Comparison of different methods for purification analysis of a green fluorescent Strep-tag fusion protein

Application

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Abstract

This Application Note describes purification of a recombinant GFP fusion protein and purity verification using fluorescent measurement, SDS-PAGE followed by Western blotting, and the Protein 200 assay.

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Introduction

This Application Note describes affinity chromatographic purification of a recombinant GFP Strep-tag® fusion protein on immobilized, engineered streptavidin (Strep-Tactin®), and purity verification using various analytical methods, including the Agilent 2100 bioanalyzer and Protein 200 LabChip® kit. The Green Fluorescent Protein (GFP) from the jellyfish *Aequorea victoria*, which fluoresces in the lower green portion of the visible spectrum, has become one of the most widely studied and exploited proteins in biochemistry, cell biology and histochemistry[1]. Strep-tag® is a short peptide (8 amino acids) with highly selective binding properties for streptavidin. Strep-tags can be genetically fused to a protein's N- or C-terminal and are ideally suited for rapid and convenient purification of recombinant proteins by affinity chromatography[2].

Experimental

All experiments described were performed according to the instructions supplied by the manufacturers. The chemicals used were obtained at the highest available quality. Strep-Tactin® POROS® and Strep-Tactin®-peroxidase conjugate were purchased from IBA GmbH (Göttingen, Germany). The expression clone for GFP, cloned into the vector pRSET was a kind gift of IBA GmbH. The Agilent 2100 bioanalyzer and Protein 200 LabChip kit were supplied by Agilent Technologies Deutschland GmbH (Waldbronn, Germany).

Results and Discussion

The GFP fused to a C-terminal Strep-tag (28 kDa) was expressed in *E. coli*, as recommended by IBA GmbH. Approximately 2 g *E. coli* cell pellet was resuspended in 40 ml lysis buffer (100 mM Tris-HCl, pH 8) containing 0.5 mg/ml lysozyme and 2 µg/ml avidin. Adding Benzonase® and a selection of different protease inhibitors facilitated the subsequent purification. Disruption of the cells was performed by sonification for 8 minutes in intervals of 10 seconds followed by a centrifugation step to pre-clear the crude extract (20 minutes, 10000 rpm, 4 °C).

The GFP fusion protein was purified employing affinity chromatography according to a purification method for Strep-tag fusion proteins developed by IBA GmbH. The chromatography was performed at a flow rate of 1 ml/min on a 1-ml Strep-Tactin POROS column. Per run, 5 ml of the pre-cleared *E. coli* extract was loaded onto the column and the separation was monitored by measuring the absorbance of the eluate at 280 nm (figure 1). The fractions 59 to 63 correspond to the column flow through and fraction 64 to 76 to a subsequent washing step. The elution (fraction 77 to 78) was initiated by applying four column volumes lysis buffer containing 5 mM desthiobiotin. Elution was followed by a washing step (fractions 79 to 88) and then by regeneration of the column (fraction 89 to 94) with lysis buffer containing 5 mM HABA (hydroxyazophenyl benzoic acid).

Selected fractions of the individual experimental steps were analyzed using three different techniques. These included fluorescence measurement, denaturing polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot, and analysis with the Protein 200 assay for comparison and quantitation.

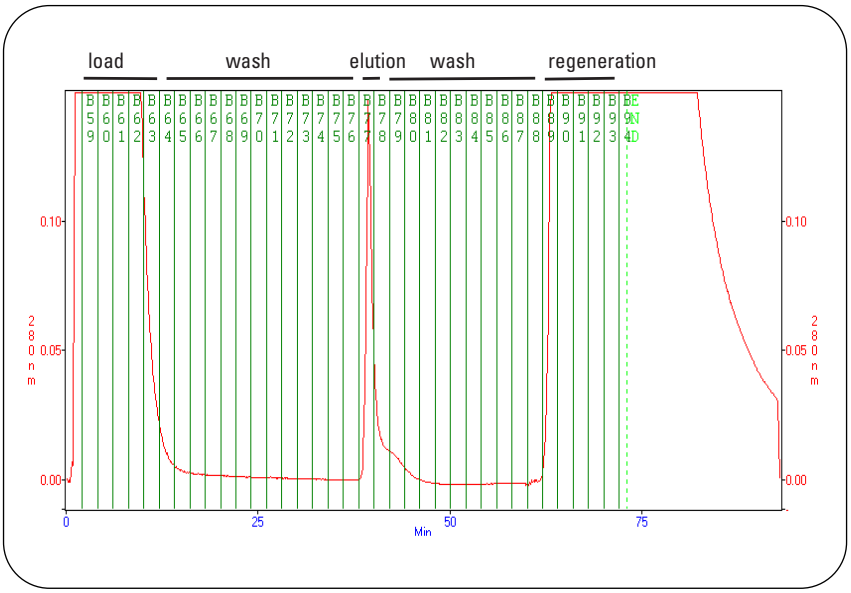

Figure 1
Affinity chromatography of the GFP fusion protein including the different experimental steps monitored by measuring the absorbance at 280 nm.

GFP detection with fluorescence measurements

Fractions containing functional GFP were identified by measuring the fluorescence of different column fractions with a fluorescence plate reader (Victor 1420 Multi label Reader, Applied Biosystems, Foster City, Ca, USA). Excitation was at 405 nm, and detection at 535 nm (figure 2).

Compared to the strong fluorescene found in the cell lysates, only low levels of fluorescence were detected in the flow through fractions 58 to 61. This indicates that the GFP bound almost completely to the affinity column. In addition, the measurements demonstrate that the washing step did not cause an unspecific elution of the GFP fusion protein from the column, because no GFP was detected in the washing fractions 64 to 76. As expected, the GFP fusion protein was eluted in the fractions 77 to 78 resulting in a strong fluorescence signal. The fractions of the column regeneration step did not contain any detectable GFP fluorescence. The yield of GFP fusion protein recovered from the column was determined by comparing the fluorescence of the initial crude extract with that of the eluted fractions 77 and 78. These measurements indicated that approximately 70 % of the loaded GFP fusion protein could be recovered.

Fluorescence measurement allows for fast determination of the relative yield of purified GFP but is not suitable for purity analysis or concentration determination. Only fluorescent proteins will be detected, prohibiting the identification of non-fluorescent, contaminating proteins.

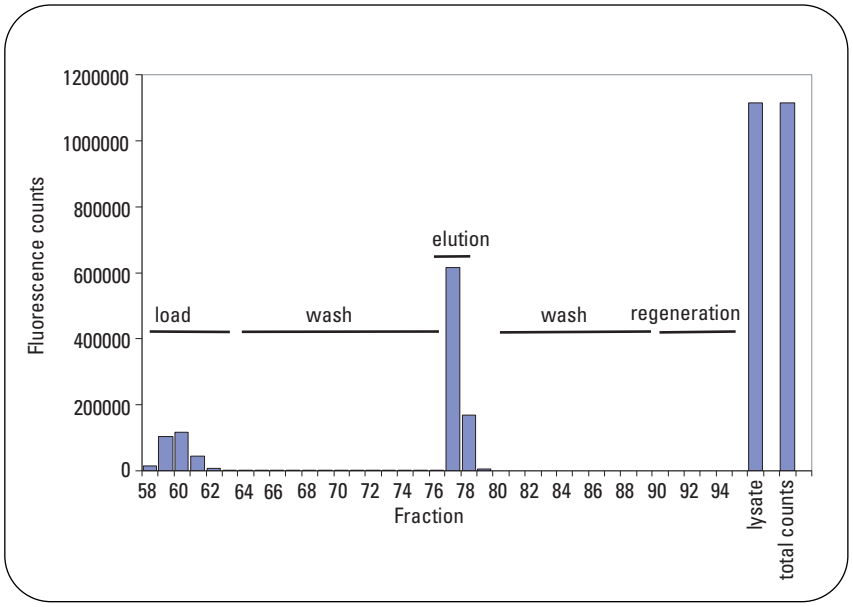

Figure 2
Fluorescent measurements demonstrating the presence of fluorescent GFP fusion protein in the different fractions obtained from the affinity chromatography.

GFP fusion protein analysis with SDS-PAGE and Western blotting

Individual column fractions corresponding to different experimental steps were analyzed using SDS-PAGE and Western blotting. The electrophoretic separation of the protein samples was performed using a 4–20 % gradient Tris-Glycine polyacrylamide gel (Invitrogen Inc., Carlsbad, CA, USA). Electrophoresis was performed in 25 mM Tris-HCl, 192 mM glycine, and 0.1 % SDS for 90 minutes at a constant voltage of 150 V. After completion of electrophoresis, one gel was stained overnight with colloidal Coomassie blue and destained for approximately five hours in double-distilled H_2O. Lane 2 in figure 3 shows the typical protein pattern of an *E. coli* cell lysate, lane 3 and 4 correspond to column fraction 60 and 62 from the loading step. A large variety of proteins can be detected in these fractions. However, it is not possible to identify the GFP fusion protein in the cell lysate and its binding to the column based on the lack of this protein in the flow through. In the elution fractions, the Coomassie-stained gel shows a strong band, which migrates between the 25 and 37 kDa bands of the molecular weight marker, which is in accordance with the expected size of the GFP fusion protein (28 kDa). A Western blot was performed to identify the GFP fusion protein based on the specific interaction with a Strep-Tactin-peroxidase conjugate, which selectively binds to the Strep-tag of the GFP. For this purpose, the separated proteins from a second SDS-PAGE experiment were transferred onto a nitrocellulose membrane using a semi-dry blotting apparatus. The transfer was performed in 25 mM Tris-HCl, 192 mM glycine, 20 % methanol, and 0.1 % SDS at a constant current of 400 mA (approximately 10-14 V). The GFP Strep-tag fusion protein was detected on the membrane by means of a Strep-Tactin-peroxidase conjugate reacting with 3-amino-9-ethylcarbazole (AEC) as substrate.

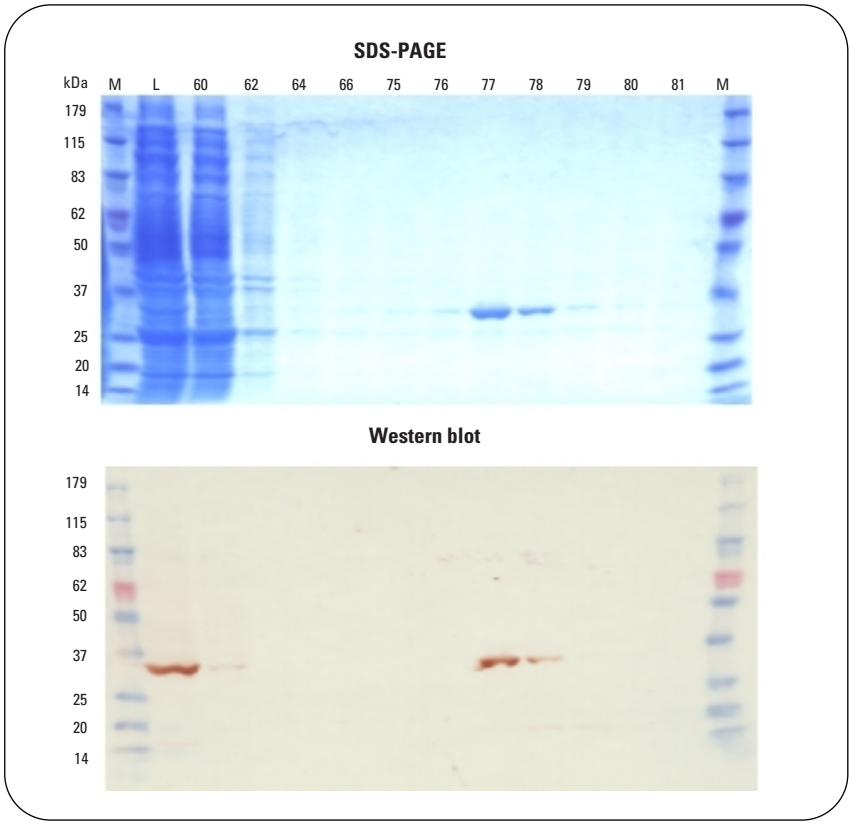

Figure 3
Analysis of GFP fusion protein purification with SDS-PAGE and Western blotting. The upper panel shows the 4-20 % gel stained with colloidal Coomassie. The lower panel shows the Western blot after detection with a Strep-Tactin-peroxidase conjugate.

The Western blot (figure 3) shows that the GFP fusion protein in the cell lysate has bound to the Strep-Tactin column matrix and that the column was not overloaded, because almost no signal was detected in the flow through fraction 60.

SDS-PAGE allows identification of column fractions containing the desired protein according to its approximate size. It can also be used to analyze relative quantity and purity. The proper identification of the protein is only possible by the highly specific interaction of the Strep-tag with the peroxidase-labeled Strep-Tactin or a protein specific antibody. However, SDS-PAGE in combination with Western blotting is laborious, time-consuming and does not provide reliable quantitative data.

Protein 200 assay

The Agilent 2100 bioanalyzer in combination with the Protein 200 LabChip kit[3] was used to analyze the cell lysate containing the GFP fusion protein, and the column fractions of the different experimental steps. This analysis system was developed by Agilent Technologies Deutschland GmbH (Waldbronn, Germany) in collaboration with Caliper Technologies Corp. (Mountain View, CA, USA) and utilizes lab-on-a-chip technology[4]. The Protein 200 assay provides sizing and relative quantitation of proteins ranging in size from 14–200 kDa under denaturing conditions, similar to SDS-PAGE[3,5] while also allowing for absolute quantitation[6]. Only 4 µl of the protein samples were analyzed according to the protocol provided by the manufacturer. The Agilent 2100 bioanalyzer software automatically determines the size of each of the proteins in kDa based on internal standards and compared to an external sizing standard. In addition, the software calculates the relative concentration (in µg/ml) based on calibration with the upper marker, which is contained in a defined concentration in each sample. Analyzing 10 protein samples took less than 45 minutes including sample preparation. The GFP fusion protein was identified with its proper size of 28 kDa. The relative concentration of this protein in the elution fraction 77 was determined to be 143.4 µg/ml with a purity of 97 % and 35.8 µg/ml with a purity of 96 % in fraction 78. This automated analysis of relative concentration and purity is a fast and easy quality control to monitor the achieved purity and yield.

Analysis with the Protein 200 assay produces results comparable to SDS-PAGE in a much shorter time frame. It is possible to determine size, purity and concentration, or the GFP fusion protein simultaneously in one single experimental step. No staining, destaining, imaging and further analysis with an imaging system and analysis software is necessary.

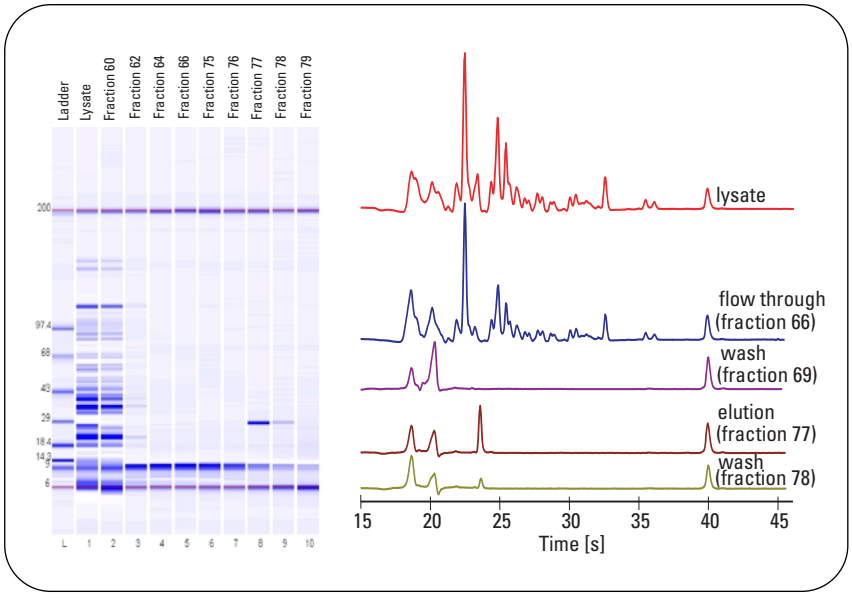

Figure 4
Analysis with the Protein 200 assay showing the gel-like image and the corresponding electropherograms.

Conclusion

The fluorescence measurement method allows fast detection of GFP in the column fractions and determination of the yield. However, this technique does not allow analysis of sample purity, which is required for optimization of the protein purification procedure. In addition, it is also limited to the analysis of fluorescent proteins. In contrast, SDS-PAGE is suitable for performing a purity analysis of the different column fractions. SDS-PAGE in combination with Western blotting is the only technique that enables true identification of the protein not only by size but also based on a specific protein-protein interaction. However, protein concentration cannot be determined with SDS-PAGE and a considerable amount of time is required to perform the different experimental steps. With the Protein 200 assay, a fast and convenient analysis of proteins or protein mixtures is possible. Information on purity, protein size and concentration for 10 protein samples is available in less than 45 minutes, in one single experimental step with minimal sample consumption and automated data analysis. Therefore, we conclude this method offers an interesting alternative to the conventional techniques such as fluorescence measurement and SDS-PAGE followed by Western blotting.

References

1.
Tsien R.Y., "The green fluorescent protein" *Annu Rev Biochem* (**1998**) 67, 509-44.

2.
Skerra A, Schmidt TG., "Use of the Strep-Tag and streptavidin for detection and purification of recombinant proteins" *Methods Enzymol.* (**2000**) 326, 271-304.

3.
Kuschel M., "Protein sizing and analysis using the Agilent 2100 bioanalyzer and Protein 200 LabChip kit" Application Note, (**2000**) Agilent publication number 5988-0975EN.

4.
Bousse L., Mouradian S., Minalla A., Yee H., Williams K., and Dubrow R., "Protein sizing on a microchip" *Analytical Chemistry* (**2001**), 73(6), 1207-1212.

5.
Kuschel M., "Differences and similarities between the Protein 200 assay and SDS-PAGE" Technical Note, (**2001**) Agilent publication number 5988-3160EN.

6.
Barthmaier P., "Absolute quantitation with the Agilent 2100 bioanalyzer and the Protein 200 LabChip kit" Application Note, (**2001**) Agilent publication number 5988-4021EN.

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Published December 1, 2001
Publication Number 5988-5025EN

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