Preparation of Transferrin Reference Material with High Purity and Function from Cohn Fraction IV

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Abstract. A mild and novel process including on one-step chromatography was developed for the preparation of transferrin reference material with high purity and function from Cohn fraction IV, by product of the Cohn fraction process. The method we have established is simple in operation and easy to enlarge. The transferrin product obtained with purity greater than 93% and a yield above 85%. Then the Tf secondary structure as well as function evaluated by circular dichroism (CD) proved no significantly varied.

1. Introduction

Tf (transferrin) is a single-chain plasmatic glycoprotein composed of 679 amino acids primarily involved in iron transport and metabolism [1-3]. Its molecular weight was 79kDa with an isoelectric point of 5.9, consisting of two subunits known as the N-lobe and the C-lobe. The plasma concentration of Tf is about 2.8 g/L and 30% of which is iron-saturated [4-6]. Several studies have shown that Tf has significant physiological function and application such as antimicrobial functions, growth factor effects on mammalian cell proliferation and differentiation [7-8]. Moreover, Tf can be used as a marker to detect some diseases with a high content in anemia patients while a low in coronary heart disease [9]. Tf mediated drug delivery system is one of the most important application [10], such as targeting iron-mediated retinal degeneration by local delivery of transferrin.

Due to widespread use of Tf-mediated drug targeting in cancer, obtaining effective activity and high purity of transferrin is particularly critical. Several methods for separating Tf have been published, however, the purity was low and process was more complicated of these method. Saçlıgil et al. purified the transferrin by magnetic immunoaffinity beads, however, the high cost of immunomagnetic beads is not suitable for large-scale. In view of the shortage of blood resources, Cohn fraction IV, byproduct of the human plasma fractionation, is a better choice as starting materials.

2. Experimental

2.1. Materials

Cohn fraction IV, the raw materials, was supplied by Hualan Biological Engineering Inc. (Xinxiang, China). Tf with nominal purity of 95% and Coomassie Blue G-250 stain were bought from Sigma-Aldrich (St. Louis, MO, USA). Capto DEAE and other chromatography media were purchased from GE
Healthcare Life Science. Prestained protein molecular weight marker was from Fermentas (Fermentas, EU). All other chemicals and reagents were of analytical grade, and the water of HPLC grade. The chromatography steps were performed on ÄKTA purifier 100(GE Healthcare Life Science, UK).

2.2. Purification

2.2.1. Anion exchange chromatography. Dissolve Cohn fraction IV in water and the supernatant was centrifuged at 10000×g for 30min at 4°C (Biofuge stratus centrifuge, Thermo scientific). The clarified supernatant after centrifugation is collected ready for following purification. Equilibrate the column with 10 ml Capto DEAE medium (10 mm x 20 cm) with 20 mM PB buffer. Load the sample onto the column and continue to equilibrate with the buffer. After equilibration to 3 column volumes, elution Tf with the buffer of 0.1 mM NaCl in 20 mM PB. And then 1 M NaCl was applied onto the column to wash the other bound proteins. The effluent fractions were characterized by HPLC.

3. Characterization

3.1. Sodium Dodecyl Sulfate Polyacryl Amide Gel Electrophoresis (SDS-PAGE)
Sodium dodecyl sulfate polyacrylamide gel electrophoresis using 12% separation gel and 5% concentrated gel. Samples were processed at 95°C for 5 min. Approximately, 5ug of each sample was loaded per lane and bands were visualized by staining with Coomassie blue. After stained, the gel was destained with 50% methanol and 10% glacial acetic acid aqueous solution. The acquisition of digital image was performed on FluorChemE (Protein Sample, USA).

3.2. HPLC analysis
The HPSEC analysis was performed on TSK-GEL G3000 SWXL (300×7.8 mm, I.D.) on the high performance liquid chromatography station (LC Solution, Shimadzu, Japan). The mobile phase used for the analysis were 20 mmol/L PB+0.1 mol/L Na2SO4 (pH7.0). The flow rate was set at 0.6 mL/min. The effluent was monitored at 280 nm. Human serum Tf obtained from Sigma was used as a reference sample.

3.3. Circular dichroism assay
Circular-dichroism (CD) measurements were performed on a spectropolarimeter J-810 (Jasco, Japan). The freeze-dried samples of Tf dissolved in pure water (0.5mg/mL), were recorded at room temperature in the 190-260 nm (1nm steps) spectral range, where each spectrum (averaged over 10 scans) was corrected with an appropriate blank. And every sample was measured 3 times.

4. Results and discussion
In an effort to optimize the elution process, a linear gradient (0-1M NaCl) was produced with 10 CVs. The Chromatogram of Cohn fraction IV on Capto DEAE column at pH7.0 using phase gradient elution and the corresponding SDS-PAGE image was shown in Fig.2, which clearly indicated that the P0 flow-through fraction mainly consists of immunoglobulins and other unbound proteins; two elution fractions (P1, P2) were eluted according to different NaCl concentrations. The P2 eluted with complete NaCl, mainly contain albumin and ceruloplasmin; the main components of P1 was the target protein, transferrin, whose purity was above 93%. Then the influence of the pH value on the separation process (pH 6.5, pH7.0, pH7.5) were studied respectively. As depicted in Fig.1, the HPLC analysis and chromatogram of the first fraction eluted from Capto DEAE column at different pH condition, the purity of the product was about 93% at pH7, while only 83% and 85% under the conditions of pH6.5 and pH7.0 based on area normalization method.

It meant that this development with 20mM PB containing 0.1M NaCl resulted in separation of Tfs from other proteins, which did not elute from the column until application of 1M NaCl. Based on the results described, the elution method of 20mM PB+0.1M NaCl (pH7.0) on Capto DEAE column was established.
In this study, several techniques were used to assess the purity of the isolated Tfs. It can be seen from SDS-PAGE (Fig. 2B, lane 2; Fig. 2B, lane 4) that the purity of product was increasing progressively with the advance of purification. As seen in Fig. 1, the final product purity exceeds 93%. These results were reasonable and well in accord with the CD profile (Fig. 3).

**Figure 1.** HPLC analysis on the effect of pH (6.5, 7.0, 7.5) on Capto DEAE chromatography. The purity is 93% at pH 7.0, which is higher than the other two by peak area calculation.

**Figure 2.** The chromatogram of AEC and SDS-PAGE analysis. (A) Transferrin was eluted from the column developed with a 0-1M NaCl gradient buffer. The P2 fraction corresponded to Tf. (B) SDS-PAGE analysis on AEC purified Tf. M: protein molecular marker; 1: Sigma Tf; 2: Pretreated sample of the Cohn fraction IV; 3: P0 in Fig. 1A, the flow-through peak mainly consists of immunoglobulins and other impurity protein; 4: P1 in Fig. 1A, corresponded to Tf; 5: P2 in Fig. 1 eluted with completed NaCl, mainly contain albumin and ceruloplasmin.
5. Conclusion
The simple procedure presented in this report permits the preparations of highly purified Tf from Cohn fraction IV with good recovery and a high purity above 93% as judged by mobility on SDS-PAGE, spectral properties, HPLC. Compared with the methods reported in other literatures, this study is more conducive to amplification, and have higher purity.

References
[1] Crichton R R, Charloateauxwauters M. Iron transport and storage [J]. European Journal of Biochemistry, 1987, 164 (3): 485-506.
[2] Deng G, Wu K, Cruce A A, et al. Binding of trivalent chromium to serum transferrin is sufficiently rapid to be physiologically relevant [J]. Journal of inorganic biochemistry, 2015, 143: 48-55.
[3] Brandsma M E, Jevnikar A M, Ma S. Recombinant human transferrin: beyond iron binding and transport [J]. Biotechnology advances, 2011, 29 (2): 230-238.
[4] Ascione E, Muscariello L, Maiello V, et al. A simple method for large - scale purification of plasma-derived apo-transferrin [J]. Biotechnology and applied biochemistry, 2010, 57 (3): 87-95.
[5] Parkkinen J, von Bonsdorff L, Ebeling F, et al. Function and therapeutic development of apotransferrin [J]. Vox sanguinis, 2002, 83 (s1): 321-326.
[6] Wu L, Wu J, Zhang J, et al. A simple method for obtaining transferrins from human plasma and porcine serum: Preparations and properties [J]. Journal of Chromatography B, 2008, 867 (1): 62-68.
[7] M.T. Andrés, J.F. Fierro, Antimicrobial mechanism of action of transferrins: selective inhibition of H+-ATPase, Antimicrob. Agents Chemother. 54 (2010) 4335–4342.
[8] Dowling P, Palmerini V, Henry M, et al. Transferrin-bound proteins as potential biomarkers for advanced breast cancer patients [J]. BBA clinical, 2014, 2: 24-30.
[9] Gupta R, Rastogi S, Nagar R, et al. Dietary and serum iron, body iron stores and coronary heart disease [J]. The Journal of the Association of Physicians of India, 2000, 48 (5): 489-492.
[10] Picard E, Le Rouzic Q, Oudar A, et al. Targeting iron-mediated retinal degeneration by local delivery of transferrin [J]. Free Radical Biology and Medicine, 2015, 89: 1105-1121.