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1 Heat treatment of curdlan enhances the enzymatic production of biologically active  
2  $\beta$ -(1,3)-glucan oligosaccharides

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18 Keywords: GH64;  $\beta$ -(1,3)-glucan oligosaccharides; curdlan

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24 Abstract: Biologically active  $\beta$ -(1,3)-glucan oligosaccharides were prepared from  
25 curdlan using GH64 enzyme (KfGH64). KfGH64 showed low activity toward native  
26 curdlan; thereby pretreatment conditions of curdlan were evaluated. KfGH64 showed  
27 the highest activity toward curdlan with heat treatment. The most efficient pretreatment  
28 (90 °C for 0.5 h) converted approximately 60% of curdlan into soluble saccharides  
29 under the optimized enzyme reaction conditions (pH 5.5, 37 °C, 100 rpm mixing speed,  
30 24 h, and 10  $\mu$ g of KfGH64/1 g of curdlan). The resulting products were predominantly  
31 laminaripentaose and a small amount of  $\beta$ -(1,3)-glucans with an average degree of  
32 polymerization (DP) of 13 and 130. The products did not contain small oligosaccharides  
33 (DP<5), indicating that the hydrolysis of heat-treated curdlan by KfGH64 is a suitable  
34 method for the production of biologically active  $\beta$ -(1,3)-glucan oligosaccharides.

35

## 36 **1. Introduction**

37 Curdlan, which is industrially produced as an exopolysaccharide of bacterium, is a  
38 water insoluble linear  $\beta$ -(1,3)-glucan polysaccharide (Maeda, Saito, Masada, Misaki, &  
39 Harada, 1967). The characteristics of curdlan such as its structure and physical  
40 properties have been studied extensively. For example, it is soluble under alkali  
41 condition and forms a gel when it is neutralized (Kanzawa, Harada, Koreeda, & Harada,  
42 1987). Curdlan displays irreversible gelation upon heating (Harada, Misaki, & Saito,  
43 1968), which has been applied in the food industry as a stabilizer (Miwa, Nakao, &  
44 Nara, 1993). In addition, curdlan has prebiotic properties for increasing *Bifidobacterium*  
45 sp., although the prebiotic activity comes from  $\beta$ -(1,3)-glucan oligosaccharides, that is,  
46 curdlan hydrolysates (Shimizu et al., 2001). Therefore, curdlan is a good resource for  
47 the production of valuable materials.

48  $\beta$ -(1,3)-Glucan oligosaccharides have biological activity and have been used in  
49 new biomedical applications (Zhan, Lin, & Zhang, 2012). They showed induction of  
50 monocytes to produce tumor necrosis factor alpha (Miyanishi, Iwamoto, Watanabe, &  
51 Oda, 2003), stimulation of the secretion of interleukin 1 $\beta$  (Jamois et al., 2005), and  
52 enhancement of defense responses in tobacco (Klarzynski et al., 2000; Fu et al., 2011).  
53 A few researches has revealed that oligosaccharides with a degree of polymerization  
54 (DP)>4 possess biological activity and work as bioactive materials (Miyanishi et al.,  
55 2003; Jamois et al., 2005). The preparations of oligosaccharides from curdlan by acid or  
56 enzyme degradation have been reported; however, the hydrolysis rate was low value of  
57 10-16% (Wu et al., 2009; Qian, Wu, Pan, & Xia, 2012; Li et al., 2013) or the main  
58 hydrolysis products possessed DP less than 5 (Kusama, Kusakabe, Zama, Murakami, &  
59 Yasui, 1984; Pang, Otaka, Suzuki, Goto, & Ohnishi, 2004). Degradation of  
60 alkali-formed curdlan gel by a fungus enzyme cocktail was successful in a high  
61 hydrolysis rate; however, the hydrolytic products were mainly small size  
62 oligosaccharides with DP<5 (Grandpierre, Janssen, Laroche, Michaud, & Warrand,  
63 2008; Fu et al., 2015). Therefore, the preparation large quantities of  $\beta$ -(1,3)-glucan  
64 oligosaccharides with a DP>4 would be much in demand.

65  $\beta$ -(1,3)-Glucan hydrolases are classified into glycoside hydrolase families (GH)  
66 16, 17, 55, 64, 81, and 128 (CAZy: [www.cazy.org](http://www.cazy.org)). Most  $\beta$ -(1,3)-glucan hydrolases are  
67 a member of GH16, but their hydrolysates are glucose and small size of  
68 oligosaccharides (DP 2–4) (Gueguen, Voorhorst, van der Oost, & de Vos, 1997;  
69 Kumagai & Ojima, 2009; Kumagai & Ojima, 2010). Therefore, hydrolases other than  
70 the GH16 enzymes are required for the production of long oligosaccharides. GH64,  
71 which is known as a family of yeast lytic enzyme, produced laminaripentaose (L5) and

72 did not produce small size oligosaccharides with DP less than 5 (Kitamura & Yamamoto,  
73 1972; Doi, Doi, Ozaki, & Fukui, 1973; Doi & Doi, 1986; Nakabayashi et al., 1998).  
74 This characteristic is suitable for producing oligosaccharides with comparatively large  
75 chain lengths; however, hydrolysis activity toward insoluble curdlan is considerably low  
76 compared with soluble substrates like laminarin. In this study, we cloned and expressed  
77 GH64 enzyme from *Kribbella flavida* NBRC 14399 (KfGH64) and investigated the  
78 suitable pretreatment conditions of curdlan and hydrolysis conditions of KfGH64 for  
79 producing biologically active  $\beta$ -(1,3)-glucan oligosaccharides. Furthermore, we  
80 evaluated the hydrolysis products obtained from our new reaction system.

81

## 82 **2. Material and methods**

### 83 **2.1. Materials**

84 Curdlan, with a molecular weight of  $8.1 \times 10^4$  Da, was purchased from Wako  
85 Pure Chemicals Industries Ltd. (Osaka, Japan); laminarin (*Laminaria digitata*) and  
86  $\beta$ -(1,3)-glucan (*Euglena gracilis*) were from Sigma-Aldrich Corp. (St. Louis, MO,  
87 USA); laminaran (*Eisenia bicyclis*) and glucan (black yeast) were from Tokyo Kasei  
88 Kogyo (Tokyo, Japan); laminaribiose–laminariheptaose were from Seikagaku Kogyo  
89 (Tokyo, Japan);  $\beta$ -glucan (barley) was from Megazyme International Ireland Ltd. (Bray,  
90 Ireland).

91

### 92 **2.2. Construction, expression, and purification of KfGH64**

93 The gene encoding L5-producing enzyme, *Kfgh64* (GenBank accession  
94 number: CP001736), was amplified by polymerase chain reaction (PCR) using genomic  
95 DNA of *K. flavida* NBRC 14399 as a template and a set of primers:

96 5'-CATATGGGGCTGACCGCCACCGTCT-3' (forward) and  
97 5'-AAGCTTTGAGTTGAACGGGTCGAGCGT-3' (reverse), where underlines indicate  
98 the restriction enzyme sites. Then, the *Kfgh64* gene was cloned into the *NdeI-HindIII*  
99 site of pET28a to construct an expression vector of pET28a(KfGH64). The recombinant  
100 protein was produced in *Escherichia coli* BL21-RIL (DE3) cells (Agilent Technologies,  
101 Palo Alto, CA, USA) harboring the pET28a(KfGH64), and purified as previously  
102 described (Kumagai, Kawakami, Mukaihara, Kimura, & Hatanaka, 2012). The protein  
103 concentrations were determined by the Bradford method (Bradford, 1976) using bovine  
104 serum albumin as the standard.

105

### 106 **2.3. KfGH64 activity assay**

107 KfGH64 activity was determined at 45 °C for 10 min in a reaction mixture (0.1  
108 mL) containing an appropriate amount of enzyme, 2% (w/v) laminarin, and 50 mM  
109 sodium acetate (pH 5.5). The amount of reducing sugars was determined using the  
110 bicinchoninic acid method (Waffenschmidt & Jaenicke, 1987). One unit of activity was  
111 defined as the amount of enzyme that liberated reducing sugars equivalent to 1.0 μmol  
112 glucose per minute. The  $V_{max}$  and  $K_m$  toward laminarin (0.5–40 mg/mL) were  
113 determined by the standard Michaelis–Menten equation using nonlinear regression  
114 (Origin Software, Lightstone Corp., Tokyo, Japan). All activity assays were performed in  
115 triplicate.

116

### 117 **2.4. Procedures for the pretreatment of curdlan**

118 Curdlan was pretreated using five procedures as follows. 1) Non-treatment as a  
119 control; 2) Alkali-neutralization–homogenization: 1 g of curdlan was slowly added into

120 100 mL of 1 M NaOH and mixed using a magnetic stirrer below 20 °C for 6 h. Then the  
121 solution was neutralized at pH 7.0 using 0.1 N HCl and insoluble curdlan was collected  
122 by centrifugation at 5,000×g for 20 min. The precipitate was washed three times with  
123 water and then homogenized (Li et al., 2013); 3) Alkali-neutralization: 1 g of curdlan  
124 was solubilized in 100 mL of 1% NaOH (pH 11) and vigorously mixed using a  
125 magnetic stirrer for 10 min, and then the pH was adjusted to 5.5 with acetic acid  
126 (Grandpierre et al., 2008); 4) Alkali-neutralization–heat treatment: 1 g of curdlan was  
127 dissolved in 30 mL of 0.1 M sodium phosphate (pH 10.5) and stirred at 30 °C for 30  
128 min. The solution was neutralized by 0.1 N HCl. After centrifugation (8,000×g for 20  
129 min), the insoluble curdlan was re-suspended in 20 mL of water and heated at 70 °C for  
130 2 h (Wu et al., 2009); 5) Heat treatment: 1 g of curdlan was dissolved in 20 mL of water  
131 and heated at 70 °C for 2 h.

132 Hydrolysis was performed in 1% or 2% pretreated curdlan and 20 µg/mL  
133 KfGH64 at 37 °C, pH 5.5, and 100 rpm mixing speed. An aliquot of the reaction  
134 solution was collected at each reaction time, and then KfGH64 was denatured by  
135 heating at 100 °C for 10 min. The supernatant was recovered by centrifugation at  
136 13,000×g for 20 min. The total amount of soluble sugar in the supernatant was  
137 evaluated by the phenol-sulfuric acid method (Masuko et al., 2005). The hydrolysis rate  
138 was determined by the following equation: hydrolysis rate=the amount of soluble  
139 saccharide in supernatant/the amount of curdlan used for reaction×100. Resultant  
140 hydrolysis products were mixture of DP-distinct carbohydrates, so that we measured  
141 their average DP from the following equation: average DP=the amount of total sugar/the  
142 amount of reducing power (both amounts were equivalent to µmol of glucose), and  
143 learned the approximate DP size of product.

144

145 ***2.5. Detailed heat treatment conditions for the pretreatment of curdlan***

146 The effects of temperature (50, 70, 90, or 100 °C), heating time (0.5, 2.0, or 4.0  
147 h), and curdlan concentration (1.0, 2.5, or 5.0%) on the heat-treatment procedure  
148 (method 4 in section 2.4) were investigated. The hydrolysis rate was estimated using 1%  
149 the heat-treated curdlan.

150

151 ***2.6. Effects of curdlan concentration and amount of KfGH64***

152 Curdlan (1 g), dissolved in 20 mL of water, was heated at 90 °C for 0.5 h  
153 (optimized condition), and used for the following analyses. To evaluate the effect of  
154 curdlan concentration on KfGH64 activity, 20 µg/mL KfGH64 was reacted with 0.5,  
155 0.75, and 1.0% of curdlan. For investigating the influence of enzyme amount on curdlan  
156 hydrolysis, 2.5, 10, and 20 µg/mL KfGH64 was incubated with 1% curdlan.

157

158 ***2.7. Evaluation of hydrolysis product***

159 The distribution of hydrolysis products in the supernatant of the reaction  
160 mixture was analyzed using high performance liquid chromatography (HPLC) with a  
161 Superdex Peptide 10/300 GL column (GE Healthcare UK Ltd., Little Chalfont, UK) and  
162 Corona Charged Aerosol Detector (Thermo Scientific Inc., Chelmsford, MA, USA). The  
163 sample prepared by the optimized enzyme reaction condition were eluted using water  
164 with a flow rate of 0.3 mL/min. Pullulan (Shodex Standard P-82: Showa Denko K.K.,  
165 Tokyo, Japan) was used as the standard of molecular mass. The distribution of  
166 oligosaccharides was analyzed using high-performance anion-exchange chromatography  
167 with pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale, CA, USA). A



168 Carbopac PA1 column (4×250 mm) (Dionex) with an isocratic flow of 640 mM NaOH  
169 and 70 mM sodium acetate was used for separation. Glucose and  
170 laminaribiose–laminarihexaose were used as standards for HPAEC-PAD analysis.

171

### 172 **3. Results and Discussion**

#### 173 **3.1. Biochemical properties of KfGH64**

174 KfGH64 was produced in an *E. coli* expression system and isolated using a  
175 TALON affinity resin (Takara Bio, Otsu, Japan). Purified enzyme (40 mg) was obtained  
176 from one liter of culture medium, and showed a single band of approximately 43 kDa  
177 on SDS-PAGE. The optimum temperature and pH were 45 °C and 5.5, respectively.  
178 KfGH64 was stable at pH 4.0–7.0 after treatment at each pH and 4 °C for 24 h. The  
179 temperature required for the half inactivation of the hydrolysis activity of KfGH64 at 30  
180 min was 45 °C. For the hydrolysis of curdlan, the long-term thermal stability was  
181 evaluated. KfGH64 was stable at 37 °C for 4 days and maintained 80% of its original  
182 activity at 40 °C for 4 days. KfGH64 showed activity for laminarin (100%), heat-treated  
183 curdlan (89%), non-treated curdlan (1.0%), and  $\beta$ -(1,3)-glucan from *E. gracilis* (0.4%)  
184 (the percentage in parenthesis is the relative initial velocity toward laminarin as 100%),  
185 and did not show any activity for laminaran from *E. bicyclis*, glucan from black yeast,  
186 or  $\beta$ -glucan from barley. The  $V_{\max}$  and  $K_m$  toward laminarin were  $442 \pm 32$   
187 ( $\mu\text{mol}/\text{mg}/\text{min}$ ) and  $21.1 \pm 3.2$  (mg/mL), respectively. The enzymatic properties of  
188 KfGH64 were almost same as those reported for GH64 enzymes (Doi, Doi, & Fukui,  
189 1973; Palumbo, Sullivan, & Kobayashi, 2003; Wu et al., 2009; Woo, Kang, Woo, & Lee,  
190 2014).

191

### 192 **3.2. Hydrolysis of Curdlan by KfGH64**

193 Alkali and/or heat-treatment curdlans were hydrolyzed by KfGH64. KfGH64 was  
194 incubated with 1.0 and 2.0% pretreated curdlan at 37 °C and pH 5.5 with shaking at 100  
195 rpm. At the indicated time, the hydrolysis rate of the curdlans was estimated from the  
196 amounts of carbohydrates in soluble fraction. The hydrolysis rates of non-heat-treated  
197 curdlans (methods 1, 2, and 3 in section 2.4) were around 3%, indicating that there were  
198 rarely different in alkali-treated and non-treated curdlan. On the other hand, the  
199 hydrolysis rates of the heat-treated curdlans (methods 4 and 5 in section 2.4) were 33  
200 and 43% for 1.0% curdlan, and 21 and 30% for 2.0% curdlan, respectively (Fig. 1).  
201 These results indicated that heat treatment was effective for increasing in the hydrolysis  
202 rate of curdlan by KfGH64. Method 5 being more effective than method 4, the treatment  
203 conditions for heating temperature, heating time, and the concentration of curdlan  
204 treated were investigated. The hydrolysis rate at 24 h-reaction of KfGH64 reached 60%  
205 by heating over 90 °C for 2 h (Fig. 2A), while it was unaffected by the heating time and  
206 the concentration of curdlan treated (Figs. 2B and 2C). We then examined effects of the  
207 concentrations of the curdlan and KfGH64 on the hydrolysis rate. Hydrolysis rates were  
208 almost similar within the range of 0.5–1.0% pretreated curdlan (Fig. 3A) that of 10–20  
209 µg/mL KfGH64 (Fig. 3B). Collectively, 1.0 mg of KfGH64 was enough for hydrolysis  
210 of 1 g of adequately pretreated curdlan.

211 Combination of the alkali-neutralization-treated curdlan gel (method 3 in section  
212 2.4) and hydrolysis using a fungus enzyme cocktail succeeded in the complete  
213 degradation of curdlan; however, most of the hydrolytic products were glucose and  
214 small size oligosaccharides with DP less than 5 (Grandpierre et al., 2008). Activity of  
215 KfGH64 toward curdlan prepared by method 3 was low (Fig. 1). It is thought that

216 hydrolysis rate of the enzymes depends on curdlan conformations. The gel  
217 conformations were different after heat treatment and alkali treatment (Zhang & Edgar,  
218 2014). The characteristic of the alkali-treated curdlan was similar to that of low-set gel  
219 (Kanzawa et al., 1987). The conformation of curdlan at room temperature is a mixture  
220 of single helix and loose triple helices (Okuyama et al, 1991; McIntosh, Stone, &  
221 Stanisich, 2005). By heating at over 80 °C, curdlan generates thermal irreversible gels  
222 (high-set gels), which are triple-stranded helices (Harada, Masada, Fujimori, & Maeda,  
223 1966; Chuah, Sarko, Deslandes, & Marchessault, 1983). The hydrolysis rate of KfGH64  
224 was increased in heat-treated curdlan (from 3% to 60%), indicating that KfGH64  
225 recognize the triple-stranded helices curdlan.

226       The hydrolysis rate of KfGH64 reached a plateau around 60% (first  
227 heating/digestion). We then attempted to further hydrolysis by the second-pretreatment  
228 of curdlan and enzyme digestion. After the first heating/digestion, the reaction solution  
229 was centrifuged at 8,000×g for 20 min to remove the supernatant (containing hydrolysis  
230 products and KfGH64), and the pellet was washed with the reaction buffer two times.  
231 Resultant pellet, suspended in the reaction buffer, was heated again at 90 °C for 0.5 h,  
232 and new KfGH64 was added (second heating/digestion). The second attempt increased  
233 the hydrolysis rate by 10% (total 70%). No degradation occurred in the first  
234 heating/digestion sample without heat treatment (data not shown). High-set gels consist  
235 of single chains interconnected with more than one triple helix (McIntosh, Stone, &  
236 Stanisich, 2005). The second heat-treatment may rebuild triple helices insusceptible to  
237 KfGH64 hydrolysis. The curdlan conformation after heating was related to its DP;  
238 heated curdlan formed fibrils, but small DP curdlan (DP 49) formed a lamellar structure  
239 (Harada, Koreeda, Sato, & Kasai, 1979), whereas disordered structures were obtained

240 with short DP <25 curdlan (Ogawa & Tsurugi, 1973). The product of the first digestion  
241 was saccharides of DP 13 and 130 (see section 3.3), which generated disordered and  
242 lamellar forms, respectively, resulting in a decrease of the hydrolysis rate by the second  
243 KfGH64 digestion. The third heating/digestion raised the hydrolysis rate by less than  
244 5%. The maximum hydrolysis rate in this study, which remained at 75% and did not  
245 reach 100%, suggested that the residual 25% curdlan formed a single helix and loose  
246 triple helices that were resistant to attack by KfGH64.

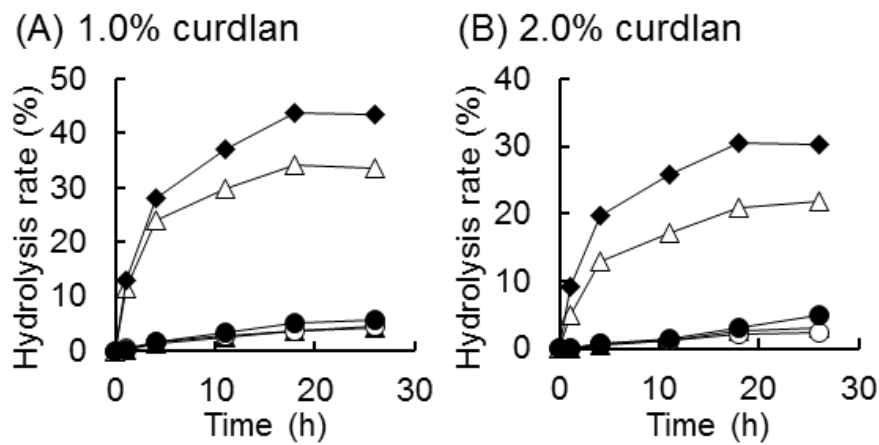


Figure 1 Kumagai et al

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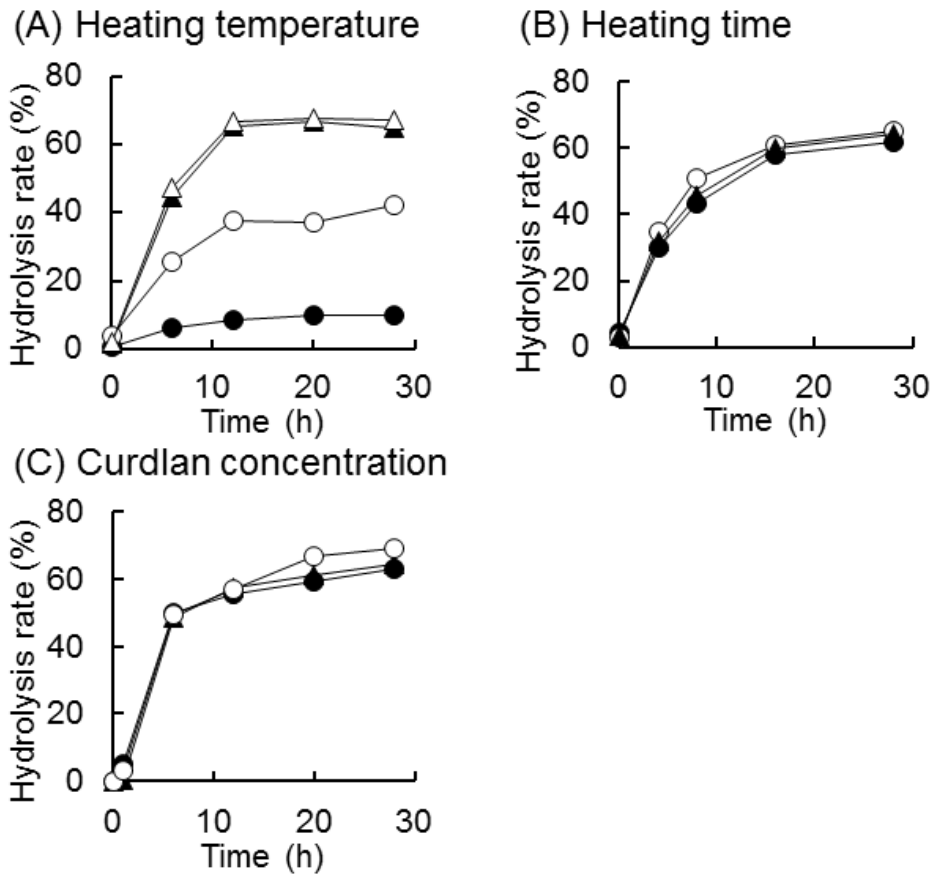


Figure 2 Kumagai et al

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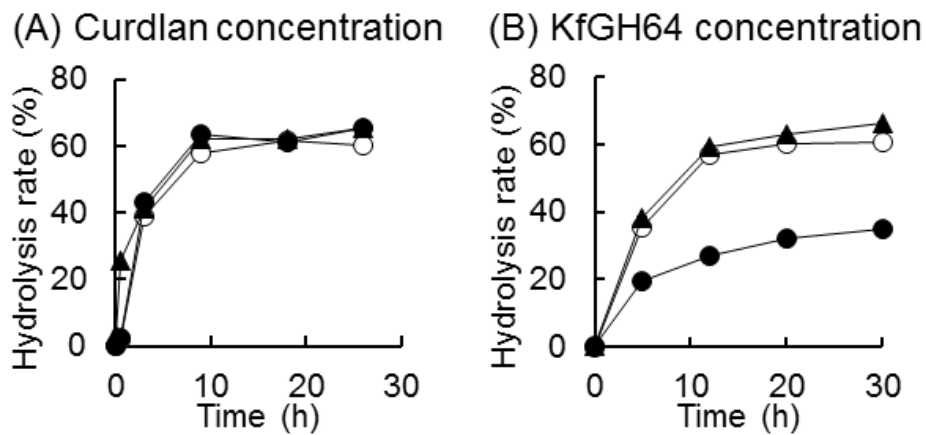


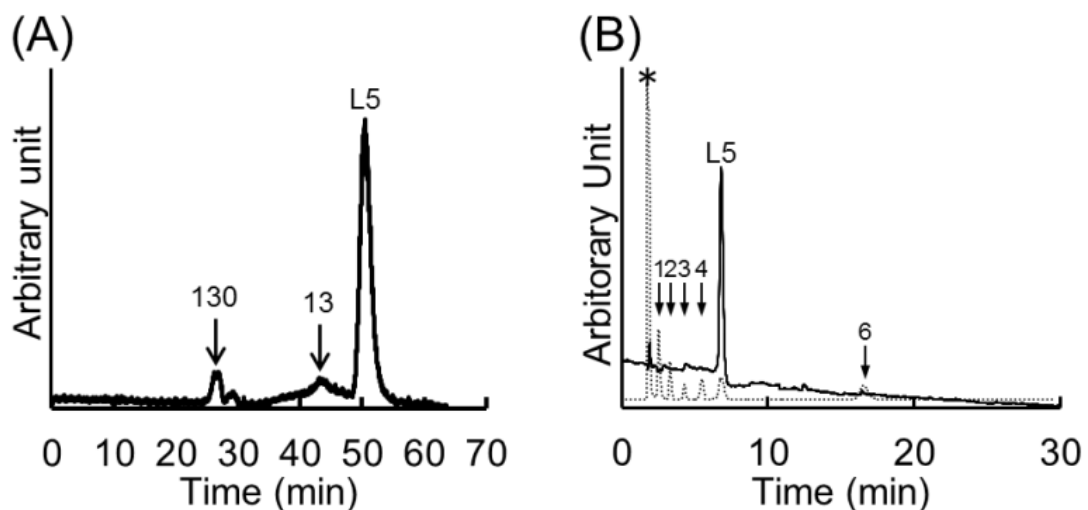
Figure 3 Kumagai et al

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252

253 *3.3. Evaluation of the hydrolysis products of curdlan*

254 The hydrolysis products of curdlan prepared by the optimized enzyme reaction  
255 condition were analyzed by gel-filtration and HPAEC-PAD. The gel-filtration analysis  
256 revealed that the main product was L5, and  $\beta$ -(1,3)-glucans with an average DP of 13  
257 and 130 were found (Fig. 4A). From the peak areas, the ratio of L5 was estimated to be  
258 approximately 80%, indicating that 0.6 g of L5 was obtained from 1 g of curdlan.  
259 HPAEC-PAD analysis revealed that glucose and small oligosaccharides (DP 2-4) were  
260 not found (Fig. 4B). The average DP of the hydrolysis products was estimated to be 7.9  
261 from chemical approach. The estimated average DP (7.9) was higher than the DP of L5,  
262 because of the coexistence of large  $\beta$ -(1,3)-glucans with an average DP of 13 and 130.  
263 The  $^1\text{H-NMR}$  spectra of the hydrolysis products were almost identical to those of  
264 standard L5 (Fig. S1), supporting that L5 was a main component. These hydrolysis  
265 products have a suitable chain length for biologically active materials.



266 Figure 4 Kumagai et al  
267

#### 268 4. Conclusions

269 Curdlan conformations affected the enzyme activity. Heat treatment converted

270 curdlan conformation into rod-like triple helices. The Rod-like triple helices structure of  
271 curdlan increased in hydrolysis rate by KfGH64. The combination of heat treatment and  
272 KfGH64 hydrolysis of curdlan was a superior approach for producing biologically  
273 active  $\beta$ -(1,3)-glucan oligosaccharides with high yield: 1 g of curdlan was hydrolyzed  
274 by 1.0 mg of KfGH64 at a hydrolysis rate of 60% in 24 h. The main hydrolysis product  
275 was L5, and the average DP of the products was 7.9. Furthermore, heat treatment is a  
276 simple method, which does not require any post-treatment using chemicals, e.g.,  
277 neutralization with acid.

278

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284

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399

#### 400 **Figure legends**

401 Figure 1. Effect of pretreatment of curdlan on the hydrolysis rate. Hydrolysis with 20  
402  $\mu\text{g/mL}$  KfGH64 was performed in 1.0% (A) and 2.0% (B) curdlan pretreated using five  
403 procedures: ●, non-treatment; ○, alkali-neutralization–homogenization; ▲,  
404 alkali-neutralization;  $\Delta$ , alkali-neutralization–heat treatment; and ◆, heat treatment.  
405 The pretreatment indicated by each symbol is explained in section 2.4.

406

407 Figure 2. Effect of heat treatment on the hydrolysis rate of curdlan by KfGH64. (A)  
408 Heating temperature. The heat-pretreatment was done for 2.0 h using 5.0% curdlan at a  
409 temperature of: ●, 50 °C; ○, 70 °C; ▲, 90 °C; and  $\Delta$ , 100 °C. (B) Heating time. 5.0%  
410 curdlan was heated at 90 °C for: ●, 0.5 h; ○, 2.0 h; and ▲, 4.0 h. (C) Concentration of  
411 curdlan. Curdlan of various concentrations: ●, 1.0%; ○, 2.5%; and ▲, 5.0%, was treated  
412 at 90 °C for 0.5 h. After pretreatment under each condition, 1% pretreated curdlan was  
413 hydrolyzed using 20  $\mu\text{g/mL}$  KfGH64 at 37 °C, pH 5.5, and 100 rpm mixing speed.

414

415 Figure 3. Effects of the concentration of curdlan and the amount of KfGH64 on the  
416 hydrolysis rate. (A) Concentration of curdlan. The pretreatment of 5.0% curdlan was  
417 performed at 90 °C for 0.5 h. After the heat treatment, ●, 0.5%; ○, 0.75%; and ▲, 1.0%  
418 of curdlan was hydrolyzed by 20 µg/mL KfGH64. (B) Amount of KfGH64. The  
419 hydrolysis of 1.0% pretreated curdlan was performed using: ●, 2.5 µg/mL; ○, 10 µg/mL;  
420 and ▲, 20 µg/mL KfGH64 at 37 °C, pH 5.5, and 100 rpm mixing speed.

421

422 Figure 4. Evaluation of the hydrolysis products. Hydrolysis products of curdlan by  
423 KfGH64 were analyzed by gel filtration (A) and HPAEC-PAD (B). The asterisk  
424 indicates the injection peak. The dotted line shows the peaks of standard sugars: glucose  
425 and laminarioligosaccharides (laminaribiose–laminarihexasaccharide). Numbers indicate the  
426 average DP of β-(1,3)-glucans (A) and DP of glucose and oligosaccharides (B).