Affinity purification of GST fusion proteins

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I. Introduction

Affinity chromatography is one of the most selective types of chromatography, and it can be a very useful technique for protein purification. It employs a specific interaction that takes place between one kind of molecule in the solute and a second molecule that is immobilized to the stationary phase. The high affinity binding that occurs between protein molecules and their specific ligands can be exploited by this technique. Examples are histidine binding to metal ions, and glutathione-*S*-transferase binding to glutathione, as will be further discussed in this protocol.

A convenient method of protein expression and subsequent purification is to fuse a protein with a glutathione-*S*-transferase (GST) domain. The DNA encoding for this 25 kDa protein domain is ligated in-frame with the gene for the desired protein so that, upon expression, your desired protein is fused to the GST domain. This is an incredible help in protein purification, since GST binds glutathione extremely strongly. The general purification strategy is thus to bind the GST fusion protein on a column of immobilized glutathione, wash away all the other stuff, and then elute the protein.

The protein can then be used directly in experiments, with the GST domain still attached, although in many cases one must then control the experiments with GST to rule out interactions between GST and other molecules. Alternatively, the GST fusion protein is often constructed with a protease cleavage site between the GST domain and the protein, so that digestion with a protease such as thrombin or blood coagulation Factor X_a and subsequent separation will remove the GST domain altogether.

II. Purification of a GST Fusion Protein

Starting with the supernatant of the cell lysis, there are two steps to GST fusion protein purification. First, the GST fusion protein is separated from all other proteins by running the supernatant over a glutathione column; the GST fusion protein binds to the glutathione column and all other proteins are washed away. The GST protein is then eluted from the column with glutathione. Second, the eluted GST protein is run over a Nap10 column to remove the glutathione, resulting in a very pure sample containing only the GST fusion protein.

A. Solutions to prepare

Buffer A (for glutathione column)

This buffer is specific to your protein and is usually specified in the literature describing the fusion protein's purification. Examples include:

For GST-KIX (100 mL)	For GST-MDM2 (100 mL)

1x PBS 1 mM DTT 0.01% Tween 1 protease inhibitor tablet fill with H_2O to 100 mL	10 mL 5 M NaCl 270 µL 1 M KCl 142 mg Na_2HPO_4 24.5 mp KH_2PO_4 200 µL EDTA 70 µL b-mercaptoethanol (bme) fill with H_2O to 100 mL adjust to pH 8.0
Glutathione Elution Buffer (15 mL) 10 mM glutathione (0.046 g glutathione) 50 mM Tris-HCl, pH 8.0 (0.75mL 1 M Tris-HCl) dH_20 (14.25 mL dH_20)	Column Regeneration Buffer 1 (pH = 8.5) (50 mL) 0.1 M Tris (5 mL 1 M Tris) 0.5 M NaCl (5 mL 5 M NaCl) dH_20 (~40 mL dH_20) pH sample to 8.5 with NaOH
Column Regeneration Buffer 2 (pH = 4.5) (50 mL) 0.1M sodium acetate (0.41g sodium acetate) 0.5M NaCl (5mL 5M NaCl) Fill with dH_20 (~45mL dH_20) pH sample to 4.5 with HCl	Storage Buffer (for Nap-10 column) This buffer is also specific to your protein and should be what you want to store your protein in for the long run.

B. Glutathione column purification

Before starting, remove and save an aliquot of lysis supernatant for later analysis on Phast Gel. The column used in the following protocol is the Bulk GST Purification Module from Amersham Pharmacia.

Column Preparation - Day 1

1. Add 1.33 mL 75% glutathione sepharose slurry to column (both 75% slurry and column are provided in kit).
2. Drain the column of its storage buffer.
3. Wash column 3 to 5 times with 3 mL Buffer A.
4. Add 1 mL of Buffer A to the sepharose. Mix so that beads are suspended and then add the slurry to the lysis supernatant in 50 mL orange-cap tube.
5. Wash column with an addition 1mL Buffer to remove any remaining sepharose and add this to the tube.
6. Incubate sample overnight at 4ºC with shaking or rotation.

Pack Column/First Elution - Day 2

All centrifugation and incubation is to be done at **4ºC**!!

1. Pour some of the sample back into the column.
2. Place the column in an orange cap tube and centrifuge at 20,000rpm for one minute at 4ºC.
3. Pour flow-through into another tube and save.
4. Add more sample to the column and centrifuge again. Repeat until all of the sample has passed through the column. As the column becomes more packed it will be necessary to centrifuge for longer amounts of time.
5. Wash column 3x with 5 mL Buffer A and save the flow-through from each wash separately.

6. Add 2 mL of Elution Buffer and incubate for one hour.
7. Collect the eluent (this is eluent 1) by centrifugation.
8. Add 2mL of Elution Buffer to column and incubate overnight.

Second Elution / Column Regeneration- Day 3

Collect eluent 2 by centrifugation. If you still have a lot of sample coming off in eluent 2, you might want to do additional elutions. If this is the first time you're doing the purification, you should probably collect additional elutions just in case.

Regenerate column:

1. Wash column with 2 to 3 volumes of alternating high pH and low pH buffers.
2. Repeat cycle 3x.
3. Wash column 2x with 1X PBS.
4. Store column in 1X PBS at 4ºC (column can be stored this way for ~1 month).

C. Evaluation of purification

At this point you will want to run a Phast Gel on the following fractions:
starting material (lysis supernatant)

1. flow through
2. wash1
3. wash 2
4. wash 3
5. eluent 1
6. eluent 2
7. other eluents

D. Removal of glutathione on a Nap-10 column

1. Equilibrate column with 3 volumes of storage buffer.
2. Add 1 mL of eluent from glutathione column, collect flow-through and save it for step 4. This is the void volume and should not have any protein in it.
3. Add 1.5 mL of storage buffer to column and collect flow-through.
4. Do "dot blot" test (blot filter paper with void and protein fractions, then stain with Coomassie blue) to ensure that your protein is in the 1.5 mL fraction.
5. Use a new Nap-10 column for each 1mL of eluent. When finished, combine fractions with protein and run a Phast Gel to check for purity.

III. Thrombin Cleavage

If the protein is desired without GST attached to it, and there is a cleavage site built in to the fusion between GST and your protein, you can use a protease to remove the GST. The following protocol describes cleavage with thrombin using the Novagen Thrombin Kit.

Thrombin is an endoprotease that cleaves at the sequence Leu-Val-Pro-Arg-❋-Gly-Ser. There are two ways to accomplish cleavage. The first (and most common) method involves carrying out cleavage while the GST fusion protein is still bound to the glutathione column. This method is excellent if you are only interested in recovering your protein, because after cleavage the GST is still bound to the glutathione and the protein elutes by itself.

If you need to recover pure GST as well, purify the sample as described above, then carry out the thrombin reaction to completion in a tube. Run the completed reaction back through a glutathione column as described above using 1x Thrombin Buffer as Buffer A. Flow-through will contain your protein plus thrombin, and then you can remove thrombin as described below. Finally, you can elute GST from column as described above. Time and amount of thrombin required for cleavage reaction is dependent on the protein. You may want to optimize the reaction conditions on a small scale first, starting with a general estimate of 1 unit of thrombin per mg of target protein.

A. Solutions to prepare

3x Thrombin Cleavage Buffer	*1X Thrombin Cleavage Buffer with 1mM DTT*
60mM Tris pH 8.5 300mM NaCl 1mM $CaCl_2$	*(to make ~50 mL)* 16mL 3x Thrombin Cleavage Buffer 32mL dH_20 Filter, then add: 7.7mg DTT 50 µL Tween (if necessary for your protein)

B. Cleavage on glutathione column

1. Add 1.33 mL of 75% glutathione slurry to column and allow to settle.
2. Drain column.
3. Wash 3x with 5 mL 1x Thrombin Cleavage Buffer.
4. Add sample in 1x Thrombin Cleavage Buffer 2-3 mL at a time. Load and incubate column in batches if sample volume is bigger than 3 mL.
5. Incubate and rotate/shake for 1 hour at 4ºC.
6. Centrifuge to pack column and save flow-through.
7. Wash column 3x with 1x Thrombin Cleavage Buffer and save washes.
8. Add biotinylated thrombin in 2 mL 1x Thrombin Cleavage Buffer (~1 unit/mg protein). Incubate and rotate/shake for 2 hours at room temperature or 4ºC, depending on robustness of target protein.
9. Remove a 20 µL aliquot from the slurry.
10. Spin down aliquot and use supernatant to run Phast Gel to determine extent of cleavage. At this time you can also run flow-through and washes on the gel.
11. If necessary, incubate overnight and/or add more thrombin
12. When complete cleavage is verified, collect protein by centrifugation. Protein will be in flow-through.
13. Elute GST as per GST purification protocol (if desired).

Collected protein will also contain thrombin. To remove thrombin, add 32 µL 50% streptavidin agarose (provided in the kit) per unit of thrombin used in the reaction. Incubate for 30 minutes at room temperature on a shaker; streptavidin will bind the biotinylated thrombin. Transfer the reaction to a spin filter and centrifuge at 2300 rpm in a microcentrifuge for 5 minutes. Filtrate should contain only your protein. Check purity by Phast Gel.

C. Small scale optimization (for cleavage off column)

1. Make 1:25, 1:50, 1:100 and 1:200 serial dilutions of thrombin in thrombin dilution buffer (provided in the kit).
2. To each of five tubes, add:
   5 µL 10x Thrombin Cleavage Buffer (provided in kit)
   10 µg target protein
   1 µL diluted thrombin (each tube receives a different dilution, to the fifth tube add 1µL of Dilution Buffer)
   dH_2O to 50 µL final volume
3. Incubate the reactions at room temperature.
4. Remove 10 µL aliquots from the reactions after 2, 4, 8 and 16 hours and put into 10 µL 2x SDS buffer.
5. Determine extent of cleavage by SDS-PAGE (or test aliquots by Phast Gel).

It may also be necessary to test cleavage at various temperatures between 4ºC and 37ºC. Once the appropriate conditions are found, scale up the reaction. For more information on factors affecting cleavage, refer to the instructions provided with the kit.

Additional information (products, handbooks and instructions as pdf files, etc.) can be found on the Amersham website at the following address: http://www1.amershambiosciences.com/aptrix/upp01077.nsf/Content/Products?OpenDocument&parentid=366157&moduleid=38861.