Characterization and Antioxidant Activity of the Exopolysaccharide Produced by *Bacillus amyloliquefaciens* GSBa-1

Wen Zhao^{1,2}, Jian Zhang^{1,2}, Yun-Yun Jiang^{1,2}, Xiao Zhao^{1,2}, Xiao-Na Hao^{1,2}, Liu Li^{1,2}, and Zhen-Nai Yang^{1,2*}

¹Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Technology and Business University (BTBU), Beijing 100048, P.R. China

²Beijing Engineering and Technology Research Center of Food Additives, Beijing Technology and Business University (BTBU), Beijing 100048, P.R. China

Received: January 8, 2018

Revised: May 29, 2018

Accepted: May 30, 2018

First published online
June 6, 2018

*Corresponding author

Phone: +86-10-6898-4870;

Fax: +86-10-6898-5456;

E-mail: yangzhennai@th.btbu.edu.cn

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2018 by
The Korean Society for Microbiology
and Biotechnology

The exopolysaccharide (EPS) produced by *Bacillus amyloliquefaciens* GSBa-1 was isolated and purified by ethanol precipitation, and DEAE-cellulose and Sepharose CL-6B chromatographies. The molecular mass of the purified EPS was determined to be 54 kDa. Monosaccharide analysis showed that the EPS was composed of predominantly glucose, and it was further confirmed by NMR spectroscopy to be α -glucan that consisted of a trisaccharide repeating unit with possible presence of two α -(1 $\rightarrow$ 3) and one α -(1 $\rightarrow$ 6) glucosidic linkages. Microstructural analysis showed that the EPS appeared as ellipsoid or globose with a smooth surface. The EPS had a degradation temperature at 240°C. Furthermore, the EPS had strong DPPH and hydroxyl radical scavenging activities, and moderate superoxidant anion scavenging and metal ion-chelating activities. This is the first characterization of a glucan produced by *B. amyloliquefaciens* with strong antioxidant activity. The results of this study suggest the potential of the EPS from *B. amyloliquefaciens* GSBa-1 to serve as a natural antioxidant for application in functional products.

Keywords: *Bacillus amyloliquefaciens*, exopolysaccharide (EPS), physicochemical properties, antioxidant activity

Introduction

Bacteria can produce polysaccharides intracellularly or extracellularly via utilization of simple to complex carbohydrate substrates. The extracellular polysaccharide, also called exopolysaccharide (EPS), can be a homopolysaccharide composed of only one type of monosaccharide such as glucan, levan, or pullulan, or a heteropolysaccharide consisting of different types of monosaccharides. Most bacterial EPSs reported are heteropolymers with multiple copies of oligosaccharide unit, such as xanthan, hyaluronic acid, heparin, etc. [1–3].

Bacterial EPSs play important roles in bacterial adherence to biosurfaces and protecting cells from adverse environment damage, and these polymers can also serve as reserve carbohydrates for subsequent metabolism when there are abundant sugar supplies [4]. During the last decade,

bacterial EPSs have been reported with different biological activities, such as lowering blood cholesterol level, and antioxidation, anticancer, and immunoregulation effects [5, 6]. These bioactivities of EPSs were found to closely relate to their monosaccharide composition, molecular weight, conformation, and glycosidic linkage [7]. EPSs with a helix structure and higher molecular mass possessed higher anti-inflammatory activity [8]. EPSs with a linear branched structure exhibited stronger antitumor and immunostimulatory activities [9].

Bacillus sp. are an important bacterial group that have several advantages over other bacteria, such as easy cultivation and preservation, and are thus conducive to industrial production [10]. *Bacillus* sp. can produce EPSs with larger quantities than lactic acid bacteria, and the same *Bacillus* strain may produce more than one type of EPS [11]. The EPS from *B. amyloliquefaciens* LPL101 was

found to be a good biomulsifier with high viscosity [10]. The EPS from *B. amyloliquefaciens* MD-B1 was reported to have excellent antitumor activity [12]. Two EPSs from *B. amyloliquefaciens* C-1 exhibited excellent antioxidant activity [13].

Recently, in our laboratory, *B. amyloliquefaciens* GSBA-1, isolated from traditional glutinous rice wine starter, was found to produce a high yield of EPS in a semi-defined medium [14]. In order to further understand the structure and function of the polysaccharide, the EPS from *B. amyloliquefaciens* GSBA-1 was purified and characterized in terms of its monosaccharide composition, microstructure, and thermogravimetry. The biological function of the EPS was studied, focussing on its antioxidant activity, for exploring the application of the EPS as a functional ingredient in the food industry.

Materials and Methods

Microorganism and Culture Conditions

B. amyloliquefaciens GSBA-1 isolated from traditional glutinous rice wine starter was kept in the authors' laboratory at -80°C with 15% (v/v) glycerol. The strain was cultured in an optimized EPS-producing medium, which consisted of sucrose (20 g/l), yeast extract (5 g/l), peptone (10 g/l), and NaCl (10 g/l), at initial pH 7.0, temperature of 37°C , and shaking at 160 rpm [14].

Isolation and Purification of the EPS

B. amyloliquefaciens GSBA-1 from the freezing stock (-80°C) was activated with three consecutive transfers and incubated at 37°C for 36 h in the medium mentioned above. Then, the fermentation broth was heated in a 100°C water bath to inactive enzymes that may degrade the EPS. Then, 80% (w/v) trichloroacetic acid was added and adjusted to 4% (w/v) final concentration, stirred for 30 min, and centrifugated at $10,000 \times g$ for 40 min at 4°C was carried out to remove proteins and free cells. To the cell-free supernatant was added twice the volume of chilled ethanol, and the mixture was kept at 4°C overnight to precipitate the polysaccharide. The precipitate was collected by centrifugation at $10,000 \times g$ for 40 min at 4°C . It was then dissolved in deionized water for fractionation with a cellulose dialysis membrane (MWco 8–14 kD) at 4°C for 48 h with changes of deionized water per 8 h. The sample was lyophilized to obtain the crude EPS powder. The EPS content was determined by the phenol-sulfuric acid method [15].

The crude EPS solution (20 mg/ml, 5 ml) was fractionated on a DEAE-cellulose column (26 mm $\times$ 40 cm), which was eluted first with deionized water, and then 0.2 and 0.5 M NaCl solutions. The polysaccharide in the fractions was detected by the phenol-sulfate method. The EPS-containing fractions were pooled, dialyzed, and lyophilized for further separation with a Sepharose CL-6B column (25 mm $\times$ 50 cm), eluted with 0.9 M NaCl solution to obtain the purified EPS.

Characterization of the EPS

Molecular weight determination. The molecular weight (M_w) was determined by gel permeation chromatography (GPC) (Shimadzu, Japan) using a TSKgel GMPWXL (TOSOH, Japan) column with a RID-20 detector. The sample (0.2 mg/ml, 200 μl) was applied and eluted with a 0.1 M NaNO_3 and 0.06% NaN_3 solution at a flow rate of 0.6 ml/min. Data were collected and processed from the standard graph, which was plotted using standard dextran (Sigma-Aldrich, USA).

Monosaccharide composition determination. The monosaccharide composition analysis was performed according to the method of Zhang *et al.* [16]. In brief, 5 mg of the EPS sample was hydrolyzed with 2 ml of 2 M trifluoroacetic acid by heating at 120°C for 2 h. The hydrolysate was co-concentrated repeatedly with N_2 gas. Then, 30 mg of NaBH_4 was used to reduce the sample, and 1 ml of acetic anhydride was added to the hydrolyzed sample for acetylation. The acetylated sample was analyzed on an Agilent Technologies 7890A GC equipped with a DB225 capillary column (30 $\times$ 0.25 mm) and flame ionization detector. Nitrogen gas was used as the carrier gas with a flow rate of 1 ml/min. The column temperature was held at 120°C for 2 min, increased at $10^{\circ}\text{C}/\text{min}$ to 220°C , kept at 220°C for 10 min, increased at $10^{\circ}\text{C}/\text{min}$ to 240°C , and held for 5 min. The eight standard sugars (D-galactose, D-glucose, L-rhamnose, L-fucose, L-arabinose, D-mannose, D-xylose, and D-trehalose) were analyzed using the same method described above without the hydrolyzation step.

Fourier-transform infrared (FT-IR) spectroscopy. The major functional groups of the purified EPS were detected using FT-IR spectroscopy [17]. The sample pellet was prepared by grinding a mixture of the EPS (1 mg) and KBr (100 mg). Then, the FT-IR spectrum was recorded on a Thermo Nicolet 6700 instrument from 400 to $4,000 \text{ cm}^{-1}$.

Scanning electron microscopy (SEM) analysis. The lyophilized EPS sample (5 mg) was fixed to the SEM stub, coated with a gold layer ($\sim 10 \text{ nm}$ thick) with double side. Then, it was imaged at 3.0 kv using a scanning electron microscope (S-4800; Hitachi Ltd, Japan).

Atomic force microscopy (AFM) analysis. The purified EPS was dissolved with distilled H_2O (1 mg/ml) and stirred for approximately 2 h at 40°C . It was then diluted to 1 $\mu\text{l}/\text{ml}$, and 10 μl of the EPS solution was dispensed onto the sample carrier surface and dried at room temperature. The AFM image was taken with a Dimension Icon microscope (Bruker Instruments Co, Germany) and analyzed using SPIP 6.0.9 (Image Metrology A/S, Denmark).

Thermogravimetric analysis (TGA). A scanning calorimeter (TA-60WS; Shimadzu, Japan) was used for determination of the pyrolysis temperature and combustion. The EPS sample (5 mg) was placed in a crucible and heated with linear heating of $10^{\circ}\text{C}/\text{min}$ under a N_2 atmosphere at an energy scan range of $20\text{--}700^{\circ}\text{C}$.

Nuclear magnetic resonance (NMR) spectroscopy analysis. The EPS sample was prepared by exchanging twice with D_2O (99.9 at %D, Cambridge Isotope Laboratories, USA) to replace the H_2O

in the original sample. Immediately after lyophilization, the sample was redissolved in D₂O for ¹H NMR (5 mg/ml) and ¹³C NMR analyses (30 mg/ml). The NMR spectra of the EPS were recorded with a Bruker AVANCE AV-600MHz spectrometer (Bruker Group, USA) operated at 27°C with a 5-mm inverse probe. The chemical shift was expressed as parts per million (ppm).

Antioxidant Properties

DPPH free radical scavenging activity (RSA). The DPPH RSA was measured following the method of Wang *et al.* [17] with slight modifications. First, 1 ml of EPS solution (0, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/ml) was intensively mixed with 2.0 ml of methanolic DPPH radical solution (0.2 mM) and incubated in the dark for 1 h (room temperature). Methanol was used as a blank. The DPPH RSA was determined by measuring the absorbance at 517 nm and was calculated as DPPH RSA (%) = $(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100\%$. The scavenging activity of L-ascorbic acid on DPPH was used as a standard.

Hydroxyl radical scavenging activity. The hydroxyl RSA was measured by a method based on the Fenton reaction [19]. Briefly, the reaction mixture containing 1.0 ml of brilliant green (0.435 mM), 2.0 ml of FeSO₄ (0.5 mM), 1.5 ml H₂O₂ (3% (w/v)), and 1 ml of EPS samples (0, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/ml) was incubated at 37°C for 1 h, stirred, and centrifuged at 4,000 ×g for 10 min. The absorbance value was taken at 624 nm. The hydroxyl RSA was calculated by the following equation: hydroxyl RSA (%) = $((A_1 - A_0) / (A - A_0)) \times 100\%$, where A₁ is the absorbance of the Fenton solution reagent with EPS samples, A₀ is the absorbance of the mixture without the samples, and A is the absorbance of only the brilliant green. L-Ascorbic acid was used as standard.

Superoxide radical scavenging activity. The superoxide RSA of the EPS was determined according to the method described by

Tang *et al.* [20]. Briefly, 1 ml of the EPS samples (0, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/ml) was mixed with 1 ml of phosphate buffer saline containing 137 mM sodium chloride, 2.7 mM potassium chloride, 4.3 mM disodium hydrogen phosphate, and 1.4 mM dipotassium phosphate (pH 8.3) and stored at 25°C for 20 min. The reaction was started by adding 0.2 ml of pyrogallol (3 mM) preheated to 25°C for 4 min. The absorbance value was measured at 325 nm every 10 sec for 5 min. The distilled water served as a blank. The superoxide anion was calculated as superoxide RSA(%) = $(1 - A_{\text{sample}} / A_{\text{blank}}) \times 100\%$, where A_{sample} is the difference of the absorbance values per 10 sec for samples, and A_{blank} is the difference of the absorbance values per 10 sec for the blank.

Ferrous ion-chelating ability. The metal ion-chelating ability of the EPS was determined according to the method of Zhao *et al.* [21]. Briefly, the reaction was performed in a mixture containing 1.0 ml of the EPS solution (0, 0.2, 0.5, 1.0, 2.0, and 5.0 mg/ml), 0.05 ml of 2 mM ferrous chloride (FeCl₂) solution, 0.2 ml of 5 mM ferrozine, and 2.75 ml of water. The absorbance value was measured at 562 nm after fully mixing and held for 10 min at room temperature. EDTA-2Na and deionized water were used as the positive control and blank, respectively. The chelating activity on ferrous ions was calculated as chelating activity (%) = $(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}} \times 100\%$.

Results and Discussion

Purification of the EPS

The crude EPS of *B. amyloliquefaciens* GSBa-1 was separated and fractionated on a DEAE cellulose 52 column (Fig. 1A). The single elution peak of the EPS by 0.2% (w/v) NaCl solution was collected and further purified by

Fig. 1. Purification of the crude exopolysaccharide from *Bacillus amyloliquefaciens* GSBa-1 by anion-exchange chromatography on a DEAE-cellulose-52 column (A), and further gel permeation chromatography on a Sepharose CL-6B column (B).

Fig. 2. Molecular weight determination by gas permeation chromatography (A), and monosaccharide determination by gas chromatography (GC) (B) of the exopolysaccharide (EPS) from *B. amyloliquefaciens* GSBa-1, and GC data of the standard sugars (C).

The retention times correspond to D-trehalose (25.701), L-fucose (26.209), L-arabinose (28.104), D-xylose (29.460), D-glucose (35.694), L-rhamnose (36.403), and D-galactose (36.772).

Sephacrose CL-6B gel permeation chromatography (Fig. 1B). As a result, the fractions corresponding to the major peak of the EPS were collected, dialyzed, and lyophilized for further analyses.

Characterization of the EPS

Molecular mass of the EPS. The molecular mass of the EPS from strain GSBa-1 was determined to be 54 kDa by the GPC-RID-20 system (Fig. 2A), which was higher than that (37.6 kDa) of the EPS from *B. amyloliquefaciens* 3MS [22]. The molecular weight of the EPS varies with the producing strain, and it might be affected by the fermentation time and medium composition [23]. The chromatogram of the EPS showing a single symmetrical peak (Fig. 2A) with the Mw/Mn ratio of the EPS of 1.78 also indicated the homogeneity of the purified EPS and its homogeneous dispersion in aqueous solution [24].

Monosaccharide composition of the EPS. Comparing the GC chromatogram of the EPS (Fig. 2B) with that of the standard sugars (Fig. 2C), the major peak of the EPS corresponding to glucose was found, indicating that the EPS from strain GSBa-1 was a glucan. The monosaccharide composition of the EPS from *Bacillus* strains varies with the strains, their culture conditions, and medium compositions [25]. *B. licheniformis* produced an EPS composed of glucose, galactose, and mannose, whereas *B. tequilensis* FR9 produced an EPS composed of glucose, galactose, mannose, arabinose, and xylose [26]. *B. amyloliquefaciens* LPL061 produced two different EPSs composed of mannose and glucose in a relative molar proportion of 96.9/3.2 and 65.3/34.7, respectively [13]. Several other bacterial strains were

Fig. 3. FT-IR spectrum of the exopolysaccharide produced by *B. amyloliquefaciens* GSBa-1.

Fig. 4. Scanning electron microscope images (A and B) and atomic force microscopy images (C and D) of the exopolysaccharide from *B. amyloliquefaciens* GSBa-1.

reported to produce dextran with different structures, such as those from *Leuconostoc mesenteroides* NRRL B-640, *B. mucilaginosus* SM-01, and *Weissella cibaria* CMGDEX3 [27–29].

FT-IR spectrum analysis of the EPS. The FT-IR spectrum reveals the major functional groups of the EPS from *B. amyloliquefaciens* GSBa-1 (Fig. 3). The strong band at $3,422.81\text{ cm}^{-1}$ was attributed to the hydroxyl stretching vibration of the polysaccharide, whereas the band at $2,893.92\text{ cm}^{-1}$ was ascribed to C-H stretching vibration [30]. The absorption at $1,647.66\text{ cm}^{-1}$ was due to the stretching vibration of the C=O bond and carboxyl groups. The peak at $1,382.83\text{ cm}^{-1}$ could be assigned to CH_3 bending. The band at $1,220.90\text{ cm}^{-1}$ was attributed to the vibration of the C-O-C bond. The intense peak at $1,032.27\text{ cm}^{-1}$ was recognized for the main character of polysaccharides. The weak stretch band at 802.64 cm^{-1} was the main character for α -D-glucose [27]. The peak in the region of 668.71 and 551.20 cm^{-1} indicated the existence of glycosidic linkage bonds between the glycosyl groups [26].

SEM analysis of the EPS. SEM was used to observe the

surface morphology and microstructure of the EPS from *B. amyloliquefaciens* GSBa-1 (Fig. 4). The EPS appeared as

Fig. 5. Thermogram curves of the purified exopolysaccharide from *B. amyloliquefaciens* GSBa-1.

uniform ellipsoid or globose with a smooth surface (Figs. 4A and 4B). A similar globose microstructure of EPS was reported earlier with the EPSs produced by *B. amyloliquefaciens* LPL061 and by oral streptococci, including *Streptococcus mutans*, *Streptococcus sanguis*, and *Streptococcus salivarius* [13, 31].

AFM analysis of the EPS. AFM was performed to elucidate the surface roughness and morphology of the EPS [32]. As

shown in Figs. 4C and 4D, the EPS from *B. amyloliquefaciens* GSBa-1 was present in many spherical lumps, with heights ranging from 0.3 to 0.7 nm in a symmetrical mode, compared with the height of the single polysaccharide chain that ranges from 0.1 to 1 nm, suggesting that there was no intra- and /or inter- molecular aggregation in the EPS solution [33].

Thermogravimetric analysis. TGA determines the

Fig. 6. ^1H NMR (A) and ^{13}C NMR (B) spectra of the exopolysaccharide from *B. amyloliquefaciens* GSBa1.

dynamic weight loss of the polysaccharide sample during heating with increasing temperature. As shown in Fig. 5, the process of weight loss of the EPS sample during heating could be divided into two phases. In the first step, about 7.44% of mass loss was observed, mainly due to the moisture loss in the EPS sample during the temperature rising from 50°C to 160°C [34]. In the second step, 43.31% of weight loss was found at around 400°C and reached a plateau with a residue weight of 18.56% of the original weight. The derivative of weight loss by temperature showed that melting of the EPS started at about 240°C, corresponding to the fastest weight-losing temperature. The relatively higher degradation temperature of the EPS from strain GSBa-1, representing higher thermal stability, indicated that it could be potentially utilized in the food industry, like xanthan gum utilized before baking bread to

decrease syneresis, and guar gum utilized in fried noodles to increase the viscosity, with all the processing temperatures well overpassing 200°C.

NMR spectroscopy analysis. NMR spectroscopy is widely used in the structural characterization of bacterial EPSs. The ^1H NMR spectrum (Fig. 6A) of the EPS from strain GSBa-1 showed three major signals in the anomeric region at 5.087, 4.935, and 4.837 ppm that were assigned as α -(1 $\rightarrow$ 6) linked glucan [35]. Existence of α -(1 $\rightarrow$ 6) linkage was also reported in the glucan from *Leuconostoc lactis* KC117496 and in the EPSs from lactic acid bacteria isolated from sour dough [36, 37]. Correspondingly, there were three distinct peaks at 102.259, 100.645, and 98.370 ppm in the anomeric region of the ^{13}C NMR spectrum of the EPS (Fig. 6B), suggesting the presence of a trisaccharide repeating unit in the EPS of strain GSBa-1. The presence of

Fig. 7. DPPH radical (A), hydroxyl radical (B), and superoxide radical scavenging activities (C), and metal ion-chelating activity (D) of the purified GSBa-1 exopolysaccharide (EPS) and the positive controls.

resonance between 95 and 102 ppm also indicated the presence of an α -anomeric carbon atom [38]. The signals at 102.259 and 100.645 ppm belong to α -(1 $\rightarrow$ 3) linkages, and the signal at 98.370 ppm was assigned as an α -(1 $\rightarrow$ 6) linkage [39, 40]. The signal at 66.33 ppm was the main character for the downfield shift of the α -(1 $\rightarrow$ 6) linked D-glucan units, similar to the classical α -(1 $\rightarrow$ 6) dextrans produced by *Leuconostoc* and *Weissella* strains [41]. No signal at 90 ppm in the ^{13}C NMR spectrum suggested that there was only pyranose ring configuration for all sugars [36]. Combining the monosaccharide composition results and 1D NMR spectral analyses, we speculated that the EPS produced by *B. amyloliquefaciens* GSBa-1 was α -glucan containing a trisaccharide repeating unit with two α -(1 $\rightarrow$ 3) and one α -(1 $\rightarrow$ 6) glycosidic linkages. However, the exact structure of the *B. amyloliquefaciens* GSBa-1 EPS requires further NMR experiments for confirmation.

Antioxidant Properties

DPPH free radical scavenging activity. The DPPH free radical is a stable radical with an unpaired valence electron at one atom of the nitrogen bridge, and its free radical character can be neutralized upon exposure to a proton radical scavenger that transfers either an electron or a hydrogen atom to DPPH [42, 43]. As shown in Fig. 7A, the scavenging activity of the EPS from strain GSBa-1 on DPPH radical increased in a concentration-dependent manner. At 0.2 mg/ml, the purified EPS had scavenging activity of $14.74 \pm 1.02\%$, but increased drastically to $76.75 \pm 3.02\%$ at 5 mg/ml, approaching that ($90.08 \pm 2.08\%$) of Vc at the same concentration. In comparison, the EPSs produced by *Enterococcus faecium* BDU7 and *Weissella cibaria* GA44 had much lower DPPH RSA, being 63.5% at 10 mg/ml and 48.9% at 4 mg/ml, respectively [44, 45]. The relatively potent DPPH radical scavenging activity of the EPS might be due to its relatively lower molecular mass (54 kDa). It was reported that a lower molecular weight of the polysaccharide might be a favorable factor for its higher antioxidant activities [46, 47]. Liu *et al.* [48] also reported lower-molecular-weight EPSs with higher DPPH RSA.

Hydroxyl radical scavenging activity. The hydroxyl radical is a powerful reactive oxygen species that can react with almost all biological molecules, such as proteins, lipids, and carbohydrates, leading to biological damage and lipid peroxidation [49]. The hydroxyl radical scavenging activities of the EPS from strain GSBa-1 and Vc are shown in Fig. 7B. At the concentration of 5.0 mg/ml, the scavenging capability of Vc was $90.08 \pm 2.49\%$, whereas the activity of the EPS reached $89.75 \pm 2.68\%$. The EPS exhibited potent scavenging

activities on both DPPH radicals and hydroxyl radicals using multiple mechanisms, such as prevention of chain initiation, decomposition of peroxides, and so on [50]. Tsiapali *et al.* [51] also stated that the structural characteristics of dextran conferred part of its radical scavenging ability. The hydroxyl groups present in the polysaccharide could donate hydrogen for binding with hydroxyl radicals to achieve the scavenging activity. In addition, 39.5% DPPH RSA and 46.6% hydroxyl RSA were reported for β -glucan from oat at 1 mg/ml, and 20% DPPH RSA and 30% hydroxyl RSA for β -glucan from *Pycnoporus sanguineus* at 5 mg/ml [52, 53]. The appreciably higher hydroxyl RSA of the EPS of this study might be due to its α -configuration.

Superoxide anion scavenging activity. The superoxide radical is a precursor of most of reactive oxidative species such as singlet oxygen, hydrogen peroxide, and hydroxyl radical, which induce tissue damage or cell death [49]. As shown in Fig. 7C, the superoxide anion scavenging activity of the EPS, $44.86 \pm 0.30\%$ at the dose of 5 mg/ml, was lower than that of Vc ($99.14 \pm 0.24\%$). Maity *et al.* [54] reported 56.58% of superoxide anion scavenging activity for the glucan extracted from edible mushroom at 1 mg/ml. Liu *et al.* [55] indicated that the molecular mass of dextran markedly influenced its superoxide anion scavenging activity; two glucans obtained from *Ganoderma lucidum* mushroom with masses of 5.2 and 15 kDa had 80% and 50% of scavenging activity, respectively. Je *et al.* [56] reported that the higher number of electron-withdrawing groups attached to the polysaccharide, the weaker was the dissociation energy of the O-H bond. Therefore, the little presence of the electrophilic groups like aldehyde or keto in the EPS might explain its relatively lower scavenging activity. However, acidic polysaccharides were shown with more efficient superoxide anion scavenging activity than that of neutral polysaccharides [57]. The acidic nature of the EPS from strain GSBa-1 might contribute to its moderate superoxide anion scavenging activity.

Ferrous ion-chelating activity. The transition metal Fe^{2+} can stimulate production of hydroxyl radicals, leading to lipid peroxidation [58]. The ferrous ion-chelating activities of the EPS from strain GSBa-1 and EDTA-2Na are shown in Fig. 7D. Ferrous ion could combine with the EPS and EDTA-2Na in a dose-dependent manner, and the ferrous ion-chelating effect of the EPS was less than that of EDTA-2Na. The metal-chelating activity increased with the EPS concentration up to 2.0 mg/ml, and further increase in concentration did not enhance the activity significantly. The scavenging activities of the EPS and EDTA-2Na at 2.0 mg/ml were $30.5 \pm 1.93\%$ and $89.87 \pm 0.05\%$, respectively.

Previously, compounds with metal-chelating activities were frequently found to contain two or more of the following functional groups: -OH, -COOH, -PO₃H₂, -SH, -NR₂, -S-, =C=O, and -O- [59]. In addition, negatively charged acidic polysaccharides containing higher uronic acid showed higher metal-chelating activity [59, 60]. Soeiro *et al.* [61] showed some dextrans with 5% of iron-chelating activity at a concentration of 2 mg/ml. Only about 10% of iron-chelating activity was found for α -glucans isolated from *Aconitum kusnezoffii* [62]. In another case, the dextran extracted from *Ganoderma lucidum* mushroom fruiting body had 50% of iron-chelating activity at 10 mg/ml [63]. Melo-Silveira *et al.* [64] showed that xylans isolated from corn cobs had high iron-chelating activity of around 80% at 1 mg/ml. It seemed that glucans generally have lower iron-chelating activity even though they might exhibit good antioxidant activity.

In conclusion, the EPS produced by *B. amyloliquefaciens* GSBa-1 was purified and characterized using several different analytical techniques, and the in vitro antioxidant property of the EPS was also evaluated. The EPS was predominantly composed of glucose, and it was confirmed to be a glucan consisted of a repeating unit of trisaccharide with two α -(1 $\rightarrow$ 3) and one α -(1 $\rightarrow$ 6) glucosidic linkages, with a molecular mass of 54 kDa and Mw/Mn ratio of 1.78. Observation by SEM and AFM showed a relatively uniform spherical shape with smooth surface of the EPS. The EPS had strong DPPH and hydroxyl RSA, and moderate superoxide anion scavenging and ferrous ion-chelating activities. These results indicated that the EPS from *B. amyloliquefaciens* GSBa-1 may be a new source of natural antioxidant with potential value for therapeutics and health foods. The EPS may also serve as a novel texturizer to improve baking or frying food quality by taking advantage of its hyperthermal resistance property. Further work on its defined structure and potential application as a novel bioactive agent is in progress.

Acknowledgments

The authors gratefully acknowledge the financial support from the National Natural Science Foundation of China (Grant No. 31571857) and Beijing Municipal Science and Technology Project (Z171100002217019).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Abid Y, Casillo A, Gharsallah H, Joulak I, Lanzetta R, Corsaro MM, *et al.* 2017. Production and structural characterization of exopolysaccharides from newly isolated probiotic lactic acid bacteria. *Int. J. Food Microbiol.* **103**: 669-675.
2. Schmid SV, Rehm B. 2015. Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. *Front. Microbiol.* **6**: 496-502.
3. Singh A, Singh S. 2004. *Organic Chemistry of Natural Products*. Campus Books International, New Delhi.
4. Nwodo UU, Okoh AI. 2012. Bacterial exopolysaccharides: functionality and prospects. *Int. J. Food Microbiol.* **13**: 14002-14015.
5. Sasikumar K, Kozhummal VD, Devendra L, Nampoothiri KM. 2017. An exopolysaccharide (EPS) from a *Lactobacillus plantarum* BR2 with potential benefits for making functional foods. *Bioresour. Technol.* **241**: 1152-1156.
6. Guo Y, Pan D, Li H, Sun Y, Zeng X, Yan B, 2013. Antioxidant and activity of immunomodulatory selenium exopolysaccharide produced by *Lactococcus lactis* subsp. *lactis*. *Food Chem.* **138**: 84-89.
7. Prado MR, Boller C, Zibetti RGM, Souza D, Pedroso LL, Soccol CR. 2016. Anti-inflammatory and angiogenic activity of polysaccharide extract obtained from Tibetan kefir. *Microvasc. Res.* **108**: 29-33.
8. Du B, Yang Y, Bian Z, Xu B. 2017. Molecular weight and helix conformation determine intestinal anti-inflammatory effects of exopolysaccharide from *Schizophyllum commune*. *Carbohydr. Polym.* **172**: 68-77.
9. Yan JK, Wang WQ, Li L, Wu JY. 2011. Physiochemical properties and antitumor activities of two α -glucans isolated from hot water and alkaline extracts of *Cordyceps* (Cs-HK1) fungal mycelia. *Carbohydr. Polym.* **4**: 753-758.
10. Han Y, Liu E, Liu L, Zhang B, Wang Y, Gui M, *et al.* 2015. Rheological, emulsifying and thermostability properties of two exopolysaccharides produced by *Bacillus amyloliquefaciens* LPL061. *Carbohydr. Polym.* **115**: 230-237.
11. Malick A, Khodaei N, Benkerroum N, Karboune S. 2017. Production of exopolysaccharides by selected *Bacillus* strains: optimization of media composition to maximize the yield and structural characterization. *Int. J. Biol. Macromol.* **102**: 539-549.
12. Chen YT, Yuan Q, Shan LT, Lin MA, Chen DQ, Li CY. 2013. Antitumor activity of bacterial exopolysaccharides from the endophyte *Bacillus amyloliquefaciens* sp. isolated from *Ophiopogon japonicus*. *Oncol Lett.* **5**: 1787-1792.
13. Yang H, Deng J, Yuan Y, Fan D, Zhang Y, Zhang R, *et al.* 2015. Two novel exopolysaccharides from *Bacillus amyloliquefaciens* C-1: antioxidation and effect on oxidative stress. *Curr. Microbiol.* **70**: 298-306.
14. Zhao W, Teng JW, Zhang J, Zhao X, Jiang YY, Yang ZN.

2017. Production of exopolysaccharide by fermentation with *Bacillus amyloliquefaciens* GSBa-1, its rheological characterization and application. *Chin. J. Food Sci.* **16**: 1-9.
15. Wang J, Zhao X, Tian Z, Yang Y, Yang Z. 2015. Characterization of an exopolysaccharide produced by *Lactobacillus plantarum* YW11 isolated from Tibet Kefir. *Carbohydr. Polym.* **125**: 16-25.
16. Zhang L, Li Y, Zhao X, Zhang X. 2013. Antioxidant activity of an exopolysaccharide isolated from *Lactobacillus plantarum* C88. *Int. J. Food Microbiol.* **54**: 270-275.
17. Wang Y, Li C, Liu P, Ahmed Z, Xiao P, Bai X. 2010. Physical characterization of exopolysaccharide produced by *Lactobacillus plantarum* KF5 isolated from Tibet kefir. *Carbohydr. Polym.* **82**: 895-903.
18. Qiao D, Ke C, Hu B, Luo J, Ye H, Sun Y, et al. 2009. Antioxidant activities of polysaccharides from *Hyriopsis cumingii*. *Carbohydr. Polym.* **78**: 199-204.
19. Zhao H, Li J, Zhang J, Wang X, Hao L, Jia L. 2017. Purification, in vitro antioxidant and in vivo anti-aging activities of exopolysaccharides by *Agroclype cylindracea*. *Int. J. Food Microbiol.* **102**: 351-357.
20. Tang W, Dong M, Wang W, Han S, Rui X, Chen X, et al. 2017. Structural characterization and antioxidant property of released exopolysaccharides from *Lactobacillus delbrueckii* ssp. *bulgaricus* SRFM-1. *Carbohydr. Polym.* **173**: 654-664.
21. Zhao H, Li J, Zhang J, Wang X, Hao L, Jia L. 2017. Purification, in vitro antioxidant and in vivo anti-aging activities of exopolysaccharides by *Agroclype cylindracea*. *Int. J. Food Microbiol.* **102**: 351-357.
22. El-Newary SA, Ibrahim AY, Asker MS, Mahmoud MG, Awady ME. 2017. Production, characterization and biological activities of acidic exopolysaccharide from marine *Bacillus amyloliquefaciens* 3MS. *Asian Pac. J. Trop. Med.* **10**: 652-662.
23. Malick A, Khodaei N, Benkerroum N, Karboune S. 2017. Production of exopolysaccharides by selected *Bacillus* strains: optimization of media composition to maximize the yield and structural characterization. *Int. J. Food Microbiol.* **102**: 539-549.
24. Zheng JQ, Wang JZ, Shi CW, Mao DB, He PX, Xu CP. 2014. Characterization and antioxidant activity for exopolysaccharide from submerged culture of *Boletus aereus*. *Process Biochem.* **49**: 1047-1053.
25. Singh RP, Shukla MK, Mishra A, Kumari P, Reddy CRK, Jha B. 2011. Isolation and characterization of exopolysaccharides from seaweed associated bacteria *Bacillus licheniformis*. *Carbohydr. Polym.* **84**: 1019-1026.
26. Rani RP, Anandharaj M, Sabhapathy P, Ravindran AD. 2017. Physiochemical and biological characterization of novel exopolysaccharide produced by *Bacillus tequilensis* FR9 isolated from chicken. *Int. J. Food Microbiol.* **96**: 1-10.
27. Matsuzaki C, Takagaki C, Tomabeichi Y, Forsberg LS, Heiss C, Azadi P, et al. 2017. Structural characterization of the immunostimulatory exopolysaccharide produced by *Leuconostoc mesenteroides* strain NTM048. *Carbohydr. Res.* **448**: 95-102.
28. Yu L, Xu X, Zhou J, Lv G, Chen J. 2017. Chain conformation and rheological behavior of exopolysaccharide from *Bacillus mucilaginosus* SM-01. *Food Hydrocoll.* **65**: 165-174.
29. Maina NH, Tenkanen M, Maaheimo H, Juvonen R, Virkki L. 2008. NMR spectroscopic analysis of exopolysaccharides produced by *Leuconostoc citreum* and *Weissella confusa*. *Carbohydr. Res.* **343**: 1446-1455.
30. Liu J, Luo J, Ye H, Sun Y, Lu Z, Zeng X. 2010. Medium optimization and structural characterization of exopolysaccharides from endophytic bacterium *Paenibacillus polymyxa* EJS-3. *Carbohydr. Polym.* **79**: 206-213.
31. Bowen WH, Burne RA, Wu H, Koo H. 2017. Oral biofilms: pathogens, matrix, and polymicrobial interactions in microenvironments. *Trends Microbiol.* **26**: 229-242.
32. Magdalena PB, Adam C, Adam W, Sabina G, Andrzej G, Justyna C. 2015. Physicochemical characterization of exopolysaccharides produced by *Lactobacillus rhamnosus* on various carbon sources. *Carbohydr. Polym.* **117**: 501-509.
33. Liu L, Qin Y, Wang Y, Li H, Shang N, Li P. 2014. Complete genome sequence of *Bifidobacterium animalis* RH, a probiotic bacterium producing exopolysaccharides. *J. Biotechnol.* **189**: 86-87.
34. Wang K, Li W, Rui X, Chen X, Jiang M, Dong M. 2014. Structural characterization and bioactivity of released exopolysaccharides from *Lactobacillus plantarum* 70810. *Int. J. Biol. Macromol.* **67**: 71-78.
35. Maina NH, Tenkanen M, Maaheimo H, Juvonen R, Virkki L. 2008. NMR spectroscopic analysis of exopolysaccharides produced by *Leuconostoc citreum* and *Weissella confusa*. *Carbohydr. Res.* **343**: 1446-1455.
36. Saravanan C, Shetty PKH. 2016. Isolation and characterization of exopolysaccharide from *Leuconostoc lactis* KC117496 isolated from idli batter. *Int. J. Biol. Macromol.* **90**: 100-106.
37. Bounaix MS, Gabriel V, Robert H, Morel S, Remaud-Siméon M, Gabriel B, et al. 2010. Characterization of glucan-producing *Leuconostoc* strains isolated from sourdough. *Int. J. Biol. Macromol.* **144**: 1-9.
38. Seymour FR, Knapp RD, Chen ECM, Jeanes A, Bishop SH. 1979. Structural analysis of dextrans containing 2-O- α -D-glucosylated α -D-glucopyranosyl residues at the branch points, by use of ^{13}C -nuclear magnetic resonance spectroscopy and gas-liquid chromatography-mass spectrometry. *Carbohydr. Res.* **71**: 231-250.
39. Maity P, Nandi AK, Manna DK, Pattanayak M, Sen IK, Bhanja SK, et al. 2017. Structural characterization and antioxidant activity of a glucan from *Meripilus giganteus*. *Carbohydr. Polym.* **157**: 1237-1245.
40. Dertli E, Colquhoun IJ, Côté GL, Le GG, Narbad A. 2018. Structural analysis of the α -D-glucan produced by the sourdough isolate *Lactobacillus brevis* E25. *Food Chem.* **242**: 45-52.

41. Bounaix MS, Gabriel V, Morel S, Robert H, Rabier P, Remaud-Siméon M, *et al.* 2009. Biodiversity of exopolysaccharides produced from sucrose by sourdough lactic acid bacteria. *J. Agric. Food Chem.* **57**: 10889-10897.
42. Kodali VP, Dr RS. 2010. Antioxidant and free radical scavenging activities of an exopolysaccharide from a probiotic bacterium. *Biotechnol. J.* **3**: 245-251.
43. Liu CF, Tseng KC, Chiang SS, Lee BH, Hsu WH, Pan TM. 2011. Immunomodulatory and antioxidant potential of *Lactobacillus* exopolysaccharides. *J. Sci. Food Agric.* **91**: 2284-2291.
44. Abdhul K, Ganesh M, Shanmughapriya S, Kanagavel M, Anbarasu K, Natarajaseenivasan K. 2014. Antioxidant activity of exopolysaccharide from probiotic strain *Enterococcus faecium* (BDU7) from Ngari. *Int. J. Biol. Macromol.* **70**: 450-454.
45. Adesulu-Dahunsi AT, Sanni AI, Jeyaram K. 2018. Production, characterization and in vitro antioxidant activities of exopolysaccharide from *Weissella cibaria* GA44. *LWT Food Sci. Technol.* **87**: 432-442.
46. You L, Gao Q, Feng M, Yang B, Ren J, Gu L, *et al.* 2013. Structural characterisation of polysaccharides from *Tricholoma matsutake* and their antioxidant and antitumour activities. *Food Chem.* **138**: 2242-2249.
47. Huang QL, Siu KC, Wang WQ, Cheung YC, Wu JY. 2010. Fractionation, characterization and antioxidant activity of exopolysaccharides from fermentation broth of a *Cordyceps sinensis* fungus. *Process Biochem.* **48**: 380-386.
48. Liu J, Luo J, Ye H, Sun Y, Lu Z, Zeng X. 2010. In vitro and in vivo antioxidant activity of exopolysaccharides from endophytic bacterium *Paenibacillus polymyxa* EJS-3. *Carbohydr. Polym.* **82**: 1278-1283.
49. Cao S, Zhan H, Fu SY, Chen L. 2007. Regulation of superoxide anion radical during the oxygen delignification process. *Chin. J. Chem. Eng.* **15**: 132-137.
50. Wang X, Shao C, Liu L, Guo X, Xu Y, Lü X. 2017. Optimization, partial characterization and antioxidant activity of an exopolysaccharide from *Lactobacillus plantarum* KX041. *Int. J. Biol. Macromol.* **103**: 1173-1184.
51. Tsiapali E, Whaley S, Kalbfleisch J, Ensley HE, Browder IW, Williams DL. 2001. Glucans exhibit weak antioxidant activity, but stimulate macrophage free radical activity. *Free Radic. Biol. Med.* **30**: 393-402.
52. Cao J, Zhang H-J, Xu C-P. 2014. Culture characterization of exopolysaccharides with antioxidant activity produced by *Pycnoporus sanguineus* in stirred-tank and airlift reactors. *J. Taiwan Inst. Chem.* **45**: 2075-2080.
53. Hussain PR, Rather SA, Suradkar PP. 2018. Structural characterization and evaluation of antioxidant, anticancer and hypoglycemic activity of radiation degraded oat (*Avena sativa*) β -glucan. *Radiat. Phys. Chem.* **144**: 218-230.
54. Maity P, Sen IK, Maji PK, Paloi S, Devi KSP, Acharya K, *et al.* 2015. Structural, immunological, and antioxidant studies of β -glucan from edible mushroom *Entoloma lividoalbum*. *Carbohydr. Polym.* **123**: 350-358.
55. Liu W, Wang H, Pang X, Yao W, Gao X. 2010. Characterization and antioxidant activity of two low-molecular-weight polysaccharides purified from the fruiting bodies of *Ganoderma lucidum*. *Int. J. Biol. Macromol.* **46**: 451-457.
56. Je JY, Park PJ, Kim EK, Park JS, Yoon HD, Kim KR, *et al.* 2009. Antioxidant activity of enzymatic extracts from the brown seaweed *Undaria pinnatifida* by electron spin resonance spectroscopy. *LWT Food Sci. Technol.* **42**: 874-878.
57. Xu R, Shang N, Li P. 2011. In vitro and in vivo antioxidant activity of exopolysaccharide fractions from *Bifidobacterium animalis* RH. *Anaerobe* **17**: 226-231.
58. Duh PD. 1999. Antioxidant activity of water extract of four Harng Jyur (*Chrysanthemum morifolium* Ramat) varieties in soybean oil emulsion. *Food Chem.* **66**: 471-476.
59. Yuan YV, Bone DE, Carrington MF. 2005. Antioxidant activity of dulce (*Palmaria palmata*) extract evaluated in vitro. *Food Chem.* **91**: 485-494.
60. Yang H, Deng J, Yuan Y, Fan D, Zhang Y, Zhang R, *et al.* 2015. Two novel exopolysaccharides from *Bacillus amyloliquefaciens* C-1: antioxidation and effect on oxidative stress. *Curr. Microbiol.* **70**: 298-306.
61. Soeiro VC, Melo KRT, Alves MGCF, Medeiros MJC, Grilo MLPM, Almeida Lima J, *et al.* 2016. Dextran: influence of molecular weight in antioxidant properties and immunomodulatory potential. *Int. J. Biol. Macromol.* **17**: 1340-1344.
62. Gao T, Ma S, Song J, Bi H, Tao Y. 2011. Antioxidant and immunological activities of water-soluble polysaccharides from *Aconitum kusnezoffii* Reichb. *Int. J. Biol. Macromol.* **49**: 580-584.
63. Li DM, Zhou DY, Zhu BW, Miao L, Qin L, Dong XP, *et al.* 2013. Extraction, structural characterization and antioxidant activity of polyhydroxylated 1,4-naphthoquinone pigments from spines of sea urchin *Glyptocidaris crenularis* and *Strongylocentrotus intermedius*. *Eur. Food Res. Technol.* **237**: 331-339.
64. Melo-Silveira RF, Fidelis GP, Viana RL, Soeiro VC, Silva RA, Machado D, *et al.* 2014. Antioxidant and antiproliferative activities of methanolic extract from a neglected agricultural product: corn cobs. *Molecules* **19**: 5360-5367.