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Large-Scale Expression and Purification of GST-Caveolin Fusion Proteins

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The Edwin P. McCabe Honors Program

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"Large-Scale Expression and Purification of GST-Caveolin Fusion Proteins"

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LARGE-SCALE EXPRESSION AND PURIFICATION OF GST-CAVEOLIN FUSION PROTEINS

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I would like to thank God. Without Him, none of this work would have been completed. I would also like to thank Dr. Michael Elliott at the University of Oklahoma Health Sciences Center at the Dean A. McGee Eye Institute. I would like to thank Dr. John Coleman of the Department of Chemistry at Langston University. I also thank Dr. and Ms. Williams of the Department of Biology at Langston University and the Undergraduate Biomedical Education Program.

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Chapter 1: Introduction

In this thesis, I will discuss the study I conducted from June 2, 2003 to July 31, 2003 at the University of Oklahoma Health Sciences Center Dean A. McGee Eye Institute, facilitated by Dr. Michael Elliott, funded by the Undergraduate Biomedical Education Program at Langston University, expressing and purifying GST-Caveolin fusion proteins on a large-scale.

Caveolins are the principle constituents of caveolae. Caveolae are flask-shaped invaginations of the plasma membrane. Caveolae are most abundant in endothelial cells, fibroblasts, and myocytes (Smart et al. 1999). These structures participate in three main areas of cell physiology: endocytosis (Henley et al. 1999), cholesterol traffic (Razani et al. 2000) and signal transduction (Okamoto et al. 1998). Currently, three different forms of caveolins are known: caveolin-1 (or VIP21), caveolin-2, and caveolin-3 (or M-caveolin). Caveolin family members share three characteristic properties: detergent insolubility at low temperatures, self-oligomerization and incorporation into low-density Triton-insoluble fractions enriched in caveolae membranes (Song et al. 1997). Other functions for caveolins include lipid transport, membrane trafficking, and tumor suppression and tumor suppression (reviewed in Okamoto et al. 1998; Liu et al. 2002).

Vision involves the conversion of light into electrochemical signals that are processed by the retina and then sent to and interpreted by the brain (see Figure 1.1). The process of converting light into electrochemical signals begins when the protein, rhodopsin, absorbs light within the retina. Photoexcitation of rhodopsin causes the cytoplasmic surface of the protein to become active. In the active state, rhodopsin then binds and activates transducin, a G-protein composed of three subunits α, β, and γ. The activated transducin protein separates into GTP-α while GTP-β and GDP-γ sections remain coupled. Tα activates phosphodiesterase (PDE),

which, in turn, hydrolyzes cyclic GMP. The decrease of cyclic GMP concentration closes the cyclic GMP-gated channels on the photoreceptor cells, causing a shift in the cell's electric potential and a neural impulse to the brain.

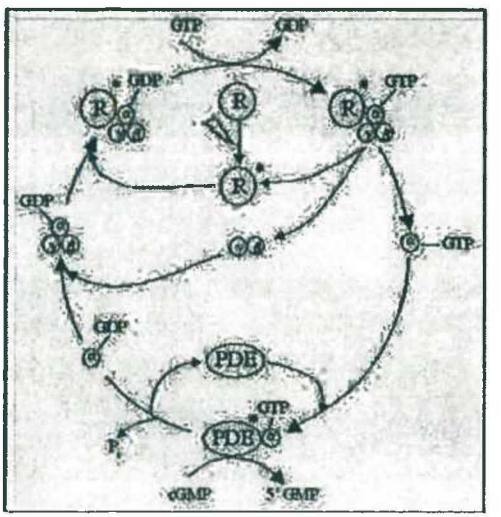

Figure 1.1. This figure depicts the activation of the phosphodiesterase cascade. Light (lightening bolt) absorbed by rhodopsin (R) converts it to an activated state (R*) that then interacts with transducin (GDP α, β, γ). Next, alpha transducin (GTP-α) dissociates both from R* and beta, gamma transducin (β,γ) to bind with an inactive phosphodiesterase (PDE) enzyme, stimulating it (PDE*) to hydrolyze cyclic GMP.

Before the expression and purification of these proteins could begin, the DNA encoding for each had to be inserted into the vector of *E. coli* bacteria DNA with Isopropyl-□D-Thiogalactoside (IPTG)-inducible promoters and an ampicillin resistance gene (see Figure 1.2). In this case, the IPTG-inducible promoter used is $lacI^q$ $LacI^q$ will prevent the E. Coli from expressing the desired protein prematurely. The addition of IPTG will initiate the expression of the desired proteins. The expressed fusion proteins will then be purified on glutathione sepharose beads and eluted with 10 mM glutathione elution buffer.

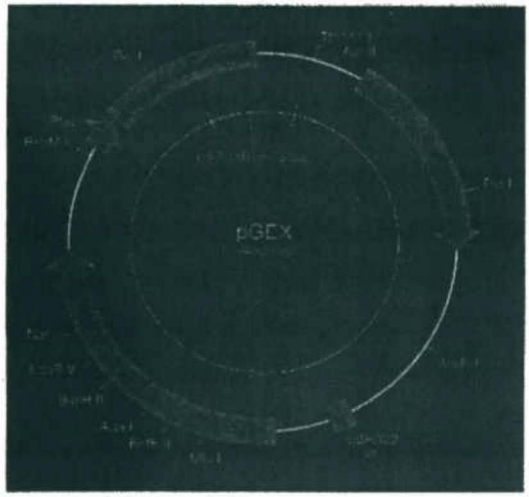

Figure 1.2. This is the pGEX vector of E. Coli bacteria DNA with IPTG-inducible promoters and an ampicillin resistance gene before the insertion of the DNA encoding each protein.

We have previously shown that Cav-1 and transducin alpha (Tα), the photoreceptor Gα subunit, interact in photoreceptors and that activation of Tα inhibits this interaction suggesting that the association may have functional consequences in photoreceptors (Elliott et al. 2003*a*). We have also shown that the Cav-1 scaffolding domain is sufficient for this interaction (Elliott et al. 2003*b*). We now want to know how this interaction influences Tα function. The potential influence of Cav-1 on visual transduction, a well-documented G-protein-coupled cascade has not been studied. To do so, I will express and purify full-length Caveolin-1 (GST-CAV-FL, amino acid residues 1-178) and Caveolin-1 deletion mutants (residues 1-140, 1-81, 61-101, 102-134, and 135-178) fused to Glutathione-S-Transferase (GST) and GST in large quantities for use in functional assays of Tα (see Figure 1.3).

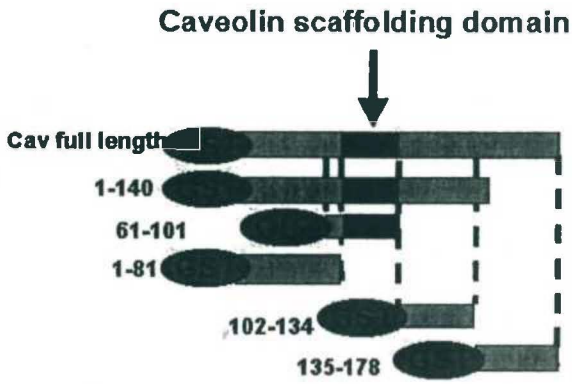

Figure 1.3. This is the diagram of GST-CAV fusion proteins.

If the proteins are required in large quantities, it is necessary to explore several different host-vector systems and purification schemes before finding one that is workable on a large-scale. This poses an objective for the expression of GST-Caveolin fusion proteins because we make an assumption before beginning the procedure that the host-vector system is appropriate for the desired proteins. This also poses an objective for the purification of GST-Caveolin fusion proteins because the procedure for doing so will need to be improved upon as the experiment is completed.

The remainder of this thesis will proceed as follows. Chapter 2 is the literature review, which will discuss what is already known about large-scale expression and purification. Chapter 3 will discuss the experiment that enabled the large-scale expression and purification of GST-Caveolin fusion proteins. Chapter 4 will encompass the results of this study. Chapter 5 will conclude the study and discuss how this investigation could be furthered.

The following are definitions of terms that will appear within the remainder of this thesis:

1. aliquot- used to describe a number or quantity that will divide another number or quantity without leaving a remainder
2. culture- to incubate in liquid media
3. dilute- thinner or weaker than at full concentration because of the addition of water or another liquid
4. supernatant- a usually clear liquid above material deposited by precipitation, centrifugation, or sedimentation

Chapter 2: Literature Review

This chapter is organized according to topics I explored before beginning the experiment as a way to troubleshoot the procedure for the large-scale expression and purification of GST-Caveolin fusion proteins.

Functions of Caveolin-1

Given that caveolin-1 interacts with both lipids and lipid anchors on proteins, it is predicted that caveolin-1 functions in lipid transport, membrane traffic, and signal transduction (Liu et al. 2002).

The first indication caveolae were involved in cholesterol traffic came from the observation that in normal human fibroblasts cholesterol moves directly to surface caveolae within minutes after being synthesized in the endoplasmic reticulum (Smart et al. 1996). The rapid transport of new cholesterol to the lymphocyte cell surface is dependent on the expression of caveolin-1. Rapid migration of caveolin to the endoplasmic reticulum after the oxidation of caveolae cholesterol (Smart et al. 1994) suggests it has a direct role in cholesterol import (Liu et al. 2002). Caveolae, which is an integral protein, is able to carry cholesterol to intracellular compartments like the endoplasmic reticulum via cystolic caveolin-1. Cholesterol esters that enter cells through caveolae appear to associate with cystolic caveolin-1. Caveolin-1 has a function in intracellular and extracellular lipid transport. This accounts for the high level of caveolin-1 expression in adipocytes (Li et al. 2001) as well as the apparent abnormalities in lipid metabolism that are seen in mice lacking caveolin-1 (Razani et al. 2002).

Caveolin-1 could attract proteins to caveolae the same way that clathrin adaptors attract transmembrane receptors to coated pits and/or function as a molecular motor that powers membrane invagination and budding (Liu et al. 2002). A recent study found that without caveolin-1, caveolae internalization is so rapid that flask-shaped caveolae are difficult to find in

thin section images unless internalization is blocked (Liu et al. 2002). In contrast, endocytosis of albumin by endothelial cells derived from mice lacking caveolin-1 is impaired (Razani et al. 2001). This study did not determine whether the impairment occurred due to malfunctioning caveolae or the absence of the albumin receptor GP60 at the cell surface. A function of caveolin-1 is to regulate the interaction of caveolae with cortical actin cytoskeleton. This enables it to control whether caveolae are at the cell surface or traveling to interior sites (Liu et al. 2002).

Immediately following the identification of caveolin-1 as a caveolae marker protein, procedures were developed for isolating caveolae from tissues as well as tissue culture cells (Anderson 1998). An outcome of using these isolation procedures was the discovery that caveolae are made up of multiple molecules that function in cellular signal transduction (Anderson 1998). A synthetic peptide matching the amino acid sequence between amino acids 81 and 101 in caveolin-1 inhibited Src kinases activity (Liu et al. 1996). A GST fusion protein containing amino acids 80 and 101 was named the caveolin-1 scaffolding domain (Liu et al. 1996). The results of over 50 studies are in agreement with the caveolin-1 scaffolding domain hypothesis. A key part of the caveolin-1 scaffolding domain hypothesis is the proposal that it functions to inactivate signaling molecules (Okamoto et al. 1998).

The Path of Caveolin-1

Caveolin-1 is inserted co-translationally into the endoplasmic reticulum membrane with its N- and C-terminal in the cytoplasm. It is incorporated into vesicles that move to the Golgi apparatus requiring amino acids 66-70 (Machleidt et al. 2000). Caveolin-1 oligomerizes and becomes detergent-insoluble requiring amino acids 91-100 and 135-140 (Lisanti et al. 1993). Vesicular transport to the cell surface requires the ability to oligomerize and amino acids 71-80. Caveolin-1 becomes incorporated into caveolae that internalize and recycle. It can enter the cytoplasm of the cell as a soluble protein embedded in a lipid particle. It then can either go to

the endoplasmic reticulum and pick up cholesterol for transport back to caveolae or enter the lumen of the endoplasmic reticulum (Liu et al. 2002). If it enters the lumen of the endoplasmic reticulum, it can either be incorporated into the particles that are secreted by the cell, remain in the cytosol, or go to the mitochondria (Liu et al. 2002).

Structure of Caveolin-1

Molecular cloning has identified three distinct caveolin genes (Glenney et al. 1992; Scherer et al. 1995; Scherer et al. 1996; Tang et al. 1996; Parton 1996; Couet et al. 1997): caveolin-1, caveolin-2, and caveolin-3. Caveolin proteins interact with themselves to form homo- and hetero-oligomers (Sargiacomo et al. 1995; Monier et al. 1995), which directly bind cholesterol (Morata et al. 1995) and require cholesterol for insertion into model lipid membranes (Monier et al.1995; Li et al. 1996). A central hydrophobic domain (amino acids 102-134) is thought to form a hairpin-like structure within the membrane. The N-terminal domain (amino acids 1-101) and the C-terminal domain (amino acids 135-178) face the cytoplasm. Amino acids 61-101 direct the formation of caveolin homo-oligomers (Sargiacomo et al. 1995), while the C-terminal domain acts as a bridge to allow these homo-oligomers to interact with each other forming a scaffold (Song et al. 1997). Recent co-immunoprecipitation and dual labeling experiments directly show that caveolin-1 and 2 form a stable hetero-oligomeric complex and are co-localized (Scherer et al. 1997).

Interactions of Caveolin-1

The scaffolding domain is required to form multivalent homo-oligomers of caveolin, mediates the interaction of caveolin-1 with G-Protein alpha subunits, Ha-Ras, Src family tyrosine kinases, and eNOS, possesses a peptide that can functionally inactivate the enzymatic activity of G-protein alpha subunits, Src family kinases, and eNOS activity, and is involved in other potential protein-protein interactions.

It is known from *in vitro* studies that aromatic residues (Trp, Phe, or Tyr) are required for the proper recognition of the caveolin-binding motif (Couet et al. 1997). These binding motifs are present within most G-protein alpha subunits. Direct interaction of caveolin with signaling molecules leads to their inactivation. Li et al. 1995 investigated the potential interaction of caveolin with heterotrimeric G proteins and found that caveolin could function to negatively regulate the activation state of heterotrimeric G proteins.

According to the literature reviewed, I am now able to begin the large-scale expression and purification of GST-Caveolin fusion proteins. The functions of caveolin-1 are lipid transport, membrane traffic, and signal transduction. The basis of these functions is reflected in the path of this protein. The structure of caveolin-1 includes a scaffolding domain (residues 82-101), which enables caveolin-1 to bind to other caveolin-1 among others proteins. This may make eluting from glutathione sepharose beads difficult. In order to examine the interaction between heterotrimeric G-proteins and caveolin, it was necessary to express and purify caveolin-1 as a GST fusion protein. Similarly, a long-term goal of this study is to know how the interaction between caveolin-1 and transducin alpha affects transducin alpha function. Now it is known that this study is necessary to achieve that goal.

Chapter 3: Method

In this chapter, I will discuss the experiment. This is the approach I decided to take in order to express and purify the GST-Caveolin fusion proteins. This chapter will enable one to repeat this study. The materials should be obtained prior to conducting this experiment and the procedures should be performed in the following order. Repeat the procedure, "Expression of GST-Caveolin Fusion Proteins" seven times in order to express GST-CAV FL (1-178), GST-CAV 1-140, GST-CAV 61-101, GST-CAV 135-178, GST-CAV 102-134, GST-CAV 1-81, and GST. To insure all seven expressions were successful, follow procedure for electrophoresis then GelCode Blue staining. Repeat the procedure, "Purification of GST-Caveolin Fusion Proteins solubilized by TX-100" two times in order to purify GST and GST-CAV 1-140. Repeat the procedure, "Purification of GST-Caveolin Fusion Proteins solubilized by N-lauroyl sarcosine five times in order to purify GST-CAV FL (1-178), GST-CAV 61-101, GST-CAV 135-178, GST-CAV 102-134, and GST-CAV 1-81. To insure all seven purifications were successful, follow procedure for elctrophoresis then GelCode Blue staining. Then, follow the procedure for electrohoresis again followed by the procedure for Immunoblot using antibodies against GST (goat polyclonal), myc-tag (monoclonal) or Cav-1 (monoclonals). Monoclonal Cav-1 antibody 2234 recognizes amino acids 1-21 and antibody 2297 recognizes amino acids 61-71 (Scherer et al. 1995).

Materials

Equipment (a detailed description of equipment appears in Appendix):

1. PowerPac 3000 Power Supply
2. Sorvall RC-5B Refrigerated Superspeed Centrifuge
3. HACH DR/3000 Spectrophotometer

4. Sonicator 715

Glutathione Sepharose 4B Resin:

1. Gently shake the bottle of Glutathione Sepharose 4B to resuspend the matrix.
2. Pipette out 6.65 mL and place into a sterile 50 mL Falcon tube.
3. Centrifuge at 1800 rpm for 5 min. using the Sorval Centrifuge and SH3000 rotor.
4. Discard supernatant.
5. Add 50 mL of cold PBS and mix.
6. Centrifuge at 1800 rpm for 5 min. using the Sorval Centrifuge and SH3000 rotor.
7. Discard supernatant.
8. Repeat steps 5-7 once.
9. Add 5 mL of cold PBS.

Glutathione Elution Buffer (50mL):

1. 10 mM reduced glutathione.
2. 50 mM Tris-HCL (pH 8.0)

1.0 M Tris-HCL (pH 8,0) (500 mL)

STE (1 L)

1. 7.5 mM Tris-HCL (pH 7.4)
2. 150 mM NaCl
3. 10% Glycerol
4. 1 mM EDTA

5. Complete Protease Inhibitor Cocktail

6. 1 mM PMSF

STE+2% TX-100 (500 mL)

50 mM Tris-HCL (pH 8.0) (500 mL)

1X Transfer Buffer:

1. 3.03 g Tris base

2. 14.4 g glycine

3. 800 mL of double distilled water

4. 200 mL of methanol

5. 0.5 mL of 10% SDS

TTBS Wash Buffer:

1. 12 g NaCl

2. 1 g Tween 20

3. 10 mL 1 M Tris-HCL (pH 7.4)

4. 1 L of double distilled water

5% BSA in TTBS:

1. 2.5 g Albunin Bovine

2. 50 mL of TTBS

Phosphate Buffered Saline (pH 7.5) (PBS):

1. Add 68.5 mL of 2 M NaCl to make a final concentration of 137 mM NaCl.
2. Add 2.7 mL of 1 M KCL to make a final concentration of 2.7 mM KCL.
3. Add 8.6 mL of 0.5 M Na_2HPO_4-$7H_2O$ to make a final concentration of 4.3 mM Na_2HPO_4-$7H_2O$.
4. Add 1.4 mL of 1 M KH_2PO_4 to make a final concentration of 1.4 mM KH_2PO_4.
5. Add additional distilled water to make a 1 L solution of PBS.

2 M NaCl (200 mL)

1 M KCL (50 mL)

0.5 M Na_2HPO_4-$7H_2O$ (100 mL)

1 M KH_2PO_4 (50 mL)

10 mg/mL ampicillin (100 mL)

1 M Isopropyl-B-D-Thiogalactoside (IPTG) (5 mL)

LB Media

Procedures

Expression of GST-Caveolin Fusion Proteins:

1. Aliquot 25 mL of LB Media into two sterile 50 mL Falcon tubes.
2. Add 250 µl of ampicillin to each Falcon tube making the final concentration 100 µg/mL of ampicillin.
3. Use flame sterilized platinum loop to transfer cells from glycerol stocks of GST-Caveolin fusion proteins (GST-CAV FL (1-178), GST-CAV 1-140, GST-CAV 61-101, GST-CAV 135-178, GST-CAV 102-134, GST-CAV 1-81, and GST) into the two Falcon tubes of LB/Ampicillin media.
4. Culture overnight at 37° C and 250 rpm.
5. Add 5 mL of 10 mg/mL ampicillin to 500 mL of LB media to make the final concentration 100 µg/mL of ampicillin.
6. Dilute (1:10 of 50 mL of culture in 500 mL of LB/Ampicillin media) with LB/Ampicillin media and continue to grow for approximately two hours at 37° C and 250 rpm.
7. Add 56 µl of 0.9 M IPTG to each diluted culture to make the final concentration 0.1 mM of IPTG and allow to grow an additional 2-2.5 hours at 37° C and 250 rpm.
8. Spin at 5000 rpm for 10 min. using the Sorval centrifuge and SLA 1500 rotor.
9. Discard supernatant.
10. Resuspend pellets in 25 mL of PBS.
11. Remove 40 µl and add to 20 µl of 3X sample buffer.
12. Transfer whole culture to sterile 50 mL Falcon tube.
13. Spin at 4000 rpm and 4° C for 10 min. using Sorval centrifuge and SH3000 rotor.

14. Discard supernatant and freeze pellet at -80° C.

Purification of GST-Caveolin Fusion Proteins solubilized by TX-100:

1. Resuspend pellet up to 10 mL of STE Buffer.
2. Add 10 mL of STE/2% TX-100 (final concentration of TX-100 = 1%)
3. Sonicate two times for 30 seconds each time using probe sonicator (keep cold).
4. Transfer sonicate to thick-walled Sorvall 50 mL tube.
5. Centrifuge at 12000 rpm/4°C/20 minutes.
6. Collect supernatant.
7. Take 100 µl aliquot for SDS gel and label "Orig Sup".
8. Resuspend pellet in ~20 mL of STE/1% TX-100 buffer.
9. Take 100 µl aliquot for SDS gel and label "Pellet".
10. To the remainder of supernatant add 0.5 mL of 50% Glut-Sepharose matrix.
11. Incubate with mixing for 30 minutes at room temperature.
12. Centrifuge at 1800 rpm for 5 min. using Sorvall Centrifuge and SH3000 rotor.
13. Collect supernatant.
14. Take 100 µl aliquot for SDS gel and label "Post Purification Sup".
15. Add 20 mL of STE/1% TX-100 buffer and mix with matrix.
16. Centrifuge at 1800 rpm for 5 min. using Sorvall Centrifuge and SH3000 rotor.
17. Discard supernatant.
18. Repeat steps 15-17 three times.
19. Add 20 mL of 50 mM Tris-HCL (pH 8.0) and mix with matrix.
20. Centrifuge at 1800 rpm for 5 min. using Sorvall Centrifuge and SH 3000 rotor.

21. Repeat steps 19-20 one time.
22. Add 0.5 mL of 50 mM Tris-HCL (pH 8.0) and mix with matrix.
23. Transfer to small tube.
24. Take 30 µl aliquot and label "Pre-Elution Beads".
25. Spin in small centrifuge for 2 min.
26. Collect supernatant and label "Pre-Elution Sup".
27. Take 2 µl aliquot and add to test tube of Biorad dye.
28. Add 0.5 mL of glutathione elution buffer and mix with matrix for 10 min. at room temp.
29. Spin in small centrifuge for 2 min.
30. Collect supernatant (~0.5 mL) and label "Eluate 1, 2, 3, 4, and 5"
31. Take 2 µl aliquot and add to test tube of Biorad dye.
32. Repeat steps 28-31 until no protein is eluted and there is no color change after addition to Biorad dye.

Purification of GST-Caveolin Fusion Proteins solubilized by N-lauroyl sarcosine:

1. Resuspend pellet up to 10 mL of STE/2X protease inhibitor.
2. Add 10 mL of STE/3% N-lauroyl sarcosine (final concentration of N-lauroyl sarcosine = 1.5%)
3. Sonicate two times for 30 seconds each time using probe sonicator (keep cold).
4. Transfer sonicate to thick-walled Sorvall 50 mL tube.
5. Centrifuge at 12000 rpm/4°C/20 minutes.
6. Collect supernatant in 50 mL Falcon tube and label "Orig Sup".
7. Add 5 mL of 20% TX-100/STE buffer.

8. Mix for 10 min. at room temperature.

9. Take 100 µl aliquot for SDS gel and label "Orig Sup".

10. Resuspend pellet in ~25 mL of STE/1% TX-100 buffer.

11. Take 100 µl aliquot for SDS gel and label "Pellet".

12. To the remainder of supernatant add 0.5 mL of 50% Glut-Sepharose matrix.

13. Incubate with mixing for 30 minutes at room temperature.

14. Centrifuge at 1800 rpm for 5 min. using Sorvall Centrifuge and SH3000 rotor.

15. Collect supernatant.

16. Take 100 µl aliquot for SDS gel and label "Post Purification Sup".

17. Add 20 mL of STE/1% TX-100 buffer and mix with matrix.

18. Centrifuge at 1800 rpm for 5 min. using Sorvall Centrifuge and SH3000 rotor.

19. Discard supernatant.

20. Repeat steps 15-17 three times.

21. Add 20 mL of 50 mM Tris-HCL (pH 8.0) and mix with matrix.

23. Centrifuge at 1800 rpm for 5 min. using Sorvall Centrifuge and SH 3000 rotor.

24. Repeat steps 19-20 one time.

25. Add 0.5 mL of 50 mM Tris-HCL (pH 8.0) and mix with matrix.

26. Transfer to small tube.

27. Take 30 µl aliquot and label "Pre-Elution Beads".

28. Spin in small centrifuge for 2 min.

29. Collect supernatant and label "Pre-Elution Sup".

30. Take 2 µl aliquot and add to test tube of Biorad dye.

31. Add 0.5 mL of glutathione elution buffer and mix with matrix for 10 min. at room

temp.

32. Spin in small centrifuge for 2 min.
33. Collect supernatant (~0.5 mL) and label "Eluate 1, 2, 3, 4, and 5"
34. Take 2 µl aliquot and add to test tube of Biorad dye.
35. Repeat steps 28-31 until no protein is eluted and there is no color change after addition to Biorad dye.

Data Collection

Electrophoresis:

1. Remove 10% tris-glycine gel from package.
2. Rinse with distilled water to remove packaging buffer.
3. Remove comb.
4. Fill and empty wells four times with TTBS Wash Buffer.
5. Fill wells with TTBS Wash Buffer.
6. Remove tape.
7. Place into PowerPac 3000 Power Supply.
8. Fill outer chamber with TTBS Wash Buffer up to bottom of bridge.
9. Fill inner chamber with TTBS Wash Buffer up to top of bridge.
10. Load wells with samples.
11. Set PowerPac 3000 Power Supply to 120 V, 120 min., and 400 mA.

GelCode Blue Staining:

1 . After electrophoresis, transfer gel to a clean tray and rinse 3 times, 5 minutes each, with 100-200 mL of deionized water. Alternatively, wash gel in 1-2 L of deionized water with gentle shaking for 15 minutes.

2. Add approximately 20 mL of GelCode Blue Stain reagent to the tray for 1 hour.

3 . Periodically, check the protein band development.

4. Replace the staining reagent with deionized water overnight.

Data collected from the SDS-Page gel staining should be processed by first scanning the gel and then analyzing the results.

Immunoblot:

1. Block with 5% BSA in TTBS overnight in cold room.

2. Incubate with anti- in 5% BSA/TTBS for 2 hours at room temperature.

3. Wash three times for 5 minutes each time with TTBS.

4. Incubate in anti- -1gG-HRP for 1 hour at room temperature.

5. Wash three times for 15 minutes each time with TTBS.

6. Wash three times for 5 minutes each time with TTBS.

7. Develop by Amersham ECL and expose to film.

The data collected from the Immunoblot technique should be processed with developing by Amersham ECL and exposing to film. Then one should assess the results.

In this chapter, I have described the different materials needed prior to conducting this experiment, the procedures, and the order that should be followed. I have provided all components necessary to replicate this experiment.

Chapter 4: Presentation

In this chapter, I will present data from this study. The data was collected following the large-scale expression and purification of full-length Caveolin-1 (GST-CAV-FL, amino acid residues 1-178) and Caveolin-1 deletion mutants (residues 1-140, 1-81, 61-101, 102-134, and 135-178) fused to Glutathione-S-Transferase (GST) and GST. The data collected from this study was able to tell us how much fusion protein was expressed, how much binded to the glutathione sepharose beads signaling successful purification, and how much was then eluted with 10 mM glutathione elution buffer. Expression and purification were assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) subjected to GelCode Blue stain and immunoblot analysis using antibodies against GST (goat, polyclonal), myc-tag (monoclonal) or caveolin-1 (monoclonals). Monoclonal caveolin-1 antibody 2234 recognizes amino acids 1-21 and antibody 2297 recognizes amino acids 61-71 (Scherer et al. 1995).

To process a GelCode Blue stain, the gel was scanned. The darkness of the line represents how much fusion protein is in the sample in each lane. The placement of the line represents the molecular weight of the protein relative to the marker.

To process an immunoblot analysis, the gel was exposed to film and developed by Amersham ECL. This data collection technique is able to relay the chemical components of the sample. The immunoblot analysis is more sensitive and, therefore, able to detect smaller amounts of fusion protein.

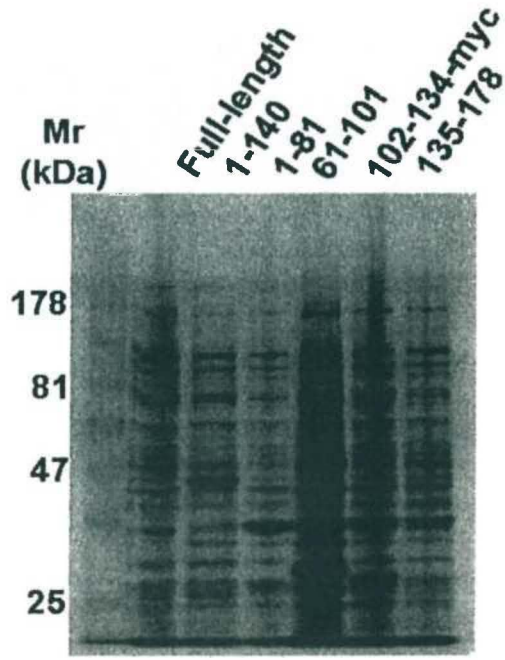

Figure 4.1. GelCode Blue staining of the bacterial media after expression of GST-Caveolin fusion proteins. It appears that all fusion proteins were expressed successfully.

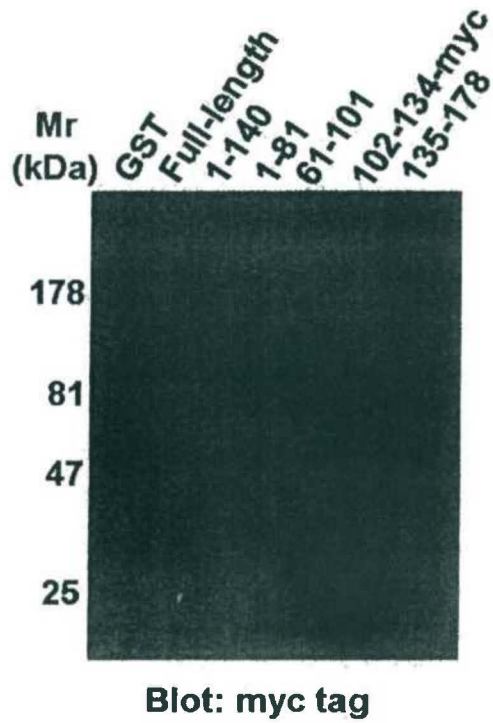

Figure 4.2 Immunoblot using an antibody against myc-tag (monoclonal) of the bacterial media after the expression of the GST-Caveolin fusion proteins. The antibody against myc-tag (monoclonal) is able to recognize GST-CAV 102-134 because it is the only fusion protein that possesses the myc-tag. This confirms that GST-CAV 102-134 was expressed successfully.

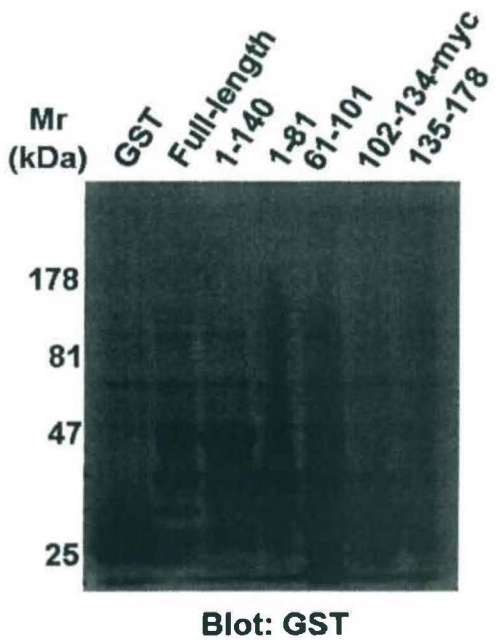

Figure 4.3. Immunoblot using an antibody against GST (goat, polyclonal) of bacterial media after expression of GST-Caveolin fusion proteins. The antibody against GST (goat, polyclonal) is able to recognize GST tagged caveolin-1 fusion proteins which tells us all proteins expressed are GST-Caveolin fusion proteins.

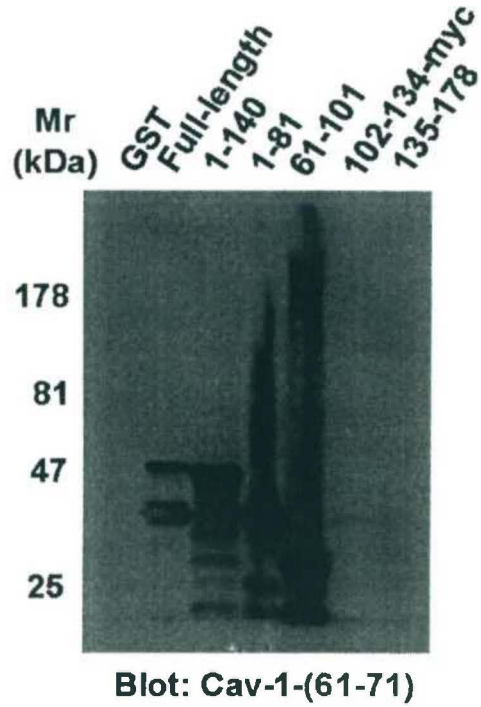

Figure 4.4. Immunoblot using antibody against caveolin-1 2297 (monoclonal) of bacterial media after expression of GST-Caveolin fusion proteins. The antibody against caveolin-1 2297 (monoclonal) is able to recognize amino acids 61-71. It, therefore, is able to tell us that GST-CAV FL, GST-CAV 1-140, GST-CAV 1-81, and GST-CAV 61-101 have been expressed successfully.

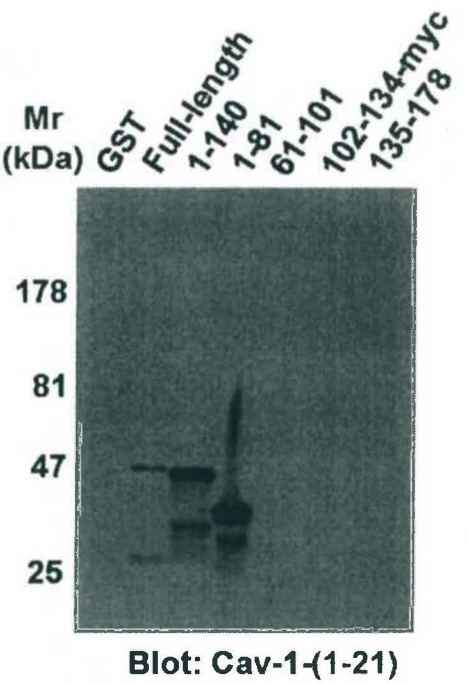

Figure 4.5. Immunoblot using an antibody against caveolin-1 2234 (monoclonal) of bacterial media after expression of GST-Caveolin fusion proteins. The antibody against caveolin-1 (monoclonal) is able to recognize amino acids 1-21. It, therefore, is able to tell us GST-CAV FL, GST-CAV 1-140, and GST-CAV 1-81 have been expressed successfully.

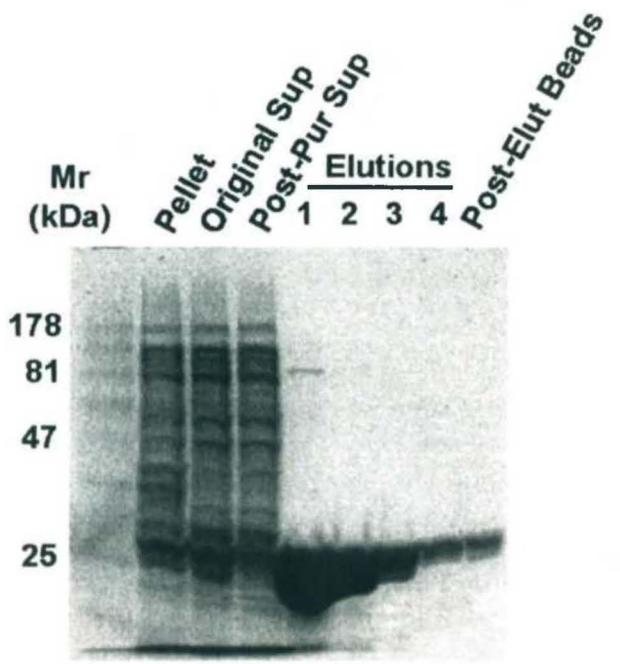

Figure 4.6. GelCode Blue staining of GST after purification. It appears the purification of GST was successful.

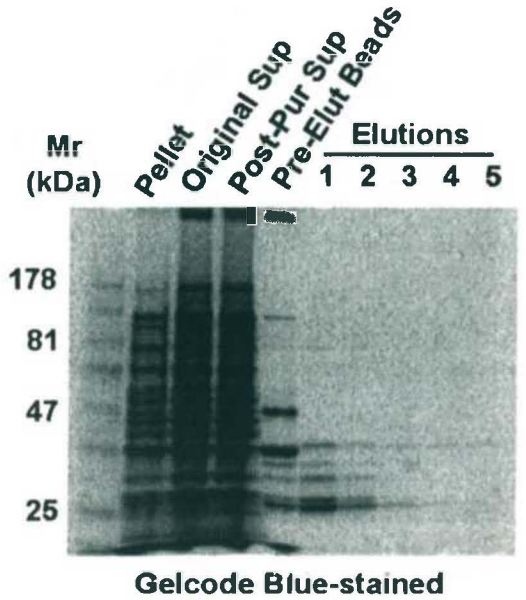

Figure 4.7. GelCode Blue staining of GST-CAV FL after purification. No detectable GST-CAV FL was eluted from the glutathione sepharose beads, but purification was successful.

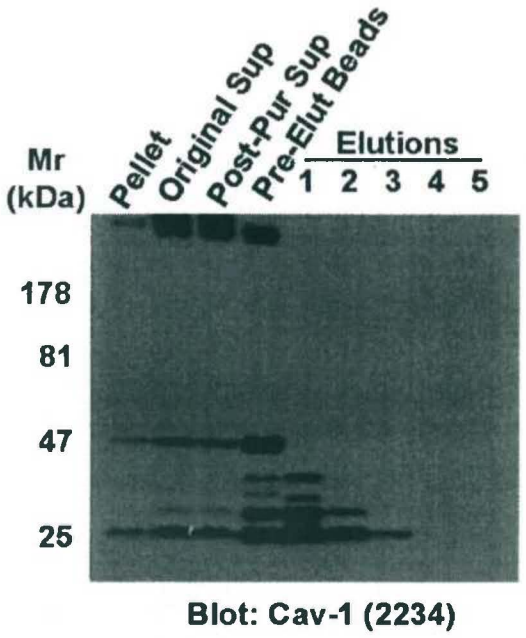

Figure 4.8. Immunoblot using an antibody against caveolin-1 2234 (monoclonal) of GST-CAV FL after purification. The antibody against caveolin-1 2234 (monoclonal) is able to recognize amino acids 1-21 and, therefore, GST-CAV FL, GST-CAV 1-140, and GST-CAV 1-81. This confirms that no detectable GST-CAV FL is eluted from the glutathione sepharose beads, but purification is successful.

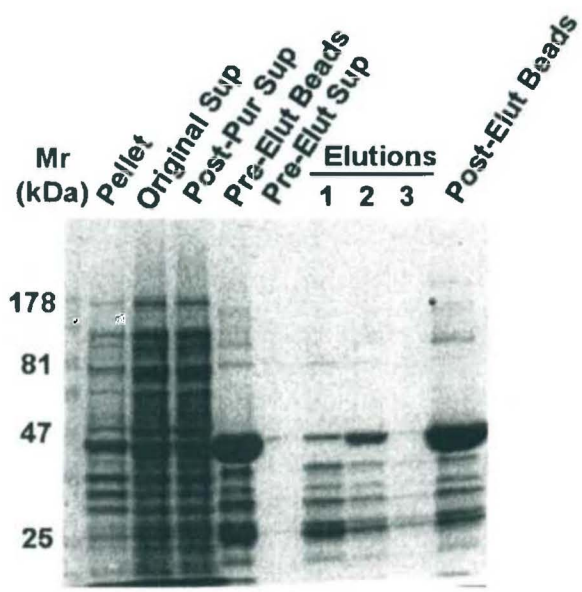

Figure 4.9. GelCode Blue stain of GST-CAV 1-140 after purification. It appears that purification of GST-CAV 1-40 was successful.

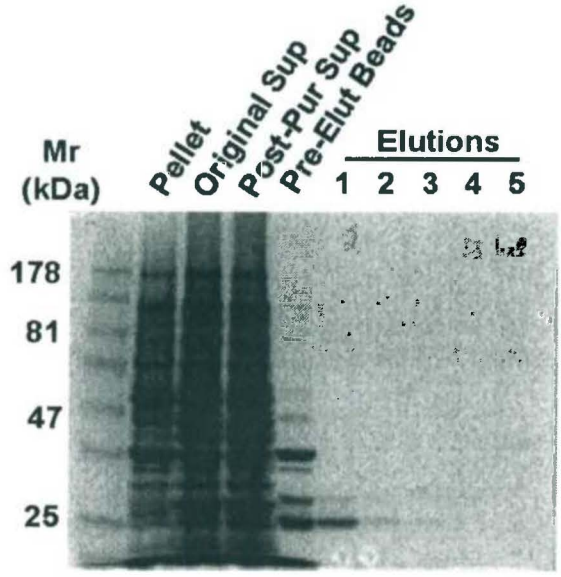

Figure 4.10. GelCode Blue stain of GST-CAV 61-101 after purification. It appears that the purification of GST-CAV 61-101 was successful.

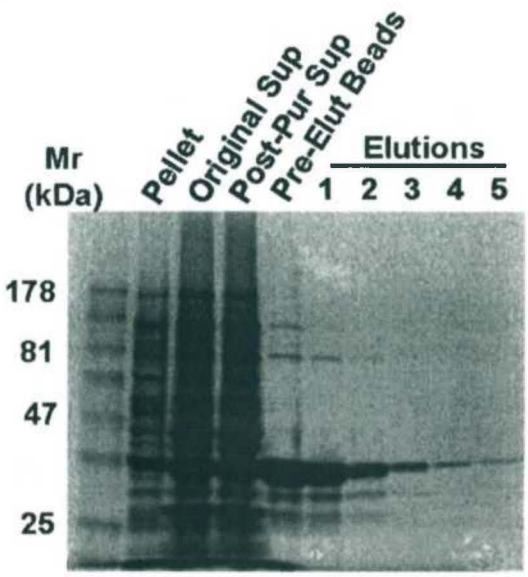

Figure 4.11. GelCode Blue stain of GST-CAV 1-81 after purification. It appears that the purification of GST-CAV 1-81 was successful.

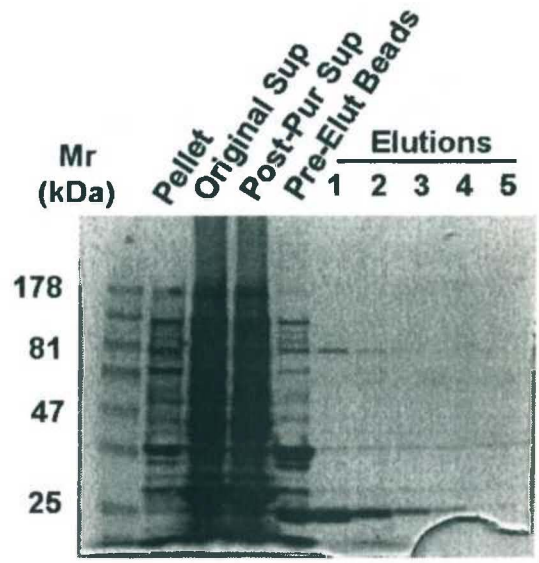

Figure 4.12. GelCode Blue stain of GST-CAV 102-134 after purification. We were unable to identify the GST-CAV 102-134 band eluted from glutathione sepharose beads after staining with GelCode Blue.

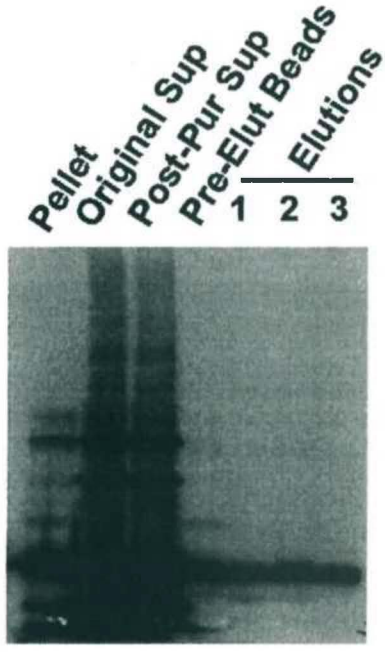

Figure 4.13. Immunoblot using an antibody against myc-tag (monoclonal) of GST-CAV 102-134 after purification. GST-CAV 102-134 was eluted from beads. There was significant degradation of the fusion protein as indicated by the abundant GelCode Blue-stained band that does not appear in the myc-tag immunoblot..

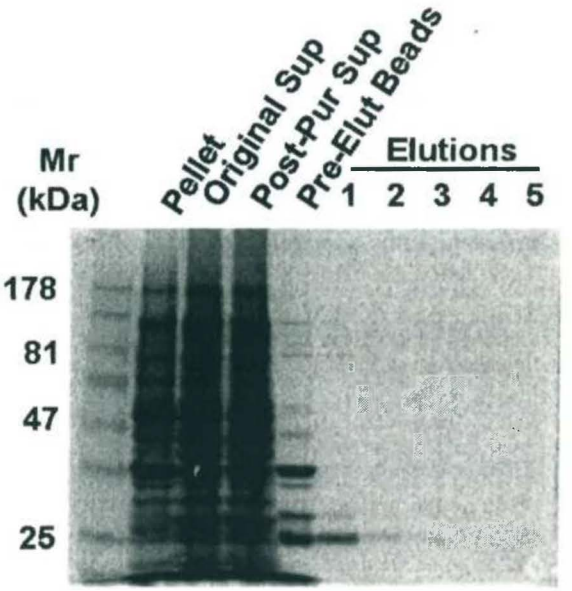

Figure 4.14. GelCode Blue stain of GST-CAV 135-178 after purification. It appears that the purification of GST-CAV 135-178 was successful.

This concludes the presentation of data from this study. As apparent, GelCode Blue stains were the first of the data collection techniques to be used and were only followed up by immunoblot analysis if necessary. Both techniques are independent of one another in what is revealed after analysis. The data collected from these techniques was able to tell us how much of the fusion proteins were expressed, which enabled us to move on to purification. The GelCode Blue stain was able to supply us with the success of purification and a rough estimate of the amount of fusion protein being eluted with 10 mM glutathione elution buffer. If it appeared in the GelCode Blue stain that no detectable fusion protein was being eluted from the glutathione sepharose beads, it was followed up with an immunoblot analysis, which is more sensitive and able to detect smaller amounts of fusion protein. The GelCode Blue stains were scanned and the immunoblot analysis was exposed to film and developed by Amersham ECL.

Chapter 5: Conclusion

Before beginning this study, the DNA encoding each protein was inserted into an IPTG inducible vector containing an ampicillin gene for expression in *E.coli.* Expression was assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using GelCode Blue and immunoblot analysis using antibodies against GST (goat polyclonal), myc-tag (monoclonal) and caveolin-1 (monoclonals). Monoclonal caveolin-1 antibody 2234 recognizes amino acids 1-21 and antibody 2297 recognizes amino acids 61-71 (Scherer et al. 1995). A GelCode Blue stain of the bacterial media after expression of the GST-Caveolin fusion proteins revealed a successful expression of all fusion proteins. The immunoblot using an antibody against GST (goat, polyclonal) of the bacterial media after GST-Caveolin fusion protein expression revealed that all proteins expressed were fusion proteins. The immunoblot using an antibody against myc-tag (monoclonal) of the bacterial media after expression of GST-Caveolin fusion proteins revealed that GST-CAV 102-134 was expressed successfully. The immunoblot using an antibody against caveolin-1 2234 (monoclonal) of the bacterial media after expression of GST-Caveolin fusion proteins revealed GST-CAV FL, GST-CAV 1-140, and GST-CAV 1-81 were expressed successfully. The immunoblot using an antibody against caveolin-1 2297 (monoclonal) revealed GST-CAV FL, GST-CAV 1-140, GST-CAV 1-81, and GST-CAV 61-101 were expressed successfully. The GelCode Blue stain and immunoblot analysis confirmed that all fusion proteins were expressed successfully and, therefore, was able to begin purification.

After each purification, a GelCode Blue staining was used to assess the amount of protein binded to glutathione sepharose beads signaling a successful purification. The GelCode Blue staining was also able to give a rough estimate of the amount of fusion protein being eluted with 10 mM glutathione elution buffer. When no detectable of small amounts of fusion protein

appeared to be eluted according to the GelCode Blue stain, it was followed up with an immunoblot analysis because it is more sensitive and able to detect smaller amounts of fusion protein. GST-CAV 102-134 is the only fusion protein that possesses myc-tag. So, when we were unable to identify the GST-CAV 102-134 band eluted from glutathione sepharose beads after staining with GelCode Blue, we conducted an immunoblot analysis using an antibody against myc-tag (monoclonal). When no detectable GST-CAV FL was eluted from glutathione sepharose beads according GelCode Blue staining, we conducted an immunoblot analysis using an antibody against caveolin-1 2234 (monoclonal), which is able to detect amino acids 1-21; GST-CAV FL, GST-CAV 1-140, and GST-CAV 1-81. The immunoblot analysis is more sensitive and able to detect small amounts of fusion protein, was conducted. After which, we were still unable to detect any GST-CAV FL being recovered from glutathione sepharose beads.

Cav-1 fusion proteins can be expressed and purified in large quantities but recovery by glutathione elution buffer may depend on the structure of Cav-1. The structure of Cav-1 includes an oligomerization domain (residues 61-101) (Sargiacomo et al. 1993), a caveolin-scaffolding domain (residues 82-101) (Couet et al. 1997), a transmembrane domain (residues 102-134) (Das et al. 1999), a caveolin-inhibitory domain (residues 135-156) (Venema et al. 1997; Razani et al. 1999), a N-terminal membrane attachment domain, and a C-terminal membrane attachment domain that enables it to bind with other Cav-1 scaffolding domains allowing the protein to form large complexes (Song et al. 1997). The Cav-1 C-terminal domain has two separate functions: membrane attachment (proximal third) and protein/protein interactions (distal third) (Schlegel et al. 2000). Homo-typic interaction of the C-terminal domain with an adjacent homo-oligomer could provide a mechanism for clustering Cav-1 homo-oligomers (Song et al. 1997) The Cav-1 fusion proteins that could be eluted from the glutathione sepharose beads did not contain the C-

terminal oligomerization domain. So, we speculate that this domain somehow interferes with the elution of Cav-1 fusion proteins from glutathione sepharose beads.

In conclusion, caveolin-1 interacts directly with G-Protein alpha subunits and can functionally regulate their activity. The proteins expressed and purified on a large-scale in this study will eventually be used in experiments on the enzyme activity of transducin alpha in photoreceptors.

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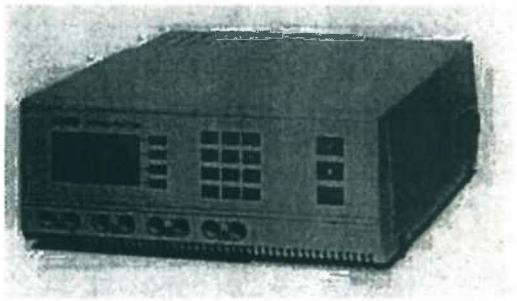
Appendix

Equipment

PowerPac 3000 Power Supply



The top-of-the-line PowerPac 3000 power supply is the most versatile power supply available, ideal for isoelectric focusing, DNA sequencing, isotachophoresis, SDS-PAGE, 2-D electrophoresis, native gel electrophoresis, western blotting, and submerged gel nucleic acid separations. With 400 W of power, the PowerPac 3000 power supply offers enough power to run the most demanding isoelectric focusing experiments, or up to four DNA sequencing cells at the same time.

The PowerPac 3000 power supply is highly sophisticated in design, yet simple to program. The numeric keypad and 4 multipurpose keys allow programming with a minimum of time and effort. The graphic LCD displays running conditions in large, easy-to-read numerals. Continuous, timed, or volt-hour controlled runs are easily programmed through this simple user interface.

The optional temperature probe allows precise automatic control of temperature between 0 and 90°C during electrophoresis, without additional control modules. This is particularly useful for DNA sequencing and single-strand conformation polymorphism (SSCP) analysis, where constant temperature is very important for superior gel resolution and reproducibility.

Sorvall RC-5B Refrigerated Superspeed Centrifuge

Easy operation that achieves desired speed and temperature without a vacuum system. It allows you to set the speed, time, and temperature in any order, and to change them at any time during a run.

Its Direct Drive system provides many years of trouble-free service with a motor that has extended life brushes, and employs no belts or gears.

It receives seven (7) fixed angle rotors for speeds up to 21,000 rpm, three (3) swinging bucket rotors with speeds up to 20,000 rpm; two (2) vertical rotors with speeds up to 20,000 rpm; one (1) zonal rotor at up to 20,000 rpm; and one continuous flow rotor at up to 20,000 rpm.

Maximum Speed 21000 rpm

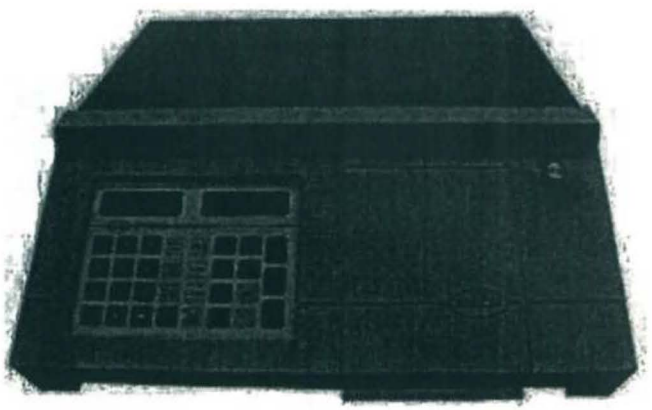
Maximum Force 49 268 g

Maximum Capacity 3 liters

Temp. Control Range +2 to +37 °C

Power Ranges: 208V, 60Hz 50A, single phase to 240, 50Hz, 30A single phase.

HACH DR/3000 Spectrophotometer



The Hach DR/3000 is a microprocessor controlled, single-beam spectrophotmeter capable of 325 to 1000 nm determinations. The DR/3000 is preprogrammed with calibrations for most water management test requirements, and it provides direct digital readouts in absorbance, transmittance, or concentration modes. Program codes are conveniently located on the bottom of the sample cell lid, and prompting lights direct the operator through tests by giving the appropriate wavelength and control key sequences for each test. Manual and best-fit programs can also be entered for user specific and custom calibrations. This unit also features an analog recorder output with adjustable voltage output, and the unit can be set to operate on 100,120, 220, or 240 volt, 50/60 Hz power. If you would like a detailed description of the features and specifications, you can download the operator's manual for this unit at http://ecommerce.hach.com/stores/hach/pdfs/Manuals/1960089.pdf .

Sonicator 715

The Sonicator 715 features one 5 cm^2 / 1 MHz applicator and a detachable applicator cable. The small applicator conforms to small and angular surfaces such as those found on the feet and hands.

Vita

Deidre L. Carroll

Candidate for the Degree of

Bachelor of Science in Chemistry

and

Completion of

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