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Purification, characterization and anticoagulant activity of the polysaccharides from green tea

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ABSTRACT

The crude tea polysaccharides were extracted from the leaves of *Camellia sinensis* using deionized water. The tea polysaccharides (TPS) were further separated and purified by anion exchange chromatograph on DEAE sepharose CL-6B column to afford TPS-1, TPS-2, TPS-3 and TPS-4. The high performance gel permeation chromatograph analysis showed that the average molecular weight of polysaccharides (TPS-1, TPS-2 and TPS-3) were 20,760, 24,230 and 250,643, respectively. TPS-4 was 689, 113 and 4150, suggesting it was heterogeneous. Monosaccharide analysis detected fucose, glucosamine, rhamnose, arabinose, galactosamine, galactose, glucose, xylose, mannose, ribose, galacturonic acid and glucuronic acid in the four polysaccharide fractions. Anticoagulant activities in vitro tests showed TPS-4 could significantly prolong APTT and TT, but not PT. The result indicated TPS-4 in the regulation of coagulation initiated via the intrinsic pathway. With current findings TPS-4 should be explored as a natural potential anticoagulant.

1. Introduction

Heparin, a sulfated polysaccharide is the first compound used clinically as an anticoagulant and antithrombotic agent (Fachina & Verli, 2008), however, it also accompanied with the side effects of bleeding and thrombocytopenia. Exploring the main structure of anticoagulant active ingredients, which are traced from natural products, as lead compounds to develop new drugs or functional foods has the advantages of low toxicity, low research and development costs, short cycle and universal health care (Cai, Gu, & Tang, 2010).

Tea, a product made up from leaf and bud of the plant *camellia sinensis*, is one of the most popular beverages consumed in the world, well ahead of coffee, beer, wine and carbonated soft drinks, next to water (Cabrera, Artacho, & Gime'nez, 2006; Chen et al., 2009; Nie & Xie, 2011). Originating from China, teas are classified into three major types in theirs green, oolong and black forms according to the different manufacturing processes, which are non, partially and fully fermented, respectively (Chan, Lim, & Chew, 2007; Mukhtar & Ahmad, 2000). Tea has a long history as a folk remedy, and is used as a traditional Chinese treatment for diabetes mellitus (Anderson & Polansky, 2002).

The leaves of *C. sinensis* contained many beneficial compounds, including polyphenols, alkloids, polysacchrides, amino acids and vitamins. Tea polysaccharides (TPS) are the main components in tea extracts, are well known to have antioxidant, hypoglycemic and immunostimulation activities (Chen, Zhang, & Xie, 2005; Han et al., 2011; Monobe, Ema, Azuma, & Maeda-Yamamoto, 2010). However, there are still only few reports on the anticoagulant effect of tea polysaccharides. In this study, we attempted to investigate the molecular weights and monosaccharide compositions, as well as the anticoagulant activity, of the polysaccharide fractions isolated from the leaves of *C. sinensis*. Different polysaccharide fractions were obtained by ion exchange chromatograph. The effects of tea polysaccharide fractions on their anticoagulant in vitro were evaluated by APTT, PT and TT assays.

2. Materials and methods

2.1. Materials

DEAE sepharose CL-6B was purchased from Pharmacia (Uppsala, Sweden). T-series dextran and the standard monosaccharides were purchased from Sigma (St. Louis, MO, USA). Activated partial thromboplastin time reagent (APTT, ellagic + bovine phospholipid), $CaCl_2$ solution, prothrombin time (PT) reagent and thrombin time (TT) reagents were purchased from Mantenuo (Changchun, China). Polyamide (80–100 mesh), trifluoroacetic acid (TFA) and all of other chemicals and reagents were analytical grade from Sinopharm Chemical Reagent (Shanghai, China).

The leaves of *C. sinensis* used for our experiments were obtained commercially from Anhui province of China. The leaves were ground to pass through a 60 mesh screen and stored at 4 °C

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in a refrigerator until the initial preparation work was carried out.

2.2. Extraction and purification of tea polysaccharide

The leaves of *C. sinensis* were pre-extracted with absolute ethanol for 24 h at room temperature and then filtered and dried at 60 °C. Subsequently, the dried residues were extracted with twenty volumes of deionized water at 60 °C for 90 min under continuous stirring, filtered through gauze and centrifuged to remove the insoluble materials. The supernatant was concentrated by rotary evaporation under reduced pressure, then mixed with four volumes of 95% ethanol and kept at 4 °C in refrigerator for 12 h. The precipitate was obtained by centrifugation and washed three times with absolute ethanol. The precipitate was dissolved in deionized water and then lyophilized in vacuum freeze dryer to obtain the crude tea polysaccharide (CTPS).

Polyamide was a polymer of hexanolactam and used for decolorization and deprotein. The protein content in polysaccharide sample was determined using a Bradford method (Bradford, 1976). The decolorization of polysaccharides was measured according to the method (Xie, Shen, Nie, Li, & Xie, 2011) with a UV–vis spectrophotometer (Shimadzu Corporation, Japan) at a wavelength of 420 nm. The CTPS were re-dissolved in deionized water and then applied to the absorption column (600 mm × 30 mm i.d.) packed with polyamide adsorbent resin. The column was eluted with deionized water at a flow rate of 1 ml/min. The main polysaccharide fractions were combined and concentrated, and then finally lyophilized. The tea polysaccharides (TPS) were obtained.

The TPS sample (100 mg) was dissolved with 0.02 mol/L phosphate buffer solution (PBS, pH 5.9) and centrifuged. The supernatant was loaded onto a DEAE sepharose CL-6B chromatograph column (600 mm × 16 mm i.d.) equilibrated with 0.02 mol/L phosphate buffer solution (pH 5.9). The column was eluted first with phosphate buffer solution and then with gradient solution (0.1 M, 0.2 M and 0.5 M NaCl) at a flow rate of 2 ml/min. The fractions were detected by the phenol–sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956) using glucose as standard and collected with automatic fraction collector. The main fraction was combined and then dialyzed (molecular weight cut off: 14,000) against deionized water for 72 h. Finally, four purified polysaccharides named TPS-1, TPS-2, TPS-3 and TPS-4 respectively were obtained by lyophilizing.

2.3. UV and FT-IR spectra analysis

UV and FT-IR spectra were performed using previous descriptions (Jiang et al., 2007). The ultraviolet spectra of the TPS solutions (100 μg/ml) were recorded with a UV–vis spectrophotometer (Shimadzu Corporation, Japan) in the region of 200–700 nm. The TPS sample (2 mg) was dried at room temperature in vacuum over P_2O_5 for 48 h prior to mixing with potassium bromide powder, and then pressed into thin disk for FT-IR measurement (Shimadzu Corporation, Japan) in the mid-infrared region at 4000–400 cm^{-1} to detect organic groups.

2.4. Determination of molecular weight

The molecular weight of polysaccharide fractions were determined by high performance gel permeation chromatograph (HPGPC) (Chen et al., 2011; Li, Yuan, & Rashid, 2009). The purified fractions were dissolved in mobile phase solution and passed through a 0.45 μm filter. The sample solution was loaded onto HPLC (Waters 600, USA) equipped with two serially linked Ultrahydrogel™ Linear columns (300 mm × 7.8 mm i.d.) and waters 2410 refractive index detector. The detailed experiment conditions were as follows: mobile phase: 0.1 M $NaNO_3$; flow rate: 0.9 ml/min; column temperature: 45 °C; injection volume: 20 μl. The column was calibrated with dextran of different molecular weight from Sigma (molecular weight: 4600, 10,000, 70,000 and 2,000,000) and the calibration curve was established before sample analysis.

2.5. Monosaccharide composition analysis

The sample (10 mg) was hydrolyzed with 2 M TFA (2 ml) for 1 h at 121 °C in sealed glass tube. After complete hydrolysis, residual TFA was evicted by vacuum concentrator. The dried sample was carried out as previously described (Yokota, Mori, Yamaguchi, Kaniwa, & Saisho, 1999) using high performance anion-exchange chromatograph with pulsed amperometric detector (HPAEC–PAD). Monosaccharide compositions of polysaccharides were performed on a Dionex Ion Chromatograph 2010i system (Dionex, USA) containing advanced gradient pump and an eluent degas module. The column used was CarboPac™PA1 analytical column (250 mm × 4 mm) coupled to a guard CarboPac™PA1 column (50 mm × 4 mm). The eluent was sodium acetate 0.15 M–sodium hydroxide 0.15 M. A mixture of each standard monosaccharide was subjected to the same condition as a reference.

2.6. Blood coagulation assays

The anticoagulant ability of the polysaccharides was assayed using APTT, PT and TT as indicators according to the method provided by Mantenuo Corporation. In the assay, normal human plasma prepared from healthy donors without a history of bleeding or thrombosis. Nine parts of human blood collected by venipuncture were drawn into one part of 0.109 M sodium citrate solution. Blood was centrifuged for 6 min at 3600 r/min, and the plasma was obtained within 2 h to complete the experiment.

For APTT clotting assay, citrated human plasma (40 μl) was mixed with the polysaccharide (10 μl), then 50 μl APTT reagent was added to the mixture and incubated at 37 °C for 3 min. Pre-incubated (37 °C) 50 μl of 0.025 mol/L $CaCl_2$ solution was added and clotting time was recorded on an automated coagulometer. In PT clotting measurement, citrated human plasma (40 μl) was mixed with the polysaccharide (10 μl) and incubated at 37 °C for 3 min,then 100 μl of PT reagent pre-incubated at 37 °C for 10 min was added and clotting time was recorded. For TT clotting test, citrated human plasma (80 μl) was mixed with the polysaccharide (20 μl) and incubated at 37 °C for 3 min. Then TT reagent at room temperature was added and clotting time was recorded. All assays were repeated three times and mean values calculated. All the polysaccharides including heparin were dissolved in water and in the control group only Saline water was used.

2.7. Statistical analysis

All the results were expressed as mean ± standard deviation (SD) of six replicated determinations and statistical analysis was performed with the Minitab 15 software (Minitab Inc., PA, USA). The efficiency of anticoagulation was compared by using the paired *t*-test. *P*-values of less than 0.05 ($P < 0.05$) were considered as significant.

3. Results and discussion

3.1. Extraction and purification

The CTPS were extracted from the leaves of *C. sinensis* and the yield was about 3.35% (lyophilized weight). The efficiency of deproteination and decolorization were 91.2% and 75.1%, separately. It

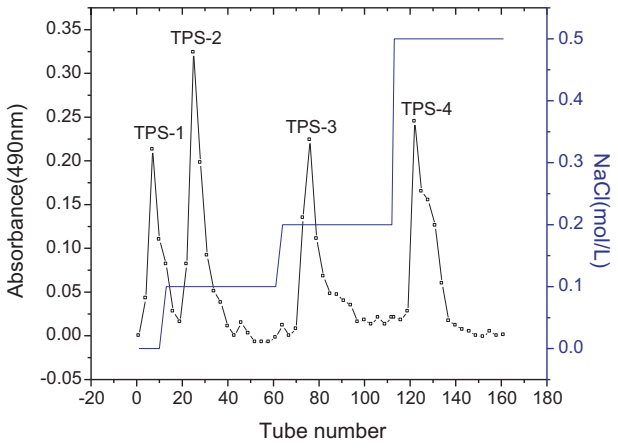

Fig. 1. Elution profile of polysaccharides extracted from the leaves of *Camellia sinensis* on DEAE sepharose CL-6B column.

may be postulated that polyamide was effective in removing proteins and pigments. The TPS obtained by polyamide column were further separated and purified by anion exchange chromatograph on a DEAE sepharose CL-6B. Four major fractions eluted with 0, 0.1, 0.2, 0.5 M sodium chloride were collected, respectively, and then concentrated, dialyzed and lyophilized to obtain TPS-1, TPS-2, TPS-3 and TPS-4 (Fig. 1). TPS-1 eluted with PBS was known as neutral polysaccharides, and TPS-2, TPS-3 and TPS-4 eluted with 0.1, 0.2 and 0.5 M NaCl were known as acidic polysaccharides.

3.2. UV and FT-IR spectra analysis

The UV and FT-IR spectra of TPS fractions are shown in Figs. 2 and 3, respectively. TPS-4 had a positive respond to absorption at 280 and 260 nm in the UV–vis spectrum, indicating presence of protein and nucleic acid. However, no absorption at 280 and 260 nm were observed for TPS-1, TPS-2 and TPS-3. The broadly intense peak between 3600 and 3200 cm^{-1} represented the stretching of the hydroxyl groups. The small band at around 2926 cm^{-1} was attributed to the C—H stretching and bending vibrations. The relatively strong absorption peak in the range of 1650–1600 cm^{-1} was due to associated water. The peaks at ground 1400–1200 cm^{-1} were also the characteristic absorptions of

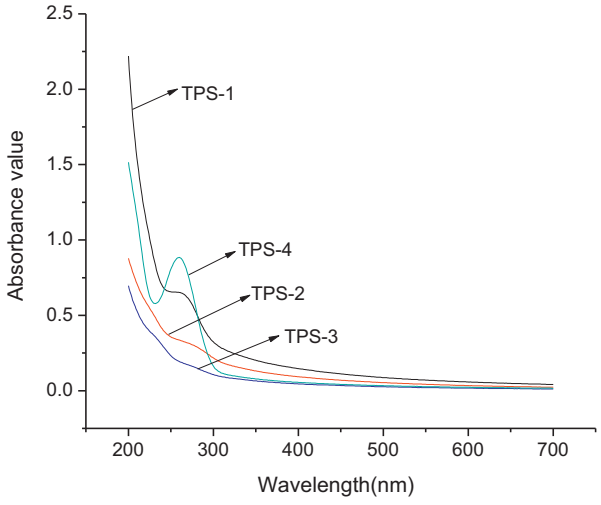

Fig. 2. UV–vis spectra of the TPS fractions in the range of 200–700 nm.

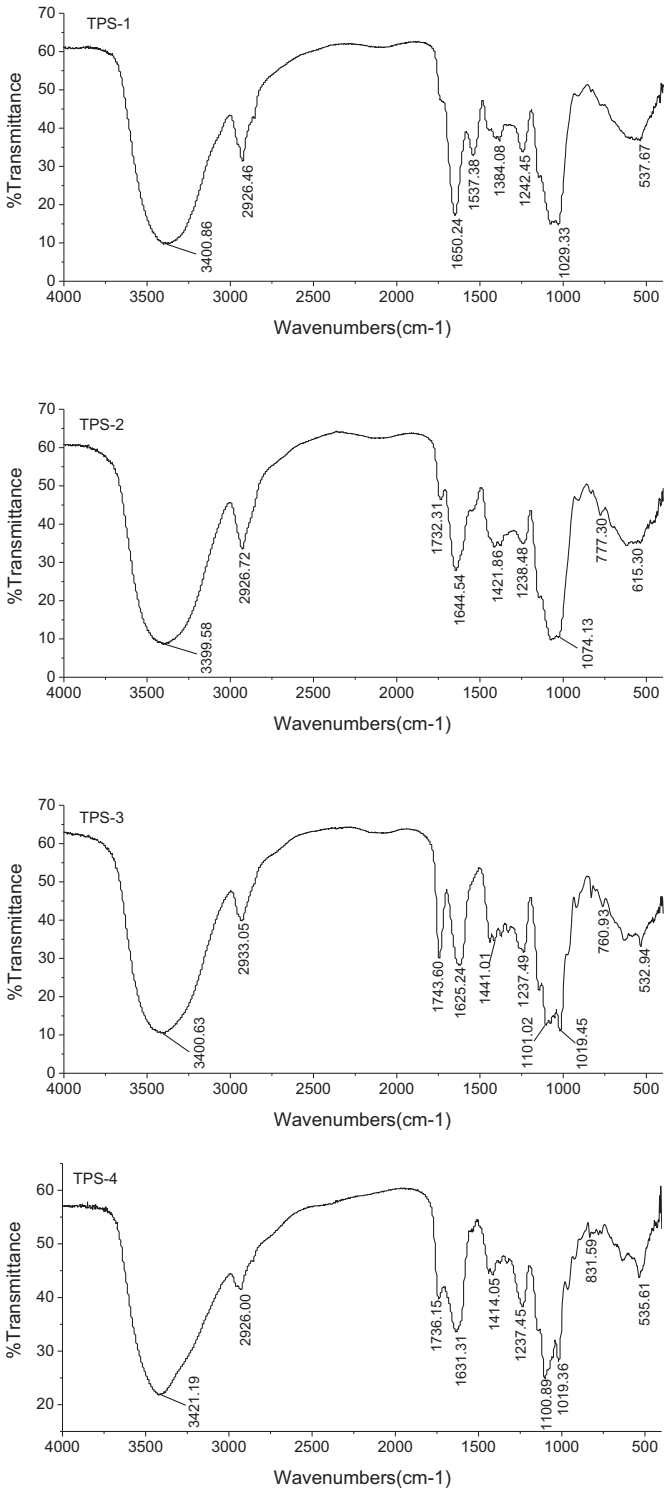

Fig. 3. FT-IR spectra of the TPS fractions in the range of 4000–400 cm^{-1}.

C—H bonds. Further, the peaks at 1200–1000 cm^{-1} range indicated the presence of C—O—H side groups and C—O—C glycosidic band vibrations. Specifically, the absorptions at 400–900 cm^{-1} (TPS-1: 537.67 cm^{-1}, TPS-2: 777.30 and 615.30 cm^{-1}, TPS-3: 760.93 and 532.94 cm^{-1}, TPS-4: 831.59 and 535.61 cm^{-1}) were assigned to skeletal modes of pyranose rings. Characterization of TPS fractions by FT-IR analysis showed the typical absorption of polysaccharides.

Table 1
Monosaccharide compositions of the polysaccharide fractions.

Fraction	Mol%											
	Fuc	GluN	Rha	Ara	GalN	Gal	Glu	Xyl	Man	Rib	GalA	GluA
TPS-1	1.3	0.1	–[a]	39.4	0.6	31.0	13.6	0.5	10.2	0.3	2.1	0.9
TPS-2	0.1	–	4.1	36.4	0.1	43.1	1.0	2.3	0.9	–	6.9	5.2
TPS-3	0.1	0.2	3.5	13.0	0.1	22.1	7.0	0.2	0.6	0.1	49.1	3.9
TPS-4	0.2	1.6	3.1	7.7	0.6	12.8	4.0	0.2	0.8	15.4	51.2	2.5

Individual components were identified and quantified based on elution of known standards.
[a] Undetected.

3.3. Molecular weight of polysaccharide fractions

The regression equation was Log Mol Wt = 12.14 – 0.392t_R with a high correlation of R^2 = 0.989973 obtained for the standard curve (Fig. 4). TPS-1, TPS-2 and TPS-3 were shown as single symmetrical peak in their respective HPGPC chromatograms, indicating that they were homogeneous (Fig. 5A–C). The average molecular weight of TPS-1, TPS-2 and TPS-3 were 20,760, 24,230 and 250,643, respectively. However, the average molecular weights of TPS-4 were 689,113 and 4150, suggesting it was heterogeneous (Fig. 5D). The molecular weight of polysaccharide fractions in this study was different from the report (Chen, Zhang, Qu, & Xie, 2008; Wang & Xia, 2006), which is probably caused by the difference in conditions of raw material or purification process.

3.4. Monosaccharide composition analysis

The monosaccharide compositions were analyzed by HPAEC–PAD after the hydrolysis of TFA. HPAEC–PAD is one of the most suitable methods because it is quantitative and does not require monosaccharide derivatization (Yokota et al., 1999). The result of HPAEC–PAD analysis shown in Table 1. The main constitutes of TPS-1 was arabinose, galactose, glucose and mannose. The results implied the dominance of rhamnose, arabinose, galactose, galacturonic acid and glucuronic acid in TPS-2. The monosaccharide compositions of the TPS-3 and TPS-4 fractions were similar. Both TPS-3 and TPS-4 were rich in rhamnose, arabinose, galactose, glucose, galacturonic acid and glucuronic acid. The content of ribose was obvious higher in TPS-4 than it in other fractions.

3.5. Blood coagulation assays

The anticoagulant activities of the polysaccharides from the leaves of *C. sinensis* were investigated by the classical coagulation assays APTT, PT and TT, and meanwhile the saline and heparin were used as negative and positive control, respectively. APTT is used to evaluate the coagulation factors such as VIII, IX, X, XII and prekallikrein in the intrinsic blood coagulation pathway while PT is used to characterize the extrinsic coagulation factors. TT is a simple screening test for the fibrin polymerization process, which measure the formation time of fibrin from fibrinogen after the addition of known amounts of thrombin to the plasma sample (Ekanayake et al., 2008; Ye, Xu, & Li, 2012).

As listed in Table 2, all the polysaccharide fractions prolonged APTT compared to saline control a dose-dependent manner.

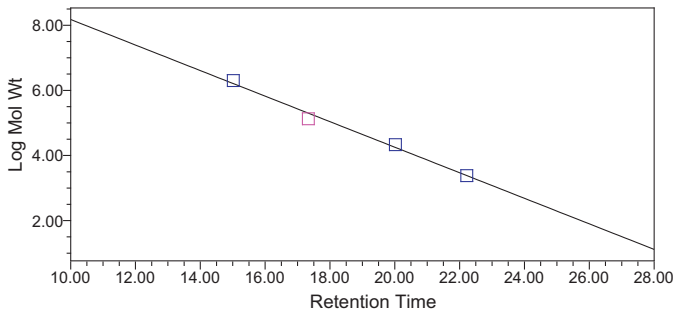

Fig. 4. Standard curve of molecular weight of polysaccharides.

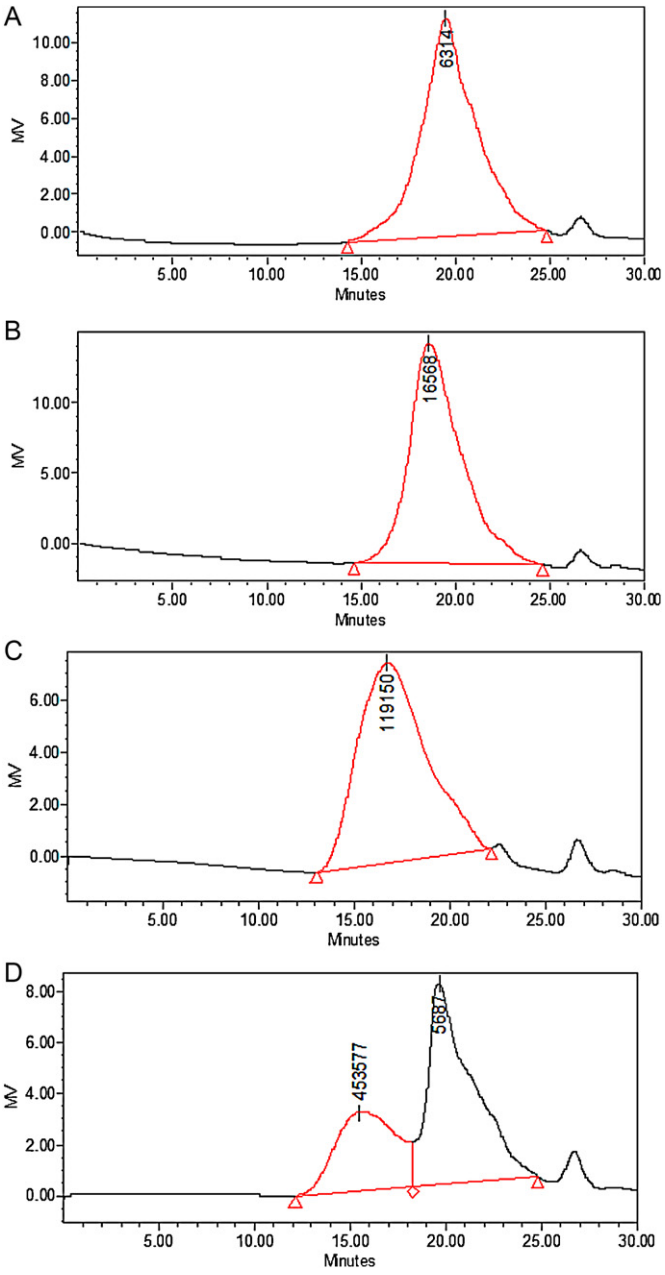

Fig. 5. Results of molecular weight of the polysaccharides TPS-1(A), TPS-2(B), TPS-3(C) and TPS-4(D).

Table 2
APTT, PT and TT of normal human plasma including the polysaccharide fractions, saline and heparin.

Sample	APTT(s) Concentration (mg/ml)			PT(s)			TT(s)		
	1	2	4	1	2	4	1	2	4
TPS	28.7 ± 1.3	30.2 ± 0.8*	31.6 ± 1.2*	10.8 ± 0.5	11.2 ± 0.6	11.9 ± 0.8	18.3 ± 0.9	18.5 ± 0.6	19.1 ± 0.5
TPS-1	27.3 ± 0.5	27.9 ± 1.2	29.8 ± 0.5*	11.4 ± 0.1	12.6 ± 0.4	12.7 ± 0.3	19.2 ± 0.4	20.4 ± 0.4	22.4 ± 0.8*
TPS-2	27.5 ± 0.7	28.0 ± 0.4	30.2 ± 0.6*	11.2 ± 0.4	12.1 ± 0.7	12.3 ± 0.5	17.3 ± 0.9	17.7 ± 1.2	18.7 ± 1.3
TPS-3	27.6 ± 1.0	28.2 ± 0.9	31.3 ± 0.6*	11.9 ± 0.2	12.4 ± 0.9	12.5 ± 0.4	18.0 ± 0.1	18.2 ± 0.3	19.2 ± 0.5
TPS-4	29.3 ± 0.8*	31.2 ± 0.8*	33.4 ± 0.7*	11.3 ± 0.7	12.5 ± 0.2	12.3 ± 0.6	20.1 ± 1.1	22.1 ± 1.2*	26.3 ± 0.8*
Saline	24.1 ± 0.9	11.2 ± 0.3	17.9 ± 0.7						
Heparin	Concentration (μg/ml)								
	1	2	4	1	2	4	1	2	4
	29.7 ± 1.0*	32.1 ± 1.4*	36.3 ± 0.9*	12.6 ± 0.6	16.7 ± 0.5*	24.3 ± 0.9*	16.8 ± 0.4	22.3 ± 0.6*	30 ± 1.2*

For each treatment the mean APTT, PT and TT time ± SD was determined for $n = 6$.
* Significantly inhibited compared with negative control ($P < 0.05$).

Although activity less than heparin, TPS-4 exhibited a little stronger anticoagulant activity than TPS, TPS-1, TPS-2 and TPS-3 with the raise of concentration in APTT experiment and showed a significant different ($P < 0.05$) from negative control. Meanwhile TPS-1 and TPS-4 showed increasing clotting time in the TT assays. But, according to the PT assay the polysaccharide fractions were no significant differences in modulating prothrombin time. The results implied that TPS-4 inhibited both the intrinsic and the common coagulation pathway and thrombin activity of controlling the fibrinogen to fibrin conversion, and did not inhibit the extrinsic pathway.

4. Conclusions

In this paper, it is concluded that leaves of *C. sinensis* contained four major polysaccharide fractions purified by DEAE sepharose CL-6B column chromatography. In addition, the anticoagulant activity in vitro investigation of TPS-4 significantly prolonged the clotting time in APTT and TT experiment. However, the detailed mechanisms of the anticoagulant activity of polysaccharides are not clear. Complex relationships existed between the structure and anticoagulant properties of polysaccharide. Based on above studies, structural characterization analysis will be carried out to elucidate the structure-function relationship in our later work.

Acknowledgements

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