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Ethanol precipitation for purification of recombinant antibodies

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ABSTRACT

Currently, the golden standard for the purification of recombinant humanized antibodies (rhAbs) from CHO cell culture is protein A chromatography. However, due to increasing rhAbs titers alternative methods have come into focus. A new strategy for purification of recombinant human antibodies from CHO cell culture supernatant based on cold ethanol precipitation (CEP) and $CaCl_2$ precipitation has been developed. This method is based on the cold ethanol precipitation, the process used for purification of antibodies and other components from blood plasma. We proof the applicability of the developed process for four different antibodies resulting in similar yield and purity as a protein A chromatography based process. This process can be further improved using an anion-exchange chromatography in flowthrough mode e.g. a monolith as last step so that residual host cell protein is reduced to a minimum. Beside the ethanol based process, our data also suggest that ethanol could be replaced with methanol or isopropanol. The process is suited for continuous operation.

1. Introduction

Purification of recombinant humanized antibodies (rhAbs) from cell culture supernatants is commonly performed by protein A chromatography followed by two to three additional chromatographic steps to obtain the purity required (Shukla et al., 2007; Sommerfeld and Strube, 2005). Depending on the rhAb the annual production volume is between 6 and 1000 kg/a with the pharmacy price value between 5000 and 400,000 US$/g (Hagel et al., 2007). In contrast, intravenous immunoglobulin (IVIG) preparations, which are usually used for the prophylactic prevention of infectious diseases in immunodeficient patients, have a significantly higher annual production volume, almost 100,000 kg/a at significantly lower cost, only ~91 US$/g (Hagel et al., 2007). The lower cost can be ascribed to the relatively simple process: The IVIG preparations are produced from collected human plasma using a series of cold ethanol precipitations (CEPs) known as the Cohn process (Buchacher and Iberer, 2006; Burnouf, 2007; Cohn et al., 1946, 1950; Moure et al., 2003; Radosevich and Burnouf, 2010). Since 1940s when this process has been first established by Cohn and coworkers, it has undergone several modifications to improve yield and purity (Buchacher and Iberer, 2006; Deutsch et al., 1946; Kistler and Nitschmann, 1962; Oncley et al., 1949). Still, the basic principle, variation of the five process parameters pH, ionic strength, ethanol concentration, protein concentration and temperature for selective precipitation of different plasma proteins, is still the same for every plasma fractionation process.

The simplicity and cost-effectiveness of the Cohn process make CEP an interesting option as an alternative, economic purification strategy for rhAbs from cell culture supernatant compared to other methods such as counter current loading (Godawat et al., 2012), counter current extraction (Rosa et al., 2013), or novel adsorption materials (Barroso et al., 2013; Borlido et al., 2013; Hilbrig and Freitag, 2012). However, the difference in the starting material (Tscheliessnig et al., 2013) makes the straight transfer of the Cohn process to the purification of rhAb from cell culture supernatant impossible: Despite the recent advances in rhAb titer in the last decade (Low et al., 2007; Shukla and Thoemmes, 2010) the concentrations of immunoglobulin (IgG) in human plasma are still higher (6–14 g/L, (Buchacher and Iberer, 2006)) than for rhAbs from cell culture supernatant (<5 g/L). Yet, due to the high complexity of the human plasma a large number of process steps are required to obtain the IVIG preparations in the required purity; this is at cost of yield (~30%) (Buchacher and Iberer, 2006). Also, the IgGs

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present in human plasma are highly heterogeneous, consisting of a range of different IgGs of various isoelectric points. In contrast the rhAbs present in the cell culture supernatant share the identical sequence thus are rather homogeneous compared to the IgGs present in human plasma (Kayser et al., 2011; Lingg et al., 2012). Only the different post-translational modifications observed result in the observed heterogeneity. This suggests that CEP could be a potential alternative for the purification of rhAbs from cell culture supernatant.

The advantage of CEP compared to other methods suggested to replace the conventional chromatography-based purification (Gagnon, 2012; Low et al., 2007) is, that besides its simplicity and cost-effectiveness, it also has a long safety record. IVIG preparations are produced using human plasma from a large pool of donors. This necessitates a tight control of potential contaminations with (known and unknown) viruses as well as prions (Buchacher and Iberer, 2006; Radosevich and Burnouf, 2010). It has been shown that the cold ethanol precipitation, also in combination with other orthogonal methods, is well capable to reach the required safety level (Buchacher and Iberer, 2006; Cai et al., 2002; Foster et al., 2000).

We present here the development of a purification platform for rhAb from CHO cell culture supernatant using CEP. In order to overcome co-precipitation of DNA with the rhAb during CEP we included $CaCl_2$ precipitation, which has been successfully established for clearance of not only DNA but also a number of protein impurities from the cell culture supernatant (Satzer et al., 2014). We established a platform based on CEP and $CaCl_2$ precipitation for the purification of four different rhAbs of different isoelectric point from CHO cell culture supernatant using a factorial design plan. The aim was a significant HCP reduction while maintaining yield. Aggregate formation is a permanent issue during CEP (Bull and Breese, 1978; van Oss, 1989; Yoshikawa et al., 2012), therefore the set-up of the CEP was designed to eliminate the detrimental effects of ethanol on proteins: good cooling of the suspension was provided and ethanol addition was slow to avoid precipitation due to excess heat caused by mixing of concentrated ethanol with an aqueous solution.

2. Materials and methods

All chemicals unless given otherwise were purchased from Merck (Darmstadt, Germany). All buffers for the analytical HPLC runs were prepared using HQ-H_2O, filtered through a 0.22 μm filter and degassed prior to use.

2.1. CHO culture supernatants

Clarified CHO culture supernatants of four rhAbs 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).rhAb1 and rhAb2 are antibodies of the same amino acid sequence but expressed in different cell lines. Their isoelectric point was around 9.2. rhAb3 and rhAb4 were more acidic antibodies with a pI of 6.7 and 6.8 respectively. rhAb3 is prone to aggregation and was used to evaluate the effect that precipitation would have on such an antibody.

2.2. Solubility curves

The alcohols used were methanol (Methanol LCMS Chromasolv, Fluka), ethanol (Ethanol 96% Emprove exp) and isopropanol (Isorpopanol LiChrosolv). rhAb3 was used to evaluate if the alcohols differ in their behavior to cause aggregation.

Aliquots of 10 ml of the clarified cell culture supernatant of rhAb3 were transferred into the reactor vessels of an Integrity 10 (Thermo Fisher Scientific) and tempered to 4 °C. The respective alcohol was added over 1 h to a final concentration of 15.0% (v/v), 22.5% (v/v) or 30.0% (v/v) using a syringe pump (Ismatec, Wertheim-Mondfeld, Germany). Simultaneously the temperature was linearly decreased to either 0 °C or −5 °C. The precipitates were collected by depth filtration (GD/X, Whatman) and dissolved in 10 ml of histidine buffer (20 mM histidine, 100 mM NaCl, pH 6.0). All experiments were performed in triplicates.

Table 1
Factors and levels used for development of the purification strategies.

Factor	Levels	
	–	+
pH	6.5	8.5
Salt type	NaCl	$CaCl_2$
Conductivity	No salt added	To 40 mS/cm with respective salt
Temperature	9 °C	−10 °C
Ethanol concentration	30% (v/v)	40% (v/v)

2.3. Factorial design plan

The set-up and evaluation of the full factorial design plan was based on Montgomery (2009). The factors and levels selected are given in Table 1. The clarified culture supernatants of rhAb1, rhAb2 and rhAb3 were first adjusted to the required pH using 12.5% HCl or 1 M NaOH. If required, 5 M NaCl or 4 M $CaCl_2$ was added to a final conductivity of 40 mS/cm and the pH verified and amended if required. Any precipitate forming was not removed prior to CEP. The CEP was performed using the Integrity 10 (Thermo Fisher, Rochford, UK) which is an automated lab reactor with 10 reaction cells for which temperature and speed of a magnetic stirrer can be independently adjusted. 2 ml of the respective solution was cooled to 4 °C and then 96% (v/v) ethanol, also cooled to 4 °C, added to obtain a final concentration of 30% (v/v) or 40% (v/v) respectively. After 2 h of incubation at 300 rpm the precipitate of each cell was removed by filtration (0.22 μm) and the supernatant analyzed for DNA, IgG and total protein. Each set-up was evaluated in triplicates.

Using the effect estimates obtained a regression model was applied to calculate the solubility of the rhAbs, DNA and total protein within the limits of the factorial design plan applied. This allowed drafting different purification strategies before experimental evaluation. The levels of the factors given in Table 1 were selected to enable a regression model where a range of different conditions could be evaluated. For pH the levels were limited by the isoelectric points of the antibodies used and for ethanol the range was limited to the concentrations which were evaluated to result in a high precipitate fraction (see Fig. 1).

2.4. Lab-scale CEP

For the $CaCl_2$ precipitation the respective supernatant or solution was adjusted to pH 8.5 using 10 M NaOH. Then 5 M $CaCl_2$ was added to a final concentration of 250 mM $CaCl_2$. If required, the pH amended to pH 8.5 using 10 M NaOH. The precipitate was removed by centrifugation (4000 rcf, 15 min, RT). For the CEP supernatant obtained after $CaCl_2$ precipitation was adjusted to pH 6.5 using 25% HCl, then transferred to the EasyMax (Mettler Toledo, Gießen, Deutschland), an automated lab reactor and tempered to 4 °C. Using the integrated syringe pump a 96% (v/v) ethanol stock was added over 4 h to obtain a final concentration of 25% (v/v). Simultaneously the temperature was linearly decreased to −10 °C. The solution was mixed by the integrated over-head stirrer (350 rpm) and temperature and turbidity monitored by a temperature probe and IR probe respectively. After incubation for 2 h post ethanol addition the precipitate was collected by centrifugation (−10 °C, 4000 rcf, 15 min)