Single-column purification of native proteins to homogeneity for structural studies – the IMAC/TAGZyme process

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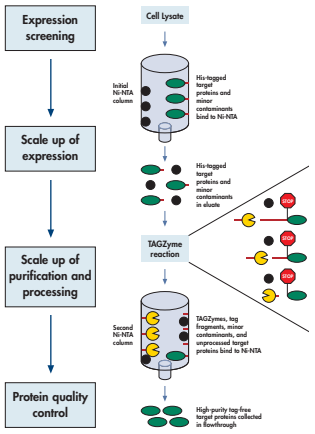
Introduction

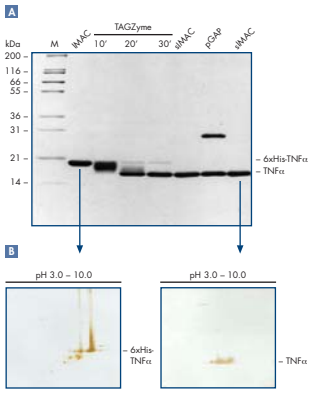
- Use of proteins for structural studies and biopharmaceuticals places high demands on protein quality, purity, and integrity. With increasing numbers of highly diverse proteins being investigated, technologies are required that avoid developing new production strategies for every target. IMAC technology for purification of 6xHis-tagged recombinant proteins [1] has been a major step towards this goal. However, using IMAC, protein purity is sometimes not sufficient for protein crystallization and vector-encoded amino acids are undesirable in biopharmaceuticals and may interfere with protein structure.
- We describe production of high-quality proteins of native structure using a single Ni-NTA IMAC chromatography column and exoproteolytic His-tag removal via the TAGZyme system followed by a reverse purification step (subtractive IMAC) to remove protease and other impurities (see flowchart). The applicability of this process is demonstrated with the production, quality control, and structural investigation of monomeric cytokine Interleukin-1beta (IL-1β [2]) and trimeric human Tumor Necrosis Factor alpha (TNFα).



The IMAC/TAGZyme strategy for protein production. The same Ni-NTA column is reused in the course of the process.

TNFα production and quality control

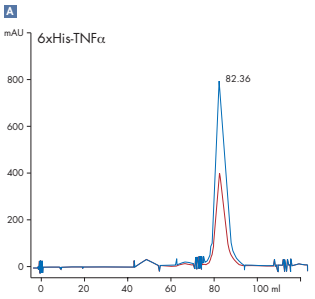
- TNFα was highly pure after single-step Ni-NTA chromatography A. However, some co-purified host cell proteins were visible upon 2D gel electrophoresis and silver staining (B left panel). These were removed by reverse Ni-NTA IMAC purification (B, right panel). The residual portion of 6xHis-TNFα which remained uncleaved by TAGZyme DAPase (30 min, A) represents formylated N-Met or N-Met-processed protein. Thus, by its selectivity for deformylated and Met-extended, i.e. in-frame substrate N-termini, DAPase acts as a filter to sort out microheterogeneities and increase product homogeneity. The sub-band pattern of TNFα in 2D gels between pI 6.7 and 5.8 is in accordance to previously reported results for the human protein produced in yeast (3).



Electrophoretic analysis of TNFα. A Ni-NTA Superflow purification, His-tag cleavage kinetics (TAGZyme), and subtractive IMAC. His-tagged TNFα was dialyzed versus TAGZyme buffer and processed by addition of TAGZyme dipeptidyl aminopeptidase I (DAPase) and glutamin-cyclotransferase (Qc-ylase) for 30 min. pyro-glutamyl-extended TNFα was recovered after reverse purification (sIMAC) and the pyro-glutamate removed by pyro-glutamyl aminopeptidase (pGAP) treatment followed by a second sIMAC step. Aliquots were analyzed by SDS-PAGE and Coomassie staining. B 2D gel electrophoresis analysis of 6xHis-TNFα eluted from the initial Ni-NTA purification step (IMAC, left panel) and of the TAGZyme-processed, final TNFα (sIMAC, right panel). Gels were silver-stained.

TNFα production and quality control

- The purity of the preparation is confirmed by gel filtration where TNFα eluted in a single sharp peak as predicted. In comparison, 6xHis-TNFα eluted slightly earlier from the gel filtration column N-terminal sequencing showed the desired N-terminus of the final preparation of TNFα (see table below).



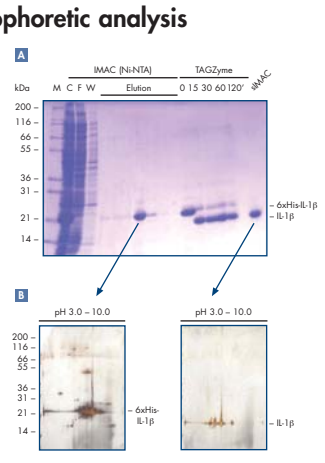
C

TNFα molecule	N-terminal sequencing
Native mature TNFα (NM00594)	VRSSSRTP...
6xHis-TNFα	MKHHHHHHQVRSSSRTP...
TAGZyme/IMAC processed TNFα	VRSSSRTP...

Quality control of TNFα. Chromatogram from size exclusion chromatography of A 6xHis-tagged and B the final TNFα sample. A Superdex 200 column was loaded with approximately 3.0 mg, respectively, of the protein samples and run with 1xTAGZyme buffer. The numbers indicates the peak elution volumes. C N-terminal sequencing of His-tagged and TAGZyme-cleaved TNFα. Sequencing was performed by MALDI-TOF MS/MS and Edman degradation.

IL-1β production and electrophoretic analysis

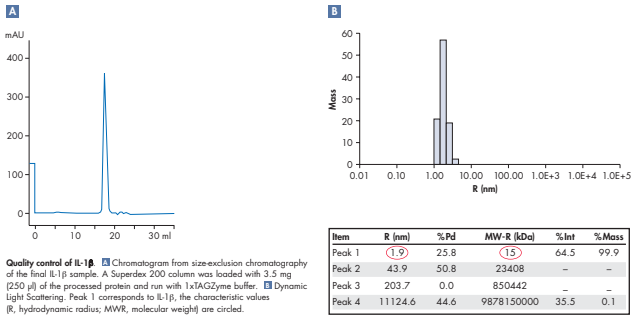
- Although apparently highly pure after initial IMAC A 2D electrophoresis of the 6xHis-IL1β elution fraction revealed impurities (B, left panel) which, however, were removed completely by subtractive IMAC (B, right panel). The different spots of IL-1β visible at different pI values seem to be an isoelectric focusing artifact as confirmed by further analyses. As for TNFα, N-Met-formylated or –processed protein was left uncleaved and was removed completely by sIMAC. 2 g of IL-1β, was produced by the process with an overall recovery of 67%, showing its suitability for industrial applications.



Electrophoretic analysis of IL-1β. A Expression, Ni-NTA Superflow purification, His-tag cleavage kinetics (TAGZyme), and subtractive IMAC. His-tagged IL-1β was dialyzed versus TAGZyme buffer and processed by addition of TAGZyme dipeptidyl aminopeptidase I (DAPase) for 120 min as described [2]. IL-1β was recovered by reverse purification (sIMAC). Aliquots were analyzed by SDS-PAGE and Coomassie staining. B 2D gel electrophoresis analysis of 6xHis-IL-1β eluted from the initial Ni-NTA purification step (IMAC, left panel) and of the DAPase-processed, detagged IL-1β (sIMAC, right panel). Gels were silver-stained.

Analysis of IL-1β reveals high-quality protein production

- IL-1β eluted in a single sharp peak from a gel filtration column at ~20 kDa A and DLS B confirmed the monodispersity of the preparation. 99.9% of the mass corresponds to peak 1 with a hydrodynamic radius of 1.9 nm equivalent for a protein of ~15 kDa. N-terminal sequencing showed the desired N-terminus of the final preparation of IL-1β (see table below).



Quality control of IL-1β. A Chromatogram from size-exclusion chromatography of the final IL-1β sample. A Superdex 200 column was loaded with 3.5 mg (250 µl) of the processed protein and run with 1xTAGZyme buffer. B Dynamic Light Scattering. Peak 1 corresponds to IL-1β, the characteristic values (R, hydrodynamic radius; MWR, molecular weight) are circled.

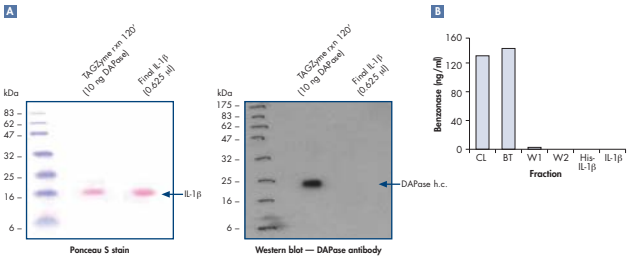
Item	R (nm)	%Pd	MW-R (kDa)	%Int	%Mass
Peak 1	1.9	25.8	15	64.5	99.9
Peak 2	43.9	50.8	23408	–	–
Peak 3	203.7	0.0	850442	–	–
Peak 4	11124.6	44.6	9878150000	35.5	0.1

IL-1β molecule	N-terminal sequencing
Native mature IL-1β (NM000576)	APVRSLN...
6xHis-IL-1β	MKHQHQHQHQHQHQAPVRSLN...
TAGZyme/IMAC processed IL-1β	APVRSLN...

N-terminal sequencing of His-tagged and TAGZyme-cleaved TNFα. Sequencing was performed by MALDI-TOF MS/MS and Edman degradation.

Enzyme depletion in IL-1β production

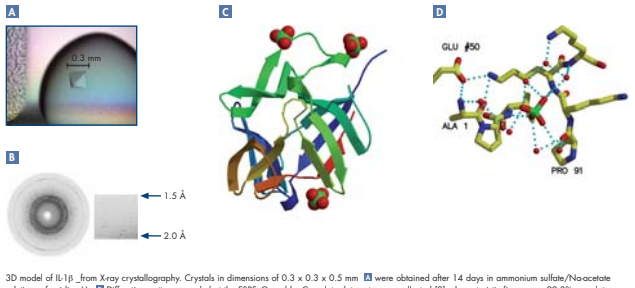
- Monoclonal antibodies versus the TAGZyme/DAPase subunits were generated and western blot analysis revealed the depletion of the protease from the preparation by subtractive IMAC (A). The absence of Benzonase nuclease used to eliminate nucleic acids and reduce sample viscosity before loading onto the Ni-NTA IMAC column was shown by ELISA (B). Additional analyses showed a low Ni (0.2 µg/mg protein, ICP-MS) and endotoxin content (0.013 ng/mg protein, LAL assay).



Quality control of IL-1β. A Immunologic analysis of TAGZyme/DAPase removal. In the Ponceau S stain (left panel), IL-1β is visible in lanes 2 and 3 at 17 kDa. In the western blot (right panel), detection of DAPase was by chemiluminescence using a monoclonal antibody generated versus the heavy chain (h.c.) and a anti-mouse HRP conjugate reporter. Corresponding aliquots from the cleavage reaction and the final IL-1β sample were loaded. B ELISA analysis of Benzonase removal. Corresponding aliquots of each fraction were analyzed. Detection limit of the assay for Benzonase is 0.2 ng/ml. **CL**, cleared cell lysate; **FT**, IMAC flow-through; **W1** and **W2**, first and second IMAC wash; **His-IL-1β**, IMAC elution; **IL-1β**, sIMAC flow-through.

Crystallization and structural analysis of IL-1β

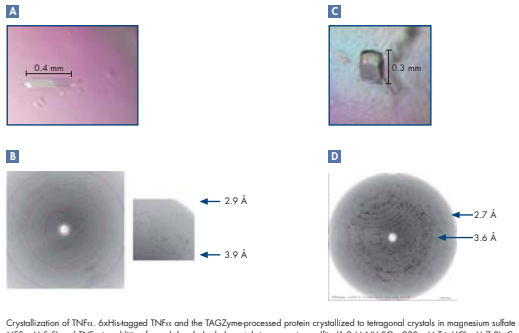
- X-ray crystallography of IL-1β confirmed the previously solved structure (pdb 9ILB) at 1.5 Å resolution. In addition to 9ILB, the built model showed the presence of three SO_4^{2-} ions in the molecule. A zoom into the structure shows that the protein is completely folded even at the termini. Heavy metal ions such as Ni^{2+} were not visible in the electron density. The fact that the N-terminus (Ala1) is engaged in intermolecular contacts within the crystal may explain why we failed to crystallize His-tagged IL-1β. The data show that the IMAC/TAGZyme strategy delivers proteins that are structurally intact, indicating that the process is well suited to produce proteins for structural studies.



3D model of IL-1β _from X-ray crystallography. Crystals in dimensions of 0.3 x 0.3 x 0.5 mm A were obtained after 14 days in ammonium sulfate/Na-acetate solutions of acidic pH. B Diffraction pattern recorded at the ESRF, Grenoble. Complete data sets were collected [2], characteristic figures are 99.3% completeness, R_{merge} 6.3 (23.5)%, I/sI 9.4 (3.1), R_{sym} 19%. C Whole molecule model. The arrows indicate beta sheets and the lines in between coiled regions. The position of the N- and C-termini are indicated. Three sulfate ions were determined and their exact positions in the vicinity of the N-terminus and their interactions are visualized. D Folding of and hydrogen bond-mediated intra- as well as intermolecular interactions at the N-terminus of IL-1β. Alanine 1 (ALA 1) of one IL-1β molecule interacts with glutamate 50 (GLU #50) of a neighboring molecule.

Crystallization and structural analysis of TNFα

- TNFα as well as 6xHis-TNFα could be crystallized. Both rhombohedral and tetragonal forms of crystals were obtained from TNFα as reported (3) with crystal sizes of 0.3 x 0.2 mm and 0.16 x 0.03 mm, respectively, with 6xHis-TNFα forming tetragonal crystals. The tetragonal TNFα crystal diffracted to 2.0 Å (synchotron) and the tetragonal 6xHis-TNFα crystal diffracted to 2.5 Å (homelab).



Crystallization of TNFα. 6xHis-tagged TNFα and the TAGZyme-processed protein crystallized to tetragonal crystals in magnesium sulfate (A and C, 2.7 M $MgSO_4$, MES, pH 5.5) and TNFα in addition formed rhombohedral crystals in ammonium sulfite (1.8 M NH_4SO_3, 200 mM Tris-HCl, pH 7.8). Crystal diffraction (B and D) was analyzed using a FR 591 Nonius Bruker homelab.

Conclusions

We show the application of a protein purification strategy which makes use of a single Ni-NTA IMAC column and the exoproteolytic removal of the His-tag by TAGZyme to generate native proteins of highest purity and structural integrity.

The only process requirement is a properly designed His tag sequence at the N-terminus of the expressed protein. Vectors of the TAGZyme pQE series may be used, or sequences may be checked by the TAGDesigner online tool on www.qiagen.com/products/protein/tagdesigner/default.aspx.

The workflow has been successfully applied for several proteins and we show data for monomeric hIL-1β and trimeric hTNFα. The process is scalable to the production range and enables generation of biopharmaceutical-grade protein [2]. Its most striking advantages are:

- Ni-NTA IMAC is the only chromatographic principle required – use in regular and reverse (subtractive) mode
- Homogenous protein preparations – free of host cell-derived impurities and process components (enzymes)
- Absolute specificity of TAGZyme processing – generate quality-proven proteins of native sequence
- High process efficiency
- Structurally intact proteins

We believe that this process is a universally applicable strategy for purification of proteins for structural studies as well as for production of biopharmaceuticals.

References

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