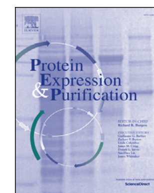
Protein Expression and Purification 134 (2017) 114–124

Contents lists available at ScienceDirect

Protein Expression and Purification

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



Development of polyol-responsive antibody mimetics for single-step protein purification

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ARTICLE INFO

Article history:
Received 24 March 2017
Received in revised form
13 April 2017
Accepted 15 April 2017
Available online 17 April 2017

Keywords:
Affinity purification
Antibody mimetics
nanoCLAMPs
Immunoaffinity
Resins
Native elution

ABSTRACT

The purification of functional proteins is a critical pre-requisite for many experimental assays. Immunoaffinity chromatography, one of the fastest and most efficient purification procedures available, is often limited by elution conditions that disrupt structure and destroy enzymatic activity. To address this limitation, we developed polyol-responsive antibody mimetics, termed nanoCLAMPs, based on a 16 kDa carbohydrate binding module domain from *Clostridium perfringens* hyaluronidase. nanoCLAMPs bind targets with nanomolar affinity and high selectivity yet release their targets when exposed to a neutral polyol-containing buffer, a composition others have shown to preserve quaternary structure and enzymatic activity. We screened a phage display library for nanoCLAMPs recognizing several target proteins, produced affinity resins with the resulting nanoCLAMPs, and successfully purified functional target proteins by single-step affinity chromatography and polyol elution. To our knowledge, nanoCLAMPs constitute the first antibody mimetics demonstrated to be polyol-responsive.

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1. Introduction

An important starting point for mechanistic, structural and functional biochemistry is the purification of enzymatically active proteins and protein complexes [1–3]. For example, structural determinations by Cryo-electron microscopy (CryoEM), depend upon purification procedures that preserve the tertiary and quaternary structure of large, multi-subunit protein complexes (reviewed in Refs. [4–6]). As another example, structure-function and other mechanistic biochemical studies often depend upon the characterization and comparison of a large number of protein variants purified under conditions that preserve enzymatic activity.

Methods of purification typically require tradeoffs between enrichment, yield, activity and convenience. For example, affinity tags enable effective, efficient and rapid purification but require genetic modification of the target protein to attach the tag (e.g., AviTag, FLAG tag, GFP, His tag, MBP, TAP-tag, and Strep-tag) [7,8]. In addition to the effort required to add the tag, this approach has the potential to disrupt the tagged protein's function, conformation, and expression level. As an alternative, immunoaffinity chromatography avoids genetic modification but requires the development of a suitable antibody. Immunoaffinity chromatography resins can be used to purify native proteins in high yield in a single step but generally require elution with pH extremes, denaturants, competing antigen or other conditions that may interfere with subsequent assays. In addition, the capture antibody can contaminate the eluate in instances when the antibody cannot be covalently crosslinked to the support. Immunoaffinity chromatography often results in a loss of activity, a disruption of protein complexes, or a need for additional purification steps to remove undesirable contaminants.

An exceptional class of monoclonal antibodies, called polyol-responsive antibodies, has a higher probability of preserving the activity and subunit interactions of target proteins or protein complexes [9–11]. Polyol-responsive antibodies have the distinctive property of enabling elution at neutral pH without denaturing agents. Although the mechanism is not well understood, polyol-responsive antibodies release antigen when exposed to neutral buffers containing propylene glycol or glycerol and a non-chaotropic salt such as ammonium sulfate. Polyol-responsive

Abbreviations used: CBM, carbohydrate binding module; SEC, size exclusion chromatography; WCL, whole cell lysate; PEB, polyol elution buffer; PG, propylene glycol; AS, ammonium sulfate.

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http://dx.doi.org/10.1016/j.pep.2017.04.008
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antibodies have been used to purify active, multi-subunit complexes such as *E. coli* RNA polymerase, eukaryotic RNA polymerase II, and the *Saccharomyces cerevisiae* Set1 complex [10,12,13]. One of these antibodies was successfully converted to a single-chain variable fragment (scFv) and was shown to retain polyol responsivity, although the affinity for the antigen was reduced in this format [14].

An emerging alternative to traditional immunoaffinity chromatography is the use of single-domain antibodies or antibody mimetic scaffold proteins. Antibody mimetics bind to targets with high specificity and affinity and can be genetically modified to add new functions or properties. The genetic modification enables precise, site-specific chemical conjugation. For example, the antibody mimetics may be engineered to facilitate the addition of a single fluorophore at a unique site or the irreversible attachment to a solid support in a defined orientation. Antibody mimetics are also typically resilient to denaturation and can be produced in high yield, microbial expression systems. Over the past 30 years, antibody mimetics based on several different scaffolds have been extensively characterized as alternatives to traditional antibodies. Examples include Affibody molecules, Affilins, Affimers, Adhirons, Affitins, Alphabodies, Anticalins, Avimers, DARPins, Fynomers, Ubvs, Nanobodies, and Monobodies [15–28]. Compared with traditional antibodies, antibody mimetics often offer advantages in speed, cost and performance, but, to our knowledge, none have been shown to be polyol-responsive [16].

To combine the advantages of polyol-responsive antibodies and antibody mimetics, we sought to develop a single-domain antibody mimetic scaffold for which polyol-responsiveness would be a general rather than an exceptional property. We screened a series of small protein domains for potential use as scaffolds for antibody mimetics and identified a scaffold enabling the isolation of antibody mimetics with the following properties: (1) Selectivity and affinity comparable to published antibody mimetics, (2) Polyol-responsiveness, and (3) High-yield production in bacteria. The scaffold we selected for further development is a beta-sandwich fold domain derived from the Type 32 carbohydrate binding module of the NagH hyaluronidase of *Clostridium perfringens* [29,30]. Because the 16 kD size is comparable to nanobodies, we call these single domain antibody mimetics nanoCLAMPs (**nano CL**ostridial **A**ntibody **M**imetic **P**roteins). This report describes the development of the scaffold, the general procedure used to isolate nanoCLAMPs against a variety of target proteins, and the use of nanoCLAMPs for the single-step capture and polyol elution of a diverse set of proteins.

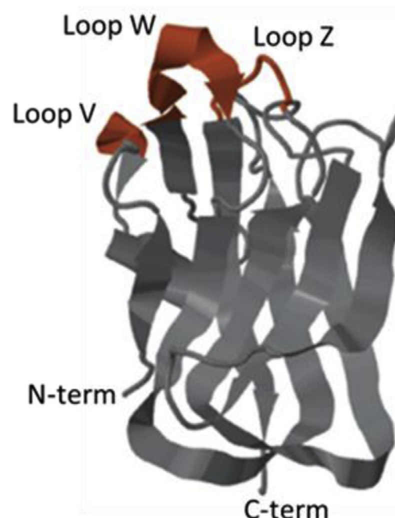

Fig. 1. Ribbon diagram of the second Type 32 carbohydrate binding module of NagH hyaluronidase of *Clostridium perfringens* (PDB 2W1Q). Loops variabilized during library construction are shown in orange.

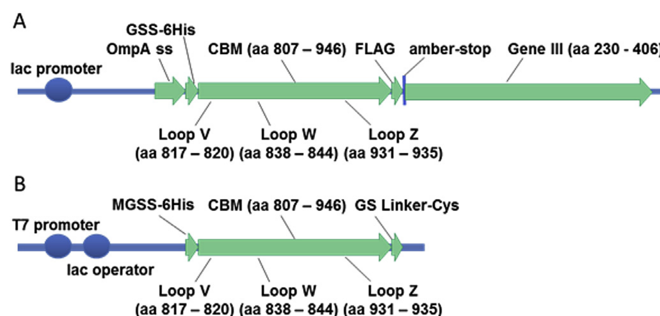

Fig. 2. Schematic of phagemid and expression constructs. A: Region of phagemid pComb3X modified to contain CBM scaffold library with randomized Loops V, W, and Z. CBM corresponds to amino acids 807 to 946 (PDB 2W1Q). The displayed GSS-6His-CBM-pIII fusion protein is approximately 36.3 kDa after cleavage of the OmpA signal sequence. B: pET expression system for nanoCLAMPs. GS-Linker-Cys amino acid sequence is GGGGSGGGGSGGGC. The expressed nanoCLAMPs are 163 aa after Met cleavage with a MW of 17.6 kDa (MW with native loops).

2. Materials and methods

2.1. Library construction

A cDNA coding for residues 807 to 946 of a carbohydrate binding module (Protein Data Bank 2W1Q) was codon optimized for expression in *E. coli* and synthesized by IDT (Coralville, IA). The cDNA was cloned into the phagemid pComb3X (Fig. 2A) such that the CBM contained an N-terminal His tag and a C-terminal FLAG tag, and was fused N-terminally to a truncated form of pIII (gene 3 product). To construct a phagemid library of variants of the scaffold CBM, we employed degenerate primers constructed using pools of 18 phosphoramidite trimers (equal mix of all amino acid codons except Cys and Met; Glen Research, Sterling, VA; TriLink Biotechnologies, San Diego, CA) at each variable position to amplify the phagemid and introduce variable positions in the loops. We amplified 1 ng of this phagemid using degenerate primer 397T-F and the non-degenerate primer 398-R (Table 1), which randomized Loop V, in a 50 μl reaction with ClonAmp HiFi PCR Mix, according to manufacturer's instructions (Takara Bio, Mountain View, CA). The reaction cycle was 98 °C for 10 s, 65 °C for 10 s, and 72 °C for 30 s, repeated 30 times. The resulting Amplicon 1 was gel purified on a 1.1% agarose gel using Qiagen (Qiagen, Germantown, MD) Qiaquick Gel Extraction Kit columns, and eluted in 12 μl elution buffer. These primers contained overlapping regions so that the resulting amplicon could be fusion cloned and ligated *in vivo* using Takara's InFusion HD Enzyme kit, with the resulting phagemid a mini-library with 4 variable codons in Loop V, which consists of residues 817 through 820. Briefly, 495 ng of the gel purified Amplicon 1 was fusion cloned in a 50 μl reaction with 10 μl of 5X InFusion HD Enzyme and incubated at 50 °C for 15 min, and then put on ice. The DNA was then concentrated and purified using a Qiaquick PCR Purification Kit column, and eluted in 10 μL EB. The DNA was then desalted on a Millipore (EMD Millipore, Billerica, MA) VSWP 0.025 μm membrane floating on 100 ml ddH_2O for 30 min, changing the water and repeating for 30 more min.

The DNA library was electroporated into electrocompetent TG1 cells (Lucigen, Middleton, WI) by adding 1 μl of DNA at 40 ng/μl to each of 6 aliquots of 25 μl of cells on ice in 0.1 cm electroporation cuvettes. The DNA was electroporated using a MicroPulser (Bio-

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