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Optimized extraction of a single-chain variable fragment of antibody by using aqueous micellar two-phase systems

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ABSTRACT

In this work, the purification of a single-chain variable fragment (scFv) of an antibody by using liquid–liquid extraction in aqueous micellar two-phase systems was optimized by means of central composite design. Protein partitioning assays were performed by using the selected system composition in previous works: Triton X-114 at 4% wt/wt, yeast fermentation supernatant at 60% wt/wt, McIlvaine buffer pH 7.00. The other system component concentrations, Cibacron Blue F3GA (CB), Fabsorbent™ F1P HF (HF) and NaCl, were selected as independent variables. ScFv recovery percentage (%R) and purification factor (PF) were selected as the responses. According to the optimization process both, scFv recovery percentage and purification factor were favored with the addition of HF and NaCl in a range of concentrations around the central point of the second central composite design (HF 0.0120% w/w, CB 0.0200% w/w, NaCl 0.200% w/w). These experimental conditions allowed the concentration and pre-purification of scFv in the micelle-rich bottom phase of the systems with a recovery percentage superior to 88% and a purification factor of approximately 3.5. These results improved the previously presented works and demonstrated the convenience of using aqueous micellar two-phase systems as a first step in the purification of scFv molecules.

Introduction

Monoclonal antibodies have played an important role in medicine and in different biotechnological fields for several years [1,2]. The most common commercially available monoclonal antibodies (mAbs) are chimeric, humanized, fully human and of murine origin. Other novel therapeutic formats, as for example single-chain variable fragments (scFv) and subtypes, are in development or are undergoing clinical testing [1]. This type of molecules, scFv antibodies, have recently gained importance in the pharmaceutical field because of the several advantages that they present respect to full-sized antibodies, such as, easy manipulation, high permeability and the fact that their immune-complexes can be quickly cleared from the human body [3].

Due to the advantageous features above mentioned, antibody fragment pipeline is now expanding [4,5], thus, its industrial production and purification is highly demanded [6]. Up to date, the production of recombinant antibodies fragments has reached high titers [7–9], which imply that the downstream unit operations delimit the cost of the whole process [7,10–12]. For example, it is known that 30% of the total purification cost corresponds to the Protein A resin, with an estimated value of 4–5 millions dollars in a scale of 10.000 L [11,13]. The reuse of this resin is also expensive (about 120.000 dollars) due to the high buffer volume consumption. This limitation represents the bottleneck for antibody fragment production at industrial levels [6,10] and as a consequence, the use of non conventional methodologies for scFv purification must be evaluated [10].

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The extraction with aqueous two-phase systems (ATPSs[1]) represents an attractive alternative as a bioseparative process [14–16]. Particularly, aqueous micellar two-phase systems (AMTPS) have several desirable features such as high enrichment factor, short-time consumption and low cost [17]. Additionally, it was demonstrated that 87% of the used surfactant can be recovered and re-used, thus reducing notoriously the purification cost [18].

The AMTPS methodology is simple and consists in incubating a mixture of sample and a surfactant solution [19] at certain temperature to allow phase separation and protein partitioning [20]. Furthermore, the partition selectivity can be improved by using mixed micelles, affinity ligands and salts [21–23].

The successful application of liquid–liquid extraction in the purification of recombinant antibodies from different sources (e.g. CHO, *Escherichia coli* cultures, etc.) has already been approached [12,24–28]. Successful purification performances, were obtained with polymer/salt and thermoseparating aqueous two-phase systems [28,29]. Additionally, Fischer and colleagues have used an AMTPS-based methodology to extract an antibody fragment from *E. coli* [18]. Recently, our research group has evaluated the feasibility of using AMTPS of Triton X-114 to extract a scFv antibody directly from *Pichia pastoris* fermentation supernatant [30]. According to our preliminary results, scFv was concentrated and partially purified in a single extractive step with recovery percentages superior to 80% and purification factors of approximately 2 [30]. Even though these results were similar or even better than those reported for other authors [30,31], a further improving of this purification performance would be desirable, thus requiring the application of statistical techniques.

According to a preliminary factorial design (data not shown), the concentration of both ligands (HF and CB) and salt (NaCl), as well as their interactions, showed to be significant factors in the scFv extraction process.

In this context, the aim of this work was to optimize the previously obtained scFv purification parameters, the scFv recovery percentage (%R) and the purification factor (PF), by studying the effect of the mentioned variables.

To identify the independent variables with statistical significance for the process and their significant interactions, the extraction experiments were performed with the aid of a central composite design [32].

Materials and methods

Chemicals

Polyethylene glycol tert-octylphenyl ether (Triton X-114) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Cibacron Blue F3GA (triazine-based dye, used as free ligand) and Fabsorbent™ F1P HF (synthetic compound, cross-linked to an agarose bed, which has been designed to specifically capture antibody fragments) were obtained from Polysciences Inc. and Prometic Biosciences respectively.

All the other reagents were of analytical grade and used as received. All the solutions were prepared in McIlvaine's buffer, pH 7.00, consisting of 1.38 mM citric acid and 5.3 mM disodium phosphate purified by filtration through a Millipore Milli-Q ion-exchange system (Bedford, MA, USA). All glassware used in the experiments was washed in a 50:50 ethanol/1 M sodium hydroxide bath, washed in a 1 M nitric acid bath, rinsed copiously with Milli-Q water and dried in an oven at 70 °C.

Biologicals

The recombinant *P. pastoris* SMD 1168 (Δpep4::URA3 Δkex::SUC2 his4 ura3, phenotype His- Mut+), an anti-LDL electronegative his-tagged single-chain antibody fragment (scFv) producing yeast (Invitrogem), was kindly provided by Professor Dulcineia Saes Parra Abdalla from the São Paulo University (Brazil) and stored at −70 °C with glycerol 20% wt/wt [33].

Yeast growth and scFv production

Buffered Glycerol Complex medium (BGCM) [30] was used to start *P. pastoris* culture. The yeast growth was initialized for 16 h in a 250-mL orbital shaker (250 rpm) at 20 °C. Afterwards, the inoculum was transferred into a 3-L bioreactor and grown for 96 h at 20 °C in BGCM medium. 1% v/v of inductor (methanol) and 1 mM of the proteases inhibitor phenylmethanesulfonylfluoride (PMSF, from Sigma–Aldrich) were added every 24 h maintaining the pH at a constant value of 6.80. The final fermented broth was then centrifuged for 10 min at 2000 rpm in order to remove yeast cells. The supernatant was storage at −4 °C before use.

Protein partitioning

Protein partitioning assays were performed by using the previously selected system composition [30]: Triton X-114 at 4% wt/wt, yeast fermentation supernatant at 60% wt/wt, McIlvaine buffer pH 7.00. The other components: CB, HF and NaCl, were added according to the experimental design described in section "Experimental design and statistical analysis". The resulting solutions were mixed at 8 °C for 1 h. Subsequently, the solutions were incubated at 17 °C for 3 h to attain partitioning equilibrium. Samples from both upper and lower phases were then taken for the determination of scFv concentration and total protein content.

Recombinant antibody fragment and total proteins assays

The quantity of single-chain antibody fragment (scFv) was determined by immobilized metal affinity chromatography (IMAC). Samples from both, yeast broth and phase systems, were passed through the chromatographic column. Bottom phases were previously diluted at least four times in PBS buffer at pH 7.40 to avoid surfactant interference. The chromatographic procedure was performed according to the manufacturer's instructions (GE Healthcare, Munich, Germany), getting a scFv sample with a final purity (P_{scFv}%) of 95 [33]. After elution, scFv concentration was estimated by using the Bicinchoninic Acid method (BCA; Pierce, Rockford, IL, USA). Bovine Serum Albumin (BSA) was used as standard. Total proteins concentration was determined with the same methodology. All experiments were run in triplicate and the medium effect was discounted.

Extraction performance parameters

The partition coefficient (K) of scFv and total proteins was calculated as follows:

$$K = \frac{C^T}{C^B} \quad (1)$$

[1] *Abbreviation used:* AMTPS, aqueous micellar two-phase systems; ATPS, aqueous two-phase systems; BCA, bicinchoninic acid; BSA, bovine serum albumin; C^0, protein concentration (scFv o TP) (mg/L) in the clarified broth; $C_{(T/B)}$, protein concentration (scFv o TP) (mg/L) in top (T) or bottom (B) phase; CB, cibacron blue F3GA; CP, cloud point; ΔCP, CP at condition A – CP at condition B (°C); HF, Fabsorbent™ F1P HF; IMAC, immobilized metal affinity chromatography; K, partition coefficient; MB, mass balance; P%, total purity; PBS, phosphate buffered saline; PEG, polyethylene glycol; PF, purification factor; scFv, single-chain antibody fragment; TP, total proteins; V^0, volume of added yeast supernatant (mL); $V_{(T/B)}$, top (T) or bottom (B) phase volume (mL); %wt/v, grams of specific compound in 100 mL of total system/solution; %wt/wt, grams of specific compound in 100 grams of total system/solution.