Multistep purification of humanized WO-2 Fab for crystallization studies using ÄKTAxpress™

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ÄKTAxpress is a suitable chromatography system for the unattended purification of poorly expressed histidine-tagged proteins from *E. coli* lysates. A single purification method was designed to incorporate four chromatography techniques (affinity, desalting, ion exchange, and gel filtration [size exclusion chromatography]) without the need for operator intervention. The resultant protein, humanized WO-2 Fab antibody fragment, was purified to a high degree of purity in a single day and subsequently crystallized overnight. These results demonstrate the opportunity to streamline protein purification schemes by combining single methods into multistep methods using an ÄKTAxpress system. We were able to diminish the bottleneck in our protein production workflow and convert protein from *E.coli* lysate to protein crystal in two days.

Introduction

Alzheimer's disease is the most common form of dementia. The amyloid-beta (Aβ) peptide (amino acids 39–43) derived from proteolytic cleavage of amyloid precursor protein, is one of the major components of amyloid plaques (1) and, as a result, is a valid target for Alzheimer's disease immunotherapy. The murine monoclonal antibody, WO-2 (mWO-2 IgG), binds the immunodominant epitope of Aβ peptide ($_3$EFRH$_6$). Murine antibody fragments (mWO-2 scFv and mWO-2 Fab) derived from mWO-2 IgG bind to the ($_3$EFRH$_6$) epitope of Aβ peptide and inhibit fibril formation *in vitro* and disaggregate preformed amyloid fibrils (2). To overcome the potential issues with human anti-mouse antibody (HAMA) responses, which generally prevent the use of murine antibodies as therapeutic agents, we have engineered a humanized version of WO-2 Fab (hWO-2 Fab). The hWO-2 Fab fragment was expressed in *E. coli,* purified using a multistep method on ÄKTAxpress, crystallized, and its protein structure solved (3). Similarly to mWO-2, hWO-2 Fab successfully inhibited amyloid fibril formation and disaggregated preformed fibrils and as such can be a good therapeutic agent against Alzheimer's disease.

Materials and methods

Gene construction, protein expression, and purification

To construct a humanized version of WO-2 Fab, the complementarity determining regions (CDRs) of the murine WO-2 IgG were grafted onto the closest matching human germline sequences (V_H, IGHV2-5*09 and V_L, IGK2-28*01). The humanized synthetic V_H and V_L genes were fused with the corresponding human constant heavy (C_{H1}) and constant light (C_L) gene fragments within the previously constructed *E. coli* expression vector, pGC-Fab, which encodes hexahistidine and FLAG™ (Sigma-Aldrich) purification tags at the C-termini of C_{H1} and C_L, respectively (2). The resulting hWO-2 Fab construct was expressed in *E. coli* TOP10F' cells as previously described (3). A periplasmic extract was prepared by sequential extraction of pelleted *E. coli* cells with ice-cold 1× TES buffer (200 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, and 500 mM sucrose) and 0.2× TES buffer (4). The periplasmic extract was clarified by centrifugation (30 min, 4°C, 48 400 × g) and filtered through 0.45 µm membrane. Imidazole (20 mM) was added to the lysate prior to loading.

Utilizing the air sensor enabled the loading feature of ÄKTAxpress; a total of 900 ml of periplasmic lysate from a 10 l volume of *E. coli* culture was purified in an automated method designed to incorporate four different chromatography steps.

A) Affinity (IMAC)

Column:	HisTrap™ FF crude, 5 ml
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4
Flow rate:	5 ml/min
Gradient:	Step gradient, 50% elution buffer in 10 CV

B) Desalting (DS)

Column:	HiPrep™ 26/10 Desalting, 53 ml
Binding buffer:	20 mM sodium acetate, 20 mM NaCl, pH 5.5
Flow rate:	10 ml/min

C) Ion exchange chromatography (IEX)

Column:	HiTrap™ SP HP, 1 ml
Binding buffer:	20 mM sodium acetate, 20 mM NaCl, pH 5.5
Elution buffer:	20 mM sodium acetate, 1 M NaCl, pH 5.5
Flow rate:	1 ml/min
Gradient:	Linear gradient, 0% to 50% elution buffer in 50 CV

D) Gel filtration (GF)

Column:	HiLoad™ 16/600 Superdex™ 200 pg, 120 ml
Elution buffer:	1× TBS, pH 7.4, (50 mM Tris-HCl, 150 mM NaCl, pH 7.4)
Flow rate:	1.5 ml/min
Fractions:	2 ml

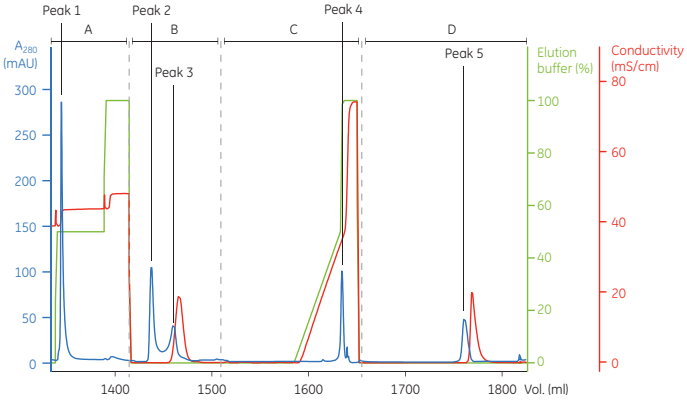

Fig 1. Unattended four-step purification of hWO-2 Fab. The four purification steps were **A)** IMAC, **B)** DS, **C)** IEX, and **D)** GF. Five peaks of interest are shown and these are described in the Results and discussion section.

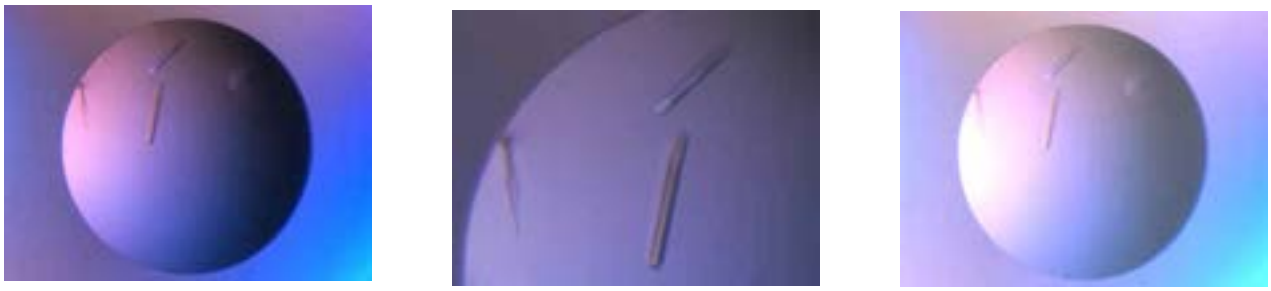

Fig 2. Typical crystals of hWO-2 Fab after purification on ÄKTAxpress and cystallization with 20% PEG 3350 and 0.2 M sodium sulfate.

Results and discussion

Purification on ÄKTAxpress

To purify hWO-2 Fab, periplasmic lysates of *E. coli* cells containing the recombinant protein were prepared and loaded directly onto a HisTrap FF crude 5 ml column to capture hWO-2 Fab from contaminating *E. coli* proteins by immobilized metal affinity chromatography, IMAC (Fig 1). After washing, the captured protein was eluted with a step gradient of 250 mM imidazole, and peak 1 (3.5 ml, from peak leading edge UV_{280}> 50 mAU to trailing edge cutoff > 100 mAU) was collected into capillary loop 1. This peak (the largest) was automatically selected and taken into the next chromatography step, an intermediate desalting step, where it was buffer exchanged using HiPrep 26/10 Desalting into 20 mM sodium acetate, 20 mM NaCl, pH 5.5 and recollected as peak 2 (8.4 ml, UV_{280}> 20 mAU) into capillary loops 2 and 3. The second peak in the desalting phase (peak 3) correlates to small breakdown products of less than M_r 5 000.

The desalted hWO-2 Fab (pI 8.0) was further purified onto a HiTrap SP HP 1 ml cation exchange column over a gradient of 0% to 50% 20 mM sodium acetate, 1 M NaCl, pH 5.5 over 50 column volumes (peak 4; 3.2 ml; UV_{280}> 30 mAU). The peak was automatically injected onto HiLoad 16/600 Superdex 200 pg gel filtration column as a final polishing step. Fractions (peak 5, UV_{280}> 20 mAU) were collected into the 96-well block fraction collector and pooled. The yield of hWO-2 Fab was determined to be 1.15 mg (7.3 ml at 0.16 mg/ml).

Crystallization studies

Protein was concentrated to ~4 mg/ml using an Ultrafree™ centrifugal concentrator (MWCO 10 000, Millipore Corp.) for crystallization studies. The crystallization was undertaken at the CSIRO Collaborative Crystallization Centre (www.csiro.au/c3/).

Typical crystals of h-WO2 Fab are shown in Figure 2. The largest dimension was 300 µm. Data were collected from crystals grown in 20% PEG 3350 and 0.2 M sodium sulfate. Crystals also grew in PEG 1500 and with other salts. All crystals were grown at 293K, in SD-2 sitting drop plates (IDEX Corp, Santa Rosa, USA). The droplets consisted of 150 nl protein and 150 nl crystallant equilibrated against a reservoir of 50 µl of crystallant. The structure of hWO-2 Fab was solved at the Australian Synchrotron with an atomic resolution of 0.22 nm (PDB:3AAZ).

Conclusions

ÄKTAxpress chromatography system was suitable for developing an unattended, multistep method to purify a poorly expressed recombinant tagged protein from *E. coli* lysate. Utilizing multistep methods afforded greater control and reproducibility in quality of protein purified for crystallization studies. The final yield of 1.15 mg of hWO-2 Fab, although low (0.1 mg/l of culture) was of a high purity, and retained functional activity. Subsequent crystallization of the protein was performed overnight.

Using ÄKTAxpress enabled a workflow from protein lysate to protein crystal in two days.

References

1. Kang, J. *et al.* The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* **325**, 733–736 (1987).
2. Robert, R. *et al.* Engineered antibody intervention strategies for Alzheimer's disease and related dementias by targeting amyloid and toxic oligomers. *Protein Eng. Des. Sel.* **22(3)**, 199–208 (2009).
3. Robert, R. *et al.* Germline humanization of a murine Aβ antibody and crystal structure of the humanized recombinant Fab fragment. *Protein Science* **19**, 299–308 (2010).
4. Instructions: Recombinant Phage Selection Module, GE Healthcare, 27-9403-01, Edition AD (2003).

Ordering information

Product	Code number
ÄKTAxpress Single chromatography system	28-9089-36
HisTrap FF crude, 5 × 5 ml*	17-5286-01
HiPrep 26/10 Desalting column, 1 × 53 ml	17-5087-01
HiTrap SP HP, 5 × 1 ml	17-1151-01
HiLoad 16/600 Superdex 200 pg, 1 × 120 ml	28-9893-35

* *Also available in 100 × 5 ml pack size, code number 17-5286-02*

For more information on purification of tagged proteins using ÄKTAxpress system, visit www.gelifesciences.com/akta