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Extended Abstract

Euro Biotechnology 2018: Production of antibody fragments with plasmid-based and genome integrated T7 E. coli expression systems: evaluation of systems performance in microtiter fed-batch like cultivations- Monika Cserjan- University of Natural Resources and Life Sciences

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Abstract

Recombinant proteins are expressed in different host organisms. Escherichia coli (E. coli) is the most commonly used nonmammalian production host for biopharmaceuticals of moderate complexity. Full-length antibodies are currently the top-selling biopharmaceuticals; however, a new, seminal class of biopharmaceuticals includes engineered antibody-derived fragments and small scaffold-binding proteins. These proteins bind specific targets, which activate or block key reactions in vivo. They are smaller than antibodies, which gives them a higher potential for tissue penetration (e.g., they can cross the blood–brain barrier); thus, the number of accessible targets is increased. Antigen-binding fragments (Fabs) are single, monovalent, binding arms derived from immunoglobulin G (IgG) molecules. Fabs are composed of a light chain (LC) and a heavy chain (HC), connected with a disulfide bond. Due to the small molecular size and reduced complexity of these fragments, E. coli -based expression systems are an attractive alternative to mammalian cell cultures.

Construction of Fab Plasmids - All enzymes and kits were purchased from New England Biolabs (NEB, Ipswich, USA). Genes that encoded Fab fragments fused to the leader signal sequence (OmpASS) were purchased from ATUM (Newark, USA). The leader and spacer sequences were constant for all Fabs, but the sequences that encoded the Fabs were codon-optimized. All Fabs had the same aa sequence in the constant region but different Fab-specific sequences in the variable region. For PB systems, we cloned the Fab sequences into the expression vector, pET30a, via the Nde I and Eco RI restriction sites. We performed polymerase chain reactions (PCRs) to replace the OmpA signal sequence (OmpASS) with the DsbA signal sequence (DsbASS).

Fab LCs and HCs were amplified separately with sense primers that carried an overhanging DsbASS sequence (LC with Nde I_DsbASS-Fab_sense, HC with Bsa I_DsbASS-Fab_sense): the antisense primer for LC amplification bound at the 3′-end of UTRlinker (Bsa I_UTRlinker_antisense).

Integration of Fabs into the E. coli Genome - Fabs were amplified from pET30a vectors with TN7/1_pET30a_for and TN7/2_pET30a_back primers (Table S1, Supporting Information). Due to the primer overhangs, the integration cassette was flanked by 50 bp sequences that would anneal to the genomic TN7-site for integration. Genome integration was performed as described by Sharan et al µ-Bioreactor Cultivation- The µ-bioreactor system, BioLector (m2p-labs GmbH, Baesweiler, Germany), was used for cultivation.

We performed two types of cultivations. Precultures were grown in 48-well Flowerplates B (m2p-labs GmbH) without optodes; the main cultures were grown in Flowerplates-BOH (m2p-labs GmbH) equipped with optodes for online measurements of the dissolved oxygen (DO) and pH value. Both plate types accommodated a working volume of 800 µL. Precultures were started from cells scratched off of a frozen research cell bank and cultivated in Luria–Bertani broth (Merck, Darmstadt, Germany) at 37 °C. The main culture was started from precultures, with an initial cell density of OD600 = 0.3, and cultivated at 30 °C. The main culture was cultivated according to protocols developed previously in our group.

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Cell Lysis:

Frozen cell pellets were thawed and resuspended in 200 µL lysis buffer (30 mM Tris, pH 8.2, 10 mM MgCl2, 30 mM ethylenediaminetetraacetic acid). Then, lysozyme (Merck) and benzonase (Sigma-Aldrich) were added to final concentrations of 10 000 U and 2.5 U, respectively. After 10 min of vortexing, 1.5% Triton X-100 was added.

The lysate was incubated on the vortex for another 10 min. Cell debris was removed with a 10 min centrifugation at 15 000 × g at 4 °C. The inclusion body (IB) fraction was washed twice with 100 mM Tris, pH 8.2, and solubilized by vortexing in 8 M urea and 100 mM Tris, pH 8.2, for 30 min. The final volumes of the soluble fraction and resolubilized IB fraction were equal. The debris was pelleted at 15 000 × g for 10 min and both the soluble and IB fractions were stored at −20 °C until analysis.

Analysis of Fab Expression:

Expression of soluble Fab was determined with a sandwich ELISA that only detects correctly assembled Fab. The capture antibody binds only the LC, whereas the detection antibody specifically recognizes the hinge region of the HC. To be in the standard curve range of the ELISA, the Fab concentration range was initially estimated by western blot (WB) analysis. Accordingly, the soluble fractions of cell lysates were adjusted with a dilution buffer (1× phosphate-buffered saline [PBS], containing 0.1% Tween 20 and 1% bovine serum albumin; Merck). Purified human Fab/κ (Bethyl P80-115; Montgomery, USA) was used from 0.78 ng mL−1 to 100 ng mL−1 to calculate a standard curve. We coated 96-well plates with anti-human IgG (Fab-specific) goat antibody diluted 1:400 in coating buffer (100 mM NaHCO3 and 40 mM Na2CO3, pH 9.6–9.8). Plates were washed with 1× PBS, containing 0.1% Tween 20, in a HydroFlex microplate washer. The samples were incubated at room temperature (RT) for 1 h to allow the capture antibodies to bind. Anti-human IgG mouse that specifically recognizes the hinge region and then anti-mouse IgG goat antibody conjugated to peroxidase were applied as detection antibodies, both diluted 1:1000, and each incubated for 1 h at RT.

Discussion:

We clearly demonstrated that plasmid-free expression systems could greatly facilitate a direct characterization of the host cell response to Fab production.

Site-directed integration of a single GOI copy into the host cell genome eliminates effects on host metabolism triggered by variations in plasmid copy numbers, plasmid-encoded antibiotic resistance gene expression, and plasmid replication. By eliminating these confounding factors, the effects of Fab production on host cell metabolism become clearly evident.

Conclusions:

We recommend that GI expression hosts should be the systems of choice for the direct characterization of host cell response to the expression of recombinant proteins. Our results confirmed that GI production systems were superior to conventional PB systems for recombinant proteins translocated to the periplasm. We demonstrated the suitability of GI systems, both as the central part of our setup, for fundamental studies, and as an attractive alternative system for protein production. Based on the knowledge gained and the platform we developed, we have provided a good basis for further detailed host cell response and process characterizations using GI production systems in benchtop fed-batch fermentations.

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