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

3

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19 **Abstract**

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

36

37 *Key words:* gastropod; sea hare; *Aplysia*; laminaribiose;  $\beta$ -glucosidase; GHF1.

38

## 39 **1. Introduction**

40  $\beta$ -1,3-Glucanase (EC 3.2.1.6) is an enzyme that hydrolyzes  $\beta$ -1,3-glucosyl  
41 linkage of  $\beta$ -glucans producing  $\beta$ -1,3-glucooligosaccharides and glucose. This enzyme  
42 distributes over fungi, bacteria, higher plants, and marine mollusks (Sova et al., 1970;  
43 Erfle et al., 1988; Tangarone et al., 1989; Hrmova and Fincher, 1993; Mrsa et al., 1993;  
44 Miyanishi et al., 2003a; Ueda et al., 2011; Aires et al., 2012). Their physiological roles  
45 are diverse and vary depending on their origins. For example, this enzyme intimately  
46 relates to seed germination and antifungal activity in some plants (Emst et al., 1992;  
47 Leubner-Metzger et al., 1995). While it plays important roles for cell division and  
48 morphogenesis in fungi (Esteban et al., 2005; Gastebois et al., 2013). On the other hand,  
49 marine molluscan enzymes in digestive fluid play an important role for saccharification  
50 of laminarin and chrysolaminarin from their dietary algae (Sova et al., 1970;  
51 Lépagnol-Descamps et al., 1998). Accordingly, the molluscan enzymes are generally  
52 called laminarinase. Higher order structure of algal laminarin is known to vary  
53 depending on algal sources (Black et al., 1951; Størseth et al., 2005; Smelcerovic et al.,  
54 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  $\beta$ -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  $\beta$ -glucosidase (Perez-Pons et al., 1994; Opassiri et al., 2004; Sanchez

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

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

90

91 **2. Materials and methods**

92

93 *2.1. Materials*

94

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

109 DEAE-650M and TOYOPEARL HW50F were from Toyo Soda Mfg. Co. (Tokyo,  
110 Japan). Other reagents were purchased from Wako Pure Chemical Industries. Superdex  
111 200 10/300 GL was purchased from GE Healthcare Life Sciences (Uppsala, Sweden).

112

## 113 2.2. Purification of laminaribiose-hydrolyzing enzyme

114

115 Laminaribiose-hydrolyzing enzyme was purified as follows. The crude enzyme  
116 (approximately 100 mL from 20 animals) from *A. kurodai* was subjected to ammonium  
117 sulfate fractionation and the precipitates formed between 40 – 60% saturation of  
118 ammonium sulfate were collected by centrifugation at  $10,000\times g$  for 10 min. The  
119 precipitates were dissolved in and dialyzed against 10 mM sodium phosphate buffer (pH  
120 6.0) and centrifuged at  $10,000\times g$  for 10 min to remove insoluble materials. The  
121 supernatant was then applied to a TOYOPEARL Phenyl-650M column ( $2\times 30$  cm)  
122 pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0) containing 40%  
123 saturated ammonium sulfate. Un-adsorbed proteins were washed out with the same  
124 buffer, and then proteins adsorbed to the column were eluted stepwisely with the buffer  
125 containing 40%, 30%, 20%, 10%, and 0% saturated ammonium sulfate. By this  
126 chromatography, laminaribiose-hydrolyzing enzyme was eluted in the 10%-saturated



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

138

### 139 *2.3. Assay for hydrolyzing activity of enzyme*

140

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

145 of laminaribiose-hydrolyzing activity was defined as the amount of enzyme that  
146 liberates 2.0  $\mu\text{mol}$  glucoses per min since two moles of glucose are produced from one  
147 mole of laminaribiose. If laminaritriose or larger substrates were used as substrates, one  
148 unit of activity was defined as the amount enzyme that liberates 1.0  $\mu\text{mol}$  glucoses per  
149 min. Hydrolyzing activity for the  $\beta$ -*p*NP-derivatives was assayed in a reaction mixture  
150 containing 2.5 mM *p*NP-derivatives and 10 mM sodium phosphate (pH 5.5) and 0.5 –  
151 1.0 mU/mL of enzyme at 30°C. *p*-Nitrophenol (*p*NP) released by the degradation of  
152  $\beta$ -*p*NP derivatives was determined with the molar extinction coefficient  $1.81 \times 10^5 \text{ M}^{-1} \cdot$   
153  $\text{cm}^{-1}$  at 410 nm. One unit of  $\beta$ -*p*NP derivative-degrading activity was defined as the  
154 amount of enzyme that released 1.0  $\mu\text{mol}$  *p*NP per min. pH dependence of the enzyme  
155 was measured at 30°C in reaction mixtures containing 5 mg/ml laminaribiose, 50 mM  
156 sodium citrate buffer (pH 3.5 – 6.0) and 50 mM sodium phosphate buffer (pH 5.5 – 8.1).  
157 Temperature dependence was measured at 4 – 70°C in a reaction mixture containing 10  
158 mM sodium phosphate buffer (pH 5.5). Thermal stability of the enzyme was assessed by  
159 measuring the activity remaining after the heat-treatment at 4 – 70°C for 15 min. All  
160 assays were triplicated and the data were indicated as average values with standard  
161 deviations.

162

163 2.4. Assay for transglycosylation activity of enzyme

164

165 Transglycosylation activity of laminaribiose-hydrolyzing enzyme was assayed at  
166 30°C in a reaction mixture containing 36.5 mM L2 (a donor substrate), 12 mM L3 (an  
167 acceptor substrate), 10 mU of enzyme and 10 mM sodium phosphate buffer (pH 5.5).  
168 The transglycosylation reaction was terminated by the addition of an equal volume of  
169 10% trichloroacetic acid. The reaction products were analyzed by thin-layer  
170 chromatography (TLC).

171

172 2.5. TLC

173

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

180

181 *2.6. SDS-polyacrylamide gel electrophoresis*

182

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.

191

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

193

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

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

201

## 202 *2.8. Determination of protein concentration*

203

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

206

## 207 **3. Results**

208

### 209 *3.1. General properties of laminaribiose-hydrolyzing enzyme*

210

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

218

### 219 3. 2. *Substrate specificity of AkLab*

220

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 ( $\beta$ -1,4-glucoside), gentiobiose ( $\beta$ -1,6-glucoside) and lactose  
229 ( $\beta$ -1,4-galactoside), but not  $\alpha$ -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  $\beta$ -D-glucoside and  
232 low activity toward *p*NP  $\beta$ -D-galactoside, but practically no activity toward other  $\beta$ -*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  $\beta$ -glucosidase-like

235 enzyme with high preference to laminaribiose.

236

### 237 3. 3. *Transglycosylation activity of AkLab*

238 As shown in Fig. 3A, substantial amounts of oligosaccharides larger than  
239 original substrate L2 were found to be produced during hydrolysis of L2. This strongly  
240 suggested that AkLab catalyzed transglycosylation among L2 molecules and produced  
241 such larger oligosaccharides. We then examined how large oligosaccharides can be  
242 produced by the transglycosylation activity of AkLab using L2 and L3 as a donor and an  
243 acceptor substrate since L2 is preferable substrates of AkLab and L2 and L3 are  
244 distinguishable in TLC. As shown in Fig. 5, L4 was readily produced in the early stage  
245 of reaction (0.3 – 1.0 h). Upon extension of reaction time to 4 – 24 h, oligosaccharides  
246 L4 – L7 appeared. During the reaction, glucose was produced concomitantly with the  
247 rapid decrease in L2 and moderate decrease in L3. These results strongly suggested that  
248 AkLab transferred glucose unit of L2 to L3 by the transglycosylation activity and  
249 produced glucose and oligosaccharides larger than L3. Interestingly, the  
250 oligosaccharides thus produced were considerably stable, i.e., they accumulated in the  
251 latter phase of reaction. This result can be interpreted by the substrate specificity of  
252 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  $\beta$ -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.

257

### 258 3. 4. *Partial amino-acid sequence of AkLab*

259

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  $\beta$ -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  $\beta$ -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- $\beta$ -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  $\beta$ -glucosidase from *Corbicula japonica* (Sakamoto et al., 2009). These results indicated  
274 that AkLab is also a member of GHF1.

275

## 276 **4. Discussion**

277

### 278 *4.1. Physiological roles of AkLab in A. kurodai*

279

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  $\beta$ -1,3-glucanase (AkLam36 and AkLam33, respectively)  
288 from the digestive fluid of sea hare *A. kurodai* (Kumagai and Ojima, 2010). Although

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

302

#### 303 4. 2. Similarity of AkLab to other enzymes

304

305 The molecular mass of AkLab, i.e., 110 kDa estimated by SDS-PAGE, was  
306 similar to those of exo- $\beta$ -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  $\beta$ -glucosidase from a marine mollusk *Corbicula japonica* (100 kDa)  
309 (Sakamoto et al., 2009). Optimum temperature and pH of AkLab, 50°C and pH 5.5,  
310 were also similar to those of *H. pomatia* (50°C and pH 5.5) and *E. maakii* (55°C and pH  
311 5.2) (Marshall and Grand, 1975; Elyakova and Shirokova, 1997). AkLab most rapidly  
312 hydrolyzed  $\beta$ -1,3-glucoside, e.g., laminaribiose, and slowly  $\beta$ -1,4-glucoside (cellobiose)  
313 and  $\beta$ -1,6-glucoside (gentiobiose) (Fig. 4, Table 2 and 3).  $\beta$ -pNPG was also a preferable  
314 substrate for AkLab (Table 4); however, laminarin was not degraded (data not shown).  
315 The exo- $\beta$ -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- $\beta$ -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,  $\beta$ -glucosidase is known  
321 to show broad substrate specificity and hydrolyze various  $\beta$ -glucosides (Hrmova and  
322 Fincher, 2007; Pesentseva et al., 2008). For example, barley  $\beta$ -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,

325 we concluded that AkLab is a  $\beta$ -glucosidase-like laminarinase-hydrolyzing enzyme.

326

#### 327 4. 3. *Transglycosylation activity of AkLab*

328

329 AkLab produced oligosaccharides larger than original substrates by its  
330 transglycosylation activity. Transglycosylation activity is known as a characteristic  
331 property of glycosyl hydrolases that split glycoside linkage with a retaining manner  
332 (Wang and Huang, 2009). Transglycosylation usually takes place in accordance with the  
333 specificity of the enzymes toward the configuration of glycosyl linkage. However, the  
334 glycosyl linkages formed by  $\alpha$ -glucosidase were not completely consistent with the  
335 hydrolytic specificity (Kato et al., 2002). Namely, not only  $\alpha$ -1,4-linked  
336 oligosaccharides but also  $\alpha$ -1,6-linked oligosaccharides were produced by the  
337 transglycosylation. Laminarinase hydrolyzes mainly  $\beta$ -1,3-glycosyl linkage; however, it  
338 can hydrolyze  $\beta$ -1,4- and  $\beta$ -1,6-linkages adjacent to  $\beta$ -1,3-linkages in certain conditions.  
339 Therefore, AkLab may also produce oligosaccharides possessing different  
340 configurations of glycosyl linkages. Actually some spots with different mobility from  
341 marker sugars were observed between L2 and L3, and below L4 in TLC (Fig. 5). In  
342 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  $\beta$ -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- $\alpha$  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.,  $\beta$ -1,3-linked glucose main chain with  $\beta$ -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 GHF1 on the basis of partial amino-acid  
360 sequences. We are now analyzing the entire primary structure of AkLab by the cDNA

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

363

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368

#### 369 **References**

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### 539 **Figure Legends**

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

541 (A) Fractions showing laminaribiose-hydrolyzing activity in TOYOPEARL  
542 Phenyl-650M chromatography were pooled and dialyzed against 10 mM sodium  
543 phosphate buffer (pH 7.0), then applied to a column of TOYOPEARL DEAE-650M (2  
544 × 45 cm) pre-equilibrated with the same buffer. (B) Fractions showing  
545 laminaribiose-hydrolyzing activity in TOYOPEARL DEAE-650M column  
546 chromatography were concentrated to less than 2 ml, then applied to Superdex 200  
547 10/300 GL column pre-equilibrated with 10 mM sodium phosphate buffer (pH 6.0)  
548 containing 300 mM NaCl. (C) Monitoring of purification of AkLab by SDS-PAGE. M,  
549 marker proteins; A, the sample after TOYOPEARL Phenyl-650M chromatography; B,  
550 the sample after TOYOPEARL DEAE-650M chromatography; C, the sample after  
551 Superdex 200 10/300 GL chromatography.

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553 Fig. 2. Optimum temperature and pH, and thermostability of AkLab. A, Optimum  
554 temperature of AkLab was measured at 4 – 70°C in a reaction mixture containing 5  
555 mg/mL of laminaribiose. B, Optimum pH of AkLab was measured at 30°C in the  
556 reaction mixtures adjusted to pH 3.5 – 6.0 with 50 mM sodium citrate buffer and pH  
557 6.0-8.1 with 50 mM sodium phosphate buffer. C, Thermostability of AkLab was  
558 assessed by measuring the activity remaining after the incubation of enzyme at 4-70°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°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.

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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  $\mu$ L of the mixture was applied to TLC. The  
583 abbreviations used are the same as in Fig. 3.

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595 Table 1. Purification of AkLab.

596		Total	Specific	Total	Purifi-	Yield
597		protein	activity	activity	cation	
598	Samples	(mg)	(U/mg)	(U)	(fold)	(%)
599	Crude	3493	0.031	107	1	100
600	AS <sup>*1</sup>	2631	0.065	172	2.1	161
601	Phenyl <sup>*2</sup>	380	0.076	28.8	2.5	27
602	DEAE <sup>*3</sup>	20	0.89	17.7	29	17
603	Superdex <sup>*4</sup>	1.62	3.4	5.5	110	5.1

604 <sup>\*1</sup>Fraction precipitated between 40 and 60% saturation of ammonium sulfate. <sup>\*2</sup>Active  
605 fraction obtained by TOYOPEARL Phenyl-650M chromatography. <sup>\*3</sup>Active fraction  
606 obtained by TOYOPEARL DEAE-650M chromatography. <sup>\*4</sup>AkLab purified by  
607 Superdex 200 gel-filtration.

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613 Table 2. Relative activity of AkLab toward laminarioligosaccharides.

614	Substrate	Specific activity (mU/mg)	Relative activity (%)
615	laminaribiose	3400 ± 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 μmol 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.

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

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

639 \*One unit of activity was defined as the amount of enzyme that produces 2  $\mu$ 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.

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649 Table 4. Substrate specificity of AkLab

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

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

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667 Fig. 1.

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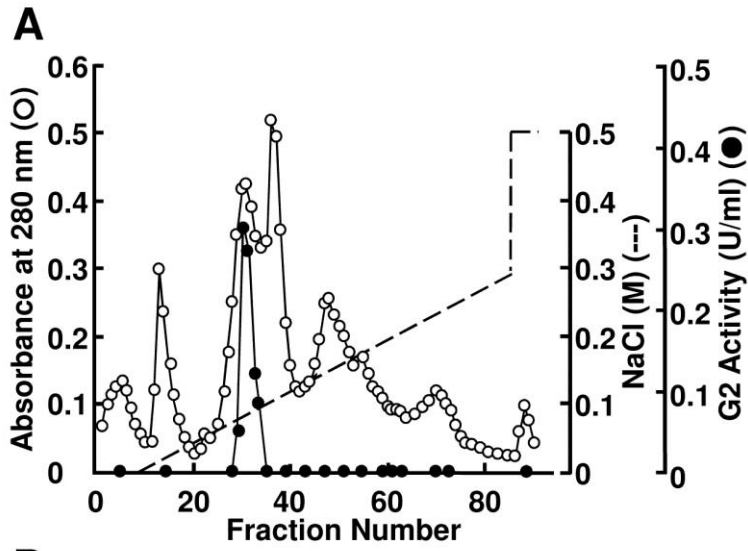
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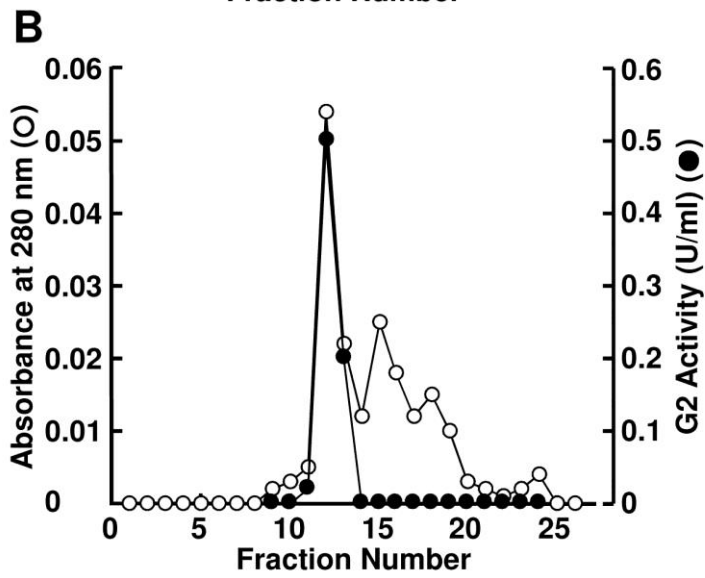
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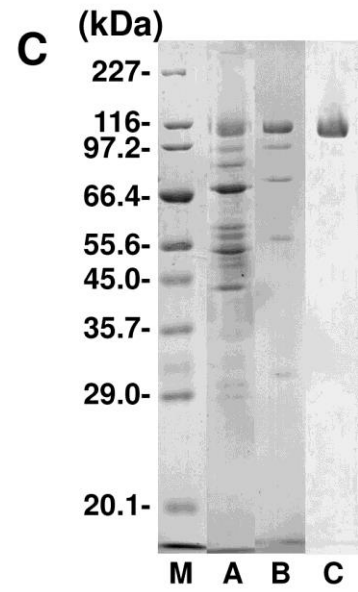


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685 Fig. 2.

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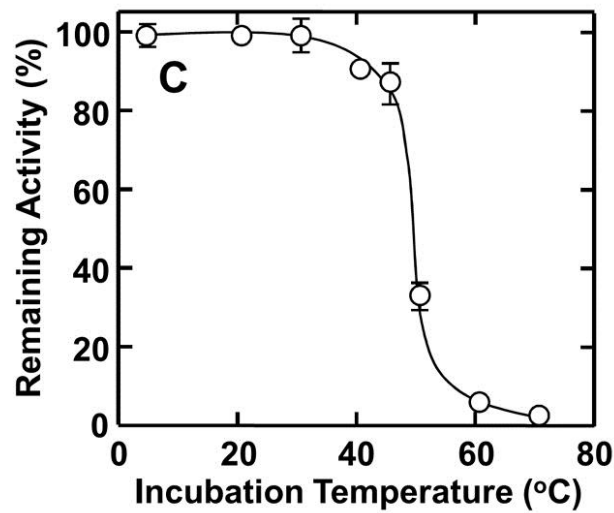
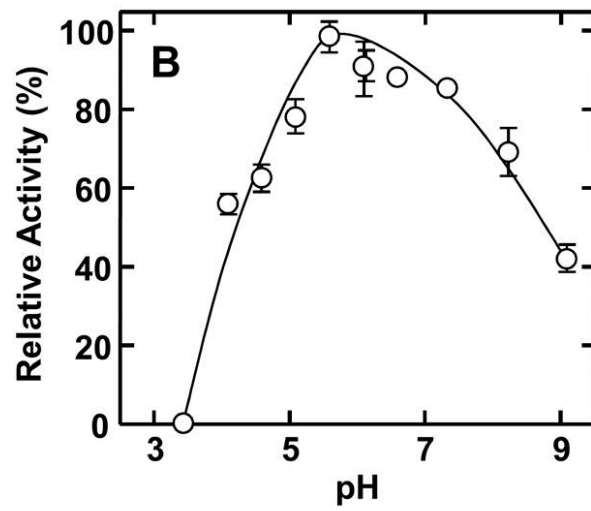
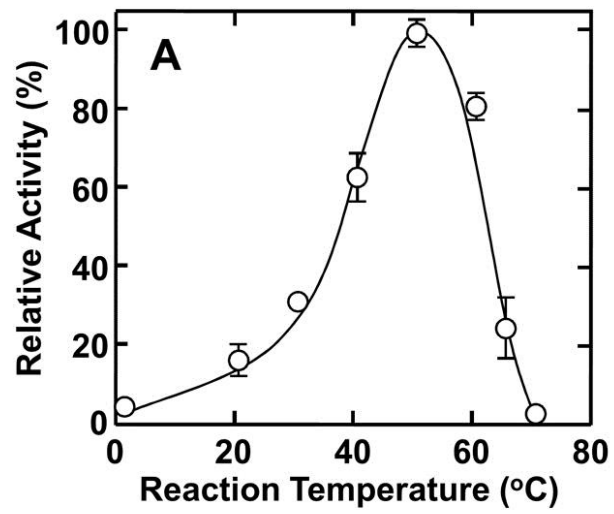
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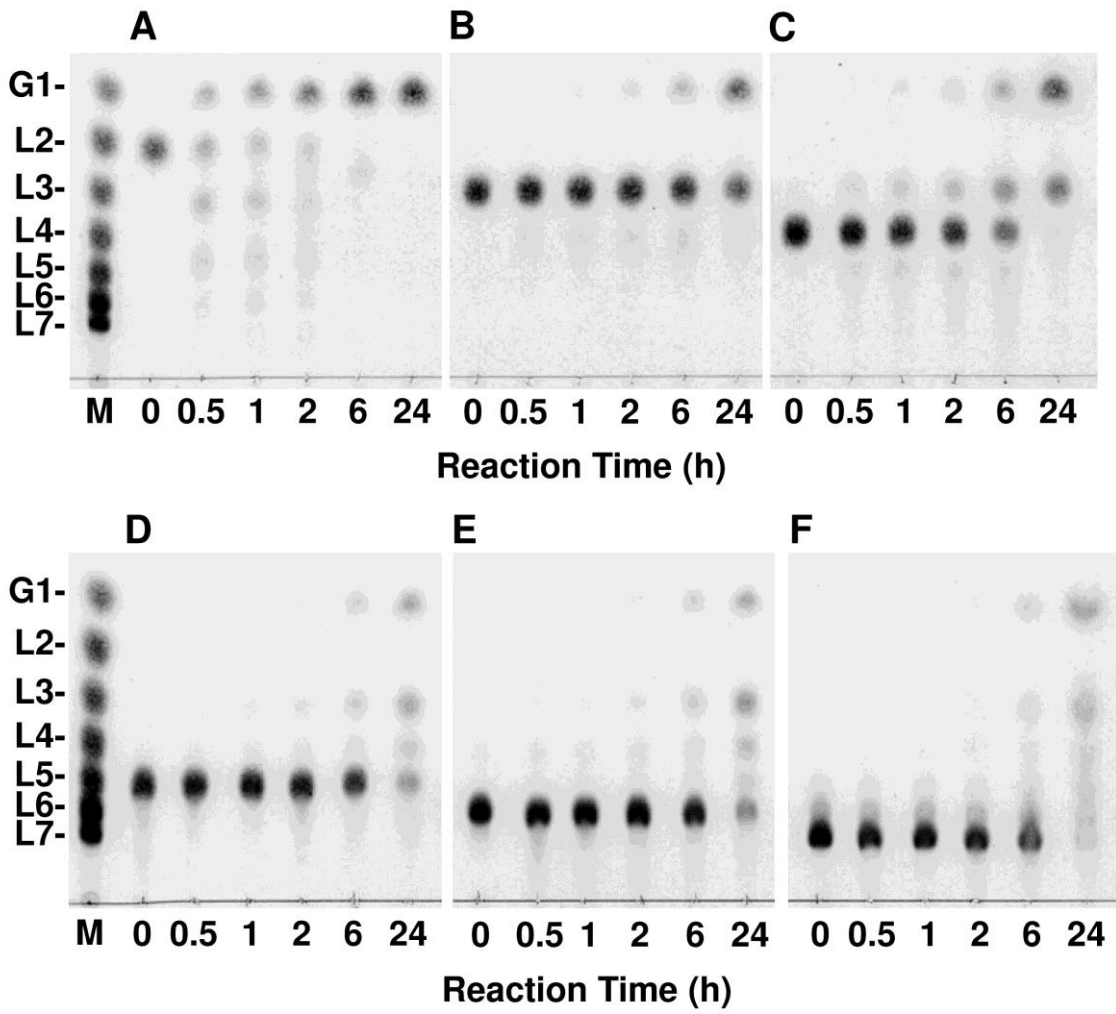
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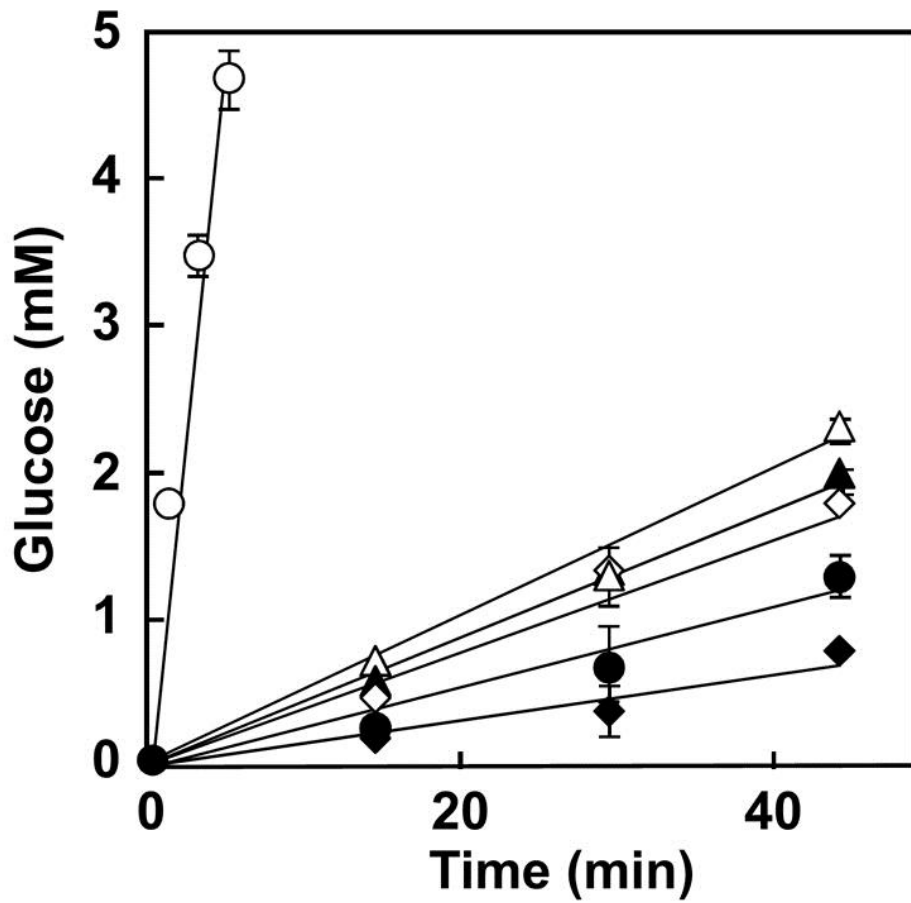
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739 Fig. 5.

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