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The chemical and digestive properties of a soluble glucan from *Agrobacterium* sp. ZX09

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ABSTRACT

A salt-tolerant strain *Agrobacterium* sp. ZX09 produced a high molecular mass, water-soluble extracellular polysaccharide (EPS) composed of D-glucose. By examining periodate oxidation, ^{1}H NMR and ^{13}C NMR spectra, it was proven that the EPS consisted of the following repeating unit: →3)-β-D-Glc*p*-(1 → 3)-[β-D-Glc*p*-(1 → 3)-β-D-Glc*p*-(1 → 3)]3-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1→. *In vitro* the EPS was indigestible by α-amylase, and strongly inhibited pancreatic α-amylase activity. Fasting mice fed on the EPS failed to increase blood glucose levels. Experimental diets containing the EPS suppressed steep increase in blood glucose concentration. These results suggest that the novel water-soluble glucan could be potentially useful for decreasing absorption of dietary carbohydrate and postprandial blood glucose level.

1. Introduction

Glucan is a polysaccharide of D-glucose monomers linked by glycosidic bonds. A few bacteria and yeasts employ large extracellular glucosyltransferase for the synthesis of high molecular mass glucans from sucrose (Freimunda, Sauter, Käppeli, & Dutler, 2003; Kim, Ryu, Choi, Rhee, & Lee, 2003; Kuramitsu, 1975). Although high similarity exists between these glucansucrase enzymes, they are able to synthesize α-glucans with different types of glucosidic linkages. These glucans can be divided into the following two groups: (i) α-glucans, which contains large amounts of glycosidic bonds of the alpha form. Dextran is typical α-glucan which consists of α-1,6 glycosidic linkages between glucose molecules in its straight chain, while branches begin from α-1,3 linkages (and in some cases, α-1,2 and α-1,4 linkages as well) (Purama, Goswami, Khan, & Goyal, 2009). (ii) β-Glucans, naturally occurring polysaccharides with poly-branched β-1,3-(D)-glucans or β-1,6-(D)-glucose side chains, are integral cell wall constituents in a variety of bacteria, yeast, fungi and cereal plant (Babíček, Čechová, Simon, Harwood, & Cox, 2007; Sener, Toklu, & Cetinel, 2007). β-(1 → 3, 1 → 4) glucan from oat could enhance resistance to *Eimera vermiformis* infection in immunosuppressed mice (Yun et al., 1996). The beta-1,3-(D)-glucan with beta-1,6-glucan linkage extracted from yeast cell wall (*Saccharomyces cerevisiae*) has been shown to act as a potent non-specific immune-activator (Hofer & Pospíšil, 1997; Jørgensen & Robertsen, 1995). Beta-glucans maybe used to lower cholesterol and control postprandial blood glucose (Demirbas, 2005; Jenkins, Jenkins, Zdravkovic, Würsch, & Vuksan, 2002; Klopfenstein, 1988).

In the family of β-1,3-D-glucan, curdlan is one of the important polysaccharide to be applied as food additive. Curdlan molecules, produced by some *Agrobacterium* species and some other bacterium (Harada & Harada, 1996; Kim et al., 2003), have as many as 12,000 glucose units (Futatsuyama, Yui, & Ogawa, 1999), and are insoluble in water, but dissolve in dilute bases (0.25 M NaOH) (McIntosh, Stone, & Stanisich, 2005). Heating aqueous suspensions of curdlan above 80 °C and then cooling it produces a high-set, thermo-irreversible gel, whereas a low-set, thermo-reversible gel is produced on heating to 55 °C (McIntosh et al., 2005). These unusual rheological properties among natural and synthetic polymers underlie curdlan's use as a biothickening and gelling agent in foods (Harada & Harada, 1996).

Recently, it has been demonstrated that β-glucan and its derivatives have multiple biological activities as antioxidant (Kogan et al., 2005), anti-virus (Zhang, Zhang, Wang, & Cheung, 2003; Zhang, Cheung, Ooi, & Zhang, 2004) with low toxicity, anti-tumor (Chen, Xu, Zhang, & Zeng, 2009; Kobayashi et al., 2005; Yoon et al., 2008) and inhibit cellular invasion (Falch, Espevik, Ryan, & Stokke, 2000), especially as a nutritional key, inserting in a specific receptor for β-1,3-glucan in macrophage, turning and activating the immune system of the macrophage (Sener et al., 2007; Tokunaka et al., 2000; Vetvicka & Yvin, 2004). However, the application of β-glucan has been limited because of its properties of insolubility in aqueous media with physiological pH (Hromádková et al., 2003; Kim et al., 2003; Ohno et al., 1999). Administration of glucan as a food additive induced no toxicity, while intravenous injection with insoluble glucan may cause undesirable side effects. There-

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fore exploring a soluble β-glucan in physiological media should be important for their application. The solubility of glucans in the media depends on their chemical structure, molecular mass and conformation (Colleoni-Sirghie, Fulton, & White, 2003). The sulfate and phosphate esters of curdlan (Koumoto et al., 2004) with increased water solubility, display enhanced biological activity (Toida, Chaidedgumjorn, & Linhardt, 2003) and, moreover, a water-soluble aminated curdlan derivative has tumorigenic properties (Seijelid, 1986).

Here, we reported a novel strain *Agrobacterium* sp. ZX09, isolated from a salt soil sample, which produced a water-soluble extracellular glucan containing large amounts of 1,3-glycosidic bonds of the beta form and a small amount of alpha form. We therefore investigated the inhibition effect of the novel glucan on α-amylase activities, and demonstrated that feeding mice containing this glucan decreased significantly the levels of postprandial blood glucose.

2. Materials and methods

2.1. Strain and the culture conditions

The strain ZX09 used in this study was isolated from a soil sample from ocean coast of Shandong, China. This isolate was accidentally found in an investigation of microbial resources from saline soils. Cultures were maintained on Htm agar consisting: NaH_2PO_4 1 g, KNO_3 3 g, $CaCl_2$ 0.07 g, $MgCl_2$ 0.2 g, $FeSO_4{\cdot}7H_2O$ 0.0125 g, $MnSO_4$ 0.003 g, $ZnCl_2$ 0.0075 g, sucrose 20 g, agar 9 g and H_2O 1000 ml, pH 7.2. Biomass production in various conditions was monitored or compared each other by reading OD at 600 nm. The characteristics of strain ZX09 were identified with reference to Bergey's manual of systematic bacteriology. Genomic DNA preparation, PCR amplification of 16S rDNA, and sequencing of the PCR products were carried out as described previously (Rainey, Ward-Rainey, Kroppenstedt, & Stackebrandt, 1996). The 16S rDNA sequence obtained in this study was deposited in the GenBank database under the accession number GU810841.

2.2. Preparation, isolation and purification of the water-soluble exo-polysaccharides (EPS)

A colony of the strain ZX09 was inoculated into a 250 ml flask containing 50 ml medium consisting of 2% sucrose and mineral salts solution (0.1% NaH_2PO_4, 0.3% KNO_3, 0.007% $CaCl_2$, 0.02% $MgCl_2$, 0.00125% $FeSO_4{\cdot}7H_2O$, 0.0003% $MnSO_4$, 0.00075% $ZnCl_2$ and 1.0% NaCl, pH 7.2). The inoculated preparation was incubated at 28 °C on a rotary shaker at 220 rpm for 24 h. A 0.5 ml portion was transferred to a 250 ml flask containing 50 ml fermentation medium as above described. The fermentation was performed on a rotary shaker at 220 rpm for 48 h unless otherwise indicated. The culture broth was diluted more than 3 times with de-ionized water and centrifuged at 12,000 × *g* for 30 min to separate cells from the supernatant. The supernatant was added to two volumes of 95% ethanol. Productivity of extracellular materials (EMS) were expressed in terms of the weight after ethanol precipitation collected by centrifugation at 6000 × *g* for 15 min and dried under reduced press. The EPS was isolated and purified according to previously described methods (Ge, Duan, Fang, Zhang, & Wang, 2009; Nakashima et al., 1987). Gel filtration chromatography was conducted with a Sepharose CL-4B (Pharmacia) column (1.5 by 60 cm), and the polysaccharides were eluted with 50 mmol/l phosphate buffer, pH 7.2, at the rate of 1 ml/min. Fractions containing polysaccharides were collected, and the total sugar content in each fraction was determined by the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

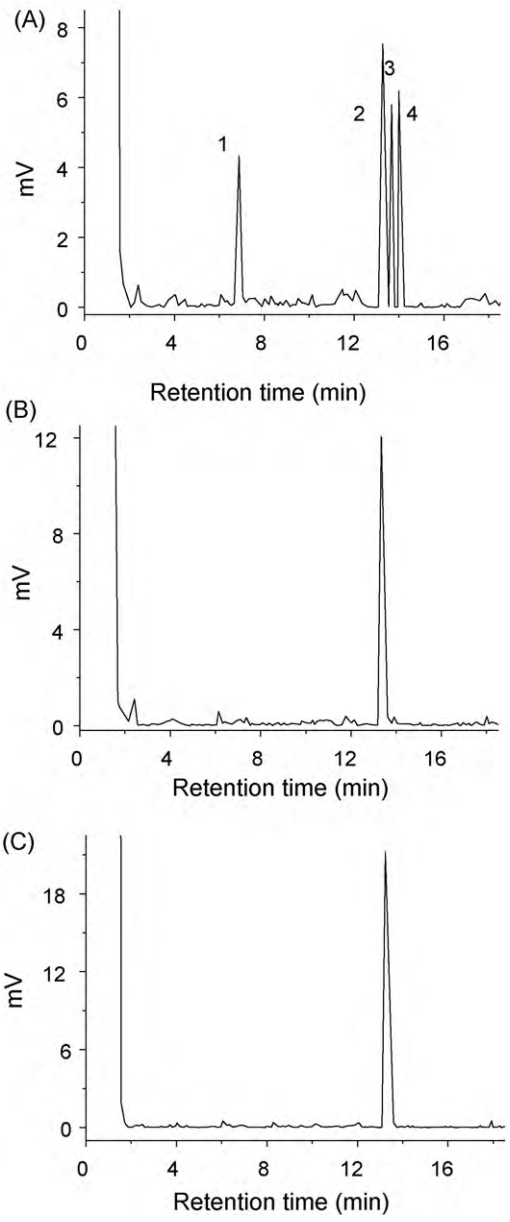

Fig. 1. Monosaccharide composition analysis of the EPS. (A) The GC profiles of monosaccharide standards. 1, D-Arabinose; 2, D-glucose; 3, D-mannose; 4, D-galactose. (B) The GC profile of the hydrolyzed EPS. (C) The GC profile of the mixed EPS and D-glucose.

2.3. Chemical analysis

The total sugar content in the EPS was measured by the method of Dubois et al. (1956) by monitoring the absorbance at 490 nm with glucose as a standard. The total protein content was estimated using the Bradford reagent (Bio-Rad, USA) with bovine serum albumin as a standard. The moisture content of the EPS was determined gravimetrically after heating the material (500 mg) in an oven at 105 °C for 24 h. The ash content of the EPS (3 g) was determined gravimetrically after dry mineralization at 600 °C for 12 h. Monosaccharide Compositions in the purified EPS were determined by TLC analysis and gas-liquid chromatography of o-methyloxime acetate derivatives obtained after acid hydrolysis of polysaccharides (6 h, 100 °C) in a 2N trifluoroacetic acid solution (Neeser & Schweizer, 1984). Periodate oxidation of the EPS were performed by Linker, Evans, and Impallomeni (2001), by monitoring the absorbance at 223 nm

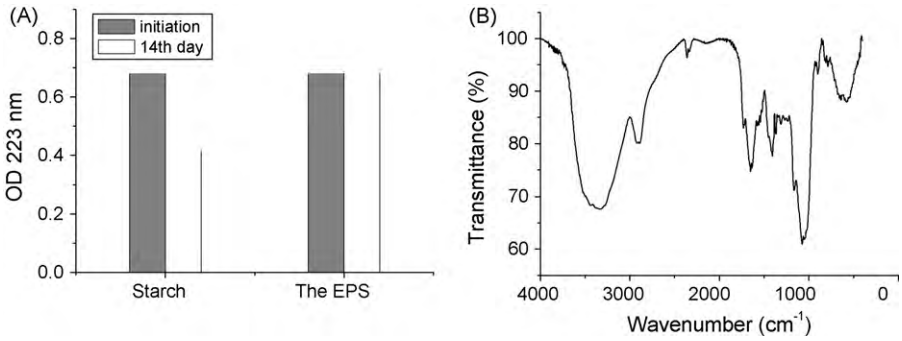

Fig. 2. The periodate oxidation (A) and the FT-IR spectrum of the EPS (B). No reaction between the EPS and periodate was observed.

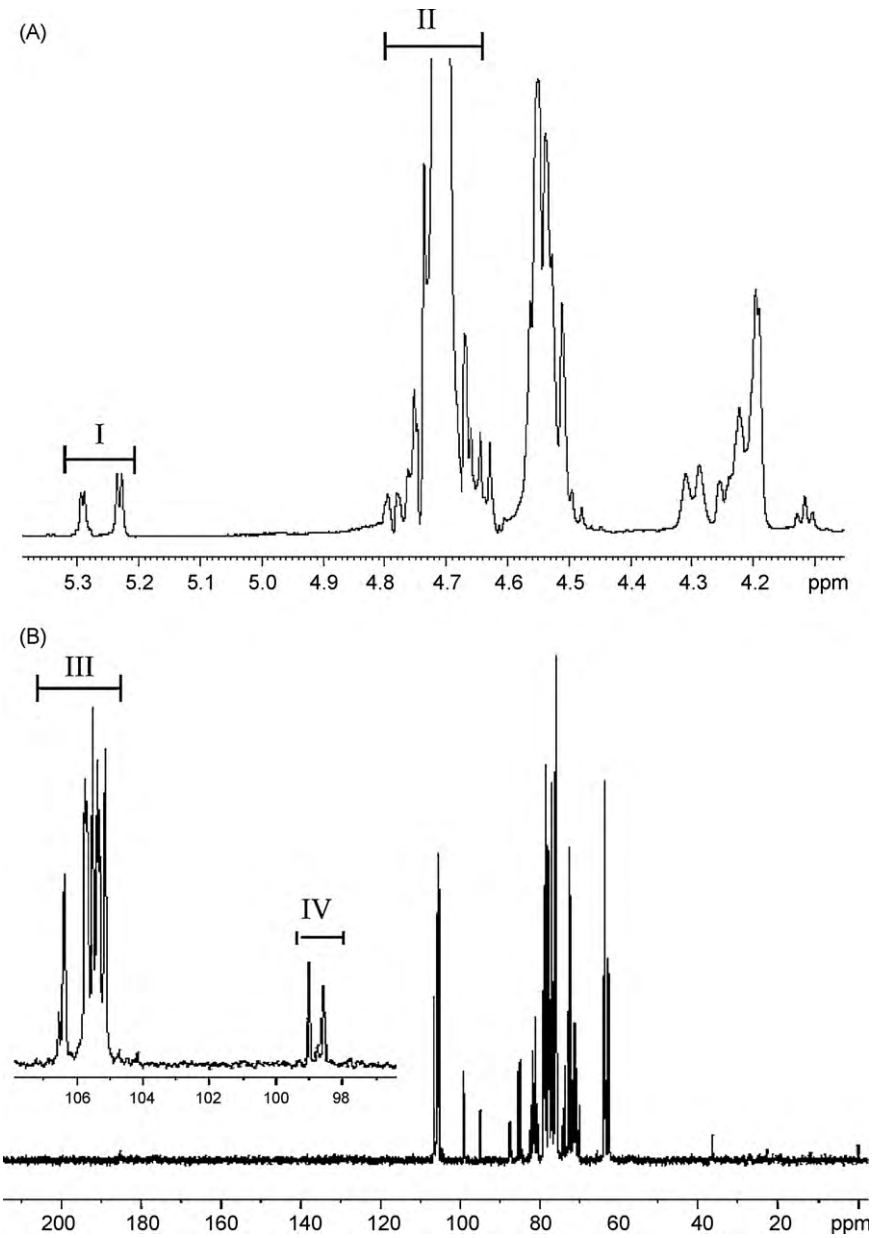

Fig. 3. ^{1}H NMR spectrum (A) and ^{13}C NMR spectrum of the EPS (B). Region I were assigned to α-glucose H1 residues (δ 5.1–5.3 ppm) with relatively weak signals; region II were assigned to β-glucose H1 residues (δ 4.6–4.8 ppm) with relatively strong signals; region III were strong signals representing β-glucoses C1 residues (104.5–106.5); and region IV were assigned to α-glucose C1 residues and terminated α or β glucose C1 (98.5–99.05 ppm) with relatively weak signals.

Table 1
The chemical shifts of ^{13}C NMR of the EPS from *Agrobacterium* sp. ZX09.

Carbon	Chemical shifts								
	Glc 1 →1,3-β-D-Glc*p*	Glc 2 →1,3-β-D-Glc*p*	Glc 3 →1,3-β-D-Glc*p*	Glc 4 →1,3-β-D-Glc*p*	Glc 5 →1,3-β-D-Glc*p*	Glc 6 →1,3-β-D-Glc*p*	Glc 7 →1,3-β-D-Glc*p*	Glc 8 →1,3-α-D-Glc*p*	Glc 9 →1,3-α-D-Glc*p*
C1	106.36	105.75	105.69	105.51	105.37	105.31	105.14	99.05	98.50
C2	75.93	75.87	75.78	75.63	75.35	74.25	74.15	74.07	73.72
C3	**87.36**	**87.16**	**85.45**	**84.86**	**84.86**	**82.34**	**82.05**	**81.80**	**81.59***
C4	71.92	71.81	71.48	71.11	71.22	71.11	70.99	70.83	70.35
C5	77.74	77.63	76.96	76.39	76.13	76.06	77.28	76.93	75.87
C6	63.94	63.83	63.83	63.56	63.43	63.40	63.35	62.78	62.67

* Chemical shifts after it was linked C-1.

with known structure starch as a positive control. Infrared spectra of the samples were recorded with a Bruker Fourier transform infrared (FT-IR) spectrometer (TENSOR 27, Germany) in the range of 4000–400 cm^{-1} using the KBr disk method.

2.4. Acid hydrolysis of the EPS for NMR analysis

The EPS was partially hydrolyzed for NMR analysis by the method of De Paula, Heatley, and Budd (1998). Briefly, the pH of an EPS solution was adjusted to 2.0 by adding 0.5 M sulfuric and heated 100 °C for 24 h. Then solution were neutralized with barium carbonate, filtered and de-ionized using an Amberrlite IR-120 column. Solution was dialyzed for 24 h against de-ionized water and freeze dried. NMR spectra were obtained on a Bruker AV-500 instrument (Switzerland) (100 MHz for ^{13}C NMR, 400 MHz for ^{1}H NMR) at 70°C. Before the analysis, EPS samples were exchanged twice in D_2O with intermediate lyophilization and then dissolved in 0.5 ml of D_2O to a final concentration of 30 mg ml^{-1}.

2.5. Analysis of amylase inhibition

Mouse pancreas α-amylase was isolated purified by standard protocol. The measurements of amylase activity were performed to according to the method of Bernfeld modified (Bernfeld, 1955). The glycemic response to the EPS was examined by monitoring glucose level after feeding the EPS or mixed EPS and starch in 15 h fasting mice. The C57/BL6 mice were orally given the EPS (1 mg/g), starch (1 mg/g) or mixed different amounts of EPS and starch by intragastric administration. Blood samples were taken from tail vein at 0, 30, 60 and 120 min. The blood glucose level was measured by a commercial Glucose Meter (Johnson & Johnson, USA). All animal maintenance and use procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee at NJUST.

3. Results

3.1. Cellular and metabolic characteristics of strain ZX09 and 16S rDNA sequence analyses

Strain ZX09, isolated from a saline soil sample, showed characteristic morphological and chemotaxonomic features of Agrobacteria. A colorless colony appeared in Htm agar after 2 days of incubation. The size of the colony increased and extended whole plate after about 5–10 days. The vegetative cells were cylindrical rods. The strain ZX09, which produced large amounts of extracellular material, had the following bacteriological characteristics: gram staining (−), growth at 10 °C (−), at 45 °C (−), at pH 9.0 (+), esculin (−), glucose (+), starch (−), sucrose (+). ZX09 could grow at NaCl concentrations ranging from 0 to 7.0%, with optimal growth occurring at 2% NaCl. The sequences of the 1357-bp 16S rDNA fragment of strain ZX09 was submitted to GenBank with an accession number GU810841. The ZX09 displayed no sensitive to NaCl concentration (up to 7%), which was distinguished from all reported *Agrobacterium* strains, reflected a possible adaptation to salt soil environment.

3.2. The chemical composition of the extracellular material

Batch fermentations of strain ZX09 were performed under standard conditions. The biosynthetic production of extracellular macromolecules continued and achieved a maximal value at 48 h. At this point, the viscosity of the fermentation broth was at its highest value (data not show). The chemical compositions of the extracellular macromolecules were sugar (77.13%), protein (6.2%), moisture (5.2%) and ash (10.28%). The results revealed that the extracellular macromolecules were composed mainly of polysaccharides. Hydrolysis of the purified exo-polysaccharide (EPS) with 2 M trifluoroacetic acid yielded D-glucopyranose as one and only constituent, which was determined by gas chromatogram (Fig. 1A–C), and conformed by thin layer chromatogram analysis.

3.3. The chemical analysis of the EPS

The crude EPS obtained from the ethanol precipitate of the cultured broth by ZX09. The average molecular weight of the purified EPS was estimated from a calibration curve of standard dextrans obtained by gel filtration on Sepharose CL-4B to be about 2×10^6. The periodate oxidation values of the EPS revealed that the EPS was resistant to periodate, indicating that the glucose units of the EPS isolated from ZX09 were linked by 1,3-glucosidic linkages (Fig. 2A). To find the configuration of glucose in the EPS, we performed an infrared (IR) spectroscopy for the EPS, and the IR spectrum showed an absorption band at 890 cm^{-1}, indicating that D-glucopyranose has a β-configuration; it was also revealed that a

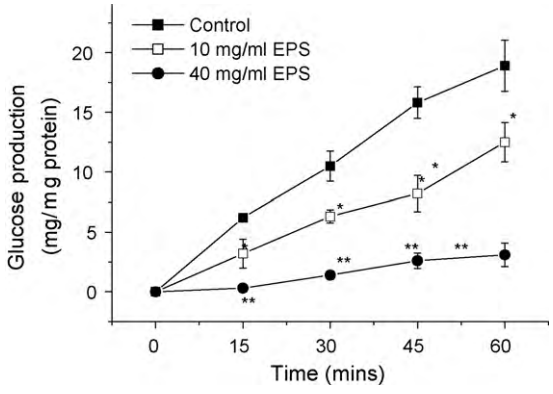

Fig. 4. *In vitro* hydrolysis of starch. The reaction mixture included pancreatic juice (α-amylase) prepared from C57/b6 mice. The ration mixture including 0.1% (w/v) soluble starch was incubated for 15, 30, 45 and 60 min at 37 °C with or without EPS. The reaction was terminated by adding the 50 mM Tris–glucose oxidase reagent. The hydrolysis activity was expressed as mg glucose production per mg protein. Vertical bars represent mean ± SEM ($n = 3$). Statistical evaluation was performed using a Student's *t*-test. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with control group.

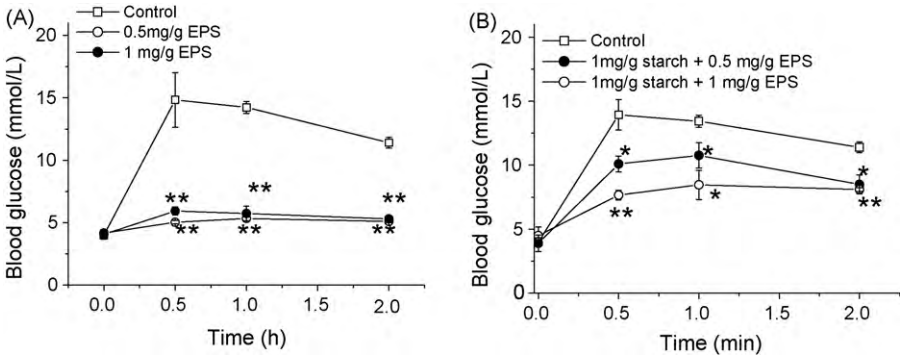

Fig. 5. Blood glucose responses of the mice to starch and the EPS. (A) No significant changes of blood glucose while the EPS present in fasting mice and (B) reducing blood glucose levels after administration of the mixture of starch and EPS in fasting mice. Each value was the mean ± SEM of 3 samples or 5–6 animals. Statistical evaluation was performed using a Student's *t*-test. $^{*}p < 0.05$ and $^{**}p < 0.01$, compared with control group or control mice.

little α-glucopyranose could exist since there was a weak characteristic absorption band at 810 cm^{-1} (Fig. 2B).

3.4. The NMR analysis of the EPS

To get clear NMR signals, the EPS were performed to hydrolyze partially with sulfuric acid as described in Section 2. The ^{1}H NMR spectrum of acid-hydrolyzed EPS showed two singlets, which were assigned to β-glucose residues (δ 4.6–4.8 ppm) with strong signal region and α-glucose residue (δ 5.1–5.3 ppm) with weak signals, respectively, and this suggested, agreement of data from IR, that glucose residues in the EPS existed both β- and α-configurations (Fig. 3A). The ^{13}C NMR spectra of the EPS were conducted, low field signals at 106.4, 105.75, 105.69, 105.51, 105.37, 105.31, 105.14 ppm were assigned to the C-1 of β-D-glucose residues, and the signals at δ 98.5 and 99.05 ppm were assigned to the C1 of α-D-glucose residues (Fig. 3B). There were no signals between 67 and 70 ppm, indicating no C-6 in glucose residues in the EPS was linked. The signals at 81–87 ppm were assigned as C-3 chemical shifts after it was linked C-1. Comparing the ^{13}C NMR and ^{1}H spectra of the EPS with data in the literature, the chemical shifts of the EPS were shown in Table 1. Based on the results of chemical and NMR analysis, the EPS consists of the following possible repeating unit: →3)-β-D-Glc*p*-(1 → 3)-[β-D-Glc*p*-(1 → 3)-β-D-Glc*p*-(1 → 3)]$_3$-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*-(1→.

3.5. Inhibition of pancreatic amylase in vitro and in vivo

Tests for the EPS as substrate of bacterial α-amylase were negative. It did not support the growth of *Bacillus subtilis* 168 when sterilized by filtration and added to growth media at a final concentration of 0.1–0.5%. *In vitro* inhibition of pancreatic α-amylase isolated from mouse pancreas was observed when the EPS present at both 0.1% and 4% concentration (Fig. 4).Then, we designed the protocols testing whether the EPS could be as starch blocker *in vivo*. Fasting mice were intragastrically administrated with the compared amounts of starch and low and high amounts of the EPS. As shown in Fig. 5A, mice feeding on starch had remarkably high fasting blood glucose level at 0.5 and 1 h after treatment of starch; the EPS at doses of 0.5 and 1 mg/g did not change significantly the blood glucose levels at multiple time points. This lack of elevated glucose level could be attributed to the absence of 1,3-glucosidic enzymes in mice. Also, the mixed EPS and starch resulted in decreasing blood glucose levels (Fig. 5B).

4. Discussion

It was reported that a variety of *Agrobacteria* strains produced the linear (1 → 3)-β-glucan, the cyclic (1 → 3, 1 → 6)-β-glucans and the side chain-branched (1 → 3,1 → 2)-β-glucan (McIntosh et al., 2005). Curdlan was a neutral, essentially linear (1 → 3)-β-glucan with a few intra- or inter-chain (1 → 6) linkages (McIntosh et al., 2005; Saito, Misaki, & Harada, 1968). In natural state, curdlan was poorly crystalline and was found as a granule, much like that of starch, being insoluble in distilled water. The EPS from *Agobacterium* sp. ZX09 was a lined glucan, which consisted of a large amount of β-1,3-D-glucosidic linkages as main bone structure, together with a small proportion of α-1,3-D-glucosidic linkages. Most glucans consisted of β-1,3 linkages displayed a water-insoluble property, and however, the EPS from ZX09 had relatively high molecular mass and water-soluble, seeming to correlate with the presence of α-1,3-D-glucan bound. The differences between β-glucan linkages and chemical structure were significant in regard to solubility and overall biological activity (Šandula, Kogan, Kačuráková, & Machová, 1999; Schmid et al., 2001).

Administration of soluble β-glucan together with a mixed meal was known to decrease postprandial glucose and insulin concentrations. Soluble fibers increased the viscosity of the alimentary bolus, and hence lengthen the gastric emptying and intestinal transit times (Cameron-Smith, Collier, & O'Dea, 1994; Johansen, Knudsen, Sandstrom, & Skjoth, 1996; Wood, 1990). In our observation, the EPS from *Agrobacterium* ZX09 delayed the rate of starch digestion by pancreatic amylases *in vitro*, which may delay the interaction of the enzyme with its substrate (Dunaif & Schneeman, 1981; Dutta & Hlasko, 1985; Hansen & Schulz, 1982). As observed in our *in vivo* experiment, feeding on mixture of the EPS and starch limited the postprandial rise in plasma glucose concentration, which might be due to reduction of the rate of starch hydrolyzation. Radiolabeled studies verified that both small and large fragments of β-glucans were found in the serum, which indicated they were absorbed from the intestinal tract (Tsukagoshi et al., 1984), where they began to interact with macrophages to activate immune function (Frey et al., 1996). Studies also demonstrated that β-D-glucan promoted as dietary supplement for weight loss (Burkus & Temelli, 2005). Thus, the EPS from *Agobacterium* ZX09, as a novel structure of β-glucan, might be potentially useful for decreasing absorption of dietary carbohydrate and postprandial blood glucose level.

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