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Structural studies of the glucuronic acid oligomers produced by *Gluconacetobacter hansenii* strain

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Abstract

Gluconacetobacter hansenii PJK, a cellulose producing bacterium recently isolated from the rotten apples, produced fair amounts of the water-soluble polysaccharides (WSPS). WSPS were studied for their monosaccharides composition after acid hydrolysis, which revealed that the hydrolysates consist only of one sugar, glucuronic acid. The structure of the WSPS was investigated using various spectroscopic techniques including FT-IR, MALDI-TOF MS and ^{1}H, and ^{13}C NMR. These studies revealed that the product is a mixture of oligomers with the α-glucuronic acid as building blocks. The possible structure of the major oligosaccharide in the mixture has been deduced.

Keywords: *Gluconacetobacter hansenii* PJK; Water-soluble polysaccharides; Glucuronic acid; Spectroscopic techniques

1. Introduction

Microbial fermentation is a source for the production of food polysaccharides, although most polysaccharides derived from plants and seaweeds are used in industry (European Commission, 2000). Bacterial exopolysaccharides (EPS) generally have unique rheological properties caused by their high purity and regular structure. Therefore, the food industry frequently uses EPS as thickening, gelling, or stabilizing agents (Kornmann, Duboc, Marison, & Stockar, 2003). The discovery of immune modulation and tumouristasis by β-D-glucans provides some novel applications of EPS (Sutherland, 1998). Biopolymers production by bacterial fermentation is a potential alternative to petrochemical and vegetal polymers due to their wide diversity and the possibility of specific structural changes induced by controlled culture conditions in bioreactors (Brou, Jaffrin, Ding, & Courtois, 2003).

Polysaccharides, including glucuronan, which contain carboxyl groups, are valuable compounds because their enveloping and/or resolving activity makes them suitable as carriers for various active substances, and as solvents, stabilizers, binders, swelling agents, and so on (Antonius & Cornelis, 1991). Conservation of the carbon skeleton in the oxidized polysaccharide is often advantageous for achieving the complexing or stabilizing activity (Antonius & Cornelis, 1991). The medical properties of oxidized celluloses to stop bleeding during surgery, to prevent the formation and reformation of post-surgical adhesions, to promote anti-bacterial activity, to promote bone regeneration, and their usefulness in periodontal therapy have been attributed to the glucuronan structure of such compounds (Kumar & Dong, 2002).

Many EPS, derived from microbial origin, contain glucuronic acid moieties in their structure (Takemura, Tabuchi, Watanabe, Tsuchida, Morinaga and Sone, 1995; Tayama, Minakami, Entani, Fujiyama, & Massai, 1985; Valla & Kjosbakken, 1981) or are the homopolymers of glucuronic acid (Courtois, Courtois, Heyraud, Colin-Morel, & Rinaudo, 1992; Courtois, Seguin, Declomesnil, Heyraud, Colin-Morel and Dantas, 1993; Dantas, Courtois, Courtois, Seguin, Gey and Heyraud, 1994; Lintner, 1999; Michaud, Courtois, Courtois, Heyraud, Colin-Morel and Seguin, 1994). The EPS produced by the *Rhizobium meliloti MN1CS* strain (Courtois et al., 1992) is a linear homopolymer of partially acetylated glucuronic acid (Courtois et al., 1993) and can be used as a substitute for pectin and alginate in the food or cosmetic industry because of its remarkable gelling or thickening properties (Lintner, 1999). The oligoglucuronans obtained by degradation of the polymer by enzymatic action present biological activities such as root growth promoting activity and bacteriostasis (Iwasaki & Matsubara, 2000; Kitamikado, Nishimura, Yamaguchi, & Tseng, 1993).

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Recently, a cellulose-producing strain isolated from rotten apples was identified as *Gluconacetobacter hansenii* based on its physiological characteristics and 16S rDNA complete sequencing method and was specifically named *G. hansenii* PJK (Park, Park, & Jung, 2003). *G. hansenii* PJK has been extensively investigated in studies of cellulose production under various experimental conditions (Jung, Park, & Chang, 2005; Jung, Park, & Park, 2003a; Jung, Park, & Park, 2003b; Park et al., 2003; Park, Hyun, & Jung, 2004; Park, Jung, & Park, 2003). This bacterium has also been used to produce water-soluble polysaccharides composed of glucuronic acid, which was revealed by only HPLC analysis (Jung et al., 2005). In the current study, we report the results of the structural analysis of these water-soluble polysaccharides using various spectroscopic techniques, FT-IR, MALDI-TOF MS and NMR spectroscopy and HPLC chromatogram.

2. Materials and methods

2.1. Bacterial culture

G. hansenii PJK (KCTC 10505BP), isolated from rotten apples and identified by 16S rDNA complete sequencing method (Park et al., 2003), was grown on a basal medium containing 10 g glucose L^{-1}, 10 g yeast extract L^{-1}, 7 g peptone L^{-1}, 1.5 mL acetic acid L^{-1}, and 0.2 g succinate L^{-1}. The agar plates, used for keeping strains, were prepared by dissolving 15 g agar L^{-1} in the basal medium. The pH of the medium was adjusted to 5.0 with NaOH or HCl. Colonies of *G. hansenii* PJK were inoculated into 50 mL medium in a 250 mL flask shaken at 200 rpm and cultured at 30 °C for 24 h. 5% of the culture broth collected from four flasks was inoculated into 3 L of the basal medium in a 5-L jar fermenter (Kobiotech Co., Korea) equipped with a six flat-blade turbine impeller at 30 °C, at an agitation rate of 500 rpm and an aeration rate of 1 vvm.

2.2. Isolation and purification of WSPS

The WSPS were isolated and measured by the modified method of Valla and Kjosbakken (1982). The supernatant, obtained by centrifuging the culture broth for 20 min at 3580 g, was treated twice with 5 volumes of ethanol for 1 h at 4 °C followed by separation of the precipitates by centrifugation each time, and finally drying at 60 °C in an oven until the constant weight was achieved. The constant weight was considered as the dry weight of WSPS.

2.3. Hydrolysis of WSPS and analysis of monosaccharides

The composition of WSPS was analyzed by measuring the monosaccharides after hydrolysis by the modified method suggested by Tajima, Uenishi, Fujiwara, Erata, Munekata and Takai (1998). A dried WSPS of 0.018 g in 10 mL of 4 M HCl was hydrolyzed for 12 h at 80 °C and dried at 60 °C in a drying oven until the weight became constant. The pure sugars in the hydrolysates were obtained by re-dissolving the dried product in 4 mL of distilled water followed by filtration through 0.45 μm membrane filter and drying it at −50 °C in a freeze-dryer. The composition of the resultant monosaccharides was determined by the HPLC (model 600E, Waters Co.) using a Sugar-Pak I column (6.5 mm × 300 mm) and an RI-detector (Model 410, Water. Co). The mobile phase was 0.5% Ca–EDTA buffer at 30 °C and the flow rate was 0.5 mL min^{-1}. The optical rotation of hydrolyzed product was also measured at 27 °C using a polarimeter (AP-100, ATAGO, USA).

2.4. Fourier transform infrared (FT-IR) spectroscopy

FT-IR spectra of the native polysaccharide sample was recorded using a Mattson Galaxy 7020A FT-IR spectrophotometer, with a resolution of 0.025 cm^{-1}, a wavelength range 4000–400 cm^{-1}, and a DTGS detector. Dried polysaccharide was ground with KBr pellets at room temperature and the measurement was taken.

2.5. Matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry

The MALDI-TOF mass spectra were acquired with a PE Voyager DE-STR biospectrometry workstation equipped with an N_2 laser (337 nm, 0.5-ns pulse width, 20-Hz repetition rate) (applied biosystems). The instrument was equipped with a delayed extraction ion source and was operated in the reflector mode with positive polarity at an accelerating voltage of 20 kV. The matrix, α-cyano-4-hydroxycinnamic acid, was prepared as a saturated solution in 50% acetonitrile and 0.1% trifluoroacetic acid solvent. For MALDI-TOF analysis, 0.5 μl of the matrix solution was mixed with 0.5 μl of the sample on the sample plate, and the mixture was air-dried.

2.6. Nuclear magnetic resonance (NMR) spectroscopy

All ^{1}H, and ^{13}C NMR spectra of the polysaccharides solution were obtained at 80 °C in D_2O (99.8% atom D; Merck, Germany) using an FT-NMR spectrometer (Varian Inova, USA), at 500 and 125 MHz, respectively, and chemical shifts were referenced to acetone. The instrument was equipped with an 11.7 T, 51 mm bore magnet.

3. Results and discussion

3.1. Analysis of the hydrolyzed products of WSPS

The WSPS produced by the *G. hansenii* PJK were isolated from the supernatant of the centrifuged culture broth by repeated precipitation with ethanol followed by centrifugation each time. The purified WSPS were dried at 60 °C until constant weight was achieved. The dried WSPS were subjected to acid hydrolysis and the hydrolysates were analyzed using HPLC. As reported previously (Jung et al., 2005) and found in this study, glucuronic acid was the only component detected in the HPLC pattern. The retention time (5.522 min) of the sole peak that appeared in the chromatogram, was the same as that

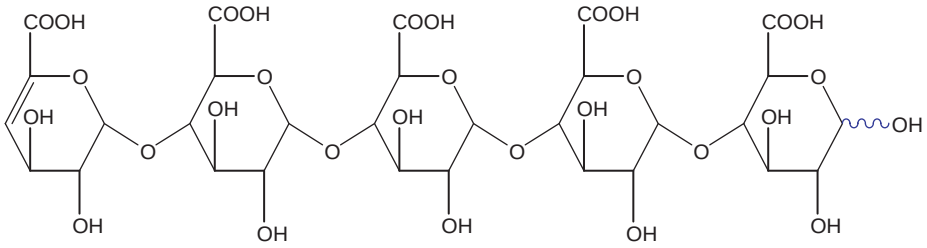

Fig. 1. The possible chemical structure of the major oligoglucuronate in the mixture of WSPS produced using the basal medium by *G. hansenii* PJK in a jar fermenter.

of standard glucuronic acid (5.520 min). The same retention time to the standard D-glucuronic acid and positive optical rotation of the hydrolyzed product means that the glucuronic acid of WSPS belongs to D series. *G. hansenii* PJK strains are known to produce a homopolysaccharide which yields glucuronic acid upon hydrolysis (Jung et al., 2005). Many EPS derived from microbial origin contain glucuronic acid moieties in the heteropolysaccharides (Takemura et al., 1995; Tayama et al., 1985; Valla & Kjosbakken, 1981) or the homopolymers of glucuronic acid (Courtois et al., 1992; Courtois et al., 1993; Dantas et al., 1994; Lintner, 1999; Michaud et al., 1994). Polysaccharides containing glucuronic acid moieties in their structures offer several novel properties of nutritional, medical, and pharmaceutical importance (Courtois et al., 1992; Courtois et al., 1993; Dantas et al., 1994; Lintner, 1999; Michaud et al., 1994).

The polysaccharides, containing glucuronic acid units, produced by *G. hansenii* PJK, may be particularly suitable for various purposes, such as carriers for drugs and other active substances, solubility enhancers for low polar or polymeric substances in water medium, support materials for separating devices, and possibly for separating optical antipodes, stabilizers, etc. (Antonius & Cornelis, 1991).

3.2. Structural analysis of the native WSPS

The structure of the native WSPS was investigated using various spectroscopic techniques including FT-IR, MALDI-TOF MS and NMR spectroscopy.

The FT-IR spectrum of WSPS produced by *G. hansenii* showed absorption bands at 3417 and 2929 cm^{-1}, respectively indicated the presence of hydroxyl (hydrogen bonded) and CH groups in the isolated polysaccharides. The absorption band at 3417 and 2929 cm^{-1} respectively indicate the presence of hydroxyl (hydrogen bonded) and CH groups in the isolated polysaccharides. The asymmetric and symmetric stretching modes of the carboxylate groups ($-COO^-$) were observed as two absorption bands at 1646 and 1528 cm^{-1}, respectively. Two other important bands at 1235 and 1061 cm^{-1} were attributed, respectively, to the stretching of C–O and the vibration of C–O–C bridge in the WSPS. Similarly, the absorption bands at 1386 cm^{-1} appeared due to the C–H bending vibration in the polysaccharide, while the shoulder in the range of the 3095–3065 cm^{-1} and two small bands at 1454 and 804 cm^{-1} are indicative of the presence of the double bond in the molecule(s) as these values correspond, respectively, to the sp^2 C–H stretching, =C–H scissoring in-plane vibration, and =C–H out of plane bending (Pavia, Lampman, & Kriz, 2001). However, the band for C=C stretching, which should have appeared at 1660–1600 cm^{-1}, might overlap with the band for the carboxylic function. The overall IR absorbencies were found to be in good agreement to previously reported values for polyuronic acids having C–O–C glycosidal bridges (Pavia et al., 2001; Pengzhan, Quanbin, Ning, Zuhong, Yanmei and Zhi'en, 2003).

The ^1H NMR spectrum from 4.2 to 4.6 ppm region of the native polymer revealed a complex spectral pattern of the ring protons. The absence of any prominent signals around 2 ppm region confirmed the absence of any of *O*-acetyl groups in the oligosaccharides of the mixture. The peak for the anomeric proton appeared as a doublet at 5.639 ppm with a small coupling constant (3.34 Hz), which was clearly indicative of the α-configuration (Kardosova, Matulova, & Malovikova, 1998; Michaud, Pheulpin, Petit, Seguin, Barbotin and Heyraud, 1997; Mulloy, Ribeiro, Alves, Vieira, & Mourao, 1994; Wawer, Piekarska-Bartoszewicz, Temeriusz, Potrzebowski, & Ciesielski, 1998) in the mixture of WSPS. It can be assumed that the polymer may be composed of or the derivatives of α-glucuronic acid rather than β as reported previously (Courtois et al., 1993). ^1H NMR spectra also exhibited an important peak at 5.848 ppm, characteristic of the H-4 of an unsaturated unit corresponding to the non-reducing terminus unit of the glucuronan (Costa, Michaud, Petit, Heyraud, Colin-Morel and Courtois, 2001; Liu, Jiang, Liao, & Guan, 2002). This suggests that some or all of the constituents of the mixture are composed of oligomers of α-glucuronic acid with unsaturation at the terminal unit of the non-reducing end. However, we could not obtain a good ^{13}C NMR spectrum of the original WSPS (data not shown) but still it displayed a peak at about 145 ppm, which confirmed the presence of unsaturation in the molecule(s) (Liu et al., 2002).

Table 1
Fragmentation analysis of the major oligoglucuronate in the mixture of WSPS produced using the basal medium by *G. hansenii* PJK in a jar fermenter

m/z	Species	Type of fragments
876.5	$C_{30}H_{36}O_{30}$	$[M+H]^+$-5H
717.15	$C_{24}H_{29}O_{25}$	$[M-B_1]^+-5H$
701.19	$C_{24}H_{29}O_{24}$	$[M-C_1]^+$-4H
542.2	$C_{18}H_{22}O_{19}$	$[M-B_2]^+-3H$
522.36	$C_{18}H_{18}O_{18}$	$[M-C_2]^+$-7H

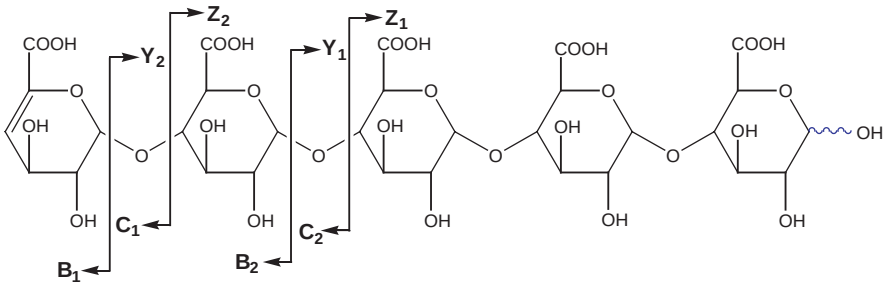

Fig. 2. MALDI-TOF MS fragmentation pattern of the major oligoglucuronate in the mixture of WSPS produced using the basal medium by *G. hansenii* PJK in a jar fermenter.

MALDI-TOF-mass spectrometry is a convenient tool for the structural analysis of oligosaccharides, because of its sensitivity and applicability to the analysis of mixtures (Mazumder, Lerouge, Loutelier-Bourhis, Driouich, & Ray, 2005). The MALDI-TOF mass spectrum of the WSPS was interpreted in light of the HPLC chromatogram of the hydrolyzed products, and the FT-IR, and the NMR data of the native polymer. By the combination of all these analytical and spectroscopic techniques, we have elucidated the structure of the major oligosaccharide (depicted in Fig. 1). The observed peaks and the corresponding species for the fragments of major oligomer are presented in Table 1 using the similar nomenclature of the fragmentation proposed by Domon & Costello (1998) as shown in Fig. 2. The MALDI-TOF mass spectrum showed a peak at *m/z* 876.5, which could be the molecular ion peak $[M+H]^+ - 5H$ for the major oligosaccharide in which all the carboxylic functions have been deprotonated, thus corresponding to the molecular weight 880 Da, which is consistent with the proposed structure (Fig. 1). Other important peaks in the MALDI-TOF MS were observed at *m/z* 717.15, 701.19, 542.28 and 522.36, which appeared to be due to the cleavage of glycosidic bridges between the glycuronosyl units. This type of fragmentation pattern is consistent with the mass pattern of acidic oligosaccharides (Lee, Park, Seo, Choi, & Jung, 2004; Schiller, Arnhold, Benard, Reichl, & Arnold, 1999).

From the spectroscopic data it is apparent that the major oligomer in the mixture of WSPS has unsaturation in its structure at the non-reducing terminus, but the HPLC chromatogram showed a single peak of glucuronic acid, as mentioned earlier. This homogeneity of the hydrolysates may possibly be due to the reduction of the double bond during hydrolysis, thus yielding all monomers as glucuronic acid.

In conclusion, this study has revealed that the so called water-soluble polysaccharides produced by *G. hansenii* PJK are actually the mixture of oligomers of glucuronic acid all having molecular weights less than 1000 Da. According to the literature search, this may be the first report of production of such type of glucuronic acid oligomers by microorganisms, although there is a report on the synthesis of some related oligomers as a result of the enzymatic activity on glucuronan rather than directly by the bacteria (Delattre, Michaud, Lion, Courtois, & Courtois, 2005). Moreover, the oligomers produced by *G. hansenii* PJK were identified in the current study to be α- linked rather than β.

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