Journal of Chromatography A, 1355 (2014) 115–124

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Phenylboronic acid as a multi-modal ligand for the capture of monoclonal antibodies: Development and optimization of a washing step

Raquel dos Santos[a,1], Sara A.S.L. Rosa[a,1], M. Raquel Aires-Barros[a], Andres Tover[b], Ana M. Azevedo[a,*]

[a] Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Department of Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisbon, Portugal
[b] Icosagen Cell Factory, Eerika tee 1, Ülenurme vald, 61713 Tartumaa, Estonia

ARTICLE INFO

Article history:
Received 1 April 2014
Received in revised form 29 May 2014
Accepted 2 June 2014
Available online 6 June 2014

Keywords:
MAbs purification
Multimodal chromatography
Phenylboronate ligands
Capture step
Wash and elution studies

ABSTRACT

In this work, phenylboronic acid (PBA) was thoroughly investigated as a synthetic ligand for the purification of an immunoglobulin G (IgG) from a clarified cell supernatant from Chinese Hamster Ovary (CHO) cell cultures. In particular, the study was focused on the development of a washing step and in the optimization of the elution step using a serum containing supernatant. From the different conditions tested, best recoveries – 99% – and purifications – protein purity of 81% and a purification factor of 16 out of a maximum of 20 – were achieved using 100 mM D-sorbitol in 10 mM Tris–HCl as washing buffer and 0.5 M D-sorbitol with 150 mM NaCl in 10 mM Tris–HCl as elution buffer. The purification outcome was also compared with protein A chromatography that revealed a recovery of 99%, 87% protein purity and 29 out of a maximum of 33 purification factor. Following the main purification, purified IgG was characterized in terms of isoelectric point, size and activity. In the end, a proof of concept was performed using two different mAbs from serum-free CHO cell cultures.

1. Introduction

Monoclonal antibodies (mAbs) are the leading family of biopharmaceutical compounds in terms of therapeutic and market potential with total sales expected to reach a value of $70 billion in 2015 [1]. Currently, more than 40 mAb-based drugs have been approved by regulatory agencies (FDA, EMEA) for the treatment of several disorders as different types of cancer, multiple sclerosis and immunological diseases such as rheumatoid arthritis and psoriasis. The combination of an ever-growing patient population with the relatively low potency of mAbs led to the need for production processes that can be rapidly developed to produce consistently large quantities of pharmaceutical-grade mAbs [2]. In order to meet the requirements, mammalian cell lines have become the dominant antibody expression system, with Chinese Hamster Ovary (CHO) cells being the main choice [3,4]. With the continuous discoveries in molecular biology and genetics, combined with new advances in media and feed development, higher cell culture productivities and antibody titers of 10 g/L were achieved with CHO expression system [5]. This drastic increase has reinforced the demand for new downstream technologies able to cope with this upward trend in productivity. In order to keep up this gain, it is now essential to design new, as well as to improve the existing downstream processes that remain an unresolved bottleneck. The established downstream processing of mAbs follows a platform-based approach that encompasses a protein A affinity chromatography capture step and two polishing steps to ensure a high clearance of impurities as host cell proteins, DNA, viruses, aggregates and low molecular clipped species [6]. The protein A chromatography exploits the specific interaction between the Fc region of the antibodies and the immobilized protein A. Given its high selectivity for antibodies, purities greater than 98% are typically achieved in a single step from a clarified complex cell culture media [7]. The major drawbacks of this chromatographic step are the high associated costs, which can represent more than 70% of the

* Corresponding author. Tel.: +351 218419065; fax: +351 218419062.
E-mail address: a.azevedo@tecnico.ulisboa.pt (A.M. Azevedo).
[1] These authors have contributed equally.

http://dx.doi.org/10.1016/j.chroma.2014.06.001
0021-9673/

total downstream process [8], ligand leaching, instability at high pH and the formation of product aggregates under low pH standard elution conditions [9].

Many attempts have been focused on the search for cheap, stable and easy-to-use alternatives for protein A. For the choice of such alternatives, the dynamic binding capacity should be evaluated in relation to specific resin properties as rigidity and porosity, resin resistance and stability throughout a number of purification cycles [10]. Novel affinity-based separations have emerged from the development of synthetic ligands including biomimetic peptides obtained by combinatorial libraries and artificial ligands generated by *de novo* process designs [11,12]. One promising synthetic ligand is phenylboronic acid (PBA), used in boronate affinity chromatography [13]. The PBA ligand is known to be a useful tool for the specific capture and effective enrichment of target *cis*-diol-containing molecules, such as carbohydrates, glycoproteins, enzymes, RNA, nucleotides and nucleic acids [14–16]. Antibodies are glycoproteins, bearing N-linked oligosaccharide at the asparagine residue of CH2 domain of the Fc portion. In the former, despite some heterogeneity, the 1,2-*cis*-diol saccharides fucose, manose and galactose can be typically found. Boronate ligands are able to form a pair of covalent bonds with molecules containing *cis*-diols *via* a reversible esterification reaction [17]. In acidic solutions, boronic acids adopt a trigonal planar form which can revert to a tetrahedral boronate anion upon hydroxylation in alkaline conditions. Both the acid and its conjugate base can bind to diol compounds [18]. However, since the equilibrium constant for the tetrahedral (K_{tet}) is usually higher than that of the trigonal form (K_{trig}), complexes are less stable in acidic conditions [19]. This esterification is stronger if the two hydroxyl groups of the diol are on adjacent carbon atoms and in an approximately coplanar configuration, such as 1,2-*cis*-diol [17]. Ligands as PBA are aromatic and thus also able to establish hydrophobic and π–π interactions. Secondary ionic interactions between boronates and diols are also possible through coulombic attraction or repulsion effects, hydrogen bonding by the hydroxyl groups and charge transfer interactions. The latter is more prone to occur in acidic conditions, since in the trigonal uncharged form, the boron atom has an empty orbital and can thus serve as an electron receptor for a coordination interaction, enabling Lewis acid–base interactions to occur [20].

The aim of the present work is to evaluate the potential of PBA as an alternative multi-modal ligand for the direct purification of different mAbs from clarified CHO cell culture supernatants. Previous work have already shown the ability of PBA to bind antibodies highlighting that non-specific interactions could play an important role in the binding of proteins to the PBA ligand [18,21]. Also, the development and optimization of a washing step will be emphasized since it is an important feature to increase the final purity. In this work, we have evaluated the selectivity of the controlled porous glass (CPG)-PBA and fully characterize the purified fractions in terms of product yield, purity and IgG activity. Special attention is given to the elucidation of the binding and elution mechanisms.

2. Materials and methods

2.1. Chemicals

Tris(hydroxylmethyl)aminomethane (Tris), sodium chloride (NaCl), D-sorbitol, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium azide (NaN_3) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium phosphate monobasic anhydrous and sodium phosphate dibasic were obtained from Panreac Quimica Sau (Barcelona, Spain), and hydrochloric acid from Fluka (Buchs, Switzerland). All other chemicals were of analytical or HPLC grade. Water used in all experiments was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. IgG production

2.2.1. Anti-human interleukin-8 monoclonal antibodies

Anti-human interleukin-8 monoclonal antibodies were produced by CHO DP-12 clone#1934 (ATCC CRL-12445) using DHFR minus/methotrexate selection method. CHO DP-12 cells were grown in 75% (v/v) serum-free media (ProCHO™5, Lonza Group Ltd, Belgium) and 25% (v/v) DMEM (Dulbecco's Modified Eagle's Medium) (Gibco®, Carlsbad, CA, USA) supplemented with 10% (v/v) ultra-low IgG fetal bovine serum (FBSUL, Gibco®). 200 nM methotrexate was present in the culture medium to maintain selective pressure. ProCHO™5 was supplemented with 4 mM L-glutamine (Gibco®), 2.1 g/L $NaHCO_3$ (Sigma–Aldrich), 10 mg/L recombinant human insulin (Lonza), 0.07% (v/v) lipids (Lonza) and 1% (v/v) antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) (Gibco®). The DMEM used contains 4.5 g/L D-glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate. After ressuspension of DMEM powder, 1.5 g/L $NaHCO_3$, 2 mg/L recombinant human insulin, 35 mg/L L-proline (all from Sigma–Aldrich), 0.1% (v/v) of a trace element A, 0.1% (v/v) of a trace element B (both from Cellgro®, Manassas, VA, USA) and 1% antibiotics (v/v) (100 U/mL penicillin and 100 μg/mL streptomycin) were added. The cultures were carried out in T-175 or T-75 flasks (BD Falcon, Franklin Lakes, NJ) at 37 °C and 5% CO_2 with an initial cell density of 2.8×10^4 cells/cm^2. Cell passages were performed every 4 days. Cell supernatants were collected by centrifugation and storage at -20 °C. In each passage, cells were washed with phosphate buffered saline (PBS, Gibco®) and detached from the flask by adding Accutase solution (Sigma–Aldrich) for 3 min at 37 °C. Cell number and viability were determined using the Trypan Blue (Gibco®) exclusion method. This culture was maintained for several months and the mAb concentration varied between 40 and 91 mg/L.

Anti-human interleukin-8 (anti-IL-8) monoclonal antibodies were also produced in serum-free media, using only ProCHO™5, prepared as described above. Cultures were performed as described for the serum-media but with cell passages every 6 days. The concentration of mAb was around 130 mg/L.

2.2.2. Anti-human recombinant hepatitis C virus (HCV) subtype 1b monoclonal antibodies

Anti-human recombinant hepatitis C virus (HCV) subtype 1b monoclonal antibodies were from mouse hybridoma cells expressing a mouse anti-recombinant hepatitis C virus subtype 1b nonstructural protein 5B (NS5B) RNA-dependent RNA polymerase (RdRp) monoclonal antibody. The mRNA of antibody variable regions was isolated and for reconstruction of full antibody, the corresponding cDNA fragments were cloned into the Icosagen Cell Factory proprietary QMCF expression vector containing IgG1 antibody constant regions. The QMCF plasmid contains the mouse polyomavirus (Py) DNA replication origin which in combination with the Epstein-Barr virus (EBV) EBNA-1 protein binding site, as nuclear retention element, ensures stable propagation of plasmids in the QMCF cells [22]. Chimeric antibody was expressed by the QMCF cell line CHOEBNALT85, developed from CHO-S cell line (Invitrogen™, Carlsbad, CA, USA) which have been adapted for suspension growth in chemically defined serum-free medium. The cells were grown in a mix of two serum-free media, (i) CD CHO medium (Gibco®) and (ii) 293 SFM II medium (Gibco®) with the addition of CHO CD efficient feed B and feed A with glutamine using an *in-house* procedure (Icosagen Cell Factory OÜ) in the presence of G418 (700 μg/mL) to select expression plasmid containing pool of the cells. After antibiotic selection, 5 vials of expression cell bank was generated (10^7 cells/vial) and kept at liquid nitrogen.