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Application of high-speed counter-current chromatography coupled with a reverse micelle solvent system to separate three proteins from *Momordica charantia*

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ABSTRACT

High-speed counter-current chromatography (HSCCC) coupled with a reverse micelle solvent system was successfully developed to separate three proteins from *Momordica charantia*. Suitable HSCCC conditions were carefully optimized as follows: the stationary phase was a reverse micellar phase composed of isooctane and 50 mM bis-(2-ethylhexyl)-1-sulfosuccinate sodium (AOT). The mobile phase contained mobile phase A (50 mM Tris–HCl buffer containing 50 mM KCl at pH 7.0) for forward-extraction and mobile phase B (50 mM Tris–HCl buffer containing 0.5 M KCl at pH 10.0) for back-extraction. The flow rate, detection wavelength and column temperature were set at 1.5 ml/min, 280 nm and 4 °C, respectively. Under these conditions, three fractions (I, II and III) were separated, which showed high purity when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The structures of these proteins were then identified by MALDI-TOF/TOF-MS/MS and compared with the NCBInr database. Fractions I and III were identified as resistance-like protein P-B and pentatricopeptide repeat-containing protein, respectively, which were found in *M. charantia* for the first time. However, fraction II, which is thought to be a new protein, was not identified, and further investigations on this fraction are required. The anticancer activities of these three proteins on the human gastric cancer cell line SGC-7901 were evaluated *in vitro*. The results indicated that fraction II has excellent anticancer activity (IC_{50} = 0.116 mg/ml for 48 h treatment). This is the first report on the use of HSCCC to isolate proteins from *M. charantia*.

1. Introduction

Momordica charantia (*Kugua* in Chinese), a well-known vegetable worldwide, belongs to the *Cucurbitaceae* family. It has also been used as a natural medicine in China for a long time. The famous ancient Chinese medicinal literary of the Compendium of Materia Medica recorded that *M. charantia* has detoxicating and hypoglycemic activities. In addition, pharmacologic research indicated that *M. charantia* has hypoglycemic, antibacterial, antiviral and anticancer effects [1–3]. The active chemicals in *M. charantia* are thought to be alkaloids, saponins, glycosides, triterpenes, fixed oils, proteins and steroids [4–6]. Many researchers have focused their attention on the proteins in *M. charantia* because of their outstanding effects on diabetes, HIV and cancers [7,8]. For example, as a single chain ribosome inactivating protein (30 kDa from *Kugua*), MAP-30 has antiviral and antitumor activities [9,10]. Therefore, the establishment of an efficient method to produce pure proteins from *M. charantia* is critically important for further research.

Many column chromatographic techniques have been developed for the separation of proteins from *M. charantia*, including hydroxyapatite chromatography, Sephadex gel chromatography, ion exchange chromatography and capillary electrochromatography [11–13]. However, these classic methods are tedious, have low separation efficiencies, are time consuming and require multiple chromatographic steps. High-speed counter-current chromatography (HSCCC) is a modern technique for the purification of natural products and has been applied in protein separation and enrichment [14–16]. For example, cross axis CCC and spiral CCC using a two-aqueous phase solvent system (polyethylene glycol (PEG)-potassium phosphate buffer system or a PEG-dextran system) have been used for separating proteins [17,18]. Although the two-aqueous phase solvent systems can provide suitable conditions for protein separation, they have drawbacks including low retention of the stationary phase, low recovery and poor resolution. In addition to two-aqueous phase solvent systems, the reverse micelle solvent

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system can also be used for protein separation [19,20]. In our previous studies, HSCCC coupled with reverse micelle solvent systems were successfully used to separate bromelain from *pineapple* fruit and C-phycocyanin from *Spirulina platensis* [21,22].

In this study, HSCCC coupled with a reverse micelle solvent system was successfully used to separate three proteins from *M. charantia*. Under optimized HSCCC conditions, two products including resistance-like protein P-B and pentatricopeptide repeat-containing protein were purified from *Kugua* for the first time. One protein with excellent anticancer activity on the human gastric cancer cell line SGC-7901 was also separated, however, this protein was not identified and further investigations on this protein are needed.

2. Materials and methods

2.1. Apparatus

The HSCCC system used in this study was a TBE-300A high-speed counter-current chromatograph (Shanghai Tauto Biotech, Shanghai, China) equipped with three multilayer coil separation columns connected in series (I.D. of tubing = 1.6 mm, total volume = 260 ml) and a 20 ml sample loop. The revolution radius or the distance between the holder axis and central axis of the centrifuge (R) was 5 cm, and the β values of the multilayer coil varied from 0.5 at the internal terminal to 0.8 at the external terminal ($\beta = r/R$, where r is the distance from the coil to the holder shaft). The revolution speed of the instrument could be regulated with speeds ranging from 0 to 999 rpm. The HSCCC system was equipped with a model TBP-50A constant-flow pump (Shanghai Tauto Biological Company, Shanghai, China), a UV–Vis detector (Model UV-8823B, Beijing, China) and a model WH500-USB workstation (Shanghai Wuhao Information Technology Co., Ltd, Shanghai, China). The experimental temperature was adjusted by an HX1050 constant temperature circulating implement (Beijing Boyikang Lab Implement, Beijing, China). The DYCZ-28B electrophoresis cell and DYY-6C electrophoresis power supply was provided by Beijing Liuyi Instrument Factory (Beijing, China). An Applied Biosystems MALDI-4800-TOF/TOF-MS/MS (ABI, USA) was used for protein identification.

2.2. Reagents

Isooctane used for HSCCC separation was of analytical grade (Sinopharm Chemical Reagent, Shanghai, China). Reverse osmosis Milli-Q water (18 MΩ) (Millipore, USA) was used for all solutions and dilutions. Bis-(2-ethylhexyl) sulfosuc-cinate sodium (AOT) was obtained from Tokyo Chemical Industry Co., Ltd. (Japan). Cetytrimethylammonium bromide (CTAB) was purchased from Xianduan Chemical Industry Co., Ltd. (Xiamen, China). Sodium dodecyl sulfate (SDS), acrylamide, N′N′-ethylene-bisacrylamide, ammonium persulfate, β-mercaptoethanol and bromophenol blue (Sigma–Aldrich Co., St. Louis, MO, USA) were used for electrophoresis.

The Coomassie (Bradford) Protein Assay Kit was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were purchased from Dalian Shenlian Chemical Reagent Co., Ltd. (Dalian, China).

2.3. Plant material

The fruits of *M. charantia* obtained from a market in Dalian (China) were authenticated by Dr. Yunpeng Diao (College of Pharmacy, Dalian Medical University, Dalian, China).

2.4. Crude protein extraction

Acid extraction: the juice of *M. charantia* (450 g) was mixed with an 8-fold volume of 50 mM acetic acid for 2 h and then subjected to centrifugation at 10,000 rpm for 20 min. Then, 1 M Tris buffer was added to the supernatant to adjust the pH to 7.6, and the mixture was left to stand overnight at 4 °C.

Alkali extraction: the fruits of *M. charantia* (450 g) were washed and pressed into a juice, mixed with a 10-fold volume of Tris–HCl buffer (50 mM, pH 8.2), and left to stand overnight at room temperature.

The resulting mixtures from the two extraction methods were subjected to centrifugation at 12,000 × g for 20 min, the precipitate was discarded, and ammonium sulfate was added to the upper phase to reach 80% saturation, and left to stand for 48 h at 4 °C. The mixtures were then centrifuged at 12,000 × g for 20 min. The precipitates were collected and then dialyzed in Tris–HCl buffer (50 mM, pH 8.2) for 48 h, yielding the crude proteins.

2.5. Preparation of the two-phase solvent system and sample solution

The HSCCC experiments were performed with a solvent system composed of a reverse micellar phase and a Tris–HCl buffer phase. The stationary phase was a reverse micellar phase (50 mM AOT/isooctane), mobile phase A was 50 mM Tris–HCl buffer containing 50 mM KCl (pH 7.0) for forward extraction, and mobile phase B was 50 mM Tris–HCl buffer containing 500 mM KCl (pH 10.0) for the backward extraction. Forward extraction is where the protein is removed from mobile phase A to the reverse micellar phase, and backward extraction is where the protein is extracted from the reverse micellar phase by mobile phase B.

In the HSCCC separation, 80 mg of the crude protein extracted by alkali extraction was dissolved in 20 ml of mobile phase A.

2.6. HSCCC purification

The multilayer coil column was filled with the stationary phase, and mobile phase A was pumped into the column at a flow rate of 1.5 ml/min, while the apparatus at 4 °C was rotating at 830 rpm. After the mobile phase was eluted from the tail outlet, and a hydrodynamic equilibrium was established in the column, the sample solution was injected through the injection valve. When the forward extraction was finished (at approximately 180 min), mobile phase A was changed to mobile phase B at a flow rate of 1.5 ml/min. The effluent was monitored by a UV detector at 280 nm. Each fraction was collected manually according to the profile.

2.7. Protein determination and purity analysis

The contents of the crude protein and the separated fractions were determined using Coomassie Brilliant Blue G with bovine serum albumin (BSA) as the standard, as previously reported [23]. The purities of the separated fractions were further analyzed by SDS-PAGE, which was carried out on a 12% separating gel and a 5% stacking gel. The gels were then stained with 0.1% Coomassie Brilliant Blue R250, and cleared by destaining solution which contained 10% glacial acetic acid and 45% ethanol.

2.8. Protein identification

The separated fractions were identified by MALDI-TOF/TOF-MS/MS. First, the bands of the denatured proteins were cut from the SDS-PAGE gel, destained with 50% ACN/25 mM NH_4HCO_3, and then reduced with 10 mM DTT at 56 °C and alkylated with 50 mM iodoacetamide in the dark for 1 h at room temperature. Then, the

gel plugs were lyophilized and immersed in 15 ml trypsin solution (10 ng/ml) in NH_4HCO_3 (25 mM) and left at 37 °C for 15 h. Finally, the tryptic peptide mixtures were first extracted with 5% TFA (100 ml) and then with the same volume of 2.5% TFA/50% ACN. Finally, the extracted solutions were blended, lyophilized, and used for MS/MS analysis as in our previous report [22]. The analytical conditions were as follows: Solid-state laser source (355 nm) and positively charged ions were analyzed in reflection mode. A 20,000 V pulse voltage and a 23,000 V reflection voltage were used. CHCA (α-cyano-4-hydroxycinnamic acid) was used as the matrix. The MS/MS spectra data of each protein was searched in the NCBI database.

2.9. Anticancer activity in vitro

The activities of the separated proteins from *M. charantia* were investigated in a human gastric cancer cell line SGC-7901 provided by ATCC (USA). The cells were cultured in RPMI-1640 containing 10% FCS at 37 °C in an incubator containing 0.5% CO_2. The cultured cells were detached by trypsinization, and re-suspended in fresh culture medium at a density of 2×10^5 cell/ml. Then, 100 μl of the cells were placed into 96-well flat-bottomed plates. After incubation in 5% CO_2–air mixture at 37 °C for 12 h, different concentrations of the separated products were added to the cells. Six identical wells were used for each concentration of the separated products, and six identical wells without drugs were used as the controls. The cells were incubated for another 12, 24 and 48 h, and then 10 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazo-liumbromide) (5 mg/ml) was added to each well. After 4 h incubation, 100 μl of 10% SDS was added and incubated overnight. Finally, the 96-well flat-bottomed plates were subjected to the MTT cell viability assay. The 50% inhibitory concentration (IC_{50}) of the separated proteins on the SGC-7901 cells after 48 h of treatment was calculated.

Cell morphology of apoptotic cells was investigated by staining cells using a combination of the fluorescent DNA-binding dyes, acridine orange (AO) and ethidium bromide (EB). Cells were plated onto 12-well plates (500 μl/well) at a density of 2×10^5 cell/ml and then incubated overnight. The investigated protein with anticancer activity at different concentrations was added to each well and incubated for 48 h. In addition, control wells were stained according to introduction of the examination agent box. Images of the cells were then obtained using an OLYMPUS fluorescence microscope (Japan).

3. Results and discussion

3.1. Selection of suitable extraction method

The extraction of crude protein from *M. charantia* is a critical step for further purification, and common extraction methods are acid and alkali extraction [24]. In the present paper, a comparison of the two extraction methods was carried out to identify the best method. Following acid extraction, 0.8 g crude protein with 18% protein content was obtained. However, 5.0 g crude protein with 17% protein content was obtained following alkali extraction. SDS-PAGE analysis indicated that more protein bands from the crude protein were obtained following alkali extraction than acid extraction. Thus, alkali extraction was selected to prepare the crude protein from *M. charantia* for further purification.

3.2. Selection of suitable surfactant

Successful separation of proteins by HSCCC mainly depends on a suitable two-phase solvent system. Thus, the reverse micelle solvent system used in this study was optimized step by step.

In the first step, a suitable surfactant and its concentration in the reverse micellar phase were optimized. Two types of surfactants, AOT and CTAB were investigated. CTAB is a cationic surfactant, suitable for separating proteins with high molecular weight and low isoelectric point [21]. When CTAB was used, products with a single band could not be separated, even if the mobile phase was changed (Fig. 1).

AOT is an anionic surfactant, and is suitable for purifying proteins with low molecular weight and high isoelectric point [25]. When the concentration of AOT is between 20 and 50 mM, proteins can be extracted into the reverse micelles [26]. Thus, in the present study, reverse micellar phases (AOT/isooctane) at different concentrations (20, 30 and 50 mM) were investigated, and the results indicated that when the AOT concentration was 20 or 30 mM, the reverse micelles in the HSCCC column were unstable. This resulted in low retention of the stationary phase, and the effluents were emulsive during the separation procedure. However, when the concentration of AOT reached 50 mM, the reverse micelle solvent system had good retention in the HSCCC column, and the effluents were limpid. Thus, 50 mM AOT was selected to prepare the solvent system for the separation of proteins from *M. charantia*.

3.3. Optimization of mobile phase A for forward-extraction

The second step was to choose a suitable buffer as mobile phase A with an appropriate pH value and ionic strength for forward-extraction. In previous reports, a solution composed of 50 mM Tris–HCl buffer and 50 mM KCl was frequently used as the mobile phase for protein separation [27–29]. Thus, 50 mM Tris–HCl buffer with 50 mM KCl was used as mobile phase A for forward-extraction.

When an anionic surfactant (AOT) is used, the pH value of the mobile phase should be lower than the isoelectric point (pI) of target proteins [30–32]. As the pI values of most proteins extracted by alkali extraction from *M. charantia* are between 8.3 and 9.5, the pH value of mobile phase A should be lower than 8.3 [33,34]. Therefore, mobile phase A at different pH values (6.5, 7.0, 7.5 and 8.0) was investigated. The results showed that when the pH value was adjusted to 7.5 or 8.0, the separated fractions were not purified (Fig. 2A and B). However, when the pH value was adjusted to 7.0, two fractions (Fig. 2C) with high purity were separated from the crude extract. Similar findings were obtained at pH 6.5 (Fig. 2D), however, higher retention of the stationary phase was achieved. Thus, 50 mM Tris–HCl buffer with 50 mM KCl and pH 7.0 was used as mobile phase A for forward-extraction.

3.4. Optimization of mobile phase B for backward-extraction

The third step was to optimize the pH value and ionic strength of mobile phase B for backward-extraction. First, 50 mM Tris–HCl buffer solution at various ionic strengths (0.1, 0.3, 0.5 and 0.7 M KCl) was tested using liquid–liquid extraction. The results showed that the proteins could be back-extracted when the concentration of KCl was higher than 0.3 M. Further experiments were performed on HSCCC, and the results (Fig. 3A) showed that only two fractions were separated when the concentration of KCl was 0.3 M. When the concentration of KCl was increased to 0.5 or 0.7 M, one more fraction with only one protein band on SDS-PAGE analysis was separated (Fig. 3B and C). Finally, 0.5 M KCl was selected due to higher recovery and short dialyzing time.

The pH value, another important factor of mobile phase B, was then considered. In the extraction process, the pH value must be higher than the pI of the protein to avoid the reverse micelles being destroyed. Thus, mobile phase B at pH 9.5 and 10.0 was investigated, respectively, and the results indicated that only two fractions were separated when the pH value was set at 9.5. However, when the pH value was 10.0, one more fraction was effectively isolated.

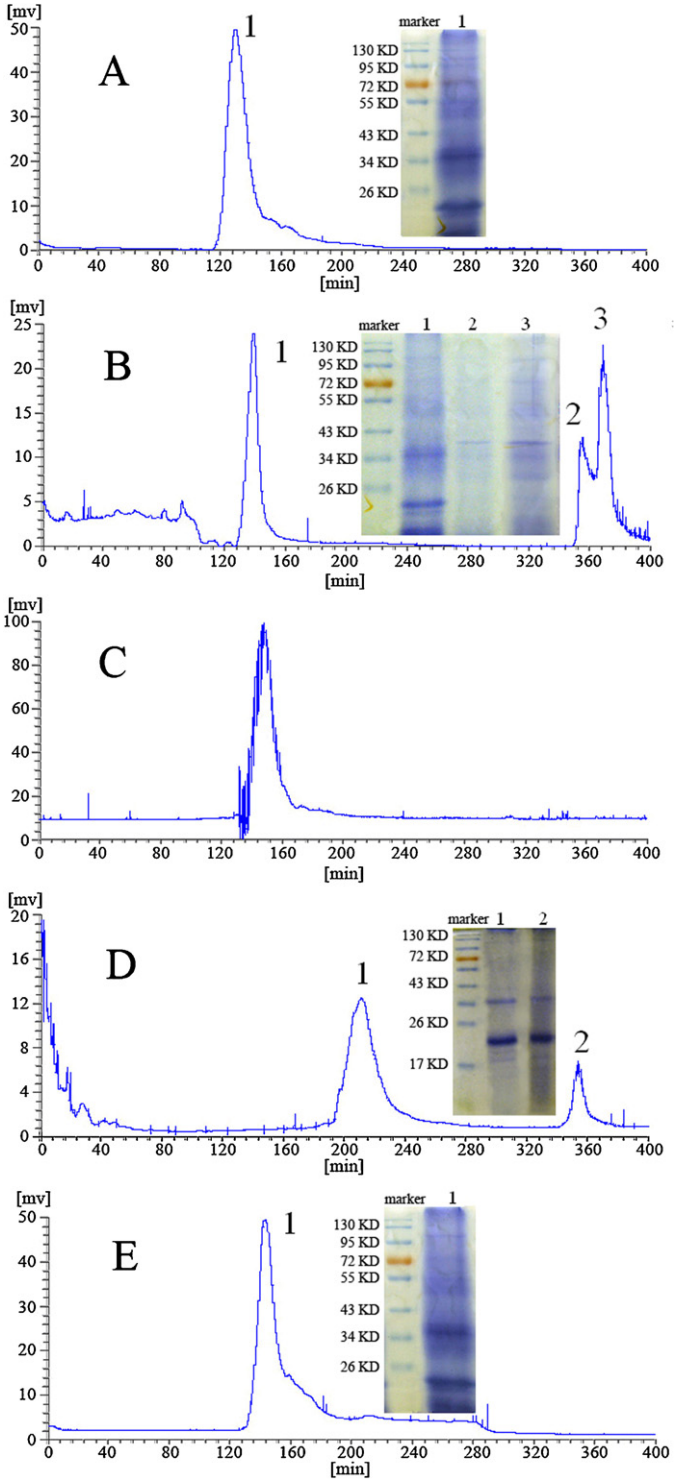

Fig. 1. Effects of CTAB in HSCCC separation and SDS-PAGE analysis of the separated fractions. Separation conditions: stationary phase: 200 mM CTAB/isooctane-hexanol. Mobile phase A: 50 mM Tris–HCl buffer containing 50 mM KCl; mobile phase B: 50 mM Tris–HCl buffer containing 1.0 M KCl. (A) mobile phase A: pH 9.0; mobile phase B: pH 6.5, (B) mobile phase A: pH 9.5; mobile phase B: pH 6.5, (C) mobile phase A: pH 10.09; mobile phase B: pH 6.5, (D) mobile phase A: pH 9.5; mobile phase B: pH 6.8, and (E) mobile phase A: pH 9.5; mobile phase B: pH 7.02.

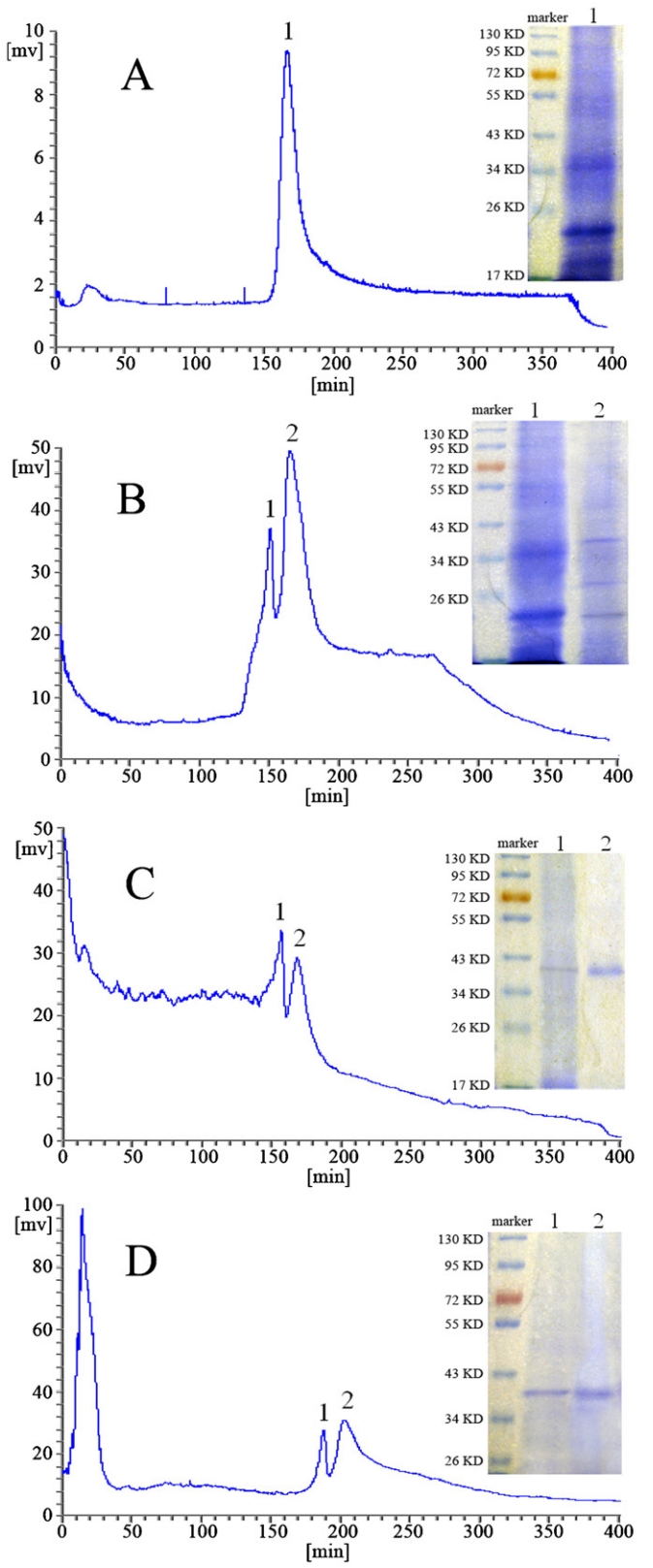

Fig. 2. Effects of pH value on forward-extraction in HSCCC separation and SDS-PAGE analysis of the separated fractions. Separation conditions: stationary phase was reverse micellar phase (50 mM AOT/isooctane), the mobile phase A was 50 mM Tris–HCl buffer containing 50 mM KCl, (A) pH 8.0; (B) pH 7.5; (C) pH 7.0; and (D) pH 6.5, while the mobile phase B was 50 mM Tris–HCl buffer containing 500 mM KCl, pH 10.0.

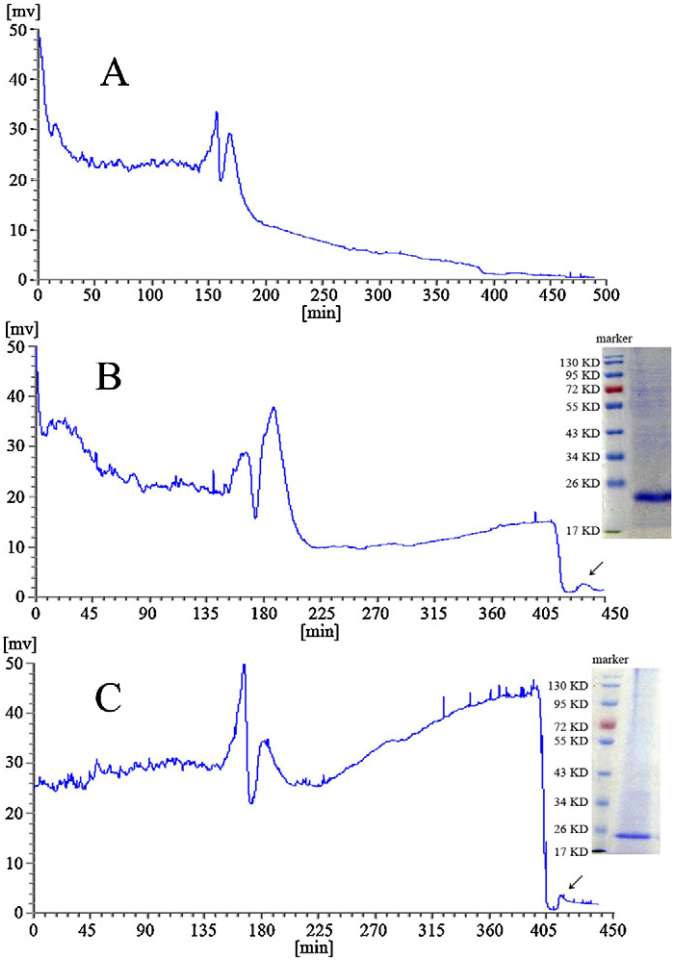

Fig. 3. Effects of ion strength on backward-extraction in HSCCC separation and SDS-PAGE analysis of the separated fractions. Separation conditions: stationary phase was reverse micellar phase (50 mM AOT/isooctane), the mobile phase A was 50 mM Tris–HCl buffer containing 50 mM KCl, pH 7.0; the mobile phase B was 50 mM Tris–HCl buffer containing (A) 0.3 M KCl; (B) 0.5 M KCl; (C) 0.7 M KCl, pH 10.0.

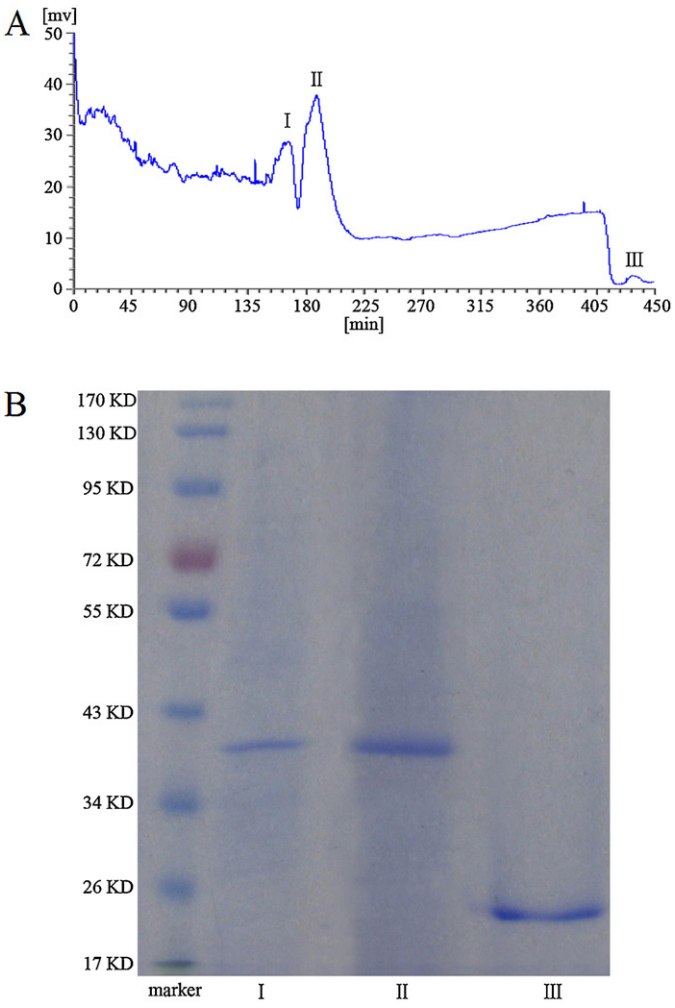

Fig. 4. HSCCC chromatogram for separating proteins from the crude extract of *M. charantia* (A). The images of the separated proteins analyzed by PAGE and stained by Coomassie blue R250 (B).

Thus, 50 mM Tris–HCl with 0.5 M KCl at pH 10.0 was used as mobile phase B.

Other factors, such as the amount of sample, flow rate of the mobile phase, revolution speed of the apparatus and separation temperature were investigated separately. The optimum values for these factors were found to be 80 mg, 1.5 ml/min, 830 rpm and 4 °C, respectively. The HSCCC chromatogram, under these optimized conditions, is shown in Fig. 4A.

In order to confirm the reproducibility of the developed method, repeated separation procedures were carried out. Although, the profiles of the HSCCC chromatograms were not uniform (data not shown), three fractions (I, II and III) were separated in each run. The amounts and purity of these products were similar. Thus, good reproducibility of the established method for separating proteins from *M. charantia* using HSCCC coupled with a reverse micelle solvent system was obtained.

Three fractions were successfully purified from *M. charantia* by HSCCC using optimized conditions, in which the stability of the reverse micelle solvent system was critically important for protein separation. Many factors including pH value, ionic strength, flow rate of the mobile phase, column temperature and rotation speed of the apparatus can affect the stability of the reverse micelle solvent system. When the pH value and ionic strength were out with the optimal range, the reverse micelle solvent system was unstable. High flow rate of the mobile phase, rotation speed and column temperature can cause loss of the stationary phase and emulsification of the reverse micelle solvent system, which can result in separation failure due to the unstable solvent system.

3.5. Protein determination and purity analysis

The protein contents of the produced fractions (I, II and III) were 0.86 g/g, 0.94 g/g and 0.93 g/g, respectively. The collected fractions were further analyzed by SDS-PAGE, and each fraction contained one protein band and showed high purity (Fig. 4B).

3.6. Protein identification

The separated proteins were identified by MALDL-TOF/TOF-MS/MS, and the data were matched with the NCBInr database. Fraction I was identified as resistance-like protein P-B (139.94 KD molecular mass and pI 7.93) with a protein score of 64. Fraction III was identified as pentatricopeptide repeat-containing protein (60.6 KD molecular mass and pI 8.67) with a protein score of 94, which can affect the expression of mitochondrial mRNA and tRNA [35]. However, fraction II was not matched with the database, and could be a new protein.

3.7. Anticancer activities of the separated proteins

In order to investigate the bioactivities of the separated proteins, growth inhibition of a human cancer cell line was determined. The separated proteins at six different concentrations (0.033, 0.067, 0.133, 0.267, 0.534 and 1.068 mg/ml) were used to treat SGC-7901 cells for 12, 24 and 48 h, respectively. The results indicated that fractions I and III did not have anticancer activity, and only fraction II showed obvious concentration- and time-dependent growth inhibition of cancer cells, and the IC_{50} value after 48 h of treatment was 0.116 mg/ml. The effect of fraction II on SGC-7901 cells was investigated under a fluorescent microscope using AO/EB double staining after the cells were exposed to various concentrations of fraction II for 48 h. The cancer cells emitted a green fluorescence and no significant death or apoptosis were observed in the control and lowest concentration groups. However, apoptosis with orange and densely cardinal red stained colors in cells treated with the higher concentration of fraction II were observed.

4. Conclusion

In the present study, a new and effective method of separating proteins from the fruit of *M. charantia* was successfully developed using HSCCC coupled with a reverse micelle solvent system. Two proteins, resistance-like protein P-B and pentatricopeptide repeat-containing protein, were separated from *Kugua* for the first time, and one unknown protein with significant anticancer activity was also separated. Thus, HSCCC coupled with a reverse micelle solvent system is a useful technique for protein separation and enrichment.

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