# Heat treatment of curdlan enhances the enzymatic production of biologically active β-(1,3)-glucan oligosaccharides

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

## 1. Introduction

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

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

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

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

## 2. Material and methods

### *2.1. Materials*

Curdlan, with a molecular weight of $8.1\times10^4$ Da, was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan); laminarin (*Laminaria digitata*) and β-(1,3)-glucan (*Euglena gracilis*) were from Sigma-Aldrich Corp. (St. Louis, MO, USA); laminaran (*Eisenia bicyclis*) and glucan (black yeast) were from Tokyo Kasei Kogyo (Tokyo, Japan); laminaribiose–laminariheptaose were from Seikagaku Kogyo (Tokyo, Japan); β-glucan (barley) was from Megazyme International Ireland Ltd. (Bray, Ireland).

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

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

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

### *2.3. KfGH64 activity assay*

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

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

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

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

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

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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.

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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.

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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

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

## 3. Results and Discussion

### *3.1. Biochemical properties of KfGH64*

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

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

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

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

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

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

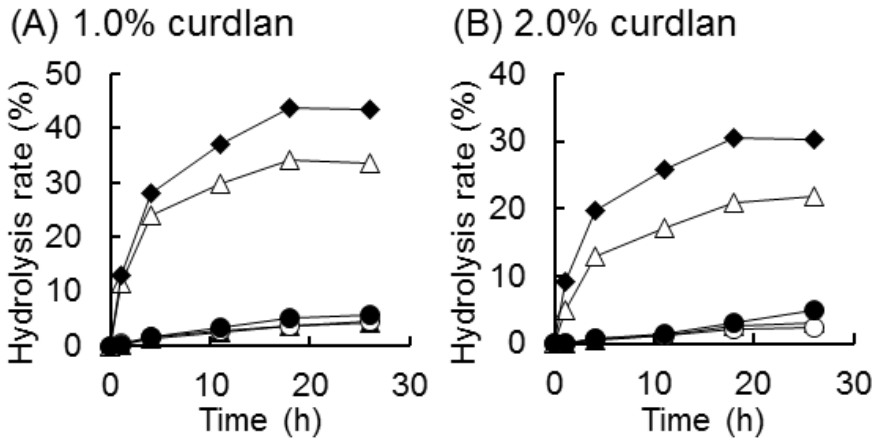


Figure 1 Kumagai et al

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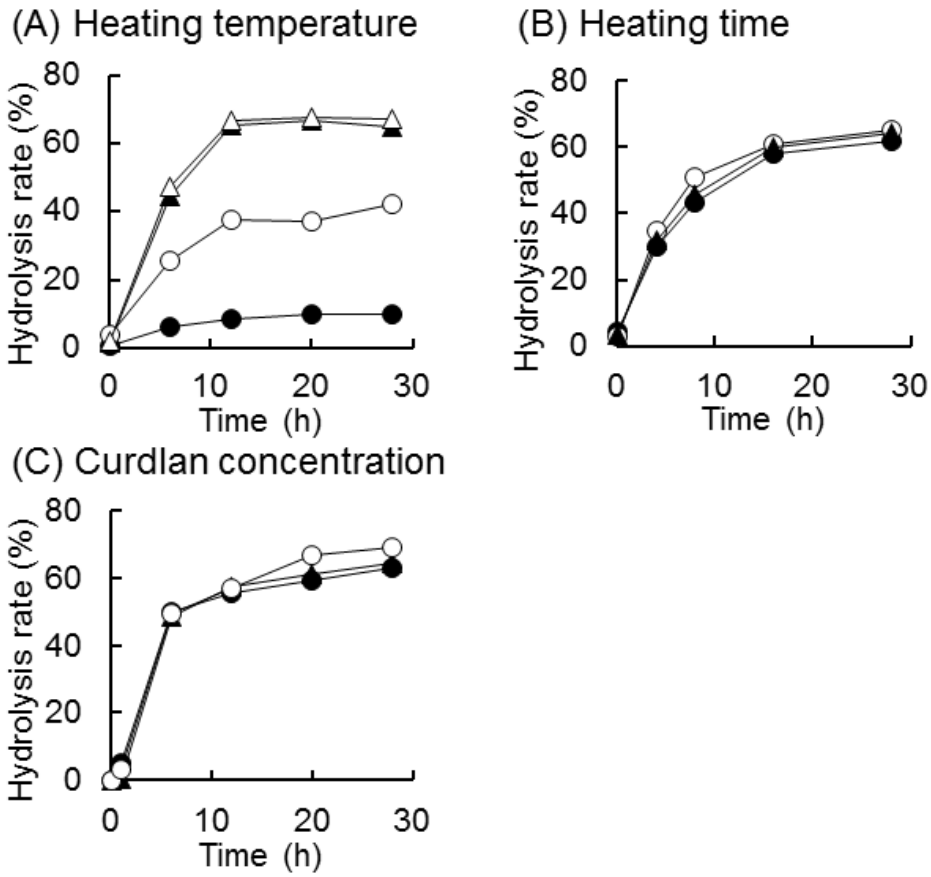


Figure 2 Kumagai et al

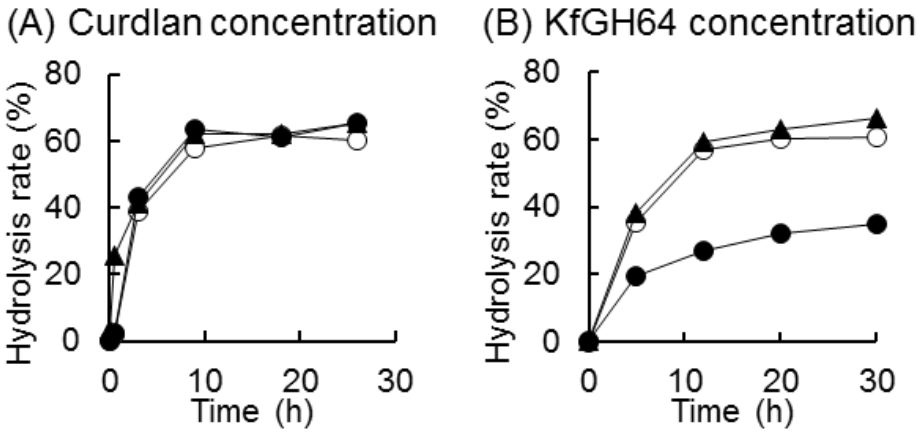


Figure 3 Kumagai et al

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

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

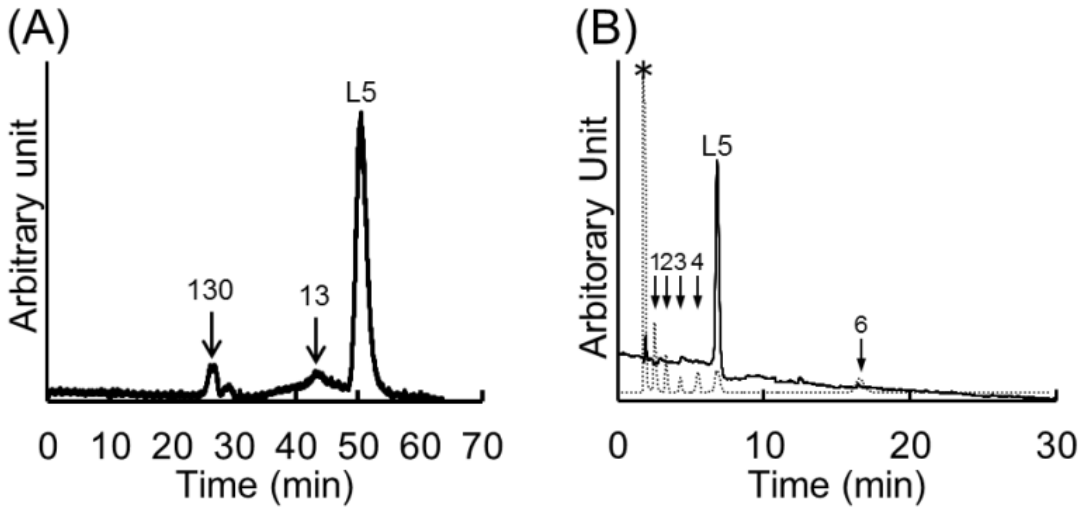


Figure 4 Kumagai et al

## 4. Conclusions

Curdlan conformations affected the enzyme activity. Heat treatment converted

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

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**Figure legends**

Figure 1. Effect of pretreatment of curdlan on the hydrolysis rate. Hydrolysis with 20 μg/mL KfGH64 was performed in 1.0% (A) and 2.0% (B) curdlan pretreated using five procedures: ●, non-treatment; ○, alkali-neutralization–homogenization; ▲, alkali-neutralization; △, alkali-neutralization–heat treatment; and ◆, heat treatment. The pretreatment indicated by each symbol is explained in section 2.4.

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

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

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