Protein Expression and Purification 128 (2016) 29–35

Contents lists available at ScienceDirect

Protein Expression and Purification

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

Development of an automated mid-scale parallel protein purification system for antibody purification and affinity chromatography

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ARTICLE INFO

Article history:
Received 16 June 2016
Received in revised form
1 August 2016
Accepted 3 August 2016
Available online 4 August 2016

Keywords:
Automation
High-throughput protein purification
Affinity chromatography
Monoclonal antibody (mAb)

ABSTRACT

Protein purification is often a bottleneck during protein generation for large molecule drug discovery. Therapeutic antibody campaigns typically require the purification of hundreds of monoclonal antibodies (mAbs) during the hybridoma process and lead optimization. With the increase in high-throughput cloning, faster DNA sequencing, and the use of parallel protein expression systems, a need for high-throughput purification approaches has evolved, particularly in the midsize range between 20 ml and 100 ml. To address this we modified a four channel Gilson solid phase extraction system (referred to as MG-SPE) with switching valves and sample holding loops to be able to perform standard affinity purification using commercially available columns and micro-titer format deep well blocks. By running 4 samples in parallel, the MG-SPE has the capacity to purify up to 24 samples of greater than 50 ml each using a single-step affinity purification protocol or a two-step protocol consisting of affinity chromatography followed by desalting/buffer exchange overnight (~12 h run time). Our evaluation of affinity purification using mAbs and Fc-fusion proteins from mammalian cell supernatants demonstrates that the MG-SPE compared favorably with industry standard systems for both protein quality and yield. Overall the system is simple to operate and fills a void in purification processes where a simple, efficient, automated system is needed for affinity purification of midsize research samples.

1. Introduction

In large molecule drug discovery, protein purification has become a bottleneck for protein and antibody production. During a typical therapeutic antibody campaign tens to hundreds of antibodies need to be purified from hybridoma cell supernatants for early characterization [1]. Additionally, as programs progress many lead variants that are generated as secreted recombinant proteins will also need purification. Because these samples are often expressed in volumes ranging between 20 and 100 ml and require only affinity purification by Protein A [2] for initial assessment, we determined that a simple, easy to use, high-throughput midscale parallel purification system capable of single-step affinity chromatography would be of significant value in early stage large molecule drug discovery efforts.

Currently, the industry standards for antibody and protein purification are the ÄKTA FPLC systems (GE Life Sciences). These systems are capable of mid-to large-scale multi-sample purification in series and can perform many complicated purification tasks such as salt gradients and size exclusion chromatography (SEC) precisely and reliably. However, standard ÄKTA FPLCs don't allow parallel purification and they are overly-complex and expensive in cases when only single-step affinity purification is required for antibodies and other affinity purification. Because of this we felt that ÄKTAs should remain the system of choice for complex multi-step purifications and large-scale lab preparations, but a simpler more cost-effective system that allowed parallel sample processing could increase efficiency when sample numbers are high and the purification required is simple on/off affinity chromatography. Over the years, many protein purification methods and systems have been developed for handling multiple samples [3–8]. However, most of these methods and systems were designed for small-scale affinity purification of 1 ml or less. Other systems have been developed that enable mid-to large-scale purification of samples in parallel. These systems do not run in series and they require a number of columns

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http://dx.doi.org/10.1016/j.pep.2016.08.005
1046-5928/

equal to the number of proteins being purified which can become costly and time consuming to set up [9]. Additionally, these systems are not setup to use micro-titer plates requiring an extensive network of tubing for sample loading and collection. Recently, an auto-sampler coupled to an ÄKTA FPLC has been reported to work very efficiently for automated two-step purification of antibodies [10].

We set out to develop a system that could run >20 mAb (or other standard affinity purifications) overnight unattended and deliver the same quality and yield as a standard single-step ÄKTA purification. The design criteria included running multiple samples in parallel to save time and using micro-titer plates to simplify sample loading and collection while facilitating compatibility with upstream and downstream automation processes. Additionally, we wanted to be able to use standard commercially available columns and have an interface that was simple and easy to use. To make the system cost-effective we determined that a UV detector was not required because of the predictability of affinity chromatography and that a gradient maker for ion exchange was not needed. After considering multiple systems we chose to modify a Gilson SPE system (GX-274) with switching valves and holding loops to accommodate GE Hi-trap affinity columns and added a unique bed layout to hold 8 micro-titer plates. In this paper we describe how the system was designed and evaluated and we present data demonstrating that it can perform single-step affinity purification with comparable quality and yield to industry standard systems in less time.

2. Materials and methods

2.1. Expression of purification of recombinant antibodies and Fc-fusions

Recombinant expression of mAbs was performed using 2936E Human Embryonic Kidney (HEK) cells in F17 media supplemented with 1X GlutaMAX, 25 μg/ml Geneticin, and 0.1% Pluronic F68 (all reagents from Thermo-Fisher, Life Technologies). Cells were grown in non-baffled shake flasks (Corning) at 150 rpm at 37 °C supplemented with 5% CO_2 and transfected at a cell density of 1.2×10^6/ml using 25 kDa linear polyethyleneimine (PEI). DNA was diluted into pre-warmed media then added to 1.5 mg/ml PEI (DNA:PEI = 1:3). Transfection complex was incubated at room temperature for 20 min prior to transfection. Following transfection cells were grown for 7 days and cell supernatants were collected and filtered through a 0.45 μm filter. For some experiments, antibodies were purified directly from supernatant as indicated.

For experiments using purified mAb added back to conditioned media, IgGs or Fc-Fusions were purified by loading supernatants on to a recombinant protein A column (MabSelect Sure; GE Life Sciences) using an ÄKTA FPLC (GE Life Sciences). Columns were washed with 10 CV of PBS followed by elution with 100 mM acetic acid. Elution was concentrated and run on a Hi-Load 26/60 Superdex size exclusion chromatography column (SEC) developed with 10 mM sodium acetate pH 5.2, 150 mM NaCl to any remove aggregate. Antibodies were quantified using Nanodrop A280 and added at indicated concentrations to F17 media.

2.2. One-step purification

For developing the general protocol to perform affinity chromatography purification, four samples were purified by the MG-SPE in a single run. One milliliter mAbSelect Sure columns (GE Life Sciences) were used to perform purification with a flow rate of 2 ml/min. Prior to loading samples, the system was primed with system fluid (10% glycerol, 0.02% NaN3) and sanitized using 0.2 N NaOH. Then the system was equilibrated with PBS buffer (pH 7.4). Forty-four milliliter cell supernatant containing a recombinant expressed mAb was loaded on to the column for each channel. After loading the samples, the system was washed with 10 ml PBS buffer (pH 7.4) and eluted with 3 ml of 100 mM acetic acid. The eluates were analyzed by non-reducing 4%–12% SDS-PAGE (Thermo-Fisher Life Technologies). Ten microliters of each eluate was loaded on to the gels shown. Gels were stained with Safe Stain (Thermo-Fisher). The quality of the purified protein was also analyzed by analytical SEC. A Superose 6 10/300 GL Column (GE Life Sciences) was used to perform the analysis. Fifty microliters of the elution was loaded onto the column and run isocratically with PBS.

The purified yield was measured by purifying antibody of known amount using MG-SPE. Seven samples containing different amounts of antibody (100 μg, 200 μg, 500 μg, 1000 μg, 2000 μg, 4000 μg) were purified and the recovery was calculated based on UV absorbance at 280 nm obtained on Nano-drop (Thermo Scientific). In order to make the comparison, same experiments were run on an ÄKTA FPLC (GE Life Sciences).

In order to test the cross contamination carry-over from previous runs, 12 samples were purified in three runs sequentially. Cell supernatant containing recombinant expressed mAb was purified in first and third runs and cell supernatant containing a recombinant expressed Fc-fusion protein was purified in second run. After purifying the first four samples, the system was cleaned with 25 ml 0.2 N NaOH and equilibrated with 25 ml PBS. Then samples 5–8 were purified by the same protocol as samples 1–4. The cleaning step was repeated prior to purification of samples 9–12. The eluates were analyzed by reducing and non-reducing SDS-PAGE.

2.3. Two-step purification including buffer exchange

After purification by affinity chromatography, the 3 ml eluates were buffer-exchanged to 10 mM sodium acetate pH 5.2, 150 mM NaCl by passing them through 8.3 ml PD-10 desalting columns by gravity flow with the addition of 4 ml additional formulation buffer. Columns (GE Life Sciences) were set up on a collection block shown in Fig. 5 and placed on the plate deck in the elution block spot. The product from a single step Protein A purification was dispensed directly on top of the column and allowed to pass into the column by gravity. To have full elution, 4 ml of additional formulation buffer was dispensed on top of the column and the ~7 ml of eluate was collected as purified product and analyzed. The product was analyzed by non-reducing SDS-PAGE. The recovery after two-step purification was evaluated using the same method described in Section 4.

3. Results and discussion

3.1. Gilson setup and modifications

The standard Gilson GX-274 SPE (Gilson Inc.) system is composed of three main parts (Fig. 1): four syringe pumps (A), a robotic arm with four channels (B), and a plate deck (C). The syringe pumps connect to the robotic arm which can transfer liquid samples on the plate deck. In order to perform on/off affinity chromatography, modifications to the standard SPE system were as follows (Fig. 1): 1) a 25-ml holding loop was installed upstream of each syringe pump to permit sample and buffer loading without entry into the pump (E); 2) a 6-port switching valve that accepts standard chromatography cartridges (including Hi-Trap columns) was placed between the robotic arm and the holding loop for each channel (D); 3) a new plate deck was added to hold eight micro-titer plates and 4–500 ml buffer reservoirs (C). Detailed plans are available from Gilson Inc. Additionally, sample blocks with standard micro-titer