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Short communication

One-step purification of α-amylase from the cultivation supernatant of recombinant *Bacillus subtilis* by high-speed counter-current chromatography with aqueous polymer two-phase systems

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Abstract

Purification of α-amylase from the cultivation supernatant of recombinant *Bacillus subtilis* by high-speed counter-current chromatography (HSCCC) in polyethylene glycol (PEG) 4000-inorganic salt aqueous polymer two-phase systems was studied. The effects of sodium chloride concentration on the partition coefficients of α-amylase and total protein were respectively tested in PEG4000-phosphate and PEG4000-citrate aqueous polymer two-phase systems to find the proper range of sodium chloride concentration for the HSCCC purification of α-amylase. α-Amylase was purified from the cultivation supernatant by HSCCC in PEG4000-phosphate system containing 2% (w/w) sodium chloride, yet with considerable loss of activity. PEG4000-citrate aqueous polymer two-phase system containing 2% (w/w) sodium chloride and supplemented with 0.56% (w/w) $CaCl_2$ as protective agent was then successfully applied to purify α-amylase from cultivation supernatant by HSCCC to homogeneity and significantly increased the recovery of α-amylase activity from around 30 to 73.1%.

Keywords: High-speed counter-current chromatography; Aqueous polymer two-phase system; α-Amylase; Purification

1. Introduction

High-speed counter-current chromatography (HSCCC) is a continuous liquid–liquid partition chromatography without solid support matrix, the stationary phase of which is retained in the separation columns by gravity and centrifugal force field [1,2], and therefore avoids the disadvantages arising from the interaction of samples with the solid support, such as absorb and denaturation of target products. HSCCC has the unique features of high recovery, high efficiency and the ease to scale-up, and has been widely used in the separation and purification of natural products, antibiotics and rare elements with organic/aqueous systems [3,4]. As for the purification of biological macromolecules, the aqueous polymer two-phase systems with mild conditions, such as high water content (70–90%) and low interfacial tension, are good choice and have been widely used for the purification of proteins, nucleic acids and cells [5–7]. The combination of aqueous polymer two-phase system with HSCCC, could further resolve the limitations of aqueous polymer two-phase system extraction, including low efficiency in single-step operation and difficulties to perform continuous extraction.

However, the high viscosity and low interfacial tension of aqueous polymer two-phase system tends to cause emulsification of the two phases and results in lower and unstable retention of stationary phase in conventional HSCCC apparatus, such as type-J coiled planet centrifuge [8]. Hence, the research on the purification of biomolecules by HSCCC with aqueous polymer two-phase system includes both the improvement of the structure of HSCCC apparatus and the optimization of the separation conditions of aqueous polymer two-phase system. A series of HSCCC apparatus suitable for aqueous polymer two-phase systems was designed by Ito

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and co-worker, including cross-axis coil planet centrifuge [9], nonsynchronous coil planet centrifuge [10] and spiral disk assembly fitted on synchronous coil planet centrifuge[11], among which, the cross-axis coil planet centrifuge has better balance between the complexity of structure and the separation efficiency, and have been used for the separation and purification of a variety of proteins including hen egg white [12], cholinesterase [13], single-strand DNA binding protein [14], glycoprotein [15], glucosyltransferase [16].

Recently, a novel self-designed HSCCC apparatus, model TBE-300V has been fabricated by Tauto Biotech, which is a vertical multi-column synchronous counter-current chromatographic apparatus with features of simplified structure, self-balance without counterweight, controllable temperature and the ease to scale-up. The HSCCC purification of α-amylase from the cultivation supernatant of recombinant *Bacillus subtilis* was studied with said apparatus in this paper. α-Amylase (1,4-α-D-glucan glucanohydrolases; EC 3.2.1.1) is a commercially important enzyme which is widely used in many industrial fields, such as starch processing, brewage, sugar refining, textile treatment, detergent and fermentation [17]. The conventional purification procedures of α-amylase include precipitation with ammonia sulphate, absorb with starch, ion-exchange chromatography and size-exclusion chromatography. The total recovery of α-amylase after above steps is only around 40% [18]. Therefore, the one-step HSCCC-PPS purification of α-amylase was performed in this paper, with the intention of increasing the efficiency and recovery of purification.

2. Experimental

2.1. Chemicals

PEG4000 (average molecular mass of 4000), dipotassium hydrogen phosphate, trisodium dihydrogen phosphate, trisodium citrate, citric acid, sodium chloride and calcium chloride were of reagent grades and from Shanghai Chemical Reagent (Shanghai, China).

2.2. Equipments

HSCCC apparatus TBE-300V was from Tauto Biotech (Shanghai, China), three coiled separation columns with six layers of coiled PTFE tube were connected in series (inner diameter of PTFE tube = 2.6 mm, revolution radius = 50 mm, β value = 0.51–0.74, total volume = 120 mL) with a 20 mL sample loop. The temperature of separation columns was controlled by water circulator (HX-1050, Boyikang Lab Instrument, Beijing, China). The rotation speed of separation columns was adjusted between 700 and 1000 r/min.

TBE-300V was coupled to Äkta Prime (GE Healthcare) for liquid pumping, sample detection and fraction collection (shown in Fig. 1).

2.3. Preparation of aqueous polymer two-phase systems

PEG4000-phosphate aqueous polymer two-phase system were prepared by dissolving 130 g PEG4000, 100 g NaH_2PO_4 and 40 g K_2HPO_4 into 730 g distilled water and then thoroughly mixing in a separatory funnel at room temperature, followed by equilibrating the system till two clear layers formed. Sodium chloride was added as required.

PEG4000-citrate aqueous polymer two-phase system were prepared by dissolving 130 g PEG4000, 120 g trisodium citrate and 20 g citric acid into 730 g distilled water and and then thoroughly mixing in a separatory funnel at room temperature, followed by equilibrating the system till two clear layers formed. Sodium chloride was added as required.

2.4. Preparation of sample solutions for HSCCC

Add 1.42 g PEG4000 and 1.53 g phase-forming salts (1.09 g NaH_2PO_4, 0.44 g K_2HPO_4 and 1.31 g trisodium citrate and 0.22 g citric acid for phosphate and citrate aqueous polymer two-phase systems, respectively) into 8 mL of the cultivation supernatant of recombinant *Bacillus subtilis* to meet the composition of the aqueous polymer two-phase system used for purification, i.e. 13.0% (w/w) PEG1000-14% (w/w) phosphate system and 13.0% (w/w) PEG1000-14%

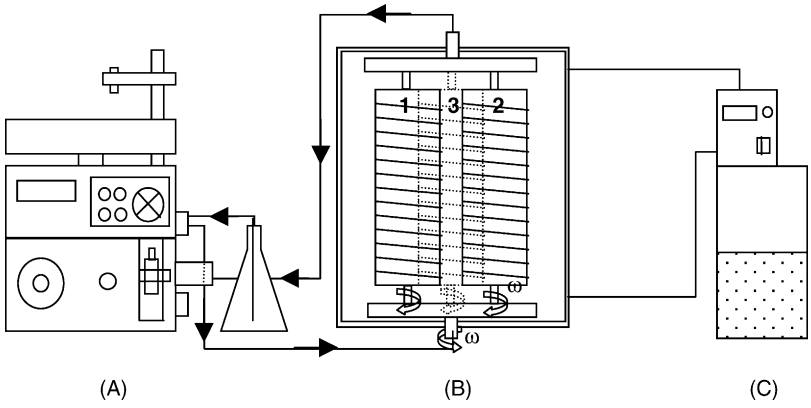

Fig. 1. Diagram of HSCCC, model TBE-300V. (A) Äkta Prime; (B) TBE-300V; (C) water bath; (1–3) coiled separation columns.

(w/w) citrate system. Sodium chloride was added as required. Gently stir until all solids dissolved.

2.5. HSCCC operation

Fill the separation columns of HSCCC apparatus with the stationary upper phase, followed by pumping mobile phase in head to tail mode at 0.4 mL/min; meanwhile the apparatus was rotated at 880 r/min. The effluent from the outlet of the column was monitored at 280 nm. After the mobile phase flowed out of the outlet and the absorbance became stable, indicating that the equilibration between mobile and stationary phase had been established in the column, note the volume of flowing-out stationary phase (V_f). The retention of stationary phase before loading sample is defined as the ratio between the volume of the station phase retained in the separation columns (V_r) and the total volume of the separation columns (V_t). The sample solution was injected through the sample loop. The elution peak was collected by the fraction collector and assayed for α-amylase activity and protein content. After purification the aqueous polymer two-phase system in the separation column was blown out with compressed air into a measuring cylinder and the retention of stationary phase after purification was recorded again.

2.6. Assays

α-Amylase activity was measured using the starch-iodine method [19]. One unit of α-amylase activity was defined as the amount of enzyme catalyzing the hydrolysis of 1.0 g soluble starch in 1 h at pH 6.0 and 60 °C.

Total protein concentration was measured by the method of Bradford [20]. The samples were read at 595 nm against the blanks with the same compositions as the samples, but without any proteins, to avoid the interference of PEG and phase-forming salt.

2.7. Electrophoresis

Fractions collected were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% (w/v) acrylamide gels according to the method of Laemmli [21]. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250.

3. Results and discussion

3.1. Effects of sodium chloride concentration on the partition coefficients of α-amylase and total protein in PEG4000-inorganic salt aqueous polymer two-phase systems

According to our previous report upon the purification of α-amylase with PEG-citrate aqueous polymer two-phase system [22], the partition coefficient of α-amylase could be efficiently adjusted by altering the sodium chloride concentration of aqueous polymer two-phase system containing PEG with high molecular mass of above 3350. The partition coefficients of α-amylase and total protein were respectively tested in PEG4000-citrate and PEG4000-phosphate aqueous polymer two-phase system with different concentration of sodium chloride to find the proper concentration of NaCl for effective separation. The pH of both aqueous polymer two-phase system were set at 6.0 by adjusting the ratio between the alkaline and acidic salts in the phase-forming salts (K_2HPO_4, NaH_2PO_4 and trisodium citrate, citric acid for phosphate and citrate aqueous polymer two-phase systems respectively), at which α-amylase is most stable and exhibit the highest activity [17]. Partition coefficient was defined as the ratio between the concentration or activity of target protein in upper phase and lower phase. The results were shown in Fig. 2.

The partition coefficients of α-amylase and total protein both increased with the increase of NaCl concentration, but at different extents in both systems, resulting in larger difference between the partition coefficients of α-amylase and total proteins. However, at the NaCl concentration of 4% (w/w), α-amylase substantially partitioned into the upper phase, which made it hard to elute with lower phase as mobile phase. The NaCl concentration between 0 and 2% was preferred to perform HSCCC purification of α-amylase, since the partition coefficient of α-amylase is around 1, which could make the contaminant proteins with lower partition coefficients to elute

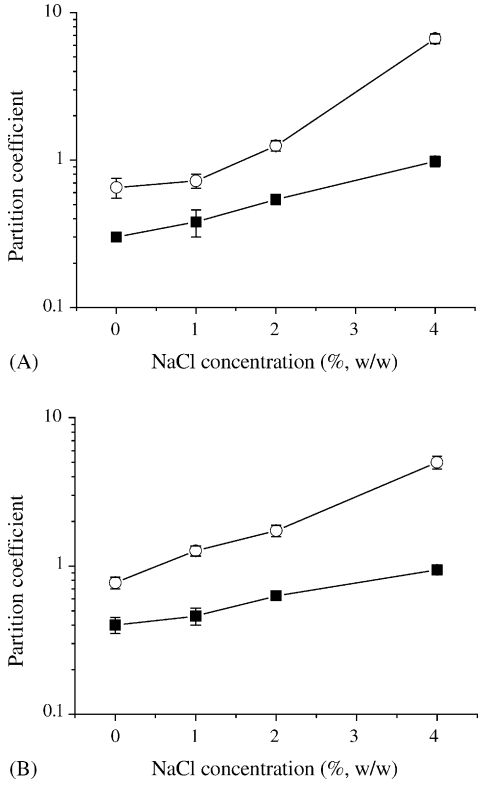

Fig. 2. Effects of NaCl concentration on the partition coefficients of α-amylase (◯) and total protein (■) in PEG4000-phosphate (A) and PEG4000-citrate (B) aqueous polymer two-phase systems, respectively.

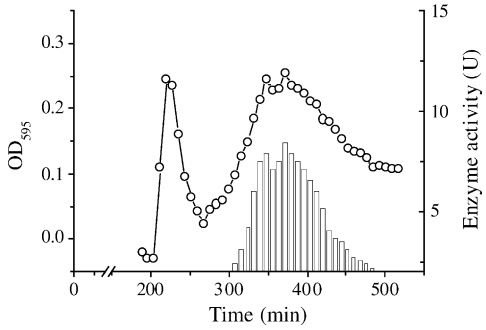

Fig. 3. Effect of NaCl concentration on the HSCCC purification of α-amylase in PEG4000-phosphate aqueous polymer two-phase system containing 2% (w/w) NaCl. (◯) Total protein concentration; (column) α-amylase activity. Experimental conditions: sample solution, 8 mL cultivation supernatant, prepared as described in Section 2; solvent system: 13.0% (w/w) PEG4000-10.0% (w/w) NaH_2PO_4-4% (w/w) K_2HPO_4 in distilled water; mobile phase: lower phase; flow rate: 0.4 mL/min; revolution: 880 r/min; retention of stationary phase before loading sample and after purification: 38 and 35%, respectively.

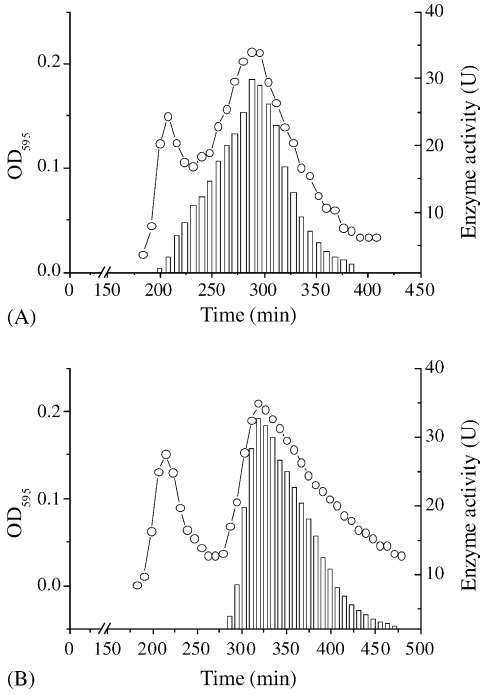

Fig. 4. Effect of NaCl concentration on the HSCCC purification of α-amylase in PEG4000-citrate aqueous polymer two-phase system: (A) 1% (w/w) NaCl; (B) 2% (w/w) NaCl; (◯) Protein concentration; (column) α-amylase activity. Experimental conditions: sample solution, 8 mL cultivation supernatant, prepared as described in Section 2; solvent system: 13.0% (w/w) PEG4000-12.0% (w/w) trisodium citrate-2% (w/w) citric acid in distilled water; mobile phase: lower phase; flow rate: 0.4 mL/min; revolution: 880 r/min; retention of stationary phase before loading sample and after purification: 38 and 35%, respectively.

firstly and the elution time of target protein to be practically short as well.

3.2. High-speed counter-current chromatographic purification of α-amylase in PEG4000-inorganic aqueous polymer two-phase systems

PEG4000-phosphate aqueous polymer two-phase systems with NaCl concentration of 0, 1 and 2% were firstly selected to perform HSCCC purification of α-amylase, due to its good phase-forming capacity and system characteristics such as lower viscosity, shorter phase-separation time (data not shown), compared with PEG4000-citrate systems. The resultant HSCCC chromatographs and α-amylase activity profiles in the presence of 2% sodium chloride were shown in Fig. 3.

Two protein elution peaks could be attained by HSCCC in PEG4000-phosphate aqueous polymer two-phase system. As the increase of NaCl concentration, the retention time of α-amylase activity increased and correlated well with the retention time of the second protein peak at the NaCl concentration of 2% (w/w), indicating that the second protein peak is mainly composed of α-amylase. Merely, the recovery of total α-amylase activity is as low as around 30%, while the one for total protein is above 90%, indicating severe loss of α-amylase activity occurred during the HSCCC separation process, which was caused by the deprivation of Ca^{2+} and may be alleviated by using PEG-citrate aqueous polymer two-phase system supplemented with Ca^{2+}, according to our previous studies [22].

PEG4000-citrate aqueous polymer two-phase systems supplemented with 0.56% (w/w) $CaCl_2$ as protective agent was further applied to perform HSCCC purification of α-amylase in order to improve the recovery of α-amylase. The effect of NaCl concentration on the HSCCC purification of α-amylase was tested in PEG4000-citrate aqueous polymer two-phase system and the results were shown in Fig. 4 and Table 1.

Two elution peaks were also attained by HSCCC in PEG4000-citrate aqueous polymer two-phase system, as in PEG4000-phosphate system (Fig. 3). α-Amylase was

Table 1
Effects of NaCl concentration on the retention time and the recovery of α-amylase activity in PEG4000-phosphate aqueous polymer two-phase system

Sample activity (U)	NaCl Concentration of solvent system (%, w/w)	Retention time of elution peak[a]		α-Amylase activity peak[b]		Recovery of total protein (%)
		Peak 1 (min)	Peak 2 (min)	Retention time (min)	Recovery (%)	
440	1	200	288	288	71.5	90.2
440	2	215	320	320	73.1	91.4

[a] Elution peaks determined by Bradford method.
[b] Elution peak determined by α-amylase activity assay.

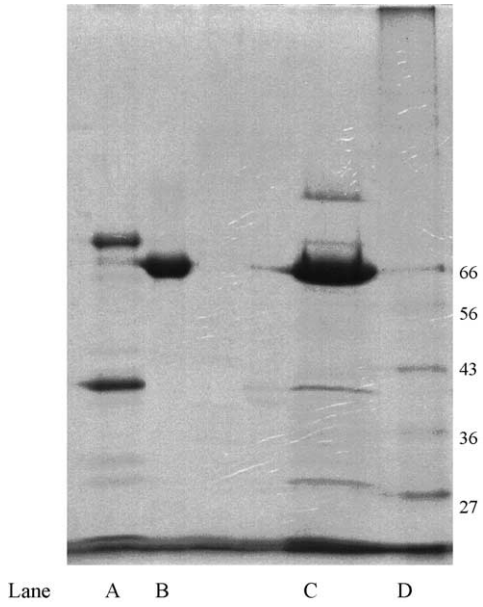

Fig. 5. Electrophoresis results of HSCCC purification of α-amylase in PEG4000-citrate aqueous polymer two-phase system. Lane A: elution peak 1 in Fig. 4, concentrated around five times by ultra-filtration; Lane B: elution peak 2 in Fig. 4, concentrated around five times by ultra-filtration; Lane C: fermentation supernatant; Lane D: molecular mass standards.

eluted after the elution of most contaminant proteins and the difference between the retention time of α-amylase and contaminant proteins increased with the NaCl concentration. In the presence of 2% (w/w) NaCl (Fig. 4B), α-amylase was substantially separated from contaminant proteins, which was indicated by the good correlation between the protein concentration and α-amylase activity of the second elution peak as well as the electrophoresis results (Fig. 5). Meanwhile, the recovery of α-amylase was greatly improved from around 30 to 73.1%, which proved our previous suggestion. The nearly 30% loss of α-amylase activity in PEG4000-citrate systems could be caused by the high ionic strength of the PEG-inorganic salt aqueous polymer two-phase systems.

4. Conclusion

PEG4000-phosphate and PEG4000-citrate aqueous polymer two-phase systems were applied to perform one-step purification of α-amylase from the cultivation supernatant of *Bacillus subtilis* by high-speed counter-current chromatography. The purification efficiency of both aqueous polymer two-phase system was similar, yet the latter aqueous polymer two-phase system supplemented with 0.56% $CaCl_2$ could greatly increase the recovery of α-amylase activity from around 30 to 73.1% by sustaining the activity with high concentration of Ca^{2+}. These results demonstrated the promising prospect of HSCCC purification of biological macromolecules in aqueous polymer two-phase system with high efficiency and recovery.

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