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Capture and intermediate purification of recombinant antibodies with combined precipitation methods

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ABSTRACT

A protocol for capturing recombinant antibodies was developed with a combination of different precipitation methods. This process comprised four different precipitations, including caprylic acid, polyethylene glycol (PEG), $CaCl_2$, and cold ethanol. The final yield and purity of the antibodies nearly reached drug substance values, with a low host cell protein concentration (<300 ppm), no high molecular weight impurities, and a yield of 70%. In fact, only the combination of caprylic acid and PEG precipitations was needed to achieve purity similar to that produced with protein A affinity chromatography. Caprylic acid was used to remove the host cell proteins and high molecular weight impurities, and the PEG precipitation separated the IgG from the remaining impurities.

1. Introduction

With sufficiently high titers, crystallization [1] and precipitation of recombinant antibodies has become an option for capture and for intermediate purification. For capture, it is important to enrich the antibody, remove host cell impurities (aggregates) for intermediate purification, and perform polishing. Emphasis has been put on aggregate removal, because aggregates might be immunogenic. Currently, the state-of-the-art process for the biopharmaceutical production of monoclonal antibodies (mAbs) [2] consists of seven to nine discontinuous processing steps, including column-based polishing and mAb capturing [3–6]. In recent years, the development of downstream processing has mainly focused on improving the column-based purification steps [7–9]. Due to the cost of producing high affinity chromatography materials, most development work has focused on improving the binding capacity and stability of protein A resins [10,11]. The aim of the present study was to replace the discontinuous process of protein A affinity capture, followed by column-based intermediate purification, with a fully continuous mAb purification process. For achieving this target a combination of different precipitation steps realized as a tubular reactor was our theoretical approach for a continuous purification process. Mixing, precipitation, and separation can be realized with static mixers, cross valves, separators or filter units connected in parallel. For this kind of reactor scale up and product output adjustments can be performed through changing tube size and flow rate. In addition a reusable tubular bench-top reactor would be timesaving solution for process development and scale up tests. Methods for precipitating polyclonal antibodies have been well established, but they are not frequently used for mAbs, which are secreted from hybridomas, or for recombinant antibodies, which are expressed in mammalian cells.

Methods have also been well established for fractionating human plasma [12] with polyethylene glycol (PEG) as the precipitant [13]. A linear relationship was observed between the logarithm of solubility and the concentration of PEG. This relationship is the same as the equation that describes the salting out of proteins [15]. The precipitation of proteins with PEG occurs in a volume exclusion reaction [16,17]. In recent years, further research was performed to determine the solubility curve of common proteins during the precipitation process [18–23]. Precipitation by PEG does not allow removal of high molecular weight impurities (HMWIs), like DNA [24] or aggregates, because the precipitation of larger proteins tends to require lower PEG concentrations and shorter

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polymer lengths [13]. The design of a protein purification process that is mostly based on precipitation requires methods with different selectivity for removing aggregates, HMWIs, and host cell impurities. For example, PEG precipitation was combined with $CaCl_2$ precipitation, which is known to precipitate HMWIs [25]. For further removal of host cell proteins (HCPs), another precipitation method must be implemented. Caprylic (octanoic) acid precipitation is commonly used for separating venom from horse plasma [26]. The precipitation parameters are described in several publications [27–30]. Caprylic acid precipitation has been combined with several purification methods to purify various proteins. For example, it was combined with ammonium sulphate precipitation to purify human immunoglobulins (IgGs) [31], and it was combined with ion-exchange chromatography to separate equine antivenom [32]. Also, a combination of caprylic acid precipitation and cation-exchange chromatography was used to remove HCPs from the supernatant of Chinese hamster ovary (CHO) cell cultures [33]. Thus, we reasoned that caprylic acid combined with PEG precipitation should be able to remove a substantial fraction of HCPs in an antibody purification process. Should the purity of PEG-precipitated mAbs fail to meet the expected purity requirements, further orthogonal precipitation methods could be applied. For instance, caprylic acid and PEG precipitation could be combined with $CaCl_2$ precipitation or with cold ethanol precipitation (CEP). CEP is used in the blood plasma industry [34] to purify plasma-derived IgGs on the scale of tons.

In this study, we explored the combination of PEG, $CaCl_2$, caprylic acid, and CEP to maximize the removal of aggregates and high and low molecular weight impurities. These combined precipitations could be performed successively, and they consisted of only two to four purification steps, depending on the required purity. These combinations might provide the basis for replacing the typical discontinuous recombinant antibody purification process with a continuous series of precipitation steps.

2. Materials and methods

All chemicals were purchased from Merck (Darmstadt, Germany). Solutions were prepared with HQ-H_2O (filtered reverse osmosis water) and filtered through a 0.22-μm (pore size) membrane. Buffers for analytical high performance liquid chromatography (HPLC) runs were degassed prior to use.

2.1. CHO culture supernatants

Four clarified CHO culture supernatants that contained mAbs were provided by Novartis Pharma AG (Basel, Switzerland). They were stored at −20 °C for long-term storage or 4 °C for short-term storage. Prior to use, the supernatants were filtered (0.22 μm membranes). The mAb1 and mAb2 antibodies had the same amino acid sequence, but were expressed in different cell lines. Their isoelectric point was around 9.2, and the IgG concentrations were 3.44 mg/mL (mAb1) and 1.31 mg/mL (mAb2).

2.2. Optimization of caprylic acid/PEG precipitations

2.2.1. pH optimization of caprylic acid precipitation

A cell culture supernatant stock solution was prepared. A sodium acetate solution (4 M) was added to the clarified cell culture supernatant to a final concentration of 50 mM sodium acetate, and the pH was adjusted to 4.0 with acetic acid. For pH screening, the supernatant stock solution was separated into four parts with pH values of 4.5, 5.0, 5.5, and 6.0. These solutions were combined with 1% caprylic acid and mixed on a rotator at 40 rpm for 60 min. The precipitates were centrifuged (Avanti J-25, Beckmann Coulter, Brea, USA) at 10,000 × *g*, 4 °C, and filtered through 0.22 μm membrane (Syringe filter 25 mm GD/X, Cat. No. 6872-2502, Whatman, Maidstone, UK). Next, PEG precipitation was performed at the indicated pH value. Yield and purity data were determined after caprylic acid and PEG precipitations. Due to the influences of low pH on measurements with analytical affinity liquid chromatography, the yield was determined with size-exclusion chromatography (SEC; Bio SEC 3, Agilent, Santa Clara, USA). The percent yield was determined by comparing the IgG peaks of the precipitated samples to those of the original cell culture supernatants. Purity was monitored by measuring HCPs (HCP ELISA, Cygnus, Rockville, USA) and HMWIs (TSKgel G3000SWxl, Tosoh, Tokyo, Japan).

2.2.2. Parameter optimizations of caprylic acid/PEG precipitations

To improve the yield of the combined caprylic acid/PEG precipitation, the parameters of the caprylic acid precipitation were varied individually to screen for the optimal value. Precipitations were performed at different pH values (4.5, 5.0, and 5.5), with different salts (sodium acetate and sodium citrate), at different salt concentrations (5, 10, 20, 40, 50, 100, 125, and 200 mM), and different caprylic acid concentrations (0.5, 1.0, 2.0, 4.0, 6.0, and 10.0%). The solution mixing parameters were also varied, including the mixing time (15, 30, 45, 60 min) and rotation speed (10, 20, 30, and 40 rpm). For each screening, the parameters that were not varied remained fixed; only the parameter selected for screening was changed. After the caprylic acid precipitation, the separation of precipitates was performed as described above (Section 2.2.1), except that the pH value was adjusted to approximately 7 (between 6.9 and 7.1) prior to the PEG precipitation. The purified protein yield (Bio SEC 3, Agilent, Santa Clara, US and CIM Protein A HLD, BIA Separations, Ljubljana, Slovenia) and the HMWI content (TSKgel G3000SWxl, Tosoh, Tokyo, Japan) were determined after both precipitation steps. The HCP content (HCP ELISA, Cygnus, Rockville, USA) was only measured after the caprylic acid/PEG precipitation.

2.3. Combining different precipitation methods

To achieve pharmaceutical-grade purification, four different precipitation methods were combined, including caprylic acid and $CaCl_2$ which precipitates impurities as well as PEG and CEP which precipitates mAbs. These precipitation methods were performed in different orders and with varying amounts of precipitations steps. The caprylic acid precipitation was performed under the optimal conditions determined in the first part of the study (sodium citrate 100 mM, pH 4.5, rotated at 30 rpm for 45 min). Next, the precipitates were centrifuged (Avanti J-25, Beckmann Coulter, Brea, USA) at 10,000 × *g*, at 4 °C, and then filtered through a 0.2 μm membrane Filter (Syringe filter 25 mm GD/X, Cat. No. 6872-2502, Whatman, Maidstone, UK). The clarified supernatant was adjusted to approximately pH 7 (between 6.9 and 7.1) for PEG6000 precipitation with 14% PEG, at 5 rpm rotation speed (SB-3, Stuart, Staffordshire, UK) for 60 min. Precipitated mAbs were centrifuged (Heraeus Multifuge X3, Thermo Fisher Scientific, Waltham, USA) at 4000 × *g*, 20 °C. For $CaCl_2$ and CEP precipitations, 300 mM Na_2HPO_4 was added to a final concentration of 4 mM PO_4^{3-}. The solution was then heated to 20 °C and 1 M NaOH was added to adjust the pH to 8.5. Next, 4 M $CaCl_2$ was added to adjust the conductivity to 29 mS/cm (final concentration $CaCl_2 \sim 120$–150 mM); then the pH was checked and amended. After approximately 5 min of equilibration, the suspension was centrifuged at 4000 g for 15 min at 20 °C, and the pellet was discarded. The supernatant was cooled to 4 °C and 96% (v/v) ethanol was slowly added over 4 h to a final concentration of 25% (v/v). Simultaneously, the solution was cooled to −10 °C. The final mixture was equilibrated for 2 h before centrifugation (4000 × *g*, 15 min, −10 °C). All mAbs purified with CEP and PEG