World J Microbiol Biotechnol (2016) 32:206
DOI 10.1007/s11274-016-2167-4

ORIGINAL PAPER

Characterization and enzymatic hydrolysis of hydrothermally treated β-1,3–1,6-glucan from *Aureobasidium pullulans*

Katsuki Hirabayashi[1,2] · Nobuhiro Kondo[1] · Sachio Hayashi[2]

Received: 9 July 2016 / Accepted: 27 October 2016 / Published online: 1 November 2016

Abstract The chemical structure of hydrothermally treated β-1,3–1,6-glucan from *Aureobasidium pullulans* was characterized using techniques such as gas chromatography/mass spectrometry (GC/MS) and nuclear magnetic resonance (NMR). The chemical shifts of anomeric carbons observed in the ^{13}C-NMR spectra suggested the presence of single flexible chains of polysaccharide in the sample. β-1,3–1,6-Glucan from *A. pullulans* became water-soluble, with an average molecular weight of 128,000 Da after hydrothermal treatment, and the solubility in water was approximately 10% (w/w). Sample (3% w/v) was completely hydrolyzed to glucose by enzymatic reaction with *Lysing enzymes* from *Trichoderma harzianum*. Gentiobiose (Glcβ1 → 6Glc) and glucose were released as products during the reaction, and the maximum yield of gentiobiose was approximately 70% (w/w). The molar ratio of gentiobiose to glucose after 1 h reaction suggested that the sample is likely highly branched. Sample (3% w/v) was also hydrolyzed to glucose by *Uskizyme* from *Trichoderma* sp., indicating that it is very sensitive to enzymatic hydrolysis.

Keywords β-1,3–1,6-Glucan · *Aureobasidium pullulans* · Hydrothermal treatment · Chemical structure · Enzymatic hydrolysis

✉ Sachio Hayashi
shayashi@cc.miyazaki-u.ac.jp

[1] Itochu Sugar Co. Ltd, 3 Tamatsuura, Hekinan-shi, Aichi 447-8506, Japan

[2] The Interdisciplinary Graduate School of Agriculture and Engineering, University of Miyazaki, 1-1 Gakuen Kibanadai Nishi, Miyazaki-shi, Miyazaki 889-2192, Japan

Introduction

β-1,3–1,6-Glucans consist of glucose residues linked by β-1,3 bonds with attached side chain glucose residues joined by β-1,6 linkages, and are found in a variety of natural resources such as fungi, yeasts, bacteria and seaweeds (Chen and Sevior 2007; Bobadilla et al. 2013; Zykova et al. 2014; Kuda et al. 2015). The importance of β-1,3–1,6-glucans as food supplements and clinical materials is increasing because of their biofunctional activities (Chen and Sevior 2007; Dalonso et al. 2015), such as antitumor, immunomodulatory, and antioxidant properties. In particular, the clinical potential of water-soluble β-1,3–1,6-glucans (*laminaran* from seaweed Kuda et al. 2015 and *schizophyllan* from *Schizophyllum commune* Zhong et al. 2015), and alkaline water-soluble *lentinan* from *Lentinula edodes*, (Sun et al. 2015) has been reported to date.

β-1,3–1,6-Glucan is also obtained by fermentation using the black yeast *Aureobasidium pullulans*, and its antitumor (Kimura et al. 2006), immunomodulatory (Tada et al. 2008; Le et al. 2010; Tanioka et al. 2013), food allergy inhibitory (Kimura et al. 2007a), and other medicinal activities (Kubala et al. 2003; Kimura et al. 2007b; Shin et al. 2007; Joo-Wan et al. 2012; Guzman-Villanueva et al. 2014; Kim et al. 2015) have been reported. *A. pullulans* β-1,3–1,6-glucan cannot be hydrolyzed completely by enzymes such as β-1,3-glucanase and/or β-1,6-glucanase because it is not water-soluble, which presents challenges for its utilization as a medical and/or bio-industrial material. Some research has been conducted on an partially isolated water-soluble, low-molecular weight fraction of *A. pullulans* β-1,3–1,6-glucan (Kimura et al. 2006, 2007a, b). However, there are no reports dealing with the enzymatic hydrolysis of intact water-soluble *A. pullulans* β-1,3–1,6-glucan.

In this paper, we first characterized and confirmed the chemical structure of hydrothermally treated β-1,3–1,6-

glucan from *A. pullulans* using techniques such as GC/MS and NMR. Next, we investigated the water-solubility of the samples. Water solubility is important because it greatly impacts the utility of this functional carbohydrate in many industrial fields.

Materials and methods

Preparation of hydrothermally treated *A. pullulans* β-1,3–1,6-glucan

Aureobasidium pullulans ATCC 20524 was cultured in liquid medium consisting of 0.6% (w/v) sucrose, 0.2% (w/v) rice bran and 0.2% (w/v) ascorbic acid at 23 °C for 72 h. β-1,3–1,6-Glucan was then harvested by the addition of burnt alum (Bulson et al. 1984; Savangikar et al. 1985; Asif et al. 2016). The obtained β-1,3–1,6-glucan was subjected to hydrothermal treatment (180 °C for 15 min at pH 5.5), concentrated by ultrafiltration, autoclaved for 15 min at 121 °C, and lyophilized for 48 h.

Monosaccharide compositional analysis

Sample (10 mg) was hydrolyzed with 3 mL of 2 N H_2SO_4 at 105 °C for 6 h and then 15 mL distilled water was added. After neutralization with $BaCO_3$ and centrifugation (13,000×*g*, 15 min), the solution was evaporated to dryness. The residue was dissolved in 2 mL of distilled water and the product was identified using high performance liquid chromatography (HPLC, Shimadzu LC-10A; Shimadzu, Kyoto, Japan, fitted with a Sugar-D column 4.6 × 25 mm; Nacalai Tesque, Kyoto, Japan) under the following conditions: temperature, 30 °C; mobile phase, acetonitrile:water (80:20, v/v); flow rate, 1 mL/min; and RI detector. The sample was also analyzed using thin layer chromatography (TLC) on silica gel plates (Silica Gel 60, Merck Co., Darmstadt, Germany) using a mixed solvent (*n*-butanol:ethanol:water = 5:2:1, v/v) for development.

Fourier transform infrared spectrometry (FT-IR) analysis

A KBr tablet containing sample powder (approx. 1%, w/w) was analyzed using FT-IR (FT/IR-300, JASCO Corporation, Tokyo, Japan).

Methylation and gas chromatograph/mass spectrometry (GC/MS) analysis

Sample (5 mg) was methylated three times with 1 mL sodium hydroxide/dimethyl sulphoxide (40 mg/mL) and 0.5 mL methyl iodide at 30 °C for 30 min. The methylated product was isolated by partitioning between $CHCl_3$ and H_2O (3:1, v/v). The organic layer containing product was washed with 3 mL of water three times and dried. The resulting partially methylated product was hydrolyzed, reduced, acetylated and analyzed using a Shimadzu GCMS-QP2010SE with an InerCap Rtx-5MS capillary column (60 m × 0.25 mm × 0.25 μm). The oven temperature was initially 180 °C during injection for 5 min, then was increased at 2 °C/min to 230 °C and held at this temperature for 5 min. The partially methylated alditol acetates were identified by their relative retention times on GC and by their fragment ions in EI-MS using NIST08 Mass Spectral Library.

Nuclear magnetic resonance (NMR) analysis

The sample was dissolved in D_2O (30 mg/mL) to exchange the active hydrogens and was then lyophilized. This process was repeated three times. The ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker DPX 400 MHz NMR for δ_H and a 100 MHz NMR for δ_C at room temperature (25 °C) in D_2O. HSQC was carried out using standard Bruker software. Chemical shifts are given as δ values with reference to 1,4-dioxsane used as an internal standard, and coupling constants are given in Hz.

Measurement of solubility

Sample solution (1.2 g/10 mL) was kept at each temperature for 30 min with reference to The Japanese Pharmacopeia Sixteen Edition and then centrifuged at 2220×*g* for 30 min at the same temperature. The obtained supernatants were dried overnight at 105 °C and the solubilities of the residues were determined as % (w/w).

Molecular weight estimation

The molecular weight of each sample (10 mg/mL) was estimated by high performance gel permeation chromatography (HPGPC, Shimadzu LC-10A; Shimadzu) fitted with a TSKgel G5000PWxL-CP column (7.8 × 30 mm; Tosoh Corporation, Tokyo, Japan) under the following conditions: temperature, 40 °C; mobile phase, 20 mmol/L phosphate buffer, pH 6.8; flow rate, 0.5 mL/min; and RI detector. The average molecular weight was measured using standard polysaccharide pullulans (Shodex STANDARD P Series, SHOWA DENKO K. K., Tokyo, Japan).

Enzymatic hydrolysis

Lysing enzymes (primarily β-glucanase, cellulase, protease and chitinase; Sigma-Aldrich Japan, Tokyo, Japan) and *Uskizyme* (primarily β-1,3-glucanase and chitinase; Wako

Pure Chemical Industries Ltd, Osaka, Japan) were used. *Lysing enzymes* were dialyzed against MacIlvain buffer (pH 7.5) to remove glucose from the preparation prior to sample hydrolysis. The reaction mixture containing 500 μL of sample as substrate (6% w/v) in 150 mmol/L MacIlvain buffer (pH 5.5) and 500 μL of *Lysing enzymes* (80 U) or *Uskizyme* (25 U) was incubated for 24 h at 40 °C. The obtained glucose and gentiobiose were determined using HPLC using the same conditions as described above. Total yields of glucose and gentiobiose are shown as the percentage of the initial substrate concentration in the reaction mixture.

Results

Monosaccharide component

The results of monosaccharide component analysis using HPLC are shown in Fig. 1. A single peak was detected from the hydrolyzed sample and the retention time was 17.35 min, essentially the same as that of standard glucose (17.24 min). The yield of glucose from the hydrolyzed sample was 98% (w/w). The hydrolyzed sample also provided a single spot by TLC whose retention factor value (R_f value) was 0.65, the same as that of standard glucose (0.65) (Fig. 2). From these results, the monosaccharide component of the sample was concluded to be glucose.

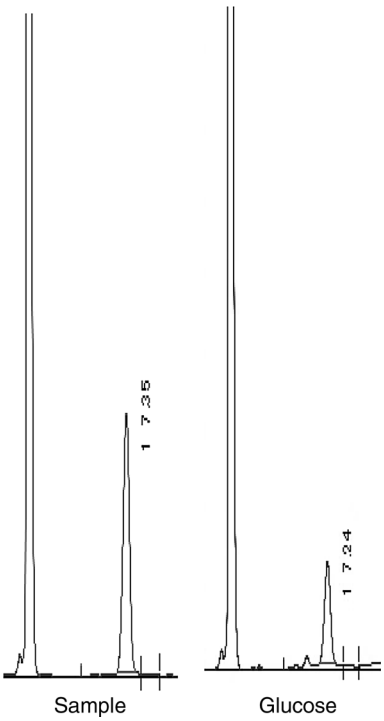


Fig. 1 Monosaccharide component analysis using HPLC

FT-IR analysis

The IR spectrum of the sample is shown in Fig. 3. Absorbances due to β-glucosidic linkage (886–900 and 950–954 cm^{-1} Mohacek-Grosev et al. 2001, 889–890 cm^{-1} Tanioka et al. 2013 and 892 cm^{-1} Novak et al. 2012), pyranose ring (1022–1079 cm^{-1} Novak et al. 2012, 1200–1206 cm^{-1} Mohacek-Grosev et al. 2001, and 2853–2922 cm^{-1} Novak et al. 2012) and hydroxyl group (3383 cm^{-1} Novak et al. 2012) were observed and identified by reference to the values provided in previous reports. From the above results, the sample was concluded to be β-glucan.

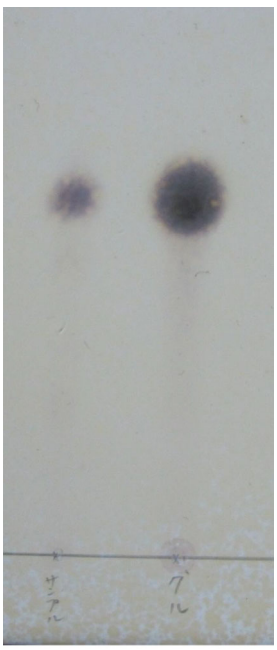
GC/MS analysis

The total ion monitor and analytical data of the methylated sample obtained by GC/MS analysis are shown in Fig. 4 and Table 1, respectively. As shown in Table 1, the linkages between the glucosyl residues were determined to be Glc-(1→, →3)-Glc-(1→, →3,6)-Glc-(1→ according to the values of mass fragments (*m/z*) described in previous reports (Mizuno et al. 1999; Rout et al. 2008). From the above results, the sample was concluded to be 1,3-1,6-glucan.

NMR analysis

The HSQC (^1H–^{13}C) spectra of the sample are shown in Fig. 5 and the values of the chemical shifts in the anomeric region of the sample are summarized in Table 2. From the chemical shifts of the anomeric carbons, the mode of the

Fig. 2 Monosaccharide component analysis using TLC



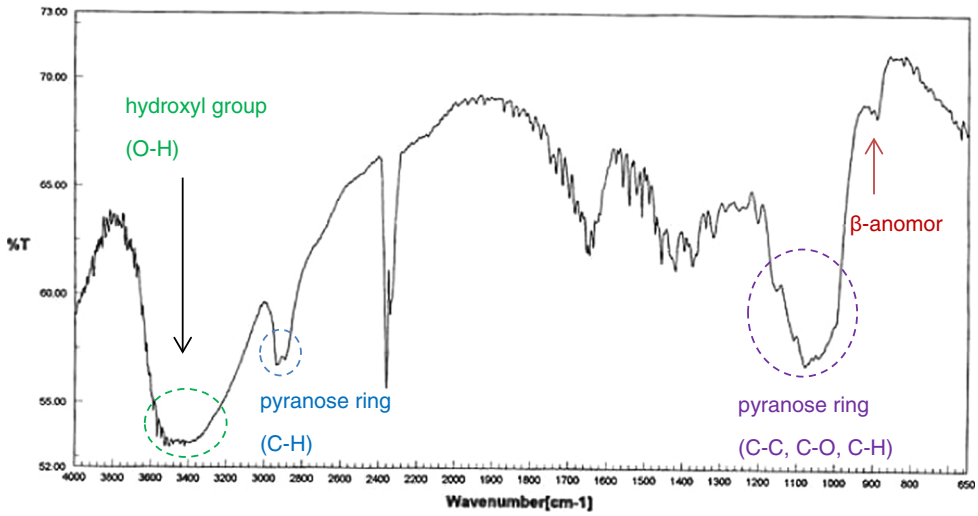


Fig. 3 IR spectra of the sample

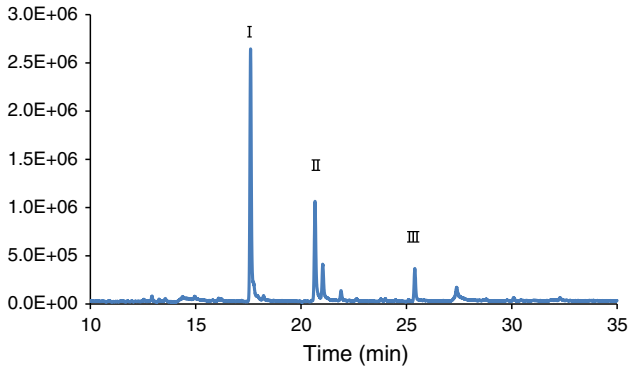


Fig. 4 Total ion monitor of GC/MS analysis of the methylated sample

glycosidic linkages of Glc-(1→, →3)-Glc-(1→, →3,6)-Glc-(1→ were determined to be β due to the detection of cross-peaks of anomeric carbons at around 103 ppm and anomeric protons at around 4.5 or 4.7 ppm. From these results, the sample was concluded to be β-1,3–1,6-glucan according to the values of chemical shifts described in previous reports (Kimura et al. 2007a; Pramanik et al. 2007; Santos-Neves et al. 2008; Sumidele et al. 2008; Tada et al. 2008; Carbonero et al. 2012; Fang et al. 2012; Ruthes et al. 2013; Dalonso et al. 2015).

Solubility

The solubility of hydrothermally treated *A. pullulans* β-1,3–1,6-glucan is shown in Fig. 6. Although an increase in solubility was not seen as the temperature increased, the maximum solubility was approximately 10% (w/w) between 50 and 70 °C.

Molecular weight

The HPGPC chromatogram of the sample is shown in Fig. 7. A wide range of molecular weights was observed, with the average value estimated to be approximately 128,000 Da.

Enzymatic hydrolysis

The time course of enzymatic hydrolysis of a 3% (w/v) (30 mg/mL) sample as substrate by *Lysing enzymes* is shown in Fig. 8. The % glucose yield to the initial substrate concentration, i.e., the hydrolysis efficiency, reached 102.2% after 1 h reaction at 40 °C, indicating complete hydrolysis of the sample. The amount of gentiobiose produced during the reaction decreased, and it was completely hydrolyzed after 24 h reaction with *Lysing enzymes*. After 1 h reaction, the amounts of glucose and gentiobiose reached 9.4 mg/mL (52.2 mmol/L) and 21.3 mg/mL (62.2 mmol/L), respectively. The yield of gentiobiose was 69.5% and the molar ratio of gentiobiose to glucose was 1.19.

The time course of the enzymatic hydrolysis of 3% (w/v) (30 mg/mL) samples by *Uskizyme* is shown in Fig. 9. The hydrolysis efficiency was 94.6% after 24 h reaction at

Table 1 Linkage analysis of the sample

Peak no.	Methylated sugars	Linkages	Major mass fragments (*m/z*)
I	2,3,4,6-Me_4-Glc	Glc-(1→	71,87,101,117,129,145,161,205
II	2,4,6-Me_3-Glc	→3)-Glc-(1→	58,71,87,101,117,129,161,233
III	2,4-Me_2-Glc	→3,6)-Glc-(1→	58,87,101,117,129,159,189,233

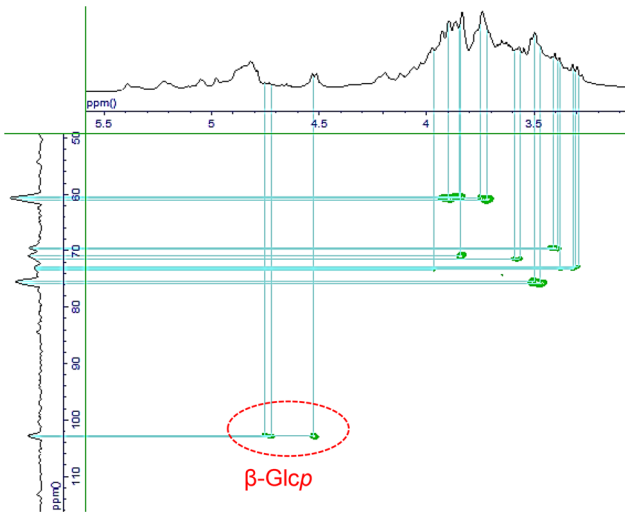


Fig. 5 HSQC NMR analysis of the sample

Table 2 Chemical shifts of anomeric carbons and protons for each type of glucose residue in the sample

Glycosyl residues	C-1 (ppm)	H-1 (ppm)
β-D-Glc-(1→	102.8	4.53
→3)-β-D-Glc-(1→	102.7	4.75
→3,6)-β-D-Glc-(1→	102.9	4.72

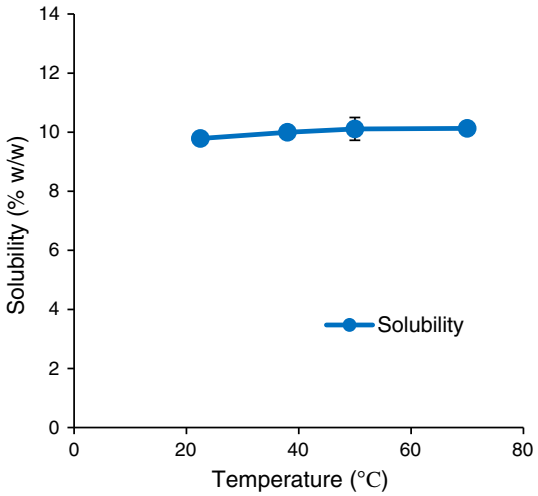


Fig. 6 Solubility of the sample

40 °C, indicating that the principal product was glucose and thus the sample was considered to be hydrolyzed by *Uskizyme*. After 3 h reaction, the levels of glucose and gentiobiose reached 14.6 mg/mL (81.0 mmol/L) and 11.5 mg/mL (33.6 mmol/L), respectively, with a total yield of 86.6%. The molar ratio of gentiobiose to glucose (0.41) was less than that shown in Fig. 8.

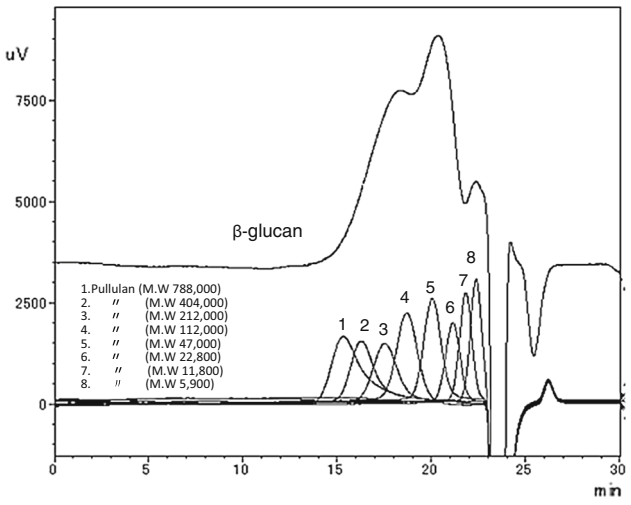


Fig. 7 Molecular weight estimation using HPGPC

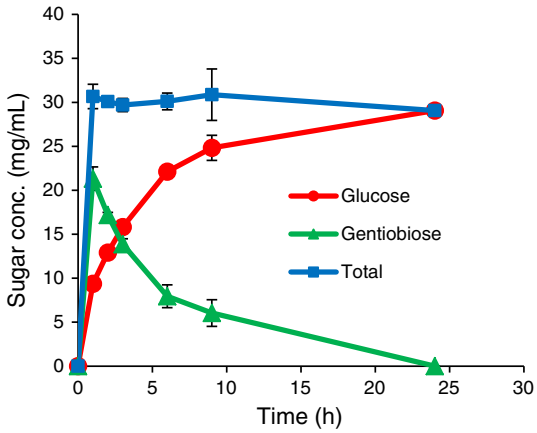


Fig. 8 Time course of the enzymatic hydrolysis of the sample by *Lysing enzymes*

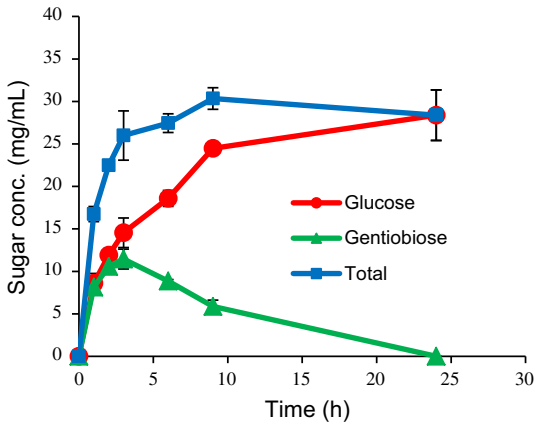


Fig. 9 Time course of the enzymatic hydrolysis of the sample by *Uskizyme*

Discussion

Previously, Zhang et al. (2002) reported that anomeric carbons underwent a chemical shift from 104.1 ppm to 103.1 ppm in the ^{13}C-NMR spectrum when the triple-helical chain of β-1,3–1,6-glucan from *Lentinus edodes* was changed to a single-flexible chain in DMSO. It is possible that the current samples contain single flexible chains, given the observed resonances below 130 ppm. However, further investigations to clarify the structure of the sample are necessary. It may be possible to manufacture pharmaceutical products and functional food materials using hydrothermally treated *A. pullulans* β-1,3–1,6-glucan, similar to the use of polysaccharides such as *Laminallan* and *Schizophyllan*, because hydrothermally treated *A. pullulans* β-1,3–1,6-glucan changed from being water-insoluble to water-soluble, which is a very important and valuable characteristic especially in the bio-industry.

The molecular weight of sample was slightly larger than that of *Schizophyllan* (76,800) and *Lentinun* (94,700), and smaller than that of *Curdlan* (>136,000) (Tanioka 2010). The molecular weight distribution of polysaccharides such as β-glucan is variable (Tsubaki et al. 2008) and wide (300,000–700,000, *Lentinun* CAS 37339-90-5). A wide distribution of molecular weights after hydrothermal treatment might be a feature of the heat treatment of polysaccharides.

The hydrolysis (15.9, 24 and 33%) of acidic polysaccharide from *Aureobasidium* by *Kitalase* (*exo*-β-1,3-glucanase) was reported previously, but no detailed reaction conditions such as the concentrations of substrate and products were provided (Hamada and Tsujisaka 1983). The detection of gentiobiose and glucose as hydrolysis products generated by *Kitalase* were also described previously (Kimura et al. 2006), but again, no information regarding the reaction conditions or the results were given. From the molar ratio of gentiobiose to glucose (1.19), the sample is likely highly branched, with branches originating from the C6 of glucose every one or two units of the main β-1,3-glucan chain. The above results suggest that the sample has a similar branching pattern to that previously reported for β-1,3–1,6-glucan from *A. pullulans* (Tada et al. 2008), and that the chemical structure of the sample is similar to that of the low-molecular weight water-soluble β-1,3–1,6-glucan from *A. pullulans* 1A1 strain (Kimura et al. 2006, 2007a). From the above results, it is concluded that the water-solubility of the sample rendered it sensitive to enzymatic catalysis. This property is very useful for the production of functional oligosaccharides such as gentiobiose because the yield of gentiobiose after 1 h reaction was approximately 70%.

In the present experiment, gentiobiose produced from the sample was also hydrolyzed by *Uskizyme* (primarily β-1,3-glucanase and chitinase). It is considered that *Uskizyme* also contained β-1,6-glucanase/β-1,6-glucosidase activities because we confirmed the complete hydrolysis of commercial gentiobiose (2% w/v, Wako Pure Chemical Industries Ltd) to glucose by the enzyme (data not shown). It would be far preferable to use purified enzymes such as β-1,3-glucanase/β-1,3-glucosidase and/or β-1,6-glucanase/β-1,6-glucosidase for the hydrolysis experiments, but these enzymes are currently not available commercially. In the near future we intend to purify the hydrolyzing enzymes from *Lysing enzymes* and/or *Uskizyme* to conduct our hydrolysis experiments more precisely.

From the above results, it is proposed that hydrothermally treated β-1,3–1,6-glucan from *A. pullulans* can be efficiently hydrolyzed by enzymatic methods. Although there are several reports on the hydrolysis of β-1,3–1,6-glucan, as described in the Introduction, this is the first evidence of the complete enzymatic hydrolysis of β-1,3–1,6-glucan from *A. pullulans*. Investigations to clarify why hydrothermal treatment rendered *A. pullulans* β-1,3–1,6-glucan water-soluble and amenable to enzymatic hydrolysis will be carried out in the near future. It is also considered that the sensitivity of the present sample to hydrolyzing enzymes is useful for the production of functional oligosaccharides such as gentiooligosaccharides (Takahashi et al. 2014; Unno et al. 2005) from *A. pullulans* β-1,3–1,6-glucan.

In the present report, we noted and described the newly obtained superior characteristics of hydrothermally treated β-1,3–1,6-glucan from *A. pullulans* and suggest that this material will help contribute to bio-industries utilizing functional carbohydrates.

References

Asif MB, Majeed N, Iftekhar S, Habib R, Fida S, Tabraiz S (2016) Chemically enhanced primary treatment of textile effluent using alum sludge and chitosan. Desalin Water Treat 57:7280–7286

Bobadilla F, Rodriguez-Tirado C, Imarai M, Galotto MJ, Andersson R (2013) Soluble β-1,3/1,6-glucan in seaweed from the southern hemisphere and its immunomodulatory effect. Carbohydr Polym 92:241–248

Bulson PC, Johnstone DL, Gibbons HL, Funk WH (1984) Removal and inactivation of bacteria during alum treatment of a lake. Appl Environ Microbiol 48:425–430

Carbonero ER, Ruth AC, Freitas CS, Utrilla P, Galvez J, Silva EV, Sassaki GL, Gorin PAJ, Lacomini M (2012) Chemical and Biological properties of highly branched β-glucan from edible mushroom *Pleutus sajor-caju*. Carbohydr Polym 90:814–819

Chen J, Sevior R (2007) Medical importance of fungal β-(1 → 3), (1 → 6)-glucans. Mycol Res 111:635–652

Dalonso N, Goldman GH, Gern RMM (2015) β-1,3–1,6-Glucans: medicinal activities, characterization, biosynthesis and new horizons. Appl Microbiol Biotechnol 99:7893–7906

Fang J, Wang Y, Lv X, Shen X, Ni X, Ding K (2012) Structure of aβ-glucan from *Grifola frondosa* and its antitumor effect by activating Dectin-1/Syk/NF-KB signaling. Glycoconj J 29:365–377

Guzman-Villanueva LT, Ascencio-Valle F, Macias-Rodriguez ME, Tovar-Ramirez DT (2014) Effect of dietary β-1,3/1,6-glucan on the antioxidant and digestive enzyme activities of Pacific red snapper (*Lutjanus peru*) after exposure to lipopolysaccharides. Fish Physiol Biochem 40:827–837

Hamada N, Tsujisaka Y (1983) The structure of the carbohydrate moiety of an acidic polysaccharide produced by *Aureobasidium* sp. K-1. Agric Biol Chem 47:1167–1172

Joo-Wan K, Cho H-R, Ku S-K (2012) Efficacy test of polycan, a beta-glucan originated from *Aureobasidium pullulans* SM-2001, on antitumor cruciate ligament transection and partial medical meniscectomy-induced osteoarthritis rats. J Microbiol Biotechnol 22:274–282

Kim KH, Park SJ, Lee YJ, Lee JE, Song CH, Choi SH, Ku SK, Kang SJ (2015) Inhibition of UVB-induced skin damage by exopolymers from *Aureobasidium pullulans* SM-2001 in Hairless mice. Basic Clin Pharmacol Toxicol 116:73–86

Kimura Y, Sumiyoshi M, Suzuki T, Sakanaka M (2006) Antitumor and antimetastatic activity of a novel water-soluble low molecular weight β-1,3-D-glucan (branch β-1,6) isolated from *Aureobasidium pullulans* 1A1 strain black yeast. Anticancer Res 26:4131–4142

Kimura Y, Sumiyoshi M, Suzuki T, Suzuki T, Sakanaka M (2007a) Inhibitory effect of water-soluble low-molecular-weight β-(1,3-1,6) D-glucan (branch β-1,6) purified from *Aureobasidium pullulans* GM-NH-1A1 strain on food allergic reactions in mice. Int Immunopharmacol 7:963–972

Kimura Y, Sumiyoshi M, Suzuki T, Suzuki T, Sakanaka M (2007b) Effect of water-soluble low molecular weight β-1,3-D-glucan (branch β-1,6) isolated from *Aureobasidium pullulans* 1A1 strain black yeast on restraint stress in mice. J Pharm Pharmacol 59:1137–1144

Kubala L, Ruzickova J, Nickova K, Sandula J, Ciz M, Lojek A (2003) The effect of (1 → 3)-β-D-glucans, carboxymethylglucan and schizophillan on human leukocytes in vitro. Carbohydr Res 338:2835–2840

Kuda T, Kosaka M, Hirano S, Kawahata M, Sato M, Kaneshima T, Nishizawa H, Kimura B (2015) Effect of sodium-alginate and laminaran on Salmonella typhimurium infection in human enterocyte-like HT-29-Luc cells and BALB/c mice. Carbohydr Polym 119:113–119

Le TH, Le KXT, Cuong PT, Cue NTK, Le TB, Ikeue Y, Watanabe Y, Agatsuma T (2010) Adjuvant effect of Sophy β-glucan on H5N1 and H5N2 vaccination using a mouse model. Tropical Med Health 38:23–27

Mizuno M, Minato K, Ito H, Kawade M, Terai H, Tsuchida H (1999) Anti-tumor polysaccharide from the mycelium of liquid-cultured *Agaricus blazei* Mill. Biochem Microbiol Int 47:707–714

Mohacek-Grosev V, Bozac R, Puppel GJ (2001) Vibrational spectroscopic characterization of wild growing mushrooms and toadstools. Spectrochim Acta Part A 57:2815–2829

Novak M, Synytsya A, Gedeon O, Slepicka P, Prochazka V, Synytsya A, Blahovec J, Hejlova J, Copikova J (2012) Yeast β(1-3)(1-6)-D-glucan films: preparation and characterization of some structural and physical properties. Carbohydr Polym 87:2496–2504

Pramanik M, Chakraborty I, Mondal S, Islam SS (2007) Structural analysis of a water-soluble glucan (Fr.I) of an edible mushroom, *Pleirotus sajor-caju*. Carbohydr Res 342:2670–2675

Rout D, Mondal S, Chakraborty I, Islam SS (2008) The structure and conformation of a water-insoluble (1 → 3), (1 → 6)-β-D-glucan from the fruiting bodies of *Pleurotus florida*. Carbohydr Res 343:982–987

Ruthes AC, Carbonero ER, Cordova MM, Baggio CH, Santos ARS, Sassaki GL, Cipriani TR, Gordon PAJ, Lacomini M (2013) *Lactarius rufus* (1 → 3), (1 → 6)-β-D-glucans: structure, antinociceptive and anti-inflammatory effects. Carbohydr Polym 94:129–136

Santos-Neves JC, Pereira MI, Carbonero ER, Gracher AHP, Alquini G, Gorin PAJ, Sassaki GL, Iacomini M (2008) A novel branched αβ-glucan isolated from the basidiocarps of the edible mushroom *Pleurotus florida*. Carbohydr Polym 73:309–314

Savangikar CV, Savangikar VA, Joshi RN (1985) Fractional coagulation of proteins from alfalfa leaf juice by use of alum. Plant Sci 95:47–49

Shin HD, Yang KJ, Park BR, Son CW, Jang H, Ku SK (2007) Antiosteoportic effect of polycan, β-glucan from *Aureobasidium*, in ovariectomized osteoporotic mice. Nutrition 23:853–860

Sumidele FR, Olsen LMO, Carbonero ER, Baggio CH, Baggio CH, Freitas RM, Santos ARS, Gorin PAJ, Iacomoni M (2008) Anti-inflammatory and analgesic properties in a rodent model of a (1 → 3), (1 → 6)-linked β-glucan isolated from Pleurotus pulmonarius. Eur J Pharmacol 597:86–91

Sun M, Ahao W, Xie Q, Zhan Y, Wu B (2015) Lentinan reduces tumor progression by enhancing gemcitabine chemotherapy in urothelial bladder cancer. Surg Oncol 24:28–34

Tada R, Tanioka A, Iwasawa H, Hatashima K, Shoji Y, Ishibashi K, Adchi Y, Yamazaki M, Tsubaki K, Ohno N (2008) Structural characterization and biological activities of a unique type β-D-glucan obtained from *Aureobasidium pullulans*. Glycoconj J 25:851–861

Takahashi H, Imamura T, Konno N, Takeda T, Fujita K, Konishi T, Nishihara M, Uchimiya H (2014) The gentio-oligosaccharide gentiobiose functions in the modulation of bud dormancy in the herbaceous *Perennial Gentiana*. Plant Cell 26:3949–3963

Tanioka H (2010) Basic and applied science of β-glucan. CMC Publication, Tokyo

Tanioka A, Tanabe K, Hosono A, Kawakami H, Tsubaki S, Hachimura S (2013) Enhancement of intestinal immune function in mice by β-D-glucan from *Aureobasidium pullulans* ADK-34. Scand J Immunol 78:61–68

Tsubaki K, Tanioka A, Hatashima K, Shoji Y, Iwasawa H, Yamazaki M (2008) Immuno-modulation activities of new β-glucan from cereal and fermentation food. In: Yagasaki K, Yamazaki M (eds) Bromacology: pharmacology of foods and their components. Research Sighpost, Kerala, pp 147–169

Unno T, Nakakuki T, Fujimoto Y, Okada G, Kainuma S, Goda T (2005) Industrial production and higher application of functional β-glucooligosaccharides having a bitter taste. J Appl Glycosci 52:59–64

Zhang L, Li X, Zhou Q, Zhang X, Chen R (2002) Transition from triple helix to coil of *Lentinan* in solution measured by SEC, vicometry, and ^{13}C NMR. Polym J 34:443–449

Zhong KZ, Tong L, Liu L, Zhou X, Zhang Q (2015) Immunoregulatory and antitumor activity of schizophllan under ultrasonic treatment. Int J Biol Macromol 80:302–308

Zykova SN, Balandina KA, Vorokhobina N, Kuznetsova AV, Engstad R, Zykova TA (2014) Macrophage stimulating agent soluble yeast β-1,3/1,6-glucan as a topical treatment of diabetic foot and leg ulcers: a randomized, double blind, placebo-controlled phase II study. J Diabetes Invest 5:392–399