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Two routes for production and purification of Fab fragments in biopharmaceutical discovery research: Papain digestion of mAb and transient expression in mammalian cells

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ABSTRACT

Fab (fragment that having the antigen binding site) of a monoclonal antibody (mAb) is widely required in biopharmaceutical research and development. At Centocor, two routes of Fab production and purification were used to enable a variety of research and development efforts, particularly, crystallographic studies of antibody–antigen interactions. One route utilizes papain digestion of an intact monoclonal antibody for Fab fragment production. After digestion, separation of the Fab fragment from the Fc (fragment that crystallizes) and residual intact antibody was achieved using protein A affinity chromatography. In another route, His-tagged Fab fragments were obtained by transient expression of an appropriate construct in mammalian cells, and typical yields are 1–20 mg of Fab fragment per liter of cell culture. The His-tagged Fab fragments were first captured using immobilized metal affinity chromatography (IMAC). To provide high quality protein sample for crystallization, Fabs from either proteolytic digestion or from direct expression were further purified using size-exclusion chromatography (SEC) and/or ion-exchange chromatography (IEC). The purified Fab fragments were characterized by mass spectrometry, SDS–PAGE, dynamic light scattering, and circular dichroism. Crystallization experiments demonstrated that the Fab fragments are of high quality to produce diffraction quality crystals suitable for X-ray crystallographic analysis.

Many research activities in biopharmaceutical discovery and development require the Fab fragments of a monoclonal antibody rather than the whole immunoglobulin molecule. Fab fragments such as Reopro have been approved as therapeutic drugs [1,2]. In addition to therapeutic uses, Fabs were widely used in diagnostics and biopharmaceutical research. As a research reagent, Fab fragments are particularly useful in analysis of antibody–antigen interactions. The crystal structures of antibody–antigen complexes give high-resolution 3-dimensional structural information, essential for analyzing the structure–function relationship of antibody–antigen binding. Due to the flexible nature of the hinge region that is located between the Fab fragments and Fc fragment of monoclonal antibodies, crystallization of whole immunoglobulin molecules has only been successful in limited cases [3–5]. Compared with the whole immunoglobulin molecule, Fab fragments without the hinge region are more amenable to crystallization [6].

Traditionally, Fab and $F(ab')_2$ fragments are produced from monoclonal antibodies by enzymatic digestion using papain or pepsin [7,8], and subsequently, the Fab fragments are purified from the digestion mix. Depending on the hinge structure of an immunoglobulin, papain digestion may require careful optimization in order to obtain homogeneous Fab fragments, which can be very time-consuming. There are a number of techniques including protein A or protein G affinity chromatography, antigen affinity chromatography, ion-exchange chromatography and size-exclusion chromatography to purify Fab fragments from the digestion mix [8]. Some disadvantages associated with these techniques limit their use. These limitations include low affinity of protein A and protein G for antibodies produced in hosts such as rat, as well as incomplete separation of Fab fragments from undigested antibody and Fc fragments when their isoelectric points are too close to resolve them by ion-exchange chromatography. Nevertheless, papain digestion of monoclonal antibodies is still a useful method to obtain Fab fragments from monoclonal antibodies.

Advances in recombinant DNA technologies have enabled the cloning of antibody genes and expression of their Fab fragments that contain two polypeptide chains, the light chain and the heavy Fd chain (VH domain and CH1 domain). Both chains need to be assembled together with an inter-chain disulfide bond to form a Fab fragment. Compared with other expression systems, mammalian cells have the appropriate cellular folding machinery (chaperones, foldase, etc.) to ensure proper folding of complicated molecules including immunoglobulin and their fragments. Although stable cell

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lines offer high titers of expressed protein, transient expression is widely used in biopharmaceutical research because it can produce several mg to several hundred mg of protein quickly.

We utilize both methods (Fig. 1) for production and purification of Fab fragments to enable a variety of research activities including protein crystallography for structure determination of Fab fragments and/or their complexes with antigens. When monoclonal antibody is available in sufficient amounts, Fab fragment is usually produced by papain digestion. For monoclonal antibodies that are in early discovery stage, their Fab fragments are routed for transient expression in HEK293 cells. As an example for each route, here we describe the production and purification of the Fab fragment of CNTO888 by papain digestion of the mAb and transient expression of the recombinant Fab in HEK293 cells.

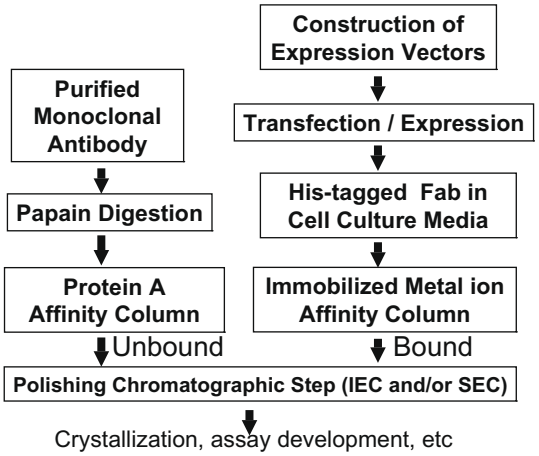

Fig. 1. Overview of two routes for Fab production and purification.

Materials and methods

Monoclonal antibody preparation

CNTO888, a human IgG1κ[1] antibody that neutralizes human CC-chemokine ligand 2 (CCL2; also known as monocyte chemotactic protein-1 or MCP-1) [9], was produced and purified in Centocor R&D Inc. (Radnor, PA). For papain digestion, the monoclonal antibody was concentrated to ~20 mg/ml and dialyzed against 10 mM EDTA, 20 mM sodium phosphate, pH 7.0.

Papain digestion and fragment purification

Immobilized papain (Pierce, Rockford, IL) was activated in digestion buffer (phosphate buffer containing 20 mM cysteine hydrochloride, pH 7.0) following manufacturer's instructions. CNTO888 monoclonal antibody was mixed with the activated papain (40 mg of mAb/ml of immobilized papain as packed resin) and the reaction was incubated at 37 °C with shaking. A 20 μl aliquot of the reaction was taken out after 4 h and 6 h, respectively, and the samples were centrifuged to pellet the immobilized papain and aggregates. Five microliters of the supernatant was analyzed by SE-HPLC. The digestion was stopped by centrifugation and filtration to remove immobilized papain from the reaction. To separate the Fab fragment from Fc fragment and intact mAb, the digestion mix was loaded onto a 10-ml MabSelect column (GE Healthcare, Piscataway, NJ) equilibrated with PBS. The MabSelect column was washed with 2 column volumes (c.v.) of PBS, and the Fab fragment was collected in the flow through and wash fractions. The Fc fragment and intact mAb were eluted from the MabSelect column with 100 mM glycine, pH 2.5.

Construction of expression vectors

Two Lonza-based vectors (Lonza Group Ltd., Switzerland), p4275 and p4208, were used to construct expression plasmids for heavy Fd chain and light chain of CNTO888, respectively. Plasmid p4275 encodes CH1 of human IgG1 heavy chain followed by a C-terminal His_6-tag, and p4208 encodes the CL domain of human kappa light chain. Heavy and light chain variable regions with secretion leader sequences were PCR amplified from CNTO888 mAb gene, and cloned into p4275 and p4208 using HindIII/ApaI and HindIII/BsiWI cloning sites, respectively. Expression vectors containing the V-regions were screened by restriction digest analysis and further characterized by DNA sequencing analysis. The expression plasmids for heavy Fd chain and light chain of CNTO888 were assigned as p4956 and p4560, respectively, and the recombinant Fab encoded by them was named CNTO8486.

Expression of His-tagged Fab in mammalian cells

The expression of CNTO8486 encoded by plasmids p4965 and p4560 was first evaluated in a small-scale test following a 7-day protocol, and cell culture materials were obtained from Invitrogen (Carlsbad, CA). Day 1 – HEK293E cells, which is a suspension adapted human embryonic kidney-293-based cell line stably expressing the Epstein–Barr virus nuclear antigen (EBNA1) [10], were seeded in a T150 flask at approximately 8×10^6 cells/flask with a volume of 30 ml. The culture media contained DMEM, 10% FBS, and 2 mM glutamine. Day 2 – 1 ml of Opti-MEM were added to two 15 ml conical tubes. 7.5 μg of each expression plasmid was added to one tube and 90 μl of Lipofectamine2000 was added to the second tube. Both tubes were incubated for 5 min at room temperature, after which one portion was mixed with the other and incubated at room temperature for 20 min. This mixture was added drop-wise to the media in a T150 flask. Day 3 – the media was removed and 30 ml 293 SFM II, 6 μM glutamine, and 5 μM sodium butyrate was added to the flask and incubated at 37 °C/5% CO_2 for 4 days. Day 7 – conditioned media was harvested and spun at 1000 rpm for 5 min to clear the culture supernatant. The expression level of Fab as secreted protein in the supernatant was measured using a Fab ELISA. After enrichment of His-tagged Fab using Talon metal affinity resins (Clontech, Mountain View, CA), the integrity of the Fab was determined by SDS–PAGE under both non-reducing and reducing conditions. Subsequently, transient expression of His-tagged Fab was scaled up to the appropriate volume (1–10 L for yield of a few mg to 100 mg Fab) depending on the expression level and desired amount of Fab. Upon harvest, the conditioned media was concentrated to 1–2 L and processed for purification.

Immobilized metal affinity chromatography (IMAC), ion-exchange chromatography (IEC), and size-exclusion chromatography (SEC)

A Ni–Sepharose HisTrap HP column (GE Healthcare) was used for immobilized metal affinity chromatography. The cell culture

[1] *Abbreviations used:* IgG, immunoglobulin G; mAb, monoclonal antibody; Fab, fragment (of antibody) that having the antigen binding site; $F(ab')_2$, dimer of the Fab fragment; Fc, fragment that crystallizes; HC, heavy chain; LC, light chain; VH, variable heavy domain; VL, variable light domain; CH1, first constant domain of immunoglobulin heavy chain; CL, constant domain of immunoglobulin light chain; MCP-1, monocyte chemotactic protein-1; mg, milligram; IMAC, immobilized metal affinity chromatography; SEC, size-exclusion chromatography; IEC, ion-exchange chromatography; PBS, phosphate buffered saline; FBS, fetal bovine serum; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE-HPLC, size-exclusion high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; DLS, dynamic light scattering; CD, circular dichroism; R_H, hydrodynamic radius; T_m, melting temperature; kDa, kiloDalton.