Protein purification and characterization using the 1100 Series purification system and the 2100 bioanalyzer

Application

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Abstract

Orthogonal methods for protein purification and purity analysis are important to determine with certainty protein identity and purity. Further, residual impurities that are not visible in the chromatographic step may be revealed, giving valuable hints for modification and optimization of the purification strategy. The Agilent 1100 Series purification system and the Agilent 2100 bioanalyzer with the Protein 200 Plus LabChip® kit provide an ideal solution that ensures efficient and reliable protein purification and characterization. This Application Note describes how these two instruments complement each other in the purification of a protein.

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Introduction

Protein yield and purity are important criteria for the success of a purification process. Besides efficient chromatographic conditions, well-established and reliable instruments also contribute to successful purifications. Due to its high resolving power, preparative reversed phase (RP) HPLC is often employed as a last polishing step in purification workflow for peptides and small hydrophilic proteins. Although the solvent conditions used in RP-HPLC are known to denature protein structure it can often be regained by adjusting favorable conditions especially for small proteins. The Agilent 1100 Series purification system represents an appropriate and well-established system for reversed phase chromatography. Its modular set-up, reliable fully automated delay volume calibration and a comprehensive software package provide a versatile platform that is the right choice for purification tasks with flow rates up to 100 mL/min.

Protein purification processes should always be tracked with orthogonal methods as they provide complementary information on protein characteristics such as molecular weight and hydrophobicity. Applying such a strategy enables determination of protein identity and purity with more confidence.

In this Application Note we show how the Agilent 1100 Series purification system and the Agilent 2100 bioanalyzer can be used together for efficient protein purification analysis. The Agilent 2100 bioanalyzer offers a reliable, fully automated analysis system capable of analyzing 10 different protein samples in less than 30 minutes. In contrast to conventional SDS-PAGE no tedious separation and staining steps are necessary. Further, the microfluidic system enables monitoring and optimizing the protein purification process fast and easily.

Experimental

Agilent 1100 Series purification system

A 56 kDa protein present in a protein mixture was subjected to purification from contaminants with the Agilent 1100 Series purification system. Due to its high resolving power RP-HPLC was chosen as the last polishing purification step. The Agilent 1100 Series purification system comprised:

- Two Agilent 1100 Series preparative pumps
- Manual prep injection valve kit with 20 mL PEEK sample loop
- Agilent 1100 Series column organizer
- Agilent 1100 Series variable wavelength detector (0.06 mm flow cell)
- Agilent 1100 Series fraction collector preparative scale
- ZORBAX 300SB-C8 column (21.2 × 250, 7 µm)
- ChemStation A09.03 software for system control

Up to 20 mL protein sample (50 mM phosphate buffer, pH 5.2, filtered 0.45 µm) solution were injected into a 20 mL PEEK sample loop mounted at a manual injector. Before each run the start position of the vial in the fraction collector could be specified through the ChemStation software. By turning the handle of the manual injector the purification run was easily started. The proteins were purified at a flow rate of 15 mL/min using water and acetonitrile as eluents, both containing 0.08 % TFA. Fraction collection was triggered on threshold and slope of the UV signal at 280 nm. Prior to purification the exact delay volume was determined by a delay volume calibration. For further details of fraction collection with the Agilent 1100 Series purification system, please refer to references 1 and 2.

Agilent 2100 bioanalyzer and the Protein 200 Plus assay
The pre-purified sample was directly analyzed using the 2100 bioanalyzer. The different fractions obtained with the 1100 Series purification system were lyophilized in order to evaporate acetonitrile and TFA. All fractions could be solubilized in 30 µl of PBS buffer. Since the Protein 200 Plus assay is compatible with a large variety of buffers and buffer components all kinds of different buffers can be used to solubilize the proteins of interest. A detailed list of compatible buffers can be found in the Protein 200 Plus reagent kit guide.

Samples and chips were prepared according to the protocol provided with the Protein 200 Plus LabChip kit. The kit includes 25 chips, spin filters and all reagents needed for the experiments including the Protein 200 Plus ladder and the upper and lower marker premixed in the sample buffer. The chip-based separations were performed on the Agilent 2100 bioanalyzer using the dedicated Protein 200 Plus assay software assay. The software automatically determines the size of each protein in kDa, its relative concentration to the upper marker and the % total of the protein. Latter gives direct information about the purity of a particular protein.

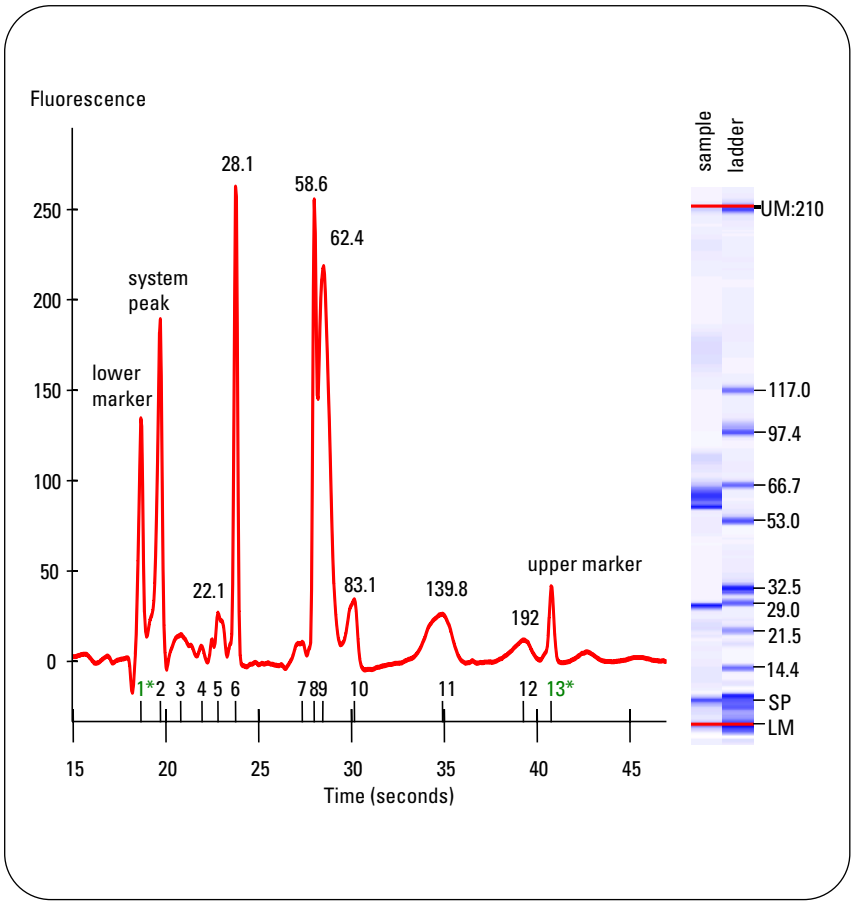

Figure 1
Analysis of the pre-purified protein sample on the Agilent 2100 bioanalyzer using the Protein 200 Plus assay. The electropherogram and gel-like image are shown. The numbers indicate the molecular weight in kDa. The 56 kDa protein of interest runs at a migration time of 28 s corresponding to 58.6 kDa.

Results and Discussion

Only 4 µL of the pre-purified sample was used in a first analytical step using the Agilent 2100 bioanalyzer to determine whether and in what amounts the 56 kDa protein of interest was present. Figure 1 shows the electropherogram and gel-like image of the analysis. The peak in the electropherogram running at 28 seconds corresponds to a 58.6 kDa protein which has an abundance of 20 % of the total protein concentration and is also visible in the gel-like image. This clearly indicates the presence of the target protein in the sample.

The remaining material was subjected to a purification step using RP-HPLC. Figure 2 shows the chromatogram of the RP-HPLC purification run of the pre-purified sample with the Agilent 1100 purification system. The vertical lines indicate start and stop of fraction collection and the horizontal line visualizes the threshold value for fraction triggering. Further, the corresponding vials in the fraction collector are denoted for each fraction.

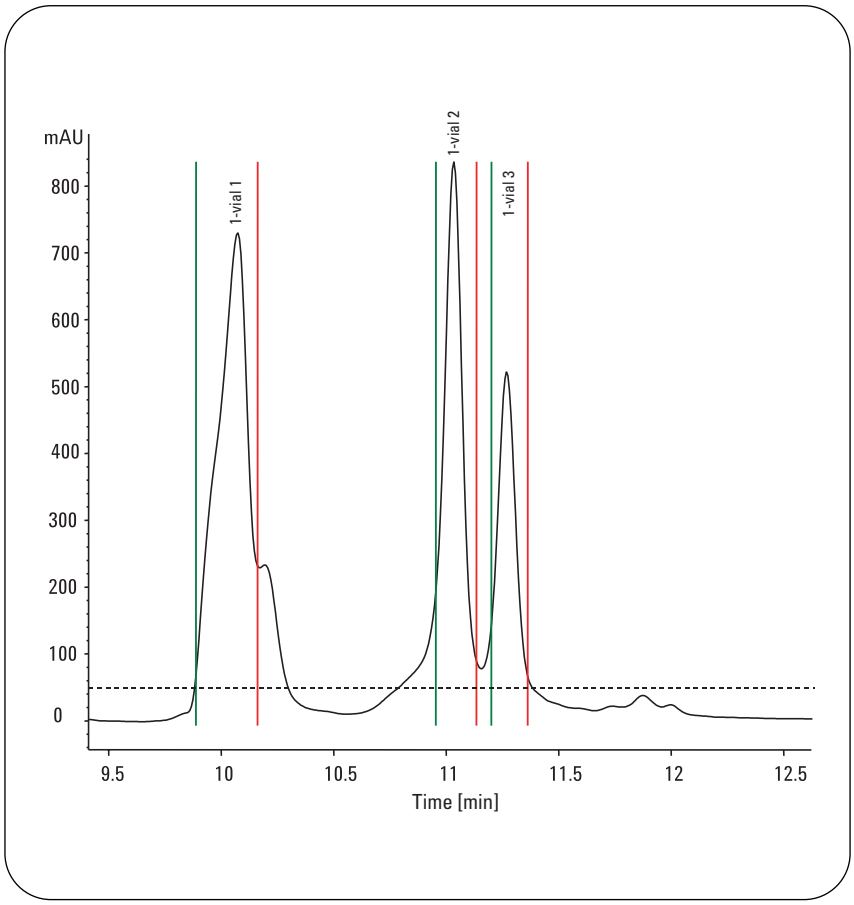

Figure 2
Chromatogram of the purification of the pre-purified protein sample. The vertical lines indicate start and stop of fraction collection, respectively. The horizontal line indicates the threshold value for peak triggering of the 280 nm UV-signal. Additionally, the numbers denote the positions of the collected fractions in the fraction collector.

To identify the fraction that contains the 56 kDa target protein all three fractions were lyophilized, solubilized in PBS buffer and analyzed with the Agilent 2100 bioanalyzer.

Figure 3A shows the electropherograms of the starting material and the three fractions. The corresponding gel-like image is displayed in Figure 3B. The data of the electropherograms and the corresponding gel-like image clearly show that the 56 kDa target protein was purified in fraction 2.

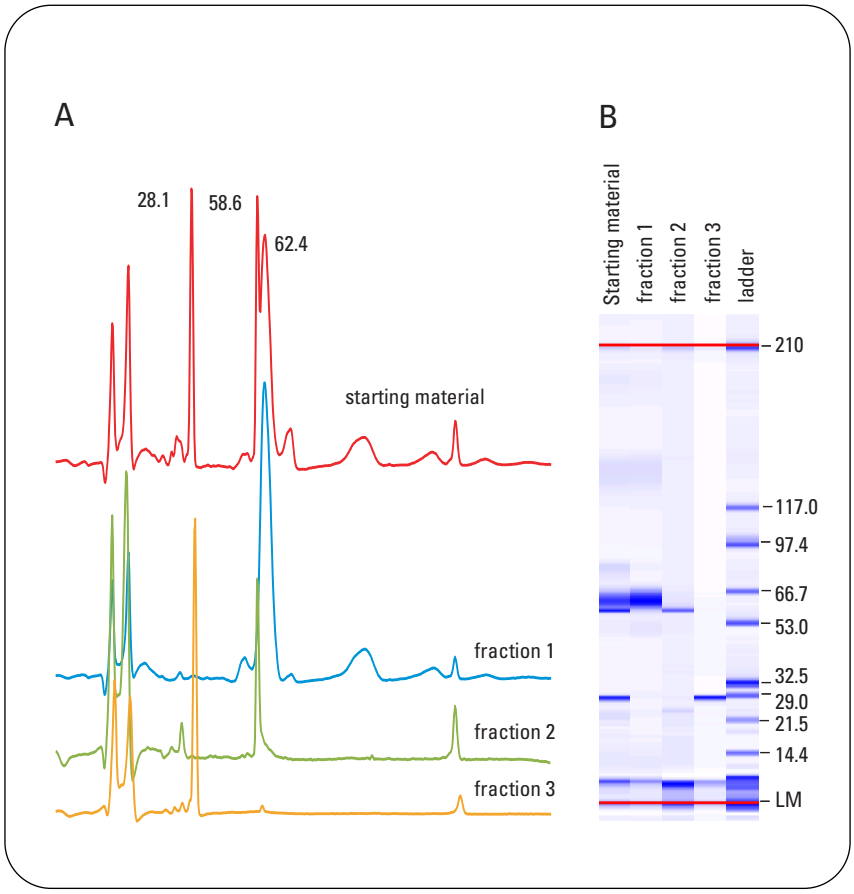

Figure 3
Analysis of the three fractions generated with the purification system on the Agilent 2100 bioanalyzer using the Protein 200 Plus assay. The electropherograms (A) and gel-like image (B) are shown. The numbers indicate the molecular weight in kDa. The 56 kDa protein of interest was located in fraction 2.

Figure 4A shows the RP-HPLC re-analysis of fraction 2. Due to a precise delay volume calibration the chromatogram of the re-analysis shows a single, symmetrical peak at about 11.5 minutes. Although this result suggests a pure protein, the electropherogram of fraction 2 (figure 4B) still shows a minor impurity around 20 kDa. The purity of the protein determined by the 2100 bioanalyzer software was 76 % with a relative concentration of that protein of 456 µg/mL. This result can be easily understood, knowing that the Agilent 2100 bioanalyzer separates proteins by a method that is orthogonal to RP-HPLC. RP-HPLC is known to separate molecules due to differences in hydrophobicity, whereas the 2100 bioanalyzer detects differences in protein size. For this reason the impurities seen in figure 4B could be revealed. In the RP-HPLC chromatogram the peak comprises two different co-eluting proteins. To yield a pure protein a further purification step with a complementary method such as ion exchange chromatography or gel filtration[5] would be necessary.

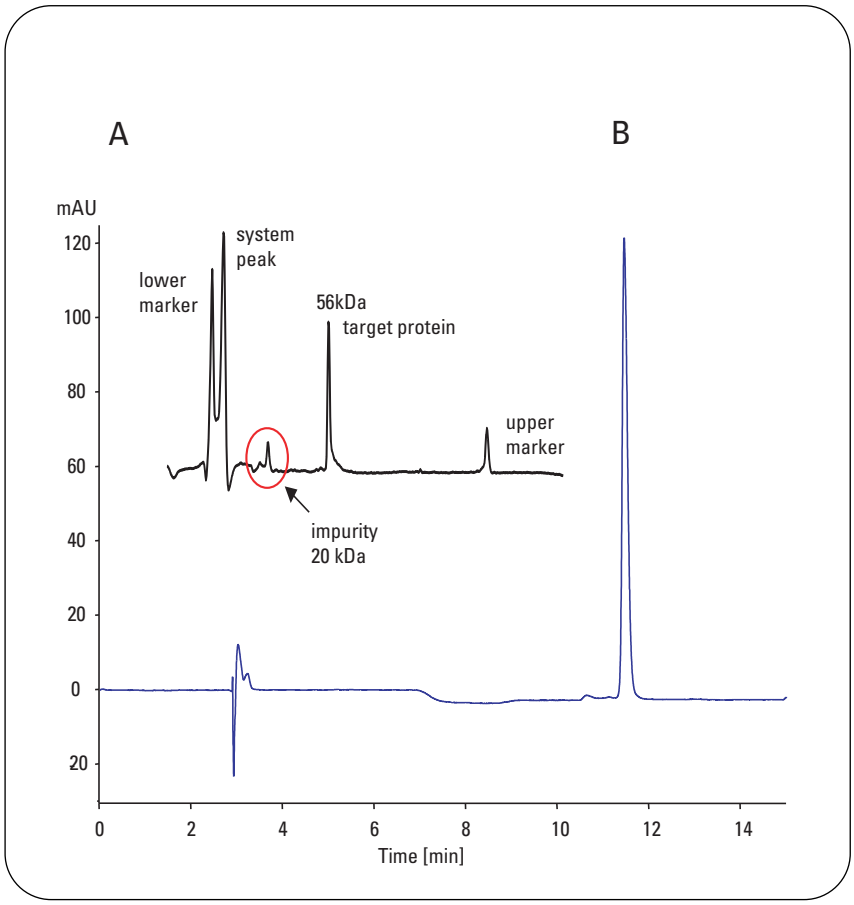

Figure 4
Chromamtogram (A) and electropherogram (B) of fraction 2. Although the RP-HPLC suggests a pure protein, the electropherogram still reveals a minor impurity around 20 kDa.

Conclusion

In this Application Note we show that the Agilent 1100 Series Purification system and the Agilent 2100 bioanalyzer complement each other perfectly in protein purification processes. The 2100 bioanalyzer not only determines protein masses faster and easier than conventional SDS-PAGE[3,4] but also serves as a complementary analytical method to RP-HPLC. Since both methods separate proteins by different physico-chemical principles they provide orthogonal information for the analyst. This is impressively demonstrated in the re-analysis of a purified 56 kDa protein. Although the target protein peak was precisely collected with the Agilent 1100 Series purification system the re-analysis of the corresponding fraction with the Agilent 2100 bioanalyzer still revealed some minor impurities not visible by RP-HPLC. If sample purity is a crucial issue it should not only be determined by a single analytical method. For this reason Agilent Technologies offers solutions — the 1100 Series purification system and the 2100 bioanalyzer — which together ensure efficient and reliable protein purification and characterization.

References

1.
"Agilent 1100 Series Purification Platform" Agilent Technologies Brochure, **2001**, publication number 5988-36EN.

2.
"Optimizing Fraction Collection with LC/MSD Systems Using an Active Splitter and Delay Sensor" Agilent Technologies Technical Note, **2002**, publication number 5988-7610EN.

3.
"Differences and similarities between the Protein 200 assay and SDS-PAGE" Agilent Technical Note, **2001**, publication number 5988-3160EN.

4.
"Comparison of different protein quantitation methods" Agilent Technologies Application Note, **2002**, publication number 5988-6576EN.

5.
"Protein analysis by aqueous size exclusion chromatography" Agilent Technologies Application Note, **2001**, publication number 5988-4266EN.

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