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Short communication

Continuous protein concentration via free-flow moving reaction boundary electrophoresis

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ABSTRACT

In this work, we developed the model and theory of free-flow moving reaction boundary electrophoresis (FFMRB) for continuous protein concentration for the first time. The theoretical results indicated that (i) the moving reaction boundary (MRB) can be quantitatively designed in free-flow electrophoresis (FFE) system; (ii) charge-to-mass ratio (Z/M) analysis could provide guidance for protein concentration optimization; and (iii) the maximum processing capacity could be predicted. To demonstrate the model and theory, three model proteins of hemoglobin (Hb), cytochrome C (Cyt C) and C-phycocyanin (C-PC) were chosen for the experiments. The experimental results verified that (i) stable MRBs with different velocities could be established in FFE apparatus with weak acid/weak base neutralization reaction system; (ii) proteins of Hb, Cyt C and C-PC were well concentrated with FFMRB; and (iii) a maximum processing capacity and recovery ratio of Cyt C enrichment were 126 mL/h and 95.5% respectively, and a maximum enrichment factor was achieved 12.6 times for Hb. All of the experiments demonstrated the protein concentration model and theory. In contrast to other methods, the continuous processing ability enables FFMRB to efficiently enrich diluted protein or peptide in large volume solution.

1. Introduction

In many cases, protein concentration in bio-sample is very diluted, such as recombinant protein [1], denature and renaturation of inclusion bodies [2], and the one after sieve chromatographic separation. Under those situations, diluted protein samples have to be concentrated before further research or purification process.

The commonly-used concentration methods include precipitation, ultrafiltration, lyophilization and chromatography technique. The "salting out" method, the primary precipitating way, always requires a dialysis step to remove salts before the next separation process [3,4]. The precipitation with the organic reagent, such as the acetone and TCA, may result in protein degradation problem [5]. Ultrafiltration is increasingly used for protein concentration. However, the filters are usually blocked by proteins [6]. Freeze-drying method can well maintain activity of the target protein, but it still has some inherent limitation, such as over drying, time consuming and low efficiency. Chromatography technique is another powerful tool for protein concentration, include ion-exchange chromatography and affinity chromatography [7,8]. However, it is a batch-like concentration method and has low processing capacity. Commercial manufacturing of enzymes and biologic drugs commonly requires the processing of thousands of liters of a starting feedstock [9]. Therefore, sustainability is an additional and increasingly demanded requirement [4].

As a continuous processing technique, free-flow electrophoresis (FFE) provides a potential tool for continuous protein concentration. FFE is an important electrophoretic technique which is capable of continuously separating bio-mixtures in mild conditions maintaining high biological activities. It has been used for separation of polypeptides, proteins, cells, cell organelles, and microorganisms [10–14]. However, FFE is rarely used for concentration purpose. In 2009, we developed the method of free-flow moving reaction

Abbreviations: C-PC, C-phycocyanin; Cyt C, cytochrome C; FFE, free-flow electrophoresis; FFMRB, free-flow moving reaction boundary electrophoresis; FFZE, free-flow zone electrophoresis; Hb, hemoglobin; MRB, moving reaction boundary; Z/M, charge-to-mass ratio.

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boundary electrophoresis (FFMRB) for high efficient concentration of amino acids [15]. In FFMRB, a huge pH gap at the moving reaction boundary (MRB) was created by two different phases. Amino acids were driven by the electric field and finally concentrated at the MRB based on the isoelectric focusing principle. In our previous work, the sample solution was injected with up to 20 inlets simultaneously and about tenfold condensation was achieved.

Compared with other concentration methods, FFMRB has several advantages. The first and foremost is its continuous processing capacity which enables FFMRB to process large volume sample. Second, its mild buffer circumstance allowing high biological activities of bio-samples. Third, there is no absorbance material used in FFE chamber resulting in quite high recovery. However, the method of FFMRB has not been used for protein or peptide concentration. Particularly, the model and theory of FFMRB have not been advanced for the protein concentration yet.

The purposes in this work are to (i) advance the model and theory of protein concentration in FFMRB, (ii) develop the relevant method of protein concentration, and (iii) demonstrate the theory and method of FFMRB by systematical experiments. To the authors' knowledge, it is the first work to develop the model and theory of FFMRB for protein enrichment.

2. Materials and methods

2.1. Proteins and chemicals

C-phycocyanin (C-PC, Mr 40 kDa, pI 5.2) from *spirulina* and Cytochrome C (Cyt C, Mr 12.2 kDa, pI 9.6) from equine heart were got from BioChemAn Biotechnology Co., Ltd. (Shanghai, China). Hemoglobin (Hb, Mr 64.5 kDa, pI 6.8) from bovine blood was bought from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Pierce® BCA Protein Assay Kit was bought from Thermo Scientific (Rockford, USA). Sodium hydroxide, formic acid, sodium formate, ammonium hydroxide, neutral red and other chemical regents were analytical reagent grade and obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Deionized water obtained from a Milli-Q system (Millipore, MA, USA) was used for all separation solutions.

2.2. Instruments

A Canon EOS 70D Digital SLR Camera (Canon, Japan) was used for recording MRB or protein concentrating conditions in FFE chamber. A Mettler Toledo delta 320 pH Meter (Mettler-Toledo, Switzerland) was used for detecting pH values in buffer preparation. A Bio-Rad Power Pac™ HV (Bio-Rad, USA) was used as power supply. A PE&ENSPIRE 2300 plate reader (PerkinElmer, USA) was used for spectrophotometric detection.

A self-balance FFE device (FFE-32, Shanghai BioChemAn Biotechnology Co., Ltd, Shanghai, China) was used for performing FFMRB. The instrument had 32 inlets and 32 outlets, and the separation chamber was 245 mm × 60 mm × 0.4 mm. The anode chamber and the cathode chamber were isolated from the separation chamber with two conductivity membranes. Two 16-channel pumps were employed for buffer or sample injection. In this system, a self-balance collector was employed to stabilize the liquid flow in separation chamber [15]. First, the solution level in collector-tubes always kept at the same horizontal level due to gratis gravity and principles of connected vessel. Second, collector-tubes had same shape and were set at same horizontal level. Therefore, solution flowed into collector-tubes at the same flow rate. Meanwhile, solution was pumped into separation chamber with the same flow rate for each inlet. Theoretically, solution pumped into the separation chamber via specific inlet just flowed into the corresponding collector-tubes, result in stable flow pattern.

2.3. Solutions and samples

Solution of 20 mM sodium formate was adjusted to pH 10.0 with 2.0 M ammonium hydroxide, used as background buffer of phase β. Solutions of 10 mM, 15 mM, 17.5 mM and 20 mM formic acid were adjusted to pH 3.5 with 4.0 M sodium hydroxide, used as background buffer of phase α, respectively. In MRB velocity studies, neutral red was added to both of the two phases as indicator with a concentration of 10 μg/mL. Solutions of 0.1 M phosphoric acid and 0.1 M sodium hydroxide were used as anode buffer and cathode buffer, respectively.

In protein concentration, the 20 mM formate-ammonium buffer (pH 10.0) was used for phase β. The solution of 17.5 mM formate buffer (pH 3.5) was used as phase α. C-PC, Hb and Cyt C were dissolved in phase α with the same concentration of 0.1 mg/mL, respectively.

2.4. Procedures of computation

In MRB velocity computation, the following physico-chemical parameters were used (25 °C). Formic acid: pKa = 3.75; hydrogen (H^+): $m_0 = 36.3 \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$; hydroxyl ($OH^-$): $m_0 = 20.5 \times 10^{-8}\ m^2\ V^{-1}\ s^{-1}$. The MRB velocity was computed according to the method in Ref. [16].

For charge-to-mass ratio (Z/M) analysis, the protein sequence information was got from the UniProtKB (http://www.uniprot.org/) and pI and Mr (molecular weight) were computed by the ExPASy (http://web.expasy.org/compute_pi/). With the sequence information, protein charge profiles under different pH circumstance was calculated by CLC Main Workbench 7.5.1 (CLC bio) [17].

In maximum processing capacity estimation, protein electrophoretic velocity in phase α (V_{pro}^{α}) was determined by free-flow zone electrophoresis (using the same FFE apparatus) [15,17]. Solution of 17.5 mM formate buffer (pH 3.5) was used as background buffer, the sample solutions with protein concentration of 0.1 mg/mL was injected as a narrow stream and a voltage of 360 V was applied. The V_{pro}^{α} was the lateral migration distance divided by the retention time.

2.5. Procedure of FFMRB

First, the electrode buffer pump was started, the anode buffer and cathode buffer circulated through the corresponding electrode chamber at a flow rate of 300 mL/h per vessel. As shown in Fig. 1A, the acid solution was continuously pumped into separation chamber at the left side creating phase α. The alkaline solution was continuously pumped into separation chamber at the right side forming phase β. Both of phase α and phase β were driven by two 16-channel pumps, which were set at the same flow rate of 0.3 mL/min per channel in order to maintain the stable fluid layer in the separation chamber. A voltage of 200 V was applied for FFMRB. The MRB profiles in the separation chamber were record with a camera for future analysis.

In the protein concentrating experiments, proteins were dissolved in phase α at the concentration of 0.1 mg/mL before the FFMRB concentration. As shown in Fig. 1B, sample solutions were continuously pumped into separation chamber with flux of 83.8, 69.5 and 126 mL/h for Hb, C-PC and Cyt C, respectively. A voltage of 250 V was applied. After an hour's electrophoresis, all the fractions were collected and the protein content in each fraction was estimated by BCA Protein Assay Kit according to product specifications.