Purification of epitope-tagged protein by chromatography methods and LC-MS/MS analysis.

Abstract

The biochemical and structural characterization of recombinant proteins depends on their purification. In this experiment, we present the purification of an epitope-tagged protein utilizing ion exchange chromatography in conjunction with nickel-nitrilotriacetic acid (Ni-NTA) chromatography. The protein was produced in Escherichia coli and tagged with a histidine tag for affinity purification. The His6-tagged protein was immobilized onto a Ni-NTA resin for purification, then washed with a solution containing imidazole to elute non-specifically bound proteins. The target protein was then extracted using imidazole at a greater concentration. The eluted protein was purified using ion exchange chromatography by separating it from other impurities according to its charge. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) were used to confirm the purity and identification of the isolated protein. The SDS-PAGE examination showed a single band with the target protein's anticipated molecular weight, indicating excellent purity. The LC-MS/MS analysis verified the protein's identification and found no additional contaminating proteins. In conclusion, the epitope-tagged protein was purified to high purity using the Ni-NTA and ion exchange chromatography procedures. SDS-PAGE was used to establish the purity of the Brefeldin A-inhibited guanine nucleotide-exchange protein 2. LC-MS/MS was used to confirm its identification and molecular weight. This study illustrates an effective and dependable technique for recombinant protein purification utilizing affinity and ion exchange chromatography.

Introduction

Protein purification is essential in many biochemical and biotechnological applications, such as drug discovery, enzyme synthesis, and protein structure analysis. Column chromatography, gel filtration chromatography, ion exchange chromatography, and mass spectrometry are some methods that can be used to purify proteins. Column chromatography separates proteins based on their interaction with a stationary phase, which can be a gel or solid support. The size exclusion concept underlies gel filtration chromatography, which separates proteins depending on their molecular weight. Proteins are separated using ion exchange chromatography according to their charge characteristics.

A potent method for protein purification known as affinity chromatography relies on a protein's unique interactions with a ligand fixed on a solid substrate. In this method, ligands that selectively attach to the epitope tag are functionalised into agarose, Sepharose, and magnetic beads.

Affinity chromatography can purify epitope-tagged proteins, which have been genetically altered to include a particular peptide sequence. The purity and identity of the eluted protein can then be determined using techniques like SDS-PAGE and mass spectrometry. Adding more purification procedures, like column chromatography, can improve the protein's purity.

One such strategy is using a recombinant protein construct with a tag attached to the target protein. By selectively binding to a particular affinity resin with this tag, the target protein can be separated from other biological components. A popular tag for observing protein distribution and dynamics in living cells is a green fluorescent protein (GFP). The fluorescent protein GFP was initially discovered in the jellyfish Aequorea Victoria. As a result of its unique ability to self-illuminate, it is a fantastic tool for researching protein localisation, gene expression, and protein-protein interactions.

Tagging valuable proteins is one of the most well-liked uses of GFP. A protein of interest can be fused with GFP to allow researchers to monitor its mobility and localisation inside cells. GFP can also be employed as a purification marker for proteins. Researchers can employ GFP affinity purification to separate and isolate the protein of interest from a complicated mixture by introducing GFP into the protein of interest. In this instance, a 6xHis-tagged recombinant construct of the GFP-tagged protein is cloned and expressed in E. coli. Using Ni-affinity chromatography, which is based on the precise binding of the 6xHis tag to the Ni-NTA affinity resin, the protein can be easily purified thanks to the 6xHis tag. A complicated combination, such as a lysate of E. coli cells producing the 6xHis-GFP protein, can be selectively purified using Ni-NTA resin because it binds specifically to the His-tagged protein. The nickel-charged beads that make up the Ni-NTA resin bind to the His-tagged proteins with preference. His-tagged proteins can be quickly and effectively isolated from complicated mixtures with this method, which is widely employed in the protein purification process.

Methods

The following protocol has been adapted from BSP83 practical handbook and http://www1.qiagen.com/literature/handbooks/PDF/Protein/Expression/QXP_QIA

We prepared cleared E. coli lysates under native conditions. Centrifugation of the bacterial lysate containing our protein was done at 10,000 x g for 10 min at RT to pellet the cellular debris. 5µl of clear supernatant from the bacterial lysate was removed and put in fresh Eppendorf tubes for gel electrophoresis, and the pellet was discarded. A certain proportion of the cellular protein, including the 6xHis-tagged protein, may remain insoluble and be in the pellet. This material was solubilized using denaturing conditions to recover the tagged protein completely. A small portion was set aside for electrophoresis analysis, and 5µl 2x SDS-PAGE sample buffer to 5 l of supernatant was added and stored at -20 °C for SDS-PAGE examination.

Purification of GFP-tagged protein by Ni-NTA tagged proteins under native conditions.

The column was installed and positioned vertically; it has a top-end and bottom-end cap. Once the column was secured and aligned to be vertical, we added 300ml of the Ni-NTA slurry, a slightly bluish colour from the Nickel ions, to the column tube and let it settle for a while. Then we added 3ml of the equilibration buffer on the top of the column. We added 5ml of wash buffer very carefully, dropwise along the inner wall of the column, 4ml to wash it clearly and 1ml later to get the accurate protein from the column. Then we uncapped the bottom and added the buffer until all the wash volume had been added, and we collected the flowthrough in four tubes. After that, when only 1-2 mm buffer remained above the gel, we capped the bottom to stop the flow. After the column was equilibrated, we loaded the sample, took 1 ml from the lysate, and stored it at –20°C for SDS-PAGE analysis.

Gel-filtration chromatography using a Sephadex G25 column.

To desalt 0.6ml of material, we poured 3ml of Sephadex G25 slurry into an empty column. The column was then mounted on the supplied platform, and a 15 ml plastic tube was positioned beneath the column outlet to collect all solvent flow during sample loading and washing. The column was then prepared by being washed with 5 ml of equilibrating buffer, which equilibrated the column. The entire green-coloured solution was then added along the column's wall. After adding the sample, the column immediately added 1 ml of equilibrating buffer and changed the receiving tube to a clean one. The desalted protein used for loading in the ion exchange chromatography was collected from the full flow-through.

The slurry was prepared with 2 ml of equilibrating buffer before being placed into an empty column. Used 2 ml of equilibrating buffer to rinse. The salt solution was made by serially diluting 1m NaCl in equilibration buffer to provide 62 mM, 125 mM, 250 mM, and 500 mM NaCl concentrations. Applied salt solutions in descending order, starting with 0.25 ml of 50 mM salt solution, then 125 mM, 250 mM, and finally 500 mM, and collected low through in a separate, clean tube. The greenest sample was mixed with 5 l of 2x SDS-PAGE sample buffer and kept at -20 °C for SDS-PAGE analysis. The greenest sample was kept at -20°C.

Immunoprecipitation of protein complexes, analysis of protein mixtures by gel electrophoresis.

The supplied lysate received 10µl of anti-Scribble antibody, incubated at room temperature for 10 minutes. Added 20µl of Protein A/G magnetic beads and used a yellow tip to aspirate the resuspended slurry. After that and 15 minutes of incubation was done at room temperature. After one minute on the magnetic rack, the supernatant was gently collected with a 1ml pipette. 1ml of washing buffer was added, and the tube was rotated several times to mix it. We carried out the washing process twice, washed with PBS without detergent, added 20µl of trypsin, and then incubated at 37°C. Separated on a magnetic rack, add 5µl of 20% formic acid was added and underwent LC-MS/MS analysis.

Results

In the experiment, we placed the column vertically as it is crucial for the resolution and effectiveness of the column; we used a single filter made of polyethylene that was placed perpendicularly to the column and inserted through the opening at the top of the column by aligning it horizontally and carefully pushing it into the bottom with a 1µl pipette.

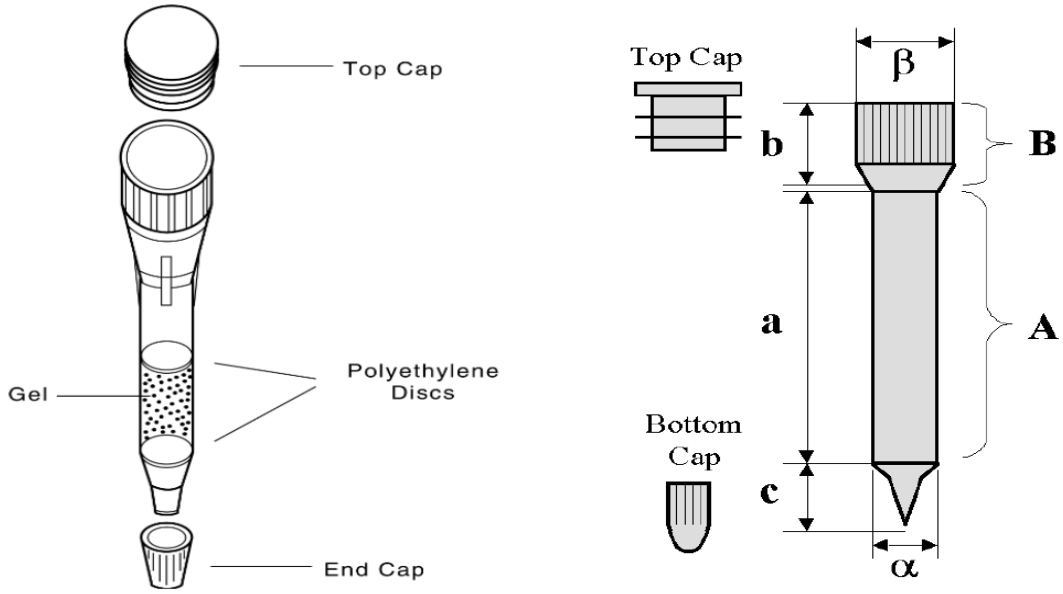

Figure 1. Schematic representation of the column adapted from B6983 practical handbook.

Our E. coli cells that produced the fusion protein yielded a protein lysate. Once the bacterial cell wall and cell membrane had been broken, the sample was obtained by enzymatic lysis using lysosomes.

All the proteins were then dissolved in the solution and extracted using a buffer containing a protease inhibitor to stop the lysate sample from degrading and prevent phosphorylation.

The unique interaction between the nickel ions on the column and the histidine residues on the protein's tag causes His-tagged proteins to attach to the column when introduced to a Ni-NTA (nickel-nitrilotriacetic acid) column. Chelation is an interaction that permits the selective separation of His-tagged proteins from a complex protein mixture. The pH, the amount of salt present, and whether imidazole is present in the buffer are just a few variables that affect how well the His-tagged protein binds to the Ni-NTA column. Imidazole competes with the histidine residues for binding to the nickel ions and is typically present at higher concentrations in the buffer that bind the protein to the column.

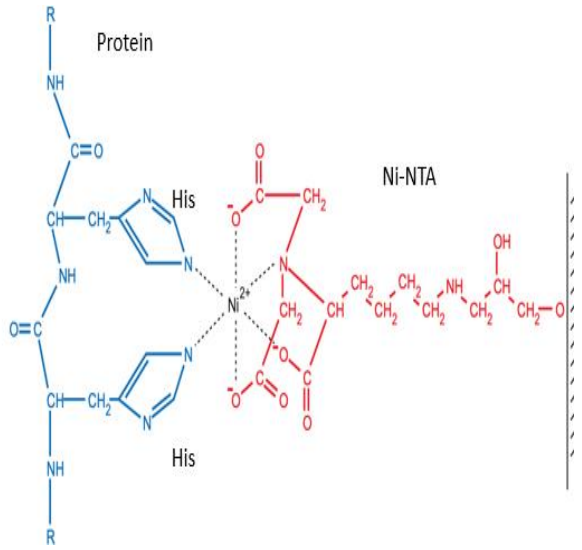

Figure 2. Representation of binding of His-tagged proteins to the Ni-NTA column, adapted from the BS983 practical handbook.

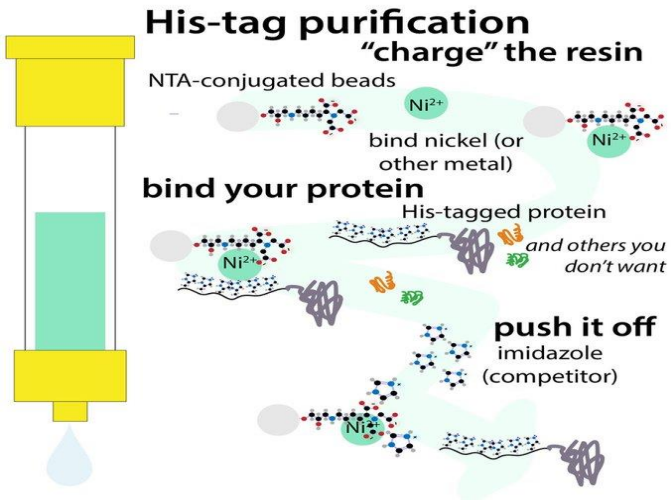

Figure 3. Showing IMAC (Immobilized Metal Affinity Chromatography), represented as a His-Tag and Ni-NTA column, is a typical form of affinity chromatography.

https://thebumblingbiochemist.com/365-days-of-science/histidine-immobilized-metal-affinity-chromatography/

The gel turned bright green as the GFP-tagged protein was correctly bound to the column. We collected the flowthrough in four fractions in four clean Eppendorf tubes and pooled together the green fractions. Eppendorf 1, 2, was our Ni-NTA elution sample.

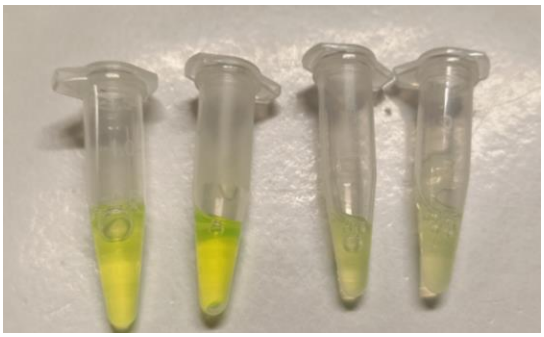

Figure 4. Ni-NTA sample.

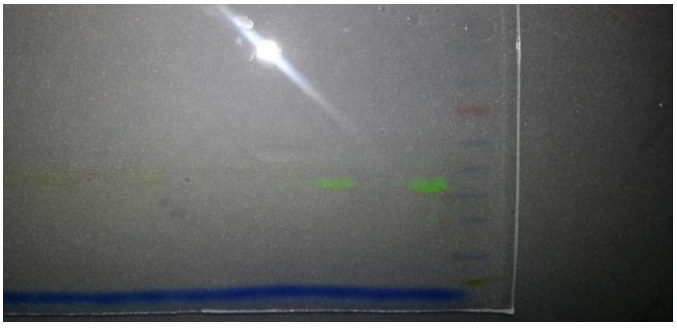

Figure 5. Image of Unstained gel with GFP-tagged protein

The result of the analysis of SDS-PAGE gels shows the following:

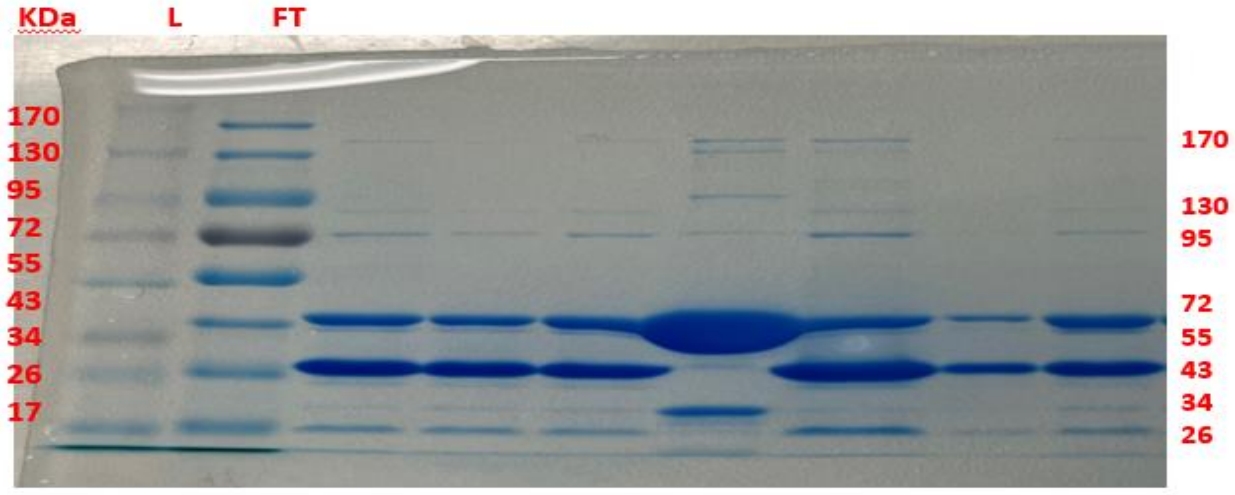

Figure 6. SDS-PAGE gel.

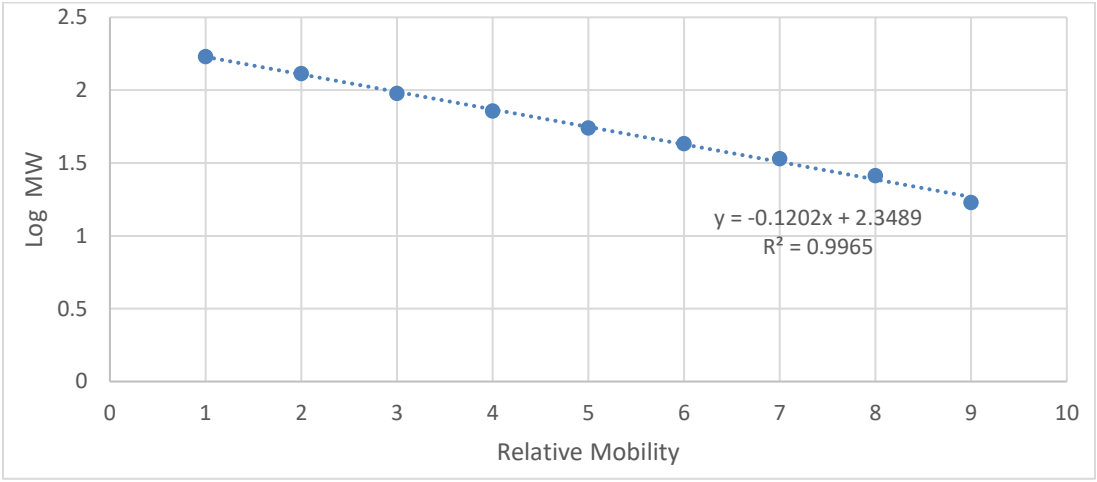

Figure 7. Determining the MW of an unknown protein by SDS-PAGE

We used identified peptide sequences to determine which part of protein X which was cloned in the expression vector where both Krt1 and Trypsin are contaminated.

Identification of protein was done by UniProtKB reviewed (Swiss-Prot), and the protein was identified as Brefeldin A-inhibited guanine nucleotide-exchange protein 2; the gene was ARFGEF2 with a total number of 1785 amino acids.

We have also identified the molecular weight with the help of Expasy, ProtParam tool and the number of amino acids was 170 and molecular weight was 18696.60 Da but we converted into 18.696 kDa by dividing with 1000.

>sp|Q9Y6D5|BIG2_HUMAN Brefeldin A-inhibited guanine nucleotide-exchange protein 2 OS=Homo sapiens OX=9606 GN=ARFGEF2 PE=1 SV=3

LEKILADKEVKRPQHSQLRRACQVALDEIKAEIEKQRLGTAAPPKANFIEADKYFLPFELACQS
KSPRVVSTSLDCLQKLIAYGHITGNAPDSGAPGKRLIDRIVETICSCFQGPQTDEGVQLQIIKALL
TAVTSPHIEIHEGTILQTVRTCYNIYLASKNLINQTTAKATLTQMLNVIFTRMENQVLQEARELE
KPIQSKPQSPVIQAAAVSPKFVRLKHSQAQSKPTTPEKTDLTNGEHARSDSGKVSTENGDAPRE
RGSSLGTDDGAQEVVKDILEDVVTSAIKEAAEKHGLTEPERVLGELECQECAIPPGVDENSQT
NGIADDRQSLSSADNLESDAQGH

Discussion

The 6xHis-tagged protein must be soluble if native conditions are desired or required for purification. Though most of the protein is contained in inclusion bodies, it typically contains some soluble substance purifiable in its original state. Copurification of associated proteins, such as enzyme subunits and binding proteins present in the expressing cells, added to the lysate before purification, or added to the Ni-NTA matrix after the 6xHis-tagged protein is bound, can be accomplished through the purification of tagged proteins under native conditions. Under native conditions, as opposed to denaturing conditions, the opportunity for unrelated, nontagged proteins to interact with the Ni-NTA resin is typically higher. The more proteins present in the first wash are evidence of this. The lysis and wash buffers might be made with a low imidazole concentration (10–20 mM) to lessen nonspecific binding. Rarely, the natural protein's tertiary structure conceals the 6xHis tag, necessitating denaturation before soluble proteins may be isolated on Ni-NTA. Always perform a parallel purification under denaturing circumstances as a control. Moving the tag to the protein's other terminal typically resolves the issue of inaccessible tags, especially if purification is only possible under denaturing conditions, and this is undesirable.

Finally, the field of proteomics uses mass spectrometry, a potent analytical tool, to locate and measure proteins in intricate biological materials. Liquid chromatography-mass spectrometry (LC/MS) is a frequently used technique that combines the sensitivity and specificity of mass spectrometry with the separation capacity of liquid chromatography. To identify the purified protein, confirm its purity, and find any potential impurities or alterations that may have happened during the purification process, LC/MS analysis can be used after protein purification. The protein sample is ionised as part of the LC/MS analysis, the ions are separated based on their mass-to-charge ratios, and the resulting mass spectrum is detected. The molecular weight, charge state, and fragmentation pattern of the protein are all revealed by the mass spectrum, which can be utilised to identify the protein and ascertain its amino acid composition.

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