Journal of Chromatography A, 1397 (2015) 73–80

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Target protein separation and preparation by free-flow electrophoresis coupled with charge-to-mass ratio analysis

Qiao-Yi Shen[a,1], Chen-Gang Guo[a,1], Jian Yan[a,b], Qiang Zhang[a], Hai-Yang Xie[a], Sharmin Jahan[a], Liu-Yin Fan[a], Hua Xiao[a,**], Cheng-Xi Cao[a,*]

[a] State Key Laboratory of Microbial Metabolism, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China
[b] Institute of Refrigeration and Cryogenics, School of Mechanical Engineering, Shanghai Jiao Tong University, Shanghai 200240, China

ARTICLE INFO

Article history:
Received 19 January 2015
Received in revised form 1 April 2015
Accepted 4 April 2015
Available online 11 April 2015

Keywords:
Preparative electrophoresis
Cytochrome C
Protein purification
Separation optimization

ABSTRACT

Herein, a novel strategy was developed to separate and prepare target protein from complex sample by free-flow electrophoresis (FFE), which mainly based on the charge-to-mass ratio (*C*/*M*) analysis of proteins. The *C*/*M* values of three model proteins, namely Cytochrome C (Cyt C), myoglobin (Mb) and bovine serum albumin (BSA) were analyzed under different pH and the separation of these proteins was predicted by CLC Protein Workbench software. Series of experiments were performed to validate the proposed method. The obtained data showed high accordance with our prediction. In addition, the chamber buffer (CB) of FFE system was optimized to improve the resolution of separation. Meanwhile, in order to evaluate the analytical performance of the proposed method, Cyt C was extracted from swine heart and further separated by FFE based on *C*/*M* analysis. Results showed that Cyt C was completely separated from the crude sample and a purity of 96.9% was achieved. The activity of prepared Cyt C was 98.3%, which indicate that the proposed method is promising in a wide variety of research areas where the native properties of proteins should be maintained for downstream analysis.

1. Introduction

Target sample purification is an important step in bioanalysis for interference free and precise outcome. In the past few decades, numerous efforts have been made toward the development of innovative, simple, robust, and inexpensive separation techniques to address this issue. As a matrix-free electrophoretic technique, free-flow electrophoresis (FFE) has been applied for the separation of numerous biological samples, such as proteins [1–3], ion-pair reagents from isolated components [4], cells [5] as well as organelles [6]. Matrix-free feature of FFE make it highly unique over conventional technique, which lead to its following merits: (i) high sample recovery [7,8]; (ii) good preservation of biological activity [7–9]; (iii) continuous separation; and (iv) low cost [8–10].

According to the size of separation chamber, there are two types of FFE, viz. large scale FFE (LS-FFE) and micro scale FFE (μ-FFE) [10]. The LS-FFE has great potential for sample preparation, due to its high throughput and continuous separation [10–12]. On the contrary, the μ-FFE was usually applied for sample analysis and detection owing to the merits of small sample demand and time-saving [13–15]. Although LS-FFE has been developed as a separation tool for about fifty years [16], this approach has not been widely used for preparative separation of biological samples. This situation may be caused by the following three issues: (i) troublesome operation procedures in LS-FFE; (ii) non-ideal Joule heating dissipation in LS-FFE, which greatly reduced its repeatability; and (iii) great difficulty in developing reasonable separation methods and experimental conditions for specific biological samples.

To address the first two issues, Chen [17] designed a novel FFE device with gas cushion injector and self-balance collector, which greatly simplified the procedure of chamber buffer (CB) injection and fraction collection. Shao et al. [18,19,10] successfully separated organic compounds, proteins, cells from biological samples through using a similar device. Yan [20] further optimized the mechanical structure of FFE device and greatly improved its performance, including a more compact separation chamber, a faster air bubble removal unit, and an anti-leakage design. Meanwhile, a thermoelectric cooling system was added to the device to control the Joule heating dissipation, which greatly improved the repeatability of the device.

* Corresponding author at: School of Life Science and Biotechnology, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, China.
Tel.: +86 21 3420 5820; fax: +86 21 3420 5820.
** Corresponding author.
E-mail addresses: huaxiao@sjtu.edu.cn (H. Xiao), cxcao@sjtu.edu.cn (C.-X. Cao).
[1] These authors have equal contribution to the paper.

http://dx.doi.org/10.1016/j.chroma.2015.04.012
0021-9673/

To solve the last issue, scientists have attempted to optimize the running buffer and electric field of FFE system to suppress band broadening [21,22]. Initially, the selection of separation condition of FFE system was random and empirical. For example, Nath [23] utilized electrophoretic titration method to predict the pH of background buffer and separated different enzymes, successfully. However, the titration method was labor-consuming and reagent-wasting. With the help of computer simulation software, scientists have tried to predict the separation results and optimize the experimental conditions. Fonslow [24] used COMSOL software to mimic the pressure profiles of fluid for the development of a new FFE device and realized the optimization of flow field in separation. Through employing MATLAB software, Xu [25] simulated the mass-transfer process and calculated the range of voltage to optimize the operational conditions. Zhang et al. [26] programmed computer software to simulate the FFE operation parameter (e.g., electric field, flow rate, and pH) for the prediction of separation results. However, the principle of fundamental design for the separation of different proteins, such as alkaline, neutral and acidic proteins, by FFE is still not clear.

Therefore, the main purpose herein is to develop a fundamental strategy for the separation of model alkaline protein via FFE. To achieve the purpose, we first used the charge-to-mass ratio analysis of CLC Protein Workbench software for the separation design of model alkaline protein cytochrome C (Cyt C, pI 9.6) from the other two model proteins, namely myoglobin (Mb, pI 6.9) and bovine serum albumin (BSA, pI 4.8). Then, we performed comprehensive experiments to confirm our theoretical design. Finally, we conducted the separation and preparation of Cyt C from swine heart to verify the efficacy of the developed strategy.

2. Experimental

2.1. Chemical reagents

Tris, dithiothreitol (DTT), N,N,N′,N′-tetramethylethylene diamine (TEMED) and sodium dodecyl sulfate (SDS) were purchased from Bio-Rad (CA, USA). Zeolite artificial (Permutit 60-80mesh), ammonium sulfate, sodium dithionite, silver nitrate (analytical reagent grade, AR) and Coomassie Brilliant Blue R-250 were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Acrylamide and N,N′-methylenebisacrylamide were got from Fluka company (St. Louis, USA). Myoglobin from equine skeletal muscle (Mb, Mr 17,000, pI 6.8–7.0), Equine heart Cyt C (Mr 13,000, pI 9.6) and bovine serum albumin (BSA, Mr 67,000, pI 4.7–4.9) were purchased from Sigma–Aldrich China (Shanghai, China). All other analytical reagent grade chemicals were purchased from local companies in Shanghai. All solutions were prepared with 18.3 MΩ ultrapure water (Merck Millipore, USA) unless otherwise noted.

2.2. Instruments and simulation software

A FFE instrument [20] (Mode FFE-24, Shanghai BioChemAn Biotechnology Co., Shanghai, China) was used for protein separation in this work. There were 12 inlets and 24 outlets connected with the separation chamber, and the size of separation chamber was 150 mm × 45 mm × 0.5 mm (L × W × H). The adjustable range of temperature of the cooling system was from 4 °C to 20 °C under the conditions of less than 1000 V and the electrical conductivity was 1.5 mS/cm.

A BIAOMA JB90-D electromotion stirrer (Shanghai Specimen and Model Factory, China) was used for stirring and mixing slurry. A Shengxi CX-250 versatile grinder (Shanghai Shengxi Pharmaceutical Machinery Co., China) was used for grinding tissue. A type-320 pH Meter (Mettler-Toledo, Switzerland) was used for adjusting the pH of solutions. During FFE run, D3100 digital camera (Nikon, Japan) was used for imaging. SDS-PAGE was carried out on Mini VE system from GE (Buckinghamshire, UK). A UMAX Powerlook 2100XL-USB (UMAX Taiwan) was used to image SDS-PAGE results. A ST360 microplate reader (Shanghai Kehua Bioengineering. Co., China) was used for protein concentration measurement.

The protein sequences of the three model proteins were retrieved from the NCBI protein database. Then CLC Protein Workbench 5.0 (CLC bio) was utilized to analyze the charge they carry at different pH value.

2.3. Buffers and samples

Tris–acetic (1 M Tris adjusted to pH 6.0, pH 8.0 and pH 10.0 by 1 M acetic acid, respectively), Tris–HCl (1 M Tris adjusted to pH 8.0 by 1 M hydrochloric acid), and phosphate buffers (1 M dibasic sodium phosphate adjusted to pH 8.0 by 1 M monosodium phosphate) were prepared as stock solution, respectively. 1 M stock solution of Tris–acetic with pH 6.0, 8.0 and 10.0 was diluted into 30 mM solutions as CBs. 21 mM (pH 8.0, electrical conductivity 1.02 mS/cm) Tris–HCl buffers, 29 mM (pH 8.0, electrical conductivity 1.02 mS/cm) Tris–acetic buffers and 5.75 mM (pH 8.0, electrical conductivity 1.02 mS/cm) phosphate buffers were prepared, respectively, to optimize the CBs of FFE system. The electrode buffer (EB) was the same as CB, except for its conductivity, which was 10 times higher than that of CB.

In order to optimize the FFE system, six buffers were used to prepare protein samples, including (A) 29 mM Tris–acetic buffer pH 8.0, (B) 30 mM Tris–acetic buffer pH 6.0, (C) 30 mM Tris–acetic buffer pH 8.0, (D) 30 mM Tris–acetic buffer pH 10.0, (E) 21 mM Tris–HCl pH 8.0, and (F) 5.75 mM phosphate buffers pH 8.0. BSA, Mb and Cyt C were prepared at the concentration of 5 mg/mL, respectively. The three protein mixture was used as the model sample for optimizing the FFE system.

2.4. Cyt C purification strategy

Fig. 1 shows the schematic diagram of Cyt C separation from swine heart tissue through 6 steps. The detailed procedures were described as follows: (1) fresh pig hearts were rinsed with PBS to eliminate excess fat and further grounded into paste by the grinder mentioned in Section 2.2; (2) the mashed heart tissue was mixed with distilled water (1:2, v/v) and the mixture was then adjusted to pH 4.0 by sulfuric acid. After stirring for 2 h at 20 °C, the mixture was titrated to pH 7.0 with 2 M ammonia. The homogenized tissue was filtrated through eight layers of gauze and the filtrates were collected. The remaining tissue residue was homogenized again and the filtrates were combined; (3) synthetic zeolite (60–80 mesh) was added to the filtrates at the ratio of 10:1 (w/v) to adsorb the proteins. After stirring for 40 min and standing for a while, the supernatant was discarded. Cyt C adsorbed on the synthetic zeolite was washed with distilled water for 3 times, 0.2% NaCl for 4 times until the eluents were clear; (4) The proteins on the zeolite were eluted with 25% ammonium sulfate solution. The concentration of ammonium sulfate was increased to 45% (w/v) to precipitate large molecular weight proteins. (5) Subsequently, transparent supernatant was collected and 25 ml of 20% trichloroacetic acid (TCA) was slowly added at the ratio of 1:40 (v/v). The precipitated proteins were dissolved in CB and then transferred into a dialysis bag, dialyzed until no sulfuric acid residual could be detected. The prepared sample was the crude mixture containing Cyt C; (6) the FFE separation of the crude mixture containing Cyt C was carried out using the optimized system to prepare high purity Cyt C products.