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Scalable downstream processing of recombinant antibody Fab fragments

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Human Fabs are versatile tools, which lend themselves to the development of targeted drugs, for both therapeutic and diagnostic applications (George and Urch, 2000). Thanks to the widespread use of phage display libraries, good human antibody fragments can be identified for almost any target (Rader and Barbas, 1997). In the course of an internal drug discovery project, three different Fabs were identified as promising candidates for diagnostic and therapeutic uses, therefore well characterized batches were required for further studies.
These Fabs were expressed in *E. coli* XL1Blue strain (Burioni et al., 1998) in a 20-L fermentor. After growth, cells were harvested by centrifugation and stored frozen until processed.
The cell paste was passed through a high-pressure homogenizer and, after centrifugation and filtration, the supernatant was purified by metal chelating affinity chromatography (Porath et al., 1975), exploiting the cloned 6xHis tag.
After concentration by ultrafiltration, the purified Fab was submitted to size exclusion chromatography to reach the required purity, followed by a further concentration step to achieve the desired concentration.
Yields depend mainly on the expression level of the Fabs. They were from 3 to 13 mg protein per litre of culture medium and the purity was > 95%.
The described method proved to be useful to purify several recombinant Fabs and may be applied to the purification of other 6xHis-tagged recombinant proteins for *in vitro* or preclinical applications.

1. Experimental procedures

1.1 Fermentation Conditions

50 mL of LB Medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) containing 0.1 mg/mL Amp was inoculated with a glycerinate stock of transformed *E. coli* XL1Blue and incubated for 8 h at 37°C in an orbital shaker. After growth, 20 mL of this pre culture was inoculated in 1 L of SY Medium (9 g/L soy peptone, 30 g/L yeast extract, 5 g/L NaCl; divided in two flasks) containing 0.1 mg/mL Amp, supplemented with 0.2% Glucose and 12.5 mL of TB40x (328.6 g/L K_2HPO_4, 46.2 g/L KH_2PO_4) per flask, and incubated 16 h (overnight) at 30°C in the shaker.

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The fermentor (Applikon BioBench 20) was prepared with 12 L of SY medium. After preparation and sterilization, 0.1 mg/mL Amp, 120 mL of 20% Glucose and 300 mL of TB40x were added to the medium using a peristaltic pump under sterile conditions.
The fermentor SY medium was inoculated with the overnight pre-culture at a final OD_{600nm} = 0.2 and fermentation was started at the following initial conditions:
temperature: 30°C; speed: 250 rpm; pressure: 500 mbar; dO2: 60%; pH: 7 ± 0.2.
Bacterial growth was followed using a TruCell2® probe (Finesse Solutions), which allowed online monitoring of cell density. The culture was automatically induced after 12 h or at the preset OD level, by adding an IPTG solution (1 mM).
The pH value was controlled at 7.0 by automatic addition of 2 M NaOH and 10% Sulfuric Acid.
At the end of the fermentation, the cells were harvested by centrifugation (15000*g* for 30 min at 4°C) and the pellet was stored at -40°C until further processing.
The total wet weight of harvested cells in this fermentation was 370 g.

1.2 Fab purification

Fab purification was achieved by two chromatographic steps after cell disruption and homogenization. However, a significant fraction of Fab was obtained after reprocessing of lower purity fractions (see below).
After every purification step, analytical samples were collected and submitted to Western blotting or SDS-PAGE analysis in order to accurately recover the purified Fab fractions.

1.2.1. Fab extraction

The bacterial pellet was suspended in 500 mL of 20 mM Tris-HCl, 100 mM NaCl, pH 8.0 and homogenized with five cycles in an EmulsiFlex C5 apparatus at a pressure of 130-150 MPa, while keeping the crude extract refrigerated on ice.
The OD_{600nm} was monitored after each cycle and the process was stopped when it was below 50. The instrument was then washed with 100 mL of the above Tris buffer to recover all the sample.
Finally the lysate was centrifuged (33000*g* for 20 min at 4°C) to remove cell debris and the supernatant was collected. To this pool 50 mL of 5 M NaCl was added to achieve approximately 0.5 M NaCl concentration, then it was adjusted to pH 8.0 with 1 M Tris and filtered on a 0.45 µm membrane.

1.2.2. Immobilized Metal Chelate Affinity Chromatography (IMAC)

Before purification, the IMAC Fast Flow (GE Healthcare) column (35 x 30 mm, 25 mL volume) was loaded with 50 mL of 100 mM $NiCl_2$ and washed with 150 mL of water. Then the column was equilibrated with 20 mM Tris-HCl, 500 mM NaCl, pH 8.0.
The clarified bacterial extract was loaded onto the column overnight at 1 mL/min. After loading, the column was washed with 20 mM Tris-HCl, pH 8.0 until the 280 nm absorbance reached the baseline, followed by 20 mM Tris-HCl 500 mM NaCl, pH 8.0. Bound proteins were eluted in four isocratic steps at 5%, 10%, 50% and 100% of 20 mM Tris-HCl, 500 mM NaCl, 500 mM Imidazole, pH 8.0.
The eluted fractions were analyzed by SDS-PAGE and Western blot.

1.2.3. Size Exclusion Chromatography (SEC)

The Fab containing fractions were concentrated to 7 mL (Amicon ultrafiltration membrane YM10) and loaded at 1 mL/min onto a Sephacryl S-100 HR (GE Healthcare)

column (2.2 x 85 cm) previously equilibrated with 20 mM Tris-HCl, 0.15 M NaCl. The eluate was collected in 3 mL fractions. The eluted fractions were analyzed by SDS-PAGE.

1.2.4. Ion Exchange Chromatography (IEX Q)

Since not all the Fab containing fractions had sufficient purity, some fractions from size exclusion chromatography were pooled, adjusted to pH 8.0 and diluted with water to achieve a conductivity of 5 mS/cm. The pool was then filtered on a 0.22 µm membrane and loaded onto a Mustang Q Cartridge (total volume 10 mL; Pall) previously equilibrated with 20 mM Tris-HCl pH 8.0 at flow rate of 10 mL/min.

Bound proteins were eluted with a gradient from 0 to 50% of 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0 in 400 mL.

The eluted fractions were analyzed by SDS-PAGE. As expected, the Fab was recovered in the flow-through fraction. It was concentrated by ultrafiltration and combined with the already pure fractions obtained from SEC.

2. Results and Discussion

2.1 Fermentation and extraction of Fab

The Fab was expressed in transformed *E. coli* XL1Blue. The growth started from a glycerinated stock and was developed in three steps. Induction started after 12 h during the third step (Figure 1).

At the end of fermentation OD_{600nm} = 13.5 was reached. Western blot analysis showed that correctly folded Fab was present in the harvested cells. The same analysis revealed the presence of some Fab in the growth medium.

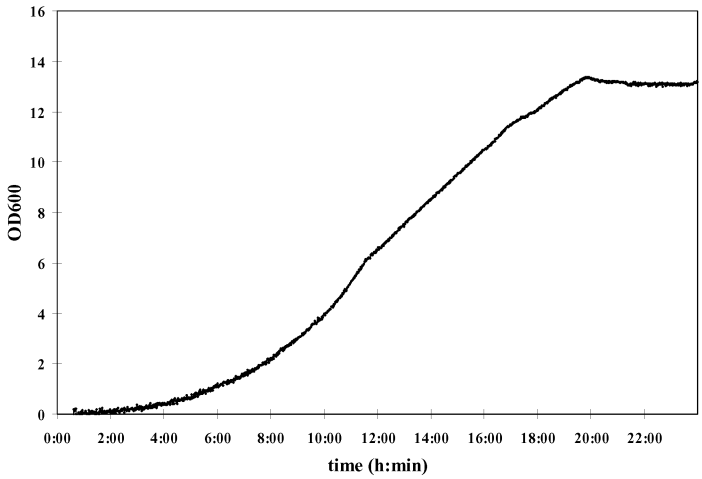
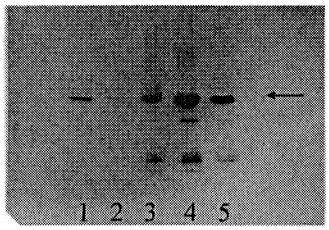


Figure 1. Cells growth curve, as recorded by the TruCell2 probe

2.2 Purification of Fab

Processing of bacterial cells with the EmulsiFlex-C5 homogenizer allowed the release of the Fab expressed in the periplasmic space. After the first metal chelating

chromatography step, the Fab was identified in the first three Imidazole containing fractions: 25, 50 and 250 mM (Figure 2).



1. Ref. Standard Fab 11 ng
2. Flow Through IMAC
3. Fraction 25 mM Imidazole
4. Fraction 50 mM Imidazole
5. Fraction 250 mM Imidazole

Figure 2. Western blot analysis of the IMAC purification

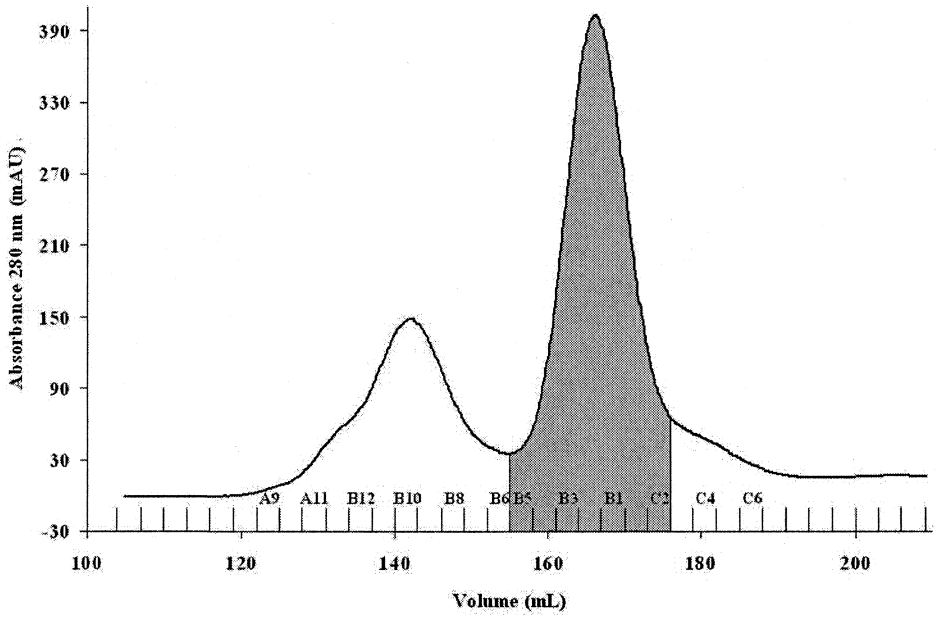


Figure 3. Size Exclusion Chromatography (SEC). Fractions from B5 to C2 were pooled.

The fraction eluted at 250 mM was concentrated to 7 mL and loaded on the SEC column (Figure 3). After SDS-PAGE analysis, the fractions from B5 to C2 were pooled as shown in Figure 3.

The presence of Fab in the 25 and 50 mM imidazole fractions was unexpected and required additional purification. These fractions were pooled and concentrated by ultrafiltration to a final volume of 7 mL and loaded onto the SEC column (Figure 4).

In order to reach a better purity, the pool from the second SEC was diluted until conductivity reached 5 mS/cm and loaded onto the IEX Q column for a third purification step. The eluted proteins were analyzed by SDS-PAGE (Figure 5).

The flow through from IEX Q column and the pool of the fractions B5-C2 of the first SEC were pooled in a final batch of purified Fab.

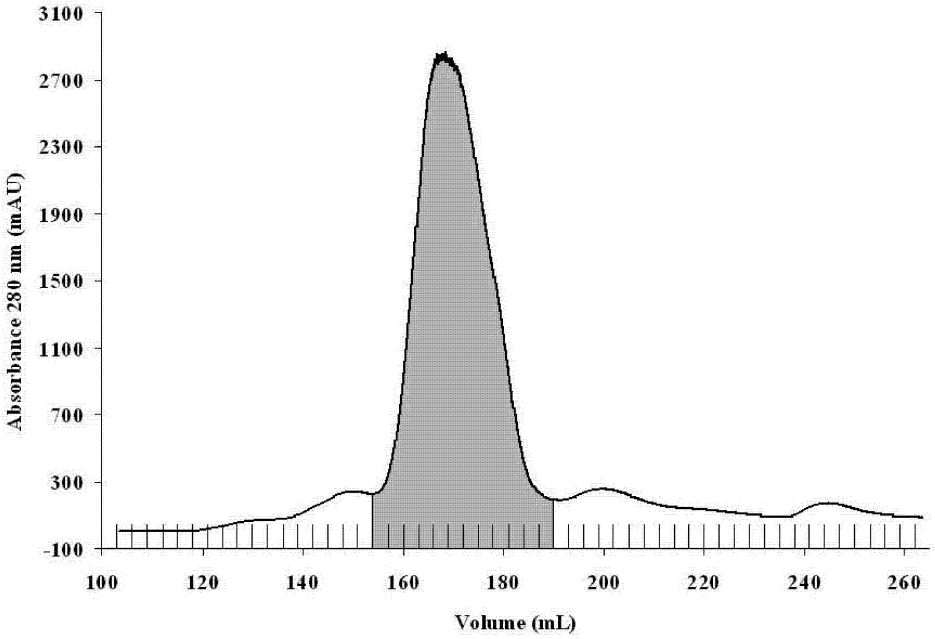


Figure 4. Second Size Exclusion Chromatography (SEC). Fractions from main peak were pooled.

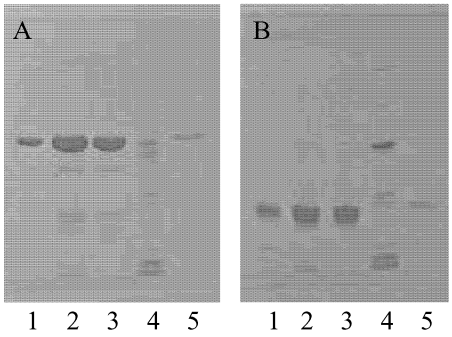
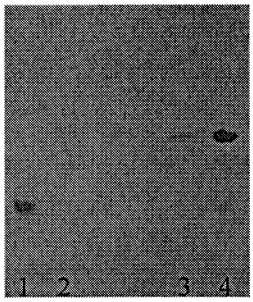


Figure 5. SDS-PAGE Analysis of IEX Q purification. Panel A not reducing and Panel B reducing conditions 1. Pool B5-C2 from first SEC; 2. Pre-column sample; 3. Flow through; 4. Salt eluted fraction; 5. Fab Reference Standard

2.3 Final Analyses of Fab

The absorbance of the Fab final batch was 1.77 cm^{-1} at 280 and 0.93 cm^{-1} at 260 nm. Based on the average extinction coefficient of 1.3 $mL \cdot mg^{-1} \cdot cm^{-1}$ and a volume of 119 mL, the calculated product yield was about 162 mg.

The purity >95% was estimated by SDS-PAGE analysis (Figure 6).



1. Fab Final batch diluted 1:2 in reducing sample buffer
2. Fab Ref. Standard 60 ng reduced
3. Fab Ref. Standard 60 ng
4. Fab Final batch diluted 1:2 in sample buffer

Figure 6. SDS-PAGE Analysis of Fab

3. Conclusions

In this case a very good yield was obtained, which was equivalent to 13.3 mg/L of culture medium. In other cases, yields from 3 to 7.5 mg/L were obtained, showing that even minor sequence differences have an impact on expression and folding capability. Due to the large amount of expressed Fab, the IMAC column was probably overloaded and some Fab, which was loosely retained, appeared in the 25 and 50 mM fractions. Usually only aspecifically bound proteins are eluted at these imidazole concentrations, while 6xHis-tagged proteins are more strongly retained.

However part of the Fab was obtained at good purity according to the programmed two-step procedure. The remaining Fab was purified separately and a single SEC run was not sufficient to achieve the desired purification. An anion-exchange chromatography step was added. This step resulted in positive adsorption of most contaminants and left the Fab in the flow-through. Therefore it can be effectively added to the purification scheme to reach a greater purity and reduce the endotoxin contamination level, if desired.

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