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Isolation of monoclonal antibody from a Chinese hamster ovary supernatant. I: Assessment of different separation concepts

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ABSTRACT

The performance of different non-affinity purification techniques commonly used for isolating CHO derived monoclonal antibodies has been investigated. Ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), aqueous-two-phase extraction (ATPE) and their integration has been evaluated in terms of yield and purity of the product obtained. The integration of chromatographic techniques comprised two steps, in which the CHO supernatant was directly injected into the IEC column to capture monoclonal IgG1 and then the isolated fraction was processed using the HIC column. To reduce the influence of the feed media on retention of the target protein, the feed mixture was on-column diluted by use of the multi-injection technique. In the coupled process of extraction and chromatography the ATPE operation was used for the pre-purification of the supernatant as well as for buffer exchange. The bottom ATPE phase containing the target protein was further purified on the HIC column without feed dilution. The influence of operating conditions on the effectiveness of different purification processes has been evaluated. The best performance with respect to the product purity was achieved for the coupled process of IEC and HIC. The experimental data acquired were exploited in subsequent investigations for determining underlying kinetic parameters and for the process prediction and optimization.

1. Introduction

In recent years an increase in the demand for biopharmaceutical products has been observed. This particularly holds true for monoclonal antibody (mAbs) constituting an important and the most expanding category of biotechnology drugs which are being applied against a number of previously incurable diseases [1,2]. Monoclonal antibodies are typically produced in Chinese hamster ovary (CHO) cell expression systems [3,4], where they are secreted from the cells. The cell culture supernatant is further processed in a series of downstream operations to remove the product contaminates, i.e., host cell protein (HCP), DNA, lipids, and product-related impurities such as high molecular weight aggregates [5,6].

Typically, few chromatographic steps are used for the purification of mAbs. The protein capture is often performed using protein-A affinity media. The product is further polished by one or two additional orthogonal chromatographic techniques [7].

The protein-A capture step is capable of performing the required separation meeting purity, yield and throughput requirements for pharmaceutical antibody purification [7–9]. However, main disadvantages of this operation include cost and resin stability [9]. Therefore, there is a growing interest in developing alternative procedures to capture the target antibody [10]. For this purpose affinity ligands that involve specific interactions with immunoglobulins have been developed, which can potentially be used to produce relatively cheap chromatographic media resistant to sanitization agents [2,11,12]. Another possibility is a combination of few non-affinity purification stages that comprise different chromatographic techniques such as ion exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), mixed mode chromatography, charge-induction chromatography or hydroxyapatite chromatography [9,10,13–15].

IEC is the most widely used technique for the separation of proteins in general and for mAbs in particular [16–20] and is very often considered as the first option in a process development program [18]. The sample with the protein mixture is loaded at low concentration of salt containing counter-ions with respect to the column matrix at pH differing from the isoelectric point [18], while elution is imposed by increase of the salt concentration or proper pH change in the direction of pI. Because of high binding capacity of cation exchangers, large volumes of the sample can be separated in a short time.

HIC constitutes an important separation technique which has also been successfully adapted to the purification of mAbs [13,21]. It enables rapid separation of proteins with high resolution and high yield without denaturation. The sample loading is realized at high concentration of a cosmotropic salt (e.g., ammonium sulfate), which enhances hydrophobic interactions between the adsorbent

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surface and proteins and, thus, the adsorption strength. The elution is executed by lowering the salt concentration.

Both IEC and HIC are frequently combined in multi-stage chromatographic purification of mAbs [9], which involves designing problems because of a number operating parameters that have to be taken into account, such as sample volumes, pH and the salt concentration in the mobile phase used for both the column loading and chromatographic elution.

Non-chromatographic alternatives for mAbs purification including membrane chromatography, tangential flow filtration, aqueous two-phase extraction (ATPE), precipitation and crystallization have also been described [22,23]. Among them ATPE gained increasing interest as a scalable technique, in which purification and concentration can be coupled in a single step under mild conditions [24,25]. Nevertheless, ATPE usually has to be complemented by selective chromatographic steps [13,21].

In this study the performance of different non-affinity separation techniques commonly used for isolation of mAbs from a CHO supernatant has been assessed. Two chromatographic techniques: IEC and HIC have been taken into account because of low cost of the media, mild operating conditions and high popularity in industrial applications. For the same reasons ATPE was considered as a non-chromatographic alternative.

Single step operations or two-step combinations of IEC and HIC have been investigated as well as the integrated process of ATPE and HIC. The multiple-injection technique has been used for the integration of IEC and HIC. The idea of the method was developed in our previous studies [26] and relies on the on-column dilution of the feed mixture in the presence of chromatographic matrix.

2. Materials and methods

2.1. Instruments and materials

2.1.1. Instruments

The HPLC instrument Äkta purifier with UV and conductometric detectors and a data station (GE Healthcare Life Sciences, Uppsala, Sweden) was used. The injector was a Rheodyne sampling valve with a few sample loops with different volumes: 0.1–5 mL.

For the electrophoresis the P81 single sided 10 cm × 10 cm system for vertical SDS-PAGE electrophoresis with the power supply EC300XL2, max. output: 300 V (Symbios (Owl), Poland) was used.

Mini-PROTEAN 3 (Bio-rad) was used for the vertical SDS-PAGE electrophoresis and for the immunotransfer onto the nitrocellulose membrane in the Western-Blot analysis.

2.1.2. Columns

Two columns I.D. = 0.4 cm, L = 12.5 cm were packed with a stationary phase Butyl Sepharose 4FF (GE Healthcare Life Sciences) for HIC and cation exchanger UNOsphere S (Bio-Rad, Warsaw, Poland) for IEC.

An analytical column SuperSW3000 TSKGel (Tosoh Bioscience LLC, Tokyo, Japan) I.D. = 0.4 cm, L = 15 cm, with a guard column I.D. = 0.4 cm, L = 3.5 cm, particle size 5 μm was used for the purity analysis by size exclusion chromatography (SEC).

A HiTrap column (GE Healthcare Life Sciences) with the recombinant protein-A, I.D. = 0.69 cm, L = 2.7 cm, was exploited in affinity chromatography.

2.1.3. Chemicals

Polyethylene glycol (PEG) – molecular weight 3350 (Sigma, Poznań, Poland), salts of analytical grade: ammonium sulphate (AS), disodium phosphate and sodium chloride (both from POCH, Gliwice, Poland), Tris, glycine, acrylamide (Carl Roth, Poland), methylenobisacrylamide, SDS, Coomasee (Sigma), Immunoglobulin G (IgG) from bovine serum (Sigma, purity 95%).

2.1.4. Biologicals

Chinese Hamster Ovary (CHO) cell supernatant was produced and delivered by ExcellGene (Monthey, Switzerland). It contained a human IgG1, directed against a human surface antigen. The origin of the cell line was a double dihydrofolate reductase (DHFR) negative mutant of CHO cells. The cells were transfected with three vectors, containing the antibody heavy chain gene, the light chain gene and a functional DHFR-gene, respectively, and a high expressing clone was isolated from hundreds of cell lines. An ExcellGene proprietary serum-free medium, containing only one protein, was used for production. Phenol red was added to the medium as a pH indicator. According to ExcellGene, the antibody titer was 100–150 mg/L as determined by enzyme-linked immunosorbent assay (ELISA). The supernatant was produced according to the same procedure as that reported by Azevedo et al. [13].

2.2. Procedures

2.2.1. ATPE systems

The ATPE systems were prepared by weighting appropriate amount of 50% [w/w] PEG solution and 35% [w/w] ammonium sulphate (AS) solution in 50 mM sodium phosphate buffer pH 6.

To determine bimodal curves two-phase systems were titrated drop-wise with PEG or AS solutions until systems became clear after vortex mixing. Positions of conodes were determined by the equilibrium composition of the bottom and upper phase for different two-phase systems. The AS salt concentration was quantified by measurements of the signal of conductometer of the Äkta purifier system, whereas the PEG content by refractive index measurements using a refractometer 39614 (Knauer, Warszawa, Poland). In the latter case the detector signal was corrected with the contribution of AS.

On the basis of the experimental analysis of the extraction equilibria for bovine serum IgG (used here as a standard) in ATPE systems, the solution PEG/AS: 20/8 [%w/w] was selected for the purification. This system was found to be optimal with respect to the selectivity and yield of the operation. Similar procedure was used by Azevedo et al. [13] but in our study ammonium sulphate was applied instead of sodium citrate. Moreover, the CHO supernatant was used as received, while in [13] it was artificially enriched with an IgG1 standard up to the concentration 1 mg/mL. The experiments of CHO extraction were performed in 15 and 50 mL centrifuge tubes, wherein all system components were mixed and then centrifuged. After the phase separation, the bottom phase of the extraction system was carefully collected for further experiments. The bottom phase from the ATPE experiment containing water instead of the CHO supernatant was used as a blank sample in the HIC elution as described below.

2.2.2. Ion exchange chromatography

A 50 mM sodium phosphate buffer, pH 5 or 7 free of the AS salt was prepared as the loading buffer. The salt buffer contained AS at concentration 1.7 M dissolved in phosphate buffer. The mobile phase was a mixture of the AS salt buffer and phosphate buffer with different volume content. Desired pH value was achieved by adding phosphoric acid to the solution. The mobile phase flowrate was 1 mL/min.

The CHO supernatant was injected into the IEC column without preconditioning (i.e., without buffer exchange or feed dilution). The column loading was realized by: (a) injecting the supernatant in a single pulse with different volumes: 0.1–5 mL; (b) injecting the supernatant using the multi-injection technique [26]; i.e., by injecting several pulses with 0.1, 0.5 or 2 mL volumes with a