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Preparation, partial characterization and bioactivity of exopolysaccharides from *Lactobacillus casei* LC2W

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ABSTRACT

Crude exopolysaccharides (LCP) were isolated from skim milk fermented by *Lactobacillus casei* LC2W and fractionated into three fractions (LCP1, LCP2 and LCP3) with the ratios of 35.74%, 12.61% and 33.34% (w/w) based on LCP, respectively. The molecular weight, polydispersity, intrinsic viscosity and radius of gyration of three fractions were determined by high-performance size exclusion chromatography, and monosaccharide compositions were analyzed by high-performance anion-exchange chromatography. The antihypertensive effects of exopolysaccharides were evaluated using spontaneously hypertensive rats (SHR). SHR were randomized into four groups (eight rats per group) and fed with the test samples (LCP1, LCP2 and LCP3) at the daily dosage of 15 mg kg^{-1} (body weight) and saline for 7 days. The results showed only LCP1 could decrease significantly the systolic blood pressure of SHR ($P < 0.01$), and LCP1, LCP2 and LCP3 had no significant effect on the heart rate of SHR.

1. Introduction

Microbial exopolysaccharides (EPS) are extracellular polysaccharides mainly involved in cell adhesion and protection, which are either associated with, and often covalently bound to, the cell surface in the form of capsules, or secreted into the extracellular environment in the form of slime (De Vuyst & Degeest, 1999). EPS produced by the food-grade lactic acid bacteria (LAB) have been widely studied for their physicochemical properties and potential health effects during the last decades (Degeest, Mozzi, & De Vuyst, 2002). LAB EPS are considered to not only play an important role in improving the rheology, texture and mouthfeel of fermented products, but also provide beneficial physiological effects on human health, such as antitumour activity, immunomodulating bioactivity and antimutagenicity (Doleyres, Schaub, & Lacroix, 2005; Van Calsteren, Pau-Roblot, Bégin, & Roy, 2002; Yang, Huttunen, Staaf, Widmalm, & Tenhu, 1999). EPS from *Lactobacillus bulgaricus* 878R had antitumoral activity, and a water-soluble polysaccharide fraction from *Bifidobacterium adolescentis* exhibited immunopotentiating activity (Ebina, Ogama, & Murata, 1995; Hosono, Lee, Amenati, Natsume, Hirayama, & Adachi, 1997). Kitazawa, Yamaguchi, Fujimoto, and Itoh (1993a), (1993b), (1996), Kitazawa et al. (1998), Kitazawa et al. (2000) reported that the phosphopolysaccharide produced by *Lactococcus lactis* subsp. *cremoris* exhibited antitumor effect and immunostimulating activity, and the phosphopolysaccharide from *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 could enhance lymphocyte mitogenicity and macrophage cytostaticity. At present, increasing understanding in structures and physicochemical properties of LAB EPS makes it possible to be exploited in food applications, but little information was reported on EPS physiological functions (Nishimura-Uemuraet et al., 2003). We investigated EPS produced by *Lactobacillus casei* LC2W and discovered their blood pressure-lowering activity. It was reported that plant polysaccharides from *lycium barbarum* and *crataegus pinnatifida* leaves and D-polymannuronic sulfate from seaweeds exhibited the antihypertensive activity in the last decade (Huang, 2001; Jia, Dong, Wu, Ma, & Shi, 1998; Zhu, Geng, & Guan, 2000). Sawada et al. (1990) found that polysaccharide–glycopeptide complexes from *Lactobacillus casei* had the blood pressure-lowering activity. However, no EPS with antihypertensive effect from *Lactobacillus* has been reported so far.

The objectives of the present work were to isolate and purify EPS produced by *Lactobacillus casei* LC2W, and to study the physicochemical properties of different fractions and their antihypertensive effects on SHR.

2. Materials and methods

2.1. Preparation and partial characterization of EPS from skim milk fermented by Lactobacillus casei LC2W

2.1.1. Bacterial strains and culture medium

Lactobacillus casei LC2W was obtained from Technology Center of Bright Dairy and Food Co., Ltd. (Shanghai, China). The growth

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medium used for EPS production was reconstituted skim milk (10% skim milk powder), supplemented with 1.0% glucose and 1.0% yeast nitrogen base.

2.1.2. Preparation of the fermented milk

Inocula were prepared as the method reported (Ai, Zhang, Guo, Chen, Wu, & Tang, 2006).

Batch cultures were performed in a 5 L capacity fermentor (Bio Tron Inc., made in Korea) using the following conditions: incubation temperature of 32.5 °C, incubation time of 26 h and inoculum concentration of 4.0%. The initial cellular density in the fermentation broth was about 3.5×10^7 cfu mL^{-1} (cfu, colony forming units), and pH values decreased from initial 6.2 to 4.3 at the end of the fermentation (Ai et al., 2006).

2.1.3. Extraction and purification of EPS

Culture medium was heated in boiling water for 10 min to inactivate enzymes, and then cooled down to room temperature, centrifuged (20 min, 10,000*g*, 4 °C) to remove cells and coagulated proteins, and the supernatant was collected. 80% (w/v) trichloroacetic acid was added to the supernatant to final concentration of 4% (w/v) with gentle stirring and kept at 4 °C for 10 h. Proteins precipitated were removed by centrifugation and then EPS were precipitated from the supernatant with three volumes of cold ethanol followed by an overnight incubation at 4 °C. After centrifugation, the precipitate was resuspended in deionized water and dialysed (molecular weight cut-off: 12,000–14,000) for 3 days. The retentate was centrifuged to remove insoluble material. The supernatant was lyophilized and white crude polysaccharides (LCP) were obtained (Richard & Maria, 2003).

LCP were fractionated by anion-exchange chromatography on a DEAE-Sepharose FF (Amersham Bioscience, Uppsala, Sweden) column (*D* 2.6 cm × 30 cm) equilibrated with 0.05 M Tris–HCl buffer (pH 7.6), and were first eluted with the buffer at a flow rate of 3.0 mL min^{-1} followed by a linear gradient of NaCl concentration (0–1.2 M). Three polysaccharide fractions were collected, respectively, with a fraction collector, concentrated using a rotary evaporator at 50 °C, dialyzed for 3 days and lyophilized. The three fractions were then loaded, respectively, onto a Sepharose CL-6B gel column (*D* 1.6 cm × 100 cm) and eluted with 0.05 M Tris–HCl buffer (pH 7.6) with 0.1 M NaCl at a flow rate of 0.3 mL min^{-1}. The eluates were pooled, concentrated, dialyzed and lyophilized. The eluting procedure was monitored by an online ultraviolet detector for proteins at the wavelength of 280 nm, and the eluate fractions were assessed by the total carbohydrate content using phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956).

2.1.4. Chemical compositions analysis

Moisture and ash contents were determined according to AOCS and AOAC methods (AOCS, Ba 2a-38; AOAC 942.05). Total carbohydrate content was measured by the reaction with phenol in the presence of sulfuric acid, using glucose as a reference (Dubois et al., 1956). The protein content was estimated by the method of binding of Coomassie Brilliant Blue G-250 to protein, using bovine serum albumin as a standard (Bradford, 1976).

2.1.5. High-performance size exclusion chromatography (HPSEC)

The molecular weight, polydispersity, intrinsic viscosity and radius of gyration of the EPS were determined using high-performance size exclusion chromatography (HPSEC) (Wang, Wood, Cui, & Ross-Murphy, 2001; Wang, Wood, Huang, & Cui, 2003). The HPSEC system consisted of a Shimadzu SCL-10Avp pump unit and automatic injector (Shimadzu Scientific Instruments Inc., Columbia, Maryland 21046, USA), Viscotek Triple detectors (Viscotek Co., Houston, TX) composed of a refractive index detector (RI, Model 200), a viscometer (DP, Model 250) and a right angle laser light scattering detector (RALLS, Model 600), and two columns in series: a Shodex Ohpak KB-806M (Showa Denko K.K., Tokyo, Japan) and an Ultrahydrogel linear (Waters, Milford, CT, USA). The mobile phase was 100 mM $NaNO_3$ with a flow rate of 0.6 mL min^{-1}, and the injection volume of sample was 100 μL. Pullulans (P-82, JM Science, Inc., NY, USA) of known molecular weight and intrinsic viscosity were used as standards and dn/dc of 0.146 mL g^{-1} as a refractive index increment was used for polysaccharides solution. The polysaccharides were dissolved in 100 mM $NaNO_3$ (50 °C, 1 h), cooled and filtered through a 0.45 μm nylon filter prior to injection into the column. The weight average molecular weight, polydispersity, intrinsic viscosity and radius of gyration were calculated using the software TriSEC provided by Viscotek.

2.1.6. Infrared spectra of EPS

The IR spectra of polysaccharides were determined using a Fourier transform-infrared spectrophotometer (Nexus 5DXC FT-IR, Thermo Nicolet, America). The sample was ground with spectroscopic grade KBr (potassium bromide) powder and then pressed into a 1 mm pellet for FT-IR measurement in the frequency range of 4000–400 cm^{-1} (Mid infrared region).

2.1.7. Monosaccharide composition of EPS

Monosaccharide composition was determined by hydrolyzing samples in 1 M H_2SO_4 at 100 °C glycerin bath for 2 h followed using a Dionex HPAEC (High-performance anion-exchange chromatography) system (DX-500) equipped with pulsed amperometric detection (PAD) (Sunnyvale, CA). The monosaccharide was eluted by a linear gradient of NaOH solutions (100 mM and 300 mM) according to the procedure as previously described by Wood, et al. (Wood, Weisz, & Blackwell, 1994). The percentage of monosaccharide in the sample was calculated from the peak areas using response factor.

2.2. Antihypertensive effect of EPS

2.2.1. Animals

Male inbred SHR (aged 17 weeks and weighing 341–357 g) were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. These animals received a standard laboratory diet and tap water ad libitum. All animals were housed in cages on a 12 h cycle of lights on and off. Temperature and humidity were controlled at 24 ± 1 °C and 55 ± 5%, respectively.

2.2.2. Experimental design

32 SHR were divided randomly into four groups (eight rats per group), i.e. three test groups and one placebo group. They were orally administrated with the test samples (LCP1, LCP2 and LCP3) at the daily dosage of 15 mg kg^{-1} body weight and saline for 7 days. The systolic blood pressure (SBP) and heart rate were measured by the tail-cuff method before the oral administration and 2, 4, 6, 8, 10 and 24 h after oral administration the first day, and before the oral administration and 2 h after oral administration on third, fifth and seventh day. Before the measurements the rats were warmed in a holder kept at 38 °C for 15–20 min (Furushiro, Sawada, Hirai, Motoiki, Sansawa, & Kobayashi, 1990).

2.2.3. Statistical analysis

The results were expressed as mean ± standard deviations. The significance of differences between the treated and placebo groups was evaluated by Student's *t*-test.

3. Results and discussion

3.1. Preparation of exopolysaccharides

The yield of white fluffy crude polysaccharides (LCP) was 153.4 mg L^{-1} based on the skim milk fermented. LCP was fractionated by ion-exchange chromatography into three fractions (LCP1, LCP2 and LCP3) (see Fig. 1). The recovery of the eluted polysaccharides was about 81.7%. The three fractions were further isolated, respectively, by gel filtration chromatography. The results revealed that each fraction showed a single peak on the chromatogram, indicating it was composed of homogeneous polysaccharide, respectively.

Yield, moisture, ash, protein and total carbohydrate content of LCP and its fractions obtained after the anion-exchange chromatography are listed in Table 1. The results suggested that LCP1 (35.74%) and LCP3 (33.34%) were two major components of EPS, followed by small amounts of LCP2 (12.61%). LCP1 and LCP2 were eluted by ion-exchange chromatography using buffer elution, indicating they are neutral polysaccharides. However, LCP3 with relatively high content of protein (60.36%) was obtained using NaCl solution of high ionic strength as eluent. The elution curve of polysaccharide in LCP3 was in agreement with that of protein in both the ion-exchange chromatography and the gel permeation chromatography. Therefore LCP3 is proposed to be a glycoprotein or carbohydrate–protein complex in nature.

3.2. High-performance size exclusion chromatography

The chromatograms from HPSEC analysis of the fractions LCP1, LCP2 and LCP3 are shown in Fig. 2, and the single symmetrical peak of each fraction further proved its homogeneity. The weight average molecular weight (M_w), polydispersity, intrinsic viscosity and radius of gyration of each fraction are summarized in Table 2. The M_w of LCP1 (1,236,000 Da) was much higher than that of LCP2 (21,000 Da) or LCP3 (17,000 Da). The M_w of LCP1, LCP2 and LCP3 were in agreement with the molecular weight reported previously, i.e. M_w of LAB EPS varying from 1.0×10^4 to 6.0×10^6 (De Vuyst, De Vin, Vaningelgem, & Degeest, 2001). Intrinsic viscosity and radius of gyration value for the three fractions, which reflected the conformation of polymers in the solvent system, decreased in the order LCP1 → LCP2 → LCP3, which were consistent with change of M_w. The polydispersity indexes (M_w/M_n where M_n was the number average molecular weight) of LCP1 (1.202), LCP2 (1.000) and LCP3 (1.003) indicated a rather narrow M_w distribution, suggesting the homogeneous molecular size distribution in each fraction.

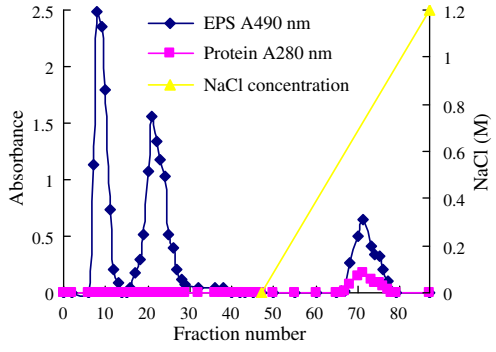

Fig. 1. Elution profile of crude exopolysaccharides on DEAE-Sepharose FF column.

Table 1
Chemical components analysis of EPS

EPS	Yield	Moisture %wt.	Ash %wt.	Protein %wt.	Carbohydrate %wt.
LCP	153.4 ± 7.61[a]	7.15 ± 0.47	4.39 ± 0.25	30.28 ± 0.67	57.77 ± 1.16
LCP1	35.74 ± 0.52[b]	7.06 ± 0.87	1.08 ± 0.29	n.d.[c]	89.79 ± 2.67
LCP2	12.61 ± 0.98[b]	7.01 ± 0.73	0.96 ± 0.67	1.32 ± 0.51	87.04 ± 1.39
LCP3	33.34 ± 1.12[b]	7.05 ± 0.89	5.85 ± 0.74	60.36 ± 1.01	22.23 ± 1.27

[a] mg L^{-1}, Based on the milk fermented.
[b] %wt., Based on LCP.
[c] Not detected.

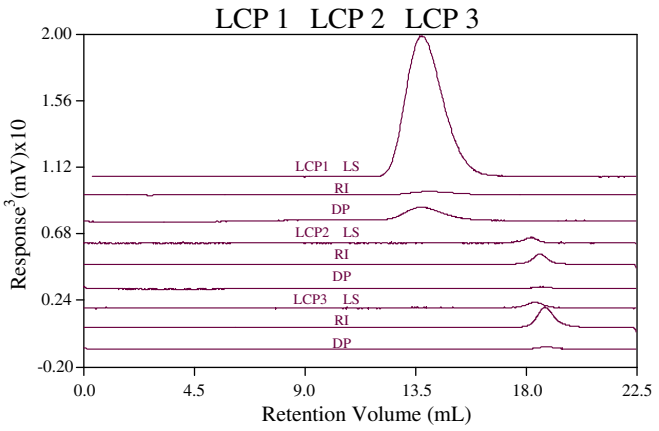

Fig. 2. High-performance size exclusion chromatograms of EPS fractions LCP1, LCP2 and LCP3.

Table 2
Molecular weight (M_w), polydispersity (Pd), intrinsic viscosity ([η]) and radius of gyration (R_g) of ESP LCP1, LCP2 and LCP3

EPS	M_w (Da)	Pd	[η] (dL g^{-1})	R_g (nm)
LCP1	1,236,000	1.202	1.875	42.72
LCP2	21,000	1.000	0.0877	3.23
LCP3	17,000	1.003	0.0564	2.30

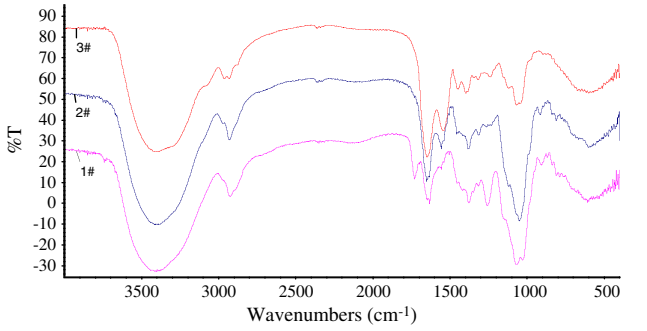

Fig. 3. Fourier transform infrared spectra of EPS fractions LCP1, LCP2 and LCP3.

3.3. FT-IR spectroscopy

The FT-IR spectra of LCP1, LCP2 and LCP3 are presented in Fig. 3. The results showed characteristic absorbance of polysaccharides. The broader band of absorption at between 3700 and 3000 cm^{-1} was due to stretching of the hydroxyl groups, –OH. Each polysaccharide showed high absorbance in the region 1200–950 cm^{-1}, which was within the so-called fingerprint region, where the position and intensity of the bands were specific for each polysaccharide, allowing its possible identification (Filippov, 1992), and this

region was dominated by ring vibrations overlapped with stretching vibrations of (C–OH) side groups and the (C–O–C) glycosidic band vibration. The remarkable difference between LCP1 and other two was the absorbance at 1732 cm^{-1} and 1258 cm^{-1}, which was characteristic peak of the esterifiable group, –COOR. The group was likely to be CH_3COOR according to LAB EPS structure reported and LCP1 being neutral polysaccharide ((De Vuyst et al., 2001).

3.4. Monosaccharide composition

The monosaccharide compositions of LCP1, LCP2 and LCP3 are shown in Table 3. LCP1 and LCP2 were composed of glucose, rhamnose and galactose in molar ratios of 4.0:1.9:1.0 and 5.0:16.2:1.0, respectively. Besides these three sugars, LCP3 contained a given amount of mannose, the monosaccharide molar ratio of Glu:Rha:Gal:Man = 1.0:2.1:3.8:3.5. In general, LAB EPS are mainly composed of similar monosaccharide species (galactose, glucose and rhamnose) with different molar ratio. The EPS containing mannose were also reported before (De Vuyst et al., 2001).

3.5. Antihypertensive activity of EPS

The effects of three fractions of EPS on SBP of SHR are shown in Fig. 4. It was observed that EPS LCP1 decreased the SBP of SHR significantly in the first day. The maximal reduction (about 20 mm Hg) was observed from 2 to 4 h after oral administration. At the moment, SBP levels in treated SHR were remarkably different from the initial ones before oral administration ($P < 0.01$) as well as those of the placebo group ($P < 0.01$). However, the blood pressure-lowering effect disappeared 24 h after the administration. Subsequently, the effect was observed in SHR fed with LCP1 consecutively for 6 days. No significant changes were found in SBP of SHR fed with EPS LCP2 or LCP3. Meanwhile, all EPS fractions showed no obvious effect on the heart rate of SHR (Fig. 5). The results suggested that LCP1 was able to lower the blood pressure of SHR without significant impact on the heart rate.

Other researchers reported that milk fermented by *Lactobacillus* species such as *Lb. helveticus* and *Lb. bulgaricus* exhibited the antihypertensive activity. However, the main functional components reported were bioactive peptides from casein such as Val-Pro-Pro and Ile-Pro-Pro instead of EPS (Fuglsang, Nilsson, & Nyborg, 2002; Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000; Leclerc, Gauthier, Bachelard, Santure, & Roy, 2002; Masuda, Nakamura, & Takano, 1996; Seppo, Kerojioki, Suomalainen, & Korpela, 2002). Moreover, these peptides decreased the blood pressure by inhibiting the Angiotensin-I-converting enzyme (ACE) activity (Nakamura, Masuda, & Takano, 1996; Takano, 1998). On the contrary, EPS LCP1 produced by *Lactobacillus casei* LC2W in skim milk was unable to inhibit ACE activity in vitro experiments.

Table 3
Monosaccharide compositions of ESP LCP1, LCP2 and LCP3

EPS	Glucose (%)[a]	Rhamnose (%)[a]	Galactose (%)[a]	Mannose (%)[a]	Total sugars (%)[b]
LCP1	57.8	27.7	14.5	n.d.[c]	88.31
LCP2	22.6	72.9	4.5	n.d.[c]	83.14
LCP3	9.6	20.2	36.7	33.5	15.18

[a] Quantities of the neutral sugar residues are given in mol.% of total sugars content.
[b] Contents of total sugars are calculated as wt.%.
[c] Not detected.

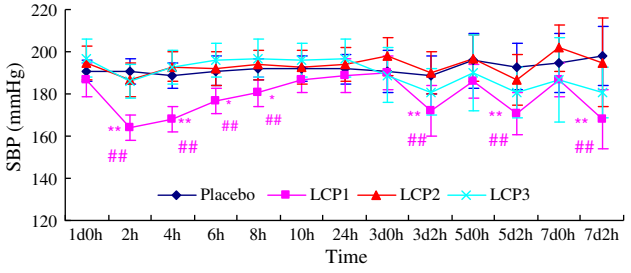

Fig. 4. Effect of oral administration of EPS fractions LCP1, LCP2 and LCP3 on the SBP of SHR.

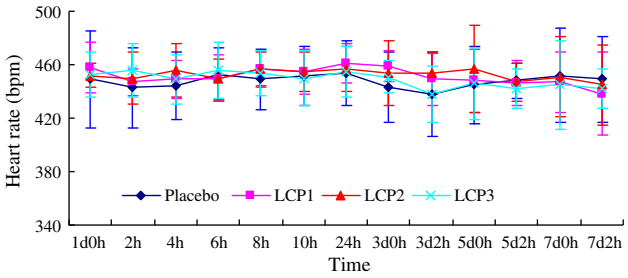

Fig. 5. Effect of oral administration of EPS fractions LCP1, LCP2 and LCP3 on the heart rate of SHR.

4. Conclusions

The EPS LCP with a yield of 153.4 mg L^{-1} were isolated from skim milk fermented by *Lactobacillus casei* LC2W and fractionated into three fractions, LCP1, LCP2 and LCP3. The ratio of each fraction was 35.74%, 12.61% and 33.34% based on LCP, respectively. The molecular weight of EPS fractions ranged from 10^4 to 10^6 and three polydispersity indexes were close to the unity, indicating the homogeneous molecular size distribution in each fraction. And intrinsic viscosity and radius of gyration of LCP1 were much higher than those of LCP2 and LCP3. All EPS LCP were hetero-EPS composed of glucose, rhamnose and galactose but in one case also of mannose. The data presented convincingly demonstrated the EPS LCP1 was able to lower the blood pressure of SHR without significant effect on the heart rate. Further studies such as structure analysis, rheological properties and antihypertensive mechanism, are in progress.

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