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Characterization of a novel exopolysaccharide with antitumor activity from *Lactobacillus plantarum* 70810

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ABSTRACT

Three methods were used to prepare the cell-bound exopolysaccharides (c-EPS) of *Lactobacillus plantarum* 70810, and the maximum yield (64.17 mg/mL) was obtained by ultrasonic extraction. After anion exchange and gel filtration chromatography, the c-EPS was fractionated as a single peak with a molecular weight of 169.6 kD. Its structural characteristics were investigated by gas chromatography (GC), methylation, Fourier-transform infrared spectroscopy (FTIR), and nuclear magnetic resonance (NMR) analysis. Based on obtained data, the novel c-EPS was found to be a galactan containing a backbone of α-D-(1 → 6)-linked galactosyl, β-D-(1 → 4)-linked galactosyl, β-D-(1 → 2,3)-linked galactosyl residues and a tail end of β-D-(1 →)-linked galactosyl residues. Preliminary *in vitro* tests revealed that c-EPS significantly inhibited the proliferation of HepG-2, BGC-823, especially HT-29 tumor cells. Our results suggested that the c-EPS produced by *L. plantarum* 70810 might be suitable for use as functional foods and natural antitumor drugs.

1. Introduction

Several lactic acid bacteria (LAB) are known to synthesize exopolysaccharides (EPS). The EPS generally exist in two forms depending on their locations: cell-bound exopolysaccharides (c-EPS) which closely adhere to the bacterial surface, and released exopolysaccharides (r-EPS) that release into the surrounding medium [1,2]. EPS are associated with microbial cells protection against the adverse environments (*e.g.* desiccation, toxic compounds and osmotic stress) [3]. Additionally, they are thought to play an essential role in the colonization of LAB to various ecosystems. For instance, c-EPS facilitate the colonization of LAB to intestinal mucosa, and thus enhance the immunity of host [4]. In the food industry, the EPS are used as biothickeners because of their stabilizing, emulsifying or gelling properties. Moreover, it has been suggested that some EPS produced by LAB may confer health benefits to the consumers such as immunomodulatory, antitumor, antibiofilm and antioxidant activity [5–8]. Among them, antitumor activity has particularly received intensive interest due to the increasing number and the high mortality of patients suffered from cancer. Though the antitumor agents used currently in chemotherapy practice possess strong activity, many doubts raised about their safety and side effects (such as hemopoetic suppression and immunotoxicity), which have focused attention on the extraction and identification of antitumor agents from natural sources [9]. EPS from safe natural sources such as LAB may serve as a good substitute to the synthetic antitumor agents.

A large number of EPS-producing LAB have been investigated [10–13]. These studies were primarily focused on dairy species of technological interest such as *Lactobacillus delbrueckii* subsp. *Bulgaricus*, *Lactococcus lactis* and *Lactobacillus casei*. *Lactobacillus plantarum* is frequently isolated from food products (vegetables, sausages, *etc.*). Recently some strains of *L. plantarum* are considered to be probiotics due to several of their properties (cholesterol-lowering, liver protection, *etc.*) [14–16]. Thus, *L. plantarum* EPS-producing strains may be interesting in two respects: as a textural agent and as a new potentially probiotic strain.

In our laboratory, we have isolated an EPS-producing strain *L. plantarum* 70810 from Chinese Paocai, which produced two different polymers including c-EPS and r-EPS. The influence of growth parameters on EPS production, and the biosorption of Pb (II) by its exopolysaccharide (r-EPS) have been described [17]. In the present work, the c-EPS from *L. plantarum* 70810 were prepared and characterized by gas chromatography (GC), gas chromatography–mass spectrometer (GC–MS) and nuclear magnetic resonance (NMR). In addition, the *in vitro* antitumor activity of c-EPS against HepG-2, BGC-823 and HT-29 cells was evaluated.

Abbreviations: c-EPS, cell-bound exopolysaccharides; LAB, lactic acid bacteria; EPS, exopolysaccharides; r-EPS, release polysaccharides; Mw, molecular weight.

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2. Material and methods

2.1. Microorganism and chemicals

The bacterial strain *L. plantarum* 70810 was isolated from Chinese Paocai [17] and maintained in a semi-defined medium (1000 mL) containing 20 g of lactose, 10 g of mannose, 10 g of soya peptone, 5 g of ammonium citrate, 4 g of yeast extract, 0.2 g of $MgSO_4$, 0.05 g of $MnSO_4$, 0.04 g of $FeSO_4$ and 1 mL of Tween 80.

DEAE-cellulose-52 was from Waterman Co. Ltd. (Springfield Mill, UK). Dialysis membranes (Mw cut-off 8000–14,000 Da) were from Solarbio Co., Ltd. (Beijing, China). Sephadex G-100 was from Pharmacia Biotech Co., Ltd. (Uppsala, Sweden). 5-Fluorouracil (5-FU), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), methyl iodide (CH_3I), arabinose, mannose, rhamnose, fucose, glucose, galactose and xylose were from Sigma Chemical Co., Ltd. (St. Louis, USA). Bovine serum albumin (BSA) and Dulbecco's modified eagle medium (DMEM) were purchased from Gibco (Gibco BRL, Grand Island, NY, USA). All other reagents used were of analytical grade.

2.2. Extraction methods of c-EPS

After inoculation at 31 °C for 24 h, 200 mL cultures were centrifuged at 15,000 × *g*, 4 °C for 10 min. The supernatant was used to isolate the r-EPS (data not given). The viscous cell precipitates were washed twice with 0.85% NaCl and then centrifuged as above. The extraction of c-EPS was carried out using three different methods as described by Yang et al. [1] and Tallon et al. [2].

Method A: the cell precipitates were resuspended in 1 M NaCl and treated by a JY92-IIN cell sonicator (Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) at 40 W, 4 °C for 3 min; Method B: the cell precipitates were resuspended in 0.5% phenol and the mixture was agitated at 60 rpm at room temperature for 4 h. Method C: the cell precipitates were resuspended in 0.05 M EDTA and the mixture was agitated at 60 rpm at 4 °C for 4 h. At the end of each assay, the supernatant fraction was concentrated after centrifugation to eliminate insoluble material. The final concentrates were mixed with three volumes of anhydrous alcohol for precipitation of c-EPS. After standing overnight at 4 °C, the precipitate was collected by centrifugation, then dialyzed in distilled water for 3 days and lyophilized using a Heto Power Dry LL3000 freeze drier (Thermo Electron Co., Bath, UK). The c-EPS yield (expressed as mg/L) was estimated by phenol-sulfuric acid method [18].

2.3. Analysis of bacterial growth and EPS production

L. plantarum 70810 was inoculated in 2000-mL Erlenmeyer flasks containing 1200 mL the semi-defined broth as mentioned above and incubated at 31 °C. Samples (approximately 50 mL) were withdrawn at different time intervals from 0 to 48 h. A pH meter (Sartorius PB-10, Germany) was used to determine the pH values. The cell viability was evaluated by dilution plating with MRS agar medium incubated at 37 °C for 48 h. EPS yields (expressed as mg/L) were estimated by phenol-sulphuric method [18] with glucose as standard.

2.4. Purification of c-EPS

Crude c-EPS solution (10 mg/mL, 10 mL) was subjected to a DEAE-cellulose-52 anion exchange chromatography column (26 mm × 500 mm). The samples were eluted with deionized water, 0.1, 0.3 and 0.5 M NaCl at a flow rate of 1 mL/min. Every 10 mL of elution was collected automatically and the carbohydrates content was determined by phenol-sulfuric acid method [18]. The obtained fraction was further purified by a Sephadex G-100 column (16 mm × 600 mm) and eluted with deionized water at a flow rate of 0.2 mL/min. A fraction containing purified c-EPS was collected, dialyzed and lyophilized.

2.5. Estimation molecular weight (Mw) of c-EPS

The Mw of purified c-EPS was determined by an Agilent 1100 series high performance liquid chromatography (HPLC) equipped with a TSK-GEL G4000SW_{XL} column (300 mm × 7.8 mm, Tosoh Co., Tokyo, Japan). The column was eluted with deionized water at a flow rate of 0.6 mL/min. Ten microliter (1 mg/mL) of purified c-EPS sample was injected into the column and the elution was monitored by an evaporative light-scattering detector (ELSD). Standard dextrans (1×10^4 to 80×10^4 Da) were used to determine the Mw of samples.

2.6. Basic components analysis of c-EPS

The total carbohydrates content and protein content was determined by phenol-sulfuric acid method [18] and Bradford method [19], respectively. Total uronic acid content was determined by spectrophotometry with *m*-hydroxybiphenyl [20]. Sulfated group content was analyzed with barium chloride-gelatin method [21].

2.7. Monosaccharide composition analysis of c-EPS

Five milligrams of purified c-EPS were hydrolyzed with 2 mL (2 M) trifluoroacetic acid (TFA) at 120 °C for 2 h. The hydrolysates were then subjected to aldononitrile acetate precolumn-derivatization gas chromatography (GC) for determination of the monosaccharide composition. GC was performed on an Agilent 6890N GC fitted with a flame ionization detector (FID) and a HP-5 capillary column (30 m × 0.32 mm i.d., 0.25 μm). The operating conditions were as follows: the N_2 carrier gas rate was 1.0 mL/min; injection and detector temperatures were 250 °C and 280 °C, respectively; column temperature was started at 120 °C for 3 min, then increased to 210 °C at the rate of 15 °C/min and maintained there for 4 min. Standard arabinose, mannose, rhamnose, fucose, glucose, galactose and xylose were prepared for comparison.

2.8. Ultraviolet (UV) and Fourier-transform infrared spectroscopy (FTIR)

For determination of the protein and nucleic acid contents in c-EPS, UV spectrum from 190 to 400 nm was recorded by a Shimadzu UV-1603 spectrophotometer (Shimadzu Co., Kyoto, Japan).

FTIR was performed for determination of the functional groups presented in c-EPS. The spectrum from 400 to 4000 cm^{-1} was recorded by a Bruker Tensor-27 FTIR spectrophotometer (Bruker Co., Ettlingen, Germany).

2.9. Methylation analysis and mass spectrometry

Five milligrams of c-EPS were completely methylated using methyl iodide and solid sodium hydroxide in dimethyl sulfoxide as described by Liu et al. [22]. After hydrolysis with 2 M TFA (2 h, 120 °C), the partially methylated monosaccharides were reduced with $NaBH_4$ for 2 h at room temperature. pH value was then adjusted to 5.5 by acetic acid solution and the samples were acetylated with pyridine-acetic anhydride (1:1, v/v) at 100 °C for 1 h. The partially methylated alditol acetates mixtures were analyzed by an Agilent 5975MSD-6890 GC-MS, equipped with a HP-5 capillary column using a temperature program of 100–250 °C at 6 °C/min and maintained there for 5 min.

2.10. Nuclear magnetic resonance (NMR) spectroscopy analysis

NMR spectrum of the polysaccharide solution was recorded using a Bruker AVANCE AV-500 spectrometer (Bruker Group, Fällanden, Switzerland) operated at 500 MHz. Both of ^{1}H and ^{13}C NMR were performed. The operated temperature was at 313.0 K. In order to improve the solubility, 17.5 mg polysaccharides were dissolved and analyzed in 500 μL D_2O-CF_3COOD (10:1, v/v). The delay (Dl) and acquisition time (AQ) was 4.00 s and 2.92 s for ^{1}H NMR spectra, and 1.08 s and 2.0 s for ^{13}C NMR, respectively. Chemicals shifts were expressed in parts per million (ppm).

2.11. Antitumor activity of c-EPS

For investigate the antitumor activity of c-EPS, human liver cancer HepG-2, gastric cancer BGC-823 cells and colon cancer HT-29 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 mg/L) under 5% CO_2 atmosphere at 37 °C. The antitumor activities of c-EPS on HepG-2, BGC-823 cells and HT-29 cells *in vitro* were evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay [20]. Briefly, 100 μL of tumor cells were incubated on a 96-well plate at a concentration of 2×10^5 cells/mL. After inoculation under 5% CO_2 at 37 °C for 24 h, the tumor cells were treated with various concentrations of c-EPS (0, 50, 100, 200, 400 and 600 μg/mL) and fluorouracil (5-FU, 50 μg/mL) for 24, 48 and 72 h, respectively. At the end of each treatment, 10 μL (5 mg/mL) of MTT was added and the tumor cells were inoculated for another 4 h. The liquid was then removed and 100 μL DMSO was added to the well. After dissolving of the formed crystal formazan, the absorbance was measured by a Synergy™ 2 microplate reader (BioTek Instruments, Inc., Burlington, VT) at 570 nm. The inhibition ratio was expressed as follows:

$$\text{Inhibition ratio}\,(\%) = \left[1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{control}} - A_{\text{blank}}}\right] \times 100$$

where A_{control} and A_{blank} were the absorbance of the system without the addition of c-EPS and cells, respectively.

2.12. Statistical analysis

All the data were expressed as mean ± standard deviation of three replicates. Tests of significant differences were determined by one way ANOVA of Tukey method (SPSS 16.0). The values were considered to be significantly different when $P < 0.05$.

3. Results and discussion

3.1. Preparation and production of EPS

Three known methods for extraction of c-EPS were applied to *L. plantarum* 70810 cells harvested at early stationary phase (see Section 2.2). Ultrasonic treatment gave the highest c-EPS yield of 64.17 mg/L compared to the other two extraction protocols, *i.e.*, 0.05 M EDTA treatment (18.42 mg/L) and 0.5% phenol treatment (10.65 mg/L). The results indicated the c-EPS yields of *L. plantarum* 70810 strongly depended on the extraction methods and ultrasonic treatment favored the extraction of c-EPS. This was in accordance with previous researches. Yang et al. [1] extracted 12 mg/L c-EPS from *Lactobacillus rhamnosus* JAAS8 by 0.5% phenol, whereas the c-EPS obtained by ultrasonic extraction from *L. plantarum* EP56 was about 73.6 mg/L [2]. Therefore ultrasonic extraction method was selected for performing the following extractions.

The growth of *L. plantarum* 70810, pH values of the medium and the EPS production at different fermentation times are shown in Fig. 1. The viable count peaked at 20 h, whereas the pH decreased throughout the fermentation periods and the final pH reached 3.71. The amount of total EPS (c-EPS and r-EPS) increased with fermentation time and reached a maximum of 581.03 mg/L in the stationary phase. However, the total EPS decreased slowly afterwards. The c-EPS yield increased rapidly during the first 12 h. After that, the production rate was slow down from 12 h to 22 h, which was corresponded to the stable phase of the bacteria growth. At 22 h, c-EPS reached a maximum concentration of 66.86 mg/L, whereas the yield dropped rapidly with further fermentation. The r-EPS yield showed a different behavior throughout fermentation, the yield continued to increase up to 48 h and reached 523.24 mg/L. Our results were in agreement with a previous study [2], however, disagreement with another study, which observed a continuing increase of the c-EPS amount throughout the fermentation periods from *L. rhamnosus* JAAS8, and the r-EPS yield decreased after reaching the maximum yields during fermentation [1]. These results indicated the amount of c-EPS largely influenced by the LAB strains and fermentation time.

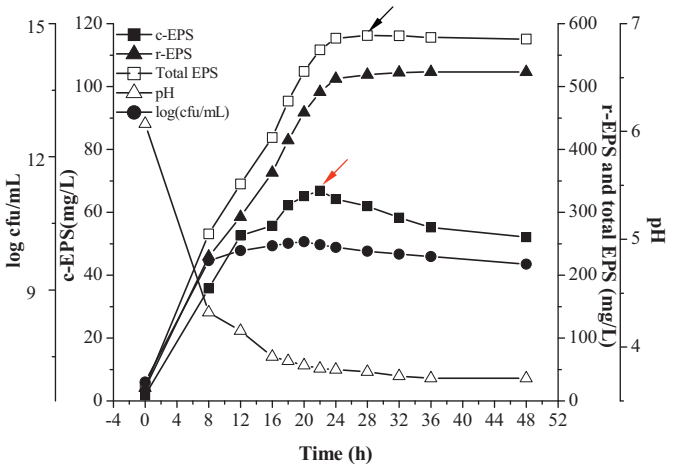

Fig. 1. Production kinetics curve of *L. plantarum* 70810 at 31 °C over time from 0 to 48 h. Red arrow and black arrow shows the maximum yield of c-EPS and total EPS, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.2. Purification and physicochemical characteristics of c-EPS

The c-EPS by ultrasonic extraction was firstly loaded to an anion exchange column, and then subjected to a Sephadex G-100 gel filtration column for further purification (Fig. 2A, B). The corresponding chromatogram showed only one peak for c-EPS, indicating c-EPS by ultrasonic extraction might be a homogeneous polysaccharide.

A further HPLC analysis of c-EPS fraction confirmed its homologeity. According to the elution curve of the standards, c-EPS was estimated a Mw of 169.6 kD. Tallon et al. [2] reported *L. plantarum* EP56 grown in a chemically defined medium produced a c-EPS of 8.5×10^5 Da, Yang et al. [1] also reported the Mw of c-EPS from *L. rhamnosus* JAAS8 was 5.5×10^5 Da. Both of them were much larger than that of the c-EPS obtained in the current study.

Total carbohydrates content of c-EPS was 95.45%. The sulfated group and uronic acid contents were 0.55% and 1.28%, respectively. It had a negative response to the Bradford test and no absorption at 260 nm or 280 nm in the UV spectrum, indicating the absence of protein or nucleic acid (data not shown). GC analysis (Fig. 3) showed that c-EPS was homopolysaccharides and composed of galactose. Yang et al. [1] reported that the c-EPS from *L. rhamnosus* JAAS8 was mainly composed of galactose. Tallon et al. [2]

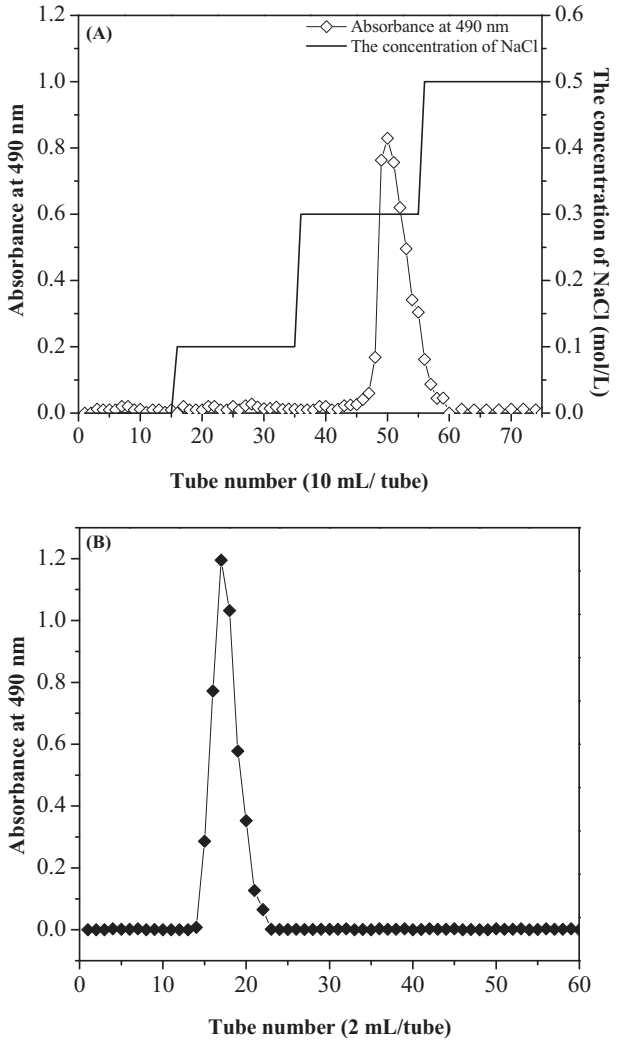

Fig. 2. DEAE-cellulose-52 anion exchange chromatogram (A) and Sephadex G-100 (B) gel filtration chromatogram of c-EPS.

mentioned galactose and glucose were the predominant components of c-EPS produced by *L. plantarum* EP56. Besides, glycerol, phosphoglycerol, *N*-acetylglucosamine and *N*-acetylgalactosamine were also reported constituted for c-EPS produced by LAB. However, it is particularly new since no previous investigations reported galactan compose of the c-EPS from LAB. It has been well accepted that monosaccharide composition of EPS produced by LAB

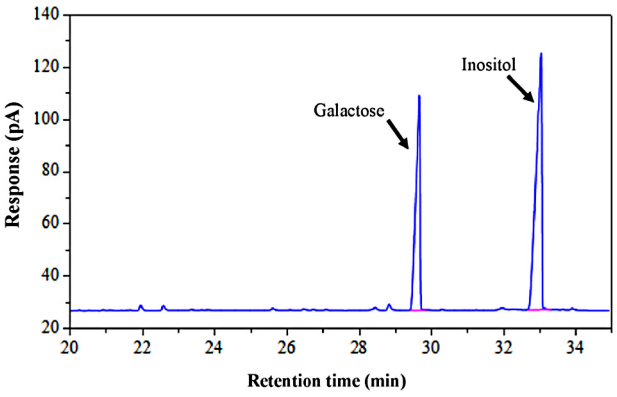

Fig. 3. GC chromatogram of hydrolyzed c-EPS.

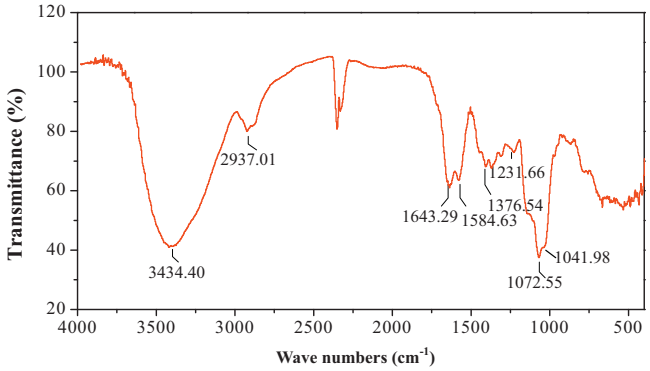

Fig. 4. FTIR spectrum of c-EPS.

was affected by the type of strains, culture conditions and medium compositions [12].

The FTIR spectrum of c-EPS is shown in Fig. 4. All the peaks obtained were in agreement with the typical absorption peaks of a polysaccharide. The broad absorption peak observed at around 3380–3440 cm^{-1} indicated the presence of intensive hydroxyl groups (O—H) stretching frequency [23]. The peak between 2930 and 2940 cm^{-1} was assigned as C—H and C—O group [24], whereas the peaks at around 1070 cm^{-1} and 1640 cm^{-1} were reported corresponded to C—O groups [24]. In addition, the absorption peak around 1580 cm^{-1} was usually assigned as vibrations of C=O from carboxylates, this was in accordance with the results of uronic acid [25]. In the fingerprint region (regions blow 1500 cm^{-1}), small peaks obtained around 1230 cm^{-1} indicated the possible presence of sulfated groups in c-EPS [26], which was consistent with our previous results.

3.3. Methylation analysis of c-EPS

The results of methylation analysis indicated 2,3,6-tri-O-methyl-galactitol, 2,3,4-tri-O-methyl-galactitol, 2,3,4,6-tetra-O-methyl-galactitol and 4,6-di-O-methyl-galactitol were the main methylated sugar derivatives with the molar percentages of 51.4:24.8:13.1:10.7, respectively. These results indicated c-EPS were mainly consisted of (1 → 4)-linked galactcosyl, (1 → 6)-linked galactcosyl, (1 → 2,3)-linked galactcosyl and tail (1→)-linked galactcosyl.

3.4. ^{1}H NMR and ^{13}C NMR analysis of c-EPS

In the ^{1}H NMR spectrum, the anomeric region (4.5–5.5 ppm) signals were often used to differentiate the anomeric protons of sugar residues in polysaccharides. The ring proton region (3.1–4.5 ppm) was assigned to protons attached to C2–C6 and usually poorly resolved due to their overlapping chemical shifts [12]. As shown in the ^{1}H NMR spectrum (Fig. 5A), the signals in 4.824 ppm ($J_{H1,H2}$ = 3.222), 4.7978 ppm ($J_{H1,H2}$ = 3.572) and 4.55 ppm ($J_{H1,H2}$ = 7.815) indicated c-EPS contained both α and β-type glycosidic linkages. In the ^{13}C NMR spectrum (Fig. 5B), two quartet signals presented between 110 and 170 ppm were considered belonged to the resonance of CF_3COOD. The ^{13}C NMR spectrum was also included anomeric carbons regions (95–110 ppm) and ring carbons (50–85) regions [12]. Based on the results of methylation analysis as well as the data reported previously [27–30], the signals in 97.67, 101.25, 104.66, 106.23 and 106.40 ppm were corresponded to 1,4-linked-α-D-Gal*p*, 1,6-linked-α-D-Gal*p*, 1,2,3-linked-β-D-Gal*p*, 1,4-linked-β-D-Gal*p* and β-D-Gal*p*-1, respectively. The results indicated FraC-2 had a backbone mainly composed of α-D-(1 → 6)-linked galactcosyl,

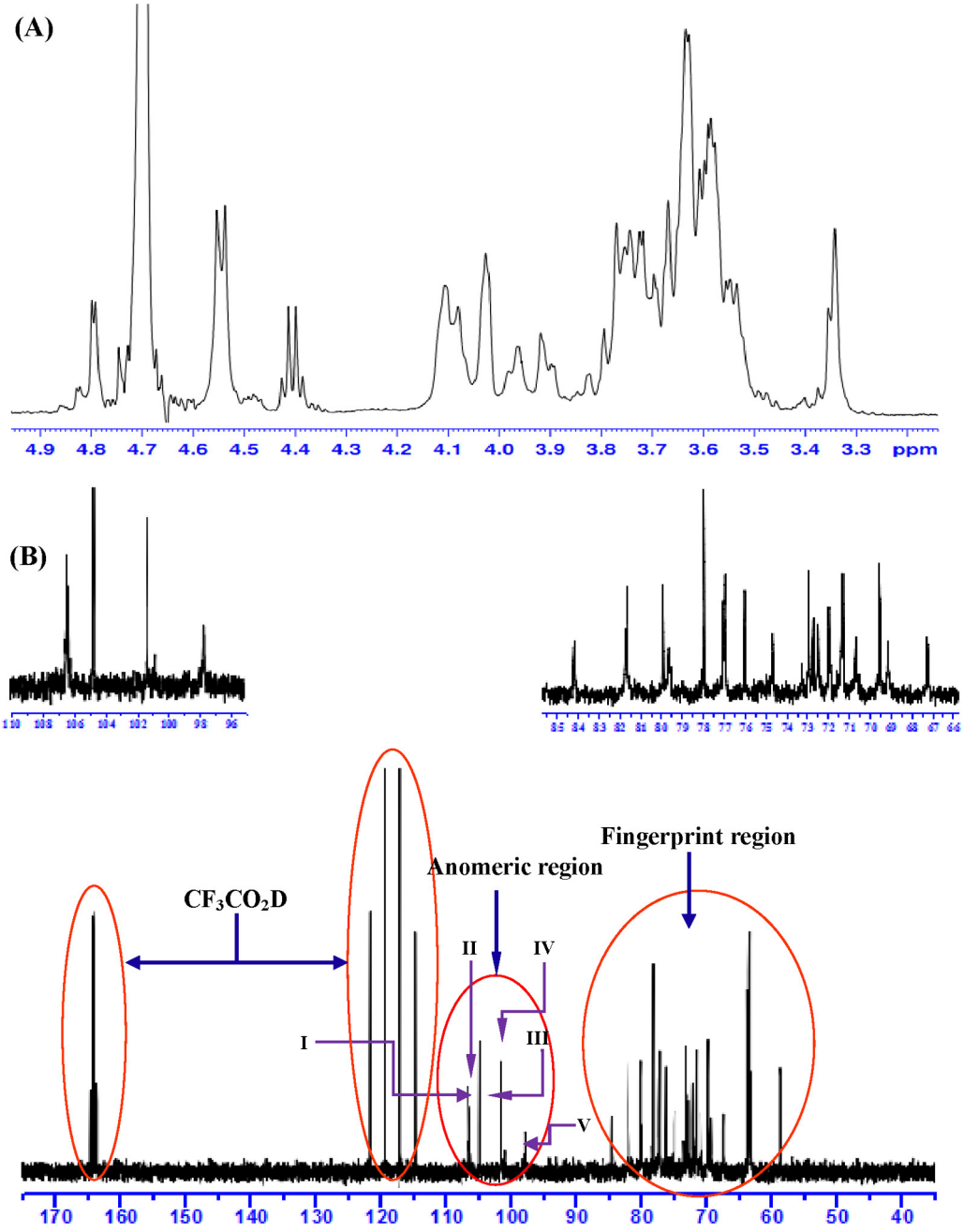

Fig. 5. ^{1}H NMR (A) and ^{13}C NMR (B) spectrograms of c-EPS. (I, 1-linked-β-D-Gal*p*; II, 1,4-linked-β-D-Gal*p*; III, 1,2,3-linked-β-D-Gal*p*; IV, 1,6-linked-α-D-Gal*p*; V, 1,4-linked-α-D-Gal*p*).

β-D-(1 → 4)-linked galactcosyl and β-D-(1 → 2,3)-linked galactcosyl residues. The tail ends were composed of β-D-(1→)-linked galactcosyl residues.

3.5. *In vitro antitumor activity of c-EPS*

The inhibition of c-EPS at different concentrations (50–600 μg/mL) and incubation periods (24, 48, 72 h) against HepG-2, BGC-823 and HT-29 cells were summarized in Fig. 6A–C. The inhibition effects of c-EPS on HepG-2 cells significantly increased along with the increasing concentrations and treated time ($P < 0.05$). At the lowest concentration (50 μg/mL) for a incubation period of 24 h, the inhibition ratio of c-EPS against HepG-2 was 10.12 ± 0.75%. This was improved about 5 folds (56.34 ± 1.07%) after treated with a more concentrated sample (600 μg/mL) and a prolonged time (72 h). However, this is not comparable to the positive control 5-FU. Xue et al. [31] reported 400 μg/mL polysaccharides from *Phellinus baumii*, when treated for 48 h, could suppress the proliferation of HepG-2 cells by 61.2%. Whereas Zhang et al. [32] reported the inhibition rate of 8 mg/mL polysaccharides from *Cordyceps militaris* on HepG-2 cells was 57.11% after 72 h incubation. This suggested c-EPS had moderate antitumor ability on HepG-2 cells. The inhibition effects of c-EPS on BGC-823 and HT-29 cells were also demonstrated good dose and time dependent matters. The highest inhibition ratio was about 61.57 ± 2.07% and 88.34 ± 1.97% for BGC-823 and HT-29 cells, respectively. The antitumor activity of c-EPS against BGC-823 was higher than that of *Radix hedysari* polysaccharide at the same concentration and time [33]. It is clear that significantly higher inhibition ratios were observed against HT-29

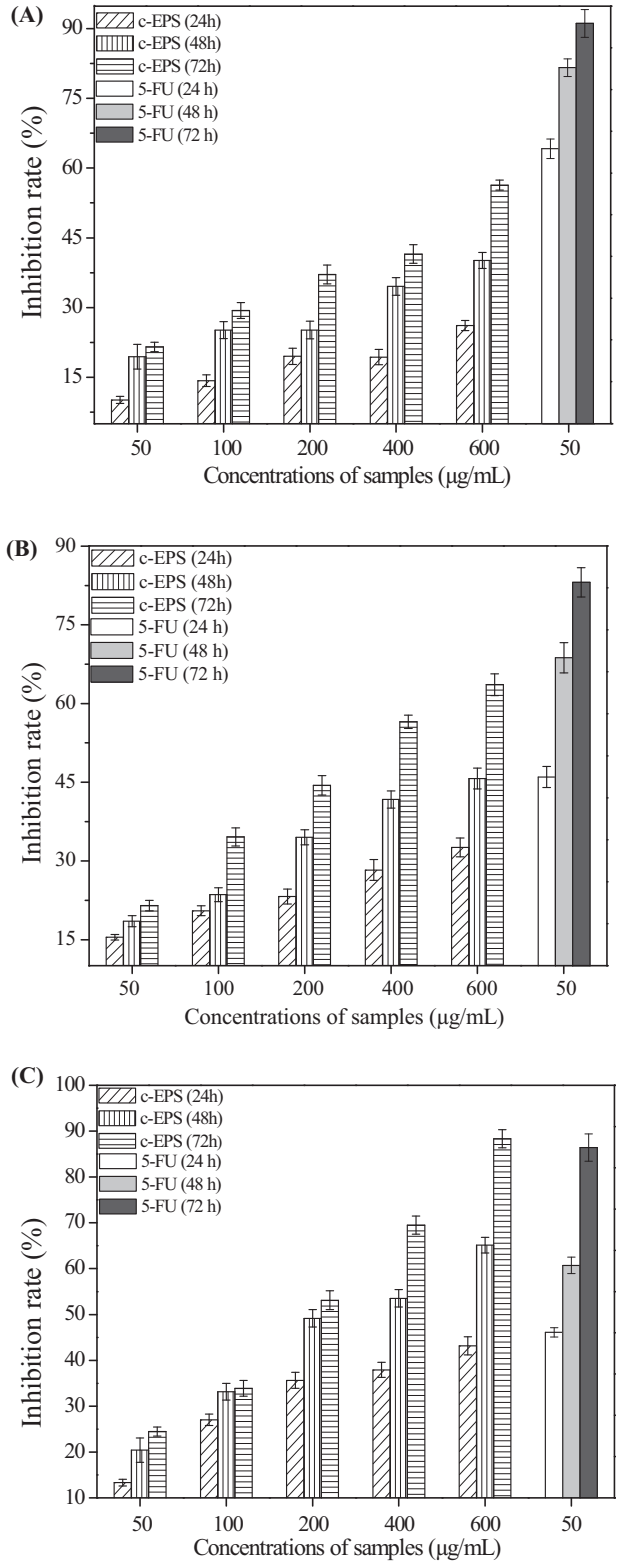

Fig. 6. Antitumor effects of c-EPS on HepG-2 (A), BGC-823 (B) and HT-29 (C) cells. All values were expressed as means ± standard deviation (SD) of three replications.

cells than that of other two cells of all c-EPS concentrations, in which the highest inhibition ratios were comparable to that of 5-FU (86.41 ± 2.98%). These results indicated the c-EPS from *L. plantarum* 70810 has moderate antitumor activity against HepG-2 cells and significant inhibition effects on BGC-823 and HT-29 cells.

4. Conclusions

In the present work the c-EPS from *L. plantarum* 70810 was prepared and characterized. The novel c-EPS was galactan with an estimated Mw of 169.6 kD. Further study revealed the c-EPS had a backbone of α-D-(1 → 6)-linked galactcosyl, β-D-(1 → 4)-linked galactcosyl, β-D-(1 → 2,3)-linked galactcosyl and a tail of β-D-(1→)-linked galactcosyl residues. *In vitro* antitumor analysis demonstrated the c-EPS had significant antitumor activities on HepG-2, BGC-823, especially HT-29 tumor cells. These results suggested that the c-EPS produced by *L. plantarum* 70810 might be suitable for use as functional foods and natural antitumor drugs.

Acknowledgment

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