Separation and Purification Technology 133 (2014) 149–154

Contents lists available at ScienceDirect

Separation and Purification Technology

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

Partitioning behavior of enhanced green fluorescent protein in nickel-chelated affinity-based aqueous two-phase micellar system and its purification from cell lysate

Shuo Wang [a], Shu Bai [a,b], Xiao-Yan Dong [a,b], Yan Sun [a,b,*]

[a] Department of Biochemical Engineering and Key Laboratory of Systems Bioengineering of the Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China
[b] Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin 300072, China

ARTICLE INFO

Article history:
Received 8 April 2014
Received in revised form 3 July 2014
Accepted 4 July 2014
Available online 10 July 2014

Keywords:
Liquid–liquid extraction
Aqueous two-phase micellar system
Nickel-chelated surfactant
Green fluorescent protein
Purification

ABSTRACT

Aqueous two-phase micellar system (ATPMS), an alternative to chromatography, has been considered as a promising liquid–liquid extraction technique for biomolecule purification. To improve the specificity of ATPMSs, a nickel-chelated surfactant (TX-Ni) has been fabricated. The affinity-based ATPMS formed by Triton X-114 (TX) and TX-Ni was characterized for the purification of recombinant hexahistidine-tagged enhanced green fluorescent protein (EGFP). The stability of EGFP in the ATPMS was first confirmed. Then, the affinity binding of EGFP to TX-Ni was proved by investigation of the partitioning behavior. Thereafter, EGFP was extracted directly from cell lysate by the Ni(II)-chelated ATPMS. It was found that, more impurities were removed to the micelle-poor phase with increasing NaCl concentration, and the increase of TX-Ni gave rise to a recovery of EGFP over 90%. Finally, ethylenediaminetetraacetic acid (EDTA) was used to back-extract EGFP, presenting a total recovery yield of 83% with a purity of 70%. The results indicate that the affinity-based ATPMS is promising for the primary separation of histidine-rich proteins.

1. Introduction

With the rapid development of biotechnology, the production scale of biomolecules has dramatically increased, which, in turn, has intensified the pressure to develop cost-effective downstream processes for the recovery and purification [1–3]. At present, chromatographic procedures make up >70% of the downstream costs, which account for 50–80% of the total manufacturing costs [3,4]. Therefore, a great deal of interest has been focused on other separation techniques. One such alternative is liquid–liquid extraction with aqueous two-phase systems (ATPSs), which is featured by cost-efficiency, ease of scale-up, process integration capability, as well as biocompatibility [5–8].

One promising type of ATPSs is the aqueous two-phase micellar system (ATPMS) formed by aqueous surfactant solutions. The high percentage of water provides a mild environment for biological substances. As for ATPMSs composed of nonionic surfactants, the initial homogenous solution spontaneously separates into a micelle-rich and a micelle-poor phase when heated above the cloud point [9]. Purification is achieved when target molecules predominantly partition into one of the phases, while contaminants partition into the opposite one [10]. Particularly, ATPMSs are convenient for the extraction of membrane proteins which are solubilized into the micelle-rich phase due to hydrophobic effects [11]. Moreover, ATPMSs are also capable of purifying hydrophilic proteins attributing to excluded-volume effects [12], electrostatic interactions [13] and/or affinity interactions [14–17].

Although ATPMSs have been widely studied in the extraction and purification of biomolecules [1,10,18–23], the lack of specificity is still one major drawback restricting their application. In our previous work [24], a novel nickel-chelated surfactant (TX-Ni) was synthesized, aiming to improve the selectivity of ATPMS, as immobilized metal-ion affinity ligands have been successfully utilized in ATPS [25,26], reverse micelle extraction [27,28], as well as immobilized metal affinity chromatography (IMAC) [29]. Encouragingly, the ATPMS composed of Triton X-114 (TX) and TX-chelated nickel (TX-Ni) exhibited a potential for the separation of recombinant hexahistidine-tagged enhanced green fluorescent protein (EGFP), a good representative of histidine-rich proteins.

This work reports the behavior of the Ni(II)-chelated ATPMS in the purification of recombinant EGFP from cell lysate. First, the

* Corresponding author at: Department of Biochemical Engineering and Key Laboratory of Systems Bioengineering of the Ministry of Education, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China. Tel.: +86 22 27404981; fax: +86 22 27403389.
E-mail address: ysun@tju.edu.cn (Y. Sun).

http://dx.doi.org/10.1016/j.seppur.2014.07.005
1383-5866/

influence of solution components on the cloud point was examined. Then, the stability and partitioning behavior of pure recombinant EGFP in the ATPMS were investigated. At last, EGFP was purified by forward and back extractions using the Ni(II)-chelated ATPMS. The results obtained were compared with those achieved by IMAC.

2. Materials and methods

2.1. Materials

Triton X-114 was obtained from Fluka (Bucks, Switzerland). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO, USA). Chelating Sepharose Fast Flow and Sephadex G25 were received from GE Healthcare (Uppsala, Sweden). Iminodiacetic acid (IDA, >97%) and benzyltriethylammonium chloride (BTAC, >99%) were from Guangfu Fine Chemical Research Institute (Tianjin, China). Tris(hydroxymethyl) aminomethane (Tris), sodium chloride, imidazole and disodium salt of ethylenediaminetetraacetic acid (EDTA) were from Sangon Biotech Co., Ltd. (Shanghai, China). The protein molecular weight marker (Blue Plus II) was from Beijing Dingguo Changsheng Biotech Co., Ltd. (Beijing, China). Other chemicals were all of analytical grade from local sources. All materials were used as received.

2.2. Synthesis of TX-Ni

TX-Ni was synthesized according to the procedure described earlier [24]. Briefly, TX was firstly activated by epichlorohydrin. Then, the intermediate epoxide was coupled with IDA. After extraction with chloroform, the dried product was chelated with nickel ions to obtain TX-Ni, which was purified by extraction and re-precipitation. Finally, TX-Ni was dried under vacuum before use.

2.3. EGFP production and chromatographic purification

Hexahistidine-tagged EGFP was expressed by cultivation of the recombinant *E. coli* harboring the vector pET28a-EGFP as described previously [30]. After centrifugation at 5000×*g* for 10 min, 1 g of cell pellet was suspended in 10 mL of 20 mmol/L Tris-HCl buffer (pH 8.0) and then disrupted by sonication. The cell lysate was centrifuged at 13,000×*g* for 15 min at 4 °C, and the supernatant was used for subsequent experiments.

To prepare a pure EGFP standard, the lysate supernatant was first purified by IMAC and gel filtration chromatography (GFC) [27]. All chromatographic experiments were performed on an ÄKTA Explorer 100 system (GE Healthcare, Uppsala, Sweden). In the IMAC, 50 mL of the clarified cell lysate supernatant was loaded onto an XK16/20 column (16 mm I.D., 20 cm in length) packed with 20 mL of Chelating Sepharose FF, which had been charged with nickel ions and equilibrated with buffer A (20 mmol/L Tris-HCl, 100 mmol/L NaCl, 30 mmol/L imidazole, pH 8.0). After washing with buffer A till reaching a UV absorbance baseline at 280 nm, the bound proteins were eluted by applying buffer B (100 mmol/L imidazole and other components the same as in buffer A). The eluate was collected and 5 mL of the collected pool was subsequently loaded onto another XK16/20 column packed with 40 mL of Sephadex G25 gel for GFC separation. By washing with 20 mmol/L ammonium acetate buffer (pH 8.0), imidazole was removed and the EGFP peak was collected. The pure EGFP was lyophilized and stored at −20 °C before use.

2.4. Phase diagram determination

TX was mixed with TX-Ni to form an affinity-based ATPMS. The solution composition was expressed by the mole fraction of TX-Ni (α), defined as:

$$\alpha = \frac{[\mathrm{TX-Ni}]}{[\mathrm{TX-Ni}] + [\mathrm{TX}]} \quad (1)$$

Phase diagrams for the aqueous solutions of TX or surfactant mixtures were measured by the cloud-point method [31]. Briefly, surfactant solutions of known concentrations were prepared and then put into a programmable temperature controller (PolyScience 9512, PolyScience, IL, USA) with temperature precision of ±0.01 °C (readout accuracy, ±0.25 °C). At beginning, each sample was cooled to 20 °C, which was low enough to make the solution exhibit a single and clear phase. The temperature was then raised at 0.2 °C/min until the solution became turbid, indicating the onset of phase separation (the temperature denoted as T_u). As soon as the clouding was observed, the temperature was lowered at 0.2 °C/min until the solution became clear again at a temperature T_d. The cloud point temperature was taken to be the average of T_u and T_d. The procedure was repeated thrice for each data point to ensure reproducibility.

2.5. Determination of protein concentration

EGFP concentration was determined by fluorometric assay with excitation and emission wavenumbers of 488 nm and 509 nm, respectively [30]. The EGFP purified by chromatography was used to determine the calibration curve. Total protein concentration was determined by the Bradford assay using BSA as a standard [32].

2.6. Influence of TX or TX-Ni on EGFP activity

In order to evaluate the effect of surfactants on EGFP activity in the ATPMS, 1 mL of 0.2 mg/mL EGFP solution was mixed with an equal volume of surfactant solutions of different TX and TX-Ni concentrations. The fluorescence intensities before and after a 2-h incubation at 37 °C were measured. The intensity of 0.1 mg/mL EGFP solution free of surfactants was measured before incubation as control. Prior to measurement, all samples were cooled to 20 °C and vibrated vigorously to obtain a clear homogenous solution.

2.7. EGFP partitioning experiments

All samples for the partitioning experiment were prepared using 20 mmol/L Tris-HCl buffer (pH 8.0). Equal volumes (1.5 mL) of surfactant and EGFP solutions were mixed and incubated at 4 °C to exhibit a clear and homogeneous phase. Then, the samples were incubated in a 37.00 ± 0.01 °C water bath to undergo phase separation. After partitioning equilibrium was attained after 2 h, the two coexisting phases were withdrawn separately, and EGFP concentrations in the micelle-rich phase ($[\mathrm{EGFP}]_r$) and the micelle-poor phase ($[\mathrm{EGFP}]_p$) were determined as above. The partitioning behavior of EGFP was quantitatively described in terms of the partition coefficient K_{EGFP} defined as:

$$K_{\mathrm{EGFP}} = \frac{[\mathrm{EGFP}]_r}{[\mathrm{EGFP}]_p} \quad (2)$$

The recovery yield (R) was calculated according to the following equation:

$$R = \frac{[\mathrm{EGFP}]_R V_R}{[\mathrm{EGFP}]_i V_i} \times 100\% \quad (3)$$

where $[\mathrm{EGFP}]_R$ and V_R are the recovered EGFP concentration and solution volume, and $[\mathrm{EGFP}]_i$ and V_i are the initial EGFP concentration and solution volume, respectively. Triplicate experiments were performed and the average values with standard deviations are presented.