Monitoring Protein Fate during Purification with the Agilent 2100 Bioanalyzer

Application Note

Production-QA/QC

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Abstract

Monitoring protein fate with respect to recovery, stability and purity in a fast and reliable manner is fundamental in protein purification. In this Application Note, we present a purification strategy developed for a tag-labeled DNA binding protein, in which the protein fate was monitored with the Agilent 2100 Bioanalyzer. Lab-on-a-chip analysis provides several advantages in quantitation, resolution, time and data handling. Therefore, we have replaced conventional SDS-PAGE by Bioanalyzer analysis.

Introduction

At the Microchemistry Core Facility of the Max-Planck Institute of Biochemistry we provide an in-house service for recombinant protein production. Our cloning, expression and purification approaches are very close to a "consensus" recently reviewed by a number of Structural Consortia, with Immobilized Metal Affinity Chromatography (IMAC) being the most common purification method.[1] Although hexahistidine and other affinity tags have simplified protein purification tremendously, there are still a number of challenges remaining including protein degradation, precipitation, soluble aggregates, and loss on hydrophobic surfaces.

Therefore, an accurate and fast protein sample analysis method is critical to optimizing a protein expression and purification strategy. In this study, we optimized our protein purification strategy for a His6-tagged DNA-binding protein with the help of the Bioanalyzer Protein 230 kit for protein sizing and impurity assessment.

Experimental

A DNA-binding protein, the protein of interest (POI), was N-terminally fused to His6-Sumo3-tag,[2] bound to Ni beads, and released from the tag and beads by treatment with His-tagged SenP2 protease.[3] Bioanalyzer chips have been prepared according to the instructions provided with the Agilent Protein 230 kit.

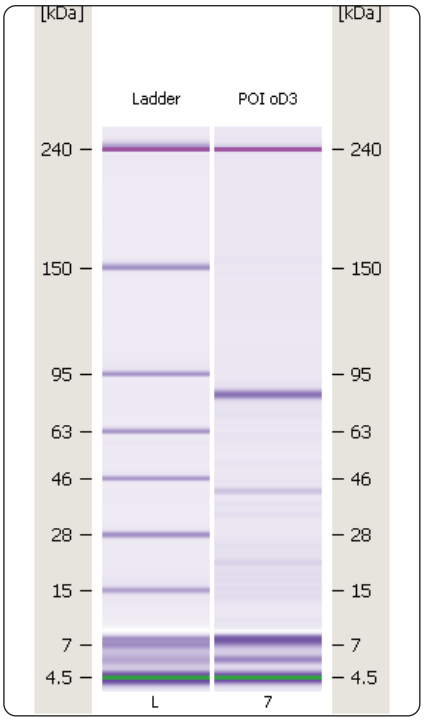


Figure 1
Gel-like image of a 14–230 kDa chip run of cell lysate. 100 µL cells were lyzed in 440 µL, 20 mM Tris pH 8.0 + 0.25% SDS for 5 min at 95 °C; Based on the relative quantification with the Bioanalyzer, the overall yield for the POI was estimated to 30 mg/g biomass.

Protein production

Expression level as determined by chip analysis (Figure 1) was 51% of the total with a relative concentration of 390 ng/µL. Relative concentration is related to an internal standard, the upper marker that migrates at 240 kDa.[4] We did not include external calibration samples, which is an option provided in the Bioanalyzer Expert software. The protein level was therefore calculated to 30 mg POI/g biomass based on this relative protein quantitation strategy.

We observed an unexpected electrophoretic behavior of the 54 kDa fusion protein with the Protein 230 Kit separating at about 80 kDa. This reproducible offset between the expected and observed molecular size could be due to some specific interaction between the protein and the linear polymer used within the Bioanalyzer protein kits. This was observed in previous studies, specifically for glycosylated proteins.[5]

Protein purification by affinity chromatography

In a first experiment, we typically use half the amount of Ni beads relative to the target protein in order to prevent background binding. Initially, we purified from 5 g cells, which corresponds to 150 mg POI (Figure 1). Empirically, less than 100% of protein detected in those SDS-buffer lyzed samples is amenable to purification. We assumed about 50% soluble protein and loaded the lysate supernatant onto Ni beads providing 40 mg binding capacity which corresponds to a 200% excess of target protein. Table 1 and Figure 2 illustrate protein recovery as determined by Bioanalyzer analysis. Less than half of POI in the total lysate was soluble and recovered in the lysate supernatant. As anticipated from the bead capacity, 45 mg were depleted from the lysate supernatant, and subsequently released by washes, SenP2 on-column cleavage and final imidazole elution. Together with the flow through, this adds up to 110 mg POI within the lysate supernatant.

Sample	Rel. Conc. [ng/µL]	% Total	Volume [mL]	POI total [mg]
Total lysate	1,368.7	48.2	20	274
Lysate supernatant	551.2	38.2	20	110
Flow through	325.1	29.9	20	65
Wash 1	435.7	48.3	30	13
Wash 2	405.9	84.1	30	12
SenP2 cleavage	4,784.3	67.4	2	10
Imidazole eluate	9,745.4	55.2	1	10

Table 1
Protein recovery dertermined by analysis with the Agilent 2100 Bioanalyzer.

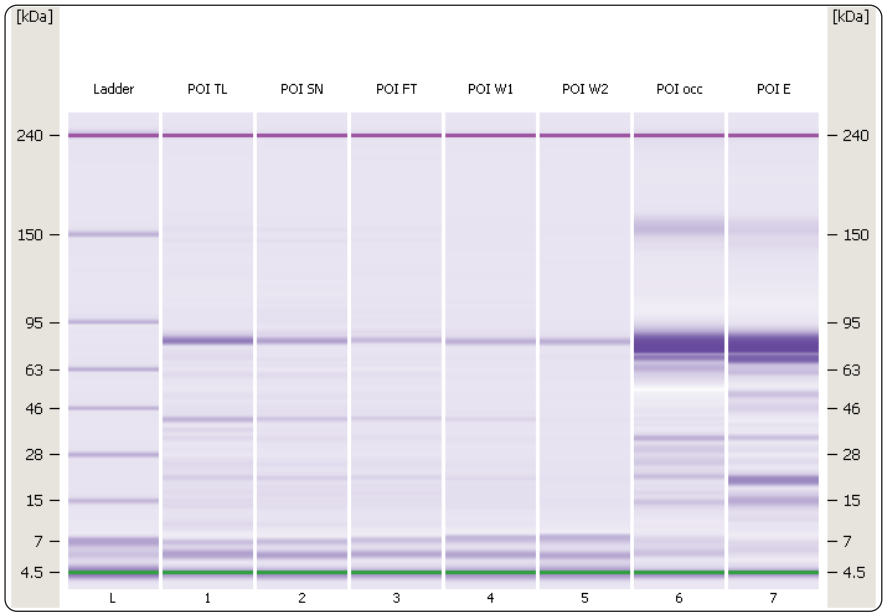


Figure 2
Gel-like image of a Protein 230 chip run monitoring the purification process. TL (total lysate) 1:10 diluted, SN (lysate supernatant) after 30 min 20.000 rpm 1:10 diluted, FT (flow through) 1:10 diluted, W 1,2 (washes), occ (on-column cleavage), E (imidazole eluate).

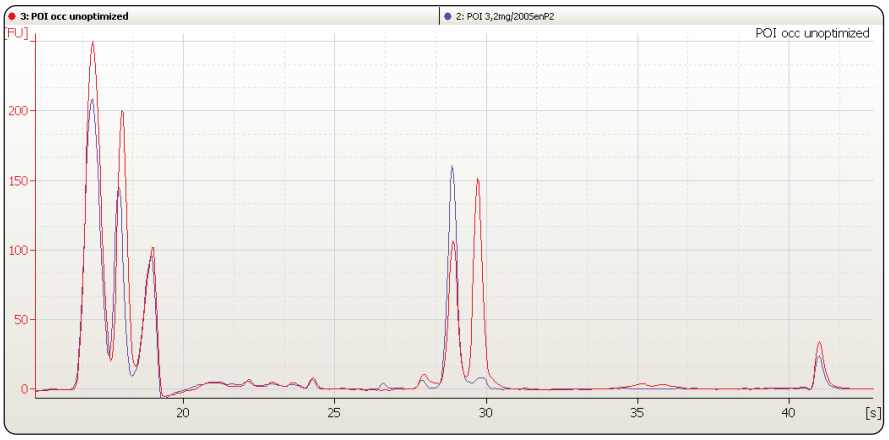
Lysate supernatant from 5 g cells was loaded to 1 mL Ni beads bound for 2 h at 4 °C, washed twice with 30 mL washing buffer containing 40 mM imidazole. On-column cleavage was performed in 2 mL cleavage buffer using 100 units SenP2 protease overnight at 4 °C. Remaining protein was released from Ni beads with 500 mM imidazole.

Optimization of on-column cleavage

Although a 100-fold molar excess of SenP2 protease was used for on-column cleavage, most of the POI released from Ni beads was still fused to its His6-Sumo3-Tag, probably due to protein aggregation on the beads. When cleaved in solution, fusion protein and tag free protein interacted very strongly and could not be separated by gel filtration, ion exchange or any other means. Therefore, the only promising strategy for separating the two forms was to improve on-column tag removal. An excess of beads was used to prevent self-interaction of the POI. An even higher excess of SenP2 protease allowed finding and cleaving its substrate on Ni beads that were only barely covered with target protein. Finally, the optimal ratio of protein-to-beads-to-protease for the best possible on-column cleavage performance was empirically determined to 1:7:100 by monitoring the percentage of cleaved protein versus its tagged precursor (Figure 3). As illustrated in the blue electropherogram (Figure 3), almost all the tagged protein can be cleaved under these conditions. The molecular weight difference of about 10 kD is sufficient to achieve a baseline separation between the tagged and tagless protein.

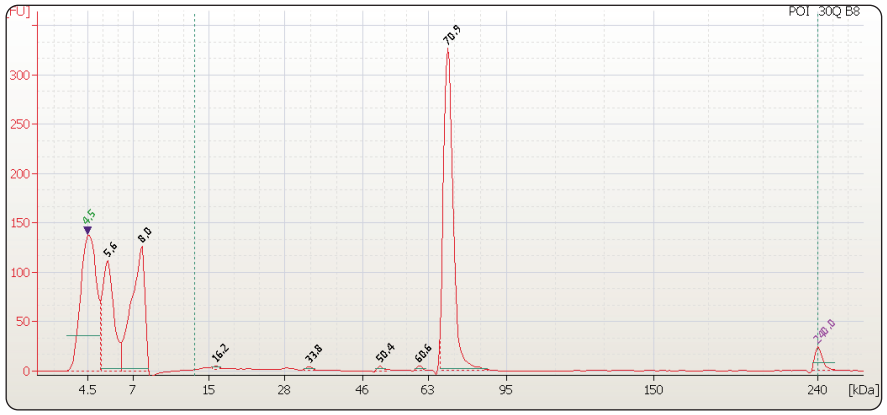
Final protein purification and QA

The POI released by SenP2 on-column cleavage at 78% purity (Figure 3) was purified to homogeneity by anion exchange chromatography. Using Bioanalyzer analysis, the final purity of the separated POI was 96.9% (Figure 4a). Our final quality control of purified proteins also includes analysis by LC-MS to assure correct molecular mass and estimate purity based on UV (Figure 4b). Figure 4 shows good correlation between HPLC separation coupled to UV/ESI-TOF versus electrophoretic separation and fluorescence detection by microfluidics in monitoring the purity of protein samples.



Sample	Size [kDa]	Rel. Conc. [ng/µL]	% Total
Unoptimized on-column	71.9	310.2	38.3
cleavage	81.9	414.7	51.3
Optimized on-column	70.9	993.0	78.0
cleavage	83.2	11.4	0.9

Figure 3
Electropherogram overlay and result table of on-column cleavage samples analyzed with the Protein 230 kit. On-column cleavage of the His-tag can easily be monitored. With the optimized protocol, POI is 78% pure while tagged POI is reduced to less than 1%.



Size [kDa]	Rel. Conc. [ng/µL]	% Total
4.5	0.0	0.0
5.6	0.0	0.0
8.0	0.0	0.0
16.2	10.9	0.7
33.8	11.8	0.8
50.4	12.8	0.8
60.6	13.0	0.8
70.9	1,484.8	96.9
240.0 (marker)	60.0	0.0
324.8	0.0	0.0

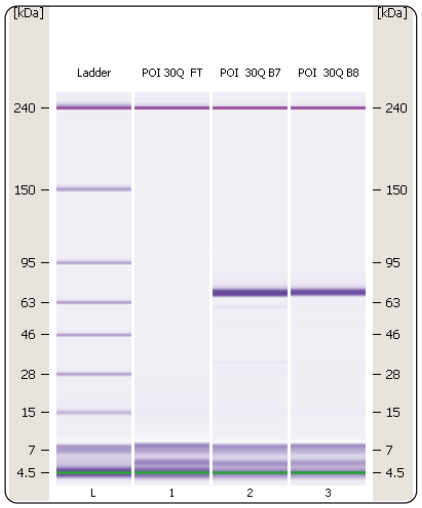


Figure 4a
Chip analysis of the POI eluted in a NaCl gradient on Source 30 Q; FT flow through, B7, B8: protein peak fractions eluted at 45 mM NaCl. Shown are gel-like image, electropherogram, and peak table, indicating that the POI is purified to roughly 97%.

Results and discussion

We have replaced conventional SDS-PAGE by Agilent 2100 Bioanalyzer chip analysis for several reasons:

- Quantitation and resolution: relative quantitation is accurate and allows reliable monitoring of protein fate during purification. Degradation products or contaminating proteins can easily be followed during the course of purification. Double bands, hardly detected in typical Coomassie stained SDS gels are separated at high resolution.
- Time: electrophoresis is finished within 20 min. Since data is displayed on-line, the interpretation of peak tables can be performed immediately. It is unnecessary to stain, destain, scan, and dry gels.
- Data handling: a comparison mode integrates data from different chip runs into one analysis. This allows the reliable comparison of individual protein peaks from different protein samples and experiments. Another helpful tool is the overlay of samples as shown here for SenP2 on-column cleavage efficiency (Figure 3).

Because Bioanalyzer protein separation is based on microfluidic capillary gel electrophoresis, relative protein migration times can differ from traditional SDS-PAGE separation. Significant differences were especially observed for proteins that are non-globular or tend to form higher structures.[5] Therefore, the first chip analysis of a POI requires some caution. Furthermore, the price per sample is higher than for ready-made SDS-PAGE. However, depending on sample throughput, the significantly improved quality of data and faster time to result may justify these drawbacks.

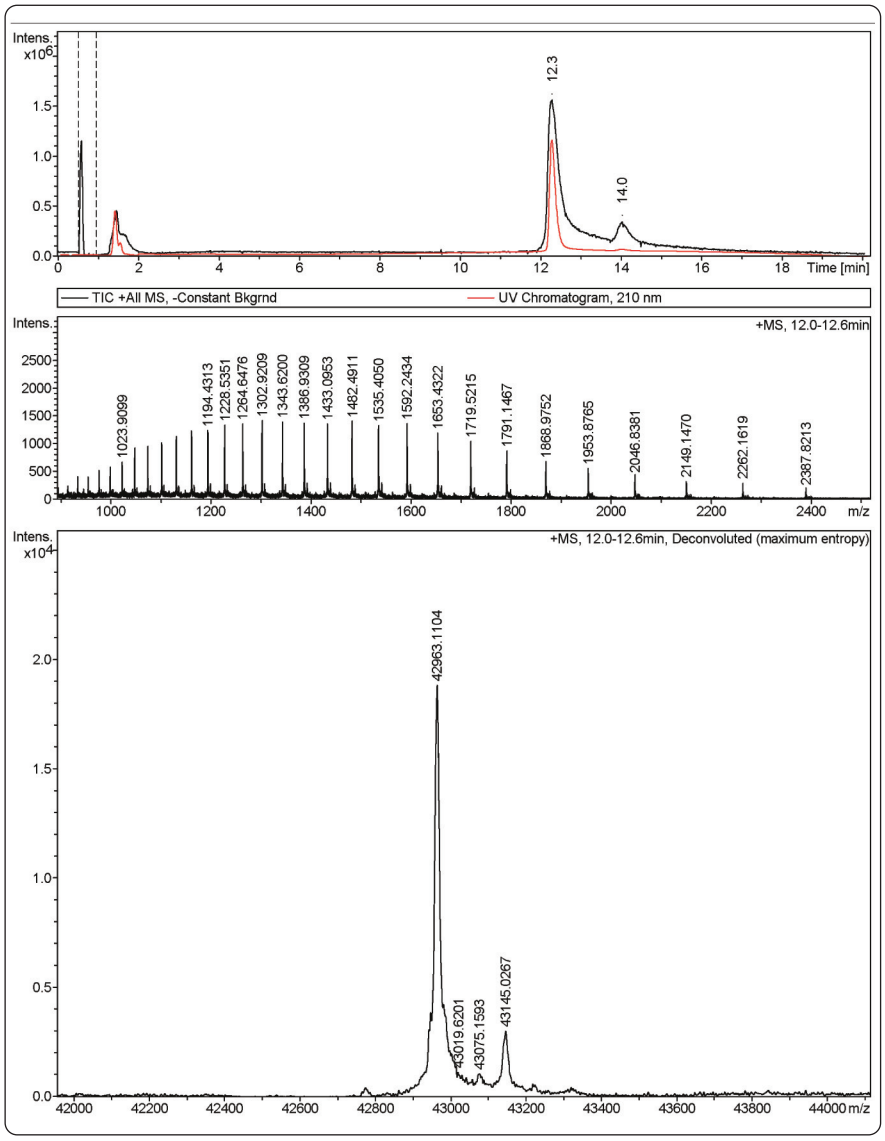


Figure 4b
LC-MS of POI measured on Agilent 1100 HPLC-Bruker microTof. UV chromatogram (red trace) returns comparable results to the Bioanalyzer electropherogram.

References

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4 Protein Sizing and Quantitation with the Agilent Protein 80 and Protein 230 Kits on the Agilent 2100 Bioanalyzer; Agilent Technical Note 5590-5721, May 2010

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