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REVIEW

Advances in Technology of Countercurrent Chromatography for Separation of Protein and Peptide

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Abstract: Countercurrent chromatography is a kind of continuous and effective liquid-liquid partition chromatography with many unique characteristics such as large load capacity, no irreversible adsorption, high recovery rate, low risk of sample denaturation and so on, which has irreplaceable advantages in the separation of proteins and peptides. This review presents the advances of several kinds of new technology of countercurrent chromatography in the separation of proteins and peptides. The development prospect of this field is also discussed.

Key Words: Countercurrent chromatography; Protein; Peptide; Review

1 Introduction

Proteins and peptides are the material basis of life. With the deep-going research on structures, functions and applications of proteins and peptides in the field of bioscience, it is becoming increasingly urgent to develop high-efficiency technology to separate proteins and peptides from complicated biosystem.

Countercurrent chromatography (CCC) is a continuous and effective liquid-liquid partition chromatographic technique, of which the stationary phase and the mobile phase are two immiscible phases. In the separation process, the stationary phase is retained in the separation column by centrifugal field, while the mobile phase is pumped through the stationary phase at a certain flow rate. The sample will partition repeatedly in the two phases after it is loaded in the system. Different components of the sample will be eluted at different time according to their partition coefficients, thus separation is achieved. CCC has been widely applied in the separation and purification of natural products[1]. Various flavonoids[2], polyphenols[3], alkaloids[4], and terpenoids[5] have been separated and prepared by CCC. CCC has many advantages such as large load capacity, no irreversible adsorption, high recovery rate and low risk of sample denaturation. The sample can be loaded in the system without complicated pretreatment. Compared with HPLC, CCC uses no expensive chromatographic column[6], thus could be developed into an important technology to effectively separate proteins and peptides from complicated biosystem. CCC has already been used in separation and purification of proteins and peptides. Table 1 summarizes some research reports on the separation and purification of proteins and peptides using CCC. According to the retaining mode of the stationary phase and the design of the separation column, CCC can be classified into two types: hydrostatic countercurrent chromatography and hydrodynamic countercurrent chromatography. The difference between the two types of instruments is whether the gravitational field generated by the instrument is constant or not. This paper reviews the

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Table 1 Application of countercurrent chromatography in the separation and purification of proteins and peptides

Proteins or peptides	Instrument	Solvent systems	Ref.
Human B19 parvo virus-like particles	Centrifugal partition chromatography	14.8% (*w/w*) PEG 400-16.28% (*w/w*) phosphate (pH 3.0)-7.5% NaCl in water	[7]
Val-tyr, trp-tyr Lysozyme, myoglobin	Centrifugal partition chromatography	1-Butanol-acetic acid-water (4:1:5, *V/V*) 12.5% (*w/w*) PEG 1000-12.5% (*w/w*) K_2HPO_4 in water	[8]
Recombinant cyanovirin-N	Centrifugal partition chromatography	13% PEG 4000-13% sodium phosphate (pH 3.0) in water	[9]
Monoclonal antibody 1, 2 and 4	Centrifugal partition chromatography	21.02% (*w/w*) PEG 400-18.99% (*w/w*) citrate in water	[10]
Gly-Gly, Gly-Tyr, Ala-Tyr, Leu-Val and Leu-Tyr	Centrifugal partition chromatography	Methyl-tert-butylether-acetonitrile-*n*-butanol-water (2:1:2:5, *V/V*). 15 mM DEHPA was added to the upper phase	[11]
Lysozyme, myoglobin	Toroidal coil countercurrent chromatography	12.5% (*w/w*) PEG 1000-12.5% (*w/w*) K_2HPO_4 in water	[12]
Enramycin-A and -B	High-speed countercurrent chromatography	1-Butanol-hexane-0.05% aqueous trifluoroacetic acid solution (43:7:50, *V/V*)	[13]
Segetalin A and B	High-speed countercurrent chromatography	Petroleum ether-ethyl acetate-methanol-water (0.5:3.5:1:5, *V/V*)	[14]
Collagenase I, II, IV, V and X	Cross-axis countercurrent chromatography	12.5% (*w/w*) PEG 1000–12.5% (*w/w*) K_2HPO_4 in water at pH 9.2	[15]
Val-tyr, trp-tyr Lysozyme, myoglobin	Spiral countercurrent chromatography	1-Butanol–acetic acid-water (4:1:5, *V/V*) 12.5% (*w/w*) PEG 1000-12.5% (*w/w*) K_2HPO_4 in water	[16]
Peptide (GIHIGPGRAFYAARK) Acyl carrier protein fragment-peptide (VQAAIDYING) Peptide (fPRGGGGNGDFEEIPEEYL, f = D-Phe) Ubiquitin-like fusion protein	Spiral countercurrent chromatography	2-Butanol-0.1% aqueous trifluoroacetic acid (1:1, *V/V*) 12.5% (*w/w*) PEG 1000-12.5% (*w/w*) K_2HPO_4 in water	[17]
Carotenoid cleavage-like enzymes	Spiral countercurrent chromatography	25% (*w/w*) PEG 1000-6.25% (*w/w*) KH_2PO_4-6.25% (*w/w*) K_2HPO_4 in water	[18]

advances of these two types of countercurrent chromatography in the separation and purification of proteins and peptides.

2 Hydrostatic countercurrent chromatography

The instrument of hydrostatic countercurrent chromatography has a single rotation axis, which can generate a constant centrifugal field. The two phases of the solvent system could not move alone in the rotating hydrostatic CCC column. A pump is needed to make the mobile phase percolate through the stationary phase. The back pressure generated in the system is relatively stronger during the run[19]. The hydrostatic countercurrent chromatography technologies mainly include centrifugal partition chromatography and toroidal coil countercurrent chromatography.

2.1 Centrifugal partition chromatography

The structure diagram of centrifugal partition chromatography (CPC) is shown in Fig.1[20]. There is a rotation axis in the central of the instrument. The separation column of CPC is a series of locules engraved in disks. The adjacent locules are connected by ducts. The entrance and exit of the tubes stretch out from the inside of the rotation axis and are connected with the outside through two rotary sealing joints. CPC has a high stationary phase retention rate for most of solvent systems. Aqueous two-phase system (ATPS) is often used in separation and purification of proteins and peptides. Sutherland *et al*[21] separated a model sample system of a mixture of lysozyme and myoglobin by CPC using an ATPS system comprising 12.5% (*w/w*) PEG-1000-12.5% (*w/w*) K_2HPO_4. At first, a laboratory-scale CPC equipped with a 500-mL column was used to separate the sample, and the resolution was 1.28. Then, a pilot-scale CPC equipped with a 6.25-L column was used to separate the sample. The resolution was 1.88, and the preparative throughput was 1.65 g h^{-1} (40 g d^{-1}). This study indicates that it is relatively easy to scale up in the process of separation and purification of proteins and peptides using CPC. The stationary phase retention rate was improved when the experiment was linearly scaled up to pilot scale. Consequently, the resolution was also improved. Oelmeier *et al*[22] purified four monoclonal antibodies by CPC using an ATPS system comprising 16.5% (*w/w*) PEG 400-17.45% (*w/w*) NaH_2PO_4-17.45% (*w/w*) K_2HPO_4. The host cell protein (HCP) clearance was improved through this method. The recoveries of the target proteins were above 98%. In Boudesocque's work[23], a kind of dipeptide (l-valyl-l-tryptophan, VW) was enriched from a complex alfalfa white protein concentrate hydrolysate using CPC by the combination of the solvent system, methyl-tert-butylether-acetonitrile-*n*-butanol-water (2:1:2:5, *V/V*) with di(2-ethylhexyl)phosphoric acid (DEHPA) cation-exchanger added in the upper phase, and two displacers: calcium chloride and hydrochloric acid. The dipeptide showed good anti-hypertensive property. The purity of the dipeptide was 10.9%, corresponding to a purification factor of 41 and a recovery of 97%. These two studies above indicate that CPC can be used in the separation and purification of proteins and