

Clean Solutions Fuel Affinity Chromatography

When using affinity chromatography to isolate proteins from solutions, ultrapure water and contamination-free chemicals are essential for reproducible data.

» by Jan Erwig, Schwann-Schleiden Research Center for Molecular Cell Biology and the Department of Plant Cell Biology, Georg-August Universitaet Goettingen, and Elmar Herbig, Sartorius Lab Instruments GmbH und Co.KG, Goettingen, Germany

Affinity chromatography is a method used to isolate proteins from solutions. The basic principle¹ has been used in laboratories for a long time. By using Fast Protein Liquid Chromatography (FPLC) systems, large volumes of high-purity target proteins can be obtained.

As part of the authors' work on the functional analysis of proteins that play a role in plant-pathogen interaction (*Arabidopsis thaliana*), this paper describes attempts to purify the protein glutathione S-transferase (GST). GST is added to proteins as a tag for later purification by affinity chromatography. GSTs play an important role in the detoxification of external organic substances. (For more detailed information on the function and regulation of GSTs in plants, see reference 2.) The purity of all substances used is essential to the purification of this protein. Therefore, it is necessary to expose all of the chemicals and solutions to ultrapure water.

Principles of affinity chromatography

To achieve purification, molecular biological methods (cloning and transformation, among others) are used to add an additional tag to the target protein, thereby producing a recombinant protein. The aim of affinity chromatography is to purify the largest volume of the recombinant protein possible. For this reason, the respective gene construct is often incorporated into bacterial systems (cloned) and overexpressed beforehand. The bacterium *Escherichia coli* usually serves as a host, but *Bacillus* strains or non-bacterial systems such as yeast, insect, mammalian or plant cells are also used³.

Purification results from the specific interaction between two reaction partners. One reaction partner is the protein tag, the other is a ligand or antibody covalently bound to a matrix. The recombinant protein (or the tag) binds specifically to these ligands. After washing out non-specific proteins, the recombinant target protein can be specifically eluted by a competitor.

The choice of the column matrix in this case is dependent on the selected protein tag. This should meet various requirements, such as high specificity to the matrix, simple detectability, elution with low molecular substances and the possibility of N- and C-terminal fusion proteins. In addition, the tags should preferably not affect the tertiary

structure or the biological activity of the protein⁴.

In the example shown here, the GST is purified from a cell extract using a Glutathione-containing matrix. GST has a size of about 26 kDa and is thus many times larger than, for example, a polyhistidine tag (1 kDa). The advantages of larger protein tags are potentially better solubility of the recombinant protein and more specific purification. Much shorter polypeptide tags are purified via matrices which, to a limited extent, can bind to non-specific proteins. The purified fraction is thus less pure as compared to the GST tag. Elution of the bound recombinant proteins is usually achieved by competitive binding of the free ligand or by changing the pH or the salt concentration. The present example demonstrates purification of the GST via protein liquid chromatography. Due to the complex structure of the system, accurate and clean working conditions and the purest materials (including water) are essential. Unless these conditions are met, fluctuating pH values and changing salt concentrations could affect binding and elution behavior and thus falsify the results. Bound GST is eluted from the column with reduced glutathione and collected in different fractions. These fractions are subsequently analyzed for their purity.

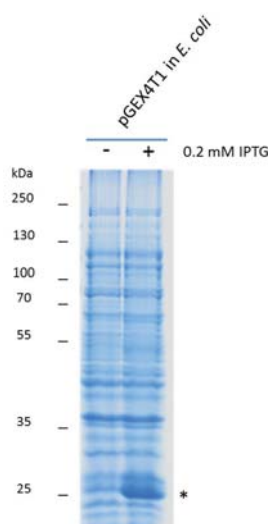


Figure 1: SDS-Page of a 10% acryl amide gel to which the total SDS cell extract of uninduced (-) and induced (+) *E. coli* cells was applied. Colloidal Coomassie Blue stained gel. GST marked with an asterisk.

Materials and methods

For purification, a liquid culture with *E. coli* TOP10 cells transformed with the expression vector pGEX4T1 was inoculated and allowed to grow to an OD of ₆₀₀ ~ 0.6. The inducer (IPTG) was added at a final concentration of 0.2 mM and the culture then further shaken for 4 hours at 28 C. One mL of culture was taken as a control both before and after the induction.

The liquid culture was then centrifuged and the pellet resuspended and lysed in cold PBS buffer containing PMSF and lysozyme. The cell lysate was then centrifuged at 24,000 x g. Control samples were taken after each step. The supernatant was transferred and degassed in an ultrasonic bath. Purification was carried out and evaluated in an affinity chromatography system. Since the system measures the UV absorbance of the sample during purification, the target protein can be identified by an increase in UV absorbance

with reduced glutathione during elution. The purified protein was collected in 200 μL fractions. For further analysis by SDS-PAGE, 20 μL of individual or two pooled peak fractions were mixed with 4 x SDS loading buffer and loaded onto a 10% polyacrylamide gel. The gel was then stained with colloidal Coomassie⁵. All buffers, solutions and media were prepared for the experiments with ultrapure water from an arium pro system (Sartorius).

Results

To investigate the expression of the target protein, uninduced and induced *E. coli* cells were lysed directly in SDS and separated by SDS-PAGE in a 10% polyacrylamide gel (Figure 1). GST has a molecular weight of about 26 kDa. The induced cells show a markedly larger volume of protein in the same size, as shown in Figure 1*. The induced cells were then lysed and the cell lysate separated by centrifugation into insoluble and soluble fractions (Figure 4). Purification of a protein by affinity chromatography requires that the protein to be purified be soluble in the aqueous phase. The solubility of GST was confirmed by SDS-PAGE followed by Coomassie staining (Figure 4).

The soluble protein fraction was used for the affinity chromatography. Using the software, the purification can be tracked in real time (Figure 3). The loading of the column with the protein sample causes an increase in UV absorption, illustrated by the blue line in Figure 3. The UV value consequently decreases when loading is completed. GST can be seen in the first third of the elution fractions as a clear peak in the UV absorption (Figure 3*).

After purification, the respective fractions were analyzed based on their protein content and purity by SDS-PAGE. The respective controls (total cell extract, cell extract after lysis, supernatant and unbound proteins) and fractions were loaded within (A7-B7) and outside (A4 and C4) the UV peaks. It can be seen in Figure 4 that the peak fractions contain the target protein in high quantity and purity (Figure 4). Compared with the controls, a distinct protein band can be seen at 26 kDa, which has the highest protein content in the peak fractions A11-B12. Compared to the supernatant used and the unbound protein, it can be seen that the expressed and soluble GST could be almost completely purified at the same time.

Conclusion

Purification and isolation and subsequent biochemical analysis of specific proteins is an important method to better understand the function of proteins. The results prove that the desired protein could be isolated as a clearly distinguishable band. Such a distinct separation can only be achieved with absolutely pure solutions. Ultrapure water, easily obtained for conducting the experiments, served as the basis for the solutions used. Such ultrapure water is characterized by its consistently high quality in terms of all the required values, such as conductivity, TOC and salinity. Identification by gel electrophoresis showed none of the interfering streaks, poor focusing or non-specific protein bands that can occur in the presence of salts or organic particles in the charged solutions⁶. To obtain reliably reproducible gel analyses, the purity of buffer and staining solutions must be ensured.

Since the experimental procedure described here involves the labor-intensive, costly and time-consuming work of growing bacteria and producing solutions, as well as carrying out chromatography runs and the subsequent gel electrophoresis, impure water/chemicals are excluded from the outset. For these reasons, a direct comparison of different ultrapure water qualities could not be performed.

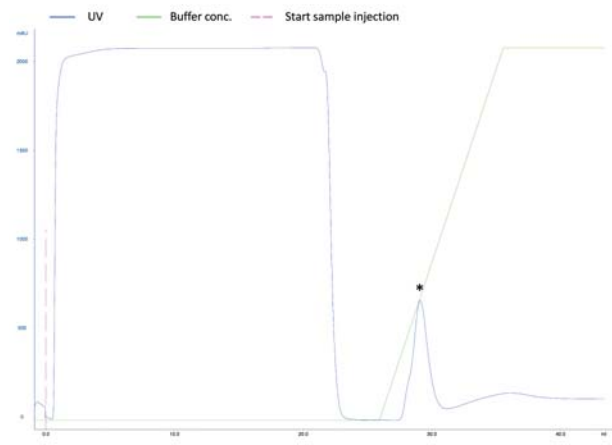


Figure 3: Flow diagram of affinity chromatography. The chromatogram allows direct analysis of the individual parameters. UV absorption (blue), concentration of the elution buffer (green) and the start of the loading of the sample onto the column are marked. Elution marked with an asterisk.

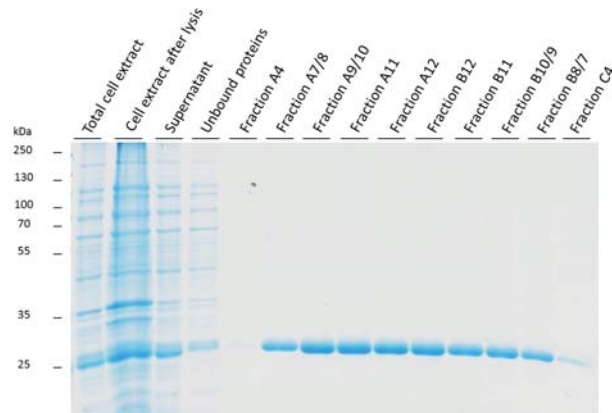



Figure 4: Colloidal Coomassie stained 10% SDS-PAGE gel showing the purified GST in non-peak and peak fractions. Controls: Total cell extract, non-soluble portion after cell lysis (cell extract after lysis), soluble portion used for chromatography (supernatant) and unbound proteins.

The freshly prepared ultrapure water should also lead to an increased number and better distribution of protein spots in a 2-D gel compared to bottled water⁶. 

References

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