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Isolation and characterization of two types of β-1,3-glucanases from the common sea hare *Aplysia kurodai*

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Abstract

Two types of β-1,3-glucanases, AkLam36 and AkLam33 with the molecular masses of 36 kDa and 33 kDa, respectively, were isolated from the digestive fluid of the common sea hare *Aplysia kurodai*. AkLam36 was regarded as an endolytic enzyme (EC 3.2.1.6) degrading laminarin and laminarioligosaccharides to laminaritriose, laminaribiose, and glucose, while AkLam33 was regarded as an exolytic enzyme (EC 3.2.1.58) directly producing glucose from polymer laminarin. AkLam36 showed higher activity toward β-1,3-glucans with a few β-1,6-linked glucose branches such as *Laminaria digitata* laminarin (LLam) than highly branched β-1,3-glucans such as *Eisenia bicyclis* laminarin (ELam). AkLam33 showed moderate activity toward both ELam and LLam and high activity toward smaller substrates such as laminaritetraose and laminaritriose. Although both enzymes did not degrade laminaribiose as a sole substrate, they were capable of degrading it via transglycosylation reaction with laminaritriose. The N-terminal amino-acid sequences of AkLam36 and AkLam33 indicated that both enzymes belong to the glycosyl hydrolase family 16 like other molluscan β-1,3-glucanases.

1. Introduction

Laminarin is a storage polysaccharide consisting of a β-1,3-linked glucose main chain and β-1,6-linked glucose branches. This polysaccharide distributes over various brown seaweeds and microalgae (Black et al., 1951; Smelcerovic et al., 2008). The higher order structure of laminarin varies depending on the origin. For example, the main chain of diatom laminarin possesses few β-1,6-linked branches (Størseth et al., 2005). While the main chain of *Laminaria digitata* laminarin possesses β-1,6-branches in a moderate frequency, e.g., the molar ratio for β-1,6-linked glucose to β-1,3-linked glucose is approximately 1:7 (Hrmova and Fincher, 1993). The main chain of *Eisenia bicyclis* laminarin possesses β-1,6-linked branches in considerably high frequency, and β-1-6-linkages are also seen in the main chain (Maeda and Nisizawa, 1968; Usui et al., 1979).

Laminarin and its degradation products exhibit certain biological activities. For example, laminarin from *E. bicyclis* stimulated immunoresponse and alleviated diabetes in human (Pang et al., 2005). On the other hand, enzymatically produced laminarin oligosaccharides promoted the secretion of TNF-α from human monocytes (Miyanishi et al., 2003). These biological activities of laminarin and its degradation products are considered to be derived from the higher order structure of laminarin, i.e., the β-1,3-linked glucose chain possessing β-1,6-linked glucose branches. However, up to now, direct correlation between the above bioactivities and the higher order structure of laminarin has been obscure.

In order to figure out the higher order structure of laminarin, it is important to analyze the local structures or unit structures of laminarin by using its limited degradation products, which can be produced by β-1,3-glucanases (EC 3.2.1.6 and EC

3.2.1.58). To date, β-1,3-glucanases have been found in bacteria, fungi, plants, insects, and invertebrate animals (Privalova et al., 1978; Takami et al., 1998; Shimizu et al., 2003; Suzuki et al., 2003; Kovalchuk et al., 2006; Suzuki et al., 2006). Recently, we isolated endolytic β-1,3-glucanases (EC 3.2.1.6) from the ezo-giant scallop *Patinopecten yessoensis* and the pacific abalone *Haliotis discus hannai* and determined their basic properties (Kumagai et al., 2008; Kumagai and Ojima, 2009). These molluscan β-1,3-glucanases seemed to be available for the structural analysis of laminarin. Namely, these enzymes degraded *L. digitata* laminarin producing 6-O-glycosyl-laminaritriose along with laminaritriose, laminaribiose, and glucose. The production of 6-O-glycosyl-laminaritriose from laminarin proves the occurrence of the β-1,6-linked glucose branches in the laminarin molecule. The molluscan β-1,3-glucanases were also useful for the synthesis of novel heterooligosaccharides comprising laminarioligosaccharides and hydroxyl compounds since these enzymes possess considerably high transglycosylation activity (Borriss et al., 2003; Giordano et al., 2004; Kumagai et al., 2008). For example, the scallop β-1,3-glucanase could produce a series of heterooligosaccharides comprising laminarioligosaccharides and various hydroxyl compounds via transglycosylation (Kumagai et al, 2008; Kumagai and Ojima, 2009). These usefulness of the molluscan β-1,3-glucanases have stimulated us to extend the research to other molluscan enzymes, which may possess some unique properties.

The common sea hare *Aplysia kurodai* is a typical herbivorous gastropod feeding mainly on Laminariales and Fucales. The sea hare grazes these seaweeds' fronds with gut teeth and digests them in stomach by using many kinds of digestive enzymes. Our preliminary experiments indicated that the digestive fluid of the sea hare could produce

glucose from the frond of *Sargassum* sp. and laminarin from *L. digitata*. These suggested that the digestive fluid of the sea hare contained β-1,3-glucanases.

Therefore, in the present study, we attempted to purify the β-1,3-glucanases from the digestive fluid of the sea hare in order to enrich general information about the molluscan β-1,3-glucanases. As a result, we succeeded to isolate two types of laminarin-degrading enzymes, i.e., an endolytic β-1,3-glucanase (EC 3.2.1.6) and an exolytic enzyme (EC 3.2.1.58). The latter enzyme was considered to be the first exolytic β-1,3-glucanase classified to glycoside hydrolase family 16 (GHF16).

2. Materials and methods

2.1. Materials

The common sea hare *Aplysia kurodai* (body length, approximately 12 cm) was collected on the coast of Hakodate, Hokkaido Prefecture, Japan, in July 2008. Digestive fluid of the animal was obtained from the gastric lumen by squeezing the stomach after dissection. Approximately 100 mL of the digestive fluid was obtained from 20 animals. The digestive fluid was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) for 2 h and centrifuged at 10,000 g for 10 min to remove insoluble materials. The supernatant was used as a crude enzyme preparation for the purification of β-1,3-glucanases. Laminarin (β-1,3:1,6-glucan) from *Laminaria digitata* and barley β-glucan (β-1,4:1,3-glucan) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Laminarin from *Eisenia bicyclis* and laminarioligosaccharides (laminaribiose – laminariheptaose, abbreviated to L2 – L7) were purchased from Seikagaku Kogyo

(Tokyo, Japan). TOYOPEARL CM-650M was from Toyo Soda Mfg. Co. (Tokyo, Japan), Mono-S 5/50GL, Superdex peptide 10/300GL, and Superdex 75 10/300GL were from GE Healthcare UK Ltd. (Little Chalfont, Bucking Hamshire, England). Other reagents were from Wako Pure Chemicals Industries Ltd. (Osaka, Japan).

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

The standard assay for β-1,3-glucanase activity was carried out at 30^{o}C in a 1 mL of reaction mixture containing an appropriate amount of enzyme, 0.2% (w/v) *L. digitata* laminarin or laminarioligosaccharides and 10 mM sodium phosphate buffer (pH 6.0). When laminarin was used as a substrate, reducing sugars produced by the reaction were determined by the method of Park and Johnson (1949) and one unit of β-1,3-glucanase was defined as the amount of enzyme that liberates reducing sugars equivalent to 1.0 μmol glucose per min under the standard conditions. When laminarioligosaccharides were used as substrates, liberated glucose was determined with Glucose CII test kit Wako (Wako Pure Chemicals Industries Ltd.) since oligosaccharide substrates themselves showed significantly high reducing power. In this case, one unit of β-1,3-glucanase was defined as the amount of enzyme that liberates 1.0 μmol glucose per min. pH dependence of the enzyme was measured at 30^{o}C using reaction mixtures containing 50 mM sodium citrate buffer (pH 2.3 – 6.0) and 50 mM sodium phosphate buffer (pH 6.0 – 9.8). Temperature dependence of β-1,3-glucanase was measured at 4 – 70^{o}C in a reaction mixture containing 10 mM sodium phosphate buffer (pH 6.0). Thermostability was assessed by measuring the activity remaining after the heat-treatment at 4 – 60^{o}C for 15 min. The kinetic parameters of the β-1,3-glucanases

for laminarin and laminaritetraose were estimated by the Lineweaver-Burk analysis. The activity was assayed in 50 mM sodium phosphate buffer (pH 6.0) containing 0.05 – 2.0 mg/mL of laminarin or 0.1 – 15 mg/mL of laminaritetraose. Transglycosylation activity of β-1,3-glucanase was assessed by measuring the amount of glucose liberated from laminarioligosaccharides during the reaction.

2.3. Thin-layer chromatography (TLC)

Degradation products of laminarioligosaccharides (L2 – L7) were analyzed by TLC as follows. Laminarioligosaccharides (5 mg/mL) were degraded with 0.01 U/mL of β-1,3-glucanases and the degradation products (1 μl) were subjected to a TLC-60 plate (Merck, Darmstadt, Germany). The sugars produced by the reaction were developed with a solvent consisting of ethyl acetate, acetic acid, and water (2:2:1 (v:v:v)) and visualized by spraying 10% (v/v) sulfuric acid in ethanol followed by heating at 130^{o}C for 10 min.

2.4. Gel filtration analysis for degradation products of laminarin

Degradation products of laminarin were analyzed by gel-filtration chromatography with a Shimadzu LC-20AT HPLC (Shimadzu, Kyoto, Japan) equipped with a Superdex peptide 10/300GL column (GE Healthcare UK Ltd.). The column was developed with 10 mM sodium phosphate buffer (pH 6.0) and elution of the sugars was detected with a Shimadzu RID-10A detector.

2.5. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 0.1% (w/v) SDS-10% (w/v) polyacrylamide slab gel (1 mm thick, 10 cm x 9 cm (width x length)) 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 the background of the gel was 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.

2.6. Determination of amino-acid sequence

The N-terminal amino-acid sequence of β-1,3-glucanase was determined with an ABI Procise 492 sequencer (Applied Biosystems, Foster City, CA, USA). Internal amino-acid sequences of β-1,3-glucanase were determined with the peptide fragments prepared by lysylendopeptidase digestion (lysylendopeptidase/sample = 1/200 (w/w), 37^{o}C, 2 h). The fragments were separated by SDS-PAGE and blotted to a polyvinylidene difluoride membrane. Several fragments well separated on the membrane were subjected to the sequencer.

2.7. 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.

3. Results

3.1. Purification of β-1,3-glucanase

β-1,3-Glucanase was purified from the crude enzyme of the common sea hare *A. kurodai* as follows: the crude enzyme (approximately 100 mL from 20 animals) was subjected to ammonium sulfate fractionation and the precipitates formed between 40 – 60% saturation of ammonium sulfate were collected by centrifugation at 10,000 x g for 10 min. The precipitates were dissolved in and dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and applied to a TOYOPEARL CM-650M column (2 x 30 cm) pre-equilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0-0.3 M NaCl (total 600 mL) and the eluent was collected as 6-mL fractions. β-1,3-Glucanase activity was detected in fractions eluted at around 0.15-0.25 M NaCl as incompletely separated two peaks. Thus, these fractions (fractions 64-78) were combined and dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and subjected to an AKTA-FPLC (GE Healthcare Bio-Science AB, Uppsala, Sweden) equipped with a Mono-S 5/50GL column pre-equilibrated with the same buffer. The adsorbed proteins were eluted with a linear gradient of 0-0.3 M NaCl (total 50 mL) and the eluent was collected as 1-mL fractions. In this chromatography, β-1,3-glucanases were completely separated to two peaks, i.e., one peak at around 0.09 M NaCl (fractions 15-16, designated Fr1) and another peak at around 0.15 M NaCl (fractions 25-27, designated Fr2). Fr1 was dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and again applied to a Mono-S 5/50GL column. The column was developed with a linear

gradient of 0-0.15 M NaCl (total 50 mL) and the β-1,3-glucanase with the molecular mass of 33 kDa was eluted at around 0.1 M NaCl. In the present study, we named this β-1,3-glucanase AkLam33 after the scientific name of the common sea hare *A. kurodai* and the molecular mass of 33 kDa (Fig. 1). On the other hand, Fr2 was concentrated to approximately 2 ml by ultrafiltration with VIVASPIN 20 (Sartorius AG, Goettingen, Germany) and applied to a Superdex 75 10/300GL column pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0) containing 300 mM NaCl. The column was developed with the same buffer and the eluent was collected as 1-mL fractions. β-1,3-Glucanase activity was detected at fractions 13-14, which correspond to the eluting positions of proteins with molecular mass of 36 kDa. These fractions were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and applied to a Mono-S 5/50GL column pre-equilibrated with same buffer. The adsorbed proteins were eluted with a linear gradient of 0-0.3 M NaCl (total 50 mL) and the β-1,3-glucanase with the molecular mass of 36 kDa was eluted at around 0.15 M NaCl. This enzyme was named AkLam36 (Fig. 1).

By the above purification procedure, AkLam33 was purified 28-fold at a yield of 0.1% and the specific activity 39 U/mg, while AkLam36 was purified 126-fold at a yield of 2.5% and the specific activity 172 U/mg (Table 1).

3.2. Basic properties of AkLam36 and AkLam33

Optimal temperatures for AkLam36 and AkLam33 were 40^{o}C and 50^{o}C, respectively, while optimal pHs were 6.0 and 5.7, respectively. The temperatures that caused a half inactivation for AkLam36 and AkLam33 during 15-min incubation were

approximately 42°C and 45°C, respectively (Fig. 2).

3.3. Mode of actions of AkLam36 and AkLam33

Specific activity of AkLam36 toward *L. digitata* laminarin was approximately 4 times higher than that of AkLam33, i.e., AkLam36 showed the activity 172 U/mg while AkLam33 showed 39 U/mg (Table 1). These results led us to consider that the substrate preferences and/or the catalytic constants are somewhat different between AkLam36 and AkLam33. Thus, at first we investigated the substrate preferences of two enzymes using several polymer and oligomer substrates. As shown in Fig. 3, AkLam36 was found to preferably degrade polymer substrates such as laminarin and barley β-glucan compared with oligomer substrates such as laminaritetraose and laminaritriose. On the other hand, AkLam33 showed higher activities toward oligomer substrates than polymer substrates and practically no activity toward barley β-glucan. Both two enzymes could not degrade laminaribiose. Thus, AkLam36 was considered to be an enzyme that degrades internal β-1,3-linkages of polymer substrates, i.e., endolytic enzyme, while AkLam33 was considered to be an enzyme that can produce glucose from oligomer substrates more efficiently than AkLam36. Both AkLam36 and AkLam33 showed no activity toward starch, carboxymethylcellulose, agar, β-1,4-mannan, β-1,4-xylan, and alginic acid. Accordingly, these enzymes were found to be specific to β-1,3-glucosyl linkages.

Then, we estimated the kinetic parameters of AkLam36 and AkLam33 for polymer-substrate laminarin and oligomer-substrate laminaritetraose. The apparent *K*m and *k*cat of AkLam36 for laminarin was approximately 1.1 mg/ml and 23.1 s^{-1},

respectively, while those of AkLam33 were 0.12 mg/ml and 0.05 s^{-1}, respectively. On the other hand, the apparent *K*m and *k*cat of AkLam36 for laminaritetraose were approximately 1.1 mg/ml and 0.21 s^{-1}, respectively, while those of AkLam33 were 0.2 mg/ml and 4.76 s^{-1}, respectively. The apparent catalytic efficiency of laminarin degradation by AkLam36 was 21.0 $s^{-1}(mg/mL)^{-1}$, which was approximately 50 times higher than that by AkLam33, i.e., 0.42 $s^{-1}(mg/mL)^{-1}$. While the catalytic efficiency of laminaritetraose degradation by AkLam36 was 0.19 $s^{-1}(mg/mL)^{-1}$, which was approximately 125 times smaller than that by AkLam33, i.e., 23.8 $s^{-1}(mg/mL)^{-1}$. It may be meaningless to directly compare the kinetic parameters for laminarin and laminaritetraose since the activities toward laminarin and laminaritetraose were estimated by measuring the different products, i.e., total reducing sugars and glucose, respectively. However, the differences in catalytic efficiencies between two substrates obviously indicated that AkLam36 and AkLam33 preferably degrade polymer and oligomer substrates, respectively.

Next, we analyzed degradation products of laminarioligosaccharides produced by AkLam36 and AkLam33 by TLC. As shown in Fig. 4, both enzymes could degrade laminarioligosaccharides larger than laminaribiose; however, the degradation processes of the substrates by the two enzymes were appreciably different from each other. Namely, AkLam36 degraded L3－L7 producing laminaribiose and glucose along with laminaritriose as major end products, while AkLam33 degraded L3－L7 producing a series of oligosaccharides with various sizes. These results suggested that AkLam36 was an endolytic β-1,3-glucanase like the scallop and abalone β-1,3-glucanases (Kumagai et al., 2008; Kumagai and Ojima, 2009). On the other hand, AkLam33 was considered as an exolytic enzyme that stepwisely releases the terminal glucose residue from the

substrates. Therefore, we further investigated the degradation process of laminarin by these enzymes by means of gel filtration through a Superdex Peptide column. As shown in Fig. 5, AkLam36 was found to produce trisaccharide, disaccharide, and glucose as major degradation products along with various sizes of intermediate oligosaccharides. This indicates that AkLam36 is an endolytic enzyme that degrades internal β-1,3-linkages of polymer laminarin. On the other hand, AkLam33 directly produced glucose from polymer laminarin without any intermediate oligosaccharides. This strongly suggests that AkLam33 exolytically cleaved off the terminal glucose residue from laminarin. On the basis of these results, we may conclude that AkLam36 and AkLam33 were an endolytic β-1,3-glucanase (EC 3.2.1.6) and an exolytic β-1,3-glucanase (EC 3.2.1.58), respectively. The split site of laminarin by AkLam33 was suggested to be located at the reducing terminus since laminaritriosylxylose was produced from laminaritetraose and xylose by this enzyme via transglycosylation reaction (data not shown, but detailed results will be published elsewhere).

3.4. Degradation of laminaribiose by AkLam36 and AkLam33 via transglycosylation

Recently, we reported that the endolytic β-1,3-glucanase, HdLam33, from the pacific abalone catalyzed transglycosylation reaction and degraded laminaribiose via transglycosylation with laminaritriose (Kumagai and Ojima, 2009). In the present study, AkLam36 and AkLam33 were also considered to possess transglycosylation activity because oligosaccharides larger than the original substrates, which were presumed to be the transglycosylation products, appeared during degradation reaction (Fig. 4). These products became prominent at reaction time 0.5 – 2 h for AkLam36 with L3 substrate

while at 2–8 h for AkLam33 with L5 substrate. Then, we examined whether or not AkLam36 and AkLam33 can degrade laminaribiose through the transglycosylation with laminaritriose. As shown in Fig. 6A, both AkLam36 and AkLam33 did not degrade laminaribiose and produced no glucose. On the other hand, AkLam36 and AkLam33 could degrade 10 mM laminaritriose and produced approximately 10 mM and 15 mM glucose, respectively. Therefore, AkLam36 and AkLam33 were considered to be capable of degrading 10 mM laminaritriose producing 10 mM glucose and laminaribiose. In addition, AkLam33 was considered to have produced additional 5 mM glucose, i.e., 15 mM glucose comprising 10 mM glucose and additional 5 mM glucose appeared to be produced. This additional glucose was considered to be derived from the degradation of laminaritetraose or laminaritriose which would be produced by the transglycosylation between the original substrate laminaritriose (a donor substrate) and a hydrolytic product laminaribiose (an acceptor substrate) (see Fig. 6B). Then, we added 30 mM laminaribiose along with 10 mM laminaritriose to the reaction mixture. As a result, approximately 15 mM and 40 mM glucose were produced by AkLam36 and AkLam33, respectively (Fig. 6A). These results indicate that AkLam36 and AkLam33 can degrade a part of laminaribiose to glucose through the transglycosylation reaction with laminaritriose. The mechanism for the degradation of laminaribiose after the formation of laminaritetraose by transglycosylation reaction is schematically represented in Fig. 6B.

3.5. N-terminal amino-acid sequences of AkLam36 and AkLam33

The N-terminal amino-acid sequences of AkLam36 and AkLam33 were determined

as GAVIFRDDFNSFGH (14 residues) and GAVIFRDDFNSFDANKWNYEVSMY (24 residues), respectively. These sequences are quite similar to each other and indicate that AkLam36 and AkLam33 are closely related isoenzymes. These amino-acid sequences of AkLam36 and AkLam33 showed 71% and 57% identity to the 121-134 and 121-146 residues, respectively, of a GHF16 β-1,3-glucanase from *Littorina sitkana* (GenBank Accession No. CAN22491). The amino-acid sequence of a lysylendopeptidyl fragment of AkLam36, YGHWPKSGEIDIM, showed 77% identity to the 178-190 residues of the abalone GHF16 β-1,3-glucanase HdLam33 (GenBank Accession No. AB488493). The amino-acid sequences of two lysylendopeptidyl fragments of AkLam33 named 33L1 and 33L2 were determined as RGQLYIKPTFT and FGTVEVRAFIPK, respectively. 33L1 showed 82% identity to the 58-68 residues of scallop GHF16 β-1,3-glucanase (GenBank Accession No. AAW34372) while 33L2 showed 83% identity to the 235-246 residues of the *L. sitkana* enzyme. These sequence identities indicate that AkLam33 and AkLam36 also belong to GHF16.

4. Discussion

4.1. General properties of AkLam36 and AkLam33

In the present study, we succeeded to isolate two β-1,3-glucanases, AkLam36 and AkLam33, from the digestive fluid of the common sea hare *A. kurodai* by ammonium sulfate fractionation followed by conventional column chromatography. Optimal temperature and pH of AkLam36 were 40^{o}C and 6.0, respectively, and the temperature that caused a half inactivation during 15-min incubation was approximately 42^{o}C. On

the other hand, AkLam33 showed the optimal temperature and pH at 50°C and 5.7, respectively, and the temperature that caused a half inactivation of AkLam33 was 45°C. Kinetic parameters of the two enzymes for laminarin and laminaritetraose indicated that AkLam36 preferably acts on polymer substrates while AkLam33 acts on oligomer substrates. By TLC and gel-filtration analyses for the degradation products of laminarin and laminarioligosaccharides, AkLam36 was regarded as an endolytic β-1,3-glucanase (EC 3.2.1.6) while AkLam33 was regarded as an exolytic β-1,3-glucanase (EC 3.2.1.58). Both enzymes possessed fairly high transglycosylation activity and could degrade laminaribiose after the formation of laminaritetraose by the transglycosylation with laminaritriose (Fig. 6 A and B).

Amino-acid sequence analysis indicated that both AkLam36 and AkLam33 are classified to GHF16 like other molluscan β-1,3-glucanases. AkLam33 appeared to be the first exolytic β-1,3-glucanase from marine invertebrates since all β-1,3-glucanases so far purified from marine invertebrates have been characterized as endolytic enzymes (Sova et al., 1970; Lépagnol-Descamps et al., 1998; Kovalchuk et al., 2006; Kumagai et al., 2008; Pesentseva et al., 2008; Zhu et al., 2008; Kumagai and Ojima, 2009; Kovalchuk et al., 2009). Besides AkLam33, exolytic β-1,3-glucanases have been reported on bacteria, yeast, insect, and a terrestrial snail (Marshall and Grand, 1975; Sánchez et al., 1982; Kulminskaya et al., 2001; Hrmova and Fincher, 1998; Igarashi et al., 2003; Genta et al., 2007); however, these enzymes were classified under GHF 3, 5, 17, and 55 (Klebl and Tanner, 1989; Chambers et al., 1993; Hrmova et al., 1996; Sakamoto et al., 2005; Ishida et al., 2009; Ooi et al., 2009). Therefore, AkLam33 appeared to be the first exolytic β-1,3-glucanase that belongs to GHF16.

4.2. Physiological significance of AkLam33 and AkLam36

Endolytic β-1,3-glucanases from marine invertebrates have been reported on sea cucumber, surf clam, scallop, and abalone (Sova et al., 1970; Kovalchuk et al., 2006; Zhu et al., 2008; Kumagai et al., 2008; Kumagai et al., 2009; Kumagai and Ojima, 2009; Kovalchuk et al., 2009). These enzymes seem to act as the digestive enzymes that degrade β-1,3-glucans in dietary algae providing laminarioligosaccharides. These oligosaccharides are usually further degraded to glucose by β-glucosidases contained in the digestive tracts.

In case of the abalone endolytic β-1,3-glucanase HdLam33, the glucose yield from laminarioligosaccharides was suggested to be greatly improved by the transglycosylation reaction (Kumagai and Ojima, 2009). In case of sea hare, endolytic enzyme AkLam36 moderately improved glucose yield by the transglycosylation (Fig. 6A), while the exolytic enzyme AkLam33 significantly improved glucose production. However, it is still unclear if the transglycosylation-catalyzing glucose production in the molluscan β-1,3-glucanases is a physiological event in molluscan digestive fluid because the transglycosylation reaction requires high concentration of laminaribiose, and the laminaribiose produced from laminarin will be readily degraded by β-glucosidases in digestive tissues. In order to evaluate the physiological significance of the transglycosylation reaction by the molluscan β-1,3-glucanases, the reaction efficiency should be investigated more precisely in the conditions similar to those in the digestive fluids.

AkLam36 showed less activity toward *E. bicyclis* laminarin compared with *L.digitata* laminarin, while AkLam33 showed fairly high activity toward both *E.*

bicyclis and *L. digitata* laminarins (Fig. 3). *E. bicyclis* laminarin contains β-1,6-linked branches in considerably higher frequency than *L. digitata* laminarin. Coexistence of AkLam36 and AkLam33 may be profitable to efficiently assimilate laminarins with different higher order structures from wide variety of seaweeds.

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Figure Legends

Fig. 1. SDS-PAGE of AkLam33 and AkLam36. M, marker; lane 1, AkLam33; lane 2, AkLam36.

Fig. 2. Temperature dependence, pH dependence, and thermostability of AkLam36 and AkLam33. (A) Temperature dependence was measured at 5 – 60^{o}C in a reaction mixture containing 0.2% laminarin and 10 mM sodium phosphate buffer (pH 6.0). (B) pH dependence was measured at 30^{o}C in reaction mixtures adjusted to pH 2.3 – 6.0 with 50 mM sodium citrate buffer and pH 6.0 – 9.8 with 50 mM sodium phosphate buffer. The activity levels were slightly different between the two buffers (approximately 10% at pH 6.0). So, we normalized the activity levels by regarding that the two activity values at pH 6.0 are same. (C) Thermostability was assessed by measuring the activity remaining after the heat-treatment at 4 – 60^{o}C for 15 min. ○, AkLam36; ●, AkLam33. All assays were performed in triplicate and the mean values are shown in the figures.

Fig. 3. Substrate specificity of AkLam36 and AkLam33. The specific activity toward polymer substrates was determined by measuring the reducing sugars produced by the reaction, while the activity toward oligomer substrates was determined by measuring the amount of glucose produced by the reaction. □, AkLam36; ■, AkLam33. The molar ratios for β-1,3-linkages to β-1,6-linkages or β-1,4-linkages are approximately 7:1 for *L. digitata* laminarin, 3:2 for *E. bicyclis* laminarin, and 1:2.3 – 2.7 for barley β-glucan (β-1,3;1,4-glucan). n.d, not detectable. All measurements were performed in triplicate and the activity values are shown as the mean values ± S.D.

Fig. 4. Thin-layer chromatography for degradation products of laminarioligosaccharides produced by AkLam33 and AkLam36. (A) Degradation by AkLam36. Five mg/mL of laminarioligosaccharides (laminaribiose – laminariheptaose, abbreviated as L2 – L7) in 10 mM sodium phosphate buffer (pH 6.0) were degraded by 0.01 U/mL of AkLam36 at 30^{o}C. The reaction was terminated at 100^{o}C for 3 min and 1 ml of the reaction mixture was applied to the TLC plates. (B) Degradation by AkLam33. 5 mg/ml of laminarioligosaccharide (L2 – L7) were degraded by 0.01 U/ml of AkLam33. Other conditions were same as in (A). M, marker; G1, glucose; L2, laminaribiose; L3, laminaritriose; L4, laminaritetraose; L5, laminaripentaose; L6, laminarihexaose; L7, laminariheptaose.

Fig. 5. Gel-filtration analysis for degradation products of laminarin. (A) 0.2% *L. digitata* laminarin was degraded by 0.01 U/mL of AkLam36 at 30^{o}C, and the degradation products obtained at reaction time 1 h and 12 h were subjected to gel filtration through a Superdex Peptide 10/300GL column. Elution of sugars was detected by a Shimadzu RID-10A detector. (B) 0.2% *L.digitata* laminarin was degraded by AkLam33 as in (A). G, glucose; L2, laminaribiose; L3, laminaritriose.

Fig. 6. Production of glucose from laminarioligosaccharides by transglycosylation activities of AkLam33 and AkLam36. (A) The reaction was carried out in the reaction mixture containing of 10 mM laminaritriose alone (○, ●), 30 mM laminaribiose alone (□, X), 10 mM laminaritriose plus 30 mM laminaribiose (△, ▲), 0.01 U/mL of AkLam36 (○, □, △) or AkLam33 (●, X, ▲) and 10 mM sodium phosphate buffer

(pH 6.0) at 30^{o}C. (B) Schematic representation for degradation of laminaribiose via transglycosylation. Open and gray circles represent the glucose residues of laminaritriose and laminaribiose, respectively. The cleavage sites (transglycosylation sites) by the enzymes were indicated by the reverse triangles. Glucose released by the reaction was shown by “G”.

Table 1. Summary for the purification of AkLam33 and AkLam36

Samples	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
Crude[*1]	470	1.4	642	1	100
AS[*2]	180	2.9	517	2	80
CM[*3]	21	8.6	182	6.3	28
AkLam33					
Mono-S (Fr1)[*4]	0.21	4.8	1	7	0.2
Mono-S[*5]	0.01	38	0.4	28	0.1
AkLam36					
Mono-S (Fr2)[*4]	1.1	80	84	58	13
Superdex[*6]	0.2	129	26	94	4.1
Mono-S[*7]	0.1	172	16.5	126	2.5

[*1]Crude enzyme after the dialysis against 10 mM sodium phosphate (pH 6.0) [*2]Fraction precipitated between 40 and 60% saturation of ammonium sulfate. [*3]Active fraction obtained by TOYOPEARL CM-650M chromatography. [*4]Active fractions 1 (Fr1) and 2 (Fr2) obtained by Mono-S chromatography. [*5]AkLam33 purified by the second Mono-S chromatography. [*6]Active fraction obtained by Superdex 75 chromatography. [*7]AkLam36 purified by Mono-S chromatography

Fig. 1

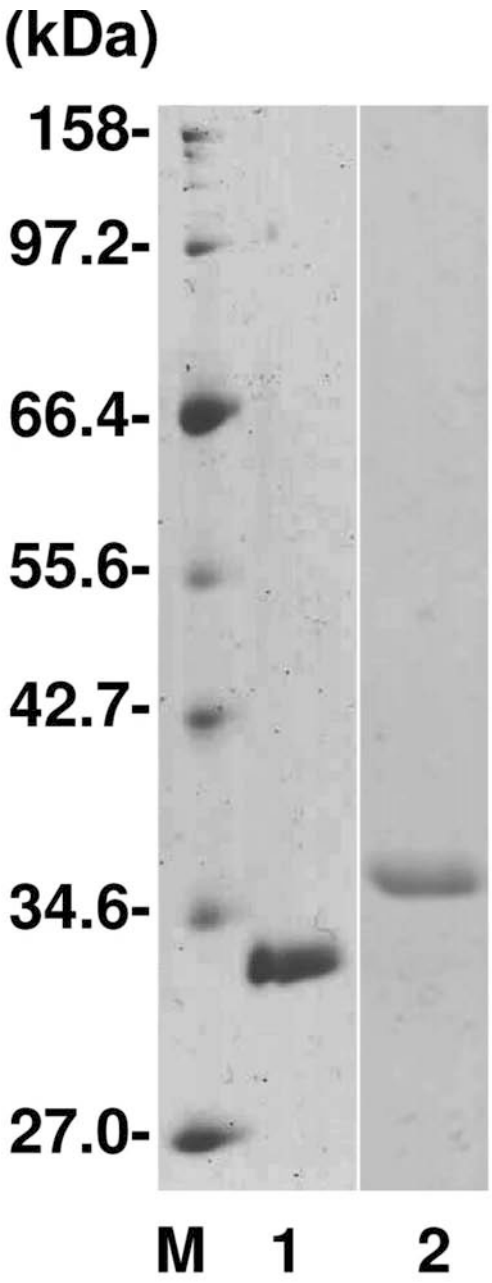


Fig. 2

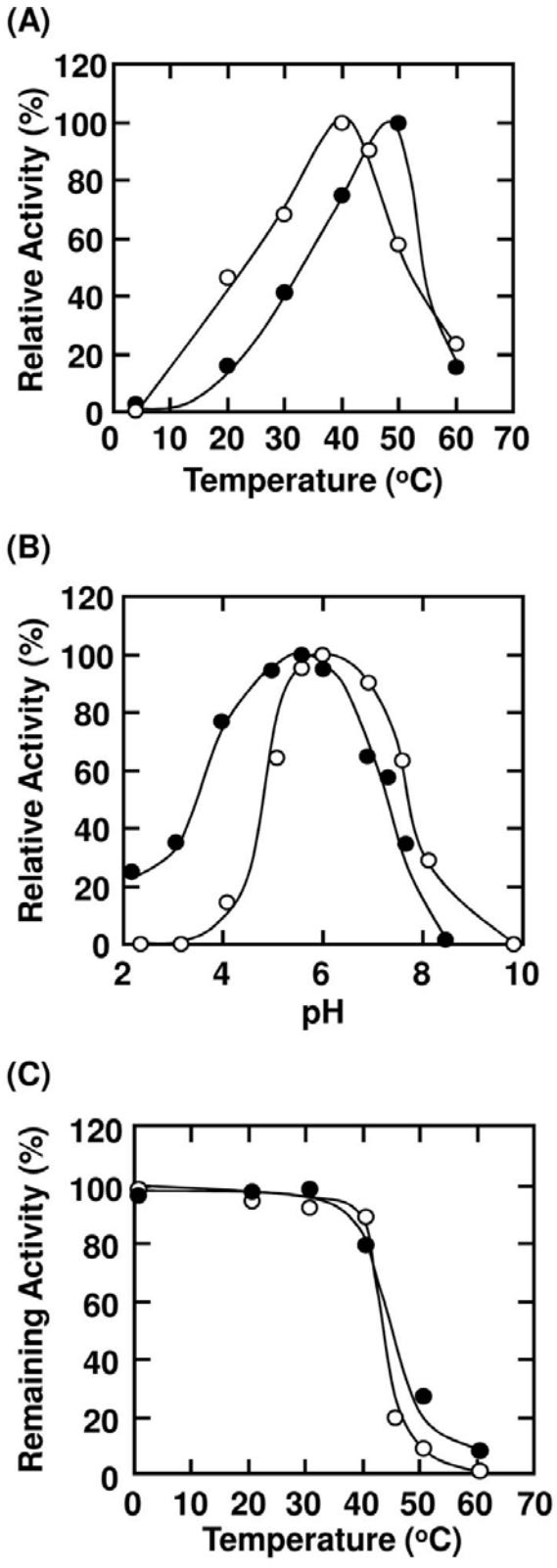

(A)
Relative Activity (%)
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70
Temperature (°C)
(B)
Relative Activity (%)
120
100
80
60
40
20
0
2 4 6 8 10
pH
(C)
Remaining Activity (%)
120
100
80
60
40
20
0
0 10 20 30 40 50 60 70
Temperature (°C)

Fig. 3

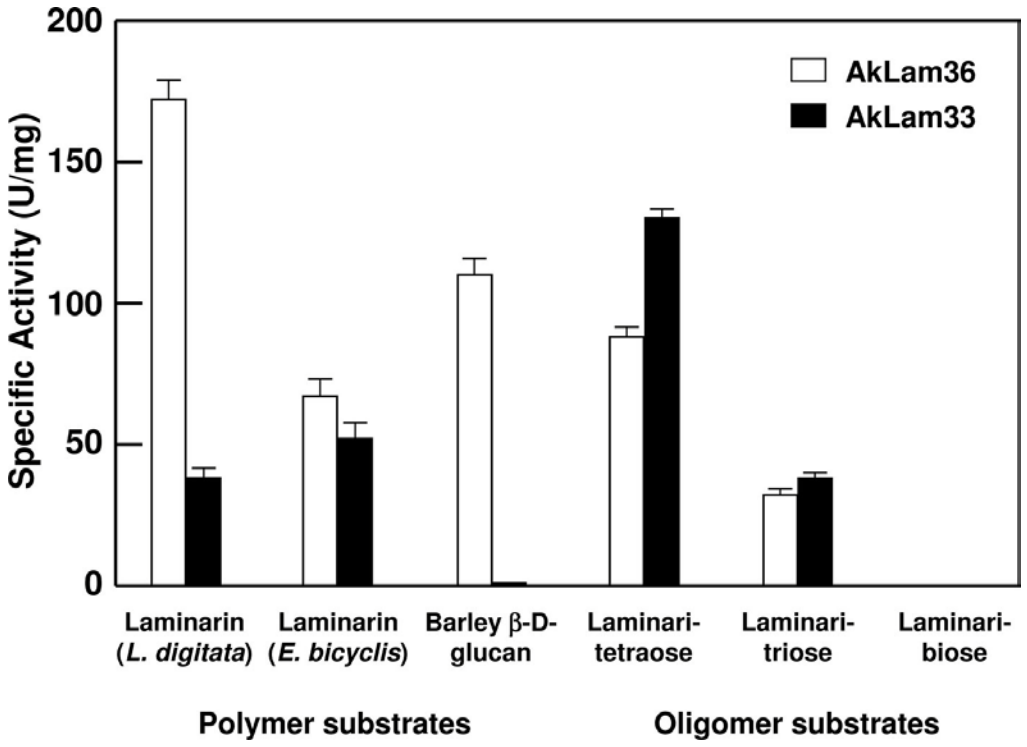

200
150
100
50
0
Specific Activity (U/mg)
AkLam36
AkLam33
Laminarin (L. digitata)
Laminarin (E. bicyclis)
Barley β-D-glucan
Laminari-tetraose
Laminari-triose
Laminari-biose
Polymer substrates
Oligomer substrates

Fig. 4

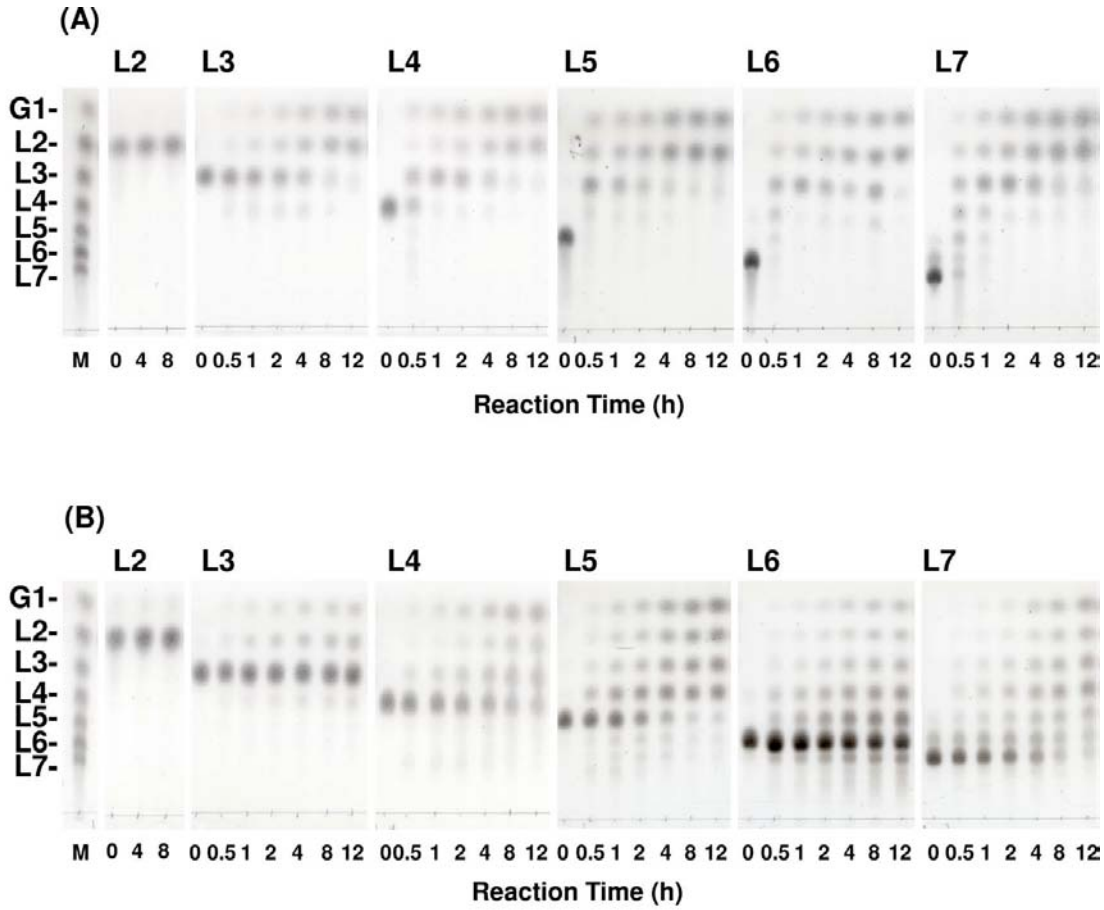

(A)
L2
L3
L4
L5
L6
L7
G1-
L2-
L3-
L4-
L5-
L6-
L7-
M 0 4 8 0 0.5 1 2 4 8 12 0 0.5 1 2 4 8 12 0 0.5 1 2 4 8 12 0 0.5 1 2 4 8 12 0 0.5 1 2 4 8 12
Reaction Time (h)
(B)
L2
L3
L4
L5
L6
L7
G1-
L2-
L3-
L4-
L5-
L6-
L7-
M 0 4 8 0 0.5 1 2 4 8 12 0 0.5 1 2 4 8 12 0 0.5 1 2 4 8 12 0 0.5 1 2 4 8 12 0 0.5 1 2 4 8 12
Reaction Time (h)

Fig. 5

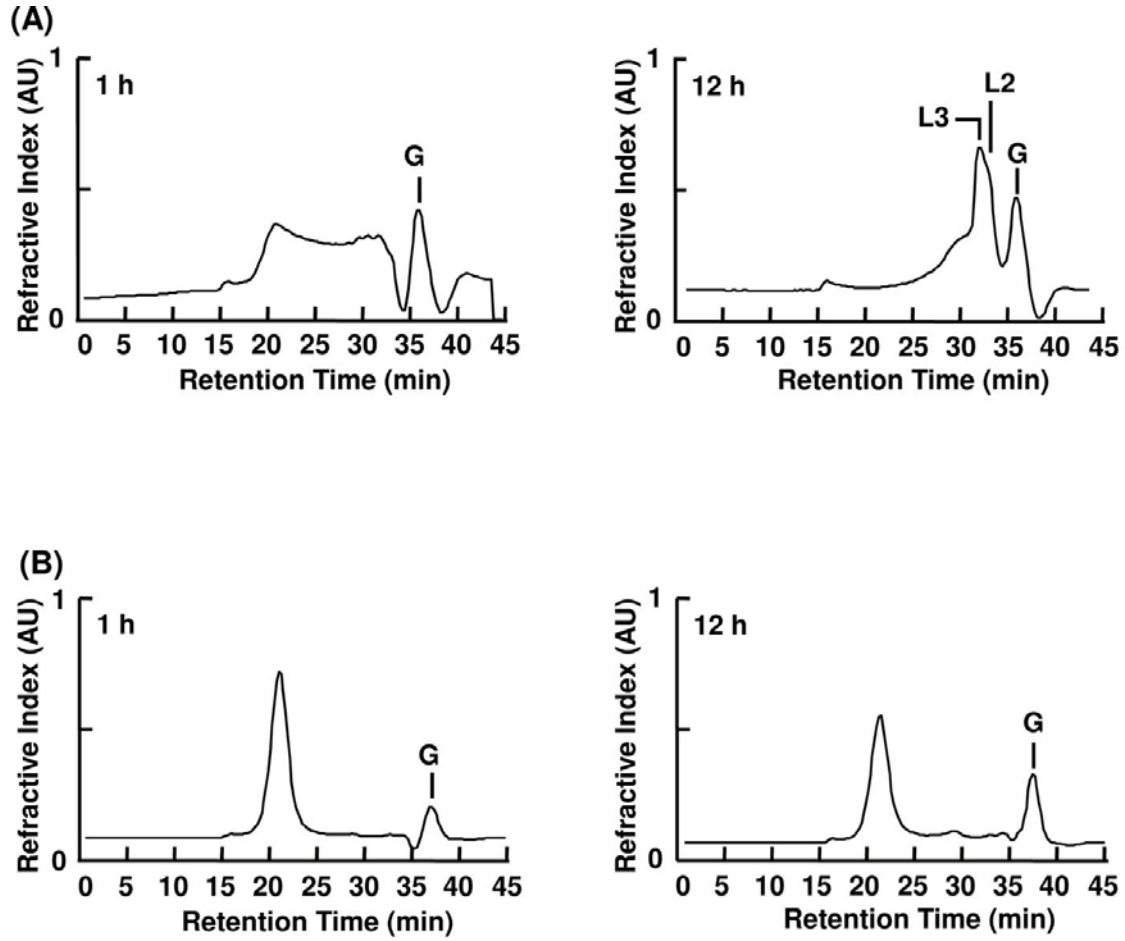
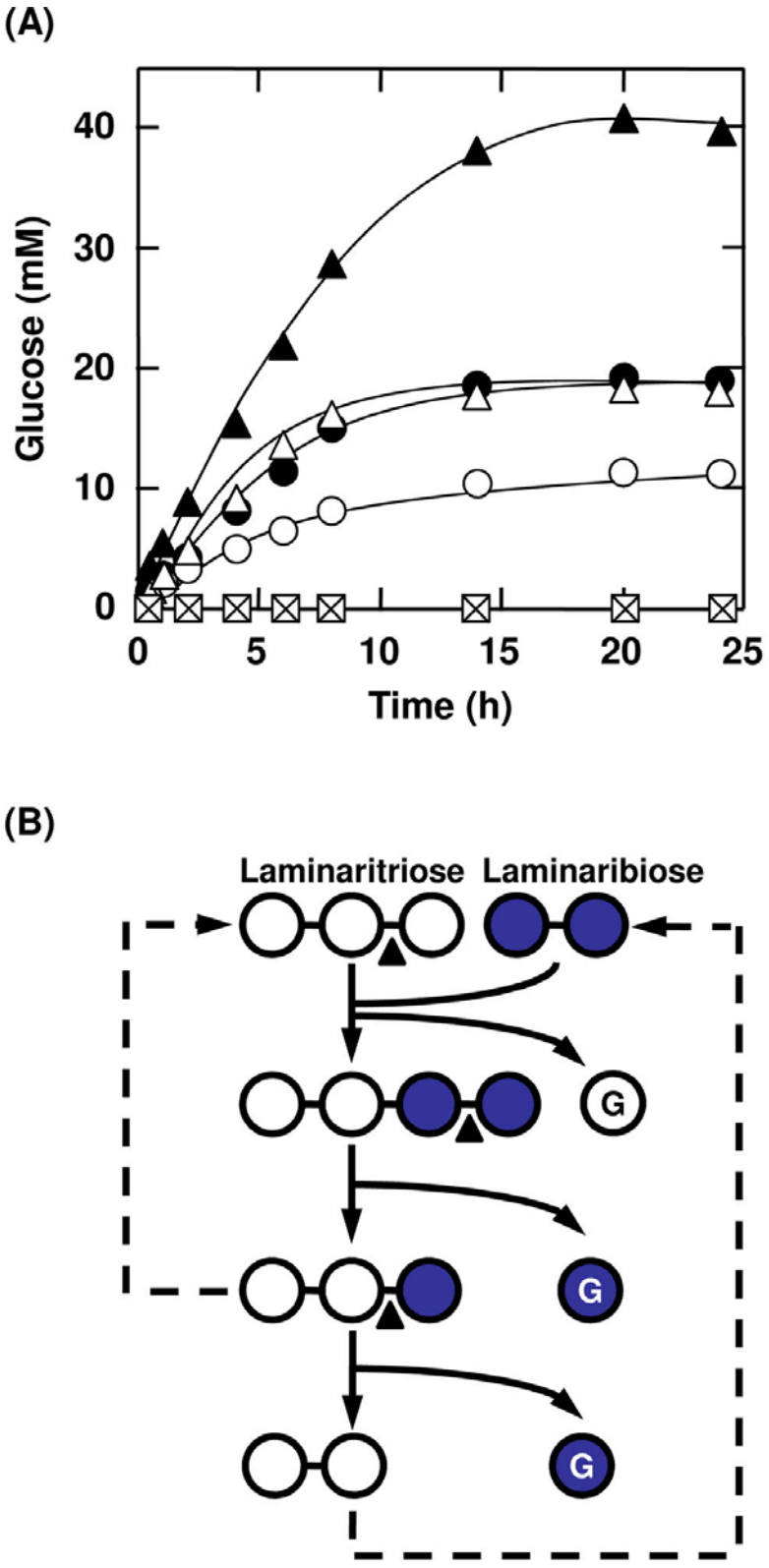

(A)
1 h
G
Refractive Index (AU)
1
0
0 5 10 15 20 25 30 35 40 45
Retention Time (min)
12 h
L3
L2
G
Refractive Index (AU)
1
0
0 5 10 15 20 25 30 35 40 45
Retention Time (min)
(B)
1 h
G
Refractive Index (AU)
1
0
0 5 10 15 20 25 30 35 40 45
Retention Time (min)
12 h
G
Refractive Index (AU)
1
0
0 5 10 15 20 25 30 35 40 45
Retention Time (min)

Fig. 6


(A)
Glucose (mM)
0
10
20
30
40
0
5
10
15
20
25
Time (h)
(B)
Laminaritriose
Laminaribiose
G
G
G