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

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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^{o}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

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

58 Molluscan laminarinases have been studied using abalone, scallop, surf clam,
59 and sea hare (Lépagnol-Descamps et al., 1998; Kozhemyako et al., 2004; Kovalchuk et
60 al., 2006; Kumagai et al., 2008; Kumagai and Ojima, 2009; Kumagai and Ojima, 2010;
61 Pesentseva et al., 2012; Zakharenko et al., 2012). These enzymes hydrolyzed laminarin
62 by an endolytic process producing laminaribiose and glucose as major end products.
63 Although these molluscan enzymes hardly hydrolyzed laminaribiose as a sole substrate,
64 they could produce glucose from the mixture of laminaribiose and laminaritetraose *via*
65 transglycosylation (Kumagai and Ojima 2009; Kumagai and Ojima 2010). In this
66 reaction, laminaribiose and laminaritetraose were used as acceptor substrate and donor
67 substrate, respectively. However, this reaction seemed to be an *in vitro* one since the
68 transglycosylation reaction required much higher concentration of donor and acceptor
69 substrates (10 – 50 mM) than those in the digestive fluid (less than 1 mM). Therefore,
70 laminaribiose in the digestive fluid of mollusks should be degraded by some other
71 ‘laminaribiose-hydrolyzing’ enzymes which have been unidentified yet. One candidate
72 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×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×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×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^{o}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^{o}C. *p*-Nitrophenol (*p*NP) released by the degradation of β-*p*NP derivatives was determined with the molar extinction coefficient 1.81×10^{5} $M^{-1}\cdot cm^{-1}$ 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^{o}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^{o}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^{o}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^{o}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^{o}C for 10 min after spraying 10% (v/v) sulfuric acid in ethanol.

181 *2.6. SDS-polyacrylamide gel electrophoresis*

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183 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was
184 carried out with 0.1% (w/v) SDS – 10 % (w/v) polyacryl-amide slab gel (1 mm thick,
185 10 cm × 9 cm (width × length)) according to the method of Porzio and Pearson (1977).
186 After the electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue
187 R-250 in 50% (v/v) methanol – 10% (v/v) acetic acid, and the background of the gel
188 was destained with 5% (v/v) methanol – 7% (v/v) acetic acid. Protein Marker, Broad
189 Range (New England BioLabs, Ipswich, MA, USA) was used as a molecular mass
190 marker.

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192 *2.7. Determination of partial amino-acid sequences*

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194 The N-terminal amino-acid sequence of laminaribiose-hydrolyzing enzyme was
195 determined with an ABI Procise 492 sequencer (Applied Biosystems, Foster City, CA,
196 USA). Internal amino-acid sequences of the enzyme were determined with the peptide
197 fragments prepared by lysylendopeptidase digestion at 37°C for 2 h
198 (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^{o}C and pH 5.5, respectively (Fig. 2A and 2B). The temperature that caused a half

217 inactivation during 15-min incubation was at around 48^{o}C (Fig. 2C).

218

219 *3. 2. Substrate specificity of AkLab*

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221 Degradation products of L2 – L7 produced by AkLab were analyzed by TLC. As
222 shown in Fig. 3, AkLab was capable of hydrolyzing all the laminarioligosaccharides
223 tested and produced glucose (Transglycosylation products are also seen. This will be
224 mentioned in the next section). Then, hydrolytic activities of AkLab for
225 laminarioligosaccharides were determined by measuring glucose-releasing rates (Fig. 4
226 and Table 2). The activity toward L2 was significantly high, i.e., it was approximately
227 20-times higher than those toward other laminarioligosaccharides. AkLab slowly
228 degraded cellobiose (β-1,4-glucoside), gentiobiose (β-1,6-glucoside) and lactose
229 (β-1,4-galactoside), but not α-glycosides like maltose and sucrose (Table 3). The
230 activities toward cellobiose and gentiobiose were 1/3 – 1/5 of the activity toward
231 laminaribiose (Table 3). AkLab showed high activity toward *p*NP β-D-glucoside and
232 low activity toward *p*NP β-D-galactoside, but practically no activity toward other β-*p*NP
233 derivatives tested (Table 4). AkLab could not degrade polymer substrate laminarin (data
234 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

253 oligosaccharides (Fig. 4 and Table 2). It is also possible to consider that the
254 transglycosylation products were not β-1,3-linked oligosaccharides but other isomers
255 with different glycosyl linkages which AkLab hardly hydrolyzes although their
256 structures were not analyzed in the present study.
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258 *3. 4. Partial amino-acid sequence of AkLab*
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260 Partial amino-acid sequence of AkLab was analyzed by the protein sequencer.
261 The N-terminal sequence of 17 residues for AkLab was determined as
262 ADLLTDKFPACFTFGVS. This sequence showed 69% identity to the 3rd – 17th
263 residues of β-galactosidase from *alpha proteobacterium* HIMB100 (GenBank accession
264 no. AFS48747), 56% identity to the 34th – 48th residues of a cellobiase from
265 *Cellulomonas biazotea* (GenBank accession no. AEM45802) (Chan et al., 2012) and
266 53% identity to the 478th – 494th residues of a β-glucosidase from *Corbicula japonica*
267 (GenBank accession no. BAG71912) (Sakamoto et al., 2009). These enzymes have been
268 classified under glycosyl hydrolase family 1 (GHF1). The amino-acid sequence of a
269 lysylendopeptidyl fragment of AkLab was determined as GPSIWDTFTSDSSHVTGG
270 (18 residues). This sequence showed 66% identity to the 68th – 87th residues of

271 Strictosidine-O-β-D-glucosidase from *Rauvolfia serpentina* (GenBank accession no.
272 CAC83098) (Xia et al., 2012) and the 50% identity to the 510th – 527th residues of a
273 β-glucosidase from *Corbicula japonica* (Sakamoto et al., 2009). These results indicated
274 that AkLab is also a member of GHF1.
275
276 **4. Discussion**
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278 *4.1. Physiological roles of AkLab in A. kurodai*
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280 We have been investigating the enzymatic properties of
281 polysaccharide-degrading enzymes from algae-feeding mollusks to understand how the
282 mollusks efficiently assimilate algal polysaccharides as carbon and energy sources
283 (Shimizu et al., 2003; Suzuki et al., 2003; Ootsuka et al., 2006; Suzuki et al., 2006;
284 Kumagai et al., 2008; Rahman et al., 2010; Zahura et al., 2010; Kumagai et al., 2013).
285 Among algal polysaccharides, laminarin, a major storage polysaccharide of brown algae
286 is known as an important glucose source for the algae feeders. Previously, we purified
287 an endo-type and an exo-type β-1,3-glucanase (AkLam36 and AkLam33, respectively)
288 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)

307 (Marshall and Grand, 1975) and *Eulota maakii* (100 kDa) (Elyakova and Shirokova,
308 1997), and β-glucosidase from a marine mollusk *Corbicula japonica* (100 kDa)
309 (Sakamoto et al., 2009). Optimum temperature and pH of AkLab, 50^{o}C and pH 5.5,
310 were also similar to those of *H. pomatia* (50^{o}C and pH 5.5) and *E. maakii* (55^{o}C and pH
311 5.2) (Marshall and Grand, 1975; Elyakova and Shirokova, 1997). AkLab most rapidly
312 hydrolyzed β-1,3-glucoside, e.g., laminaribiose, and slowly β-1,4-glucoside (cellobiose)
313 and β-1,6-glucoside (gentiobiose) (Fig. 4, Table 2 and 3). β-*p*NPG was also a preferable
314 substrate for AkLab (Table 4); however, laminarin was not degraded (data not shown).
315 The exo-β-1,3-glucanase from *H. pomatia* was reported to be capable of hydrolyzing
316 laminarin, laminaribiose and gentiobiose, but not cellobiose (Marshall and Grand, 1975).
317 While the exo-β-1,3-glucanase from *E. maakii* could hydrolyze laminarin and
318 laminarioligosaccharides, but not cellobiose and gentiobiose (Elyakova and Shirokova,
319 1997). Therefore, AkLab appeared to be different from such terrestrial molluscan
320 enzymes with respect to substrate specificity. On the other hand, β-glucosidase is known
321 to show broad substrate specificity and hydrolyze various β-glucosides (Hrmova and
322 Fincher, 2007; Pesentseva et al., 2008). For example, barley β-glucosidase degrades not
323 only cellooligosaccharides but also laminarioligosaccharides. However, activity toward
324 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

343 resistant to AkLab (Fig. 5). These oligosaccharides may contain the glycosyl linkages
344 distinct from β-1,3-linkage. Although the structures of transglycosylation products have
345 not been analyzed yet, these results suggested that AkLab was available for the
346 synthesis of artificial glycosides and oligosaccharides with beneficial functions. Beside
347 the transglycosylation products, hydrolytic products of laminarin are also known as
348 functional materials. For example, enzymatically produced laminarioligosaccharides
349 were found to promote TNF-α secretion from human monocytes (Miyanishi et al.,
350 2003b; Pang et al., 2005). The activities of laminarioligosaccharides are considered to
351 be resulted from the specific higher order structures of laminarin and
352 laminarioligosaccharides, e.g., β-1,3-linked glucose main chain with β-1,6-linked
353 glucose branches (Willment et al., 2001; Adams et al., 2008). However, it is still obscure
354 what kinds of structures are responsible for the activities of laminarin and
355 laminarioligosaccharides. To reveal the functional structure of laminarin, studies using
356 various laminarioligosaccharides with known structures are necessary. In this context,
357 AkLab is a promising enzyme since it can produce laminarioligosaccharides with
358 various sizes by its transglycosylation activity.

359 AkLab was considered as a member of GHF1on the basis of partial amino-acid
360 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.

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538
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^{o}C in a reaction mixture containing 5 mg/mL of laminaribiose. B, Optimum pH of AkLab was measured at 30^{o}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^{o}C

559 for 15 min in 10 mM sodium phosphate buffer (pH 5.5). Average values for the
560 triplicated measurements are shown with standard deviations.
561
562 Fig. 3. Thin-layer chromatography for the degradation products of
563 laminarioligosaccharides produced by AkLab. Five mg/mL of laminarioligosaccharides
564 (A – F, correspond to L2-L7) in 10 mM sodium phosphate buffer (pH 5.5) were
565 degraded with 4.4 mU/mL of AkLab at 30^{o}C. The reaction was terminated at
566 appropriate time intervals by mixing with an equal volume of 10% (w/v) trichloroacetic
567 acid and 2 μL of the mixture was applied to TLC. M, marker sugars (G1, glucose; L2 –
568 L7, laminaribiose – laminariheptaose).
569
570 Fig. 4. Degradation rates of laminarioligosaccharides by AkLab.
571 Degradation of laminarioligosaccharides by AkLab was carried out in the same
572 conditions as in Fig. 3 with the following substrates: L2 (○), L3 (●), L4 (△), L5 (▲), L6
573 (◇), and L7 (◆). The amount of glucose released by the reaction was determined with
574 glucose CII-test kit Wako. Average values for the triplicated measurements are shown
575 with standard deviations.
576

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

Table 1. Purification of AkLab.

Samples	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (fold)	Yield (%)
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.

Table 2. Relative activity of AkLab toward laminarioligosaccharides.

Substrate	Specific activity (mU/mg)	Relative activity (%)
laminaribiose	3400 ± 164*	100
laminaritriose	105 ± 3	3.1
laminaritetraose	190 ± 2	5.6
laminaripentaose	160 ± 5	4.7
laminarihexaose	150 ± 3	4.4
laminariheptaose	65 ± 3	1.9

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

Table 3. Relative activity of AkLab toward various disaccharides.

Substrate (linkage)	Specific activity (mU/mg)	Relative activity (%)
laminaribiose (β-1,3)	3400 ± 164*	100
cellobiose (β-1,4)	1054 ± 23*	31
gentiobiose (β-1,6)	1190 ± 58*	35
lactose (β-1,4)	510 ± 35	15
maltose (α-1,4) *	0	0
sucrose (α-1,2)	0	0

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

Table 4. Substrate specificity of AkLab

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

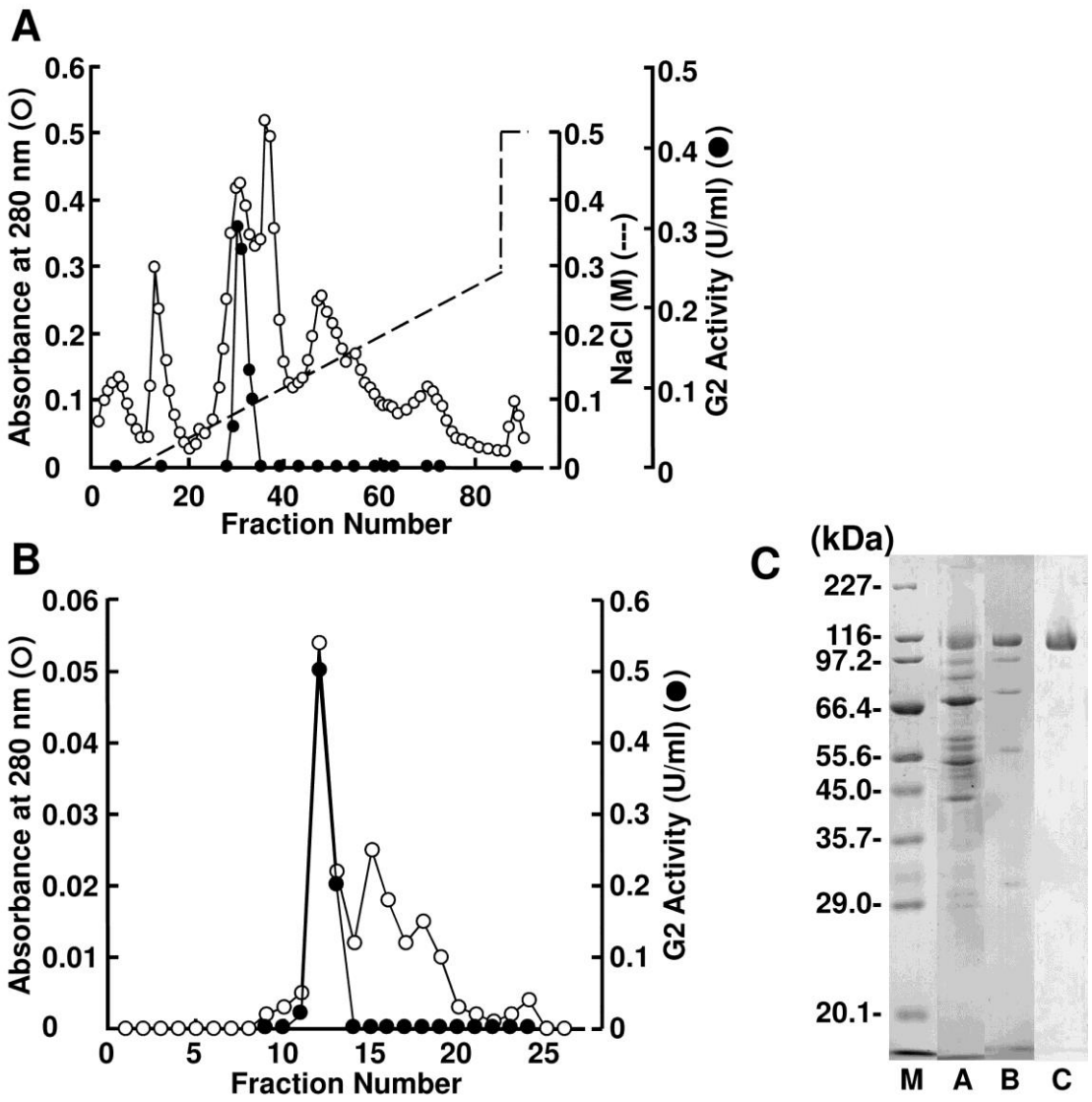
A
Absorbance at 280 nm (O)
NaCl (M) (---)
G2 Activity (U/ml) (●)
Fraction Number
B
Absorbance at 280 nm (O)
G2 Activity (U/ml) (●)
Fraction Number
C
(kDa)
227-
116-
97.2-
66.4-
55.6-
45.0-
35.7-
29.0-
20.1-
M
A
B
C

Fig. 2.

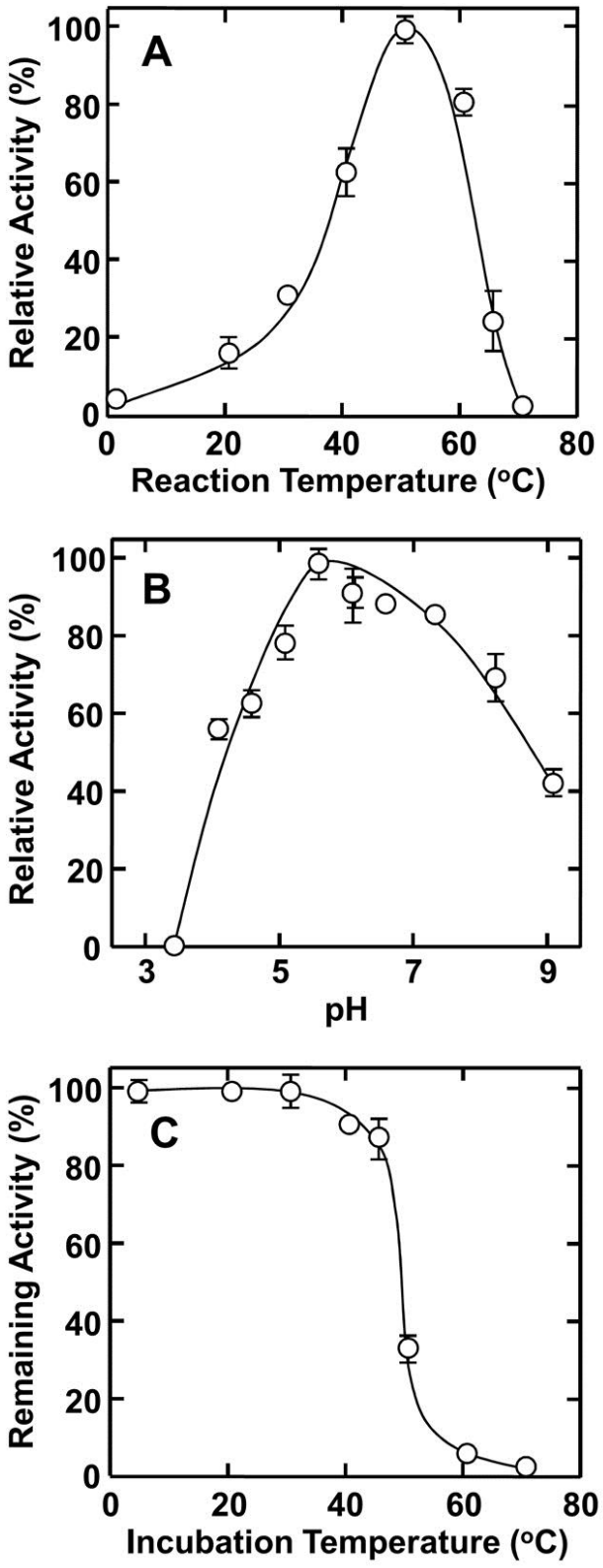
A
Relative Activity (%)
Reaction Temperature (°C)
B
Relative Activity (%)
pH
C
Remaining Activity (%)
Incubation Temperature (°C)

Fig. 3.

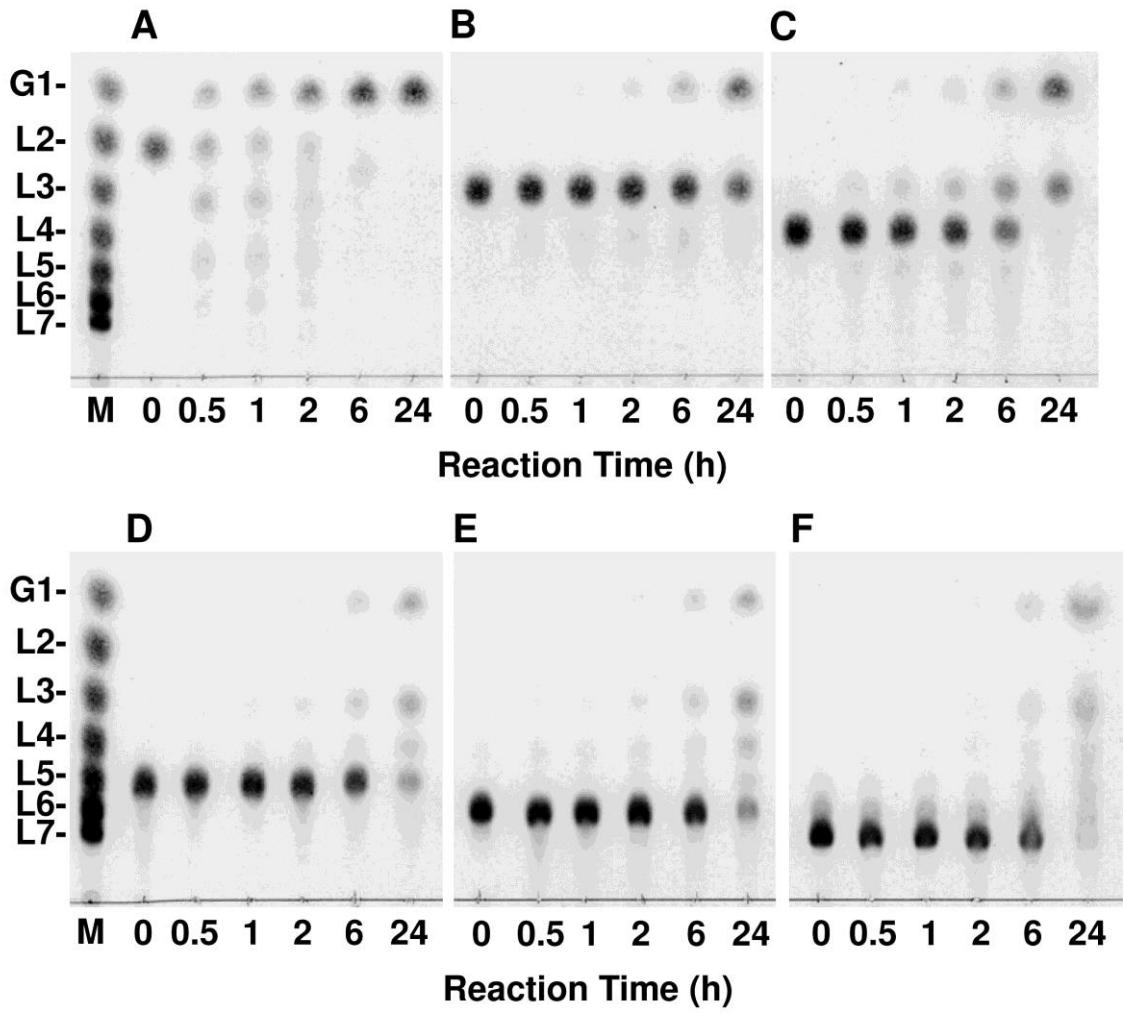
A
B
C
G1-
L2-
L3-
L4-
L5-
L6-
L7-
M 0 0.5 1 2 6 24
0 0.5 1 2 6 24
0 0.5 1 2 6 24
Reaction Time (h)
D
E
F
G1-
L2-
L3-
L4-
L5-
L6-
L7-
M 0 0.5 1 2 6 24
0 0.5 1 2 6 24
0 0.5 1 2 6 24
Reaction Time (h)

Fig. 4.

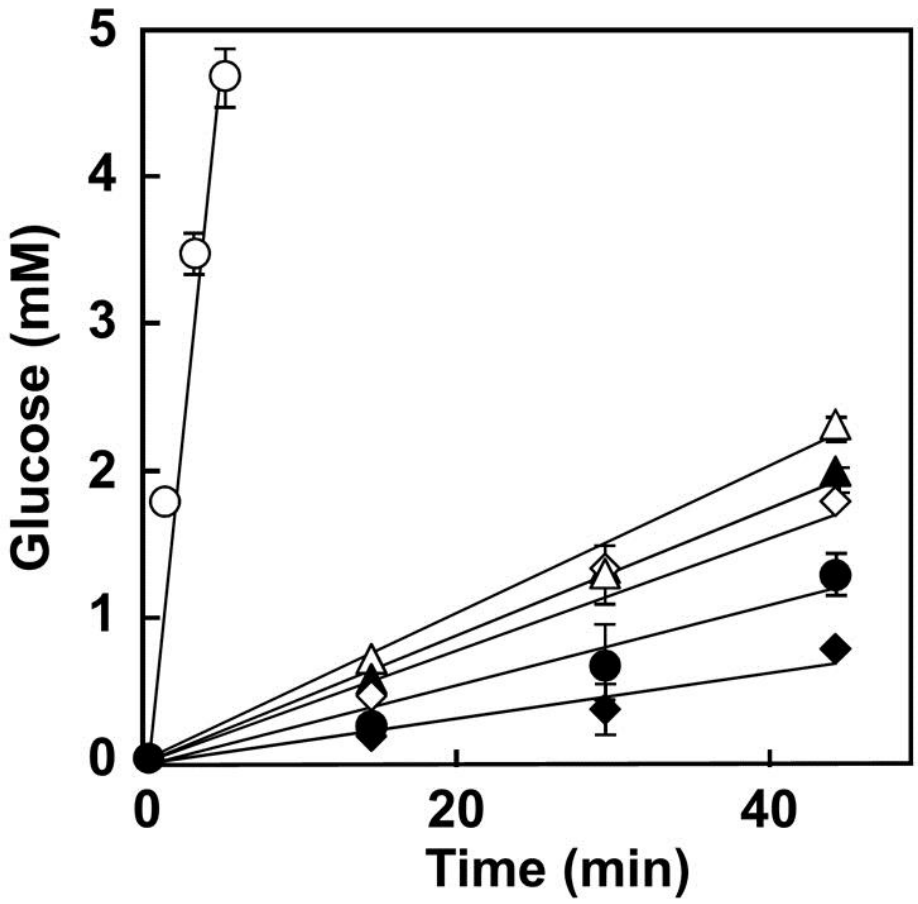

Fig. 5.