



HOKKAIDO UNIVERSITY

Title	A laminaribiose-hydrolyzing enzyme, AkLab, from the common sea hare <i>Aplysia kurodai</i> and its transglycosylation activity
Author(s)	Kumagai, Yuya; Satoh, Takuya; Inoue, Akira et al.
Citation	Comparative biochemistry and physiology b-biochemistry & molecular biology, 167, 1-7 https://doi.org/10.1016/j.cbpb.2013.07.008
Issue Date	2014-01
Doc URL	https://hdl.handle.net/2115/55292
Type	journal article
File Information	AkLab_Text(rev)HUSCUP.pdf



1 A laminaribiose-hydrolyzing enzyme, AkLab, from the common sea hare *Aplysia*
2 *kurodai* and its transglycosylation activity

3
4 Yuya Kumagai, Takuya Satoh, Akira Inoue and Takao Ojima*

5
6 Laboratory of Marine Biotechnology and Microbiology, Graduate School of Fisheries
7 Sciences, Hokkaido University, Minato-cho 3-1-1, Hakodate 041-8611, Japan

8
9 *Corresponding author: Tel: +81 138 40 8800; fax: +81 138 40 8800.

10 *E-mail address:* ojima@fish.hokudai.ac.jp (T. Ojima)

Abstract

Endo- β -1,3-glucanases (laminarinase, EC 3.2.1.6) from marine molluscs specifically degraded laminarin from brown algae producing laminaribiose and glucose, but hardly degraded laminaribiose. For the complete depolymerization of laminarin, other enzymes that can hydrolyze laminaribiose appeared to be necessary. In the present study, we successfully isolated a laminaribiose-hydrolyzing enzyme from the digestive fluid of a marine gastropod *Aplysia kurodai* by ammonium sulfate fractionation followed by conventional column chromatographies. This enzyme, AkLab, named after the scientific name of this animal and substrate specificity toward laminaribiose, showed an approximate molecular mass of 110 kDa on SDS-PAGE, and optimum pH and temperature at around pH 5.5 and 50°C, respectively. AkLab rapidly hydrolyzed laminaribiose and *p*-nitrophenyl- β -D-glucoside, and slowly cellobiose, gentiobiose and lactose, but not sucrose and maltose. AkLab showed high transglycosylation activity and could produce a series of laminarioligosaccharides larger than laminaritetraose from laminaribiose (a donor substrate) and laminaritriose (an acceptor substrate). This enzyme was suggested to be a member of glycosyl hydrolase family 1 by the analysis for partial amino-acid sequences.

Key words: gastropod; sea hare; *Aplysia*; laminaribiose; β -glucosidase; GHF1.

1. Introduction

β -1,3-Glucanase (EC 3.2.1.6) is an enzyme that hydrolyzes β -1,3-glucosyl linkage of β -glucans producing β -1,3-glucooligosaccharides and glucose. This enzyme distributes over fungi, bacteria, higher plants, and marine mollusks (Sova et al., 1970; Erfle et al., 1988; Tangarone et al., 1989; Hrmova and Fincher, 1993; Mrsa et al., 1993; Miyanishi et al., 2003a; Ueda et al., 2011; Aires et al., 2012). Their physiological roles are diverse and vary depending on their origins. For example, this enzyme intimately relates to seed germination and antifungal activity in some plants (Emst et al., 1992; Leubner-Metzger et al., 1995). While it plays important roles for cell division and morphogenesis in fungi (Esteban et al., 2005; Gastebois et al., 2013). On the other hand, marine molluscan enzymes in digestive fluid play an important role for saccharification of laminarin and chrysolaminarin from their dietary algae (Sova et al., 1970; Lépagnol-Descamps et al., 1998). Accordingly, the molluscan enzymes are generally called laminarinase. Higher order structure of algal laminarin is known to vary depending on algal sources (Black et al., 1951; Størseth et al., 2005; Smelcerovic et al., 2008). Concomitantly, properties of molluscan enzymes also seemed to vary depending

on the laminarin structures from dietary algae (Kovalchuk et al., 2006; Kumagai and Ojima, 2010). In this respect, molluscan laminarinases appears to be useful materials for comparative studies on β -1,3-glucanases.

Molluscan laminarinases have been studied using abalone, scallop, surf clam, and sea hare (Lépagnol-Descamps et al., 1998; Kozhemyako et al., 2004; Kovalchuk et al., 2006; Kumagai et al., 2008; Kumagai and Ojima, 2009; Kumagai and Ojima, 2010; Pesentseva et al., 2012; Zakharenko et al., 2012). These enzymes hydrolyzed laminarin by an endolytic process producing laminaribiose and glucose as major end products. Although these molluscan enzymes hardly hydrolyzed laminaribiose as a sole substrate, they could produce glucose from the mixture of laminaribiose and laminaritetraose via transglycosylation (Kumagai and Ojima 2009; Kumagai and Ojima 2010). In this reaction, laminaribiose and laminaritetraose were used as acceptor substrate and donor substrate, respectively. However, this reaction seemed to be an *in vitro* one since the transglycosylation reaction required much higher concentration of donor and acceptor substrates (10 – 50 mM) than those in the digestive fluid (less than 1 mM). Therefore, laminaribiose in the digestive fluid of mollusks should be degraded by some other ‘laminaribiose-hydrolyzing’ enzymes which have been unidentified yet. One candidate for this enzyme is β -glucosidase (Perez-Pons et al., 1994; Opassiri et al., 2004; Sanchez

C., 2009) and another is laminaribiose-specific hydrolase (laminaribiase, EC 3.2.1.21). To date, only one β -D-glucosidase that can degrade laminaribiose has been isolated from *Littorina kurila* (Pesentseva et al., 2012). To enrich information on degradation of laminaribiose in mollusks, it seems necessary to investigate laminaribiose-hydrolyzing enzymes using as many mollusks as possible. In addition, such enzymes are attractive from the viewpoint of practical applications since they possibly catalyze transglycosylation reaction that is available for the synthesis of artificial heterooligosaccharides with beneficial functions (Mackenzie et al., 1998).

To date, the authors have been studying on the polysaccharide-degrading enzymes from marine mollusks to understand the assimilation processes for algal polysaccharides as carbon and energy sources in mollusks (Suzuki et al., 2003; Shimizu et al., 2003; Ootsuka et al., 2006; Kumagai et al., 2008, 2009, 2010 and 2013; Rahman et al., 2010; Zahura et al., 2010). In the present study, we focused on a laminaribiose-hydrolyzing enzyme from the common sea hare *Aplysia kurodai*. This enzyme showed significantly high laminaribiose-hydrolyzing activity and also transglycosylation activity. Analysis for partial amino-acid sequences indicated that this enzyme belongs to glycosyl hydrolase family 1.

2. Materials and methods

2.1. Materials

The common sea hare *A. kurodai* (body length, approximately 12 cm) was collected on the shore of Hakodate, Hokkaido Prefecture, Japan, in July 2011. Digestive fluid was harvested from the gastric lumen of 20 specimens. The digestive fluid (approximately 100 mL) was dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and centrifuged at $10,000\times g$ for 10 min to remove insoluble materials. The clear supernatant with brownish color (crude enzyme) was used for the isolation of laminaribiose-hydrolyzing enzyme(s). Laminarin (from *Laminaria digitata*) and gentiobiose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Laminarioligosaccharides (laminaribiose – laminariheptaose, abbreviated to L2 – L7), cellobiose, *p*-Nitrophenyl β -D-glucopyranoside (β -*p*NPG) and other β -*p*NP derivatives (D-galactoside, D-mannoside, D-fucoside, D-N-acetyl glucosaminide and D-xyloside) were purchased from Seikagaku Kogyo (Tokyo, Japan). Sucrose and maltose were purchased from Kanto Kagaku (Tokyo, Japan). Lactose was purchased from Wako Pure Chemical Industries (Osaka, Japan). TOYOPEARL Phenyl-650M, TOYOPEARL

DEAE-650M and TOYOPEARL HW50F were from Toyo Soda Mfg. Co. (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Industries. Superdex 200 10/300 GL was purchased from GE Healthcare Life Sciences (Uppsala, Sweden).

2.2. Purification of laminaribiose-hydrolyzing enzyme

Laminaribiose-hydrolyzing enzyme was purified as follows. The crude enzyme (approximately 100 mL from 20 animals) from *A. kurodai* was subjected to ammonium sulfate fractionation and the precipitates formed between 40 – 60% saturation of ammonium sulfate were collected by centrifugation at $10,000 \times g$ for 10 min. The precipitates were dissolved in and dialyzed against 10 mM sodium phosphate buffer (pH 6.0) and centrifuged at $10,000 \times g$ for 10 min to remove insoluble materials. The supernatant was then applied to a TOYOPEARL Phenyl-650M column (2×30 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0) containing 40% saturated ammonium sulfate. Un-adsorbed proteins were washed out with the same buffer, and then proteins adsorbed to the column were eluted stepwisely with the buffer containing 40%, 30%, 20%, 10%, and 0% saturated ammonium sulfate. By this chromatography, laminaribiose-hydrolyzing enzyme was eluted in the 10%-saturated

ammonium sulfate fractions. These fractions were pooled, and dialyzed against 10 mM sodium phosphate buffer (pH 7.0), and then subjected to a TOYOPEARL DEAE-650M column (2×45 cm) pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 300 mM (Fig. 1A). Laminaribiose-hydrolyzing enzyme was eluted at around 100 mM NaCl. The fractions were pooled and concentrated with VIVASPIN 20 (Sartorius AG, Goettingen, Germany) and subjected to AKTA FPLC (GE Healthcare Life Science) equipped with a Superdex 200 10/300GL column, and the proteins were eluted with 300 mM NaCl – 10 mM sodium phosphate (pH 6.0) (Fig. 1B). The active fraction in this chromatography showed a single band with a molecular mass of approximately 110 kDa on SDS-PAGE (Fig. 1C).

2.3. Assay for hydrolyzing activity of enzyme

The laminaribiose-hydrolyzing activity was assayed at 30°C in a reaction mixture containing 5 mg/mL of laminaribiose (L2), 0.5 – 1.0 m unit (U)/mL of enzyme and 10 mM sodium phosphate buffer (pH 5.5). The amount of glucose released from L2 was determined by the glucose CII-test kit Wako (Wako Pure Chemical Industries). One unit

of laminaribiose-hydrolyzing activity was defined as the amount of enzyme that liberates 2.0 μmol glucoses per min since two moles of glucose are produced from one mole of laminaribiose. If laminaritriose or larger substrates were used as substrates, one unit of activity was defined as the amount enzyme that liberates 1.0 μmol glucoses per min. Hydrolyzing activity for the β -*p*NP-derivatives was assayed in a reaction mixture containing 2.5 mM *p*NP-derivatives and 10 mM sodium phosphate (pH 5.5) and 0.5 – 1.0 mU/mL of enzyme at 30°C. *p*-Nitrophenol (*p*NP) released by the degradation of β -*p*NP derivatives was determined with the molar extinction coefficient $1.81 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 410 nm. One unit of β -*p*NP derivative-degrading activity was defined as the amount of enzyme that released 1.0 μmol *p*NP per min. pH dependence of the enzyme was measured at 30°C in reaction mixtures containing 5 mg/ml laminaribiose, 50 mM sodium citrate buffer (pH 3.5 – 6.0) and 50 mM sodium phosphate buffer (pH 5.5 – 8.1). Temperature dependence was measured at 4 – 70°C in a reaction mixture containing 10 mM sodium phosphate buffer (pH 5.5). Thermal stability of the enzyme was assessed by measuring the activity remaining after the heat-treatment at 4 – 70°C for 15 min. All assays were triplicated and the data were indicated as average values with standard deviations.

2.4. Assay for transglycosylation activity of enzyme

Transglycosylation activity of laminaribiose-hydrolyzing enzyme was assayed at 30°C in a reaction mixture containing 36.5 mM L2 (a donor substrate), 12 mM L3 (an acceptor substrate), 10 mU of enzyme and 10 mM sodium phosphate buffer (pH 5.5). The transglycosylation reaction was terminated by the addition of an equal volume of 10% trichloroacetic acid. The reaction products were analyzed by thin-layer chromatography (TLC).

2.5. TLC

Degradation products of laminarioligosaccharides (L2 – L7) were analyzed by TLC. Substrate laminarioligosaccharides (5 mg/mL) were degraded with 4.4 mU/mL of enzyme, and the degradation products were subjected to a TLC-60 plate (Merck, Darmstadt, Germany). The degradation products were developed with a solvent comprising ethyl acetate, acetic acid and water (2:2:1 (v:v:v)) and visualized by heating the plate at 130°C for 10 min after spraying 10% (v/v) sulfuric acid in ethanol.

2.6. SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 0.1% (w/v) SDS – 10 % (w/v) polyacryl-amide slab gel (1 mm thick, 10 cm × 9 cm (width × 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.

2.7. Determination of partial amino-acid sequences

The N-terminal amino-acid sequence of laminaribiose-hydrolyzing enzyme was determined with an ABI Procise 492 sequencer (Applied Biosystems, Foster City, CA, USA). Internal amino-acid sequences of the enzyme were determined with the peptide fragments prepared by lysylendopeptidase digestion at 37°C for 2 h (lysylendopeptidase/protein = 1/200 (w/w)). The fragments were separated by

SDS-PAGE and blotted to a polyvinylidene difluoride membrane. The fragments well separated on the membrane were excised with a scissors and subjected to the sequencer.

2.8. Determination of protein concentration

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

3. Results

3.1. General properties of laminaribiose-hydrolyzing enzyme

By the procedure described under the ‘Materials and methods’, a laminaribiose-degrading enzyme with the molecular mass of approximately 110 kDa was purified 110-fold at a yield of 5.1% and the specific activity 3.4 U/mg (Table 1). We named this enzyme AkLab after the scientific name of the animal and its laminaribiose-hydrolyzing activity. Optimum temperature and pH of AkLab were 50°C and pH 5.5, respectively (Fig. 2A and 2B). The temperature that caused a half

inactivation during 15-min incubation was at around 48°C (Fig. 2C).

3. 2. Substrate specificity of AkLab

Degradation products of L2 – L7 produced by AkLab were analyzed by TLC. As shown in Fig. 3, AkLab was capable of hydrolyzing all the laminarioligosaccharides tested and produced glucose (Transglycosylation products are also seen. This will be mentioned in the next section). Then, hydrolytic activities of AkLab for laminarioligosaccharides were determined by measuring glucose-releasing rates (Fig. 4 and Table 2). The activity toward L2 was significantly high, i.e., it was approximately 20-times higher than those toward other laminarioligosaccharides. AkLab slowly degraded cellobiose (β -1,4-glucoside), gentiobiose (β -1,6-glucoside) and lactose (β -1,4-galactoside), but not α -glycosides like maltose and sucrose (Table 3). The activities toward cellobiose and gentiobiose were 1/3 – 1/5 of the activity toward laminaribiose (Table 3). AkLab showed high activity toward *p*NP β -D-glucoside and low activity toward *p*NP β -D-galactoside, but practically no activity toward other β -*p*NP derivatives tested (Table 4). AkLab could not degrade polymer substrate laminarin (data not shown). From these results, we concluded that AkLab is a β -glucosidase-like

enzyme with high preference to laminaribiose.

3. 3. *Transglycosylation activity of AkLab*

As shown in Fig. 3A, substantial amounts of oligosaccharides larger than original substrate L2 were found to be produced during hydrolysis of L2. This strongly suggested that AkLab catalyzed transglycosylation among L2 molecules and produced such larger oligosaccharides. We then examined how large oligosaccharides can be produced by the transglycosylation activity of AkLab using L2 and L3 as a donor and an acceptor substrate since L2 is preferable substrates of AkLab and L2 and L3 are distinguishable in TLC. As shown in Fig. 5, L4 was readily produced in the early stage of reaction (0.3 – 1.0 h). Upon extension of reaction time to 4 – 24 h, oligosaccharides L4 – L7 appeared. During the reaction, glucose was produced concomitantly with the rapid decrease in L2 and moderate decrease in L3. These results strongly suggested that AkLab transferred glucose unit of L2 to L3 by the transglycosylation activity and produced glucose and oligosaccharides larger than L3. Interestingly, the oligosaccharides thus produced were considerably stable, i.e., they accumulated in the latter phase of reaction. This result can be interpreted by the substrate specificity of AkLab that hydrolyzes laminaribiose in much higher rate than any other

oligosaccharides (Fig. 4 and Table 2). It is also possible to consider that the transglycosylation products were not β -1,3-linked oligosaccharides but other isomers with different glycosyl linkages which AkLab hardly hydrolyzes although their structures were not analyzed in the present study.

3. 4. Partial amino-acid sequence of AkLab

Partial amino-acid sequence of AkLab was analyzed by the protein sequencer. The N-terminal sequence of 17 residues for AkLab was determined as ADLLTDKFPACFTFGVS. This sequence showed 69% identity to the 3rd – 17th residues of β -galactosidase from *alpha proteobacterium* HIMB100 (GenBank accession no. AFS48747), 56% identity to the 34th – 48th residues of a cellobiase from *Cellulomonas biazotea* (GenBank accession no. AEM45802) (Chan et al., 2012) and 53% identity to the 478th – 494th residues of a β -glucosidase from *Corbicula japonica* (GenBank accession no. BAG71912) (Sakamoto et al., 2009). These enzymes have been classified under glycosyl hydrolase family 1 (GHF1). The amino-acid sequence of a lysylendopeptidyl fragment of AkLab was determined as GPSIWDTFTSDSSHVTGG (18 residues). This sequence showed 66% identity to the 68th – 87th residues of

Strictosidine-O- β -D-glucosidase from *Rauvolfia serpentina* (GenBank accession no. CAC83098) (Xia et al., 2012) and the 50% identity to the 510th – 527th residues of a β -glucosidase from *Corbicula japonica* (Sakamoto et al., 2009). These results indicated that AkLab is also a member of GHF1.

4. Discussion

4.1. Physiological roles of AkLab in *A. kurodai*

We have been investigating the enzymatic properties of polysaccharide-degrading enzymes from algae-feeding mollusks to understand how the mollusks efficiently assimilate algal polysaccharides as carbon and energy sources (Shimizu et al., 2003; Suzuki et al., 2003; Ootsuka et al., 2006; Suzuki et al., 2006; Kumagai et al., 2008; Rahman et al., 2010; Zahura et al., 2010; Kumagai et al., 2013). Among algal polysaccharides, laminarin, a major storage polysaccharide of brown algae is known as an important glucose source for the algae feeders. Previously, we purified an endo-type and an exo-type β -1,3-glucanase (AkLam36 and AkLam33, respectively) from the digestive fluid of sea hare *A. kurodai* (Kumagai and Ojima, 2010). Although

these enzymes efficiently degraded laminarin and laminarioligosaccharides larger than disaccharide, they could not hydrolyze laminaribiose. Therefore, another laminaribiose-hydrolyzing enzyme like laminaribiase (EC 3.2.1.21) was considered to participate in the complete depolymerization of laminarin in *A. kurodai*. The information about laminaribiase from marine mollusks is extremely limited, i.e., only one literature on *Littorina kurila* enzyme is currently available (Pesentseva et al., 2012). Therefore, to understand the whole process for laminarin assimilation in mollusks, more detailed studies on laminarin-degrading enzymes seemed to be necessary. In the present study, we succeeded to purify the laminaribiose-hydrolyzing enzyme AkLab from the common sea hare *A. kurodai*. This enzyme showed significantly higher activity toward laminaribiose than other oligosaccharides. Thus, AkLab was considered to be the enzyme responsible for the hydrolysis of laminaribiose that had been produced by AkLam36 and AkLam33 in the digestive fluid of *A. kurodai*.

4. 2. Similarity of AkLab to other enzymes

The molecular mass of AkLab, i.e., 110 kDa estimated by SDS-PAGE, was similar to those of exo- β -1,3-glucanases from terrestrial snails *Helix pomatia* (82 kDa)

(Marshall and Grand, 1975) and *Eulota maakii* (100 kDa) (Elyakova and Shirokova, 1997), and β -glucosidase from a marine mollusk *Corbicula japonica* (100 kDa) (Sakamoto et al., 2009). Optimum temperature and pH of AkLab, 50°C and pH 5.5, were also similar to those of *H. pomatia* (50°C and pH 5.5) and *E. maakii* (55°C and pH 5.2) (Marshall and Grand, 1975; Elyakova and Shirokova, 1997). AkLab most rapidly hydrolyzed β -1,3-glucoside, e.g., laminaribiose, and slowly β -1,4-glucoside (cellobiose) and β -1,6-glucoside (gentiobiose) (Fig. 4, Table 2 and 3). β -pNPG was also a preferable substrate for AkLab (Table 4); however, laminarin was not degraded (data not shown). The exo- β -1,3-glucanase from *H. pomatia* was reported to be capable of hydrolyzing laminarin, laminaribiose and gentiobiose, but not cellobiose (Marshall and Grand, 1975). While the exo- β -1,3-glucanase from *E. maakii* could hydrolyze laminarin and laminarioligosaccharides, but not cellobiose and gentiobiose (Elyakova and Shirokova, 1997). Therefore, AkLab appeared to be different from such terrestrial molluscan enzymes with respect to substrate specificity. On the other hand, β -glucosidase is known to show broad substrate specificity and hydrolyze various β -glucosides (Hrmova and Fincher, 2007; Pesentseva et al., 2008). For example, barley β -glucosidase degrades not only cellooligosaccharides but also laminarioligosaccharides. However, activity toward polymer substrate is modest (Hrmova et al., 1996). Taking these facts into consideration,

we concluded that AkLab is a β -glucosidase-like laminarinase-hydrolyzing enzyme.

4. 3. *Transglycosylation activity of AkLab*

AkLab produced oligosaccharides larger than original substrates by its transglycosylation activity. Transglycosylation activity is known as a characteristic property of glycosyl hydrolases that split glycoside linkage with a retaining manner (Wang and Huang, 2009). Transglycosylation usually takes place in accordance with the specificity of the enzymes toward the configuration of glycosyl linkage. However, the glycosyl linkages formed by α -glucosidase were not completely consistent with the hydrolytic specificity (Kato et al., 2002). Namely, not only α -1,4-linked oligosaccharides but also α -1,6-linked oligosaccharides were produced by the transglycosylation. Laminarinase hydrolyzes mainly β -1,3-glycosyl linkage; however, it can hydrolyze β -1,4- and β -1,6-linkages adjacent to β -1,3-linkages in certain conditions. Therefore, AkLab may also produce oligosaccharides possessing different configurations of glycosyl linkages. Actually some spots with different mobility from marker sugars were observed between L2 and L3, and below L4 in TLC (Fig. 5). In addition, the oligosaccharides produced by the transglycosylation were considerably

resistant to AkLab (Fig. 5). These oligosaccharides may contain the glycosyl linkages distinct from β -1,3-linkage. Although the structures of transglycosylation products have not been analyzed yet, these results suggested that AkLab was available for the synthesis of artificial glycosides and oligosaccharides with beneficial functions. Beside the transglycosylation products, hydrolytic products of laminarin are also known as functional materials. For example, enzymatically produced laminarioligosaccharides were found to promote TNF- α secretion from human monocytes (Miyanishi et al., 2003b; Pang et al., 2005). The activities of laminarioligosaccharides are considered to be resulted from the specific higher order structures of laminarin and laminarioligosaccharides, e.g., β -1,3-linked glucose main chain with β -1,6-linked glucose branches (Willment et al., 2001; Adams et al., 2008). However, it is still obscure what kinds of structures are responsible for the activities of laminarin and laminarioligosaccharides. To reveal the functional structure of laminarin, studies using various laminarioligosaccharides with known structures are necessary. In this context, AkLab is a promising enzyme since it can produce laminarioligosaccharides with various sizes by its transglycosylation activity.

AkLab was considered as a member of GHF1 on the basis of partial amino-acid sequences. We are now analyzing the entire primary structure of AkLab by the cDNA

method, which will provide the bases for future protein-engineering study of this enzyme.

Acknowledgements

This study was supported in part by the grants for project research (Construction of Marine Science Bases in Tohoku) of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

- Adams, Z.L., Rice, P.J., Graves, B., Ensley, H.E., Yu, H., Brown, G.D., Gordon, S., Monteiro, M.A., Papp-Szabo, E., Lowman, D.W., Power, T.D., Wempe, M.F., Williams, D.L., 2008. Differential high-affinity interaction of Dectin-1 with natural or synthetic glucans is dependent upon primary structure and is influenced by polymer chain length and side-chain branching. *J. Pharmacol. Exp. Ther.* 325, 115–123.
- Aires, R.S., Steindorff, A.S., Ramada, M.H.S., Siqueira, S.J.L., Ulhoa, C.J., 2012. Biochemical characterization of a 27 kDa 1, 3- β -D-glucanase from *Trichoderma*

379 *asperellum* induced by cell wall of *Rhizoctonia solani*. Carbohydr. Polymers 87,
 380 1219–1223.

381 Black, W.A.P., Cornhill, W.J., Dewar, E.T., Woodward, F.N., 1951. Manufacture of algal
 382 chemicals. III. Laboratory-scale isolation of laminarin from brown marine algae. J.
 383 Appl. Chem. 1, 505–517.

384 Chan, A.K., Wang, Y.Y., Ng, K.L., Fu, Z., Wong, W.K.R., 2012. Cloning and
 385 characterization of a novel cellobiase gene, *cba3*, encoding the first known
 386 β -glucosidase of glycoside hydrolase family 1 of *Cellulomonas biazotea*. Gene
 387 493, 52–61.

388 Elyakova, L.A., Shirokova, N.I., 1997. An $\text{exo-}\beta$ -1, 3-glucanase from the snail *Eulota*
 389 *maakii*. Bioorg. Khim. 3, 1656–1662.

390 Emst, D., Schraudner, M., Langebartels, C., Sandermann, H. Jr., 1992. Ozone-induced
 391 changes of mRNA levels of β -1, 3-glucanase, chitinase and ‘pathogenesis-related’
 392 protein 1b in tobacco plants. Plant Mol. Biol. 20, 673–682.

393 Erfle, J.D., Teather, R.M., Wood, P.J., Irvin, J.E., 1988. Purification and properties of a 1,
 394 3-1, 4- β -D-glucanase (lichenase, 1, 3-1, 4- β -D-glucan 4-glucanohydrolase, EC
 395 3.2.1.73) from *Bacteroides succinogenes* cloned in *Escherichia coli*. Biochem. J.
 396 255, 833–841.

397 Esteban, P.F, Rios, I., Garcia, R., Duenas, E., Pla, J., Sanchez, M., Aldana, C.R.V., Rey,
 398 F., 2005. Characterization of the *CaENG1* gene encoding an endo-1, 3- β -glucanase
 399 involved in cell separation in *Candida albicans*. *Cur. Microbiol.* 51, 385–392.

400 Gastebois, A., Aïmanianda, V., Bachellier-Bassi, S., Nasseir, A., Firon, A., Beauvais, A.,
 401 Schmitt, C., England, P., Beau, R., Prevost, M.C., d'Enfert, C., Latge, J.P.,
 402 Mouyna, I., 2013. SUN proteins belong to a novel family of β -(1, 3)-glucan
 403 modifying enzymes involved in fungal morphogenesis. *J. Biol. Chem.* 288,
 404 13387–13396.

405 Hrmova, M., Fincher, G.B., 1993. Purification and properties of three
 406 (1 $\rightarrow$ 3)- β -D-glucanase isoenzymes from young leaves of barley (*Hordeum*
 407 *vulgare*). *Biochem. J.* 289, 453–461.

408 Hrmova, M., Harvey, A.J., Wang, J., Shirley, N.J., Jones, G.P., Stone, B.A., Hoj, P.B.,
 409 Fincher, G.B., 1996. Barley β -D-glucan exohydrolases with β -D-glucosidase
 410 activity – purification, characterization, and determination of primary structure
 411 from a cDNA clone –. *J. Biol. Chem.* 271, 5277–5286.

412 Hrmova, M., Fincher, G.B., 2007. Dissecting the catalytic mechanism of a plant
 413 β -D-glucan glycohydrolase through structural biology using inhibitors. *Carbohydr.*
 414 *Res.* 342, 1613–1523.

415 Kato, N., Suyama, S, Shirokane, M., Kato, M., Kobayashi, T., Tsukagoshi, N., 2002.
 416 Novel α -glucosidase from *Aspergillus nidulans* with strong
 417 transglycosylation activity. Appl. Environ. Microbiol. 68, 1250-1256.

418 Kovalchuk, S.N., Sundukova, E.V., Kusaykin, M.I., Guzev, K.V., Anastiuk, S.D.,
 419 Likhatskaya, G.N., Trifonov, E.V., Nurminski, E.A., Kozhemyako, V.B.,
 420 Zvyagintseva, T.N., Rasskazov, V.A., 2006. Purification, cDNA cloning and
 421 homology modeling of endo-1, 3- β -D-glucanase from scallop *Mizuhopecten*
 422 *yessoensis*. Comp. Biochem. Physiol. B 143, 473–485.

423 Kozhemyako, V.B., Rebrikov, D.V., Lukyanov, S.A., Bogdanova, E.A., Marin, A.,
 424 Mazur, A.K., Kovalchuk, S.N., Agafonova, E.V., Sova, V.V., Elyakova, L.A.,
 425 Rasskazov, V.A., 2004. Molecular cloning and characterization of an endo-1,
 426 3- β -D-glucanase from the mollusk *Spisula sachalinensis*. Comp. Biochem.
 427 Physiol. B 137, 169–178.

428 Kumagai, Y., Inoue, A., Tanaka, H., Ojima, T., 2008. Preparation of β -1, 3-glucanase
 429 from scallop mid-gut gland drips and its use for production of novel
 430 heterooligosaccharides. Fish. Sci. 74, 1127–1136.

431 Kumagai, Y., Ojima, T., 2009. Enzymatic properties and the primary structure of a β -1,
 432 3-glucanase from the digestive fluid of the Pacific abalone *Haliotis discus hannai*.

433 Comp. Biochem. Physiol. B 154, 113–120.

434 Kumagai, Y., Ojima, T., 2010. Isolation and characterization of two types of

435 β -1,3-glucanases from the common sea hare *Aplysia kurodai*. Comp. Biochem.

436 Physiol. B 155, 138–144.

437 Kumagai, Y., Satoh, T., Inoue, A., Ojima, T., 2013. Enzymatic properties and primary

438 structures of two α -amylase isozymes from the Pacific abalone *Haliotis discus*

439

440 Lépagnol-Descamps, V., Richard, C., Lahaye, M., Potin, P., Yvin, J.C., Kloareg, B.,

441 1998. Purification and determination of the action pattern of *Haliotis tuberculata*

442 laminarinase. Carbohydr. Res. 310, 283–289.

443 Leubner-Metzger, G., Frundt, C., Vogeli-Lange, R., Meins, F. Jr., 1995. Class I β -1,

444 3-glucanases in the endosperm of tobacco during germination. Plant Physiol. 109,

445 751–759.

446 Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement

447 with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

448 Mackenzie, L.F., Wang, Q., Warren, R.A.J., Withers, S.G., 1998. Glycosynthases:

449 mutant glycosidases for oligosaccharide synthesis. J. Am. Chem. Soc. 120,

450 5583-5584.

451 Marshall, J.J., Grand, R.J.A., 1975. Comparative studies on β -glucan hydrolases
 452 isolation and characterization of an exo(1-3)- β -glucanase from the snail *Helix*
 453 *pomatia*. Arch. Biochem. Biophys. 167, 165–175.

454 Miyanishi, N., Hamada, N., Kobayashi, T., Imada, C., Watanabe, E., 2003a. Purification
 455 and characterization of a novel extracellular β -1, 3-glucanase produced by
 456 *Bacillus clausii* NM-1 isolated from ezo abalone *Haliotis discus hannai*. J. Biosci.
 457 Bioeng. 95, 45–51.

458 Miyanishi, N., Iwamoto, Y., Watanabe, E., Oda, T., 2003b. Induction of TNF- α production
 459 from human peripheral blood monocytes with β -1,3-glucan oligomer prepared
 460 from laminarin with β -1,3-glucanase from *Bacillus clausii* NM- 1. J. Biosci.
 461 Bioeng. 95, 192–195.

462 Mrsa, V., Klebl, F., Tanner, W., 1993. Purification and characterization of the
 463 *Saccharomyces cerevisiae* BGL2 gene product, a cell wall endo- β -1, 3-glucanase.
 464 J. Bacteriol. 175, 2102–2106.

465 Ootsuka, S., Saga, N., Suzuki, K., Inoue, A., Ojima, T., 2006. Isolation and cloning of
 466 an endo-beta-1,4-mannanase from Pacific abalone *Haliotis discus hannai*. J.
 467 Biotechnol. 125, 269-280.

468 Opassiri, R., Hua, Y., Wara-Aswapati, O., Akiyama, T., Svasti, J., Esen, A., Cairns,

469 J.R.K., 2004. β -glucosidase, exo- β -glucanase and pyridoxine transglucosylase
 470 activities of rice BGlu1. *Biochem. J.* 379, 125–131.

471 Pang, Z, Otaka, K, Maoka, T, Hidaka, K, Ishijima, S, Oda, M, Ohnishi, M., 2005.
 472 Structure of β -glucan oligomer from laminarin and its effect on human monocytes
 473 to inhibit the proliferation of U937 cells. *Biosci. Biotechnol. Biochem.* 69,
 474 553–558.

475 Perez-Pons, J.A., Cayetano, A., Rebordosa, X., Lloberas, J., Guasch, A., Querol, E.,
 476 1994. A β -glucosidase gene (*bgl3*) from *Streptomyces* sp. strain QM-B814:
 477 Molecular cloning, nucleotide sequence, purification and characterization of the
 478 encoded enzyme, a new member of family 1 glycosyl hydrolases. *Eur. J. Biochem.*
 479 223, 557–565.

480 Pesentseva, M.S., Kusaykin, M.I., Anastyyuk, S.D., Sova, V.V., Zvyagintseva, T.N., 2008.
 481 Catalytic properties and mode of action of endo-(1 $\rightarrow$ 3)- β -D-glucanase and
 482 β -D-glucosidase from the marine mollusk *Littorina kurila*. *Carbohydr. Res.* 343,
 483 2393–2400.

484 Pesentseva, M.S., Kovalchuk, S.N., Anastyyuk, S.D., Kusaykin, M.I., Sova, V.V.,
 485 Rasskazov, V.A., Zvyagintseva, T.N., 2012. Endo-(1 $\rightarrow$ 3)- β -D-glucanase GI from
 486 marine mollusk *Littorina sitkana*: Amino acid sequence and ESIMS/MS-estimated

487 features of transglycosylation and hydrolysis reactions in comparison to
 488 analogous enzyme LIV from *Pseudocardium sachalinensis*. J. Mol. Cat. B:
 489 Enzymatic 75, 73–79.

490 Porzio, M.A., Pearson, A.M., 1977. Improved resolution of myofibrillar proteins with
 491 sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Biochim. Biophys.
 492 Acta 490, 27–34.

493 Rahman, M.M., Inoue, A., Tanaka, H., Ojima, T., 2010. Isolation and characterization of
 494 two alginate lyase isozymes, AkAly28 and AkAly33, from the common sea hare
 495 *Aplysia kurodai*. Comp. Biochem. Physiol. B 157, 317–325.

496 Sakamoto, K., Uji, S., Kurokawa, T., Toyohara, H., 2009. Molecular cloning of
 497 endogenous beta-glucosidase from common Japanese brackish water clam
 498 *Corbicula japonica*. Gene 435, 72–79.

499 Sanchez, C., 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi.
 500 Biotechnol Adv. 27, 185–194.

501 Shimizu, E., Ojima, T., Nishita, K., 2003. cDNA cloning of an alginate lyase from
 502 abalone, *Haliotis discus hannai*. Carbohydr. Res. 328, 2841–2852.

503 Smelcerovic, A., Knezevic-Jugovic, Z., Petronijevic, Z., 2008. Microbial
 504 polysaccharides and their derivatives as current and prospective pharmaceuticals.

505 Curr. Pharm. Des. 14, 316–395.

506 Sova, V.V., Elyakova, L.A., Vaskovsky, V.E., 1970. The distribution of laminarinases in
 507 marine invertebrates. Comp. Biochem. Physiol. B 32, 459–464.

508 Størseth, T.R., Hansen, K., Reitan, K.I., Skjermo, J., 2005. Structural characterization of
 509 β -D-(1, 3)-glucans from different growth phases of the marine diatoms
 510 *Chaetoceros mülleri* and *Thalassiosira weissflogii*. Carbohydr. Res. 340,
 511 1159–1164.

512 Suzuki, K.I., Ojima, T., Nishita, K., 2003. Purification and cDNA cloning of a cellulase
 513 from abalone *Haliotis discus hannai*. Eur. J. Biochem. 270, 771–778.

514 Suzuki, H., Suzuki, K., Inoue, A., Ojima, T., 2006. A novel oligoalginate lyase from
 515 abalone, *Haliotis discus hannai*, that releases disaccharide from alginate polymer
 516 in an exolytic manner. Carbohydr. Res. 341, 1809-1819.

517 Tangarone, B., Royer, J.C., Nakas, J.P., 1989. Purification and characterization of an
 518 endo-(1, 3)- β -D-glucanase from *Trichoderma longibrachiatum*. Appl. Environ.
 519 Microbiol. 55, 177–184.

520 Ueda, M., Yamaki, K., Goto, T., Nakazawa, M., Miyatake, K., Sakaguchi, M., Inouye,
 521 K., 2011. Purification and characterization of 1, 3- β -D-glucanase from *Eisenia*
 522 *foetida*. Carbohydr. Polymers 86, 271–276.

523 Wang, L.X., Huang, W., 2009. Enzymatic transglycosylation for glycoconjugate
 524 synthesis. *Curr. Opin. Chem. Biol.* 13, 592–600.

525 Willment, J.A., Gordon, S., Brown, G.D., 2001. Characterization of the Human
 526 β -Glucan Receptor and Its Alternatively Spliced Isoforms. *J. Biol. Chem.* 276,
 527 43818–43823.

528 Xia, L., Ruppert, M., Wang, M., Panjikar, S., Lin, H., Rajendran, C.,
 529 Barleben, L., Stockigt, J., 2012. Structures of alkaloid biosynthetic glucosidases
 530 decode substrate specificity. *ACS Chem. Biol.* 7, 226–234.

531 Zahura, U.A., Rahman, M.M., Inoue, A., Tanaka, H., Ojima, T., 2010. An
 532 endo- β -1,4-mannanase, AkMan, from the common sea hare *Aplysia kurodai*.
 533 *Comp. Biochem. Physiol. B* 157, 137–143.

534 Zakharenko, A.M., Kusaykin, M.I., Kovalchuk, S.N., Sova, V.V., Silchenko, A.S., Belik,
 535 A.A., Anastyuk, S.D., Ly, B.M., Rasskazov, V.A., Zvyagintseva, T.N., 2012.
 536 Catalytic properties and amino acid sequence of endo-1 $\rightarrow$ 3- β -D glucanase from
 537 the marine mollusk *Tapes literata*. *Biochemistry (Mosc)* 77, 878–888.

539 **Figure Legends**

540 Fig. 1. Purification of AkLab from the digestive fluid of sea hare

(A) Fractions showing laminaribiose-hydrolyzing activity in TOYOPEARL Phenyl-650M chromatography were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.0), then applied to a column of TOYOPEARL DEAE-650M (2 × 45 cm) pre-equilibrated with the same buffer. (B) Fractions showing laminaribiose-hydrolyzing activity in TOYOPEARL DEAE-650M column chromatography were concentrated to less than 2 ml, then applied to Superdex 200 10/300 GL column pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0) containing 300 mM NaCl. (C) Monitoring of purification of AkLab by SDS-PAGE. M, marker proteins; A, the sample after TOYOPEARL Phenyl-650M chromatography; B, the sample after TOYOPEARL DEAE-650M chromatography; C, the sample after Superdex 200 10/300 GL chromatography.

Fig. 2. Optimum temperature and pH, and thermostability of AkLab. A, Optimum temperature of AkLab was measured at 4 – 70°C in a reaction mixture containing 5 mg/mL of laminaribiose. B, Optimum pH of AkLab was measured at 30°C in the reaction mixtures adjusted to pH 3.5 – 6.0 with 50 mM sodium citrate buffer and pH 6.0-8.1 with 50 mM sodium phosphate buffer. C, Thermostability of AkLab was assessed by measuring the activity remaining after the incubation of enzyme at 4-70°C

for 15 min in 10 mM sodium phosphate buffer (pH 5.5). Average values for the triplicated measurements are shown with standard deviations.

Fig. 3. Thin-layer chromatography for the degradation products of laminarioligosaccharides produced by AkLab. Five mg/mL of laminarioligosaccharides (A – F, correspond to L2-L7) in 10 mM sodium phosphate buffer (pH 5.5) were degraded with 4.4 mU/mL of AkLab at 30°C. The reaction was terminated at appropriate time intervals by mixing with an equal volume of 10% (w/v) trichloroacetic acid and 2 µL of the mixture was applied to TLC. M, marker sugars (G1, glucose; L2 – L7, laminaribiose – laminariheptaose).

Fig. 4. Degradation rates of laminarioligosaccharides by AkLab.

Degradation of laminarioligosaccharides by AkLab was carried out in the same conditions as in Fig. 3 with the following substrates: L2 (○), L3 (●), L4 (△), L5 (▲), L6 (◇), and L7 (◆). The amount of glucose released by the reaction was determined with glucose CII-test kit Wako. Average values for the triplicated measurements are shown with standard deviations.

577

578 Fig. 5. Transglycosylation products produced by AkLab. Transglycosylation reaction
579 was carried out at 30°C in the reaction mixture containing 10 mM sodium phosphate
580 buffer (pH 5.5), 36.5 mM L2 (donor), 12 mM L3 (acceptor), and 10 mU of AkLab.
581 Transglycosylation reaction was terminated by the addition of an equal volume of 10%
582 (w/v) trichloroacetic acid. Two µL of the mixture was applied to TLC. The
583 abbreviations used are the same as in Fig. 3.

584

585

586

587

588

589

590

591

592

593

594

Table 1. Purification of AkLab.

		Total	Specific	Total	Purifi-	Yield
		protein	activity	activity	cation	
Samples	(mg)	(U/mg)	(U)	(fold)	(%)	
Crude	3493	0.031	107	1	100	
AS ^{*1}	2631	0.065	172	2.1	161	
Phenyl ^{*2}	380	0.076	28.8	2.5	27	
DEAE ^{*3}	20	0.89	17.7	29	17	
Superdex ^{*4}	1.62	3.4	5.5	110	5.1	

^{*1}Fraction precipitated between 40 and 60% saturation of ammonium sulfate. ^{*2}Active fraction obtained by TOYOPEARL Phenyl-650M chromatography. ^{*3}Active fraction obtained by TOYOPEARL DEAE-650M chromatography. ^{*4}AkLab purified by Superdex 200 gel-filtration.

613 Table 2. Relative activity of AkLab toward laminarioligosaccharides.

614	Substrate	Specific activity (mU/mg)	Relative activity (%)
615	laminaribiose	$3400 \pm 164^*$	100
616	laminaritriose	105 ± 3	3.1
617	laminaritetraose	190 ± 2	5.6
618	laminaripentaose	160 ± 5	4.7
619	laminarihexaose	150 ± 3	4.4
620	laminariheptaose	65 ± 3	1.9

621 *One unit of laminaribiose-hydrolyzing activity was defined as the amount of enzyme
622 that produces 2 μ mol glucose per min since the degradation of 1 mol disaccharide
623 releases 2 mol glucose. The activities were represented as average values for triplicate
624 measurements with standard deviation.

625

626

627

628

629

630

631 Table 3. Relative activity of AkLab toward various disaccharides.

632	Substrate (linkage)	Specific activity (mU/mg)	Relative activity (%)
633	laminaribiose (β -1,3)	$3400 \pm 164^*$	100
634	cellobiose (β -1,4)	$1054 \pm 23^*$	31
635	gentiobiose (β -1,6)	$1190 \pm 58^*$	35
636	lactose (β -1,4)	510 ± 35	15
637	maltose (α -1,4) *	0	0
638	sucrose (α -1,2)	0	0

639 *One unit of activity was defined as the amount of enzyme that produces 2 μ mol
640 glucose per min since degradation of 1 mol disaccharide releases 2 mol glucose.
641 Enzyme activity was assayed at 30°C and pH 5.5 in reaction mixtures containing 5
642 mg/mL of substrates. The activities were represented as average values for triplicate
643 measurements with standard deviation.

644

645

646

647

648

Table 4. Substrate specificity of AkLab

Substrate	Specific activity (mU/mg) *	Relative activity (%)
<i>p</i> NP β-D-glucoside	1220 ± 37	100
<i>p</i> NP β-D-galactoside	268 ± 3	22
<i>p</i> NP β-D-mannoside	24 ± 0.3	2
<i>p</i> NP β-D-fucoside	8.2 ± 0.1	0.67
<i>p</i> NP β-D-N-acetyl glucosaminide	5.5 ± 0.1	0.45
<i>p</i> NP β-D-xyloside	2.3 ± 0.1	0.19

*One unit of activity was defined as the amount of enzyme that released 1 μmol *p*NP per min. The activities were represented as average values for triplicate measurements with standard deviation.

Fig. 1.

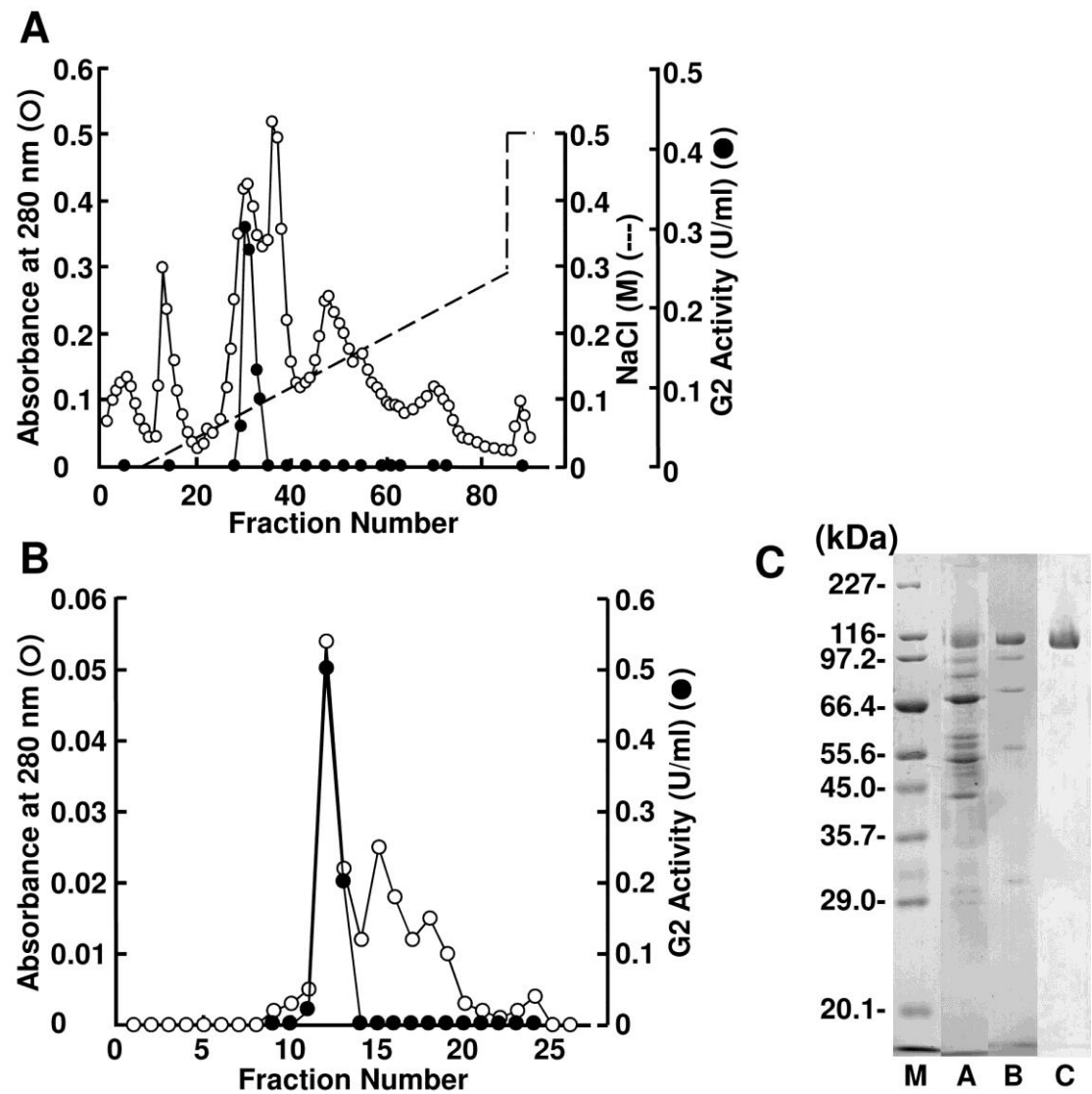


Fig. 2.

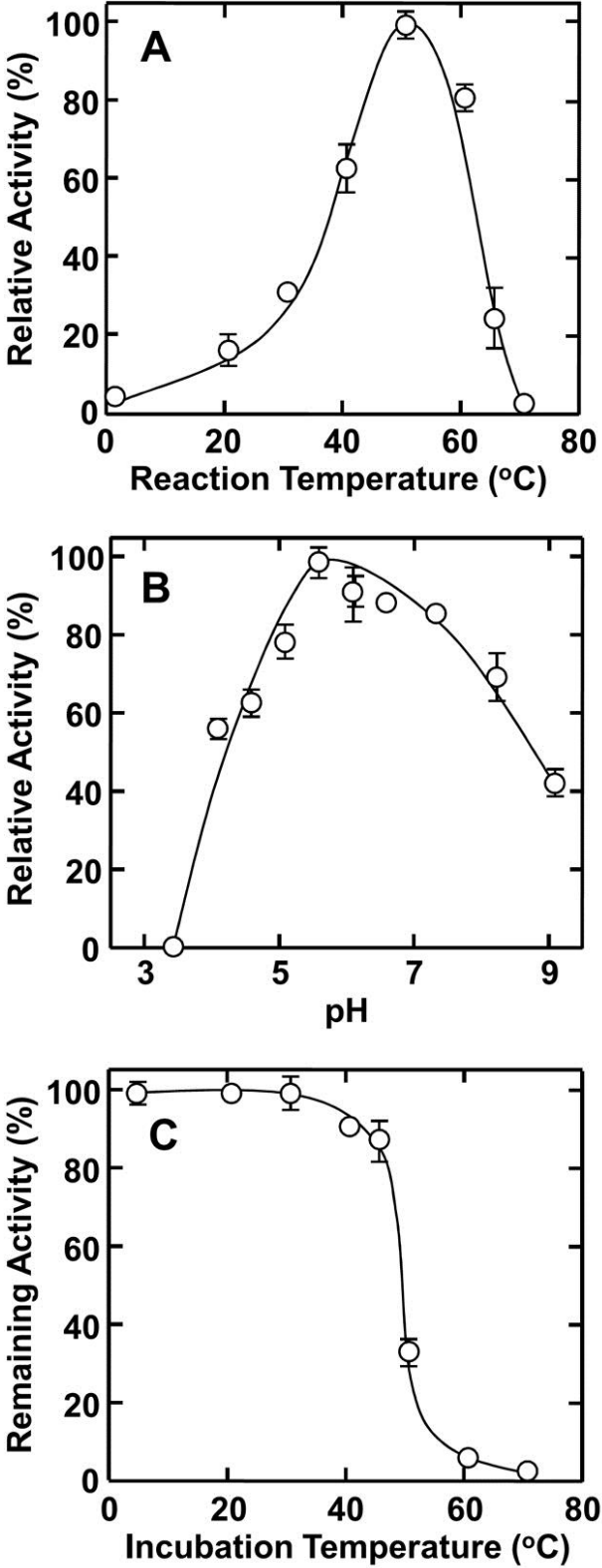


Fig. 3.

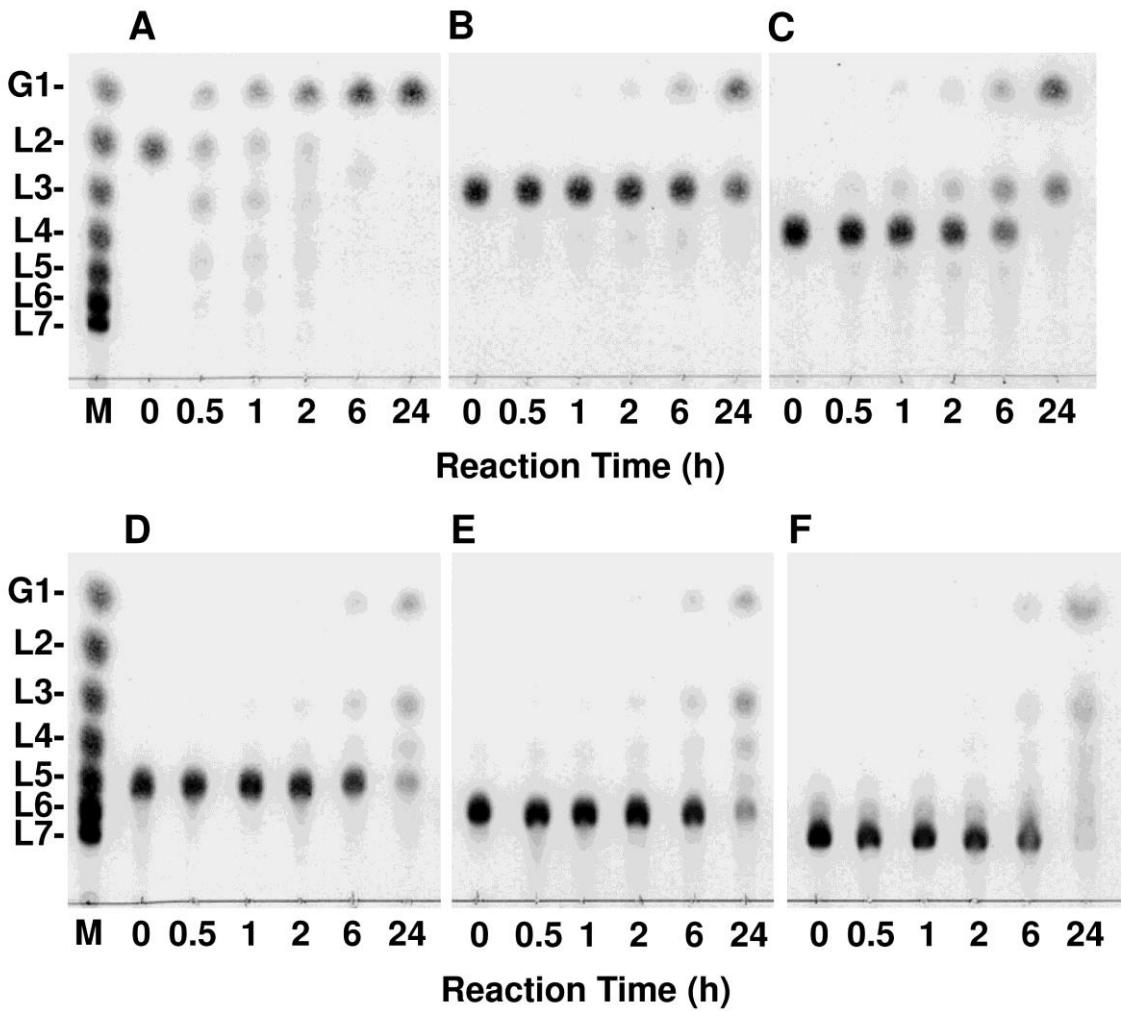


Fig. 4.

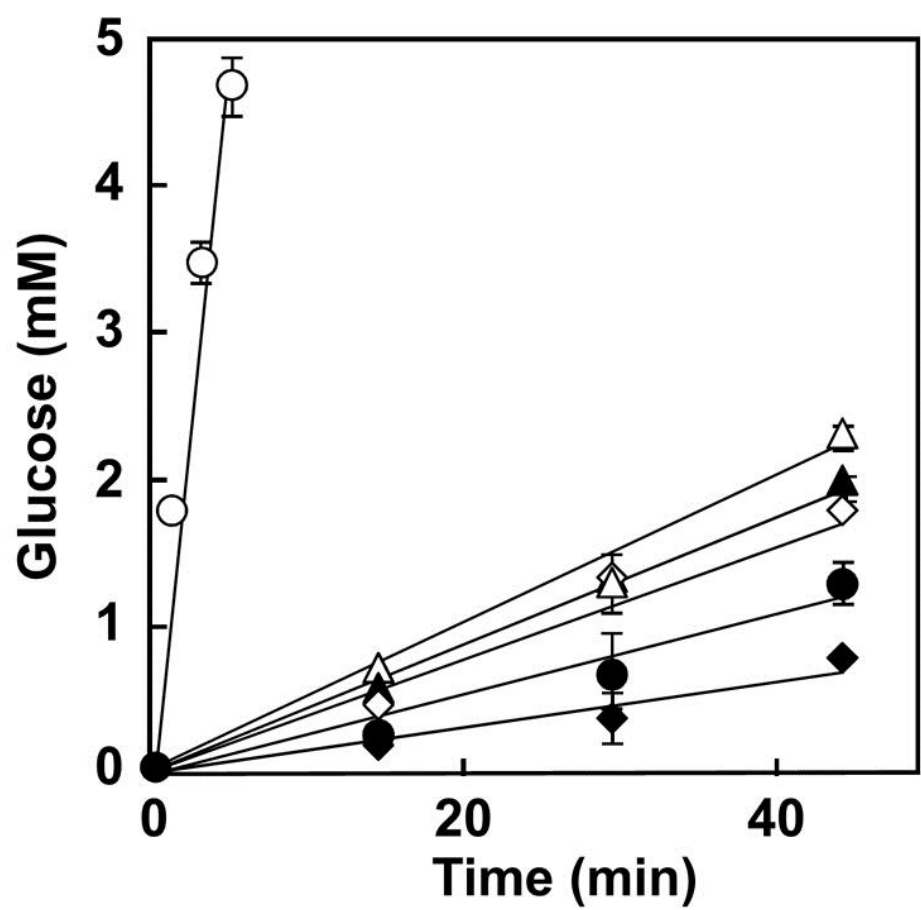


Fig. 5.

