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Protein Expression and Purification 47 (2006) 348–354
Protein Expression & Purification
www.elsevier.com/locate/yprep

Conformation-specific affinity purification of proteins using engineered binding proteins: Application to the estrogen receptor

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Received 15 September 2005, and in revised form 18 October 2005
Available online 14 November 2005

Abstract

Affinity chromatography coupled with an "affinity tag" has become a powerful and routine technology for the purification of recombinant proteins. However, such tag-based affinity chromatography usually cannot separate different conformational states (e.g., folded and misfolded) of a protein to be purified. Here, we describe a strategy to separate different conformations of a protein by using "tailor-made" affinity chromatography based on engineered binding proteins. Our method involves: (i) engineering of a binding protein specific to a particular conformation of the protein of interest, and (ii) production and immobilization of the binding protein to prepare conformation-specific affinity chromatography media. Using "monobodies," small antibody mimics based on the fibronectin type III domain, as the target-binding proteins, we demonstrated the effectiveness of our method by separating the active form of the estrogen receptor α ligand-binding domain (ERα-LBD) from a mixture of active and misfolded species and by discriminating two different conformations of ERα-LBD bound to different ligands. Our strategy should be generally applicable to the preparation of conformationally homogeneous protein samples.

Keywords: Affinity reagent; Peptide aptamer; Protein conformation; Combinatorial library

The preparation of a chemically and functionally homogenous sample is a fundamental step in biochemical and biophysical characterization of proteins and in the production of proteins for pharmaceutical and industrial applications. Among many purification methods available, affinity chromatography is a preferred method, because of its high specificity and capacity. Recently, affinity chromatography coupled with an "affinity tag" has become the method of choice for recombinant proteins. Affinity tags, such as oligohistidines and glutathione *S*-transferase (GST)[2] and epitope tags (e.g., c-myc) are fused to the protein of interest, and the fusion protein is purified using affinity chromatography specific to the tag [1].

Although the affinity tag-based protein purification is robust, it has limitations: (i) for some applications, the tag needs to be removed from the protein; (ii) because the method operates almost solely based on the properties of the affinity tag, it does not usually separate different conformations of the protein (e.g., folded and misfolded species). For example, the widely used maltose-binding protein (MBP) serves as both a solubility enhancer and an affinity tag, and an affinity purified MBP-fusion protein often contains a fraction of misfolded protein as "soluble aggregates" [1,2]. Such a sample would need to be further purified using a different method. It is particularly important to develop complementary technologies to affinity

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[2] *Abbreviations used:* GST, glutathione *S*-transferase; MBP, maltose-binding protein; LBD, ligand-binding domain; ERα, estrogen receptor α; IPTG, isopropyl-1-thio-β-D-galactoside; SRC-1, steroid receptor coactivator 1; E2, 17β-estradiol; OHT, 4-hydroxytamoxifen.

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doi:10.1016/j.pep.2005.10.021

tag-based purification for proteins prone to misfolding such as eukaryotic proteins expressed in *Escherichia coli*.

Classical affinity chromatography, which exploits the natural function of the protein of interest such as immobilized substrate analog, is highly specific to the functional form of the protein. Thus, it is perfectly suited for the preparation of a functionally (and thus conformationally) homogenous sample. However, such affinity chromatography is available for only a small subset of proteins. Immunoaffinity chromatography using a monoclonal antibody specific to the functional form is a powerful method. But screening and preparation of a monoclonal antibody suitable for such an application are laborious and expensive [3]. In this work, we describe a strategy to develop custom, conformation-specific affinity chromatography, which takes advantage of recent progress in engineering of binding proteins.

Over the last decade, there have been dramatic advances in protein engineering technologies. Methods such as phage display and yeast two-hybrid coupled with combinatorial DNA libraries make it possible to generate a very large library of protein variants and identify ones that bind to a protein of interest [4,5]. Using this principle, many groups have developed recombinant antibodies, antibody mimics, and short peptides specific to a wide variety of target molecules. However, very few have reported the use of such affinity reagents for protein purification [6,7]. In these instances, affinity reagents were produced using previously purified target proteins, limiting their applicability. An additional reason for the scarce application of affinity reagents for protein purification may be difficulties in producing such a reagent in large quantities and/or a short shelf life of affinity resin made with an affinity reagent due to low stability. For example, antibody fragments tend to have low stability and solubility, and disulfide-constrained peptides are easily denatured in a reducing environment.

Our group has developed antibody mimics, "monobodies." Monobodies are based on a small (~10 kDa) β-sandwich protein, the 10th fibronectin type III domain of human fibronectin [8]. Residues in surface loops of the monobody scaffold are diversified to create combinatorial libraries (Fig. 1), and monobodies specific to a given target are identified using the phage display or yeast two-hybrid techniques [9]. We envisioned that monobodies are suitable as an immobilized ligand for affinity chromatography, because of its small size, high stability, and robust purification method.

We have previously engineered monobodies that bind to specific conformations of the ligand-binding domain (LBD) of the estrogen receptor α (ERα) [9,10]. The ERα-LBD is a ~260-residue domain that contains a ligand-binding cavity [11,12]. Different classes of ligands (i.e., agonists and antagonists) induce different conformations of the LBD, which are responsible for the transcription regulation function of the receptor. The anti-LBD monobodies are generally specific to a particular ligand/LBD complex, indicating that they recognize a particular conformation of the ligand/LBD complex [9]. The ERα-LBD is expressed as a mixture of folded and misfolded species in *E. coli*, and we wished to develop a convenient method for preparing conformationally homogeneous samples of the ERα-LBD for biophysical characterization.

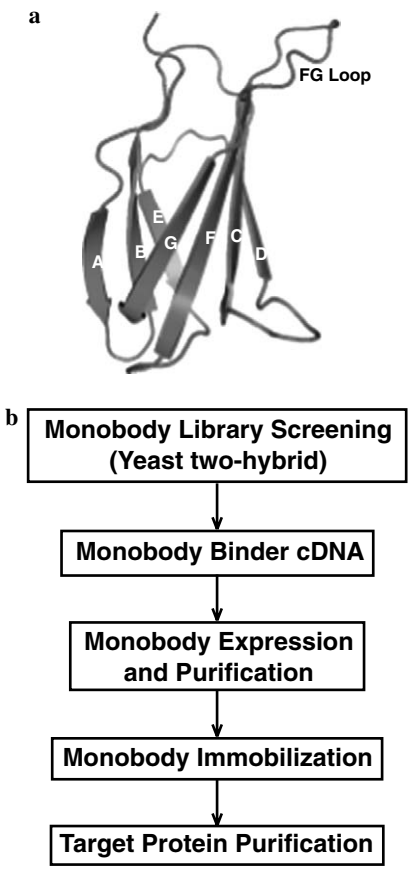

Fig. 1. (a) A schematic drawing of the monobody structure. The β-strands are labeled as A–G. The monobodies used in this work contain residues responsible for target binding in the FG loop that connects β-strands F and G. (b) An overview of the strategy of monobody-affinity chromatography for purifying a protein in a specific conformation.

In this paper, we describe the preparation of monobody-affinity chromatography media and demonstrate their effectiveness by purifying the active LBD from a mixture of active and misfolded proteins and by discriminating different ligand/LBD complexes.

Materials and methods

Plasmid construction

The yeast two-hybrid vector for a monobody, pYT45, and its variants encoding anti-LBD monobodies have been described previously [9]. An *E. coli* expression vector pHFT1 was constructed by inserting a synthetic DNA duplex into the multiple cloning sites of pET24a (Novagen). pHFT1 contains His_6, FLAG epitope tag, and a TEV protease recognition site immediately upstream of the multiple cloning sites (Fig. 2).