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Isolation, purification and immunobiological activity of a new water-soluble bee pollen polysaccharide from *Crataegus pinnatifida Bge.*

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ABSTRACT

A novel water-soluble polysaccharide was obtained from bee pollen of *Crataegus pinnatifida Bge.* Two fractions of this polysaccharide, CPP-1 and CPP-2, were first extracted by hot-water and purified. The average molecular weight of CPP-1 and CPP-2 were approximately 3.7×10^5 Da and 7.8×10^4 Da, and their chemical structures were studied by gas chromatography (GC), Fourier transform-infrared (FT-IR) spectroscopy, methylation analysis. We evaluated the effects of CPP-1 and CPP-2 on the basis of phagocytosis of macrophage assay, natural killer cells cytotoxicity assay and spleen lymphocyte proliferation assay. The results showed CPP-1 and CPP-2 significantly induced phagocytic rates and phagocytic indexes by peritoneal macrophages. Moreover, these two fractions caused a significant stimulation of rat spleen cell proliferation. At 50 μg/mL, CPP-2 activated NK cells more significantly than CPP-1. These findings suggest that they should be explored as a novel potential immunostimulants.

1. Introduction

In the past few years, outbreak of severe acute respiratory syndrome (SARS), Bird Flu virus, continuing spread of HIV/AIDS and emergence of resistance against current drugs prompt researchers to look for new protective measures against these threats. Innate immune stimulation has been shown to be effective to keep away from emerging infectious diseases. In order to reduce damage to the human body, much effort has thus been made in the search for natural immunomodulation agents (Hackett, 2003). Polysaccharides are known as biopolymers and shown to play key roles in therapeutic agents. Published data indicates that numerous polysaccharides isolated from plants, epiphytes and animals have been proven to be fewer side effects and possess a wide range of biological functions such as anti-tumor, immunomodulation and anti-oxidation properties (Amarowicz, Naczk, & Shahidi, 2000; Fujii, Maeda, Suzuki, & Ishida, 1978; Jimenez-Medina et al., 2008; Liu, Ooi, & Chang, 1997; Ooi & Liu, 2000; Yen & Chen, 1996). Consequently, large numbers of Chinese herbal medicines and edible mushrooms are extensively studied and some of them have been applied in the clinical treatment. For example, the polysaccharides of *Glycyrrhiza uralensis Fish* (Cheng, Wan, Wang, Jin, & Xu, 2008), *Lentinus edodes*, (Kosaka, Wani, Hattori, & Yamashita, 1982) and *Lingzhi* (Di, Chan, Leung, & Huie, 2003).

In the present study, we isolated a new immunostimulating polysaccharide from bee pollen of a fruit *Crataegus pinnatifida Bge.*, commonly called Hawthorn, which is a well-known Chinese herbal medicine used for the treatment of various diseases as well as a tonic medicine for thousands of years. Bee pollen is the male gametophyte of gymnosperm and angiosperm. It contains all nutrients which are necessary for plant growth and development and also targets the entire human body, which is a special boost to the reproductive, immune, and nervous systems (Kroyer & Hegedus, 2001; Serra Bonvehi, Soliva Torrento, & Centelles Lorente, 2001; Uzbekova, Makarova, Khvoynitskaya, & Slepnev, 2003). In the USA, bee pollen is defined by the Dietary Supplement Health and Education Act of 1994 as Dietary Supplement used to supplement the diet by increasing the total dietary intake. Since 20 years ago, interests on the pollen have been increasing and there are numerous studies on its nutrition contents and extracts. However, most of the researches are concentrated on the application for their development. There is little information about the bee pollen polysaccharide and biological functions. From this standpoint, we investigated a new bee pollen polysaccharide which is seldom mentioned in the previous papers.

Although the polysaccharide has been recognized as one of the main chemical components of immunomodulatory, there is rare attention about polysaccharide of bee pollen on the immune system both *in vivo* and *in vitro*. In this paper, we modified the previous method to isolate the carbohydrate compounds from the bee pollen of *C. pinnatifida Bge.*, and found a novel water-soluble polysaccharide, which had scarcely been reported. We named it

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C. pinnatifida Bge. polysaccharide (CPP). Moreover, we adopted a synthesis method to break wall of bee pollen, including the mechanic way, the temperature difference way and the enzyme way. This paper mainly describes isolation and purification of the CPP; mean molecular weight survey of the CPP by HPGPC, structural characterizations of the CPP and also refers to some preliminary immune stimulating properties. Further studies on the CPP will be summarized in our next paper. The results suggest it may be a potential immunomodulation agent which can play a decisive role in innate immune defense against virus-infected and malignant cells by virtue of their ability to recognize and destroy abnormal cells.

2. Materials and methods

2.1. Materials

The bee pollen of *C. pinnatifida Bge.* was commercially available in Beijing, China. The standard monosaccharides (D-glucose, L-rhamnose, D-xylose, D-galactose, D-ribose, D-mannose, D-arabinose, L-fucose, D-glucuronic acid and L-galacturonic acid) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), concanavalin A (ConA), pectinase and trypsin were purchased from Amresco Co. (USA) and Sigma Chemical Co. (St. Louis, MO, USA) and medium RPMI-1640, fetal calf serum from Gibco Invitrogen Co. (Gibco, USA). Dextrans of different molecular weights, DEAE–Sepharose CL-6B and Sephadex G-100 were purchased from the Pharmacia Co. (Uppsala, Sweden). All other reagents used were of analytical grade.

2.2. Isolation of the polysaccharide

The dried bee pollen of *C. pinnatifida Bge.* was ground into powder (100 g) and was preserved by freezing for 48 h and defatted with 1 L of 9:1 ethyl ether–ethanol (v/v) at 35 °C for 6 h under stirring, and the supernatant was removed. The residue was kept below 20 °C again for 24 h, and then put it into boiling distilled water under stirring for 30 min, and cooled the water to 60 °C. After mixing with 1% (m/m) pectinase the residue was extracted with distilled water (m/v = 1:10) for 3 h. After each 3-h period of water extraction and inactivating pectinase, the water extracts were collected and the residues were extracted again for three cycles. The combined extracts were pooled, concentrated in a rotary evaporator under reduced pressure at 50 °C and filtered. Then the filtrate was precipitated by adding 95% ethanol (five times the volume of aqueous extract) at 4 °C, followed by centrifugation at 5000 rpm for 20 min. The precipitate was dissolved in 400 mL of water and deproteinized with 10% (m/m) trypsin at 65 °C, pH = 7, 3 h (Song, Meng, & Xu, 2007). After inactivating trypsin, the resulting aqueous fraction was extensively dialyzed against tap water for 2 days and then distilled water for 2 days, then the crude polysaccharide fraction was obtained by precipitation in four fold volume of anhydrous ethanol and washed with acetone and ethyl ether three times to yield the crude polysaccharide (8.5 g) known as CPP in the subsequent description.

2.3. Purification of the polysaccharide

The CPP (400 mg) was dissolved in distilled water, centrifuged, and then the supernatant was applied to a column of DEAE–Sepharose CL-6B (2.6 × 40 cm) equilibrated with 25 mmol/L Tris–HCl buffer (pH = 8.0), and eluted with 25 mmol/L Tris–HCl buffer (pH = 8.0), followed by 0.02 to 1 mol/L NaCl Tris–HCl buffer (25 mmol/L, pH = 8.0) linear gradient at a flow rate of 1 mL/min. The yielded fractions were measured according to the total carbohydrate content quantified by the phenol–sulfuric acid method at 490 nm. The elution profile detected by the phenol–sulfuric acid assay showed two big elution peaks, and the protein and the nucleic acid were measured by Varian Cary100 Bio UV/visible spectrophotometer (USA). The collected fractions, which had been analyzed for their total sugar contents were pooled, dialyzed and lyophilized. The whole procedure is shown in Fig. 1.

To test the homogeneity of the purified polysaccharides, a column of Sephadex G-100 (1.0 × 100 cm) was used, loading 100 mg of the above purified two fractions for each run. The column was equilibrated with 0.1 mol/L NaCl Tris–HCl buffer (25 mmol/L, pH = 8.0) and eluted with a flow rate of 0.3 mL/min. The obtained fractions were pooled, dialyzed and freeze dried to obtain purified polysaccharides. The products were subjected to the subsequent analyses.

2.4. Analysis of physico-chemical characteristics of polysaccharide

Total sugar content was determined as anhydroglucose by the modified phenol–sulfuric acid method as D-glucose equivalents (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acids were analyzed colorimetrically by the sulfuric acid–carbazole analysis using galacturonic acid as standard (Dische, 1947). Proteins were estimated by the Bradford assay, (Bradford, 1976) using bovine serum albumin as the standard. Fehling's reagent reaction, iodine–potassium iodide reaction and Ferric chloride reaction were used to analyze the polysaccharide.

2.5. High performance gel permeation chromatography (hpgpc) analysis

The homogeneity and the molecular weight of CPP-1 and CPP-2 were determined by high performance gel permeation chromatography (HPGPC), (Wei & Fang, 1989) which was performed on a Shimadzu LC-10ATvp HPLC system fitted with TSK-GEL G3000PWxl 7.8 cm (i.d.) × 30.0 cm gel filtration columns and a Shimadzu RID-10A detector set at 40 °C. A Millennium32 version Workstation was used for the calculation of average molecular weights. A sample solution (20 μl of 0.3% polysaccharide) was injected in each run, with 0.7% Na_2SO_4 as the mobile phase at 0.5 mL/min, with 1.6 MPa. Standard dextrans (T-130, T-80, T-50, T-25 and T-10) were passed through the calibrated column, and then the elution volumes were plotted against the logarithm of their respective molecular weights. The elution volume of the purified polysaccharide was plotted in the same graph, and the molecular weight was determined.

2.6. Monosaccharide analysis

Monosaccharide analysis was investigated according to the previous methods with some modifications (Jones & Albersheim, 1972; Oades, 1967), the polysaccharide CPP-1 and CPP-2 (20 mg) were hydrolyzed separately with 2 mol/L CF_3COOH (1 mL) for 3 h at 120 °C. The acid in excess was completely removed by distilled water. Then the hydrolyzed products were reduced with $NaBH_4$ (50 mg), followed by acidification with acetic acid. It was then co-distilled with methanol to remove the excess boric acid; the reduced sugars (alditols) were acetylated with 1:1 pyridine–acetic anhydride in water bath for 1 h at 90 °C to give the alditol acetates. The alditol acetates of authentic standards (D-glucose, L-rhamnose, D-xylose, D-galactose, D-ribose, D-mannose, L-arabinose, L-fucose, D-glucuronic acid and L-galacturonic acid) with myo-inositol as the internal standards were prepared and subjected to GC analysis separately as mentioned above. Gas chromatography (GC) of the alditol acetate derivatives of monosaccharides was done on an Varian 3400 GC (Hewlett-Packard Component, USA) with a capillary column SP-2320 (30 m × 0.32 mm i.d.), a GC (HP3365) fitted with a flameionization detector. The chromatographic conditions were used: high-purity nitrogen was used as the carrier gas at a flow

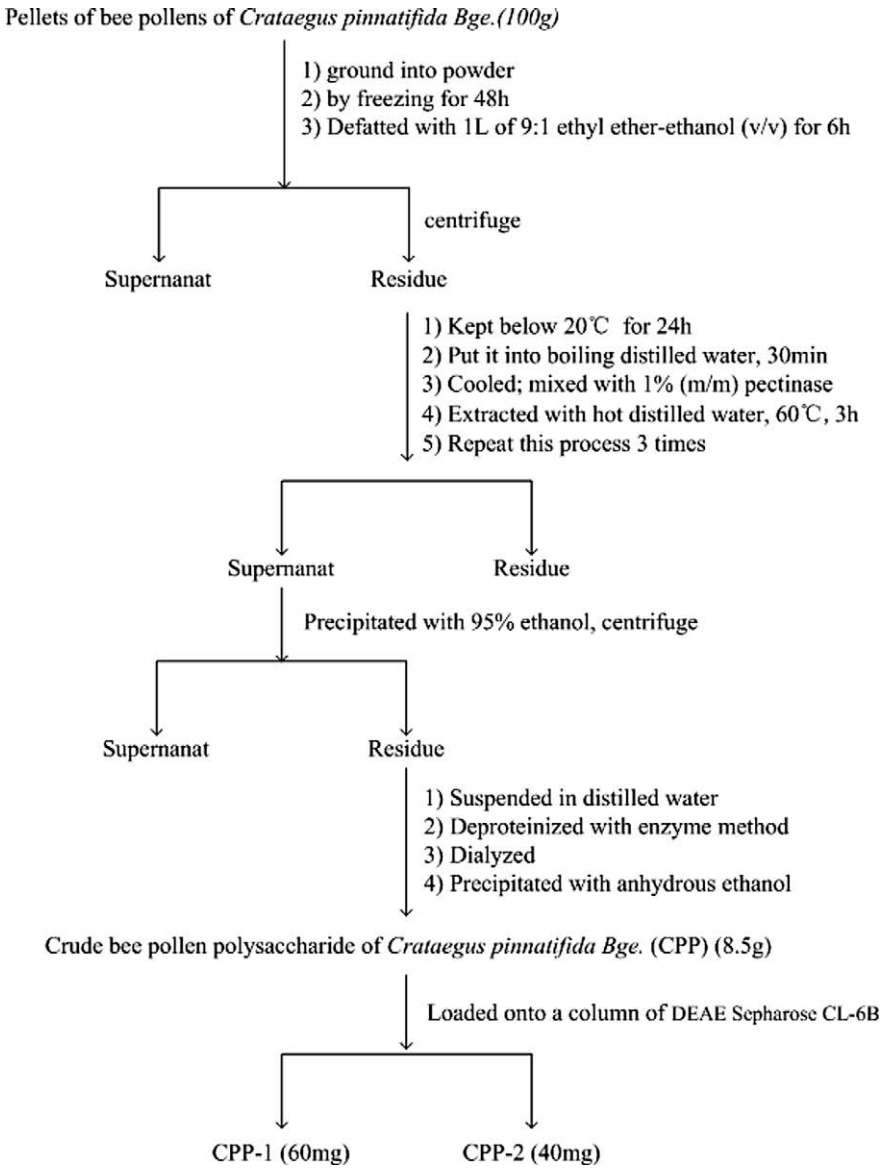

Fig. 1. Summarized extraction scheme of CPP from bee pollen of *Crataegus pinnatifida Bge.*

rate of 8 cm^3/min. The temperature of the injector and detector was 280 °C. An initial column temperature held at 130 °C for 2 min, then programmed at a rate of 10 °C/min to 250 °C and kept at 250 °C for 20 min.

2.7. Methylation analysis

The CPP-1 (20 mg) was methylated thrice according to the previous method (Hakomori, 1964). Complete methylation was conformed by the disappearance of the OH band (3200–3700 cm^{-1}) in the IR spectrum. The methylated products were hydrolyzed, reduced and acetylated (Sweet, Shapiro, & Albersheim, 1975). The partially methylated alditol acetates were analyzed by gas chromatography–mass spectrometry (GC–MS). GC–MS was done on a HP5890 (II) instrument (Hewlett-Packard Component, USA) with an HPS quartz capillary column (25 m × 0.22 mm × 0.2 nm), and at temperatures programmed from 120 to 140 °C at 1 °C/min.

2.8. FT-IR spectroscopy

Infrared spectra of polysaccharides were recorded on a Thermo Electron Nexus 8700 FT-IR spectrophotometer (USA). Samples were dried at 35–44 °C in vacuum over P_2O_5 for 48 h prior to making pellet with KBr powder.

2.9. Assay of immunobiological activity

2.9.1. Animals

Specific pathogen free (SPF) BABL/c mice (18.0–20.0 g, 6–8 weeks old, female) were purchased from the Animal Research Center, Center for The Academy of Military Medical Sciences, China. They were randomized in polycarbonate cages containing sawdust bedding (five in each cage). They received standard mouse chow and water ad libitum. The room conditions were maintained at 22 ± 2 °C with relative humidity of 50 ± 5% and 12 h light/dark cycle at least 9 days before experiment.

2.9.2. Phagocytosis of macrophage assay

A complete culture medium having culture medium RPMI 1640 supplemented with HEPES buffer 10 μmol/L, 2 mmol/L L-glutamine, 100 μg/mL penicillin, 100 μg/mL streptomycin, 5×10^{-5} mol/L 2-mercaptoethanol and heat-inactivated fetal calf serum was used for phagocytosis of macrophage assay. Animals were randomly divided into various groups with five mice each. The polysaccharides

were dissolved in saline and administrated intraperitoneally to mice of experimental groups at different doses of 10, 50, 100 mg/kg body weight. The drug of 0.5 mL dose was given daily one time for 9 days. Control animals were given same volume of sterile saline alone. Macrophages were prepared from BALB/c mice according to the previous procedure (Kim, Choi, Lee, & Park, 2004). Chicken red blood cells (CRBC) were used to assess the phagocytosis of macrophage. Briefly, mice were sacrificed by cervical dislocation 1 h after intraperitoneally injected 0.5 mL 1% CRBC, then injected 2.5 mL Hank's. Activated Macrophages were obtained by lavage of the cavity into microscope slide. After centrifugation at 1500 rpm/min for 10 min, the supernatant was removed and the free CRBC were lysed by sterile 0.16 mmol/L NH_4Cl lysing buffer. Macrophages were dyed with Giemsa-PBS buffer within 3.0 min right after the microscope slide was air-dried. Microscope slide were washed by PBS and counted with microscope. The phagocytosis index and phagocytosis rate were measured by counting the number of phagocytosed CRBC per 200 macrophage cells. Each experiment was performed in quintuplicate.

2.9.3. Assay for NK cells cytotoxicity

The BALB/c mice were sacrificed by cervical dislocation and spleens were collected under aseptic conditions in RPMI-1640. Spleen cells of mice were prepared by gently mincing and grinding the spleen fragment in RPMI-1640 medium on a fine steel mesh, and centrifuged at 1500 rpm/min at 4 °C for 10 min, and then removed the supernatant. The residue dissolved in 10 mL Tris–NH_4Cl for breaking red blood cells and kept it at room temperature for 5–10 min with shaking and centrifuged at 1500 rpm/min at 4 °C for 10 min again. The supernatant was gently removed and the precipitate was transferred to a sterile container and washed with RPMI-1640. At last, the cells were resuspended in 5 mL RPMI-1640 medium, and cells viability exceeded 95%. NK cell cytotoxicity was assayed as previous described with a minor modification (Sarangi, Ghosh, Bhutia, Mallick, & Maiti, 2006). Briefly, an aliquot of 100 μL of splenocytes named effector cells at 5×10^6 cells/mL was seeded into each well of a 96-well flat-bottom microtiter plate, thereafter Con A (positive control group, final concentration 5.0 μg/mL), the medium (negative control group), the polysaccharides (10, 20, 30, 40, 50, 100 μg/mL, final concentration) were added giving a final volume of 200 μL, and then incubated at 37 °C in a humidified 5% CO_2 incubator. The target cells of K562 were resuspended in the RPMI-1640 medium with the final concentration of 5×10^5 cells/mL. One hundred microliters of K562 target cells and 100 μL of effector cells were added to 96-well plates to give the effector-to-target cell (E:T) ratio of 10:1. After incubation for 4 h at 37 °C, 5% CO_2 and saturated humidity, MTT was added into each well and centrifuged (1500 rpm/min, 5 min) to remove the untransformed MTT carefully by pipetting. The plate was incubated for another 4 h, followed to each well a total of 100 μL including 10% SDS and 50% DMSO was added to fully dissolve the colored material. The $OD_{(570nm)}$ was measured on an ELISA reader (Model 550, Bio-Rad Instruments). Each experiment was performed in quintuplicate.

The following formula was used to calculate% cytotoxicity:

$$\%\ \text{cytotoxicity} = \frac{OD_E + OD_T - OD_{(E+T)}}{OD_T} \times 100\%$$

Note: OD_E – the effector cell wells $OD_{(570nm)}$; OD_T – the target cell wells $OD_{(570nm)}$; $OD_{(E+T)}$ – the effector-to-target cell wells $OD_{(570\ nm)}$.

2.9.4. Assay for spleen lymphocyte proliferation

Cell proliferation was assessed by using 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay (Mosmann, 1983). Splenocytes from the mice were prepared as described in previous studies. Briefly, cells of different splenocyte populations in 96-well plates (5×10^6 cells/well) were exposed to Con A, the medium and the polysaccharides in incubator at 37 °C, 5% CO_2 for 72 h. The MTT solution (1 mg/mL of phosphate buffer saline) was added to 96-well plates and diluted to 5 μg/mL in every well, and then further incubated for 6 h at 37 °C. After aspirating the supernatant from the wells, 200 μL of DMSO was added and shaken for 20 min. The absorbance of each well was then read at 570 nm using an ELISA reader (Model 550, Bio-Rad Instruments). Each experiment was performed in quintuplicate.

2.10. Statistics

The data were expressed as means ± standard errors (SE) and examined for their statistical significance of difference with Student's *t*-test. *P*-values of less than .05 were considered to be statistically significant.

3. Results

3.1. Isolation and purification of polysaccharides

The crude CPP was obtained as a water-soluble light yellow powder from the bee pollen of *C. pinnatifida Bge.* by hot water extraction. Wall-breaking treatments for this kind of bee pollen were carried out by synthesizing three ideal ways. It was demonstrated that wall-breaking rate was more significantly improved using this synthesis method as compare with using only one of them. The total yield of water-soluble polysaccharides was 8.5% by this isolation procedure, much higher than the yield of 1.5% from the pollen of *Brassica napus L.* (Yang, Guo, Zhang, & Wu, 2007). This yield of 8.5% was also higher than the yield of 6.92% from pollen of timothy grass (*Phleum pratense L.*) extracted by 0.1 mol/L NH_4HCO_3 (Brecker et al., 2005). Therefore, the adopted conditions of the isolation procedure here were helpful to obtain water-soluble polysaccharides from the bee pollen of *C. pinnatifida Bge.* with an ideal yield before further optimizing. The CPP was separated and purified by gel filtration in DEAE–Sepharose CL-6B column. Two fractions of CPP-1 and CPP-2 were eluted as shown in Fig. 2. CPP-1 and CPP-2 peaks appeared between 7–14 and 71–78 tubes, respectively. CPP-1 is white powder and CPP-2 is light yellow powder, soluble in hot water with characteristic absorption of polysaccharide at 190 nm, and no absorption at 280 and

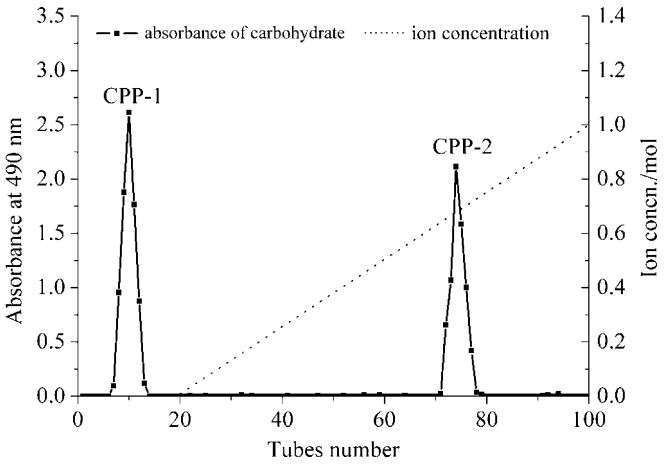

Fig. 2. DEAE–Sepharose CL-6B elution profile of bee pollen polysaccharide of *Crataegus pinnatifida Bge.* The column was eluted with Tris–HCl buffer (25 mmol/L, pH = 8.0), followed by 0.02–1 mol/L linear gradient of NaCl Tris–HCl buffer (25 mmol/L, pH = 8.0) at a flow rate of 1 ml/min.

260 nm for protein and nucleic acid. Both of CPP-1 and CPP-2 were further chromatographed on the Sephadex G-100 column (gel-permeation chromatography). The single and symmetrical peaks were shown in Fig. 3.

3.2. Analysis of physico-chemical characteristics of polysaccharides

The polysaccharide contents CPP, CPP-1 and CPP-2 were found to be 8.31%, 26.07% and 26.59%, respectively, as determined by the phenol–sulfuric acid method. Except CPP, they had a negative response in the Bradford test, indicating the absence of protein. The positive results of Sulfuric acid–carbazole reaction suggest that uronic acid were present in CPP, CPP-1 and CPP-2. The results of Fehling' test, ironic chloridate reaction and iodine reaction are shown in Table 1.

3.3. Molecular mass

High performance size-exclusion chromatography of the CPP-1 and CPP-2 on Shimadzu LC-10ATvp HPLC system suggests that the polymers are homogeneous. The results were consistent with the Sephadex G-100 column described. Based on calibration with standard dextrans, the apparent molecular weight of the polysaccharides would be 3.7×10^5 Da and 7.8×10^4 Da.

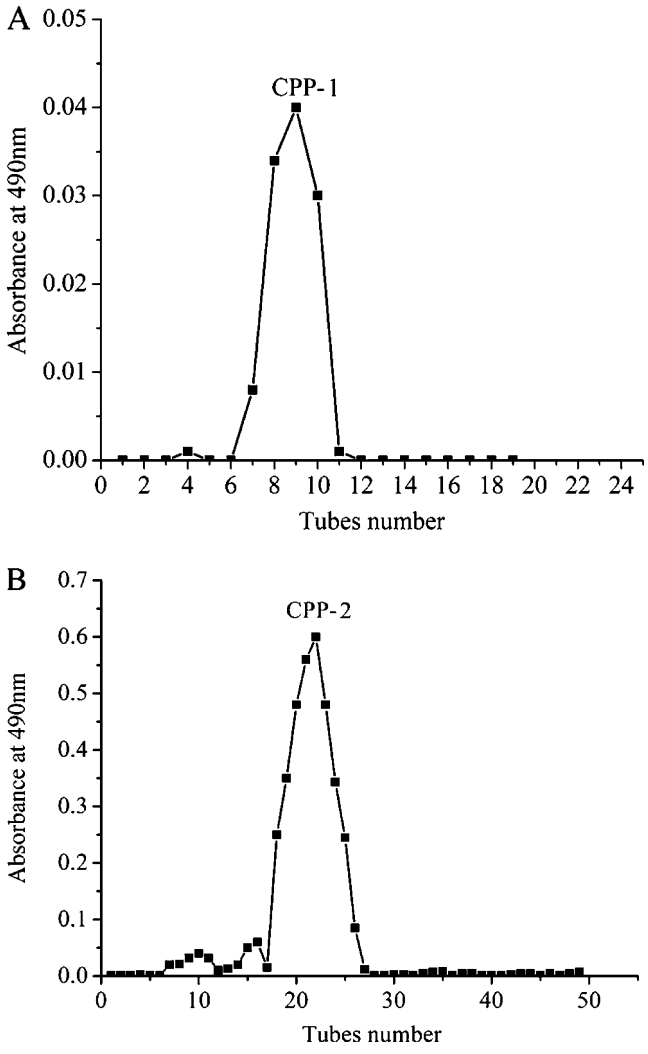

Fig. 3. (A) CPP-1 and (B) CPP-2 profile on Sephadex G-100. The column was eluted with 0.1 mol/mL NaCl at a flow rate of 0.3 ml/min.

Table 1
Physico-chemical characteristics of the polysaccharides from bee pollen of *Crataegus pinnatifida Bge*.

	CPP	CPP-1	CPP-2
Fehling's reagent reaction	–[a]	–	–
Ferric chloride reaction	–	–	–
Sulfuric acid–carbazole reaction	+[b]	+	+
Iodine reaction	–	–	–
Coomassie brilliant blue reaction	+	–	–

[a] Undetectable.
[b] Detectable.

3.4. Monosaccharide analysis

Usually, GC analysis could give the accurate content of sugars in the polysaccharides. On hydrolysis by 2 mol/L CF_3COOH, two kinds of polysaccharide were detected by GC analysis. The CPP-1 was composed of L-rhamnose, L-fucose, D-arabinose, D-xylose, D-mannose, D-galactose and D-glucose, the relative proportions were estimated by GC as 0.67:0.44:10.16:0.66:1.03:5.51:9.94, respectively. While CPP-2 composition was found by GC to consists of five kinds of monosaccharide: L-rhamnose, D-arabinose, D-mannose, D-galactose and D-glucose, whose molar ratios were 2.12:9.82:3.60:0.89:5.56, respectively, and also with small amounts of uronic acid. Both of CPP-1 and CPP-2 were rich in D-arabinose and D-glucose, and also contained D-mannose. Generally, there is the close relationship between D-mannose and immune activity. Hence the main study was focused on immune stimulation.

3.5. Methylation analysis of polysaccharides

The CPP-1 and CPP-2 were individually methylated and hydrolyzed, and the products were then converted into alditol acetates for GC–MS analysis. The combination of the fragmentation pattern and retention time of the CPP-1 and CPP-2 suggested that the reduced polysaccharide were made of 1,5-linked D-arabinoside residues. Methylation results are given in Table 2, CPP-1 was concluded from Table 2 that: (a) it was mainly composed of 1,5-L-Ara*f*, T-, 1,6-, 1,3,6-, 1,4-, 1,4,6-D-Gal*p*, T-, 1,3-D-Man*p*, T-, 1,4-D-Glc*p*; (b) the mole ratio of T-, 1,3-D-Man*p* was 45.8:54.2, the backbone of mannan consisted of Man*p* (54.2%) residues with 1 → 3 glycosidic linkage. The other Man*p* residues (45.8%) existed as non-reduced ends, which indicated that all T-Man*p* were not linked with mannan, they could locate in galactan branch or directly in the backbone of CPP-1; (c) the mole ratio of T-, 1,6-, 1,3,6-, 1,4-, 1,4,6-D-Gal*p* was 35.5:32.8:26.2:2.0:3.5, Gal*p* residue (62.5%) was mainly linked by 1 → 6 glycosidic linkage. Branched chains linked with the oxygen atom at the third site of Gal*p* residue (26.2%). In addition, some Gal*p* residues (2.0%) were linked with 1 → 4 glycosidic linkage, the other Gal*p* residues (35.5%) existed as non-reduced ends; (d) Ara*f* was the main glycosyl unit of CPP-1. There were about 89.6% Ara*f* residues linked with 1 → 5 glycosidic linkage. Oxygen substitution at the third site of Ara*f* (9.6%) would take place and 0.9% Ara*f* residues existed as non-reduced ends. Meanwhile, as summarized in Table 3, CPP-2 was mainly composed of 1,5-L-Ara*f*, T-L-Rha*p*, T-, 1,3-D-Man*p*, T-, 1,4-D-Glc*p*. The mole ratio of T-, 1,3-D-Man*p* was 35.2:64.8, the backbone of mannan consisted of Man*p* (64.8%) residues with 1 → 3 glycosidic linkage. The other Man*p* residues (35.2%) existed as non-reduced ends. Ara*f* was also the main glycosyl unit of CPP-2. There were about 92.8% Ara*f* residues linked with 1 → 5 glycosidic linkage. Oxygen substitution at the third site of Ara*f* (6.0%) would take place and 1.2% Ara*f* residues existed as non-reduced ends. Moreover, there were few amounts of D-Glc*p* in CPP-1 and D-Glc*p*, L-Rha*p* in CPP-2 (Inngjerdingen et al., 2005; Li, Nakagawa, Nevins, & Sakurai, 2006).

Table 2
Methylation analysis of CPP-1 and CPP-2.

Monosaccharide residues	Glycosyl linkage	Methylated sugar	Mol.%[a]		Mol.%[b]		Retension time (min)	
			CPP-1	CPP-2	CPP-1	CPP-2	CPP-1	CPP-2
D-Man*p*	T-	2,3,4,6-Me_4-Man*p*	45.8	35.2			20.22	19.85
	1,3-	2,4,6-Me_3-Man*p*	54.2	64.8			23.24	22.01
	Total		100.0	100.0	6.49	9.08		
D-Glc*p*	T-	2,3,4,6-Me_4-Glc*p*	65.7	70.5			24.23	24.02
	1,4-	2,3,6-Me_3-Glc*p*	34.3	29.5			17.49	17.33
	Total		100.0	100.0	5.21	3.00		
D-Gal*p*	T-	2,3,4,6-Me_4-Gal*p*	35.5	–			12.31	–
	1,6-	2,3,4-Me_3-Gal*p*	32.8	–			18.25	–
	1,3,6-	2,4-Me_2-Gal*p*	26.2	–			25.02	–
	1,4-	2,3,6-Me_3-Gal*p*	2.0	–			22.05	–
	1,4,6-	2,3-Me_2-Gal*p*	3.5	–			27.55	–
	Total		100.0	–	13.2	–		
L-Rha*p*	T-	2,3,4-Me_3-Rha*p*	–	100.0			–	11.39
	Total		–	100.0	–	4.22		
L-Ara*f*	T-	2,3,5-Me_3-Ara*f*	0.9	1.2			7.02	7.22
	1,5-	2,3-Me_2-Ara*f*	89.5	92.8			10.75	10.45
	1,3,5-	2-Me-Ara*f*	9.6	6.0			15.44	14.95
	Total		100.0	100.0	75.1	83.7		

[a] Relative molar ratio, calculated from the ratio of peak areas.
[b] Mol.% of the parent CPP-1 and CPP-2, calculated from the ratio of peak areas.

3.6. FT-IR spectroscopy

In order to investigate the functional groups of the purified CPP, the spectra were recorded at the absorbance mode from 4000 to 400 cm^{-1} (mid infrared region). Typical IR spectra for the two fractions are presented in Fig. 4. At least triplicate spectra were recorded for each sample. There are two types of end carbon-glucoside bonds: α- and β-styles, which can be judged by IR. In IR spectra, the C–H bond in α-style has an absorption peak nearby 844 cm^{-1}, while that of the C–H bond is in β-style nearby 891 cm^{-1} (Barker, Bourne, Stacey, & Whiffen, 1954). The FT-IR spectrum of CPP-1 and CPP-2 showed a strong band between 950 and 1160 cm^{-1} attributed to the stretching vibrations of pyranose ring. A characteristic absorption at 895 cm^{-1} was also observed, indicating the β-configuration of the sugar units. There was no absorption at 850 cm^{-1} for the α-configuration. While in the anomeric region (905–876 cm^{-1}), both CPP-1 and CPP-2 exhibited the obvious characteristic absorption, suggesting the existence of β-D-glucopyranosyl (Barker et al., 1954).

Table 3
Effects of the polysaccharides from bee pollen of *Crataegus pinnatifida Bge.* on macrophage phagocytosis in BABL/c mice ($n = 10$ mice per study group).[a]

Study group	Concentration (mg/kg)	Phagocytic index	Phagocytic rate
Control	–	0.22 ± 0.016	9.42 ± 1.28
CPP-1	10	0.43 ± 0.029[d]	11.83 ± 1.97[b]
	50	0.48 ± 0.026[d]	14.17 ± 2.07[c]
	100	0.54 ± 0.031[d]	16.38 ± 1.49[c]
CPP-2	10	0.42 ± 0.027[d]	11.92 ± 2.13[b]
	50	0.52 ± 0.018[d]	16.75 ± 1.08[d]
	100	0.58 ± 0.043[d]	19.50 ± 2.26[d]

Purified polysaccharide fraction 1 was named as CPP-1.
Purified polysaccharide fraction 2 was named as CPP-1.
[a] Results are represented as means ± SD based on three independent experiments.
[b] $P < .05$, significantly different from the control.
[c] $P < .01$, significantly different from the control.

A characteristic absorption band appeared at 1650 cm^{-1} and was assigned to the stretching vibration of the carboxyl group (C—O) of the CPP-1 and CPP-2, meanwhile another absorption band between 2930 and 2932 cm^{-1} was intensified and attributed to the stretching vibration of the methylene group (C–H). In addition, a continuous absorption beginning at approximately the region of 3400 cm^{-1} is characteristic of a carbohydrate ring (Jong Min Lim et al., 2005; Yanfei Peng, Zeng, & Xu, 2006).

3.7. Immunobiological activity

3.7.1. Effect of macrophage assay

As a first step towards understanding the immunomodulatory activity of the polysaccharide CPP, we investigated the effect on phagocytic rates and phagocytic indexes of macrophage. As summarized in Table 3, compared with those in the control group, the phagocytic rates and indexes of the CPP-1 and CPP-2 were significantly higher in the groups treated with10, 50 mg/kg d (for phagocytic rates and phagocytic indexes: $P < .05$) and 100 mg/kg d (for phagocytic rates and phagocytic indexes: $P < .01$).

3.7.2. Effect of NK cells cytotoxicity

NK cells make up 10–15% of peripheral blood lymphocytes in humans (Campbell & Colonna, 2001) and represent the first line of defense against many pathogens, especially viruses. Upon activation, NK cells begin to proliferate and secrete cytokines as a means of communication with other components of the immune system, in particular T cells. So we investigated the effect of NK cells cytotoxicity. As shown in Fig. 5, CPP-2 had higher effect on NK cell for its cytotoxicity effect with the concentration of 50 μg/mL ($P < .01$). Compared with the control group, significant enhancements in CPP-1 (50 μg/mL, $P < .01$) was observed. We can draw a conclusion that CPP-1 and CPP-2 at suitable dose can enhance effect of NK cells cytotoxicity. Generally, polysaccharide inducing NK cells activation increases with increasing concentration of polysaccharide. While interestingly, CPP-1 and CPP-2 inducing NK cell activation decreased with increasing concentration

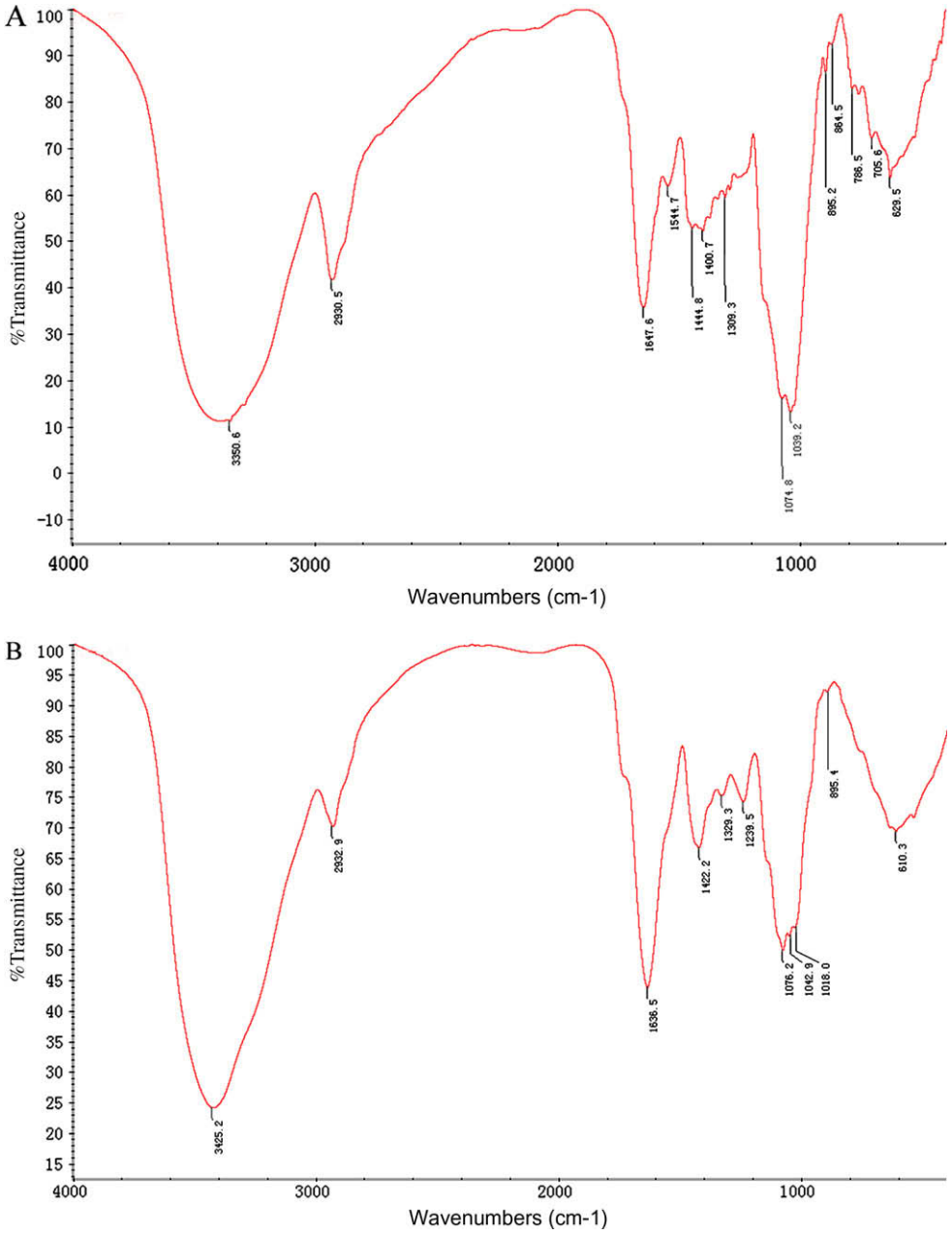

Fig. 4. (A) FT-IR spectrum of CPP-1; (B) FT-IR spectrum of CPP-2.

when the concentration was greater than the optimal concentration. It was observed that the high concentrations of CPP-1 and CPP-2 resulted in the formation of over-saturated solution. The solution enhanced the absorption at 570 nm and decreased the NK cell activation finally. Meanwhile, NK-cell mediated cytotoxicity was modulated by various cytokines, including IL-1, IL-2, IL-12, and interferon (Chichen Michael Chien et al., 2004), so further study of the effect of the high concentration of CPP-1 and CPP-2 on these cytokines is in order ongoing to immunotherapeutic strategies that will enhance the anti-tumor activity with NK cells.

3.7.3. Splenocyte proliferation

Lymphocytes are the key effector cells of mammalian immune system and our studies show that the different subpopulations of lymphocytes are activated by purified CPP at varying levels. The purified CPP-1 and CPP-2 were considerably able to proliferate splenocytes as shown in Fig. 6. At 50 μg/mL of CPP-2, splenocyte proliferation index was maximum as compared to CPP-1. CPP-2 can be considered as efficient splenocyte proliferators.

4. Discussion

Since 20 years ago, interest on the bee pollen has been increasing and there are numerous researches on their nutrition contents and extracts. In fact, bee pollen was known as its health and medical functions, including anti-tumor function and immunostimulation (Hackett, 2003). However, many investigations were achieved directly with bee pollen or bee pollen's extractions, but bee pollen's chemical components of immunostimulation remain not very clear. In this study, we have succeeded in isolating the polysaccharide CPP from bee pollen of *C. pinnatifida Bge*. Two water-soluble polysaccharide fractions, termed CPP-1 and CPP-2, were purified and the molecular weights were approximately 3.7×10^5 Da and 7.8×10^4 Da, respectively. FT-IR and quantitative carbohydrate

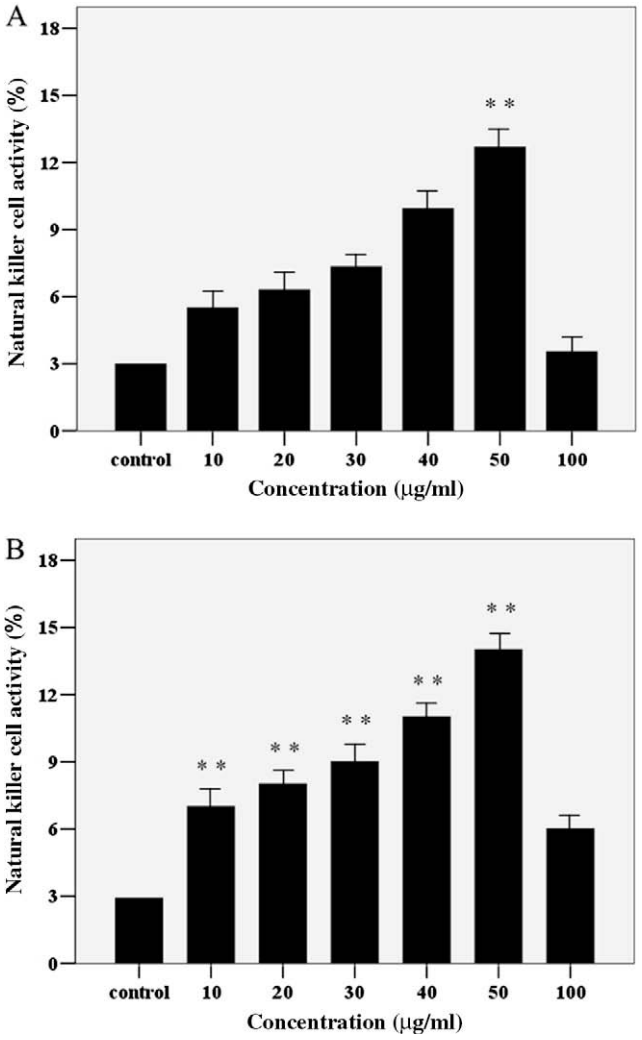

Fig. 5. Effect of the polysaccharides CPP-1 (A) and CPP-2 (B) at various concentrations, on rat natural killer cell *in vitro* and control group received PBS. Natural killer cell activity was expressed as the absorbance at 570 nm using an ELISA reader. The % cytotoxicities were represented as means ± SD of six different observations and compared against PBS control by using Student's *t* test. Data reported (**) $P < .01$ were considered significant.

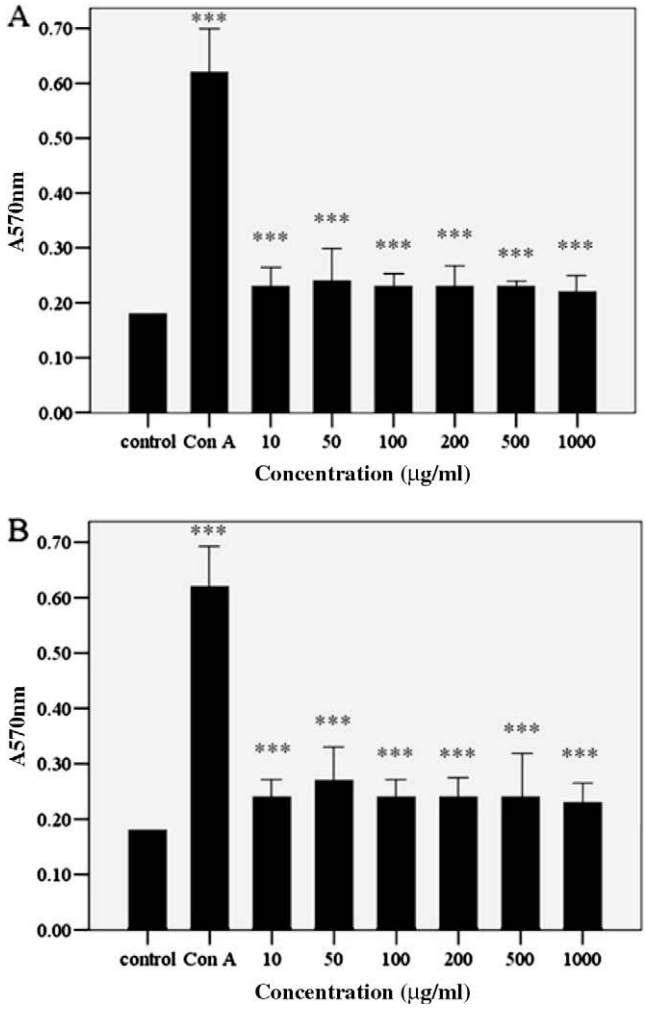

Fig. 6. Effect of the polysaccharides CPP-1 (A) and CPP-2 (B) at various concentrations, on rat spleen cell proliferation *in vitro* and control group received PBS. Proliferation activity was expressed as the absorbance at 570 nm using an ELISA reader. The results were represented as means ± SD of six different observations and compared against PBS control by using Student's *t* test. Data reported (***) $P < .001$ were considered significant.

composition analysis indicated that CPP-1 and CPP-2 were β-type heteropolysaccharides with pyran group, among which CPP-1 was composed of L-rhamnose, L-fucose, D-arabinose, D-xylose, D-mannose, D-galactose and D-glucose at the molar ratio of 0.67:0.44:10.16:0.66:1.03:5.51:9.94, respectively; CPP-2 consisted of L-rhamnose, D-arabinose, D-mannose, D-galactose, D-glucose, whose molar ratio was 2.12:9.82:3.60:0.89:5.56, respectively. Methylation results of polysaccharides shows that both of CPP-1 and CPP-2 were made of 1,5-linked D-arabinoside residues. CPP-1 was mainly composed of 1,5-L-Ara*f*, T-, 1,6-, 1,3,6-, 1,4-, 1,4,6-D-Gal*p*, T-, 1,3-D-Man*p*, T-, 1,4-D-Glc*p*; CPP-2 was primarily composed of 1,5-L-Ara*f*, T-L-Rha*p*, T-, 1,3-D-Man*p*, T-, 1,4-D-Glc*p*. These provide the basic information on this new water-soluble polysaccharide for future their fine structure and determine the structure activity relationships (Deters, Lengsfeld, & Hensel, 2005).

In this study, we also discussed the biological activity of CPP for the first time. The immune system is the human's ultimate defense against infectious diseases, tumor and cancer growth. A healthy immune system contains elements that are in balance with one another and if this balance is broken, our immune system will be unable to protect the body against harmful agents or processes. For above reasons, we investigated three aspects of immunomodulatory activity. Purified CPP-1 and CPP-2 showed higher activation on phagocytic rates and phagocytic indexes of macrophage, and the results indicated CPP-1 and CPP-2 were dose-dependent polysaccharides. Additionally, Tumor cell elimination is known to be mediated in part by the cytotoxic activity of natural killer (NK) cells. We therefore measured the enhanced cytotoxic activity of NK cells as compared with normal mice NK cells activity. Surprisingly, these two fractions were dose-independent and the optimal effect was found at 50 μg/mL ($P < .01$). And also the result of splenocyte proliferation was very ideal, especially for 50 μg/mL ($P < .001$). While discussing the biological activity of CPP, we find CPP-2 possessed higher immunomodulatory activity than CPP-1; and concluded that it is associated with compositions of the each monosaccharide. In the previous studies, many complex polysaccharides from plants, epiphytes and animals have been shown to influence the phagocytosis of macrophage, NK cells cytotoxicity and lymphocyte proliferation (Bao, Liu, Fang, & Li, 2001; Sun et al., 2008), although the assays they used were different. We could not compare the activity of natural polysaccharides on phagocytosis of macrophage, NK cells cytotoxicity and lymphocyte proliferation due to the lack of standardized assays.

Although the exact underlying mechanism of CPP is yet unknown, based on the results presented above, we can conclude that CPP has immunomodulatory activity by proliferating splenocyte, and also macrophages and NK cells involved in nonspecific immunity were primarily activated. Further investigations on the relationship between immunomodulatory activity and characteristic structure are presently underway in our laboratory.

5. Conclusions

In this study, crude polysaccharide CPP was successfully isolated from bee pollen of *C. pinnatifida Bge.* by synthesizing three ideal wall-breaking effects including the mechanic way, the temperature difference way and the enzyme way. Two fractions CPP-1 and CPP-2 were purified by DEAE–Sepharose CL-6B column chromatography. The GC–MS analysis suggested that CPP-1 and CPP-2 were made of 1,5-linked D-arabinoside residues. The complete structure of CPP-1 and CPP-2 were not obtained, but some important structural features were established. Furthermore, the polysaccharides significantly enhanced phagocytic rates and phagocytic indexes of macrophage. Considerable effects were also observed on splenocytes proliferation and the NK cells cytotoxicity, especially with CPP-2. Above all, these two fractions possessed immunostimulatory activity and can be utilized as immunostimulants for food and pharmaceutical industries.

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