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Short communication

Counter-current chromatographic separation of glycoprotein components from *Morchella esculenta* (L.) with a polymer phase system by a cross-axis coil planet centrifuge

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Abstract

Using a cross-axis coil planet centrifuge, glycoproteins were separated from fermentation media of *Morchella esculenta* (L.) by high-speed counter-current chromatography. The performance of the apparatus was optimized with four standard proteins including pepsin, lysozyme, ovalbumin and hemoglobin and a polymer phase system composed of 12.5% (w/w) polyethylene glycol 8000 and 25% (w/w) potassium phosphate in distilled water at various pH values. Separations were performed by eluting the lower phosphate-rich phase at a flow-rate of 1.0 ml/min. Under the optimized conditions three glycoprotein components in *Morchella esculenta* (L.) were resolved within 6 h.

Keywords: Counter-current chromatography; *Morchella esculenta*; Glycoproteins; Proteins

1. Introduction

The mushroom of *Morchella esculenta* (L.) contains glycoproteins which show anti-carcinogenic activity as well as an enhancement of the immune system by stimulating leucocyte production [1,2]. Since counter-current chromatography (CCC) performs separation without the solid support matrix [3], adsorptive loss and denaturation of proteins at the liquid–solid interface are minimized, it is an excellent alternative for the bio-molecular separation. This paper described the separation of glycoproteins from *M. esculenta* using a polymer phase system introduced by Albertsson [4].

2. Experimental

2.1. Apparatus

The cross-axis coil planet centrifuge (X-axis CPC) (Model 96 X-a instrument made at the Beijing Institute of New Technology Application, Beijing, China) holds a pair of horizontal rotary shafts

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symmetrically, one on each side of the rotary frame at a distance of 12.5 cm from the central axis of the centrifuge. A spool-shaped column holder is mounted on each rotary shaft at a lateral location 14.5 cm away from the midpoint. Each multilayer coil was prepared from 2.6 mm I.D. PTFE (polytetrafluoroethylene) tubing by winding it onto a 7.6 cm diameter holder forming multiple layers of left-handed coils between a pair of flanges spaced 5 cm apart. The column consists of six layers of the coil with a 150-ml capacity. The design of the instrument is similar to that described elsewhere [5].

The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Institute of New Technology Application). Continuous monitoring of the effluent was achieved with a Model 8823A-UV monitor (Beijing Institute of New Technology Application) operating at 280 nm. A manual sample injection valve with a 2- or 20-ml loop (Tianjin High-New Science and Technology, Tianjin, China) was used to introduce the sample into the column and a Model FC-95 auto fraction collector (Beijing Institute of New Technology Application) for fractionation. A portable recorder (Yokogawa Model 3057, Sichuan Instrument Factory, Chongqing, China) was used to draw the chromatogram.

2.2. Reagents

Polyethylene glycol (PEG) 8000, monobasic and dibasic potassium phosphates were of analytical grade and purchased from Beijing Chemical Factory (Beijing, China) while acetonitrile was of HPLC grade. Pepsin (porcine mucosa), lysozyme (chicken egg white) ovalbumin (chicken egg white) and bovine hemoglobin were purchased from Sigma (St. Louis, MO, USA).

2.3. Preparation of glycoprotein samples from M. esculenta

The culture liquid (1000 ml) of *M. esculenta* from deep layer fermentation was treated by ultracentrifugation to remove low-molecular-mass components, defatted with hexane and decolorized by passing through activated carbon, then followed by concentration under reduced pressure at 0°C. The concentrate was further purified by ultra centrifugation and repeated precipitation with ethanol to obtain 580 mg of crude glycoprotein.

2.4. Preparation of polymer two-phase solvent systems and sample solution

Aqueous polymer phase systems were prepared by either dissolving 125 g of PEG 8000 and 250 g of dibasic potassium phosphate in 1625 ml of distilled water or dissolving 125 g of PEG 8000 and 300 g of potassium monobasic phosphate in 1575 ml of distilled water. The pH of these solvent systems was adjusted to a desired value with HCl or NaOH. Each solvent mixture was thoroughly equilibrated in a separatory funnel at room temperature by repeating vigorous shaking and degassing several times, allowing the mixture to completely separate into two clear layers before use.

Sample solution was prepared by dissolving each standard protein in 1 ml lower phase of the two-phase solvent system used for separation. For glycoprotein separation 100 mg of the crude sample was dissolved in 1 ml of lower phase of the two-phase solvent system used for separation.

2.5. Measurement of partition coefficients of standard protein samples

The partition coefficient of each standard protein was determined spectrophotometrically using a simple test tube procedure. A 2-ml volume of each phase of the equilibrated two-phase solvent system was delivered into a test tube to which 1 mg of the sample was added. The contents were thoroughly mixed and allowed to settle at room temperature. After the two clear layers were formed, a 1-ml aliquot of each phase was diluted with 2 ml of distilled water and the absorbance was measured at 280 nm using a spectrophotometer (Model UV-2501PC, Shimadzu). The partition coefficient was obtained by dividing the absorbance value of the upper phase by that of the lower phase.

2.6. CCC separation of proteins

For each separation, the coiled column was first completely filled with the stationary upper phase.

This was followed by injection of the sample solution through the sample port. Then the mobile phase was eluted through the column at 1.0 ml/min while the apparatus was rotated at 500 rpm. The effluent from the outlet of the column was continuously monitored with an 8823 A-UV detector at 280 nm with a Yokogawa 3057 recorder and collected into test tubes (5.0 ml/tube) with an FC-95 fraction collector.

2.7. Analysis of CCC fractions of glycoprotein

All collected fractions were analyzed by high-performance liquid chromatography (HPLC) using a methyl linoleate-silica-based polymer-bonded column. The 7 μm support (Langzhou Institute of Chemistry and Physics, Chinese Academy of Sciences, Beijing, China) was suspended in 1,4-dioxane–carbon tetrachloride (1:2, v/v) and packed by the downward flow method into a stainless steel cartridge (150 mm×4.6 mm I.D.) using ethanol as a packing solvent. Elution was performed with mobile phase A: 0.1% trifluoroacetic acid (TFA) in 100% deionized water and mobile phase B: 0.1% TFA in 100% acetonitrile applying a 20-min linear gradient from 5% B to 100% B.

2.8. Molecular mass estimation of glycoprotein

Molecular mass of fractionated glycoprotein was estimated by high performance size-exclusion chromatography (HPSEC). The HPSEC system consisted of a Waters 600 controller pump, an injector fitted with a 20-μl loop, HPSEC columns (Ultrahydrogel 2000 and 500 coupled in series) and a Waters 410 interference refractometer. The column temperature was maintained at 30°C. Six PEG molecular mass standards including M_r 963 000 (WAT 011576), M_r 531 000 (WAT 011584), M_r 89 600 (WAT 011578), M_r 42 900 (WAT 011576), M_r 24 800 (WAT 011574) and M_r 9000 (WAT 011568) were used, and a 0.1 mol/l phosphate buffer solution (pH 6.8) was eluted at a flow-rate of 0.6 ml/min.

3. Results and discussion

CCC is a liquid–liquid partition method where the separation is based on the difference in solute partition coefficients. The polymer phase systems useful for partitioning proteins may be divided into two types, i.e., PEG–inorganic salt and PEG–dextran systems [6,7]. The PEG–salt systems have relatively high interfacial tension and low viscosity and provides suitable partition coefficient values for many protein samples while viscous PEG–dextran systems are mainly used for partitioning cell particles. One advantage of the PEG–phosphate system is that low molecular mass compounds are mostly partitioned unilaterally either in the upper or the lower phase while macromolecules such as proteins are distributed rather evenly between the two phases, hence contamination of small molecules are largely eliminated from the fraction. In the present experiments, the PEG–potassium phosphate system was used and the partition coefficient values of proteins were optimized by manipulating the pH of the solvent system. Fig. 1 shows a chromatogram of four standard proteins including pepsin, ovalbumin, lysozyme and hemoglobin. Using the optimized solvent system at pH 9, pepsin and hemoglobin showed the baseline separation while lysozyme and ovalbumin were only partially resolved. The partition coefficient (K) of these proteins are indicated in the Fig. 1 caption.

Fig. 2 shows a chromatogram of *M. esculenta* glycoprotein (100 mg) together with the HPLC

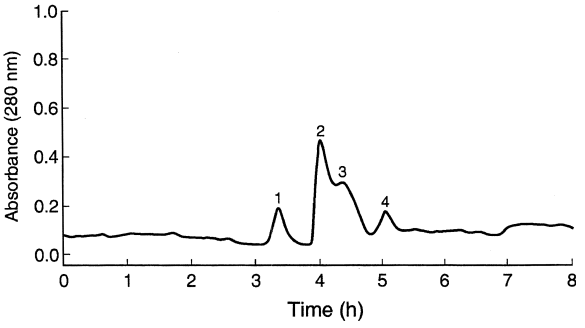

Fig. 1. Chromatogram of proteins obtained by the X-axis CPC. Experimental conditions: 10–15 mg each in 1 ml solvent; solvent system: 12.5% (w/w) PEG 8000–25% (w/w) K_2HPO_4 (pH 9.0) in distilled water; mobile phase: lower phase; flow-rate: 1 ml/min; revolution: 500 rpm. Sample: 1, pepsin (K=1.849); 2, ovalbumin (K=0.298); 3, lysozyme (K=0.530); 4, hemoglobin (K=0.980) where the partition coefficient is expressed by solute concentration in the upper phase divided by that in the lower phase.

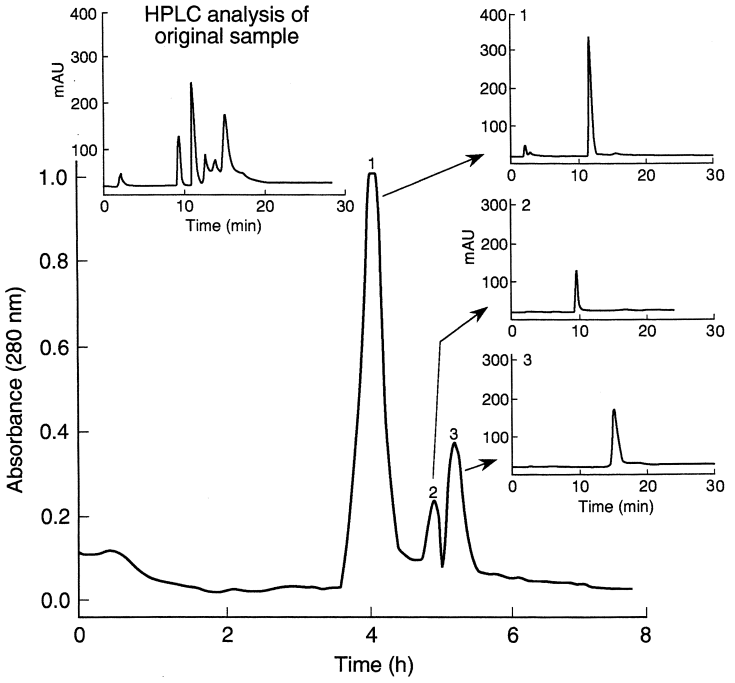

Fig. 2. Chromatogram of glycoprotein obtained by the X-axis CPC. Experimental conditions: 100 mg *Morchella esculenta* glycoprotein in 1 ml solvent. Solvent system: 12.5% (w/w) PEG 8000–25% (w/w) K_2HPO_4 (pH 9.0) in distilled water; mobile phase: lower phase; flow-rate: 1 ml/min; revolution: 500 rpm. HPLC conditions are as follows: methyl linoleate-silica-based polymer-bonded column (7 μm, 150×4.6 mm) (laboratory-made), mobile phase A: 0.1% TFA in 100% deionized water, mobile phase B: 0.1% TFA in 100% acetonitrile, 20 min gradient from 5% B to 100% B, flow-rate: 1.0 ml/min. Sample size: 10 μl of 10 mg/ml (original crude sample, left) and 4 mg/ml (each CCC fraction, right).

analysis of the original sample and CCC peak fractions. Three glycoprotein components 1, 2 and 3 were resolved and eluted within 6 h. The resolution between neighboring peaks are 2.0 (peaks 1 and 2) and 0.9 (peaks 2 and 3). The amount of each component recovered from 100 mg of the crude sample was 49.1 mg (glycoprotein 1), 10.2 mg (glycoprotein 2), and 22.3 mg (glycoprotein 3). It is interesting to note that the elution order of three glycoproteins is different between CCC and HPLC: the middle peak (peak 2) in CCC was eluted first in HPLC. The molecular masses of three glycoproteins in CCC fractions were determined by HPSEC as shown in Fig. 3 as follows: 65 700 for glycoprotein 1, 22 400 for glycoprotein 2 and 11 300 for glycoprotein 3. Although the difference in molecular shape between the glycoprotein and PEG used as the molecular mass standards may cause some uncertainty about the precise molecular mass of each glycoprotein component, these results indicate that the glycoprotein components were eluted in the order of decreasing molecular masses in CCC.

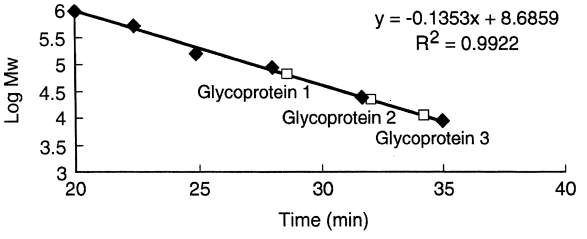

Fig. 3. Molecular mass profile of glycoproteins by HPSEC. Experimental conditions: Waters HPLC system. Columns: Ultrahydrogel 2000 and 500 coupled in series. Column temperature: 30°C mobile phase: 0.1 mol/l phosphate buffer (pH 6.8); flow-rate: 0.6 ml/min. Solid points indicate standards.

4. Conclusion

The overall results of our studies indicate that the cross-axis CPC can be efficiently used for fractionation of glycoprotein components from a crude sample mixture of *Morchella esculenta* (L.) by adjusting the composition and pH of a PEG–potassium phosphate biphasic system.

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