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New downstream processing strategy for the purification of monoclonal antibodies from transgenic tobacco plants

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ABSTRACT

Affinity chromatography on immobilized Protein A is the current method of choice for the purification of monoclonal antibodies (mAbs). Despite its widespread use it presents certain drawbacks, such as ligand instability, leaching, toxicity and high cost. In the present work, we report a new procedure for the purification of two human monoclonal anti-HIV (human immunodeficiency virus) antibodies (mAbs 2G12 and 4E10) from transgenic tobacco plants using stable and low cost chromatographic materials. The first step of the mAb 2G12 purification procedure is comprised of an aqueous two-phase partition system (ATPS) for the removal of polyphenols while providing an essential initial purification boost (2.01-fold purification). In the second step, mAb 2G12 was purified using cation-exchange chromatography (CEX) on S-Sepharose FF, by elution with 20 mM sodium phosphate buffer pH 7.5, containing 0.1 M NaCl. The eluted mAb was directly loaded onto an immobilized metal affinity chromatography column (IMAC, Zn^{2+}–iminodiacetic acid–Sepharose 6B) and eluted by stepwise pH gradient. The proposed method offered 162-fold purification with 97.2% purity and 63% yield. Analysis of the antibody preparation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), enzyme immunosorbent assay (ELISA) and western blot showed that the mAb 2G12 was fully active and free of degraded variants, polyphenols and alkaloids. The effectiveness of the present purification protocol was evaluated by using a second transgenic human monoclonal anti-HIV mAb 4E10. The results showed that the same procedure can be successfully used for the purification of mAb 4E10. In the case of mAb 4E10, the proposed method offered 148-fold purification with 96.2% purity and 36% yield. Therefore, the proposed protocol may be of generic use for the purification of mAbs from transgenic tobacco plants.

1. Introduction

Over the last decade, plant-based expression systems have emerged as a serious competitive force in the large-scale production of recombinant proteins [1–3] exhibiting enormous potential as bioreactors for expressing active mAbs for human disease therapy. Full-size immunoglobulins, as well as additional antibody derivatives, including Fab fragments, scFvs, bispecific Fvs, diabodies, minibodies, single variable domains, antibody fusion proteins, large single-chain antibodies and camelid heavy chain antibodies have been successfully expressed in a variety of plant-based expression platforms [4]. The first plant-derived proteins have already reached the market and many others are in the final stages of development [5]. The use of plants as bioreactors for the production of therapeutic proteins has a number of advantages, including the lack of animal-derived media supplements, low production cost, and enormous potential for scale-up compared to other currently available conventional production systems, such as mammalian cells or bacteria. This can be translated into enormous consumer benefits. Several different plants have been investigated for production of recombinant proteins, including leafy crops (tobacco, alfalfa, soybean, and lettuce), cereals (rice, wheat and maize), legumes (pea and soybean), and also fruit and vegetable crops (potatoes, tomatoes, and bananas) [6–9]. Tobacco is one of the strongest candidates for commercial production of pharmaceutical proteins [1] partly due to the well-established technology for gene transfer and expression. In addition, it can produce the highest amount of biomass per cultivable area of all known crops resulting in high product yields and is neither a food nor a feed crop so the risk of inadvertent contamination is low. However, the high concentration of potentially harmful products of secondary metabolism, such as polyphenols and alkaloids [10], are a downside, as well as the need

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for immediate processing of the biomass in order to avoid product degradation or modification.

Many diseases require high doses or continuous treatment with monoclonal antibodies, therefore creating the need for an affordable production platform. Any potential economic benefits derived from the use of plant-derived bioreactors can certainly be annulled in the absence of an economic downstream processing technology and strategy, creating a bottleneck in the production of an affordable commercial product [6]. The most commonly used downstream processing approach to the purification of monoclonal antibodies include Protein A affinity chromatography. This is the most selective method for antibody purification and is often used as an initial biospecific step to facilitate antibody purification and concentration [11,12]. However, it suffers from problems, such as ligand instability, leaching and high cost since Protein A resin is very expensive and readily amenable to chemical and biological degradation. In addition, leaching of the Protein A ligand is also a major concern since it is known to cause immunogenic responses in humans and has proven toxic in several clinical trials [13,14]. On the other hand, alternative conventional chromatographic methods, such as ion exchange and immobilized metal affinity chromatography are suitable for large-scale chromatography, since they are inexpensive. The respective adsorbents resistant to chemical or biological degradation, and display high protein-binding capacity, however, lack the necessary selectivity.

In the present work, we report an efficient, novel procedure for the purification of two human monoclonal anti-HIV antibodies (mAbs 2G12 and 4E10) from transgenic tobacco plants. The approach is based on a combination of three widely used conventional techniques: aqueous two-phase partition system (ATPS), cation-exchange chromatography (CEX) and immobilized metal ion affinity chromatography (IMAC). Each technique has been previously used for the purification of antibodies from complex biological sources [8,15–19]. They are cost-effective techniques and possess unique advantages for the development of a facile and scalable purification protocol from transgenic plant biomass.

2. Materials and methods

2.1. Materials

Chinese hamster ovary cell (CHO)-derived and purified human monoclonal anti-HIV 2G12 and 4E10 antibodies were kindly donated by Dr. Dietmar Katinger at Polymun Scientific. Polyethylene glycol (PEG) (molecular masses 1500, 3000 and 6000), potassium phosphate monobasic, potassium phosphate dibasic, polyclonal antibody goat anti-human-IgG (γ-chain) (GAH-IgG), polyclonal antibody goat anti-human-IgG (κ-chain) (GAH-IgG&AP) conjugated to alkaline phosphatase, iminodiacetic acid–Sepharose 6B FF and S-Sepharose FF were obtained from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Plant growth

Transgenic *Nicotiana tabacum* seeds were pre-germinated on plates, on filter paper, Whatman 3MM (soaked in distilled water). The plates were kept for 72 h at 30 °C. After germination, they were transferred into plastic pots (1–2 plants in each pot), in soil. The plants were grown in a controlled environment (12 h day/12 h night cycle, at 25 °C day/21 °C night-regime and 65% humidity and watered with deionized water every 2 days.

2.2.2. Extraction of mAbs 2G12 and 4E10 from transgenic tobacco leaves

Aqueous extraction of mAbs 2G12 and 4E10 from transgenic tobacco leaves was typically carried out by mixing 5 g of tobacco leaves with 15 ml of 50 mM sodium phosphate buffer pH 5.0. The mixture was disintegrated in a blender (total 10 min, breaking every 15 s) and placed on a rotary mixer for 60 min at 4 °C. The mixture was subsequently centrifuged at 10,000 × *g* for 30 min (4 °C). The supernatant was collected and passed through a 0.45 μm filter. The amount of protein and monoclonal antibody extracted was determined by Bradford assay and ELISA, respectively.

2.2.3. Aqueous two-phase system

Phase diagrams for the behaviour of PEG–phosphate systems have been reported [20,21]. Phase systems were prepared from stock solutions of PEG (50%, w/w) and phosphate (40%, w/w). The phosphate stock solution was prepared by mixing appropriate amounts of K_2HPO_4 and NaH_2PO_4 until the desired pH was achieved. Aqueous two phase systems (5 g) were prepared in the following manner: tobacco extract (final concentration 7.5%, w/v), water and phosphate buffer were added first, followed by gradual addition of the appropriate amount of 50% (w/w) PEG in order to avoid protein precipitation due to increased PEG concentration. Total protein was added to a final concentration of 0.3–0.35 g/l. The systems were mixed thoroughly and allowed to equilibrate at room temperature for 5 min after which they were centrifuged at room temperature for 2 min at 200 × *g* to speed up phase separation. Alternatively, phase separation can be achieved without centrifugation for longer times. The centrifuged samples were allowed to settle for 30 min. ATPS without protein (containing extraction buffer) were used for reference and comparison. Visual estimates of the volumes of top and bottom phases were made in graduated centrifuge tubes and were used to estimate the volume ratio [V_r = volume of top phase (V_t)/volume of bottom phase (V_b)]. Samples were carefully removed from both phases and analyzed to determine total protein and monoclonal antibody concentrations by Bradford and ELISA, respectively. Parameters evaluated included: (a) yield of mAb 2G12, defined as the amount of mAb 2G12 in the bottom phase/initial mAb 2G12 added into the whole system and (b) purification factor, defined as the ratio: $([\text{mAb 2G12}]/[\text{total tobacco protein}])_{\text{bottom phase}}/([\text{mAb 2G12}]/[\text{total tobacco protein}])_{\text{crude extract}}$.

2.2.4. Full central composite design

The partitioning behaviour of tobacco proteins and mAb 2G12 was studied using three two-level full central composite design (CCD) [22] to study the effect of two independent variables, namely PEG and phosphate buffer (Pi) concentrations in different PEG MW conditions. The variables were studied at two levels and for each variable studied, high (coded value: +1) and low (coded value: −1) set points were selected based on results obtained from previous experiments [23] and considering the experimental conditions required for phase separation. The ranges of factors are PEG molecular weight (1500–6000), PEG concentration (16–20%, w/w for PEG 1500, 15–20%, w/w for PEG 3000 and 17.5–20% for 6000) and phosphate buffer concentration (8.5–11.5%, w/w for PEG 1500, 7–12%, w/w for PEG 3000 and 6000) at pH 5 and transgenic tobacco extract load 7.5% (w/w). Thirteen experiments were included in each model, with four factorial, five center and four axial points. Factorial points are the experiments run with combinations of high and low levels of each factor that allow the user to determine the main effects and interactions between them. Center points are experiments run at the center level of each of the factor ranges and are used to assess the background variability in the process. Axial points are experiments that are set to a level outside the