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Short Communication

Isolation, purification, and immunomodulatory activity *in vitro* of three polysaccharides from roots of *Cudrania tricuspidata*

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Three water-soluble polysaccharides (CTPS-1A, CTPS-2B, and CTPS-3A) were obtained from roots of *Cudrania tricuspidata* (Carr.) Bur. in this study. The homogeneity of polysaccharides was determined, and the average molecular weight, ultraviolet, infrared, monosaccharide composition, and methylation analyses were carried out. Immunomodulatory activity assays *in vitro* showed that the three polysaccharides could directly stimulate the proliferation of mouse splenocytes alone or combining with concanavalin A or lipopolysaccharide. Furthermore, their stimulating activities were higher than that of the widely clinically used lentinan at optimal concentrations. CTPS-1A and CTPS-2B also enhanced the pinocytic activity of mouse peritoneal macrophages.

Keywords *Cudrania tricuspidata*; polysaccharide; immunomodulatory activity; purification

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Introduction

In recent years, plant polysaccharides have emerged as an important class of bioactive natural products. A wide range of polysaccharides has been reported to exhibit anti-tumor [1], immunostimulatory [2–6], anti-complementary [7–9], anti-inflammatory [10], anti-coagulant, and fibrinogenic [11] activities. Most polysaccharides derived from higher plants are relatively non-toxic and do not cause significant side effects that are a major problem associated with immunomodulatory bacterial polysaccharides and synthetic compounds [12]. Thus, plant polysaccharides are ideal therapeutic candidates with immunomodulatory and antitumor effects and low toxicity. Recently, lentinan (LNT), schizophyllan, and krestin have been accepted as immunomodulators in several oriental countries [12].

Cudrania tricuspidata (Carr.) Bur. belongs to the Moraceae family and is a deciduous shrub or tree widely distributed in China, Korea, and Japan. The root, stem, and root bark of this species have been used as traditional Chinese medicines. Its roots are applied in clinic for the treatment of digestive apparatus tumors, especially gastric carcinoma, and are also used to manage gonorrhea, rheumatism, jaundice, boils, scabies, bruising, and dysmenorrhea [13]. It has become one of the most important folk remedies for cancer in Korea during the past few decades, and has also shown antioxidant and anti-inflammatory activities [14]. However, the research on effective constituents from roots of *C. tricuspidata* has mainly been focused on small-molecular compounds, especially xanthones and flavonoids [15,16]. In a previous study, the crude polysaccharide was prepared from *C. tricuspidata* and the elementary activity test showed that the crude polysaccharide could activate the peritoneal macrophage in mice [17]. In the present study, the water-soluble crude polysaccharide was subjected to ion exchange and gel column chromatography, resulting in three purified polysaccharides, namely CTPS-1A, CTPS-2B, and CTPS-3A, and their immunomodulatory activities *in vitro* were investigated.

Materials and Methods

Materials

The roots of *C. tricuspidata* (Carr.) Bur. were collected in Bozhou City, Anhui Province, China, in October 2008, and identified by Professor Fang Shi of Botany at the College of Life Science, Qufu Normal University, China. A voucher specimen (No. 20081004) has been deposited in the College of Life Science, Qufu Normal University, China.

Glucose, arabinose, galactose, mannose, rhamnose, xylose, galacturonic acid, trifluoroacetic acid (TFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (Con A), lipopolysaccharide (LPS, from *Salmonella abortus equi*), RPMI 1640 medium were purchased from Sigma (St Louis, USA). Dextran T-series, DEAE-Cellulose, DEAE-Sephadex A-50, and Sephadex

G-100 were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Fetal bovine serum (FBS) was from Gibco (Grand Island, USA). LNT was provided by Nanjing Zhenzhong Bioengineering Co. Ltd, China. The LNT was endotoxin free with limulus amebocyte lysate (LAL) test. All other chemicals and solvents used were of analytical grade.

Extraction, isolation, and purification of polysaccharides

The air-dried roots of *C. tricuspidata* (Carr.) Bur. (2.5 kg) were extracted with 95% (*v*/*v*) ethanol at 90°C for 16 h under reflux to remove fat and pigment. The residue was dried naturally at room temperature and weighed 2.2 kg. One kilogram residue was extracted with boiling water three times (8 L × 3) and filtered through gauze. The aqueous solution was concentrated at 45°C in a rotary evaporator under reduced pressure, and then centrifuged at 7500 *g* for 15 min. The supernatant was precipitated with four volumes of 95% (*v*/*v*) ethanol at 4°C overnight. The precipitate was collected by centrifugation at 7500 *g* for 20 min, and washed sequentially with anhydrous ethanol, acetone, and ether. The resulting precipitate was suspended in water and dialyzed against distilled water (cut-off M_W 3500 Da). The retentate was frozen at −20°C, then thawed and centrifuged again to remove insoluble materials. After freeze–thaw for six times, the supernatant was lyophilized to afford crude polysaccharide (CTPS, 10.2 g). CTPS (8.0 g) was dissolved in distilled water, centrifuged, and loaded on to a DEAE-cellulose column. The column was eluted with water, followed by 0.1, 0.3, 0.5 M NaCl solution, respectively. Fractions of 10 ml were collected and monitored for the presence of carbohydrate using the phenol-sulfuric acid assay [18]. Fractions containing carbohydrate were pooled, concentrated, dialyzed, and lyophilized to give four fractions. The fraction eluted with 0.1 M NaCl was further subjected to DEAE-Sephadex A-50 column with 0.1 M NaCl, dialyzed, and lyophilized to yield CTPS-1A (0.17 g). The fraction eluted with 0.3 M NaCl was further chromatographied on a Sephadex G-100 gel filtration column with 0.1 M NaCl, dialyzed, and lyophilized to yield CTPS-2B (0.30 g). The fraction eluted with 0.5 M NaCl was further purified on Sephadex G-100 column with 0.1 M NaCl, dialyzed, and lyophilized to give CTPS-3A (0.24 g). The three purified polysaccharides (CTPS-1A, CTPS-2B, and CTPS-3A) were endotoxin free with the LAL test.

Spectroscopic and elemental analyses

Ultraviolet-visible spectra of 2.0 mg/ml polysaccharide solution (dissolved in distilled water) was scanned with a Cintra 5 UV-visible spectrophotometer (GBC, Sydney, NSW, Australia) under 190–400 nm. Fourier transform infrared spectra of polysaccharide (2.0 mg) were measured using a KBr pellet on a NEXUS 470 FTIR infrared spectrometer (Nicolet, Madison, USA) in the range of 4000–400 cm^{-1}. The elemental analysis of polysaccharide was carried out on a VarioEL Ⅲ elemental analyzer (Elementar, Hanau, Germany).

Homogeneity, molecular weight, monosaccharide composition, and linkage analyses of polysaccharides

The total sugar content was estimated by the phenol-sulfuric acid assay using glucose as standard [18]. Uronic acid content was determined by the *m*-phenylphenol method using galacturonic acid as standard [19]. The homogeneity and average molecular weight was determined by the high performance gel permeation chromatography performed on a Waters HPLC system equipped with a Waters Ultrahydrogel 250 column (7.8 mm × 300 mm) and a Waters 2410 differential refractometer. The mobile phase was 0.003 M NaOAc and the flow rate was 0.5 ml/min. The sample (4 mg) was dissolved in the mobile phase (0.2 ml) and centrifuged. A 20 μl sample was injected in each run. The molecular weight was estimated by reference to the calibration curve made from a Dextran T-series standard of known molecular weight (T-500, T-110, T-80, T-70, T-40, and T-9.3) [13]. Monosaccharide composition was analyzed according to the following procedure (inositol as the internal standard): the polysaccharide sample (3 mg) was hydrolyzed with 4 ml of 2.0 M TFA at 110°C for 3 h, followed by evaporation to dryness. The residue was redissolved in water (0.2 ml), with 5 μl of the solution used for thin layer chromatography analysis. The other portion was successively reduced with $NaBH_4$, acetylated with Ac_2O at 100°C for 1 h, and the resulting alditol acetate was examined by gas chromatography (GC) on a Shimadzu GC-14B system (Kyoto, Japan) equipped with a 3% OV-225/AW-DMCS-Chromosorb W column (2.5 m × 3 mm) and a flame-ionization detector. N_2 was used as the carrier gas (1.3 ml/min). The injector temperature was kept at 250°C (split injection 40:1). The operation was performed at a column temperature program from 110°C to 190°C at 5°C/min, holding for 5 min at 190°C, then increasing to 250°C at 10°C/min and finally holding for 5 min at 250°C.

The polysaccharide sample (5.0 mg) was dried for 2 days in vacuo (P_2O_5), then dissolved in 4Å molecular sieve-dried DMSO (2.0 ml), and methylated four times using the modified Ciucanu method [13]. The completeness of methylation was confirmed by the disappearance of the hydroxyl absorption in FTIR spectrum (Nujol). The permethylated polysaccharide was depolymerized with 90% (*v*/*v*) formic acid (100°C, 4 h), followed by hydrolysis with 2.0 M TFA (100°C, 6 h). The hydrolysate was converted into partially methylated alditol acetate and analyzed by GC-MS [13].

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Nuclear magnetic resonance (NMR) experiments

The polysaccharide sample (50 mg) was deuterium-exchanged twice and dissolved in 0.5 ml of D_2O (99.9% D). The ^1H and ^{13}C NMR spectra were measured at room temperature with a Bruker AM 400 NMR spectrometer, with acetone as internal standard (δ 31.50 for carbon). All chemical shifts were reported downfield relative to Me_4Si.

Animal experiment

BALB/c mice (Grade II, 6 weeks old) weighing 18–22 g were provided by Shanghai Institute of Materia Medica (Certificate No. 200606080), and acclimatized for 1 week before use. Half of them were male and the others were female. Rodent laboratory chow and tap water were provided *ad libitum* and maintained under controlled conditions with temperature of $24 \pm 1°C$, humidity of $50 \pm 10\%$, and 12/12 h light–dark cycle. All the procedures were in strict accordance with the guidelines on the use and care of laboratory animals that were established by Shanghai Institute of Materia Medica and approved by the Institute Committee for animal experiments.

Proliferation assay of mouse spleen cells

Spleen cells were prepared from BALB/c mice as previously described [20,21]. Briefly, BALB/c mice were killed by cervical dislocation. The spleens were removed and cut into small pieces, and then pressed through a stainless-steel mesh (100 meshes) to obtain a suspension of isolated spleen cells. Red cells in the suspension were lysed with a solution of Tris-HCl-NH_4Cl (pH 7.2). The cell suspension was further diluted with a 5-fold excess of RPMI 1640 medium, and after mixing and centrifugation, cell pellets were finally resuspended in RPMI 1640 medium and adjusted to 3×10^6 cells/ml. Aliquots (100 μl) of the cell suspension were added to each well of a 96-well plate in the presence of mitogen Con A (final concentration 2.5 μg/ml) or LPS (final concentration 10.0 μg/ml). The polysaccharides (CTPS-1A, CTPS-2B, and CTPS-3A) at different concentrations (final concentration 6.25, 12.5, 25, 50, and 100 μg/ml, respectively) were incubated with the above mouse spleen cells in the presence of Con A or LPS. Negative controls were treated only with RPMI 1640 medium and positive controls with 25 μg/ml LNT. After incubation at 37°C in a 95% humidified atmosphere containing 5% CO_2 for 72 h, 20 μl of MTT was added to each well, and the incubation was continued for another 6 h. The optical density (OD) of the converted dye was measured at 490 nm with a Multiskan Plate Reader (Multiskan EX; Labsystem, Vantaa, Finland).

Pinocytic activity assay of mouse peritoneal macrophages

Macrophages were prepared from BALB/c mice as described previously [22,23]. In brief, peritoneal macrophages were harvested from two to three BALB/c mice injected intraperitoneally with 3 ml of thioglycollate 3 days before sterile peritoneal lavage with 10 ml of Hank's balanced salt solution. The collected cells were seeded and cultured in RPMI 1640 containing 10% heat-inactivated FBS, 100 IU/ml penicillin and 50 μg/ml streptomycin at a density 1.0×10^6 cells/ml. The cells were allowed to adhere for 3 h to 96-well plate at 37°C in a 95% humidified atmosphere containing 5% CO_2 incubator. Then the cultures were washed twice with RPMI 1640 to remove non-adherent cells prior to the addition of 1.0 ml of fresh RPMI 1640 containing 10% FBS. The purity of the adherent macrophages was assessed by Giemsa staining (>95%) [24].

The phagocytic ability of macrophage was measured by neutral red uptake [25,26]. After cells were cultured with the polysaccharides (CTPS-1A, CTPS-2B, and CTPS-3A) at different concentrations (final concentration 6.25, 12.5, 25, 50, and 100 μg/ml, respectively) or LNT (final concentration 50 μg/ml) for 48 h, 100 μl neutral red solution (0.1% in 10 mM phosphate buffer solution (PBS)) was added and incubated for 2 h. The supernatant was discarded and the cells were washed with PBS twice to remove the neutral red that was not phagocytized by macrophage. Then cell lysate (ethanol and 0.01% acetic acid at the ratio of 1:1, 100 μl/well) was added. After the mixture was incubated at room temperature overnight, the OD at 540 nm was measured.

Statistical analysis

Quantitative data were expressed as mean ± SD. All statistical comparisons were carried out using one-way ANOVA (analysis of variance) test followed by Tukey's test. *P*-values of <0.05 were considered to be statistically significant.

Results

Monosaccharide analysis and characterization of polysaccharides

The yield of the total polysaccharide CTPS was 1.0% of the plant raw material, and the yields of three purified polysaccharides were 2.1%, 3.8%, and 3.0% for CTPS-1A, CTPS-2B, and CTPS-3A of the parent crude polysaccharide CTPS, respectively. The UV-visible spectra showed that three polysaccharides all had an absorption peak at 190 nm only, which is the characteristic UV absorption peak for a polysaccharide. There was no absorption at 260 and 280 nm, indicating that the polysaccharides

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Table 1 Structural information of the three homogeneous polysaccharides

Polysaccharide	Molecular weight (Da)	$[\alpha]_D$ (*c* 0.50, H_2O)	Monosaccharide composition/molar ratio
CTPS-1A	2.2×10^4	−28.5°	Glc:Gal:Ara:Xyl = 11.7:3.4:2.5:1.0
CTPS-2B	6.8×10^4	−22.8°	Gal:Ara:Rha:Glc:GalA = 1.2:3.5:1.4:1.0:1.3
CTPS-3A	1.0×10^5	−35.8°	Ara:Rha:Gal:GalA = 1.0:0.9:1.6:0.4

contained no nucleic acid, protein, or polypeptide. The elemental analysis showed that three polysaccharides contained no nitrogen or sulfur. This result undoubtedly proved that they contained no protein. Furthermore, the three polysaccharides showed negative Fehling's reagent and iodine–potassium iodide reactions, indicating that they did not contain reducing sugars or starch-type polysaccharides. The total neutral sugar content of CTPS-1A, CTPS-2B, and CTPS-3A was 96.0%, 82.1%, and 81.6%, respectively.

FTIR spectra (not shown) indicated that the three polysaccharides possessed characteristic absorption peaks for polysaccharide. Taking CTPS-1A as an example, the strong peak at 3405.7 cm^{-1} was the absorption of the O–H bond and the peak at 2929.4 cm^{-1} was the absorption of C–H bond. The peak at 1612.2 cm^{-1} was the absorption of hydrated water in CTPS-1A. The peaks at 1200–1000 cm^{-1} were the absorption of C–O bond.

The specific rotation ($[\alpha]_D$) and the average molecular weight of CTPS-1A, CTPS-2B, and CTPS-3A are shown in **Table 1**. In this study, all polysaccharide samples were hydrolyzed with TFA, and then GC analysis of hydrolysates was performed by precolumn-derivatization techniques using the derived alditol acetates to identify the component monosaccharides released from the polysaccharide. Total uronic acid content was determined by the *m*-phenylphenol method using galacturonic acid as standard. The standard curve showed that the good linearity was obtained by regression analysis between A_{520} (absorbance) and C (μg/ml), and the regression equation is as below: $A_{520} = 0.0314C + 0.0104$ ($r = 0.999$) within 0–40 μg/ml. The uronic acid content of CTPS-2B and CTPS-3A was 14.7% and 14.2%, respectively. And CTPS-1A contained no uronic acid. The monosaccharide composition is shown in **Table 1**.

After methylating four times using the modified Ciucanu method, the permethylated polysaccharides were depolymerized and converted into partially methylated alditol acetate. GC-MS analysis showed that CTPS-1A had a backbone mainly consisting of 1,4-D-glucosyl residues, and the terminal units were galactose, glucose, and arabinose residues. Minor branches were attached to galactosyl and glucosyl residues, and CTPS-1A also contained a trace of xylose. CTPS-2B had many types of linkages. The arabinose residues were 1-, 1,5-, and 1,3,5-linked, and the galactose residues were 1,6-, 1,3-, 1,4-, and 1,3,6-linked. CTPS-3A also had many types of linkages. The arabinose residues were 1- and 1,5-linked. The galactose residues were 1,6-, 1,3,6-, 1,4-, 1-linked. The anomeric protons and carbons in NMR spectra were consistent with the methylation and monosaccharide analysis results for three polysaccharides, respectively (see Online supplementary material).

Effect of polysaccharides on splenocyte proliferation

Immunostimulation is one of the several functions associated with polysaccharides [27]. The three homogeneous polysaccharides could directly stimulate the proliferation of mouse splenocytes (MSs) alone or combining with Con A or LPS *in vitro*.

As shown in **Fig. 1(A)**, CTPS-1A, CTPS-2B, and CTPS-3A directly stimulated the proliferation of MSs within concentrations of 6.25–100 μg/ml, compared with the control group. Furthermore, effects of CTPS-1A and CTPS-2B at 50 and 100 μg/ml were better than that of LNT at the optimal concentration of 25 μg/ml. Effects of CTPS-3A at 25, 50, and 100 μg/ml were better than that of LNT. The results showed that CTPS-1A, CTPS-2B, and CTPS-3A could significantly promote the proliferation of MSs, suggesting that three polysaccharides may possess the mitogen-like activity.

As shown in **Fig. 1(B)**, CTPS-1A, CTPS-2B, and CTPS-3A stimulated the proliferation of MSs combining with Con A within concentrations of 6.25–100 μg/ml. The effect of CTPS-1A at 50 μg/ml was better than that of LNT. The effects of CTPS-2B at 25, 50 μg/ml were better than that of LNT. Effect of CTPS-3A at 12.5 μg/ml was better than that of LNT, in particular, the stimulation concentration was lower than that of LNT. The results showed that CTPS-1A, CTPS-2B, and CTPS-3A could significantly promote the proliferation of MSs combining with Con A.

As shown in **Fig. 1(C)**, effects of CTPS-1A at 50, 100 μg/ml on the proliferation of MSs with LPS were higher than those of the control group and LNT. CTPS-2B stimulated the proliferation of MSs combining with LPS within concentrations of 6.25–100 μg/ml. Effects of CTPS-2B at 6.25, 50, 100 μg/ml were better than that of LNT at 25 μg/ml. CTPS-3A enhanced splenocyte proliferation within concentrations of 6.25–100 μg/ml. Effect of CTPS-3A at 25 μg/ml was better than that of LNT. The

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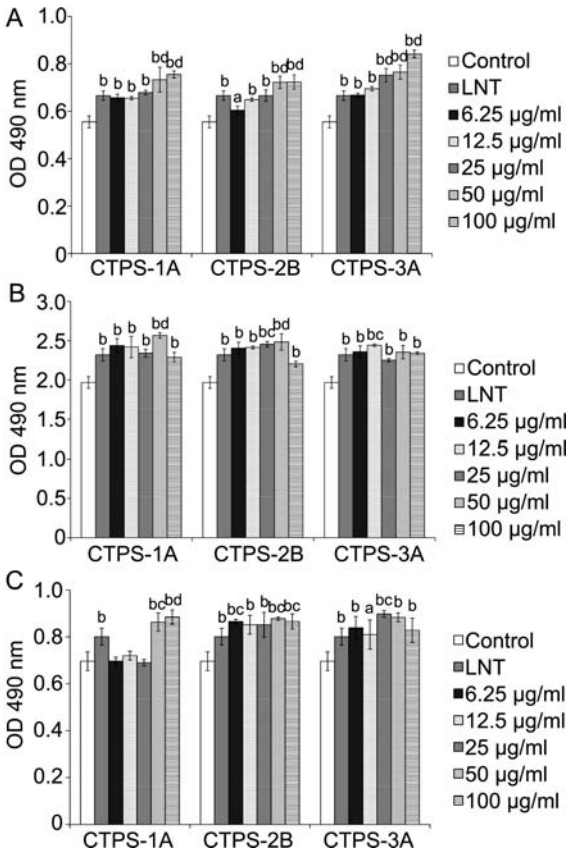

Figure 1 Stimulation effects of the three homogeneous polysaccharides from roots of *C. tricuspidata* on splenocyte proliferation in mice The effects of the three homogeneous polysaccharides at various concentrations on the proliferation of MSs alone (A), MSs with ConA (B), and MSs with LPS (C) were measured by the MTT method. The values were presented as mean ± SD ($n = 8$) and evaluated by one-way ANOVA followed by the Student–Newman–Keuls test to detect inter-group differences. Optimal concentration of LNT of 25 μg/ml was used. $^{a}P < 0.05$ and $^{b}P < 0.01$ compared with the control group (RPMI 1640 medium alone); $^{c}P < 0.05$ and $^{d}P < 0.01$ compared with the LNT group. LNT, lentinan (positive control, a widely used polysaccharide in clinic which possesses strong immunomodulatory activity).

results showed that CTPS-1A, CTPS-2B, and CTPS-3A could significantly enhance the proliferation of MSs combining with LPS.

Effect of polysaccharides on pinocytic activity of mouse peritoneal macrophages

One of the most distinguished features of macrophage activation would be an increase in pinocytic activity. Pinocytic activity of polysaccharides-activated macrophages was examined by the uptake of neutral red (0.1%). The results (**Fig. 2**) showed that enhancement of pinocytic activity was observed in macrophages treated with CTPS-1A at concentrations of 50, 100 μg/ml and CTPS-2B at 100 μg/ml, respectively. There was no difference between the effect of CTPS-1A at 50 μg/ml and that of LNT at the optimal

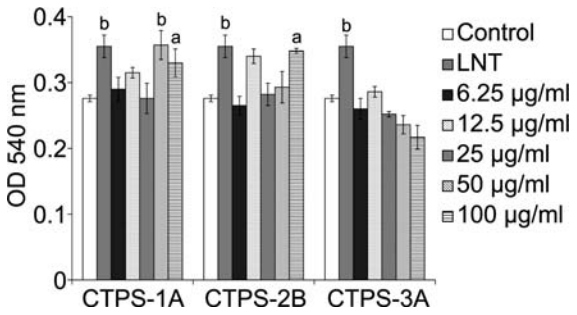

Figure 2 Phagocytotic function of macrophages stimulated with the three homogeneous polysaccharides from roots of *C. tricuspidata* Phagocytosis of peritoneal macrophage stimulated by the three homogeneous polysaccharides was measured by the neutral red colorimetric method. In this study, the optimal concentration of LNT of 50 μg/ml was used. $^{a}P < 0.05$ and $^{b}P < 0.01$ compared with the control group (RPMI 1640 medium alone); $^{c}P < 0.05$ and $^{d}P < 0.01$ compared with the LNT group. LNT, lentinan (positive control, a widely used polysaccharide in clinic which possesses strong immunomodulatory activity).

concentration of 50 μg/ml. There was no significant difference between the effect of CTPS-2B at 100 μg/ml and that of LNT. CTPS-3A showed no effect on pinocytic activity of mouse peritoneal macrophages.

Discussion

The search for novel polysaccharides that show immunomodulatory activities—without severe side effects to the host—has become an important goal in the phytochemistry and biomedical research. Several studies have been carried out with polysaccharides isolated from plant sources and different immunomodulating properties were reported. The potential usefulness of immunomodulating polysaccharides in the treatment of some diseases has been demonstrated in preclinical and clinical studies. While the activity of some polysaccharides has been known over the last decades, the lack of further purification and comprehensive structural information has limited the effort to study their potential for clinical use [2].

In our previous study [13], we purified and characterized a water-soluble α-(1→4)-glucan from roots of *C. tricuspidata*. Stimulation effects of this glucan on splenocyte proliferation in mice directly or combined with Con A or LPS were not found. However, in this study, the three homogeneous polysaccharides showed high activity and might be better immunomodulators than LNT.

The three polysaccharides were composed of multiple monosaccharide, and CTPS-2B and CTPS-3A contained uronic acid. Based on the structural information and the activity observed among four homogeneous polysaccharides, it was presumed that complex branches and uronic

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acid somewhat had significant effect on immune activity for polysaccharides. CTPS-1A, CTPS-2B, and CTPS-3A from *C. tricuspidata* which showed high activity were highly branched polysaccharides or possessed uronic acid at non-reducing terminals. In contrast, the α-(1→4)-glucan with few branches and without uronic acid showed no activity.

The purification and immunomodulatory activity of polysaccharides from higher plants has been reported in recent years. With regard to polysaccharides from *C. tricuspidata*, little research has been reported and a previous study has focused on the crude extract [17]. In the present study, three homogeneous polysaccharides with complicated structures showed good immunostimulatory activities, even better than LNT. Therefore, they have potential to act as biological response modifiers.

In summary, three homogeneous polysaccharides (CTPS-1A, CTPS-2B, and CTPS-3A) were isolated from roots of *C. tricuspidata*. Immunomodulatory activity assays *in vitro* showed that the three polysaccharides could both directly stimulate the proliferation of MSs and stimulate the proliferation of MSs combining with Con A or LPS in the range of concentrations used in these experiments. Furthermore, at some concentrations, their stimulating activities were higher than LNT. In addition, CTPS-1A and CTPS-2B also enhanced pinocytic activity of mouse peritoneal macrophages. Using polysaccharides alone or in conjunction with other already existing chemical alternatives is of great interest for therapeutic applications and will be investigated.

Supplementary data

Supplementary data are available at *ABBS* online.

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