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Purification of monoclonal antibody from tobacco extract using membrane-based bioseparation techniques

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ABSTRACT

Transgenic plants offer a promising system for large-scale production of therapeutic proteins such as monoclonal antibodies (mAbs). This paper describes a membrane-based process suitable for purification of a humanized mAb expressed in tobacco. Most monoclonal antibody purification schemes rely on the use of Protein A as the affinity ligand for antibody capture. The main objective of our work was to develop non-Protein A-based purification methods to avoid some of the problems and limitations associated with this ligand, e.g. cost, immunotoxicity, and antibody aggregation during elution. Ion exchange membrane chromatography (IEMC) was used for primary capture and preliminary purification of the mAb from tobacco juice. Hydrophobic interaction membrane chromatography (HIMC) was then used for high-resolution purification, followed by ultrafiltration for polishing, desalting and buffer exchange. Using this scheme, both high mAb purity (single peak in size exclusion chromatogram, i.e., ca. 100% purity) and high recovery (77% of mAb spiked into the tobacco extract) were achieved. Membrane chromatography is generally considered unsuitable for resolving bound proteins by gradient elution and is therefore commonly used in the bind and elute mode with a single-step change of mobile phase. We show that the gradient elution process in the HIMC step can be optimized to increase the resolution and thereby obtain product of high purity.

1. Introduction

Molecular farming of recombinant proteins by expression in genetically modified plants provides an alternative production route for biopharmaceuticals such as monoclonal antibodies (mAbs) [1–8]. This approach offers reduced contamination risks from animal viruses, potential for reduced cost and simplified scale-up of the manufacturing process [9–11]. Bioseparation is recognized as the bottleneck in molecular farming since the target protein has to be separated from impurities such as starch, cellulose, sugars, alkaloids, pigments, polyphenols, native plant proteins, and nucleic acids which are present in the plant homogenate [12]. Furthermore, recombinant protein purification from genetically modified plants is challenging due to the low expression levels of transgenic proteins *in planta* (i.e., mg/kg), resulting in the need for processing huge quantities of biomass. This implies that the overall purification process must combine high-resolution with high-throughput.

Protein A- and/or Protein G-based column chromatography is widely used for purifying mAbs from mammalian cell-culture supernatant as well as polyclonal antibodies from serum. The major limitations with column chromatography, particularly those processes employing soft gel-based media are the need to use low flow-rates to avoid back pressure, bed compaction, high buffer consumption, low product throughput, and scalability problems. Protein A/G-based affinity media is widely available in the soft-gel bead format. In such chromatographic media, diffusion-based mass transport within the gel beads predominates and this makes the separation process slow. Furthermore, specific problems associated with the use of Protein A include the leakage of this ligand, which is immunotoxic, into the product [13] and the need to elute column bound mAb under acidic conditions, which may lead to antibody aggregation [14].

Membrane-based bioseparation processes can be made to combine high-resolution separation with high product throughput, which may be conducted under relatively benign operating conditions thereby ensuring product stability [15]. With membrane chromatography this is achieved by combining the advantages of membrane technology (which gives high throughput) with those of chromatography (which gives high resolution). In column chromatography, both binding efficiency and separation are dependent

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on diffusive transport, thus mobile phase flow rate may restrict productivity and make scale-up difficult. In contrast, binding and separation in membrane chromatography depend upon convective transport (i.e., are generally independent of flow-rate), over a wide operating range [15]. High flow-rates, shorter process times and easy scale-up are therefore achievable in membrane chromatography.

Different types of membrane chromatographic processes have been successfully used for purification of both serum-based polyclonal and cell culture-based monoclonal antibodies (i.e., Protein A-based affinity membrane chromatography [16,17], ion exchange membrane chromatography (IEMC) [18,19], and hydrophobic interaction membrane chromatography (HIMC) [20–23]). However, only one paper discusses purification of recombinant monoclonal antibodies from plants using membrane chromatography (combination of Protein A and ion exchange membrane chromatography) [24]. This previous paper described the purification of mAb from transgenic tobacco leaves using IEMC in combination with Protein A membrane chromatography (PAMC). As discussed earlier, the use of a Protein A leads to some limitations and there is a growing trend in the bio-industry to examine and develop alternatives to Protein A-based purification methods. The current work deals with purification of humanized mAb from tobacco extract using a combination of three non-Protein A-based methods, i.e., IEMC, HIMC and ultrafiltration (UF). A simulated transgenic tobacco juice feedstock was used in our current work. This was prepared by spiking juice obtained from wild-type tobacco leaves with humanized monoclonal antibody hIgG1-CD4. In our purification scheme IEMC was used to capture and enrich the mAb, HIMC was then used to further purify the mAb, while ultrafiltration was used as the last step to polish and desalt the purified protein. Each of these separation steps were extensively optimized to obtain high resolution. The current work examines the possibility of fine tuning the gradient elution process in the HIMC for increasing the separation efficiency.

2. Experimental

2.1. Materials

Humanized mAb hIgG1-CD4 (batch 12) by CHO cell culture was kindly donated by the Therapeutic Antibody Center, University of Oxford, UK. The samples were shipped in dry ice (each vial containing 10 mg mAb in 2.1 mL buffer) and were used as received. Wild-type tobacco plants were grown in a greenhouse (University of Guelph, Ontario, Canada), the leaves were harvested from healthy plants and stored in a freezer at −20 °C. Chemicals including sodium phosphate (mono- and di-basic), ammonium sulphate, sodium citrate, citric acid and sodium chloride were purchased from Sigma–Aldrich, St. Louis, MO, USA. All buffers and protein solutions were prepared using ultra-pure water (18.2 MΩ cm) obtained from a Diamond Nanopure water purification unit (Barnstead International, Dubuque, IA, USA) and micro-filtered through 0.45 μm membranes (product# DS0210-4045; Nalgene Nunc, Rochester, NY, USA). Sartobind S membranes (catalogue number 94IEXS42-001) used for IEMC were purchased from Sartorius AG, Goettingen, Germany; polyvinylidine fluoride (PVDF) membrane discs (0.22 μm GVWP14250) from Millipore, Billerica, MA, USA; 70 kDa polyethersulfone (PES) ultrafiltration membrane (part number OT070) from Pall, East Hills, NY, USA.

2.2. Preparation of tobacco juice

The frozen wild-type leaves were manually crushed into small pieces within their plastic storage bags and then mixed with extraction buffer (40 mM phosphate buffer, pH 7.0, containing 50 mM ascorbic acid as antioxidant, 50 μM leupeptin as protease inhibitor, and 10 mM disodium EDTA as chelating agent). The leaves were ground in a jar type blender (51BL32, Waring Commercial, Torrington, CT, USA) for 3 min and ground further in a homogenizer (ULTRA TURRAX T25 Basic, IKA Works, Staufen, Germany) for another 3 min. The homogenate was pre-filtered through cheese cloth to remove large particles, centrifuged at 10,528 × g using a Allegra refrigerated centrifuge (X22R, Beckman, Fullerton, CA, USA) for 30 min and micro-filtered through a 42-mm diameter membrane disc (0.45 μm pore size, PES) housed in a custom-designed stirred cell at a permeate flux value of 3.0×10^{-5} $m^3/(m^2\ s)$. The clarified tobacco juice was stored at −20 °C and thawed just prior to purification.

2.3. Ion-exchange membrane chromatography

The membrane discs were housed in a custom-designed membrane module [25] having a 18-mm effective diameter. The module was integrated with an AKTA prime liquid chromatography system (GE Healthcare Biosciences, Uppsala, Sweden). The effluent from the membrane module was continuously monitored for absorbance (280 nm), pH and conductivity; the data was logged into a computer using Prime View software (GE Healthcare Biosciences). The system pressure was also continuously monitored and recorded by the AKTA prime system.

IEMC was carried out using a membrane module which contained a stack of 15 Sartobind S discs, each having an effective diameter of 18 mm. The pH of the feed solution was first optimized using 20 mM sodium phosphate buffer (PB) adjusted to different pH values. The corresponding elution buffers were prepared by adding 0.5 M NaCl to the binding buffer. The effect of pH on mAb binding was studied by injecting 5 mL of 18.9 μg/mL hIgG1-CD4 solution prepared in the appropriate binding buffer. The binding of tobacco proteins on the ion exchange membrane was tested by injecting 5 mL of tobacco juice diluted 1:1 (v/v) with the binding buffer. The effect of feed conductivity on mAb and tobacco protein binding on the membrane was examined at the optimized pH value, with the conductivity of the buffer being adjusted by addition of sodium chloride. The flow rate used in the pH and conductivity optimization experiments was 1 mL/min. Simulated transgenic tobacco feed solution was prepared by spiking wild-type tobacco juice with hIgG1-CD4 (10 mg of hIgG1-CD4 per kg of frozen leaves). Prior to the purification experiments, the tobacco simulated feed solution was diluted 1:1 (v/v) with the appropriate binding buffer (i.e., at optimized pH and conductivity). Different volumes of the simulated transgenic tobacco feed were injected and eluted to determine mAb binding efficiency from the corresponding feed solution. The flow rate used in these experiments was 2 mL/min since this gave good separation at reasonably low transmembrane pressure. In these experiments, the membrane module was first equilibrated with binding buffer followed by injection of the feed. The unbound material was then removed from the membrane module by washing with binding buffer followed by removal of bound material using eluting buffer. The effects of applying different types of NaCl gradients on the purity of eluted mAb were examined. The flow-through and "elution peak" samples from the IEMC experiments were collected and analyzed by analytical Protein A affinity chromatography and SDS-PAGE.

2.4. Hydrophobic interaction membrane chromatography

Chromatography system used for HIMC was the same as IEMC. Membrane modules for HIMC had both 18- and 42-mm effective diameters. Feed solutions for HIMC were prepared by mixing in 1:1 ratio the IEMC eluate with concentrated ammonium sulfate