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Purification of monoclonal antibody against Ebola GP1 protein expressed in *Nicotiana benthamiana*

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Abstract

Monoclonal antibodies (mAbs) are one of the fastest growing drug molecules targeting the treatment of diseases ranging from arthritis, immune disorders, and infectious diseases to cancer. Due to its unique application principle, antibodies are commonly produced in large quantities. Plants, such as *Nicotiana benthamiana*, offer a unique production platform for bio-therapeutics due to their ability to produce large amounts of biomolecules in a relatively quick manner. However, purification of a target protein from plant is an arduous task due to the presence of toxic compounds in ground plant tissue and the large quantities of plant tissues to be processed. Here, a process was developed prior to the chromatographic purification of a mAb against Ebola GP1 protein expressed in *N. benthamiana*. The process includes a diafiltration step and a charged polyelectrolyte precipitation. The diafiltration step significantly improved the precipitation efficiency, reducing the usage of polyelectrolyte by more than 2000 fold while improving the native plant protein removed from 60% to 80%. The mAb can then be purified to near homogeneity judging from SDS-PAGE by either Protein A affinity chromatography or a tandem of hydrophobic interaction chromatography and a hydrophobic charge induction chromatography. The purified mAbs were shown to retain their binding specificity to irradiated Ebola virus.

Keywords

Monoclonal antibody; Transgenic plant; *Nicotiana benthamiana*; Antibody purification; Ebola virus; Transient expression

1. Introduction

Antibodies are the fastest growing class of biotherapeutics in the past decade. Mammalian cell culture is the predominant expression system for monoclonal antibody (mAb) production, yielding mAbs with proper folding and glycosylation [1,2]. However, the substantial resource requirements and viral clearance present challenges for this production system [1]. On the other hand, plants offer a unique production platform for mAbs and have shown in recent years that they are able to express high levels of antibody (5 g/kg) in just a matter of days [3,4]. The advantages of using plant systems are the low cost of biomass

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generation and minimized risk of mammalian/human pathogens [2,5]. The lack of industrial use thus far is mainly attributed to the long lead time for generating transgenic plants. However, the development of transient plant expression systems has allowed the rapid accumulation of mAbs in plant cells at extremely high levels for clinical studies [6,7].

Ebola virus (EBV) infection causes Ebola hemorrhagic fever (EHF), and the recent outbreak (July 2014) in West Africa has brought more attention to this devastating virus, with some strains leading to fatality rates up to 90% [8]. Currently no approved vaccines or therapeutics are available to prevent or treat BEV [9]. In a remarkable development, *Nicotiana benthamiana* plant-produced mAbs were used to treat two American Ebola patients and showed promising results [10]. One of these mAbs (6D8) is a protective mAb against EBV GP1. When administered together with other two mAbs, plant-produced 6D8 protected mice and nonhuman primates with superior potency over the mammalian cell-produced equivalent mAbs [11].

The purification of a target protein from plant tissue has proven to be a challenging task. Although antibodies can be purified well by affinity chromatography columns such as Protein A, studies showed that due to the presence of native compounds such as phenolics and alkaloids, direct loading of crude plant extract onto a Protein A media will result in column fouling and/or poor binding [12–15]. The goal of this research is to develop a pre-chromatographic purification process to treat plant extracts so that the samples can be readily processed by various chromatographic methods for the purification of mAbs. A process that without the use of the costly Protein A affinity chromatography was also explored in this study. The incorporation of the diafiltration step to drastically improve the polyelectrolyte precipitation efficiency in removing native plant proteins prior to any chromatographic processes is particularly novel.

2. Materials and methods

2.1. Materials

Poly(acrylic) acid was obtained from Polysciences, Inc. (Warrington, PA). Polyethylenimine (PEI) was purchased from Sigma (St. Louis, MO). Other laboratory reagents were purchased from Fisher Scientific (Pittsburgh, PA, USA) or Sigma. Bio-Rad Protein Assay reagents were purchased from Bio-Rad Laboratories (Hercules, CA). Bovine serum albumin and 10 K molecular weight cut-off (MWCO) ultrafiltration/diafiltration (UF/DF) devices were purchased from Pierce (Rockford, IL). Kanamycin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were obtained from Bioworld (Dublin, OH). All sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) products were purchased from Invitrogen (Carlsbad, CA). Microcon centrifugal filter devices and ProSep-vA High Capacity were purchased from Millipore (Bedford, MA). All antibodies and HRP substrate were obtained from Bethyl Laboratories (Montgomery, TX). Hydrophobic resins were purchased from GE Healthcare (Piscataway, NJ). MEP HyperCel™ was purchased from Pall Life Sciences (Exton, PA). An AKTA explorer with UNICORN software was used for all chromatography experiments, and columns were purchased from GE Healthcare.

2.2. *Agrobacterium tumefaciens* infiltration

The plant codon-optimized gene sequences for heavy (HC) and light chain (LC) of 6D8 mAb [16] were cloned into MagnICON expression vectors pICH10990 and pICH21595, respectively [4]. They were separately introduced into *A. tumefaciens* GV3101 by electroporation as described previously, and positive clones were selected with carbenicillin and rifamycin as antibiotics [7]. Agroinfiltration was performed as previously described [17].

2.3. Plant proliferation and protein extraction

N. benthamiana plants were grown in a Plant Growth Chamber (Percival Scientific, Perry, IA) at 25 °C in soil and watered frequently. After agroinfiltration, plants were grown at 25 °C for 4 days before harvest [18].

Protein extraction was carried out in the extraction buffer (25 mM sodium phosphate, 10 mg/mL sodium ascorbate, 0.1% Triton X-100, 1 mM EDTA, 0.3 mg/mL PMSF, 10 µg/mL leupeptin at pH 6.6) at a ratio of 5:1 of buffer (mL) to fresh leaf tissue (g) with a PowerGen 700 homogenizer (Fisher Scientific, Waltham, MA). Samples were then centrifuged at $17,000 \times g$ for 20 min. The supernatant was filtered through Mirca cloth, while the precipitate was discarded.

2.4. Polyelectrolyte precipitation

For initial precipitation study using polyacrylic acid (PAA, average molecular weight ca. 500 kDa), protein extracts (~1.94 mg/mL) were first obtained from non-transgenic *N. benthamiana* leaves, and then immunoglobulin G (IgG, 1% of the total soluble protein) was added to simulate the extract from agroinfiltrated leaves. The extract was titrated to pH 5, incubated on ice for 10 min, and centrifuged for 10 min at $17,000 \times g$ to remove potential precipitates [19]. Varying amounts of PAA (from a stock solution of 14 mg/mL in the extraction buffer at pH 5.0) were added to the clarified solution (0, 1, 5, and 10 mg/mg IgG). The sample was mixed vigorously using a vortex for 10 s. The precipitation occurred on ice for 30 min. After precipitation, the samples were centrifuged in a Fisher Scientific Marathon 15KM centrifuge for 20 min at $17,000 \times g$. The supernatant was removed and saved for later analysis. Extraction buffer of 0.2 mL with 1.5 M NaCl was used to re-suspend the precipitate. This mixture was centrifuged for 10 min at $17,000 \times g$. The supernatant was removed and saved for later analysis. The fractions were analyzed using ELISA (Section 2.6.1).

Polyethylenimine (PEI, average molecular weight 750 kDa) was used to precipitate acidic proteins and leave IgG in the supernatant after centrifugation. Stock PEI solution from the vendor (50% w/v) was diluted to 10 mg/mL in DI water and adjusted to pH 7.0 with concentrated HCl prior to precipitation experiments. In the screening experiments, dosages of 0, 50, 200, 400, 800 mg PEI/mg total soluble protein (TSP) were added to the extract. The precipitation experiment was carried out under the same conditions as described above. The precipitate was re-suspended with the same re-suspension buffer as above with a brief sonication. Analysis was conducted as described above for PAA precipitation. In later experiments, a DF with a 10 K MWCO membrane (through batch dialysis against extraction

buffer at pH 7.0) was used prior to precipitation. Dosages of PEI after DF were 0.009 ($n = 4$), 0.09 ($n = 4$), 0.18 ($n = 5$), and 0.36 mg PEI/mg TSP ($n = 7$).

2.5. Chromatography

After precipitation, the protein extract was subjected to either (1) hydrophobic interaction chromatography (HIC, phenyl sepharose 6 fast flow high substitution) followed by hydrophobic charge induction chromatography (HCIC, MEP HyperCel™), or (2) Protein A affinity chromatography (ProSep-vA High Capacity). All columns had a bed volume of 5 mL packed in a 1 cm diameter glass column (GE Healthcare). The flow rate for all chromatographic experiments was 1 mL/min. For HIC, 25% (w/v) ammonium sulfate in 50 mM sodium phosphate pH 7.0 was used as the equilibration buffer, and a wash step (20 min) using a combination of the equilibration buffer (buffer A, ~40%) and 50 mM sodium phosphate pH 7.0 (buffer B, ~60%) was applied prior to a linear gradient of 20 min to 100% buffer B for protein elution. The HCIC column was equilibrated with 50 mM Tris-HCl pH 8.0. After a wash step at pH 5.6, the antibody was eluted with step elution at pH 5.0 followed by immediate neutralization using 1.5 M Tris-HCl pH 7.0.

In Protein A affinity chromatography, the loading buffer was 50 mM PBS with 150 mM NaCl pH 7.4. A wash of 25 mM sodium phosphate with 500 mM NaCl and Tween-20 at pH 5.0 was used. Elution was accomplished using 100 mM glycine at pH 2.5. Neutralization of the elution fractions occurred immediately using 1.5 M Tris-HCl pH 7.0.

2.6. Analytical methods

2.6.1. Protein assays—Total soluble protein (TSP) concentration was determined by the Bio-Rad protein assay with bovine serum albumin (BSA) as the standard. Enzyme-linked immunosorbent assay (ELISA) was utilized to determine IgG concentration in the samples. A goat-anti human IgG Fc region and a HRP-conjugated goat anti-human IgG kappa LC were used as the capture and the detection antibody, respectively. Absorbance measurements were performed at 450 nm.

2.6.2. SDS-PAGE—Samples were denatured for 10 min at 70 °C and run on 4–12% Bis-Tris gels under non-reducing or reducing conditions with MOPS as the running buffer. Staining protocols followed that suggested by SimplyBlue™ SafeStain or the SilverXpress Silver Staining kit (Life Technologies, Grand Island, NY). The gels were scanned with a Bio-Rad ChemiDoc XRS imager and analyzed using Quantity One Software (Hercules, CA).

2.6.3. Irradiated Ebola virus affinity assay—The antigen-binding capacity of 6D8 was examined by incubating various concentrations of 6D8 mAb with irradiated EBV immobilized on an ELISA plate. Specifically, 96-well ELISA plates were coated with 50 µL of 1:2000-diluted irradiated EBV (a gift from Dr. W. Pratt, USAMRIID) at 37 °C for 1 h and blocked with TBST containing 5% skim milk at room temperature for 1 h. After washing with TBST, the plates were incubated with various concentrations of *N. benthamiana*-produced 6D8 mAb or a *N. benthamiana*-produced anti-West Nile virus (WNV) mAb (hE16) as a negative control [20] at room temperature for 1 h. Subsequently, the plates were

incubated with HRP-conjugated goat anti-human IgG at 37 °C for 1 h and developed with TMB substrate (KPL Inc., Gaithersburg, MD). The OD_{450nm} was measured with a Bio-Tek Power Wave microplate reader (BioTek, Winooski, VT).

3. Results and discussion

3.1. mAb expression

The 6D8 mAb was expressed in *N. benthamiana* with titers of 87.2 ± 25.2 µg/g fresh leaf weight (FLW), while the expression of TSP after extraction with a 1:5 leaf mass to buffer ratio was 1.94 ± 0.37 mg/mL. The IgG percentage of TSP was 0.75 ± 0.35 . These values were low when compared to those achieved in other laboratories, which can be up to 0.5 mg/g FLW [16]. Studies have shown that conditions affecting plant growth, e.g. temperature, light intensity and fertilizer are the most likely factors in causing variability of recombinant protein yield among production batches and among laboratories [21]. Thus, one possible reason for the significant discrepancy in 6D8 yield is that the soil components and other conditions for plant growth are not identical compared with those reported previously, and thus not optimal for achieving high levels of mAb accumulation [22].

3.2. Polyelectrolyte precipitation and the effect of diafiltration

As a negatively charged polymer, PAA was considered to be able to interact with the positively charged mAb ($pI \sim 8.2\text{--}8.5$) under neutral pH to form precipitate for antibody enrichment and purification. However, PAA precipitation was proved to be ineffective. Previous study showed a similar result when PAA was used to precipitate a basic protein, lysozyme, from tobacco extract [19]. As indicated by the earlier study, the interference from polyphenolic compounds may be responsible for the ineffective precipitation [19].

PEI, a positively charged polyelectrolyte at neutral pH, however, was effective in precipitating the majority of host cell impurities while leaving 6D8 mAb in the supernatant. Initially, PEI was applied immediately to the crude extract. As the dosage increased, the amount of TSP in the supernatant decreased, but the effect of PEI dosage beyond 200 mg PEI/mg TSP becomes less significant (Fig. 1). This result is not surprising, as it was reported that native tobacco proteins are primarily of acidic nature [23], and *N. benthamiana* is a close relative of tobacco and likely has very similar protein contents. Therefore, under neutral pH, a large portion of *N. benthamiana* proteins will likely be negatively charged to interact with PEI to form precipitates. As shown in Fig. 1, more than 60% of native plant proteins can be removed by this single precipitation step, while under all of the PEI dosages studied, majority of the antibody remained in the supernatant (data not shown). However, applying the resultant supernatant directly to a chromatography column was disadvantageous, since there was still a large portion of host cell material remaining.

To improve the performance of PEI precipitation and to allow direct loading of the supernatant to a chromatography column after precipitation, a diafiltration (DF) step using a 10 K MWCO membrane was introduced prior to the precipitation step. Our result indicated that the PEI precipitation became much more effective in two facets after the DF was instituted. First, the sample was almost completely clarified, indicating the removal of

pigments and possibly majority of the phenolic compounds. The second advantage was that, after optimization, significantly less polyelectrolyte was required to effectively remove more plant proteins (Fig. 2A). Specifically, about 80% of the plant proteins were removed at 0.36 mg PEI/mg TSP, in stark contrast to the ~60% removal of plant proteins at 800 mg PEI/mg TSP when DF was not used (Fig. 1). The dramatic improvement on this precipitation step by DF, together with the results reported previously [19], suggests that the phenolic compounds interfere with all precipitations regardless of the type of polyelectrolytes used. From the SDS-PAGE gel profiles of different protein fractions after PEI precipitation at various dosages, it is evident that the antibody remained in the supernatant, and all plant protein bands became lighter with the increase of PEI dosage (Fig. 2B). It is worth to point out that the mAb did suffer some loss when the PEI dosage was increased (Fig. 2A), about 20% at 0.36 mg PEI/mg TSP. The loss probably can be attributed to some degree of interaction between PEI and the mAb, as there are still localized negative charges on the mAb to allow its weak interaction with the polyelectrolyte at high dosages, despite its overall basic *pI*. Nevertheless, based on the results in Fig. 2A, it is possible to select a precipitation condition that balances the mAb recovery and plant protein removal. To demonstrate that the mAb can be purified, 0.36 mg PEI/mg TSP was chosen to prepare samples for further processing.

3.3. mAb purification by chromatographic methods

The supernatant after PEI precipitation was directly applied to two chromatography columns in tandem, an HIC column followed by HCIC, or a single chromatography column, Protein A affinity chromatography column. The antibody recovery for the HIC step was $71.9 \pm 6.3\%$ ($n = 2$), and the biggest loss of antibody occurred in HCIC step with an average recovery of $35.5 \pm 11.6\%$ ($n = 4$) under a step elution at pH 5.0. It is critical to optimize HIC and HCIC, particularly HCIC, to increase the overall antibody yield when the tandem chromatographic methods are to be used to purify the protein. On the other hand, the antibody recovery for the Protein A affinity chromatography was high around 85% with a wash step at pH 5.0 (with 0.5 M NaCl and 0.1% Tween-20) and a step elution at pH 2.5. SDS-PAGE experiments were done to compare the performance of the two purification processes. The mAb was purified to near homogeneity by both the tandem chromatography and the Protein A affinity chromatography (data not shown).

Finally, the mAb purified by the tandem chromatography process was characterized for its bioactivity by testing its binding specificity to irradiated EBV and compared to Protein A purified mAb. As shown in Fig. 3, the binding of the antibody, purified from either method, to inactivated EBV increased with the concentration of 6D8 in the reaction, whereas the negative control IgG (a plant-produced anti-WNV IgG, hE16) showed no specific binding. This result indicates that not only the specific affinity for EBV GP1 protein is retained by the plant-derived 6D8 but also the purification processes had minimal impact on the protein's bioactivity.

4. Conclusions

The recent application of plant-derived mAbs in treating EBV patients highlights the importance of plants as a production system for mAb-based human biologics. Downstream

process development is a critical issue for protein purification from plants, where the protein extract is often of large quantity. Although Protein A affinity chromatography has been the gold standard in mAb purification, its high cost and undesired non-specific binding of plant secondary metabolites encourage the development of purification processes based on non-specific chromatographic methods. In this work, an anti-EBV mAb was transiently expressed in *N. benthamiana*, and a process prior to chromatographic methods was developed including diafiltration and polyelectrolyte precipitation. The diafiltration step significantly improved the efficiency of precipitation, which in turn was able to remove more than 80% of the plant proteins at a small polyelectrolyte dose. The 6D8 mAb can be purified to near homogeneity by either a non-specific tandem chromatographic method including HIC and HCIC or a single step Protein A affinity chromatography. The Protein A affinity chromatography gave a very high yield of 6D8, but the nonspecific tandem chromatography method presents a potentially alternative way to purify the mAb at a relatively lower cost. Furthermore, neither purification process seems to have a negative impact on the antibody's activity, as the antibody product retains comparable binding capability toward Ebola virus.

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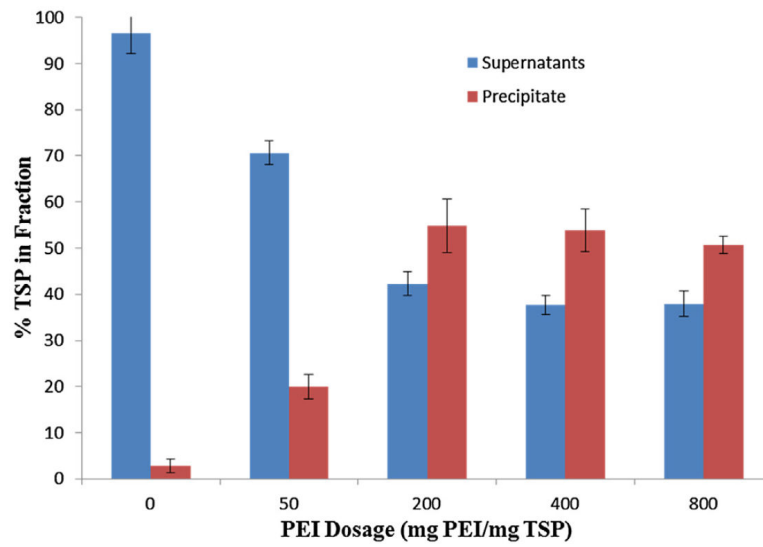


Fig. 1. Distribution of plant proteins in supernatant and precipitate at different PEI dosage after precipitation. Antibody remained mostly in the supernatant under all PEI dosages. Error bars were determined from four repeats of each experiment.

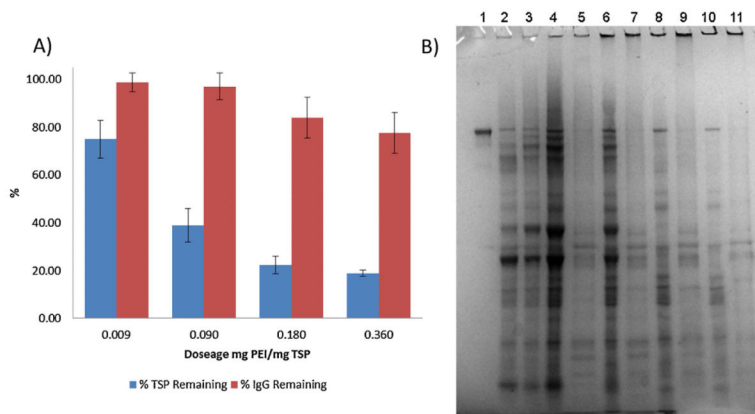


Fig. 2. PEI precipitation after diafiltration operation. (A) Percentage of antibody and TSP remaining in supernatant changes as the PEI dosage (mg PEI/mg TSP) increases. As dosage increased, the amount of TSP remaining in the supernatant decreased. The dosage that was the most effective in removing the most plant impurities was 0.36 mg PEI/mg TSP; however, a tradeoff was that the loss of some antibody increased. (B) SDS-PAGE gel profile of various supernatants and precipitates. Lane 1, positive control, human IgG, approximate size of 150 kDa; 2, crude plant extract; 3, soluble proteins after diafiltration; 4, supernatant at 0.009 mg PEI/mg TSP; 5, re-suspended precipitate at 0.009 mg PEI/mg TSP; 6, supernatant at 0.09 mg PEI/mg TSP; 7, precipitate at 0.09 mg PEI/mg TSP; 8, supernatant at 0.18 mg PEI/mg TSP; 9, precipitate at 0.18 mg PEI/mg TSP; 10, supernatant at 0.36 mg PEI/mg TSP; 11, precipitate at 0.36 mg PEI/mg TSP.

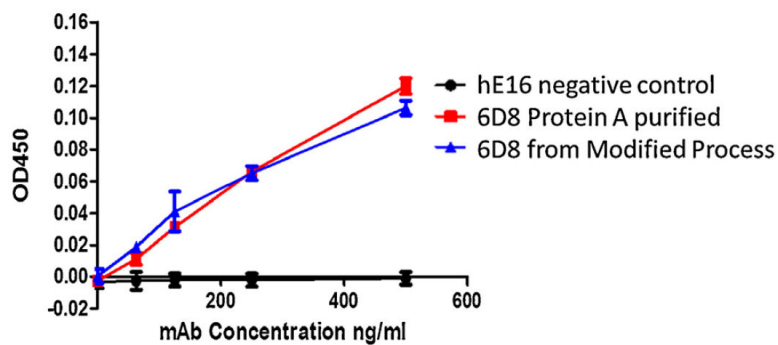


Fig. 3. Comparison of the antibody binding to irradiated Ebola virus. Various concentrations of purified 6D8 mAb from *N. benthamiana* (1–600 ng/mL) were incubated with irradiated EBV immobilized on an ELISA plate, along with a *N. benthamiana*-produced anti-WNV mAb hE16 as the negative control. The amount of mAbs bound to DEV was detected with an HRP-conjugated anti-human IgG and measured by absorption at OD_{450nm}. Results (means \pm standard deviation) were obtained from three independent experiments.