Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 52 (2007) 219–229
Protein Expression & Purification
www.elsevier.com/locate/yprep

Optimization of a mouse recombinant antibody fragment for efficient production from *Escherichia coli*

Ashwini Nadkarni, Laura-Lee Clancy Kelley, Cory Momany *

Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, Room 372, Wilson Pharmacy Building, University of Georgia, Athens, GA 30602-2352, USA

Received 11 August 2006, and in revised form 11 October 2006
Available online 26 October 2006

Abstract

A mutagenized mouse recombinant antibody fragment (rFab) that recognized HIV capsid protein was isolated from *Escherichia coli* at a level of 12 mg per liter of culture using standard shake flask methods. This is one of the highest yields of a modified antibody fragment obtained using non-fermentor-based methods. Recombinant Fab was isolated directly from the culture medium, which lacked complex materials such as tryptone and yeast extract. Fab isolated from the periplasm was not as homogeneous as that isolated directly from the culture medium. Optimization of the culture medium using recently developed media, the use of *E. coli* cell lines that contained rare tRNA codons, and mutagenesis of the Fab to improve the stability of the Fab were important factors in producing high-levels of the Fab. An isolation protocol easily adaptable to automation using a thiophilic–sepharose column followed by metal–chelate chromatography and the introduction of a non-traditional metal binding site for metal–chelate purification that bypasses the conventional hexahistidine tag cleavage step (to prevent the purification tag from interfering with crystallization) are additional features of this approach to produce a highly homogenous preparation of rFab. The resulting rFab binds to its antigen, p24, equivalent in character to the monoclonal from which the rFab was originally derived.

Keywords: Antibody fragment; Fab; Secretion; p24; HIV capsid

The application of antibody fragments such as Fabs and scFvs in biotechnology has expanded in recent years due to their strong affinity for other biomolecules, their inherent stability yet potential for tremendous diversity, and their ability to be attached to powerful selection systems like phage display [1,2]. A limiting factor in the wide use of recombinant Fabs (rFabs)[1], whether made in bacteria like *Escherichia coli*, fungi or higher eukaryotes in cell culture, has been the low yields of the Fab. A standard approach used in bacterial production of rFabs is to grow cells to high density, then isolate the Fab from the periplasm where it is designed to be exported from the bacterial cytoplasm by inclusion of secretory signal peptides on the Fab light and heavy chains. Export to the oxidizing environment of the periplasm is necessary for optimal folding and disulfide bond formation [3]. This is convenient in the context of a phage display system because filamentous phage such as M13 utilize a secretory system (Sec) to target the phage for extrusion through the bacterial membrane. In some Fab production schemes, the Fab is not exported, but instead is refolded after purification of the denatured Fab from overexpressed inclusion bodies in the cytoplasm [4]. The recent introduction of mutant *E. coli* strains, such as the Origami strain, lacking functional glutathione reductase and thioredoxin has been applied to the production of intracellular Fabs as well [5,6]. In these bacterial strains, the intracellular milieu is now much more oxidizing, making disulfide formation more favorable. But this approach

* Corresponding author. Fax: +1 706 542 5358.
E-mail address: cmomany@mail.rx.uga.edu (C. Momany).

[1] *Abbreviations used:* rFab, recombinant antibody fragment; Fab, antibody fragment; IPTG, isopropylthiogalactose; PMSF, phenylmethylsulphonylfluoride.

1046-5928/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2006.10.011

has not been readily adopted. In all cases, the yields are low, with on average only a few milligrams of rFab produced per liter of cell culture.

Our interest in efficient recombinant Fab production stems from our development of a high-throughput approach of making Fabs for co-crystallization of other biological molecules, as well as an interest in incorporating Fabs into pharmaceutical delivery systems, such as immunomicelles. While huge investments have been made in scaling the machinery of structure determinations, solving atomic structures of macromolecules is still a difficult task because the crystallization screening process is empirically driven. Many macromolecules, especially membrane and glycosylated proteins or RNA, still remain difficult to crystallize on a routine basis. One approach to addressing the crystallization problem is to co-crystallize a target protein with another protein that specifically recognizes the target so that a stable complex is formed. The earliest application of this approach was the use of antibody fragments such as Fabs and scFvs to crystallize several difficult proteins [7–9]. Non-immune-based molecular libraries have now been created for this task [10]. Antibodies and other co-crystallization proteins (CCPs) may be effective because they can control non-specific aggregation and modify the surfaces of molecules in ways favorable for crystallization. With the goal of creating a recombinant antibody system for co-crystallization, we recently built a non-immune (synthetic) rFab phage display library with a nominal diversity of 1.16×10^7 [11]. The evaluation, characterization and optimization of the expression of the rFab mutants that lead to the development of this library are described herein.

Materials and Methods

Construction of recombinant Fabs

Complementary DNA encoding the heavy and light chain variable and constant domains of Fab25.3 were obtained by performing RT-PCR using total RNA isolated from monoclonal Mab25.3 cells (gift from Ladislau Kovari and Michael Rossmann, Purdue University) with PCR primers complementary to the IgG1 heavy chain (MscvH253-F and MscIgG1-R, Table 1) and Kappa light chain (MscvK253-F and MscKappa-R) DNA sequences of Mab25.3 (Ladislau Kovari and Michael Rossmann, unpublished results) in two separate reactions following standard phage display procedures [12]. The primers were designed to include the appropriate restriction enzyme sites (*Sac*I/*Xba*I for the heavy chain, and *Spe*I/*Xho*I for the light chain) developed for insertion of mouse antibody fragments into the pCOMB-3H vector [12]. The resulting cDNAs were cloned into the pCOMB-3H plasmid by treating the heavy chain PCR product with the restriction enzymes *Sac*I/*Xba*I and the light chain cDNA with *Spe*I/*Xho*I, gel purifying the digested DNAs, and ligating the DNAs into correspondingly digested and purified pCOMB-3H in two steps, with the light chain introduced first. The resulting plasmid, pCOMB-Fab25.3-gIII contained the heavy chain gene of the Fab in frame with the phagemid gene III. A *Spe*I/*Nhe*I restriction enzyme digestion (which removed the DNA encoding the gene III phage protein), followed by gel purification of the cut DNA and ligation, produced the low-level expression plasmid pCOMB-Fab25.3. To obtain a Fab construct having a hexahistidine purification tag on the C-terminus of the heavy chain, the pCOMB-Fab25.3-gIII DNA was digested with *Spe*I and *Not*I and gel purified. The annealed oligonucleotides, PCBFab2-F and PCBFab2-R were ligated with the plasmid to make pCOMB-Fab2. To make a more efficient protein expression plasmid, the light and heavy chain sequences from pCOMB-Fab2 were transferred into pET28b (Novagen). This was performed in two steps by first digesting both pET28b and pCOMB-Fab2 with the restriction enzymes *Eco*RI and *Not*I, gel purifying the two cut DNAs, and ligating the *Eco*RI/*Not*I Fab light and heavy chain DNA with the cut pET28b. The resulting DNA was then amplified by PCR using primers Fab2-F and Fab2-R, which contained Eam 1104 restriction sites to orient the light chain appropriately behind the promoter of the pET vector. Digestion with restriction enzyme Eam 1104 concurrent with the presence of T4 ligase using the "Seamless Cloning" kit of Stratagene produced the plasmid pET28b-Fab2. The secretory leader sequences within the pCOMB-3H vector were thus transferred in the process, as well as the internal ribosome binding site encoded upstream from the heavy chain sequence. Further modifications of the pET28b-Fab2 DNA to make pET28b-Fab3 and pET28b-Fab4 were introduced using the QuikChange™ Site Directed Mutagenesis kit from Stratagene. The respective mutation primers used to create these constructs are shown in Table 1. Fab3, which encoded four less histidines at the heavy chain C-terminus and two histidines added to the C-terminus of the light chain relative to Fab2, was created in two mutagenesis steps using the primers: Fab3LΔH-F and Fab3LΔH–R, and then Fab3HΔH-F and Fab3HΔH–R. Fab4 was created from primers, Fab4ΔLeu-F and Fab4ΔLeu–R, and was designed to encode a Fab heavy chain in which a leucine was deleted near the amino-terminus of the protein. Plasmids were purified using Qiagen miniprep kits and transformed by electroporation into *E. coli* XL1-blue (Stratagene) for subcloning or into BL21(DE3) or BL21(DE3)-RIL (Stratagene) strains for high-level protein expression using the pET28-based plasmids. Transformed colonies were screened for Fab production via ELISA [12] using goat and rabbit anti-mouse Fab′$_2$ antibodies conjugated to alkaline phosphatase (Pierce Biotech). Sequencing of the DNA constructs was performed by the Integrated Biotechnology laboratory at the University of Georgia.

Production of rFabs by IPTG induction and auto-induction

"Defined" medium when used with IPTG induction contained: 4 g glucose, 2 g NH_4Cl, 6 g KH_2PO_4, 13.6 g