Process Development: Scaling Up Human IL-1β Production, Tag Removal, and X-Ray Crystallography

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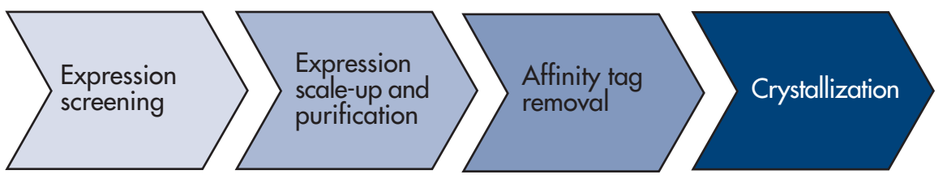
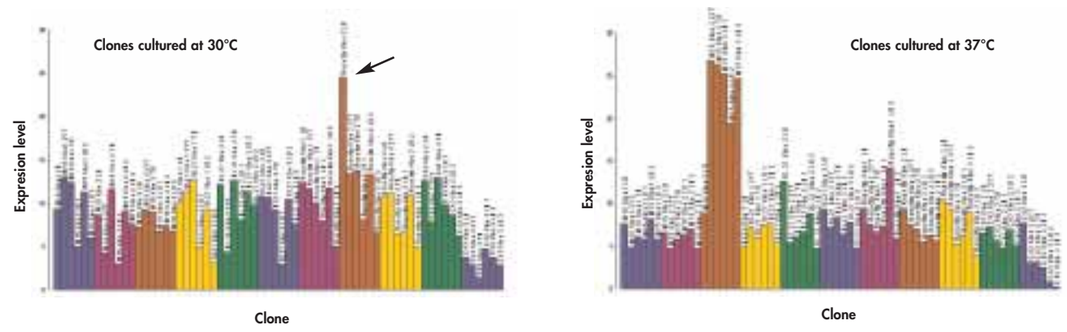
Introduction

- We describe an efficient strategy to produce gram quantities of high-quality protein using a single large IMAC chromatography column and exoproteolytic His-Tag removal using the TAGZyme system.
- The process started with high-throughput screening of expression clones, which were cultured and analyzed using an automated system.
- The scaling up process used increasingly larger volumes of Ni-NTA Superflow, up to a 100 ml pilot-scale column that enabled purification of 2 g protein from an 18-liter fermentation.
- This protein was processed using DAPase exopeptidase, which — together with unprocessed target protein — was removed by subtractive Ni-NTA IMAC.
- Numerous analyses — including electrophoresis, ELISA, ICP-MS, dynamic light scattering, and Edman N-terminal sequencing — were performed to confirm the purity, homogeneity, and structural integrity of the target protein.



High-throughput expression clone screening

- To optimize expression, a high-throughput screening of different conditions was performed.
- Four *E. coli* strains, three different culture media, two temperatures, two induction time points, and three inductor concentrations were tested for each of three clones.
- The screening was performed in 24 deep-well blocks (5 ml culture volume) and protein from 1 ml of each culture was purified on a BioSprint 96 instrument (QIAGEN).
- Expression efficiency was analyzed by SDS-PAGE and the Bradford method.



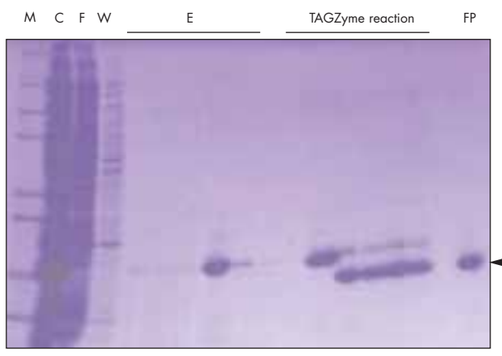
Relative efficiency of protein expression from expression clones cultured under different conditions. The arrowed clone (Rosetta [DE3] pLysS clone 2), cultured in LB medium at 30°C with induction at OD_{600} = 1.0 with 1 mM IPTG for 4 hours, was selected for scaled up expression.

Expression scale up and tag removal

- The chosen clone was expressed in scales from 1 ml culture (micro-scale) to an 18-liter fermentation.
- The 2 g protein obtained in the large-scale purification was treated with TAGZyme enzymes to remove its affinity tag.

Matrix	Matrix volume	Culture volume	Yield	Recovery (%)	Purity*
Ni-NTA Magnetic Agarose Beads (micro-scale)	100 µl	1 ml	33 µg	~ 90%	~ 97%
Ni-NTA Superflow (small-scale)	500 µl	320 ml	6 mg	~ 80%	~ 96%
Ni-NTA Superflow (medium-scale)	10 ml	1.7 l	109 mg	~ 80%	~ 98%
Ni-NTA Superflow (large-scale)	100 ml	18 l	2 g	> 88%	~ 97%

* Determined using Agilent Bioanalyzer (Protein 50 LabChip Kit)



Tag removal process step	Protein recovery	Yield
Ni-NTA IMAC	100%	2.00 g
Desalting (dialysis)	94%	1.88 g
Tag removal + second IMAC	72%	1.35 g
Overall process	67%	1.35 g

Scaled-up expression and purification of His-tagged IL1-β that delivered a total of 2 g protein. M: markers; **C**: cleared lysate; **F**: flow-through; **W**: wash; **E**: elution fractions; **TAGZyme reaction**; time course of tag removal; **FP**: final product.

The TAGZyme principle

Proteins with intrinsic DAPase stop points

- Some proteins have a natural DAPase stop point within their native sequence causing the enzyme to release its substrate (i.e., the detagged mature protein), as soon as this amino acid occurs in the N-terminal position.
- The cleavage process and enzyme removal by subtractive IMAC are typically complete within 45 minutes.

Proteins without intrinsic DAPase stop points

- A glutamine (Gln, Q) residue is introduced between the last cleavable dipeptide and the first amino acid of the mature protein.
- DAPase cleavage is performed in the presence of excess Qcyclase which catalyzes cyclization of the N-terminal glutamine (Q) to pyroglutamate.
- Converted, pyroglutamyl-extended protein is no longer accessible to DAPase. DAPase and Qcyclase are removed by subtractive IMAC and the N-terminal pyroglutamate residue is removed by addition of pGAPase which is removed by a second round of subtractive IMAC.
- This procedure is typically finished within 120 minutes.

Crystallization and solving the structure

- IL-1β protein crystals were obtained by the sitting-drop method in a 96-well microplate using one of the conditions in the EasyXtal AmSO4 crystallization suite (QIAGEN).
- The largest crystals (0.3 x 0.3 x 0.5 mm) — obtained by mixing equal amounts of reservoir (2.0 M $(NH_4)_2SO_4$, 0.1 M Na-acetate, pH 4.6) and protein (28 mg/ml) — diffracted to 2 Å.
- Crystals were coated with Paraton N and data sets were collected from IL-1β single crystals using a MAR 300 image plate (mar Research), integrated using Mosflm, and scaled (Scala).

Cell	
Spacegroup	$P4_3$
a,b	54.6 Å
c	75.8 Å
Data	
Resolution	2.0 (2.11) Å
Nmeasured (unique)	46889 (14952)
Completeness (%)	99.3 (99.3)
Rmerge	6.3 (23.5) %
I/sigI	9.4 (3.1)

Model atoms	
Protein	1219
Water	200
Sulfate	15
Geometry	
Rmsd bond length	0.006 Å
Rmsd bond angles	1.51°
Rmsd dihedral angles	24.7°
Bav	24.6 Å²
Validation	
Rwork (free 5%)	19.5 (23.9) %
Est. error (cross-validated)	0.16 (0.18) Å

Summary

- The solved structure agreed with that already published, indicating that the protein was correctly folded, and confirming the complete removal of the affinity tag without disruption of the N-terminal structure.

X-ray crystallography of IL-1β crystals. A Crystals of 0.3 x 0.3 x 0.5 mm were obtained after 14 days in ammonium sulphate/Na-acetate solutions of acidic pH. The bar indicates 0.3 mm. B Diffraction pattern obtained at 100 K from crystals diffracting to 2 Å. C Ribbon structure of IL-1β showing β-sheets, α-helices, and three sulfate ions. D Detail of the IL-1β N-terminus and a hydrogen-bonded (cyan broken line) sulfate ion. Atom color key: Carbon = yellow; Nitrogen = blue; Oxygen = red; Sulfur = green.

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