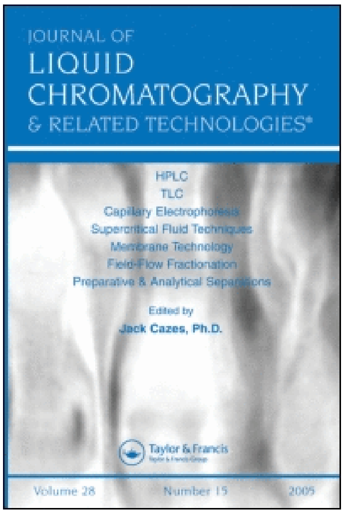

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Zhimao Chao[a]; Yoichi Shibusawa[b]; Heisaburo Shindo[b]; Yoichiro Ito[c]
[a] Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine, Beijing, P.R. China [b] Department of Analytical Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo, Japan [c] Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

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Countercurrent Chromatographic Purification of Polysaccharides from *Achyranthes bidentata* with an Aqueous Two-Phase System Using a Cross-Axis Coil Planet Centrifuge

Zhimao Chao,[1,*] Yoichi Shibusawa,[2] Heisaburo Shindo,[2] and Yoichiro Ito[3]

[1]Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine, Beijing, P.R. China
[2]Department of Analytical Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo, Japan
[3]Laboratory of Biophysical Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland, USA

ABSTRACT

Polysaccharides were purified from a high molecular weight complex of *Achyranthes bidentata* Bl. (Fam. Amaranthaceae) root by high speed countercurrent chromatography (HSCCC) using a cross-axis coil planet centrifuge (*X*-axis CPC). The purification was performed with an aqueous

*Correspondence: Zhimao Chao, Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine, 18 Beixincang, Dongcheng, Beijing 100700, P.R. China; E-mail: chaozhimao@sina.com.

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polymer two-phase system composed of 12.5% (w/w) polyethylene glycol (PEG) 1000, 8% (w/w) potassium dihydrogen phosphate, and 8% (w/w) dipotassium hydrogen phosphate buffer at pH 6.8. The preparative separation of the crude sample (2 g) was successfully performed, yielding 1.1 g of polysaccharides at high purity.

Key Words: Countercurrent chromatography; Cross-axis coil planet centrifuge; Polysaccharides; Partition coefficient.

INTRODUCTION

Some polysaccharides from Chinese traditional drugs are widely used in the clinic for treating various diseases such as hepatitis, malignancy, gastric ulcer, intoxication, and immunodeficiency. The curative effect of polysaccharide is usually greater by injecting the preparation than its oral administration. For example: a polysaccharide from the dried sclerotium of *Polyporus umbellatus* (Pers.) Fries (Fam. Polyporaceae) can inhibit the growth of sarcoma 180 cells in mouse by intravenous injection with a small dosage of 1 mg/kg, three times per week for two weeks, but only a certain inhibition can be observed if taken orally with a large dosage of 1000 mg/kg/day.[1] For injection preparation, high purity polysaccharide is required by thoroughly removing toxic impurities such as proteins and proteoglycans.

The dried root of *Achyranthes bidentata* Bl. (Fam. Amaranthaceae) is one of the commonly used Chinese traditional drugs. It has many useful indications including the treatment of soreness of the lumbar and knee joints with weakness in the legs, amenorrhea with mass formation in the abdomen, and dizziness due to hyperactivity of the liver. Several investigators have reported that polysaccharides, isolated from the roots of *A. bidentata*, produced some immunopotentiating and antitumor effects.[2,3]

In this paper, we report the purification of polysaccharides from the high molecular weight complex, extracted from *A. bidentata* root, with an aqueous polymer two-phase system. The separation was performed by high speed countercurrent chromatography (HSCCC) using a cross-axis coil planet centrifuge (*X*-axis CPC).

EXPERIMENTAL

Apparatus

The HSCCC purification of polysaccharides from high molecular weight complex of *A. bidentata* was performed using our type-XL cross-axis coil planet centrifuge (*X*-axis CPC).[4,5] The apparatus holds a pair of horizontal rotary shafts symmetrically, one on each side of the rotary frame, at a distance of 10 cm from the centrifuge axis. A spool shaped column holder is mounted on each rotary shift at a lateral location 10 cm away from the midpoint. The apparatus was fabricated by Mr. Jimmie L. Slemp at the NIH machine shop. The revolution speed of the apparatus is regulated at 500 rpm with a speed control unit (Bodine Electric, Chicago, IL).

Preparation of Separation Columns

A pair of multilayer coils with 2.6 mm I.D. standard wall polytetrafluoroethylene (PTFE) tubing (Zeus Industrial Products, Raritan, NJ) was prepared by winding it onto a 15.2 cm diameter holder hub forming four layers of left-handed coils between a pair of flanges spaced 5 cm apart. Each column consists of four layers of coiled layers with about 170 mL capacity. A pair of columns mounted on the rotary frame was connected in series using a flow tube (PTFE, 0.85 mm I.D.) to make up a total column capacity of 340 mL.

Reagents and Materials

Polyethylene glycol (PEG) 1000 (average molecular mass: 950–1050), monobasic and dibasic potassium phosphates, all used for the preparation of aqueous polymer two-phase systems, were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sulfuric acid was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), anthrone from Wako Pure Chemical Industrial Co., Inc. (Tokyo, Japan), and Coomassie protein assay reagent from Pierce Company (IL, USA). Other chemicals were all of analytical reagent grade. Dried and cut roots of *A. bidentata* Bl. were purchased from Tokyo Business Store of Tochimoto Tenkaidou (Tokyo, Japan), and identified by Prof. Zongwan Xie of the Institute of Chinese Materia Medica, China Academy of Traditional Chinese Medicine. A voucher specimen was deposited in Department of Chemistry, Institute of Chinese Materia Medica, China Academy of Traditional Medicine. Spectra/Por molecular dialysis tubing, purchased from Spectrum Medical Industries, Inc. (Laguna Hills, CA), is No. 3 membrane with 3500 of molecular weight cut-off (MWCO), 54 mm of flat width, 34 mm diameter, and 9.3 mL/cm of vol/length.

Preparation of CCC Sample Solution

The roots (1000 g) of *A. bidentata* were immersed in distilled water for 24 h each for three times at 23°C. The extracts were combined and centrifuged at 500 rpm for 20 min at 4°C to remove some precipitates, and freeze-dried to obtain a yellowish powder (616 g). A 100 g aliquot of this powder was dialyzed for 77 h with molecularporous membrane tubing (MWCO 3500) until no silver chloride precipitation reaction was formed. This high molecular weight complex was freeze-dried to obtain 17.41 g of white powder, yielding 10.7% of *A. bidentata* root.[6] Our studies have indicated that this complex showed the cytotoxicity against P388 leukemia cells in vitro, and the main components of this complex were polysaccharides and proteoglycans.[6]

The sample solution for HSCCC was prepared by dissolving 2 g of the high molecular weigh complex into 5 g of each phase of 12.5% (w/w) PEG-1000-16% (w/w) potassium phosphate buffer (pH 6.8) solvent system.

Preparation of Polyethylene Glycol-Potassium Phosphate Aqueous Two-Phase System

An aqueous two-phase solvent system for CCC separation, composed of 12.5% (w/w) PEG-1000 and 16% (w/w) potassium phosphate aqueous solution, was prepared by dissolving 125 g of PEG-1000, 80 g of potassium dihydrogen phosphate (KH_2PO_4), and 80 g of dipotassium hydrogen phosphate (K_2HPO_4) in 715 g of distilled water. After being allowed to stand, two clear layers were obtained.

Measurement of Partition Coefficients

About 2.0 mL of each phase of 12.5% PEG 1000–16% potassium phosphate buffer (pH 6.8) aqueous two-phase solvent system was delivered into a test tube to which about 10 mg of the sample was added. The contents were thoroughly mixed and then allowed to settle at room temperature. After two clear layers were formed, an aliquot (0.5 mL) of each phase was pipetted and diluted with 1.0 mL of distilled water to determine the absorbance at 280 nm using a Shimadzu UV-1200 spectrophotometer (Shimadzu, Kyoto, Japan). Another aliquot (0.1 mL) of each phase was pipetted, mixed with 5 mL of 0.2% anthrone sulfuric acid solution in test tubes, allowed boil for 10 min, cooled with running water, and the absorbance was determined at 550 nm. The partition coefficient values ($K_D = C_U/C_L$) were obtained by dividing the absorbance value of the upper phase by that of the lower phase.

CCC Fractionation of Polysaccharides from High-Molecular Weight Complex

The column of the cross-axis CPC was first entirely filled with the potassium phosphate rich lower phase as a stationary phase, and the CCC sample solution was injected into the column through a sample port from a tail of the column. Then, the apparatus was rotated at 500 rpm while the PEG 1000 rich upper phase (mobile phase) was pumped into the column from the tail at a flow rate of 0.58 mL/min by an MP-311 micro pump (Lab-Quatec, Tokyo, Japan), in the P_{II}-T-I elution mode given by a combination of the P_{II} (clockwise) planetary motion, tail-head elution mode, and inward–outward direction.[7] After the desired peaks were eluted, the flow was reversed in the P_I-H-O elution mode given by a combination of the P_I (counterclockwise) planetary motion, head-tail elution mode, and outward-inward direction, using the original stationary phase as the mobile phase to facilitate rapid elution of solutes still remaining within the column. The effluent from the outlet was continuously monitored at 280 nm with an ISCO UA-5 absorbance monitor (Instrumentation Specialties, Lincoln, NE) and 7 mL fractions were collected with an LKB 2112 Redirac fractions collector (LKB Instruments, Bromma/Stockholm, Sweden).

Analysis of CCC Fractions

We applied the protein detection method to proteoglycans. An aliquot (0.4 mL) of each fraction was pipetted and diluted with 1.0 mL of distilled water, and the absorbance was measured at 280 nm with a Shimadzu UV-1200 spectrophotometer. Coomassie protein assay reagent and element analysis of nitrogen were used to check the existence of proteoglycans in the CCC fractions.

We applied an anthrone sulfuric acid method to detect the polysaccharide contents. The 0.2% anthrone sulfuric acid solution was prepared by mixing 300 mL of sulfuric acid with 100 mL of distilled water, and dissolving 0.8 g of anthrone shortly before use. An aliquot (0.1 mL) of each fraction was pipetted, mixed with 5 mL of the 0.2% anthrone sulfuric acid solution in test tubes, and allowed to stand in a boiling water bath for 10 min. Then, the test tubes were cooled with running water, and the absorbance was determined at 550 nm with a Shimadzu UV-1200 spectrophotometer.

RESULTS AND DISCUSSION

Partition Coefficients of Polysaccharides and Proteins

High-speed countercurrent chromatography is a two-phase procedure where the separation is based on the difference in partition coefficient of solutes within the two-phase solvent system. To achieve efficient separation of polysaccharides and proteoglycans in a high molecular weight complex, it is essential to obtain a large difference in partition coefficient between

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polysaccharides and proteoglycans. Because there are some properties of the protein in proteoglycans that is not present in polysaccharides, we directly determined the absorbance of protein at 280 nm in ultraviolet, which represents the concentration of proteoglycans, while the partition coefficient of polysaccharides was determined by the color reaction of anthrone sulfuric acid. Our results showed that the partition coefficients of polysaccharide and proteoglycans in the aqueous two-phase solvent system composed of 12.5% PEG 1000–16% potassium phosphate buffer (pH 6.8) were 0.44 and 2.45, respectively. The separation factor ($\alpha = K_1/K_2$, $K_1 < K_2$) between these two compounds is as large as 5.6, indicating that they are easily resolved by HSCCC with this polymer phase system. This also meant that the polysaccharides of *A. bidentata* root had higher affinity to the potassium phosphate rich lower phase than to the PEG rich upper phase, and the proteoglycans had just contrary affinity. In addition, it was more beneficial to obtain the polysaccharides in the lower phase rich in potassium phosphate, which is easily removed by dialysis.

CCC Purification of Polysaccharides

From the results of the partition experiment described above, a polymer aqueous two-phase solvent system composed of 12.5% PEG 1000–16% potassium phosphate buffer (pH 6.8) was selected for the HSCCC purification of polysaccharides from the high molecular weight complex of *A. bidentata* roots.

Figure 1 shows the chromatogram of the high molecular weight complex with the above polymer phase system using a separation column with a total capacity of 340 mL. The sample solution contained 2 g of the high molecular weight complex, and 5 g of each phase of the polymer phase system. The separation was performed at 500 rpm at a flow-rate of 5.8 mL/min, using the upper phase as the mobile phase. The retention of the stationary phase after the run was completed was 46.8% of the total column capacity, for the solvent front emerging in the 25th tube. Fractions 26–30 corresponding to the first peak (Fig. 1) showed the absorbance of proteins at 280 nm and that of polysaccharides at 550 nm (anthrone reaction). Fractions 75–105 corresponding to the second peak, eluted by the traversed elution, showed the absorbance of polysaccharides at 550 nm (anthrone reaction) but lacked the protein absorbance at 280 nm. The results clearly indicated that polysaccharides and proteoglycans were completely separated from the high molecular weight complex.

The fractions 26–30 were pooled together, dialyzed (MWCO 3500) for 10 d to thoroughly remove PEG, and freeze-dried to obtain some

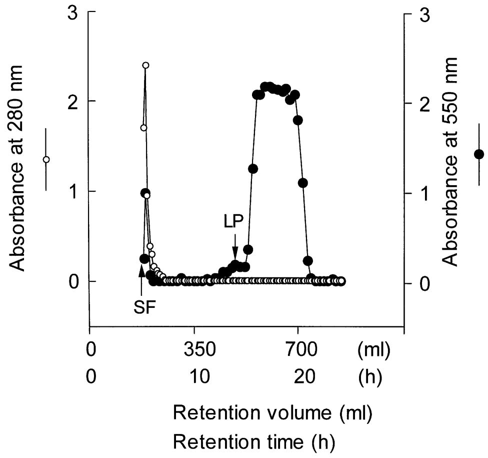

Figure 1. Purification of polysaccharides from high molecular weight complex of *A. bidentata* roots by HSCCC. Experimental conditions: apparatus: the XL cross-axis coil planet centrifuge; column: a pair of 2.6 mm-ID Teflon multilayer coils with a total capacity of 340 mL; solvent system: 12.5% (w/w) PEG 1000–16% (w/w) potassium phosphate at pH 6.8; stationary phase: phosphate-rich lower phase; mobile phase: PEG-rich upper phase; sample solution: a mixture of 2 g of high-molecular weight complex, and 5 g of each phase of the solvent system; flow rate: 0.58 mL/min; revolution: 500 rpm. SF, solvent front; LP, lower phase eluted in the reversed direction.

proteoglycans (0.391 g of white powder) with a yield of 19.6% of the high molecular weight complex sample and a yield of 2.1% (w/w) of *A. bidentata* roots.

Fractions 76–105 were pooled together, similarly dialyzed (MWCO 3500) for 10 d to thoroughly remove potassium phosphate and a small amount of PEG, and freeze-dried to obtain polysaccharides (1.103 g of white powder) at high purity. This final product lacked the absorbance at 280 nm, showed no protein reaction by Coomassie protein assay reagent, and did not contain nitrogen in the elemental analysis, and had a high yield of 55.2% of the crude sample of the high molecular weight complex or a yield of 5.91% of *A. bidentata* roots. The above yield of polysaccharides from *A. bidentata* roots obtained by the HSCCC purification technique more than doubled the yield (2.44%) of polysaccharides obtained by Sephacryl S-200 gel filtration chromatography from the same high molecular weight complex sample of *A. bidentata* roots.[6]

The overall results of our studies demonstrated that it is possible to purify the polysaccharides at a high purity, and a high yield, from the high molecular

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weight complex of *A. bidentata* roots by HSCCC using an aqueous two-phase system. The results also suggest that other polysaccharides from various plants or sclerotia such as *P. umbellatus* may be similarly carried out by the present method.

The contents of polysaccharides in a usual injection vial is only 1–2 mg, while the output of one separation by HSCCC amounts to 1.103 g of the polysaccharides, indicating that the dose for 550–1100 injections can be produced by a single HSCCC operation. Consequently, this technology may be industrialized as a small batch production in the future.

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