-terminus of HdLam33 protein and predicted as a signal peptide for secretion by the SignalP software (<http://www.cbs.dtu.dk/services/SignalP/>). Accordingly, the mature HdLam33 was concluded to consist of 329 residues with the calculated molecular mass of 36,866 Da. However, this molecular mass was appreciably greater than that estimated by SDS-PAGE for HdLam33 protein (33,000 Da). This may be due to some post-translational modifications of HdLam33 such as cleave off of the C-terminal region. Therefore, we determined the molecular mass of the HdLam33 protein by MALDI-TOF-MS. As a result, the molecular mass of the HdLam33 protein was determined as 36,855 Da which was well consistent with the molecular mass calculated from the primary structure. This indicates that the inconsistency in molecular masses between that calculated from the primary structure and that estimated by SDS-PAGE was due to the irregular mobility of HdLam33 on SDS-PAGE. The amino-acid sequence of the mature HdLam33 showed 56-61% identity with the sequences of β -1,3-glucanases from marine invertebrates which have been classified under GHF16 (see Fig. 7).

4. Discussion

4.1. Characterization of HdLam33

In the present study, we purified a β -1,3-glucanase HdLam33 from the digestive fluid of the Pacific abalone *H. discus hannai*. HdLam33 showed optimal temperature and pH at around 50°C and 6.0, respectively, and preferably degraded β -1,3-glucan such as laminarin and lichenan. These properties were similar to those of scallop β -1,3-glucanase, PyLam38, which we previously isolated (Kumagai et al., 2008). Whereas, thermal stability of HdLam33 was significantly higher than that of PyLam38, i.e., the temperature that caused a half inactivation of PyLam38 during 15-min incubation at pH 6.0 was 35°C, while that for HdLam33 was 50°C. The difference in thermal stability between HdLam33 and PyLam38

may be attributable to the molecular adaptation to their different habitat temperatures. Namely, suitable habitat temperatures for abalone and scallop were around 17°C and 10°C, respectively.

HdLam33 degraded laminarioligosaccharides, laminarin, and lichenan but not CMC, xylan, mannan, and agar. Since lichenan is comprised of β -1,3-linked cellotriose units, HdLam33 was considered to be capable of degrading β -1,4-glucosyl linkages adjacent to β -1,3-linkages. Actually, HdLam33 produced no cellotriose, cellobiose, and glucose from lichenan, but produced a trisaccharide which was definitely distinguished from cellotriose and laminaritriose on HPLC with a Sugar-D column (Nacalai Tesque, Kyoto, Japan) (data not shown). Thus, the trisaccharide from lichenan was tentatively identified as cellobiosyl- β -1,3-glucose. Confirmation of the identification for this trisaccharide by NMR is under way.

HdLam33 degraded laminarioligosaccharides larger than laminaribiose producing laminaribiose and glucose, while it degraded laminarin producing a small amount of tetrasaccharide along with laminaribiose and glucose. The tetrasaccharide is considered to be 6-*O*-glycosyl-laminaritriose derived from the β -1,6-linked glucose branching region of laminarin since the branching regions are hardly degraded by β -1,3-glucanases (Petersen et al., 2000; Kawai et al., 2006). From these results, we may conclude that HdLam33 is a typical endo-1,3;4- β -glucanase (EC 3.2.1.6).

HdLam33 catalyzed transglycosylation reaction and was considered to degrade laminaribiose through the transglycosylation reaction with laminaritriose (Figs. 5 and 6). Namely, HdLam33 transferred the laminaribiose unit of laminaritriose to laminaribiose to produce laminaritetraose and glucose. The laminaritetraose was then hydrolyzed to laminaritriose and glucose. A part of laminaritriose may be degraded to laminaribiose and glucose and another part of laminaritriose may be returned to the first step of the reaction. The laminaribiose may be introduced to the first step of the reaction. Repeating this reaction cycle, HdLam33 produces glucose efficiently from laminaritriose and laminaribiose. In this reaction, approximately 3 times larger amount of glucose was produced, i.e., 45 mM in the presence of both laminaribiose and laminaritriose *versus* 15 mM in the presence of laminaritriose alone (Fig. 5A). Although the physiological significance of this mechanism is still obscure; we now consider that this is an advantageous property of HdLam33 for the production of glucose since this mechanism requires no other enzyme like β -glucosidase. Beside HdLam33, we have not obtained any enzymes that can rapidly degrade laminaribiose from the digestive

fluid of *H. discus hannai*.

4.2. Primary structure of HdLam33

The primary structure of HdLam33 was aligned with those of β -1,3-glucanases from scallop, surf clam, and sea urchin using Clustal X software (Fig. 7). Two glutamate residues which are known to constitute the catalytic site of GHF16 enzymes were conserved among these enzymes. In addition, two cysteine residues, which were reported to form disulfide bond in the scallop enzyme (Kovalchuk et al., 2006), were also conserved among the marine invertebrate enzymes. The sequence homology between HdLam33 and the enzymes from scallop, surf clam, and sea urchin, were 61%, 56%, and 55%, respectively. These results indicate that HdLam33 also belongs to GHF16 like the other invertebrate β -1,3-glucanases. Between the scallop and surf-clam enzymes, amino-acid sequence identity was significantly high, i.e., approximately 90%; however, those between HdLam33 and bivalve enzymes were the lowest level, i.e., 56-61%. Thus, the structural deviation appears to be much prominent in HdLam33 compared with that among bivalve enzymes. This may cause differences in enzymatic properties between gastropod and bivalve enzymes. Further study focusing on the difference between gastropod and bivalve enzymes using site-directed mutagenesis technique is now underway.

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Figure Legends

Fig. 1. Purification of β -1,3-glucanase from abalone. (A) TOYOPEARL Phenyl-650M column chromatography of proteins precipitated between 20 and 60% saturation of ammonium sulfate. (B) TOYOPEARL CM-650M column chromatography of the β -1,3-glucanase fraction obtained by Toyopearl Phenyl-650M chromatography. (C) Superdex 75 10/300 GL column chromatography of β -1,3-glucanase fraction obtained by Toyopearl CM-650M chromatography. Protein elution and enzyme activity are shown with open and closed circles, respectively.

Fig. 2. Monitoring of purification of β -1,3-glucanase from the digestive fluid of the abalone *H. discus hannai* by SDS-PAGE. Lane 1, marker proteins; lane 2, the sample after ammonium sulfate fractionation; lane 3, the sample after TOYOPEARL Phenyl-650M chromatography; lane 4, the sample after TOYOPEARL CM-650M chromatography; lane 5 the sample purified

by Superdex 75 10/300 GL chromatography.

Fig. 3. Temperature dependence, pH dependence, thermostability, and substrate specificity of HdLam33. (A) Temperature dependence of HdLam33 was measured at 10-70°C in a reaction mixture containing 0.2% laminarin and 10 mM sodium phosphate buffer (pH 6.0). (B) pH dependence of HdLam33 was measured at 30°C in reaction mixtures adjusted to pH 3.5-6.0 with 50 mM sodium citrate buffer and pH 6.0-9.5 with 50 mM sodium phosphate buffer. (C) Thermostability of HdLam33 was evaluated by measuring the activity remaining after the incubation at 4-70°C in 10 mM sodium phosphate buffer (pH 6.0) for 15 min. The remaining activity was measured at 30°C. (D) Substrate specificity for HdLam33 was measured in a reaction mixture containing 0.2% laminarin (○), lichenan (△), CMC, xylan, mannan, and agar (□). Since no release of reducing sugar was detected in CMC, xylan, mannan, and agar, the result obtained with CMC is shown as a representative.

Fig. 4. Thin-layer chromatography for the degradation products of laminarioligosaccharides and laminarin produced by HdLam33. Five mg/mL laminarioligosaccharides or 2 mg/mL laminarin in 10 mM sodium phosphate buffer (pH 6.0) were degraded with 0.01 U/mL HdLam33 at 30°C. The reaction was terminated at appropriate times by mixing with an equal volume of 10 % (w/v) trichloroacetic acid and 1 µL of the mixture was applied to TLC. (A-F) Degradation products of laminarioligosaccharides (G1, glucose; L2-L7, laminaribiose to laminariheptaose) produced by HdLam33. (G) Degradation products of laminarin produced by HdLam33. M, oligosaccharide markers comprising G1 and L2-L7.

Fig. 5. Production of glucose by HdLam33 from laminaribiose, laminaritriose, and mixture of laminaribiose and laminaritriose. (A) Production of glucose from laminaribiose alone (□), laminaritriose alone (○), and mixture of laminaribiose and laminaritriose (●). The concentration of laminaribiose and laminaritriose were 30 mM and 10 mM, respectively. (B) TLC analysis for the degradation products of laminaribiose and laminaritriose produced by HdLam33. G1, glucose; L2-L5, laminaribiose-laminaripentaose.

Fig. 6. Schematic representation for degradation of laminaribiose through transglycosylation. Open and gray circles indicate the glucose residues of laminaritriose and laminaribiose, respectively. Closed reverse triangles indicate the cleavage (transglycosylation) sites. Dotted

arrows indicate the returning pathways of laminaritriose and laminaribiose to the first step of the reaction. Released glucose residues are indicated by “G”.

Fig. 7. Alignment of amino-acid sequences for HdLam33 and other laminarinases from marine invertebrates. The amino-acid sequence of HdLam33 is aligned with those of *Mizuhopecten yessoensis* enzyme (GenBank accession no. AAW34372), *Pseudocardium Sachalinensis* enzyme (GenBank accession no. AAP74223), and *Strongylocentrotus purpuratus* enzyme (GenBank accession no. AAC47235). The positions of identical residues, highly conserved substitutions, conservative substitutions, and gaps are indicated by asterisk (*), colon (:), dot (.), and dash (–), respectively. Residues participating in catalytic action as nucleophiles or proton donors in GHF16 enzymes, i.e., E186 and E191 are boxed. Cysteine residues conserved among marine invertebrate enzymes, i.e., C117 and C125 are indicated by broken-line boxes.

Table 1

Purification of HdLam32

Samples	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude	945	0.2	212	1	100
AS ^{*1}	44.3	1.9	82	10	39
Phenyl ^{*2}	16.6	4.6	77	23	36
CM ^{*3}	4.7	14	67	72	32
Superdex	0.5	68	35	340	17

^{*1}Fraction precipitated between 20 and 60% saturation of ammonium sulfate. ^{*2}Active fraction obtained by TOYOPEARL Phenyl-650M chromatography. ^{*3}Active fraction obtained by TOYOPEARL CM-650M chromatography.

Table 2

DNA primers used for amplification of HdLam33-cDNAs

Name	Sequence ^a
F1	5'-GGNAAAYACNGTTTTYGARGA-3'
F2	5'-TTYAAAYWSICAYCARYTIAAYCC-3'
R1	5'-CCARTCNCCYTTNGGDATYTTICC-3'
R2	5'-GGDATYTTNGCNACIACYTC-3'
5RaceRT	5'-GTGTGATTGAGGC-3'
5RaceF1	5'-GCGGCGCAAGACAATGGTTGCC-3'
5RaceF2, 3RaceF	5'-GCCAGGGAGCTCAGATTCCACC-3'
5RaceR1	5'-CTTGTCAGCAGTGAATGTCGG-3'
5RaceR2	5'-GAGAACGCCATTCTTGATGTAC-3'
FullF	5'-GGAAGCGATGGAATATTCAGTG-3'
FullR	5'-CCAATATTCCTTCCCCACGCTTC-3'

^a D, A/G/T; I, Inosine; N, A/C/G/T; R, A/G; W, A/T; Y,C/T;

Fig. 1.

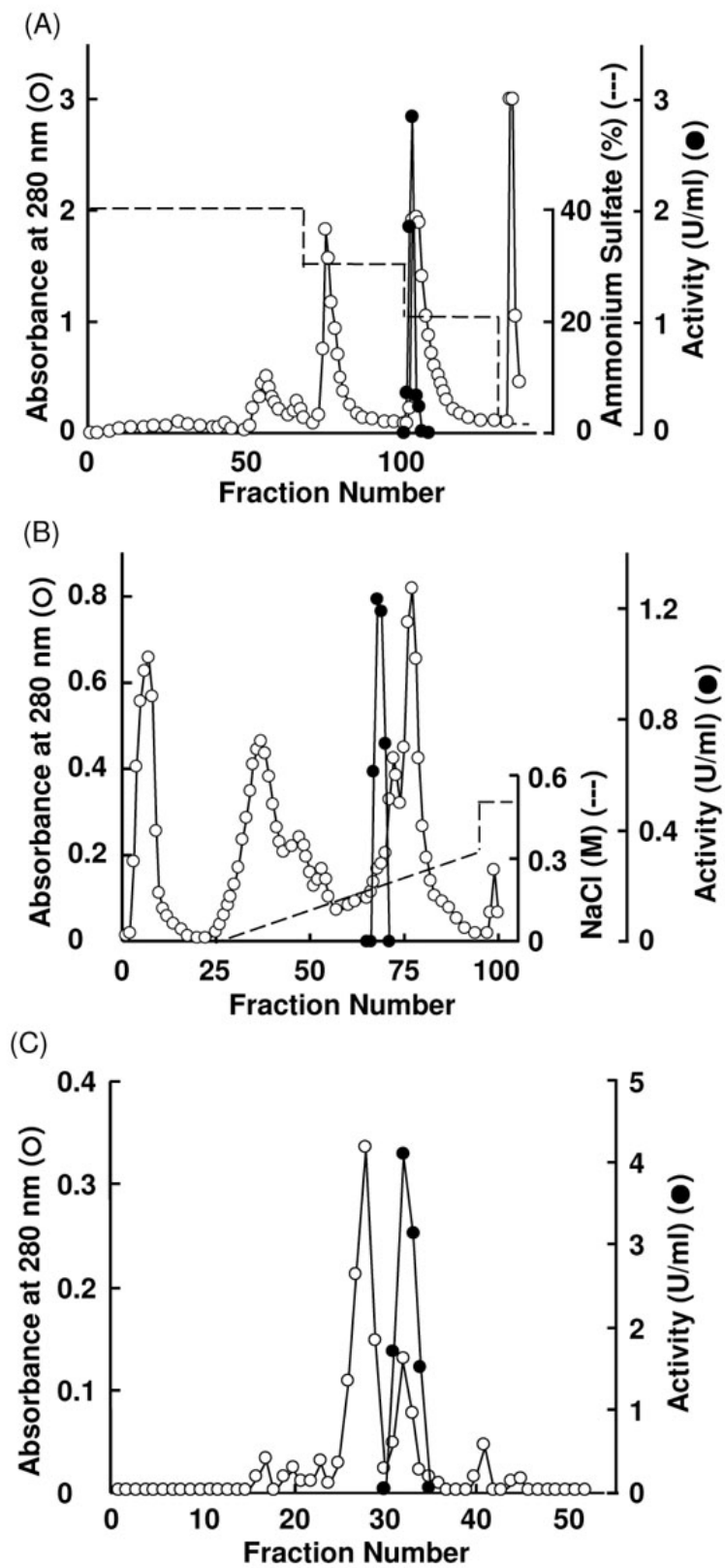


Fig. 2.

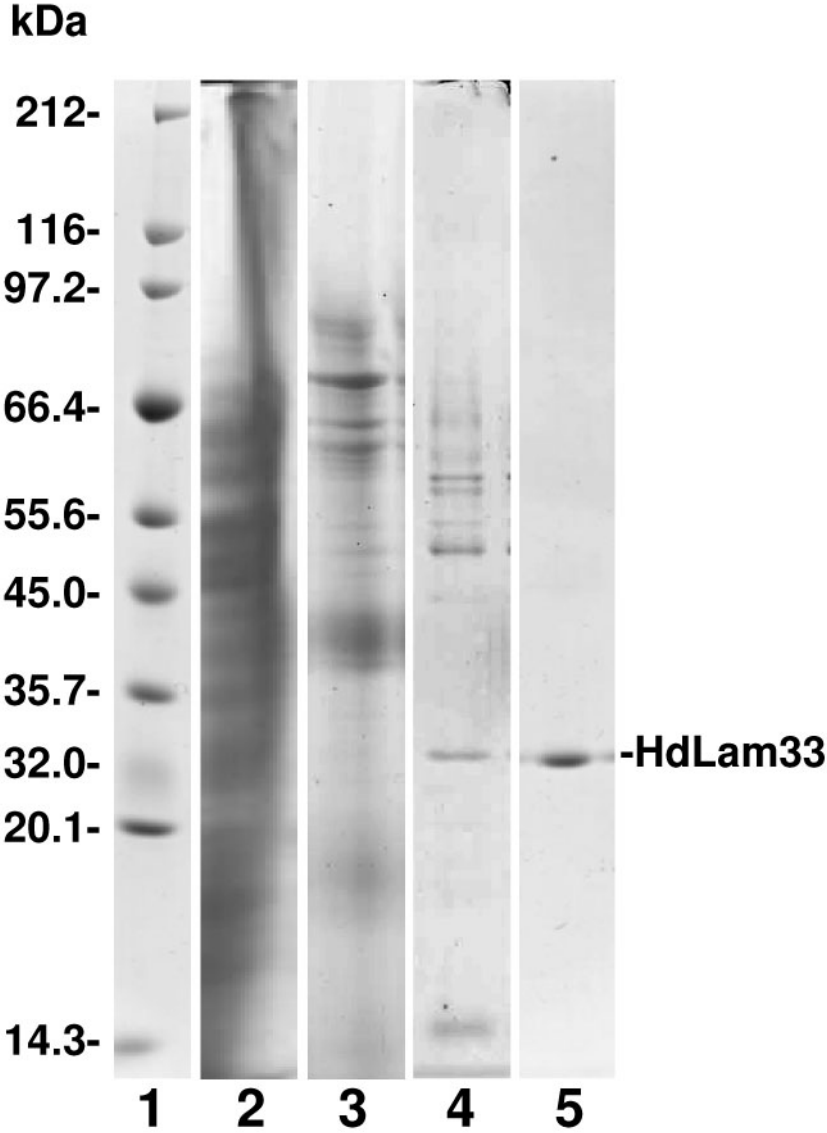


Fig. 3.

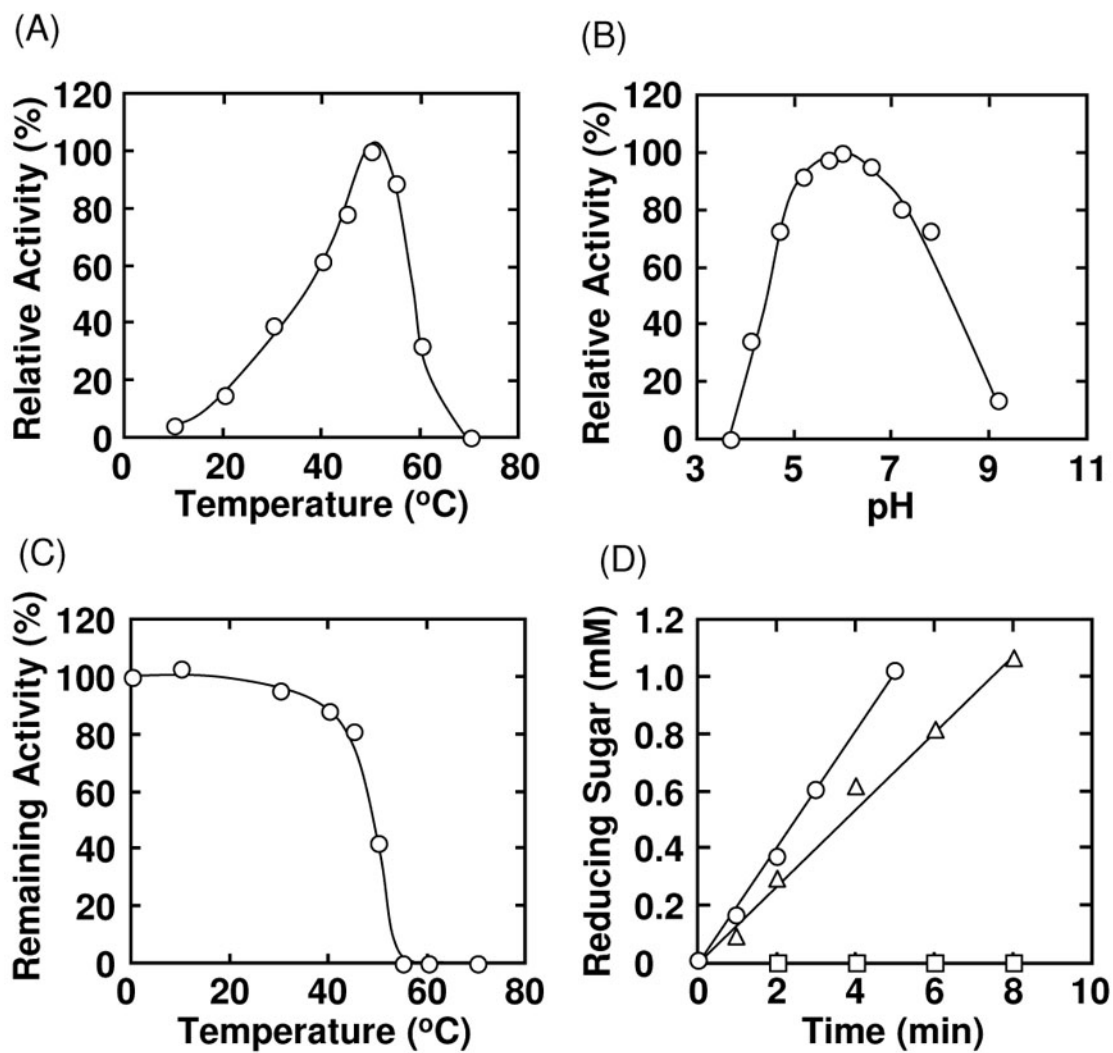


Fig. 4.

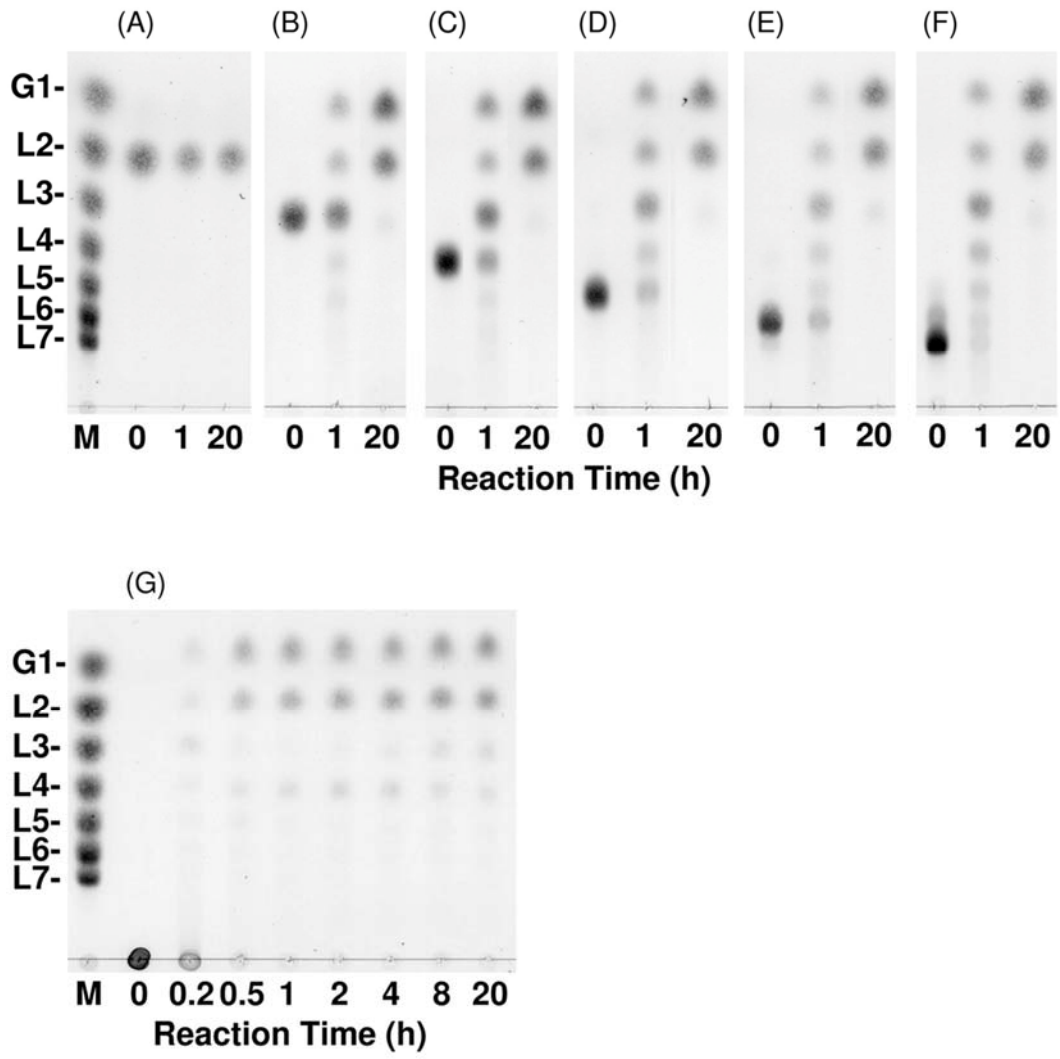


Fig. 5.

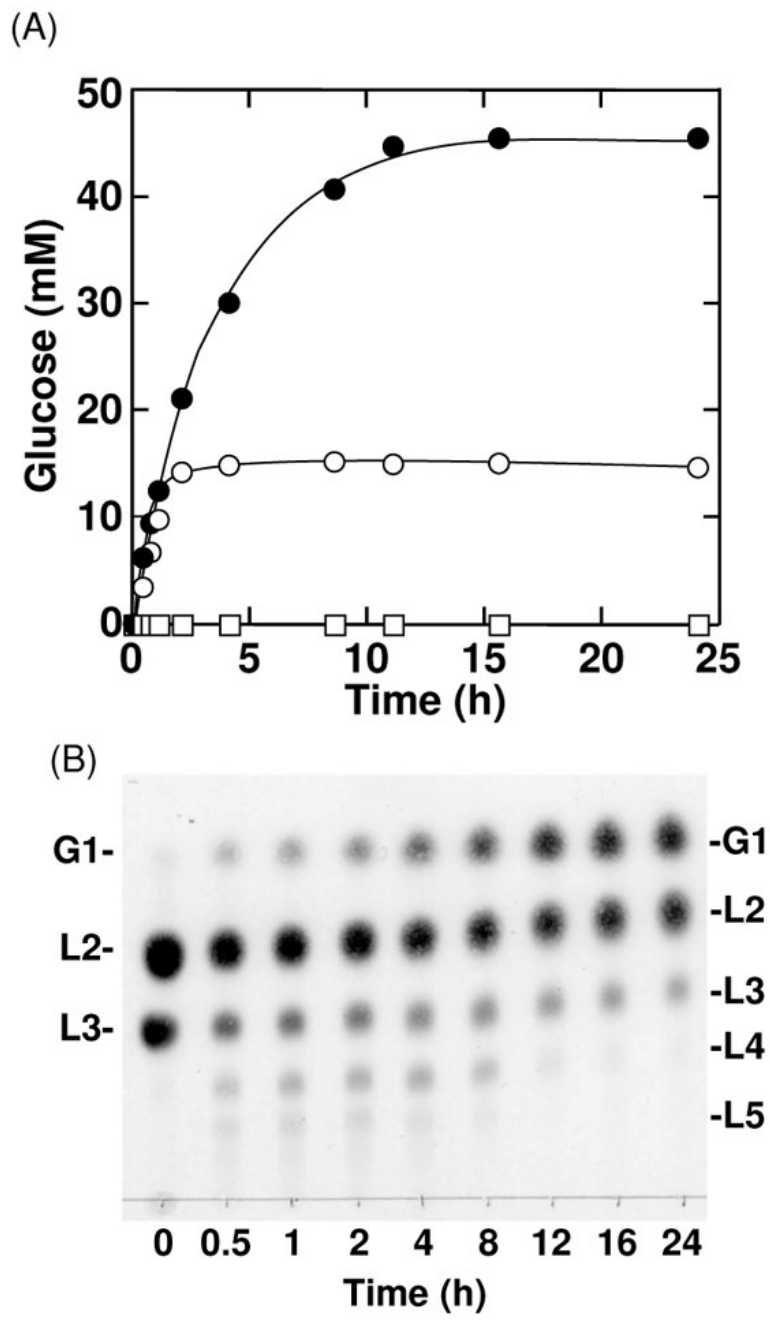


Fig. 6.

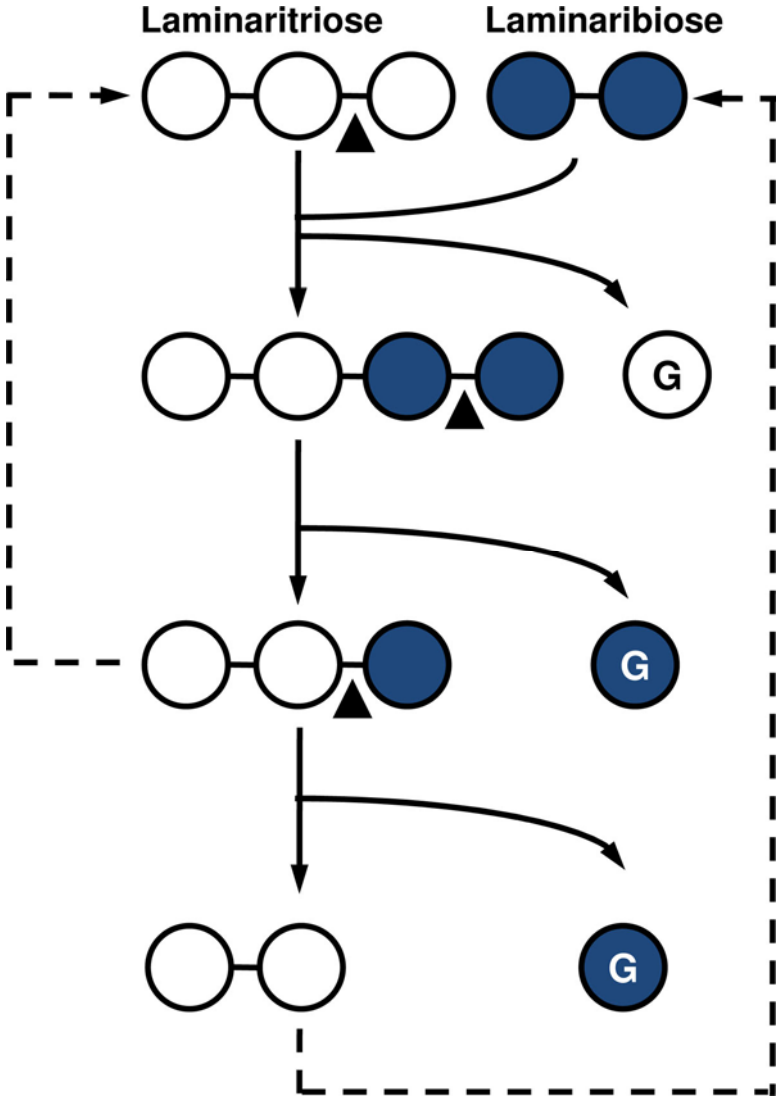


Fig. 7.

HdLam33	-----MEYSVKYLSASG-----DVVY	16
PyLam38	-----	
<i>P. sach.</i>	SFGESNHQGFHQNTDSRWYHRNHEAKYNGDKIKYRIMVALD-----GKTI	95
<i>S. purp.</i>	GVGAGQYNDVTTTDEYFVHENRDVDVENGDVVYVYVYTYGLGYQLTQSWTASET	120
HdLam33	HVDGVFTIP--AASSLSPRL-----YRRGNTV	41
PyLam38	-----MDP--LLCLVLLPLV-----AGAG--	17
<i>P. sach.</i>	ETNGKLTGPGETGVAVLPPQK-----VRRGTVV	122
<i>S. purp.</i>	EAPATNPPATESPVTNAPATESPNPGTGTQSSGGGTSQCSMYPDAACDMSTPPCNGLI	180
	*	
HdLam33	FEDSFNSHQLNPKHWHHEITCWGGNGEFQMYTPEAANTYIKNGVLYLKPTFTAD--KFG	99
PyLam38	FRDDFT--TWDPDYQIEVSAWGGNHEFQVFTPEPSNLFVRNGNLYIKPTFTRDSAHFN	75
<i>P. sach.</i>	FRDDFNG-AFDPAGWNYEVSMYGGYNWEVQAYVPDARNIFTRNGHLFIKPTLTDDHPNYN	178
<i>S. purp.</i>	FQEEFDS--FNLDIWEHEMTAGGGNWEFEYTYNNRSNSYVRDGKLFIKPTLTDD--KLG	239
	:. : : * : : * * * : : * : : * : : * : : * * * * * : : *	
HdLam33	DDFFQHGVLVVK--QWGSCTAAQDNCGRRQ--G--AQIPPIMSSKVFVSA--SITHGR	150
PyLam38	DGSLYYGTMDVN--SLWHRCTQHDNNGCHKQSYGGDSEILPPVMMSGKITTNF--AMTYGR	131
<i>P. sach.</i>	DGNLSATMDLT--ALYGYGTNADRYGQIREGRNG---ILPPVMMSGKIKSKK--TRFGK	236
<i>S. purp.</i>	EGLSSTGLDLWGSSPANLCTGNAWYCGSRTGSND--NLLNPIQSARLRTVESFSFKYGR	292
	: : : : * : * * * : * * * : * : * : * : : * : : * :	
HdLam33	VEVVAKIPKGDWIWPAIWLLPPGWPWKYGAWPASGEIDIMESRGNVHLSSEANGATQGVDR	210
PyLam38	VNVRAKIPKGDWLWPAIWMLPR--DWSYGGWPRSGEIDIMESRGNTKAILG--GQNSGVNY	188
<i>P. sach.</i>	VEARCRIPRGDWIWPAIWMLPR--DSVYGGWPRSGEIDIMESRGNTVARDGSGHNHGVNE	292
<i>S. purp.</i>	LEVEAKLPTGDWLWPAIWLLPK--HNGYGEWPASGEIDLVESRGNADIKDADGLSAGVDQ	352
	: : : : * * * * * : * * * * * : * * * * * : * * * * * : * * * * * :	
HdLam33	VLSTIHYGASPSQ--HRQGGDSKTSKTGTTWADSFHTYSVDWTAGHIRMIDNQPVMAWT	268
PyLam38	VASTLHWGPAYNHNAFAKTHASKRKYGGDDVHG--WHTYSLDWTADHIIITYVDNEMMRIN	247
<i>P. sach.</i>	VG-HLHWDQMP---VIIDSVRRRTGDLDG--DWSHAMHTYRLDWTIDHIIQVFDNRHIMNIP	347
<i>S. purp.</i>	MGSTMHWGPFWPLNGYPKTHATKFYVDDLELLNVDPATGF--WDLGEFENDAPG-----	403
	: : * : * : :	
HdLam33	TPSQGYWSYSHQSGTNPVWVWSSQGGNDAPFDGKMSLILNVAVGATNGYFQDSWHNTPHAKPWK	328
PyLam38	TPSQSFWGGGFDGNNIWASGGKNAPFDKPFHLILNVAVGG--DYFGNGEYDVP--KPWD	303
<i>P. sach.</i>	QSRKVFGLSEDLVDPFIGAVEPKAAPFDKQFYLILNVAIAGTNGFFPDNWTYD--QPKPW	406
<i>S. purp.</i>	-----IDNPWAYNPNKLTPTFDQEFYILNVAVGGVN-YFGDGLTYTP-AKPWS	450
	. : * * * : * * * * : * * * * : * * * * : * * * * :	
HdLam33	NNSPTAMMDFWKSQQWQSTWHGEDVAMKVKSVKMIQY-----	366
PyLam38	NHNP--MRSFWEARHSWEHTWGGDEVALVIDYIEMIPH-----	339
<i>P. sach.</i>	SNSPTELQDFWNARFQWLQTVHGDDVAMECDYVEMTQY-----	444
<i>S. purp.</i>	NDSPTASKDFWSDFNTWYPTWNGEEAAMQVNYVRYXEPGQTTYXLRDR	499
	. . . * . . * . * * * : * : . : :	