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Optimisation of aqueous two-phase extraction of human antibodies

Ana M. Azevedo, Paula A.J. Rosa, I. Filipa Ferreira, M. Raquel Aires-Barros *

IBB - Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

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Abstract

The purification of human antibodies in an aqueous two-phase system (ATPS) composed of polyethylene glycol (PEG) 6000 and phosphate was optimised by surface response methodology. A central composite design was used to evaluate the influence of phosphate, PEG and NaCl concentration and of the pH on the purity and extraction yield of IgG from a simulated serum medium. The conditions that maximise the partition of IgG into the upper phase were determined to be high concentrations of NaCl and PEG, low concentrations of phosphate and low pH values. An ATPS composed of 12% PEG, 10% phosphate, 15% NaCl at pH 6 was further used to purify human monoclonal antibodies from a Chinese Hamster Ovary (CHO) concentrated cell culture supernatant with a recovery yield of 88% in the upper PEG-rich phase and a purification factor of 4.3. This ATPS was also successfully used to purify antibodies from a hybridoma cell culture supernatant with a recovery yield of 90% and a purification factor of 4.1.
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1. Introduction

Antibodies are highly specific, naturally evolved molecules that recognize and eliminate pathogenic and disease antigens (Brekke and Sandlie, 2003). Both, monoclonal and polyclonal antibodies play an important role in medicine as well as in analytical biotechnology. After being used predominantly for analytical purposes, over the last years the number of therapeutic applications is growing fast. Presently, the US Food and Drug Administration (FDA) has approved 21 monoclonal antibody-based therapeutics, to treat different kind of disorders, including cancer, transplant rejection, auto-immune diseases, asthma, cardiovascular diseases and infectious diseases (Brekke and Sandlie, 2003; Leone et al., 2006; Nayeem and Khan, 2006; Zhu et al., 2006).

In addition to the therapeutic monoclonal antibodies, the importance of polyclonal antibodies has also grown over the last years (Josic and Lim, 2001). Plasma-derived human IgGs are increasingly used to treat of genetic and acquired immune deficiencies as well as several auto-immune diseases (Dwyer, 1987). Antibodies have also been used extensively as diagnostic tools in many different formats. Moreover, antibody-based immunoassays are the most commonly used type of diagnostic assay and still one of the fastest growing technologies for the analysis of biomolecules (Borrebaeck, 2000).

The need to develop effective, economical and rapid purification methods for both monoclonal and polyclonal antibodies from a variety of biological fluids is becoming imperative for both *in vitro* and *in vivo* application. What is more, the downstream processing can undercut some of the advances in cell line productivity that are being achieved through molecular biology (Thiel, 2004). The downstream processing has therefore been considered the bottleneck in providing antibodies at reliable costs, being, in fact, the major cost factor with 50–80% of total production costs (de Haan et al., 2006; Roque et al., 2004). The critical steps have been identified to be the high resolution technologies such as affinity chromatography (Thiel, 2004). It has been demonstrated that it is possible to use liquid–liquid extraction technology as the first downstream purification step, allowing simultaneously the separation and concentration of the target protein (Rito-Palomares, 2002). Moreover, extraction in aqueous two-phase systems (ATPSs) has attracted interest for

* Corresponding author. Tel.: +351 218419065; fax: +351 218419062.
E-mail address: rabarros@alfa.ist.utl.pt (M.R. Aires-Barros).

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many years and has been developed as a primary stage unit operation in the downstream processing of different biological products, such as cells (Bradley and Scott, 2004; Kumar et al., 2001), virus (Benavides et al., 2006), RNA (Staub et al., 1995), plasmids (Frerix et al., 2005; Kepka et al., 2004; Trindade et al., 2005) and proteins (Cunha et al., 2003; Everberg et al., 2004).

The practical application of ATPSs in the recovery of biological products has been exploited for several years. ATPSs are formed when combinations of hydrophilic solutes (polymers or polymer and certain salts) display incompatibility in aqueous solution above critical concentrations (Rito-Palomares, 2002). Well-studied two-phase systems include poly(ethylene glycol) (PEG)/dextran and PEG/phosphate, where each phase generally contains 80–90% (w/w) water.

ATPSs provide a suitable environment to maintain biological activity and protein solubility (Schügerl and Hubbuch, 2005). This is due to the high biocompatibility, high water content and low interfacial tension of these systems that minimises product degradation (Nucci et al., 2001). On the other hand, good resolution and yields can be obtained simply by varying certain experimental conditions such as pH, ionic strength, and polymer molecular weight. What is more, ATPSs are an ideal technology where clarification, concentration, and partial purification can be integrated in one step (Schügerl and Hubbuch, 2005).

The use of aqueous two-phase extraction in the downstream processing of monoclonal antibodies has been reported in few research studies dating from the 1990's. Zijlstra and co-workers (1996) used a functionalised PEG/Dextran system to recover IgG from hybridoma cells grown in the dextran-rich phase (Zijlstra et al., 1996). At the same time, the group of Asenjo, used a polymer/salt ATPS to successfully recover IgG from hybridoma cell supernatants (Andrews et al., 1996). The feasibility of using ATPSs for the purification of human antibodies from a mixture of proteins containing human serum albumin and myoglobin was shown in a previous work from our group (Rosa et al., 2007). A recovery yield of 101% and a purity of 99% were obtained with an ATPS composed of 8% PEG 3350, 10% (w/w) phosphate and 15% (w/w) NaCl. In the present work, the application of ATPSs is expanded to the initial recovery of human antibodies from both Chinese Hamster Ovary (CHO) and hybridoma cell culture supernatants using a system composed of PEG 6000, phosphate and NaCl.

2. Materials and methods

2.1. Materials

Polyethylene glycol (PEG) with molecular weight of 6000 and 20,000 were purchased from Fluka (Buchs, Switzerland) and were used without further purification. Potassium phosphate dibasic anhydrous (K_2HPO_4), sodium phosphate monobasic anhydrous (NaH_2PO_4) and sodium chloride (NaCl) were obtained from Sigma. Human IgG for therapeutic administration (product name: Gammanorm) was purchased from Octapharma (Lachen, Switzerland), as a 165 mg/ml solution containing 95% of IgG. Albumin, from human serum (HSA), and myoglobin, from horse skeletal muscle (Myo), were obtained from Sigma. All other chemicals were of analytical grade.

The Chinese Hamster Ovary (CHO) cell supernatant, produced and delivered by ExcellGene (Monthey, Switzerland), contains a human IgG1, which is directed against a human surface antigen. The origin of the cell line is a double $DHFR^-$ 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 was used for production. Phenol red has been added to the medium as a pH indicator. According to Excellgene the antibody titre was 110 mg/l as determined by ELISA. Due to the low concentration of IgG, the supernatant was concentrated about 4.5 times in an Amicon Ultra centrifugal filter device form Millipore (Billerica, USA).

Hybridoma culture supernatant was generously provided by Dr. Marcelo Silva, from the Centre for Studies on Malaria and Tropical Diseases (IHMT, Lisbon, Portugal). The antibody produced was an IgG2b, directed against a CD4 protein. The cell line GK 1.5 was grown in a Roswell Park Memorial Institute (RPMI) 1640 Media from Invitrogen (Carlsbad, CA, USA) and contained 10% of foetal bovine serum. Different antibody titres were analysed, e.g., 480 and 1046 mg/l.

2.2. Aqueous two-phase extraction

Batch biphasic systems of 5 g were prepared by weighing out appropriate amounts of 50% (w/w) PEG, 40% (w/w) phosphate buffer, sodium chloride (NaCl), protein feed stock solution and water in order to achieve the desired final system composition. A feed stock loading of 40% was used unless otherwise stated. The 40% (w/w) phosphate buffer solutions with different pH values were prepared by using a mass ratio of potassium phosphate dibasic anhydrous (K_2HPO_4) to sodium phosphate monobasic anhydrous (NaH_2PO_4) of 0.79 for pH 6, 2.13 for pH 7 and 7.33 for pH 8. Slight adjustments to the final pH value were performed with a 40% (w/w) solution of K_2HPO_4. The pH of the system was assumed to be equal to the original phosphate buffer stock solution.

The phase components were mixed on a Vortex agitator (Ika, Staufen, Germany), equilibrated in a water bath at 25 °C for 12 h and then centrifuged for 5 min in a fixed angle rotor centrifuge (Eppendorf, Hamburg, Germany) at $1400 \times g$ to ensure total phase separation. Upper and lower phases were then carefully separated and the volume of each phase measured. Samples from both upper and lower phases were taken for the determination of IgG concentration, protein content and for SDS–PAGE analysis. The partition coefficient, K_P, was defined as the ratio of the IgG concentration in the upper phase to that in the lower phase. The extraction yield of IgG, Y_{IgG}, was defined by the ratio between the mass of IgG in the upper phase and the mass of IgG added to the system. Phase composition was determined using phase diagrams reported by Albertsson (1986).

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