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In vivo macrophage activation and physicochemical property of the different polysaccharide fractions purified from *Angelica sinensis*

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Abstract

The water-soluble crude polysaccharide (CAP) was extracted from *Angelica sinensis* and was further fractionated by Sephacryl S-400 column chromatography, giving three polysaccharide fractions termed APF1, APF2 and APF3. The obtained results showed that the uronic acid linkages in the polysaccharides were more stable and resistant to thermal hydrolysis than neutral sugar linkages. APF2 exhibited higher thermal stability, followed by APF1, APF3 and CAP in decreasing order. APF1 contained the highest carbohydrate content (80.5%) and the lowest uronic acid content (28.4%) in comparison with APF2 (77.7% and 39.2%) and APF3 (70.1% and 34.6%). Further bioactive investigation showed that intraperitoneal administration of the polysaccharide fractions significantly induced peritoneal macrophage to release nitric oxide (NO), reactive oxygen species (ROS) and enhanced cellular lysosomal enzyme activity ($p < 0.05$) and the highest activity was achieved by APF2. Taken together, APF2 had the high thermal stability and immunostimulatory activity and so should be explored as a novel potential immunostimulants.

Keywords: *Angelica* polysaccharides; Isolation and purification; Physicochemical property; Peritoneal macrophage; Immunostimulatory activity

1. Introduction

Angelica roots in Asia, such as *Angelica sinensis* (Oliv.) Diels (Chinese Danggui), *Angelica acutiloba* (Japanese Danggui) and *Angelica gigas* (Korean Danggui), are the important traditional medicine belonging to the Umbelliferae family (Lao et al., 2004; Lu et al., 2004). They have been intensively studied in their biological activities (Kim, Ahn et al., 2005; Kim, Bang, Choi, Han, & Kim, 2005) and were widely used as health foods and medical products for women's care in Asia (Tieraona, 2005), and were marketed in Europe and America as a dietary supplement (Deng et al., 2006; Zhao et al., 2003). In recent years, the *Angelica* polysaccharides rich in the roots of Chinese Danggui have drawn the attention of researchers and consumers due to their nutritional and health protective value in gastrointestinal protection (Cho et al., 2000; Ye, Koo, Li, Matsui, & Cho, 2001, 2001c), anti-ulcer (Ye, So, Liu, Shin, & Cho, 2003), antitumor (Shang et al., 2003), anti-hepatic injury (Ye, Liu et al., 2001), radioprotective action (Mei, Tao, Zhang, Duan, & Chen, 1988) and immunostimulating activity (Sun, Tang, Gu, & Li, 2005). In recent years, polysaccharides isolated from various traditional medicinal plants have been shown to profoundly affect the immune system both *in vivo* and *in vitro*, and therefore have the potential as immunomodulator with a wide application. Furthermore, polysaccharides represent a class of high-value polymers and also have many industrial applications, such as efficient prescription, gelling, thickening, stabilizing agents and emulsifiers.

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It should be emphasized that the potential use of polysaccharide products often involves some type of heat processing, such as hot-water isolation, heating concentration, drying dehydration and high temperature sterilization. Such processing may affect their original structure and cause an irreversible modification, which may induce the changes in their proposed physiological and pharmacological properties (Bonvehi & Coll, 1993; Diisterhoft, Posthumus, & Voragen, 1992; Jung, Valdez, Hatfield, & Blanchette, 1992). Thus it is clear that the study on effect of heat treatment on polysaccharide stability is favorable for predicting the processing problem and the rational application. Moreover, an understanding of the basic physicochemical properties of polysaccharides such as carbohydrate content and molecular size, could contribute to our understanding of their practical applications (Chang, Wang, Feng, & Liu, 2006; Cohen & Yang, 1995). Unexpectedly, at present very little is known about the effects of heat treatment on the change in carbohydrate content of *Angelica* polysaccharides. Therefore, the investigation on basic properties of *Angelica* polysaccharides was particularly necessary to better find their functional properties for the wide application in food and pharmaceutical industries.

The present experiments were set up to isolate and purify the different polysaccharide fractions from the roots of *A. sinensis* and further investigate their basic physicochemical characterization. In addition, the immunostimulatory activities of these polysaccharide fractions were also investigated in murine peritoneal macrophages *in vivo* in order to most effectively acquired high-performance polysaccharide products and exploit the applied potential of *Angelica* polysaccharides.

2. Materials and methods

2.1. Materials and chemicals

The roots of *A. sinensis* were purchased from Minxian County, Gansu Province, China in 2002 and identified according to the identification standard of Pharmacopeia of the People's Republic of China. A GPC column of Sephacryl S-400 HR (0.5 cm i.d. × 100 cm) was obtained from Pharmacia Biotech Co. (Shanghai, China). Glucose, glucuronic acid and 2′,7′-dichlorofluorescin diacetate (DCF-DA) were obtained from Sigma Co. (USA). Coomassie brilliant blue G250 and bovine serum albumin (BSA) were purchased from Huamei Biochemistry Co. (Shanghai, China). *m*-Hydroxydiphenyl was from Sigma Co. (USA). MTT (Thiazolyl blue), dimethyl sulfoxide (DMSO), RPMI1640, phosphate buffer saline (PBS) and fetal bovine serum (FBS) were the product of Gibco BRL (Gaithersburg, MD, USA). All other chemicals were of the analytical grade available.

2.2. Preparation of the crude extraction

Angelica polysaccharide extracts were isolated by hot-water extraction and ethanol precipitation. The dried roots of *A. sinensis* (550 g) were defatted with 95% alcohol and then extracted with distilled water (g/ml = 1:10) for 3 h. After each three-hour period of water extracting, the water extracts were collected and the residues were extracted again for 3 cycles. The combined extracts were pooled, concentrated to 30% of the original volume under a reduced pressure and then centrifuged at 3000 rpm for 15 min. The supernatant was collected and 3 volume of 95% alcohol was added slowly by stirring to precipitate the polysaccharides, and then kept at 4 °C overnight and finally the polysaccharide pellets were obtained by centrifugation at 4000 rpm for 15 min. The polysaccharide pellets were completely dissolved in appropriate volume of distilled water and intensively dialyzed for 2 days against distilled water (cut-off M_w 8000 Da). The retentate portion was concentrated, deproteinated by freeze–thaw process for repeating seven times and centrifuged to remove insoluble material. Finally the supernatant was lyophilized in the freeze–dry apparatus (BenchTOP, Virtis Co., USA) to give crude *Angelica* polysaccharides (CAP) with a nearly colorless fluffy shape.

2.3. Sequential fractionation of polysaccharides

One gram of the CAP was dissolved in 0.1 M NaCl and filtered through a filter paper (0.45 μm). Then the solution was applied to Sephacryl S-400 gel filtration column chromatography and eluted with 0.1 M NaCl solution. Each eluted solution (5 ml) was collected and monitored by carbohydrate content based on phenol-sulfuric acid method at 490 nm absorbance. Finally, the eluted solution was concentrated and lyophilized to yield different white *Angelica* polysaccharide fractions (APF) with different molecular size according to the elution profile.

2.4. Measurement of carbohydrate and protein contents

Total carbohydrate contents in CAP as well as purified APF1, APF2 and APF3 samples were determined by phenol-sulfuric acid colorimetric method using glucose as the standard (Dubois, Gilles, Haamilton, Rebers, & Smith, 1956). Total uronic acid contents were measured by *m*-hydroxydiphenyl method using glucuronic acid as the standard (Filisetti-Cozzi & Carpita, 1991) and Fourier transform infrared (FT-IR) spectroscopy was also performed to obtain information of the uronic acid by the program that samples were incorporated into KBr powder and then pressed into a 1 mm pellet for FT-IR measurement in the frequency range of 4000–400 cm^{-1} on a Bruker Equinox55 FT-IR spectrometer. In addition, proteins in the polysaccharides were quantified according to the Lowry method using bovine serum albumin (BSA) as the standard (Lowry, Rosebrough, Farr, & Randall, 1951), combined with the method of UV absorption on a DU-800 spectrophotometer (Beckman, USA) and elementary analysis on a PE2400 instrument (PE, USA).

2.5. Size-exclusion chromatography

The molecular sizes of the polysaccharide fractions were determined by high-performance size-exclusion chromatography (HPSEC) combined with phenol-sulfuric acid method (Alsop & Vlachogiannis, 1982). The polysaccharide solution at a concentration of 1 mg/ml were eluted on a Sephacryl S-400 gel filtration column (5×90 cm) with a flow rate of 0.8 ml/min at room temperature and 0.1 M NaCl was used as the eluant. The eluted fractions were collected and analyzed for total carbohydrates by the phenol-sulfuric method. The average molecular weight (M_w) was estimated by reference to the calibration curve from a set of Dextran T-series standards of known molecular weight (6100, 16,500, 26,290, 40,000, 84,000, 158,000). In addition, the specific optical rotation was measured at 20 °C on a PE-343 digital polarimeter (PE, USA) in water (C: 1.0 mg/ml, H_2O).

2.6. Stability experiment of polysaccharides

Experimental solution (37.5 μg/ml) of CAP and purified samples were prepared by dissolving appropriate polysaccharides sample in deionized water (pH 6.5–7.0). Five milliliters of the solution was added to each capped tube (10 ml) and was incubated in a thermostatic oil bath and heated at 60 °C, 70 °C, 80 °C, 90 °C, 100 °C, 120 °C and 150 °C for 2 h or at 80 °C for different time (0, 2, 4, 6, 8, 10 and 12 h), respectively. The tubes were removed at appropriate time intervals and then centrifuged at 3000 rpm for 15 min. The supernatant was collected, intensively dialyzed and lyophilized to yield a white tested sample for polysaccharide analysis. All measurements were performed in triplicate and the average results are presented in this paper.

2.7. Preparation of peritoneal macrophages and measurement of immunoactivity

BALB/c mice, weighing 17–22 g and about 6–8 weeks old, were used for the present study. Mice were kept in cages and maintained with free access to food and water under a 12 h light/dark cycle at least 6 days before experiments. Animals were randomly divided into various groups with five mice each. The polysaccharides was dissolved in saline and administrated intraperitoneally to mice of experimental groups at different doses of 100, 300 mg/kg body weight, respectively. Control animals were given same volume of sterile saline alone. Macrophages were prepared from BALB/c mice as described previously (Kim, Choi, Lee, & Park, 2004). Briefly, peritoneal macrophages were harvested from the mice, which had been injected intraperitoneally 8 h before sterile peritoneal lavage with 10 ml of Hank's balanced salt solution. The collected cells were seeded and cultured in RPMI1640 containing 10% heat-inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin at a density 2×10^6 cells/well. The cells were allowed to adhere for 3 h to a 96-well culture plate at 37 °C. Then the cultures were washed twice with RPMI1640 to remove nonadherent cells prior to the addition of 1 ml of fresh RPMI1640 containing 10% FBS and the adhered macrophages were cultured for another 24 h.

Nitric oxide (NO) production was determined indirectly by assaying the culture supernatant for accumulated nitrite as previously described (Green et al., 1982). Briefly, 100 μl of isolated supernatants were allowed to react with Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) at room temperature for 10 min. Nitrite products were determined by measuring absorbance at 550 nm versus a $NaNO_2$ standard curve and the results were shown as nmol/10^6 cells.

Reactive oxygen species (ROS) levels were determined by measuring the oxidative conversion of the sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) to fluorescent 2′,7′-dichlorofluorescein (DCF) as described by Wang and Joseph (1999). DCFH-DA readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterases to form non-fluorescent 2′,7′-dichlorofluorescein (DCFH) which is then rapidly oxidized to form highly fluorescent DCF in the presence of ROS, and the fluorescence intensity is proportional to ROS production. Here, at the end of incubation, cells in 96-well plate at a density of 2×10^6 per well were washed two times with PBS and then loaded with 5 μl DCFH-DA (final concentration 10 μm in DMSO) at 37 °C for 35 min in dark. Then the cells were resuspended with PBS. The fluorescence intensity was recorded at 485 nm excitation and 535 nm emission. Relative ROS production was expressed as a percentage of DCF fluorescence of control.

Cellular lysosomal enzyme activity was measured according to the procedure of Suzuki et al. (1990). Macrophage monolayers in 96-well culture plates (2×10^6 cells/well) were solubilized by adding 25 ml of 0.1% Triton X-100 and incubated for 30 min at room temperature. Then 150 μl of 10 mm *p*-nitrophenyl phosphate was added to per well as a substrate for acid phosphatase, followed by the addition of 0.1 M citrate buffer (50 μl, pH 5.0). After the incubation for 1 h at 37 °C, 0.2 M borate buffer (50 μl, pH 9.8) was added to the mixture to stop the reaction. Optical densities were measured at 405 nm using an enzyme-linked immunosorbent assay reader.

2.8. Statistical analysis

The results were reported as the mean and standard deviation. Statistical differences between the treatment and non-treatment groups were assessed by the unpaired Student's *t*-test. A value of $p < .05$ was considered statistically significant.

3. Results and discussion

3.1. General characterization of the polysaccharides

In the present study, the water-soluble crude *Angelica* polysaccharides (CAP) were isolated from the *A. sinensis* through repeated ethanol precipitations. As a result, the extraction yield of the CAP may reach to approximately 10.0% (w/w) of the dried *A. sinensis*. To obtain high-purity and homogeneity polysaccharide products for stable functional property, the CAP were further purified by Sephacryl S-400 gel permeation chromatography into different fractions according to their molecular size (Fig. 1). The results showed that CAP was mainly composed of three sub-fractions, namely APF1, APF2 and APF3. Each fraction was eluted as a single symmetrical peak, which indicated that APF1, APF2 and APF3 were three homogeneous polysaccharide sub-fractions with different molecular sizes and APF2 was the predominately fraction with a percent amount of about 65% (area %). At the same time, APF1, APF2 and APF3 were estimated to have the following average molecular weights (M_w): 1.2×10^5, 5.2×10^4 and 1.6×10^4 Da, respectively, according to the calibration curve using dextran and glucose as the standards by HPSEC combined with phenol-sulfuric acid method. The negative reaction results of APF1, APF2 and APF3 with iodine–potassium iodine reagent indicated that the fractions were non-starch polysaccharides. All the three products were white and easily soluble in water. In addition, the detection results of optical activity showed that there was a great difference in specific rotation (C: 1.0 mg/ml, H_2O) of 66.8 for APF1, 143.6 for APF2 and 111.9 for APF3, respectively (Table 1).

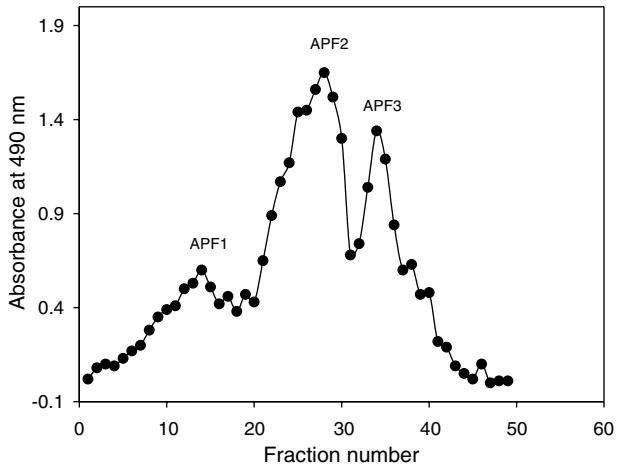

Fig. 1. Elution profile of *Angelica* polysaccharides on Sephacryl S-400 gel filtration column (5 × 90 cm). The crude polysaccharides was dissolved in 0.1 M NaCl solution and applied to the column. The eluted solution was collected and the carbohydrate content of collected fraction was monitored by phenol-sulfuric acid method.

Table 1
General analysis of the polysaccharides from the roots of *Angelica sinensis*

Samples	Total sugar (w/w%)	Uronic acid (w/w%)	Protein (w/w%)	N (%)	$[\alpha]_D^{20}$
CAP	62.8	29.3	2.7	2.30	102.6
APF1	80.5	28.4	–	–	66.8
APF2	77.7	39.2	–	–	143.6
APF3	70.1	34.6	2.4	1.85	111.9

3.2. Carbohydrate and protein contents of polysaccharides

One of the objectives of this study is to obtain higher purity of polysaccharides, hence the total carbohydrate content, total urinic acid content and protein content in CAP, purified APF1, APF2 and APF3 were determined. The linearities of glucose, glucuronic acid and BSA were assessed by preparing five calibration solutions ($n = 5$), respectively. As a consequence, the good linearities were obtained by regression analysis between A (absorbency) and C (standard concentration, μg/ml) and regression equations were as follows: $A = 0.014329C - 0.00195$ ($r = 0.9991$) for glucose at the concentration of 12.5–62.5 μg/ml, $A = 0.015569C - 0.0026$ ($r = 0.9999$) for glucuronic acid at 10.0–60.0 μg/ml and $A = 0.014517C - 0.016166$ ($r = 0.9988$) for BAS at 2.0–15.0 μg/ml.

The total carbohydrate content (indicating polysaccharide content), total uronic acid content and protein content of CAP, APF1, APF2 and APF3 were summarized in Table 1. The results showed that APF1 contained the highest total carbohydrate content (80.5%) and the lowest uronic acid content (28.4%) compared with APF2 (77.7% and 39.2%) and APF3 (70.1% and 34.6%), respectively. All the polysaccharide fractions appeared to be the heterogeneous polysaccharides, which consisted of both neutral sugars and uronic acid. In addition, FT-IR spectroscopy also was used to estimate uronic acid in the APF samples. As shown in Fig. 2, APF2 and APF3 presented intense absorbance peaks at 1150, 1100, and 1020 cm^{-1}, which are characteristic peaks of acidic polysaccharides and a higher characteristic absorbance of carboxy groups (COOH) in 1700 ~ 1730 cm^{-1} suggested that APF2 and APF3 were of the acid-rich polysaccharides (Gnanasambandam & Proctor, 2000; Sun et al., 2005). In comparison to APF2 and APF3, the highest molecular-weight APF1 was a low acidic polysaccharide because of the present of weak characteristic peaks mentioned above and the results were in agreement with the conclusion obtained by the chemical analysis of uronic acid (Table 1).

Furthermore, the protein contents of CAP and purified polysaccharide fractions were measured by the method of Bradford assay and the results were also shown in Table 1. It was evident that no proteins were undetected in APF1 and APF2 and there also were no absorbance peaks at 280 nm on the UV spectra (data not shown), indicating that APF1 and APF2 were not contaminated with proteins. Contrarily, CAP contained the highest amount

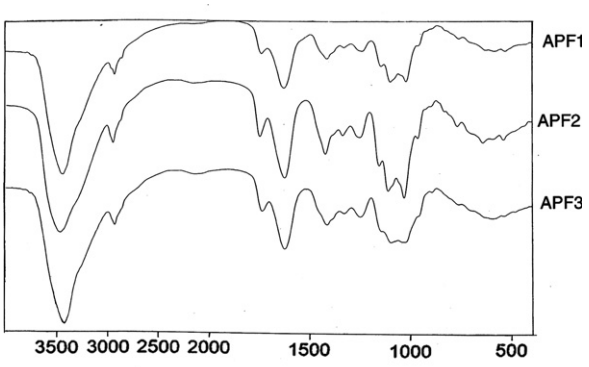

Fig. 2. IR spectra of APF1, APF2 and APF3 at wavelengths of 4000–400 cm^{-1}.

of protein (2.7%, w/w), followed by APF3 with protein (2.4%, w/w). Moreover, measurements by elemental analysis for nitrogen (N) contamination also revealed that APF1 and APF2 were free of proteins and trace proteins were present in CAP and APF3 (Table 1). The fact that uronic acid and protein were mainly present in APF3 suggested that APF3 was a protein-bound polysaccharide and the protein might be bound to the polysaccharide chains via electrostatic force interaction (Roudsari, Nakamura, Smith, & Corredig, 2006). From these results, it could be concluded that APF1 and APF2 were approved as the high-purity polysaccharide fractions.

3.3. Thermal stabilization of the polysaccharides

The thermal treatment experiments were carried out at seven different temperature 60 °C, 70 °C, 80 °C, 90 °C, 100 °C, 120 °C and 150 °C for 2 h to investigate the polysaccharide stability (the data detected at room temperature as a control) and the loss in polysaccharide content (namely total carbohydrate content containing uronic acid) were present in Fig. 3. As shown in Fig. 3, while samples were heated for 2 h at 60–80 °C there was not the significant change ($p > .05$) in polysaccharide loss compared with control samples and unexpectedly, the flocculent precipitate was observed in CAP and APF3 samples containing proteins, indicating that CAP and APF3 possessed lower thermal stability due to heating precipitation of proteins in comparison to APF1 and APF2. As heating temperature up to 90 °C, significant carbohydrate losses ($p < .05$) were firstly observed in all the tested samples and a further increase in the temperature range of 90–150 °C resulted in a dramatic loss from 1.6% to 10.3% for APF1, from 1.2% to 9.7% for APF2, especially for CAP and APF3 with a more high loss percentage of 7.7–16.3% and 3.5–15.8%, respectively. As shown in Fig. 4, the similar changes of uronic acid contents were also observed and interestingly, the same heat treatment temperature gave a lower loss of uronic acid in all the investigated samples (in the range of 4.5–8.3% for CAP, 0.7–4.0% for APF1, 0.4–4.0% for APF2 and 2.5–7.4% for APF3, respectively) than that of

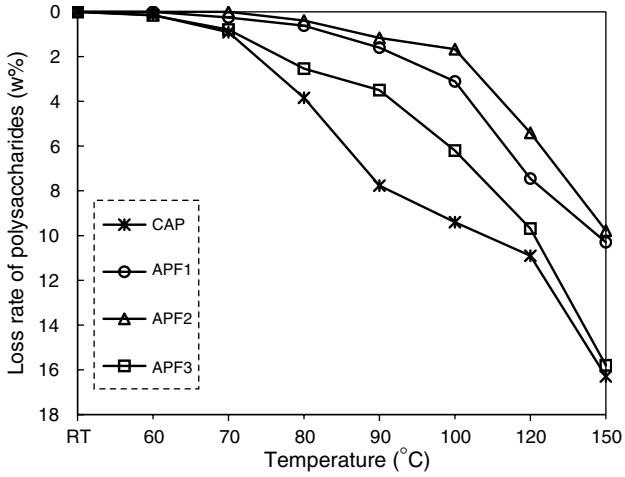

Fig. 3. Effects of treatment temperature on the loss of total carbohydrate content in *Angelica* polysaccharides in heating for 2 h. A loss (w/w%) was obtained by comparing weight of component in treated sample with that of untreated sample (at room temperature, RT).

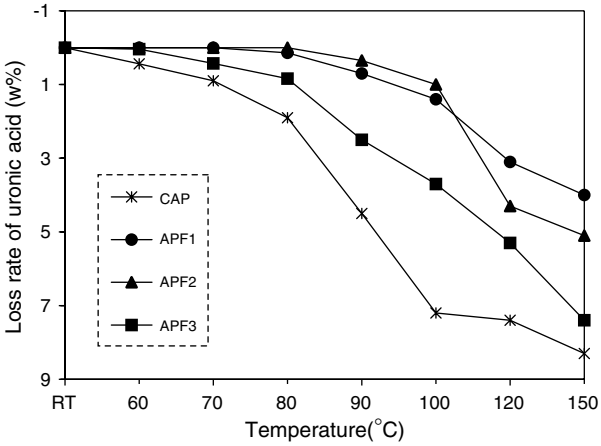

Fig. 4. Influence of heating temperature on the loss of total uronic acid content in *Angelica* polysaccharides.

total carbohydrate ($p < .05$), suggesting uronic acid linkages in the polysaccharides were more stable and resistant to thermal hydrolysis than neutral sugar linkages. It is also clear that APF2 exhibited the highest thermal stability, followed by APF1 (close to APF2), APF3 and CAP in decreasing order.

In order to further confirm the influence of heat treatment on the polysaccharides, different heating time at 80 °C was studied and the results were depicted in Fig. 5. As expected, no significant difference in carbohydrate and uronic acid losses occurred in heating for 2 h or 4 h at 80 °C. When the heating time prolonged from 6 h to 12 h, carbohydrate loss clearly increased in the range of 2.6–6.5% for APF1, 2.5–6.2% for APF2 and 6.9–10.2% for APF3, respectively. At the same time, the loss percentage of uronic acid (in the range of 2.3–5.4% for APF1, 1.7–4.5% for APF2 and 3.9–7.3% for APF3, respectively) also was lower than that of total carbohydrate, which was in

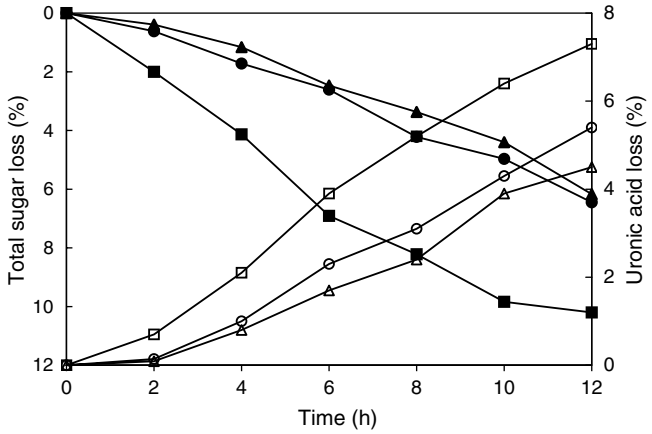

Fig. 5. Influence of heating time on the loss of polysaccharide content in *Angelica* polysaccharides at constant 80 °C. Loss rate in total carbohydrate content of APF1 (●), APF2 (▲) and APF3 (■); loss rate in total uronic acid content of APF1 (○), APF2 (△) and APF3 (□).

agreement with the effects of different temperatures on polysaccharide stability. From these results, it could be concluded that heat treatment accelerated thermal degradation of *Angelica* polysaccharides and such effects depended strongly on polysaccharide composition and temperature and time of heat treatment. It is clear that the heating temperature below 90 °C for a short time of 2–4 h may provide the ideal stability for *Angelica* polysaccharides. The similar effect has also been observed by Chang et al. (2006) in the component polysaccharides in gel juice from *Aloe vera* Miller.

However, it should be taken into consideration that hot-water extraction, which is associated with the processing for long time and high temperature, has been used for classical method for the preparation of polysaccharides and was canonized in industrial application because of the dominant superiority such as simple, economical and easily popularization.Therefore, it should be emphasized that during the heating extraction of *Angelica* polysaccharides, heating temperature below 90 °C for a short time was recommended and the concentration process before drying should be carried out under a decompress condition in order to maintain the function and activity of the polysaccharide as possible. At the same time, the processing at high temperature should be avoided in view of the wide application of *Angelica* polysaccharides in the formulation of food and traditional medicine products as an efficient constituent. In addition, filtration–sterilization should be preferred instead of heating sterilization at high temperature.

It was well known that uronic acid residues in polysaccharides can alter physicochemical property and modify the solubility of the polysaccharides, which is correlated to the wide application in food industry as functional component, gelling, thickening and stabilizing agents (Gancz, Alexander, & Corredig, 2005; Roudsari et al., 2006). Among the isolated fractions, APF2 as main fraction in the polysaccharides was uronic acid-rich polysaccharides and possessed high thermal stabilization and therefore it was approved as the potential source for specific applications, especially in the food industry.

3.4. In vivo macrophage activation of the polysaccharides

At present, study of polysaccharides mainly focus on their immunomodulatory function. Lysosomal enzyme levels are crucial aspect of phagocytic activity in the response of macrophages to invading macroorganisms (Jeong, Jeong, Yang, & Song, 2006). In this study, the effects of the various polysaccharide fractions on the cellular lysosomal enzyme activity in peritoneal macrophages *in vivo* were investigated. As shown in Table 2, intraperitoneal administration with APF1, APF2 and APF3 at the concentrations of 100 and 300 mg/kg to BABL/c mice not only improved markedly lysosomal enzyme activities in macrophages but also promoted the generation of NO and ROS, potent macrophage-derived effector molecules, respectively ($p < .05$ versus control). Among the three polysaccharide fractions, APF2 proved to be most potent as an activator of murine macrophages, where NO release, cellular ROS levels and lysosomal enzyme activity in tested mice administrated by APF2 (100 mg/kg) were increased to 37.7 ± 5.6 nmol/10^6 cells, $308.3 \pm 37.5\%$ and $368.4 \pm$

Table 2
In vivo effect of the polysaccharides on NO release, ROS levels and lysosomal enzyme activity in murine peritoneal macrophages (values are mean ± SD)[a]

Treatment (mg/kg)		Lysosomal enzyme activity (% of control)[b]	NO release (nmol/10^6 cells)[b]	Intracellular ROS (% of control)[b]
Control (saline)		100 ± 9.9	6.9 ± 0.9	100 ± 12.5
LPS	5	311.7 ± 42.7*	44.8 ± 7.9*	297.6 ± 38.4*
APF1	100	263.6 ± 22.8*	28.4 ± 4.1*	224.6 ± 34.4*
	300	313.6 ± 40.5*	33.5 ± 4.5*	289.9 ± 33.7*
APF2	100	368.4 ± 33.1*	37.7 ± 5.6*	308.3 ± 37.5*
	300	417.8 ± 52.9*	45.9 ± 7.7*	335.7 ± 42.4*
APF3	100	324.7 ± 43.2*	32.5 ± 5.5*	273.9 ± 34.5*
	300	371.4 ± 41.5*	43.3 ± 7.4*	319.5 ± 39.4*

[a] Peritoneal macrophages were isolated 8 h after intraperitoneal administration of BALB/c mice with the indicated amount of the polysaccharides and the adhered cells were cultured for 24 h.

[b] NO accumulation and the relative activity of celluar lysosomal enzyme in the culture supernatant was measured as described in text, and the same cell suspension used to study ROS release evaluated by relative fluorescence intensity.

* $p < .05$ indicates statistically different from control (saline).

33.1, in comparison with 6.9 ± 0.9 nmol/10^6 cells, 100 ± 12.5 and 100 ± 9.9 in the control groups, respectively. Such increase became more significant in high dose (300 mg/kg) of polysaccharides-treated animals. However, a low dose of LPS (5 mg/kg) as a potent activator of macrophages could be obtained the close level of response in comparison to APF2 (100 mg/kg). From the obtained results it was evident that the polysaccharide fractions strongly induced the activation of macrophage. Especially, APF2 rich in uronic acid possessed higher immunomodulatory potential.

4. Conclusions

In this study, three water-soluble polysaccharide fractions, termed APF1, APF2 and APF3, were purified from *A. sinensis*. Among these fractions, high molecular weight APF1 possessed the highest carbohydrate content and medium molecular weight APF2 contained the highest uronic acid content without protein presence. Contrarily, trace amount of proteins were present in low molecular weight APF3 as well as CAP extract. Furthermore, the polysaccharide stabilization was associated with sugar conformation, treatment temperature and time, and heating process below 90 °C for a short time was recommended. APF2 presented the highest thermal stability, followed by APF1, APF3 and CAP in decreasing order. Moreover, the polysaccharide fractions significantly induced peritoneal macrophage to release NO and ROS, and enhanced cellular lysosomal enzyme activity *in vivo*. Taken together, APF2 possessed the high thermal stability and immunostimulatory activity and so should be explored as a novel potential immunostimulants for the food and pharmaceutical purpose, which would contribute towards the sustainable use of plant resources.

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References

Alsop, R. M., & Vlachogiannis, G. J. (1982). Determination of the molecular weight of clinical dextran by gel permeation chromatography. *Journal of chromatography, 246*, 227–240.

Bonvehi, J. S., & Coll, F. V. (1993). Study of the carbohydrate fraction of the principal varieties of Tarragona hazelnuts (Corylus avellana L.). *Food Chemistry, 46*, 285–288.

Chang, X. L., Wang, C. H., Feng, Y. M., & Liu, Z. P. (2006). Effects of heat treatments on the stabilities of polysaccharides substances and barbaloin in gel juice from *Aloe vera* Miller. *Journal of Food Engineering, 75*, 245–251.

Cho, C. H., Mei, Q. B., Shang, P., Lee, S. S., So, H. L., Guo, X., et al. (2000). Study of the gastrointestinal protective effects of polysaccharides from *Angelica sinensis* in rats. *Planta Medica, 66*(4), 348–351.

Cohen, J. S., & Yang, T. C. S. (1995). Progress in food dehydration. *Trends in Food Science and Technology, 6*, 20–26.

Deng, S. X., Chen, S. N., Yao, P., Nikolic, D., van Breemen, R. B., Bolton, J. L., et al. (2006). Serotonergic activity-guided phytochemical investigation of the roots of *Angelica sinensis*. *Journal of Natural Products, 69*, 536–541.

Diisterhoft, E. M., Posthumus, M. A., & Voragen, A. G. J. (1992). Nonstarch polysaccharides from sunflower (*Helianthus annuus*) meal and palm kernel (Elaeis guineenis) mealsInvestigation of the structure of major polysaccharides. *Journal of the Science of Food and Agriculture, 59*, 151–160.

Dubois, M., Gilles, K. A., Haamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*, 350–356.

Filisetti-Cozzi, T. M. C. C., & Carpita, N. C. (1991). Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry, 197*, 157–162.

Gancz, K., Alexander, M., & Corredig, M. (2005). Interactions of high methoxyl pectin with whey proteins at oil/water interfaces at acid pH. *Journal of Agricultural and Food Chemistry, 53*, 2236–2241.

Gnanasambandam, R., & Proctor, A. (2000). Determination of pectin degree of esterification by diffuse reflectance Fourier transform infrared spectroscopy. *Food Chemistry, 68*, 327–332.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate in biological fluids. *Analytical Biochemistry, 126*, 131–138.

Jeong, S. C., Jeong, Y. T., Yang, B. K., & Song, C. H. (2006). Chemical characteristics and immuno-stimulating properties of biopolymers extracted from *Acanthopanax sessiliflorus*. *Journal of Biochemistry Molecular Biology, 39*, 84–90.

Jung, H. J., Valdez, F. R., Hatfield, R. D., & Blanchette, R. A. (1992). Cell wall composition and degradability of forage stems following chemical and biological delignification. *Journal of the Science of Food and Agriculture, 58*, 347–355.

Kim, G. Y., Choi, G. S., Lee, S. H., & Park, Y. M. (2004). Acidic polysaccharide isolated from *Phellinus linteus* enhances through the up-regulation of nitric oxide and tumor necrosis factor-a from peritoneal macrophages. *Journal of Ethnopharmacology, 95*, 69–76.

Kim, H. H., Ahn, K. S., Han, H., Choung, S. Y., Choi, S. Y., & Kim, I. H. (2005). Decursin and PDBu: Two PKC activators distinctively acting in the megakaryocytic differentiation of K562 human erythroleukemia cells. *Leukemia Research, 29*, 1407–1413.

Kim, H. H., Bang, S. S., Choi, J. S., Han, H., & Kim, I. H. (2005). Involvement of PKC and ROS in the cytotoxic mechanism of anti-leukemic decursin and its derivatives and their structure-activity relationship in human K562 erythroleukemia and U937 myeloleukemia cells. *Cancer Letters, 223*, 191–201.

Lao, S. C., Li, S. P., Kan, K. K. W., Li, P., Wan, J. B., Wang, Y. T., et al. (2004). Identification and quantification of 13 components in *Angelica sinensis* (Danggui) by gas chromatography–mass spectrometry coupled with pressurized liquid extraction. *Analytica Chimica Acta, 526*, 131–137.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *The Journal of biological chemistry, 193*, 265–275.

Lu, G. H., Chan, K., Chan, C. L., Leung, K., Jiang, Z. H., & Zhao, Z. Z. (2004). Quantification of ligustilides in the roots of *Angelica sinensis* and related umbelliferous medicinal plants by high-performance liquid chromatography and liquid chromatography–mass spectrometry. *Journal of Chromatography A, 1046*, 101–107.

Mei, Q. B., Tao, J. Y., Zhang, H. D., Duan, Z. X., & Chen, Y. Z. (1988). Effects of *Angelica sinensis* polysaccharides on hemopoietic stem cells in irradiated mice. *Acta Pharmacologica Sinica, 9*(3), 279–282.

Roudsari, M., Nakamura, A., Smith, A., & Corredig, M. (2006). Stabilizing behavior of soy soluble polysaccharide or high methoxyl pectin in soy protein isolate emulsions at low pH. *Journal of Agricultural and Food Chemistry, 54*, 1434–1441.

Shang, P., Qian, A. R., Yang, T. H., Jia, M., Mei, Q. B., Cho, C. H., et al. (2003). Experimental study of anti-tumor effects of polysaccharides from *Angelica sinensis*. *World Journal of Gastroenterology, 9*(9), 1963–1967.

Sun, Y. L., Tang, J., Gu, X. H., & Li, D. Y. (2005). Water-soluble polysaccharides from *Angelica sinensis* (Oliv.) Diels: Preparation, characterization and bioactivity. *International Journal of Biological Macromolecules, 36*, 283–289.

Suzuki, I., Tanaka, H., Kinoshita, A., Oikawa, S., Osawa, M., & Yadomae, T. (1990). Effect of orally administered beta-glucan on macrophage function in mice. *International Journal of Immunopharmacology, 12*, 675–684.

Tieraona, L. D. (2005). Menopause: A review of botanical dietary supplements. *The American Journal of Medicine, 118*, 98S–108S.

Wang, H., & Joseph, J. (1999). Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. *Free Radical Biology and Medicine, 27*, 612–616.

Ye, Y. N., Koo, M. W. L., Li, Y., Matsui, H., & Cho, C. H. (2001). *Angelica sinensis* modulates migration and proliferation of gastric epithelial cells. *Life Sciences, 68*(8), 961–968.

Ye, Y. N., Liu, E. S. L., Li, Y., So, H. L., Cho, C. C. M., Sheng, H. P., et al. (2001). Protective effect of polysaccharides-enriched fraction from *Angelica sinensis* on hepatic injury. *Life Sciences, 69*(6), 637–646.

Ye, Y. N., Liu, E. S. L., Shin, V. Y., Koo, M. W. L., Li, Y., Wei, E. Q., et al. (2001c). A mechanistic study of proliferation induced by *Angelica sinensis* in a normal gastric epithelial cell line. *Biochemical Pharmacology, 61*(11), 1439–1448.

Ye, Y. N., So, H. L., Liu, E. S. L., Shin, V. Y., & Cho, C. H. (2003). Effect of polysaccharides from *Angelica sinensis* on gastric ulcer healing. *Life Sciences, 72*(8), 925–932.

Zhao, K. J., Dong, T. T. X., Tu, P. F., Song, Z. H., Lo, C. K., & Tsim, K. W. K. (2003). Molecular genetic and chemical assessment of radix *Angelica* (Danggui) in China. *Journal of Agricultural and Food Chemistry, 51*, 2576–2583.