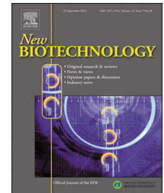
New BIOTECHNOLOGY 44 (2018) 31–40

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

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



Full length Article

Quality and cost assessment of a recombinant antibody fragment produced from mammalian, yeast and prokaryotic host cells: A case study prior to pharmaceutical development

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ARTICLE INFO

Keywords:
Therapeutic antibody fragment
Fab
Pharmaceutical development
Economic analysis

ABSTRACT

Monoclonal antibody fragments (Fab) are a promising class of therapeutic agents. Fabs are aglycosylated proteins and so many expression platforms have been developed including prokaryotic, yeast and mammalian cells. However, these platforms are not equivalent in terms of cell line development and culture time, product quality and possibly cost of production that greatly influence the success of a drug candidate's pharmaceutical development. This study is an assessment of the humanized Fab fragment ACT017 produced from two microorganisms (*Escherichia coli* and *Pichia pastoris*) and one mammalian cell host (CHO). Following low scale production and Protein L-affinity purification under generic conditions, physico-chemical and functional quality assessments were carried out prior to economic analysis of industrial scale production using a specialized software (Biosolve, Biopharm Services, UK). Results show higher titer production when using *E. coli* but associated with high heterogeneity of the protein content recovered in the supernatant. We also observed glycoforms of the Fab produced from *P. pastoris,* while Fab secreted from CHO was the most homogeneous despite a much longer culture time and slightly higher estimated cost of goods. This study may help inform future pharmaceutical development of this class of therapeutic proteins.

1. Introduction

Major rapid advances in antibody engineering and biopharmaceutical manufacturing have made antibodies and their derivatives the fastest growing class of therapeutics [1,2]. However, pharmaceutical development is not an easy task and the choice of an efficient expression platform is essential to guarantee quick and economically viable production [3]. In regard to antibody fragments, a number of expression systems ranging from bacterial hosts to transgenic animals have been developed but none of them is regarded as optimal [4]. Today, prokaryotes remain the workhorse for the expression of antibody fragments, because most do not require post-translational glycosylation or, where this is not the case, have previously been modified to avoid these issues [5]. *E. coli* K12 is the most attractive prokaryotic host, well ahead of Gram-positive bacteria and two of the FDA approved Fabs (certolizumab pegol and ranibizumab) are produced in *E. coli* [6,7]. This bacterium requires simple genetic manipulation, molecular cloning and economical media. The recombinant protein can be exported to the periplasm via the “SEC-dependent pathway” using appropriate signal sequences. Chaperones and the oxidizing environment of the periplasm favor correct folding and disulfide bridge formation [8]. However, expression quality may greatly vary from product to product. Large amounts of unprocessed antibody fragments may aggregate in the cytoplasm and, to a more limited extend, in the periplasm [9]. New *E. coli* expression platforms which allow the secretion of fully folded functional recombinant proteins into the culture medium have been developed [10]. Gram quantity per liter of fermentation broth can be achieved without requiring cell disruption and thus with lower

Abbreviations: CMC, Chemistry, Manufacturing and Controls; CoG, cost of goods; CRO, Contract Research Organization; DTT, dithiothreitol; DSP, downstream process; Fd, heavy chain fragment; GP, glycoprotein; HRP, horseradish peroxidase; IEF, Isoelectrofocusing; Mr, relative molecular weight; PBS, phosphate buffer saline; PpL, *Peptostreptococcus* protein L; PPP, platelet poor plasma; PRP, platelet rich plasma; RT, retention time; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEC-HPLC, size-exclusion high pression liquid chromatography; SPI, Soybean Trypsin Inhibitor; Ve, elution volume

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https://doi.org/10.1016/j.nbt.2018.04.006
Received 13 February 2018; Received in revised form 31 March 2018; Accepted 20 April 2018
Available online 22 April 2018
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amounts of contaminants and endotoxins, which often complicate the downstream process (DSP) [11].

Yeast have been used as well for expression of biotherapeutics since the early 1980s. *Pichia pastoris* has become the most popular strain for industrial scale production because it can achieve a high biomass concentration within a reasonable fermentation time [12]. Recently, variants making possible strong expression even with just glycerol or glucose as the sole carbon source outperformed conventional methanol-inducible promoter systems [13]. In this way, the yeast expression platform is regarded as an alternative when antibody fragments are difficult to express in bacteria. Yeast are even more robust than *E. coli*. Typical expression levels can reach 2 to 5 g L^{-1} after five days fermentation processes in the absence of endotoxins. However, while yeasts have a consistent glycosylation capability, the glycosylation pattern may differ significantly from human and this is a disadvantage. In addition, incomplete or incorrect N-terminal processing is sometimes observed resulting in product impurities [6,14].

Today, the preferred expression systems for producing complex multimeric proteins and glycoproteins such as whole antibody molecules are mammalian cells that have glycosylation repertoires most similar to humans [15]. In the biopharmaceutical industry, stably transfected cells generated within five to seven months are the standard procedure, due to high batch-to-batch consistency and yields [16]. Chinese hamster ovary (CHO) cells have been predominantly used for expression of full length IgGs ahead of mouse myeloma (Sp2/O and NSO) cells and human embryo kidney (HEK-293) cells, while embryonic retinoblasts of human origin (PER.C6) are emerging as a viable alternative [17]. Upstream processes have been optimized to reach typical yields of 5 g L^{-1} within 10–15 days [18]. However, when considering antibody fragments, the advantage of mammalian cells for expression is not obvious [19]. Up to now, two commercially available therapeutic antibody fragments are expressed in mammalian cells, namely idarucizumab, produced in CHO cells, and abciximab which is generated by papain cleavage of the intact chimeric IgG produced in Sp2/O cells.

Several less common systems, including algae, whole plants and plant cell culture are catching up while others such as insect cells have never experienced the expected industrial development [20]. An interesting alternative platform for biopharmaceutical production is the use of transgenic animals [21]. They are capable of authentic glycosylation patterns and offer an economically feasible alternative. Several companies are now developing this technology and several antibodies have been produced, mainly in transgenic goats [22]. However, this approach still remains tedious for several reasons including the risk of transmission of infections.

Within this context, the most successful and leading expression platforms remain *E. coli*, *P. pastoris* and CHO cells, which have proved to be robust and efficient enough for expression of recombinant proteins that meet all US and EEC regulatory requirements. Because selection of a good production platform is a key issue for a commercially viable process, here we have undertaken a case study and analytical early assessment. An Fab fragment (ACT017) was produced under generic conditions from the three hosts developed by Contract Research Organization (CRO) platforms and dedicated to high scale production of biopharmaceuticals. We carried out an early developability screen and analyzed the purity, integrity, homogeneity and activity of the secreted recombinant proteins. Each platform was found suitable for expression of soluble and functionally active protein. However, quality assessment revealed subtle differences that may have to be considered before developing a Chemistry, Manufacturing and Controls (CMC) strategy and moving forward to human *in vivo* applications.

2. Materials and methods

Materials

The humanized Fab fragment ACT017 is a drug candidate dedicated to the treatment of acute ischemic stroke and related diseases. It targets the glycoprotein (GP) VI of human platelets and inhibits collagen-induced platelet aggregation [23]. Its sequence was reported previously [24]. ACT017 reference material was produced at NVH-Medicinal from CHO-S cells transiently transfected with vector pBIC-PS (NVH-Medicinal, FR) under standard conditions and then purified using Capto L affinity chromatography [24]. Fab fragments assessed here were produced at Contract Research Organizations (CRO) at a pre-industrial scale by fermentation of recombinant *E. coli* (3 L), *P. pastoris* (0.4 L) or by stably transfected pool of CHO cells (1.4 L) using proprietary signal peptides and host preferable codons for efficient gene expression [25–27]. Expression and secretion were carried out under generic conditions without any upstream process optimization. Cell free media were collected after centrifugation and filtered over 0.8/0.2 μm membranes. Buffer exchange to 100 mM Na-Phosphate buffer with 150 mM NaCl (PBS), pH 7.2 was carried out on a desalting column or after extensive dialysis. Samples were aliquoted and stored at −20 °C before further blind analysis.

Recombinant GPVI-Fc comprising the extracellular domain of human GPVI fused to human IgG_1 Fc domain, was produced and purified at Syngene International Ltd (IN), after transient expression in HEK 293-6E cells and affinity chromatography using MAbselect matrix (GE Healthcare, 17519901) followed by a polishing chromatography on Nuvia™ HR-S cation exchange resin (BioRad, 156051).

Methods

Protein concentration

Protein concentrations were determined after micro BCA titration (ThermoFisher, 23235) using abciximab (Reopro®) as a standard for calibration, since its composition matches that of ACT017 (recombinant humanized Fab). Alternatively, the concentration of the purified Fab was evaluated after measuring absorbance using an extinction coefficient of 1.541 M^{-1} cm^{-1} at 280 nm.

Electrophoresis

Samples (0.5–4 μg/lane) were analyzed by SDS-PAGE under reducing (DTT 50 mM and heating for 5 min at 95 °C) or non-reducing conditions, either on a 10% PAGE homogeneous gel (Invitrogen, NW00100BOX) or a 4–20% gradient gel (Life technologies, 25269) and stained with Colloidal Blue Staining kit (Invitrogen, LC6025). Gels were run at 4 °C under 200 V for 50 min using running buffer (Invitrogen, NP0001), sample buffer (Invitrogen, B0007) and molecular weight ladders (Invitrogen, 10747-012).

Alternatively, gels were glyco-stained using the Pierce glycoprotein staining kit (Pierce, 24562). In this case, 10 μg of proteins were loaded onto the gel. Horseradish peroxidase (HRP) was used as positive control and Soybean Trypsin Inhibitor (STI) as a negative control.

For Isoelectrofocusing (IEF) analysis, samples (500 ng/lane) were analyzed on a pH 3–10 IEF protein gel (Invitrogen, EC6655 box) and stained with colloidal blue staining kit (Invitrogen, LC6025). Gels were run at 4 °C under successive voltages (100, 200 and 300 V) for 60 min each, using appropriate running buffers (Invitrogen, LC5300 and LC5310), sample buffer (Invitrogen, LC5311) and standard IEF markers (Serva, 39212;01).

Western blot

Western blotting was carried out after SDS-PAGE under reducing or non-reducing conditions on a 4–12% PAGE gradient gel (Invitrogen, NW0321BOX) or 4–20% gradient gel (Life technologies, 25269) and electrical transfer onto PVDF membrane on ice for 70 min at 100 V

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