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# Vesicle-Membrane Tethering: The Interaction of Sec1 and Exocyst Subunit Sec6

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# Vesicle-Membrane Tethering: The Interaction of Sec1 and Exocyst Subunit Sec6

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**Abstract**:

The exocyst complex is a multi-subunit tethering complex that is used in the process of exocytosis. There are eight subunits in the complex, and these subunits interact with each other as well as proteins outside of the complex to facilitate membrane fusion. Prior research has shown that the exocyst subunit Sec6 and Sec1 from the Sec1/Munc18 (SM) family, a known regulator of membrane fusion, interact with each other. In this experiment, the goal was to crosslink the two proteins and in turn work to identify the interacting amino acid residues that are responsible for the proteins' interaction. To do this, Sec1 and Sec6 were purified individually. Binding assays were then performed on the purified proteins to confirm that they folded properly and would interact. Crosslinking experiments were performed and resulted in the appearance of multiple new bands on the gel indicating that crosslinking had occurred. The proteins present in these bands will be analyzed by mass spectrometry to determine the interacting residues of the two protein subunits. Mutational and structural analysis will be performed in the future to confirm that the amino acids identified by mass spectrometry analysis are truly interacting. If the interacting residues are mutated, then the interactions between the two proteins should be weakened or even eliminated. This research is important in furthering our understanding of the exocyst complex and how it interacts with other proteins to facilitate the complex process of exocytosis.

**Introduction**:

All living organisms are comprised of a common microscopic subunit, the cell. Within the eukaryotic cell, the nucleus contains all of the genetic information used to create proteins that carry out the basic functions of the cell. Cells are very complex units, and there are many processes that occur within a cell that are not fully understood. An important process that is not fully understood is cellular trafficking. Cellular trafficking is the process of moving proteins and other biological molecules throughout the cell. Molecules can be trafficked from organelle to organelle, or they can be trafficked to the cell membrane and removed from the cell through a process known as exocytosis. Vesicles are organelles composed of a lipid membrane that play a vital role in trafficking, and vesicles deliver their cargo to its destination. For example, some vesicles travel to the cell membrane and release their cargo into the extracellular matrix. In order for the vesicles to be able to deliver cargo from one location to another, they first have to bind and fuse with another membrane.

Membrane fusion has been shown to involve several key proteins, notably SNARE (soluble N-ethylmaleimide-sensitive fusion (NSF) attachment protein receptors) proteins, Rabs, and tethering complexes.[1] To initiate the process of exocytosis, which is one form of cellular trafficking, a tethering complex on the plasma membrane "grabs" the vesicle, holding it close to the plasma membrane.[2] Then, the SNARE proteins present on the vesicle (v-SNAREs) come into close proximity with SNARE proteins on the plasma membrane (t-SNAREs; "t" indicating the target membrane). The alpha-helical SNARE proteins effectively "zipper" together, bringing the vesicle closer to the plasma membrane to promote fusion.[1] Tethering complexes aid in the fusion of membranes by holding them in close proximity to one another so that the SNAREs can interact more efficiently and with greater fidelity (Figure 1).

Large multi-subunit complexes, tethering complexes, that include COG, CORVET, Dsl1, GARP/VFT, HOPS, TRAPPI, TRAPPII, and the exocyst, are important for membrane fusion.[3] Tethering complexes can be categorized based on their function. The exocyst, Dsl1, COG, TRAPPI, and TRAPPII are all used in secretion pathways, while the other three, CORVET, GARP/VFT, and HOPS, are used for protein sorting in vacuoles.[2] These categories are limiting, however, because some of these proteins have functions outside of their particular category. For instance, the HOPS complex is also used in vacuole-vacuole fusion, and the exocyst also is required for polarized growth of the cell and aids in tethering of vesicles to membranes.[2] While SNARE proteins are said to be the fundamental machinery of cellular trafficking, vesicle tethering and fusion to the membrane is greatly diminished in experiments where tethering complexes are removed.[4]

SNARE proteins and tethering complexes alone are not solely responsible for vesicle-membrane tethering and fusion. Sec1/Munc18 (SM) proteins have also been found to be important in membrane fusion, as they interact with both SNARE proteins and tethering complexes.[3] The interactions between these subsets of proteins are known to be functionally relevant for proper membrane recognition and fusion, but the biochemistry of these interactions has yet to be elucidated.

**The Exocyst Complex**

The exocyst is a multi-subunit tethering complex (MTC) required for vesicle-membrane fusion in the process of exocytosis. It is a member of the CATCHR, or Complex Associated with Tethering Containing Helical Rods, family.[5] The exocyst complex contains eight subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84.[6] These subunits create two stable modules of four subunits each that make up the complex as a whole. One module is comprised of Sec8,

Sec6, Sec5, and Sec3, in that order. The other module is comprised of Sec15, Sec10, Exo84, and Exo70, in that order. This is depicted in Figure 2, along with the strength of the connections between the subunits. Thick lines indicate strong connections, thin lines intermediate, and dashed lines weak connections between the subunits. There are many pairwise interactions between the two modules that are critical to the stability of the complex, along with other more minor interactions of the modules.[7] It was determined that the complex is stable, composed mainly of helical bundles, and that many of its binding partners are not required for its assembly or stability.[5] Studies have also shown that the presence of most of the exocysts' subunits are necessary for its stability and function.[7] In many cases, if one of the subunits was disturbed by mutational analysis, it would lead to the dissociation of the entire complex.[7] There are no subcomplexes, which had previously been thought to be present.[5]

The three-dimensional structure of the exocyst complex has not yet been determined. Stable exocyst complexes from yeast have been visualized via negative stain cryo-electron microscopy (Figure 3); however, the individual subunits cannot be identified in it.[7] Figure 3 depicts subunit Exo70, of helical bundle structure, superimposed onto the micrograph. Although it is unknown exactly where Exo70 or any of the subunits fit exactly, this depicts how a subunit may fit into the structure of the exocyst.

**Exocyst and SNARE Proteins**

SNARE proteins are intracellular proteins that aid in facilitating membrane fusion.[8] The assembly of SNAREs from different membranes pulls the two membranes close together, which can begin the fusion reaction. SNAREs function by "zippering" four α-helical protein coils, three of which are from the plasma membrane and one is from the vesicle. There have been intermediates discovered in the formation of the SNARE complexes.[4] These intermediates form

in the zippering process of the SNAREs, after the v- and t-SNAREs join but before they are fully complexed and may be important in regulation and fusion of membranes.[4] Near the plasma membrane, SNARE proteins interact with the exocyst complex to facilitate membrane fusion. It has been observed that exocyst subunit Sec6 and t-SNARE protein Sec9 interact.[9] In the Sec6-Sec9, Sec6 promotes SNARE complex assembly and can bind to the Sso1-Sec9 binary SNARE complex.[10] Disrupting this interaction resulted in growth defects in yeast.[10] This confirmed that the exocyst probably has mechanisms similar to other multi-subunit tethering complexes which aid in the fusion of membranes. However, the exocyst appears to be more necessary in some cell types than others; for example, they are not required for synaptic vesicle fusion in neurons, while they are a key component in exocytosis for yeast cells.[4] Although the exocyst has never been visualized to physically tether membranes, its actions have been inferred due to research in which it was removed or mutated and membrane fusion decreased.

**Tethering Complexes and Sec1/Munc-18 (SM) Proteins**

Sec1/Munc-18 (SM) proteins are critical to the process of membrane fusion. Many SM proteins have been observed to bind to SNARE proteins, but their exact role in membrane fusion (although necessary) is not entirely clear. They are hydrophilic proteins that reside in the cytosol and are generally 60-70 kDa in size with a conserved amino acid sequence of approximately 600 amino acid residues.[3,8] SM proteins spatially and temporally regulate SNAREs after vesicles dock. Munc-18 binds to the closed conformation of syntaxin-1, a mammalian SNARE protein, to stabilize it. The closed conformation of syntaxin-1 is important because prevents the SNARE from forming complexes with other SNAREs.[8] Yeast Sec1, another SM protein, binds to the fully assembled exocytotic SNARE complex to stabilize the complex.[8] SM proteins also act as clasps binding to v-SNAREs and t-SNAREs. They get the name "SNAREpins" because they

organize t-SNARE complexes both temporally and spacially.[3] The structure of SM proteins is representative of their function as they are roughly in a U-shape that looks like a clasp which can be seen in Munc-18 a SM protein that has a crystal structure that has been determined (Figure 4).[11] In addition to binding to the SNARE complexes, SM proteins also interact with tethering complexes, and this interaction affects the fidelity of the SNARE interactions. For example, Sec1, an SM protein, interacts with the exocyst subunit Sec6. The structure of the C-terminal end of Sec6 has also been crystallized and comprised mostly of alpha helices (Figure 5).[12] Sec1 is located near to the plasma membrane and binds only to fully assembled SNARE complexes. This suggests that Sec1 serves as a stabilizing factor in the exocytotic process because by binding to the SNAREs it is holding them together which keeps the two membranes in close proximity leading to fusion (Figure 6).[8]

The exocyst complex and SM proteins are critically important in the process of exocytosis and understanding how they regulate membrane fusion. The SM protein Sec1 has been observed to immunoprecipitate with the exocyst complex.[13] Sec1 is also known to bind to SNARE proteins and stimulate membrane fusion. Interactions of Sec6 of the exocyst and Sec1 have been investigated in regards to the regulation of exocytosis by coordinating SNARE complex formation.[14] Sec1 is recruited to sites of secretion, which in turn coordinates SNARE complex formation as well as vesicle fusion.[14] Although SM proteins and tethering proteins, such as the exocyst, are different families of proteins, they are both involved in the late stages of membrane fusion. For this reason, their interactions need to be further investigated. The exact interaction of Sec1 and Sec6 remains an important and unsolved question. This is because it will give more insight into exocyst function, as well as a greater understanding of similar complexes.

**Other Exocyst-Associated Proteins**

The exocyst binds to many other proteins, and these interactions are critical for proper tethering and fusion events. For example, coat proteins are present on the vesicles and have been shown to interact with tethering proteins.[2] The coat proteins aid in recognition of the vesicle by the membrane, but must be shed prior to the membranes fusing. Another group of proteins that are necessary for membrane fusion are the Rab GTPases. Rabs are involved in many phases of endocytosis as well as exocytosis.[2] The exocyst complex is a Rab effector, meaning that it uses the GTP-bound Rab to facilitate the recruitment of its subunits. The GTPases that the exocyst binds to for this purpose include Sec4, Rho1, Rho3, Cdc42, and RaIA.[6] The interaction of the exocyst Sec15 subunit with GTP-bound Rab Sec4 on the vesicle membrane is used for recognizing specific secretory vesicles, as well as localization of the complex and its stable assembly.[5,15] GTP-bound Rho proteins interact with Sec3 of the exocyst, and this interaction is vital in defining the polarized membrane domains and budding in yeast.[15] Another Rab, RalA, may be important for exocyst subunit assembly.[5]

**Experimental Question and Approach**

Exocyst subunit Sec6 and SM protein Sec1 are both important in aiding in the process of exocytosis in yeast, and previous *in vitro* studies have shown that these proteins interact with one another.[15] Since the exact orientation of the exocyst subunits are not yet known, understanding how Sec6 is oriented with respect to Sec1 could give insight into how it is positioned within the exocyst complex. This experiment was performed with the purpose of determining the interacting residues in the Sec1-Sec6 interaction. In order to determine the amino acids that participate in this protein-protein interaction, Sec1 and Sec6 were purified separately. *In vitro* binding assays were performed to confirm the previously observed Sec1-Sec6 interaction.

Crosslinking experiments were optimized to find the most suitable concentration of crosslinker in the assay. Finally, these crosslinked proteins will then be sent for mass spectrometry to determine the interacting amino acid residues. Determining how these proteins interact is an important step in understanding how Sec1 and Sec6 function to facilitate membrane fusion and exocytosis.

**Methods**:

**Expression and Purification of Sec6**

A plasmid encoding Sec6-$His_6$ (pMM301) was transformed into BL21 (DE3) *E. coli* cells on ice for 1 hour. The cells were then plated on an LB carbicillin agar plate and incubated overnight at 37°C. A small-scale (100mL) culture was started by scraping plate with a sterile stick and putting colonies into 100mL of LB media with 100µL of AMP 1000X. The culture was incubated at 37°C at 200rpm until it reached an $OD_{600}$ of approximately 0.4. Six 1L flasks of LB media were prepared and 1mL of AMP 1000X was added to each and they were placed in the incubator to warm to 37°C. Then each flask was inoculated with approximately 16mL of small culture and grown at 37°C at 200rpm until an $OD_{600}$ of approximately 0.4 was reached. A 50µL uninduced sample with an $OD_{600}$ of approximately 0.4 was then taken, spun down, and the pellet was frozen. The flasks were then moved to an 18°C incubator and were induced with 1mL IPTG (0.5mM) 30 minutes later. The flasks were left to induce and express overnight at 18°C and 200rpm. The next morning a 50µL induced sample with an OD of 2.72 was taken, spun down, and the pellet was frozen. The flasks of cells were then poured into 9 centrifuge bottles and spun at 5000rmp for 10 minutes at 4°C in an SLC6000 rotor of the centrifuge. The supernatant was poured off and the pellets were scrapped out, combined in a 50mL conical tube, and placed in the -80°C freezer.

To purify Sec6, 1mL DTT, 35mg PMSF dissolved in 2mL of 100% ethanol, 1 protease inhibitor tablet, and DNase was added to 200mL of lysis buffer (300mM NaCl, 50mM $NaH_2PO_4$). The pellet was then removed from the -80°C freezer and added to the lysis buffer solution. A blender was used to homogenize the sample, and the solution was then pipetted into a new beaker to check for clumps missed by the blender. This was then run through the cell

disrupter. The sample was divided into 9 centrifuge tubes and spun down in the SS34 rotor of a centrifuge for 30 minutes at 13,000rpm at 4°C. While this was running, 5mL of Ni-NTA agarose slurry was spun down at 1000xg for 5 minutes at 4°C. The supernatant was removed and 10mL of lysis buffer was added to the slurry. This was then spun down at 1000xg for 5 minutes at 4°C, and the supernatant was removed. The supernatants from the centrifuge tubes were then combined, and the washed Ni-NTA agarose resin was added. It was then nutated at 4°C for 1 hour.

Next, the solution was spun down for 5 minutes at 1000xg at 4°C. The supernatant was then removed with a pipette. 30mL of wash buffer (50mM $NaH_2PO_4$, 300mM NaCl, 20mM imidazole) with 5mM BME was then added to the pelleted resin and incubated on ice for 5 minutes. It was then spun down at 1000xg for 5 minutes. The 30mL wash and spin down was then repeated. The supernatant was removed and 10mL of elution buffer (50mM $NaH_2PO_4$, 300mM NaCl, 250mM imidazole) with 5mM BME was then added to the pelleted resin and incubated on ice for 5 minutes. This solution was then poured onto a BioRad column and the flow through (elution product) was collected (~12.5mL). An equal amount of Buffer A (10mM Tris pH 8) was added to the elution product as well as 1mM DTT. A MonoQ 10/10 column was prepped and ~23.5mL were loaded onto the super loop and 2mL fractions were collected. The molecular weight marker, supernatant, pellet, flow through, wash1, wash 2, elution, and eleven fractions were loaded on a 10% gel (Figure 8). Fractions 40-58 from the MonoQ 10/10 column were pooled as they were observed to have the most protein in the gel. The sample was then concentrated using the 15mL Amicon Ultra (30,000 MWCO) filter units. The concentrated sample was loaded onto a buffer exchange column (PD-10) equilibrated with 25mL exchange buffer (10mM $K_2HPO_4$, 100mM $KH_2PO_4$, 140mM KCl). The 2.3mL of concentrated protein was

added to the column and eluted with 3.5mL of exchange buffer and 5 drop fractions were collected. The fractions were wat spotted and fractions 15-24 were pooled. The concentration was measured on an $A_{280}$ on NanoDrop 2000. The sample was then further concentrated using another spin concentrator to get a more concentrated sample. The final volume of Sec 6 was 1.25mL and this was made into 50μL aliquots, flash frozen in liquid nitrogen, and stored at -80°C.

**Expression and Purification of Sec1**

To express the Sec1 construct, two 100mL (SC-URA+2% glucose) seed cultures were inoculated with Sec1-His6-V5 and incubated overnight at 30°C and 200rpm. Two 2L (SC-URA+2% glucose) were then inoculated with 100mL of seed culture and incubated at 30°C and 200rpm until an OD of approximately 0.6-0.7 was reached. The cultures were then spun down at 4000rpm for 12 minutes in a swinging bucket centrifuge. The supernatant was removed to discard all glucose-containing media. The pellets were then resuspended in two 2L flasks of SC-URA and 2% galactose media and incubated overnight at 30°C and 200rpm. The next morning the cells were spun down at 4000rpm for 12min in the swinging bucket centrifuge, and the media was poured off and the pellets were ready to be carried on to purification.

Two different methods for the purification of Sec1 were used: lysing with noodle grinding and lysing with the cell disrupter. The noodle-ground Sec1 was used for the binding assays and crosslinking experiments, and the steps for that method will be described here. The cell pellet was washed by resuspending it in 50mL of deionized water over ice. The resuspended solution was then split into two 50mL conical tubes and spun down at 4000rpm for 5 minutes. The supernatant was then pipetted off. This process was then repeated. The pellet was then resuspended over ice with a volume of deionized water equal to the volume of the pellet. This

was then spun down for 15 minutes at 4000rpm and the supernatant was aspirated. The pellet was then spun down again for 15 minutes at 4000rpm to ensure the removal of all deionized water. At this point the pellet is a dry and thick paste. To noodle the cells, liquid nitrogen was placed in a Styrofoam container with a conical tube stand and a 50mL conical tube. This was then topped with aluminum foil with a hole for conical tube. The conical tube was then filled to the top with liquid nitrogen. Using a spatula the cell past was removed from the other conical tube and placed into a 30mL syringe. The cell paste was pressed out of the syringe into the liquid nitrogen in the conical tube. Once all of the cell paste was in the conical tube the liquid nitrogen was decanted from the tube, via holes in the cap of the conical tube. The conical tube was stored at -80°C. To grind the noodles the 50mL grinder was partially submerged in liquid nitrogen and the noodles were poured in. The cover was placed on the grinder and liquid nitrogen was poured over the lid repeatedly until the lid became shiny. The grinder was then removed from the liquid nitrogen and the bottom was wiped off and it was placed into the grinding machine. The tripod was placed on top of the lid and tightened and the machine was run. The grinding machine was run a total of 6 times, and in between each run the sides of the grinder were scrapped and the lid replaced and rechilled with liquid nitrogen and placed back into machine. If there were too many noodles to fit into the grinder, this entire grinding process would have to be repeated. After grinding, the powder was stored at -80°C.

To purify 30mL of lysis buffer (50mM HEPES, 150mM KCl, 30mM imidazole, 10% glycerol) was prepared, 15mL of lysis buffer is used per liter of cells. Then, 1mM PMSF, 1 protease inhibitor tablet, a small scoopful of DNase, and 0.5mM BME was added to the lysis buffer. The cell powder was then resuspended in this solution. It was spun down in an SS34 rotor at 13000rpm for 30 minutes. The supernatant from the spin was removed and 50μL PMSF was

added to each tube prior to ultracentrifugation. The supernatant was then spun down in the ultracentrifuge at 37000rpm for 35min (50.2 Ti rotor, precooled in ultracentrifuge under vacuum). During the ultracentrifugation step, the Ni-NTA resin was prepared. A 1mL slurry of the resin was washed twice with lysis buffer. The supernatant from the ultracentrifugation was saved and incubated for 1hr with Ni-NTA resin at 4°C. After an hour, the resin was spun down at 1000xg for 10 minutes. The majority of the supernatant was removed as flow through. Then, the resin was resuspended, put into a BioRad column, and the rest of the flow through was collected. The resin was then washed with a solution 3mL lysis buffer and 1µL BME. This wash was collected in a conical tube. The resin was then eluted with 3mL of Buffer B (50mM HEPES, 150mM KCl, 250mM imidazole, 10% glycerol) with 1µL BME added to it. During the elution 0.5mL fractions were collected. The fractions were wat spotted and run on a 10% gel. The most protein was seen in Fractions 2-4 and they were pooled (Figure 10). The sample was then buffer exchanged using a NAP-25 column into the exchange buffer. Fractions were collected and wat spotted and fractions 6-13 were pooled (approximately 2000µL). 100µL aliquots were flash frozen in liquid nitrogen and stored at -80°C.

**Binding Assays**

A binding assay was performed with the previously purified Sec6 and Sec1. A 1:5 dilution of the Sec6 was used. The control had no Sec1 present, 30µL Protein G sepharose, 2.5µL anti-V5 antibody, and 67.5µL binding buffer (50mM HEPES, 150mM KCl, 0.5% Igepal, 1mM DTT, 10% glycerol, pH 7.4). The experimental sample had 30µL Protein G sepharose, 2.5µL anti-V5 antibody, 9.6µL Sec1, and 57.9µL binding buffer. This pull down method works because Protein G sepharose binds to IgG antibodies, including the anti-V5 antibody, and Sec1 has a V5 tag that interacts with the anti-V5 antibody. The prepared samples were incubated,

nutating at 4°C for 90 minutes. The samples were then washed three times with binding buffer. Then, 100µL of 1:5 diluted Sec6 was added to each sample. This was incubated nutating at 4°C for 2 hours. The samples were then spun down, and the supernatant saved. The sepharose was then washed three times. The samples were then resuspended in 10µL of 1X dye. Inputs were also made for the gel with 9.6µL Sec1 with 9.6µL 2X dye for the Sec1 input and 10µL 1:5 dilution Sec6 with 10µL 2X dye for the Sec6 input. All of the samples were then bead boiled at 95°C for 10 minutes and run on an 8% gel with 3µL of molecular weight marker (Figure 11).

**Crosslinking Experiments**

To crosslink Sec1 and Sec6, the crosslinker $BS^3$ was used. To determine the optimal amount of crosslinker to use in order to achieve crosslinking, different concentrations of $BS^3$ were tested. The total volume of all samples was 100µL. The concentration of $BS^3$ and amounts of other samples used in each experiment can be seen in the table below (Table 1).

*Table 1*: Volumes of $BS^3$, Sec1, Sec6, and binding buffer used in the crosslinking experiment

| Concentration of $BS^3$ (µM) | Volume of 2mM $BS^3$ (µL) | Volume of Binding Buffer (µL) | Volume of Sec1(µL) | Volume of Sec6(µL) |
|---|---|---|---|---|
| 100 | 5 | 65 | 20 | 10 |
| 150 | 7.5 | 62.5 | 20 | 10 |
| 200 | 10 | 60 | 20 | 10 |
| 250 | 12.5 | 57.5 | 20 | 10 |
| 300 | 15 | 55 | 20 | 10 |

These samples were then incubated for 30 minutes at room temperature on a nutator. The solutions were then quenched with 5µL of 1M Tris-HCl and were incubated for 15 minutes at room temperature on a nutator. The crosslinked solutions were then added to 30µL of prewashed Protein G sepharose beads. To this, 2.5µL of anti-V5 antibody was added. This was nutated at 4°C for 90 minutes. The beads were then washed three times and then 10µL of 1X SDS buffer dye was added to the beads. The samples were then bead boiled at 95°C for 10 minutes. The

samples were run on a 7% gel separate the protein bands. Onto the gel, 3μL of molecular weight marker, 20μL of Sec1 input, and 10μL of Sec6 input were loaded, as well as the total volume of the five crosslinked samples (Figure 12).

**Results**:

**Sec6 Expression and Purification**

The transformation of Sec6 into the *E. coli* cells was successful, as observed by the presence of colonies on the LB carb plate after overnight incubation. The expression of Sec6 was detected in a gel of uninduced and induced Sec6 samples. Prior to the induction, there was a faint band of Sec6 protein, but after the induction there is a more prominent band of Sec6.

To perform the binding assays, the proteins first had to be purified. Sec6 was expressed with a histidine tag and was purified using a BioRad column packed with Ni-NTA agarose resin. The histidine tag of the Sec6 protein specifically interacts with the chelated nickel ion of the resin and can be eluted from the column with imidazole, which competes with the histidine tag for binding to the nickel. After this first purification step, the protein was further purified on an anion-exchange column (i.e., a MonoQ 10/10 column), which separates proteins based on their charge. The protein fractions from each step of the purification were run on a 10% polyacrylamide gel (Figure 8), and fractions from the MonoQ column containing the Sec6 protein were pooled and concentrated. Upon concentration, the final concentration of Sec6 was 2.168mg/mL (before adding DTT and glycerol).

**Sec1 Expression and Purification**

To express the V5-tagged Sec1, a new expression method was first attempted. Historically, Sec1 has been expressed using a galactose-based promoter, and this well-established protocol requires an 18-hour period of induction and a media change, which increases the risk of contamination of the yeast culture. To avoid these issues, a new yeast construct was made that would induce Sec1 using β-estradiol. The β-estradiol induction of Sec1 expression was attempted many times, but the Sec1 protein that was recovered was low in

concentration and, even after purification, not pure. Also, the yeast cells with the β-estradiol plasmid were prone to contamination. After attempting this protocol many times, the protocol using galactose induction was used to express Sec1. This method for Sec1 expression was successful the first time with no contamination of the yeast cultures.

In order to purify yeast-expressed proteins, the yeast cells first need to be lysed. Yeast cell lysis is more challenging than bacterial cell lysis, as the yeast cell well is more rigid due to its carbohydrate composition. Typically, yeast cells are lysed using a high-pressure technique that breaks open the cells. The disadvantages of this technique are that a higher pressure must be used to lyse the more rigid cell walls of the yeast (compared to bacterial samples), and often more than one pass through the cell disrupter is required for maximally efficient lysis. This process heats the sample, and proteins that are sensitive to temperature are prone to degradation. Thus, two different methods of cell lysis were used to determine which method would be most suitable for the purification of Sec1.

The two methods of lysis of Sec1 used were cell disruption (a high-pressure technique) and noodle-grinding. Noodle-grinding involved the washed cell pellet being injected into liquid nitrogen by a syringe. This process produced "noodles" and the noodles were kept at -80°C until they were ground. To grind the noodles, they were placed in a steel canister with three steel balls (the canister sits in liquid nitrogen). The canister was then placed in the grinder for 90-second intervals. In between each round of grinding, the canister was placed back into the liquid nitrogen and the sides were scrapped down to ensure all of the noodles were ground. This method is advantageous compared to cell disruption to lyse the cells because it gets the pellet cold fast and keeps it cold giving the protein less time to degrade. After each method was performed, the cell lysates were run on gels (Figure 9, Figure 10). These gels demonstrated that

the noodle-grinding produced more concentrated and more highly purified Sec1 protein. The noodle-grinding sample may be more pure because there was less time for the protein to degrade as it was quickly frozen in liquid nitrogen rather than being run through a cell disrupter multiple times enabling the sample to warm.

To purify the V5-tagged Sec1 after noodle-grinding, only a Biorad column with Ni-NTA agarose beads was needed. For the purposes of the binding assays and cross-linking ssays to be completed, other purification steps were not needed. One reason for this is because the binding assays utilized the V5-tag of Sec1, which would isolate the Sec1 on the Protein G sepharose resin. Any protein without the tag (and that does not interact with Sec1) would – in theory – not bind to Sec1. Thus, in the binding assays, all molecules that were not Sec1 would be eluted from the resin.

**Binding Assays**

Many binding assay trials were conducted in order to confirm that the purified Sec6 and Sec1 were binding as expected. In the binding assay Protein G sepharose is used to immobilize Sec1. This is possible because of the anti-V5 antibody, which binds to both Protein G sepharose and Sec1. Protein G sepharose binds to IgG antibodies, which includes anti-V5 antibody. Also, the Sec1 purified has a V5 tag, which interacts with the anti-V5 antibody. Without the Sec1 the Sec6 should not bind to either the Protein G sepharose or the anti-V5 antibody. A control was run with Sec6, and no Sec1, to ensure that no Sec6 was binding. In the experimental sample Protein G sepharose, anti-V5 antibody, Sec1, and Sec6 were all present. In the experimental sample the beads should pull down both Sec1 and Sec6 if Sec1 and Sec6 interact. A doublet should appear on the gel, as Sec1 and Sec6 should dissociate on the heat block. It was observed that purified V5-Sec1 and His-tagged Sec6 did bind. This can be observed in Lane 7 of Figure

11. When Sec1 is not present in the binding assay, there was some protein observed in the pellet, as seen in lane 5, but most of the protein gets washed away. When both Sec1 and Sec6 are present, they bind to each other. When the complex on the beads is boiled in the presence of gel loading dye, the proteins dissociate from each other, and the heavy chain of the anti-V5 antibody and a doublet is seen (Figure 11), Lane 7, representing both Sec1 and Sec6.

**Crosslinking Experiments**

First, the optimal amount of crosslinking reagent was determined. Initially, concentrations of $BS^3$ ranging from 0μM-60μM were used to crosslink. These concentrations were not high enough to crosslink the protein. In the next set of crosslinking experiments, a larger range of $BS^3$ concentrations were tested, ranging form 0μM -200μM. At 200μM of $BS^3$, a higher molecular weight band appeared, and the concentration of Sec1 decreased which suggested that the 200μM concentration was an appropriate starting concentration. A third and fourth round of crosslinking experiments were performed with $BS^3$ concentrations ranging from 200μM-600μM and 100μM-300μM. In the third crosslinking experiment crosslinking was observed in all lanes 200μM-600μM, however there was crosslinking at higher molecular weights than expected in the 400μM-600μM and less crosslinking observed in the expected molecular weight range. The fourth crosslinking experiment from 100μM-300μM had a substantial amount of crosslinking at all $BS^3$ concentrations, with the majority of crosslinking occurring at approximately 120kDa.

When crosslinking experiments were performed, a new band of protein appeared on the gel (Figure 12). This band was higher on the gel than either the Sec1 and Sec6 proteins alone, and therefore a larger molecule. Presumably, this band represents crosslinked Sec1-Sec6. The crosslinker does not enable the two proteins to dissociate when heated (as they did in binding

experiment) due to the covalent nature of the crosslink. At higher concentrations of crosslinker, a band appeared even higher on the gel (indicating a larger complex of proteins and crosslinking reagent). The composition of the high molecular weight complex is unknown, but it could be multiple copies of Sec1 and Sec6 crosslinked to one another due to the higher concentration of crosslinker.

There was crosslinking observed in all experimental lanes with the darkest band of crosslinked protein at 250μM. Bands were excised from this gel and sent for mass spectrometry.

**Discussion**:

Previous research has shown that Sec1 and Sec6 interact during the process of exocytosis, and this interaction has been observed *in vitro* as well.[15] The focus of this research was to crosslink Sec1 and Sec6 with the objective of determining the amino acids involved in the interaction. To do this, the proteins were purified individually, and binding assays and crosslinking experiments were performed to covalently bind the interacting interface of the two proteins. These interacting amino acid residues could then be determined using mass spectrometry.

**Sec6 and Sec1 Purification**

The Sec6 purification was successful with 23μM of concentrated Sec6 obtained. After the Ni-NTA agarose resin column, there were still many contaminating proteins, and before binding to Sec1, a cleaner sample of Sec6 protein was needed. For this reason, the protein was run on the MonoQ 10/10 column as well, which produced a significantly more pure sample that could be used in the binding assays (Figure 8).

The Sec1 purification was much more complex than anticipated. First, a new expression method was attempted using a different Sec1 construct. This method used yeast cells that had a promoter for the induction of Sec1 expression that was activated by a chemical agent, β-estradiol, rather than galactose. This method was used in many purification attempts. However, there were low levels of Sec1 obtained, and the yeast cell cultures were repeatedly becoming contaminated. It is possible that the plate on which the yeast cells expressing the Sec1 construct were plated was contaminated. Although this method did not work in this experiment, it is a method that should be attempted and refined in the future. If this method were to work properly, it would (ironically) decrease the risk of contamination, as the media would not need to be

changed (from a glucose-based media to the expression-inducing galactose media). This method also requires less time for protein expression and could potentially decrease the amount of degradation typically observed with the Sec1 protein during its expression.

A second expression protocol was then used in an attempt to avoid the previously encountered contamination issues. Using a yeast strain with a galactose-inducible promoter for the expression of Sec1, there were no issues with contamination. Upon successful expression, two methods to lyse the yeast cells were used: the cell disrupter technique and a technique called noodle-grind. Previously, the cell disrupter method has been used to lyse open the yeast cells to purify the proteins within, but it was seen in this purification that the noodle-grind lysis produced more pure Sec1 and greater amounts of the protein (Figure 9, Figure 10). In the cell disrupter method, the cells are resuspended in a lysis buffer solution and then pumped through the cell disrupter at high pressure multiple times to lyse the cells. Due to the yeast cells rigid cell wall, higher pressures are required and often two or more passes through the disrupter are required for optimal cell lysis. This takes time (approximately 30 minutes to an hour, depending on the size of the sample) and warms the sample, giving the protein a prime opportunity for degradation. The noodle-grind method, however, keeps the cells and resulting lysate colder, minimizing degradation. For the noodle-grind method, the cell pellet is washed and spun down, and then extruded into a conical tube of liquid nitrogen from a syringe. The process creates the "noodles." The noodles can then be ground with the grinding machine, which is equipped with steel canisters containing steel balls. The grinder shakes the canister at high speeds. The noodles are ground for approximately 90 seconds six separate times, but, in between each run, the canister containing the ground noodles is placed into the liquid nitrogen to keep the cell lysate cold. Once

the yeast cell noodles have been ground into a powder, the powder can be resuspended in lysis buffer solution (see Methods) and purified as described.

**Binding Assays**

Once the purified proteins were obtained, binding assays were performed to confirm that the purified Sec1 and Sec6 bound to each other as expected. It was observed after running the samples on a polyacrylamide gel that the Sec1 and Sec6 proteins did bind as expected (Figure 11). To explain our expected results based on the known interaction of Sec1 and Sec6, we used the first two lanes after the molecular weight marker for the inputs of Sec1 and Sec6, and they represent the amount of Sec1 and Sec6 that were used in each binding reaction assay. The next lane is the supernatant of the binding reaction in which no Sec1 was added. Without the Sec1, Sec6 should not bind to the beads or anti-V5 antibody, and we see similar amounts of Sec6 in this lane as in the input lane, which is expected because all Sec6 should be washed off of the beads. The next lane is the pellet from the same reaction (which contains the proteins boiled off of the beads), and since Sec6 does not bind to the beads without Sec1 there should be no Sec6 present in this lane. This is because with no Sec1 present there is no way for the Sec6 to bind to the sepharose or the anti-V5 antibody. There is a small amount of Sec6 observed in this lane, but it is very low in comparison to the supernatant and may be due to inadequate washing of the pellet. Binding buffer containing a higher concentration of salt or a small amount of detergent could prevent this non-specific binding in future experiments. The next lane is the supernatant of the binding reaction when both Sec1 and Sec6 are added. There appears to be less Sec6 in this lane as compared to the input lane. The next lane is the pellet of the same reaction. There is a band of both the Sec1 and the Sec6 in seemingly equal amounts. This doublet confirms that Sec1 and Sec6 interact. There is also a band present at around 50kDa in both of the pellet samples; this

is the heavy chain of the anti-V5 antibody that is eluted from the resin in the pellet when the sample is subjected to boiling in the presence of the gel loading buffer. The binding assay is an important control for the crosslinking because it confirms that Sec1 and Sec6 interact with each other.

**Crosslinking of Sec1 and Sec6**

Many cross-linking trials were run with different concentration of $BS^3$ crosslinker, and the best crosslinking appeared to occur in the range of 100μM -300μM of the $BS^3$ reagent (Figure 12). Crosslinking is a method by which two proteins are covalently bound together with a reagent that specifically reacts with a subset of molecules. The two residues that are crosslinked must be within a certain distance of each other due to length of the crosslinker used. This enables one to make assumptions about the protein complexes' three-dimensional structure when coupled with mass spectrometry.[16] This is because if the residues are interacting, they must be near each other and, with this information in hand, structural arrangements can be investigated. In order for the interaction to be mapped, the cross-linked protein is first enzymatically degraded into peptide fragments. Mass spectrometry can sequence the crosslinked fragments.

The crosslinker to be used in this experiment is $BS^3$ (*bis*[sulfosuccinimidyl]suberate), which is an amine-reactive crosslinker.[16] $BS^3$ has two reactive groups both of which are sulfo-NHS esters, which are reactive with primary amines and can covalently link lysine residues. The spacer arm length of $BS^3$ is 11.4 Å and it has a molecular weight of 572.43 g/mol (Figure 7).[17]

The crosslinking assays show that there are new, higher molecular weight bands present in the sample (Figure 12). In particular, there is a faint crosslinking band around 130kDa and another band present at a much higher molecular weight. The molecular weight of the complex is

slightly lower in molecular weight than was expected, given the combined molecular weights of Sec1 and Sec6. It is possible that the due to the size and configuration of the molecules that crosslinked complexes ran differently on the gel than would be expected. For example, it is possible that the crosslinked complex is more compact than either protein individually. Compact proteins are able to navigate through the polyacrylamide matrix of the gel than more elongated proteins. Thus, this discrepancy in molecular weight may simply be a function of the crosslinking process.

**Current and Future Directions**

Crosslinked bands from the gel were excised for mass spectrometry and will be analyzed in the coming months. Based on the mass spectrometry data, the Sec1 and Sec6 amino acid residues that are interacting will be identified. Mutations would be made systematically to the interacting residues of Sec1 and Sec6 to determine if such mutations reduce or eliminate complex formation. A reduction in binding would indicate that the residues suggested from the mass spectrometry data collected were in fact biochemically relevant to complex formation. These mutations could also be introduced into a strain of yeast to determine the biological effects of such mutations.

Another objective of this project was to begin to work towards determining the three-dimensional structure of the Sec1-Sec6 complex through crystallization and X-ray diffraction methods. To crystallize the complex, it would first be purified via size-exclusion chromatography and concentrated. The protein complex would then be screened in different conditions (i.e., buffers, salts, detergents, and varying temperatures) to assess protein crystal formation and growth. Structural analysis of any generated crystals would occur by way of X-ray

diffraction, and the collected data would be analyzed to determine the structure of the Sec1-Sec6 complex.

There is still much work to be done to understand how the Sec1 and Sec6 interaction facilitates membrane fusion and exocytosis. The findings from this research project have confirmed a previously observed interaction between Sec1 and Sec6 and have shown that purified samples of these proteins can be crosslinked. Future work will serve to identify the specific amino acid residues that participate in this interaction. Understanding this interaction will help to determine how Sec1 and Sec6 are structurally and spatially arranged as well as how they will be oriented to interact with other proteins. For example, understanding what amino acid residues of Sec6 interact with Sec1 with provide us with information about how Sec6 is interacting with the other subunits of the exocyst, as we presume that Sec1-Sec6 interface does not overlap with the Sec6-exocyst interface. In addition, determining how the exocyst subunits interact with other proteins can give insight into how they can fit together with each other to form this large complex.

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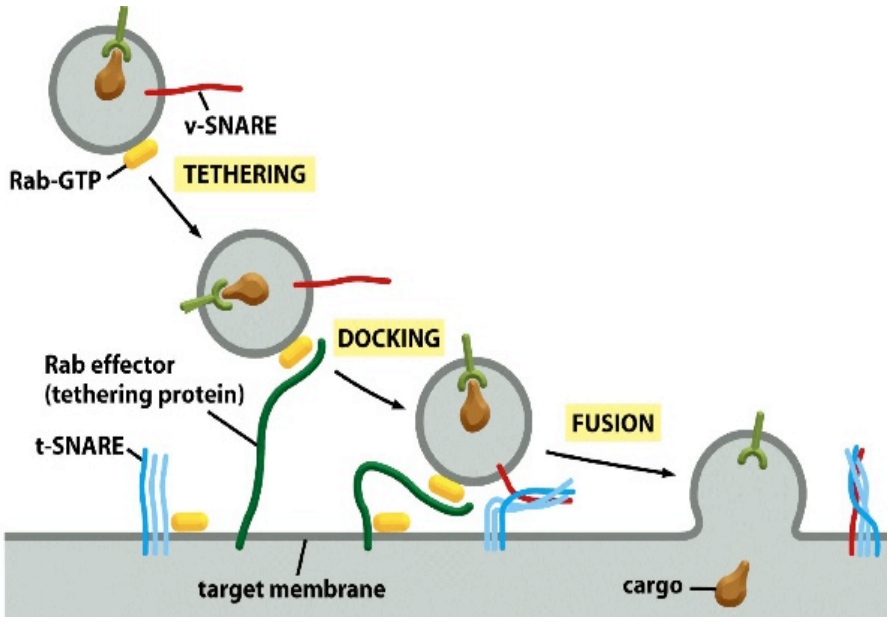


**Figure 1: Pictorial representation of the process of tethering, docking, and fusion of cell membranes using Rabs, tethering proteins, and SNAREs.[18]**

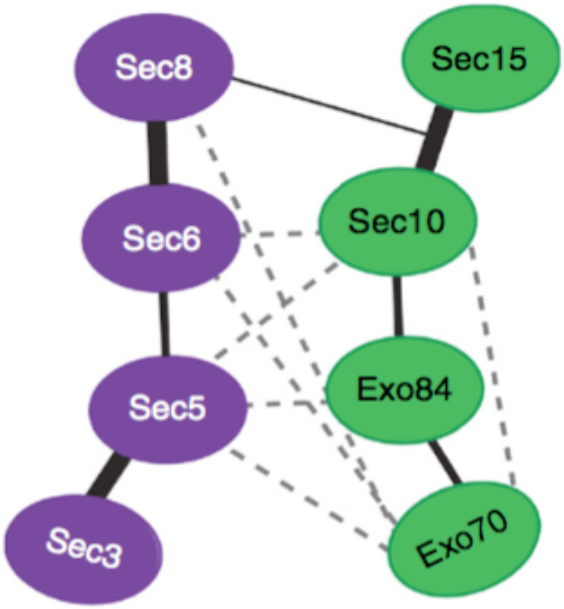


**Figure 2: Interactions of the exocysts' two modules with thicker lines indicating stronger connections and dashed lines indicating weaker connections.[7]**

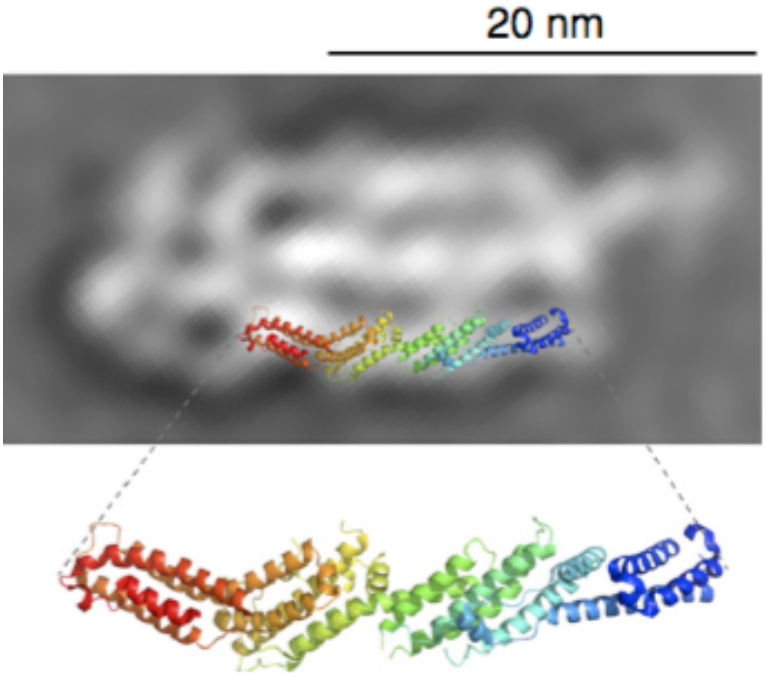


**Figure 3: Structure of the yeast exocyst complex, with Exo70 ribbon diagram overlaid to depict how it may possibly fit.[7]**

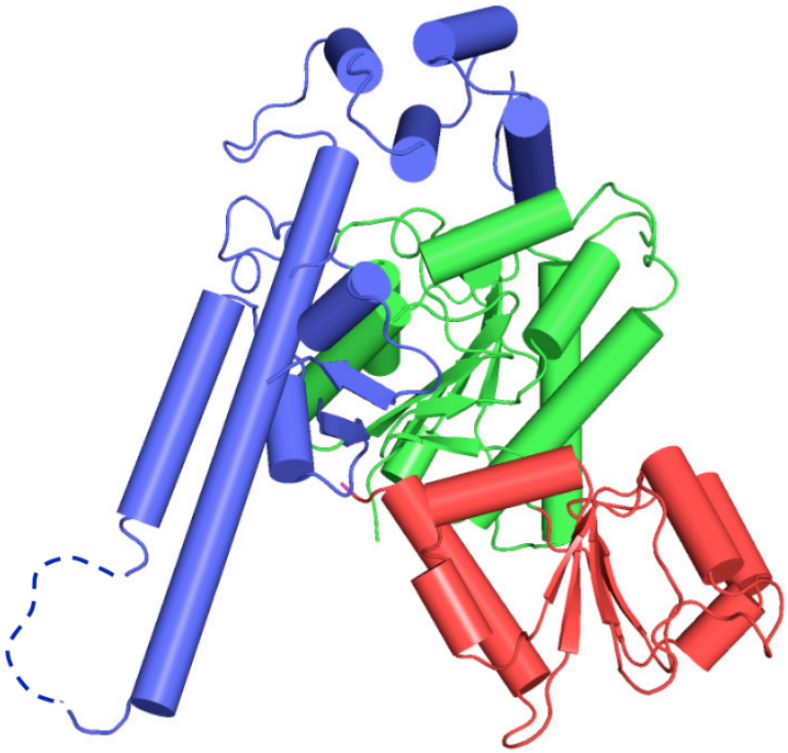

**Figure 4: Structure of Munc18 and SM protein. The structure of Sec1 itself has not yet been determined, but the SM proteins that have been crystallized have similar structure. The cylinders represent alpha-helices.[11]**

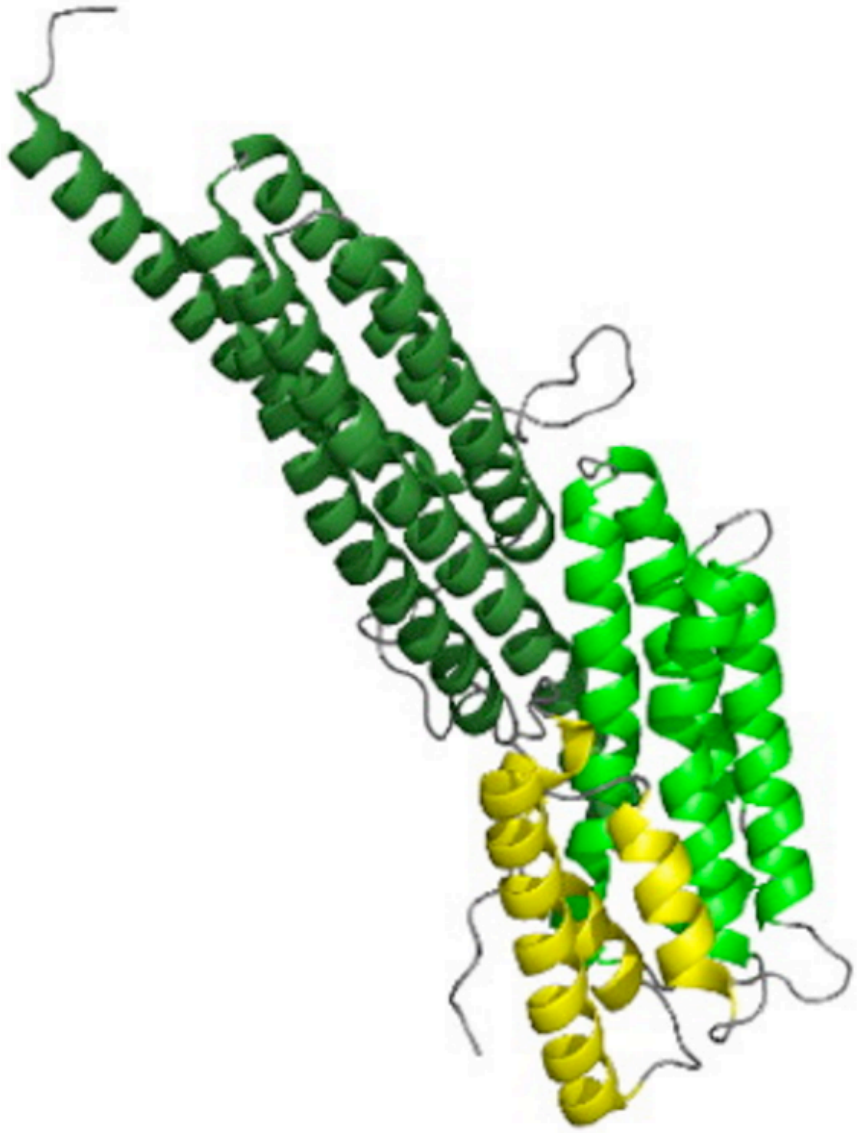

**Figure 5: C-terminal structure of Sec6 comprised primarily of alpha helices. This portion of the protein is more stable than the protein as a whole.[12]**

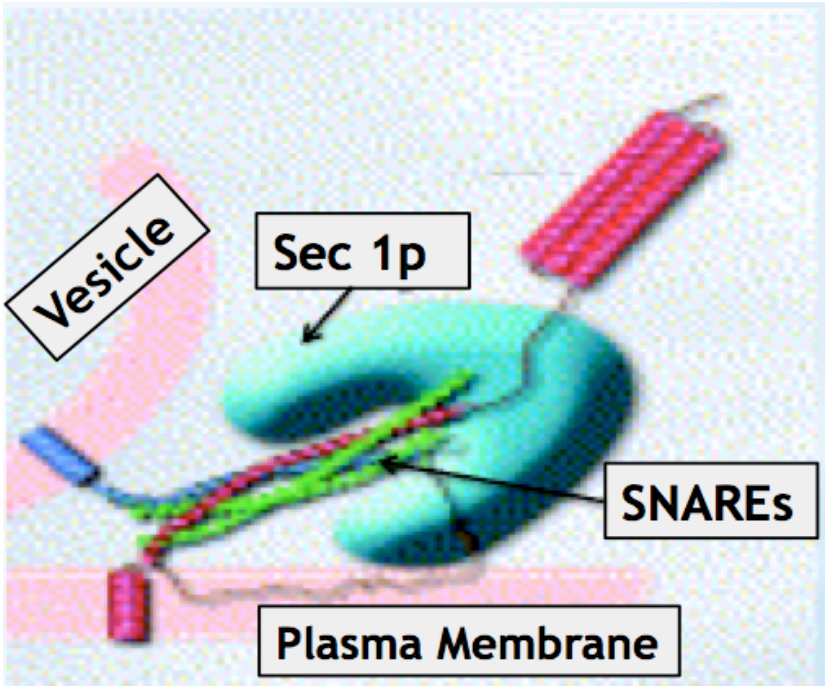


**Figure 6: Sec1 interaction with SNARE proteins.[19]**

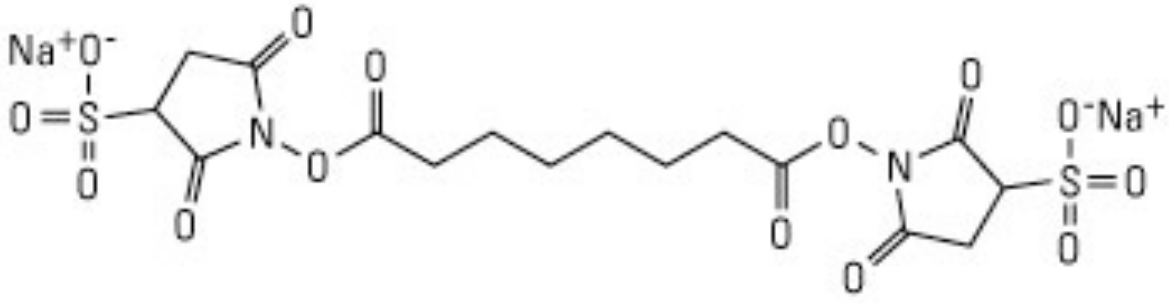


**Figure 7: The chemical structure of BS$^3$ crosslinking reagent.[17]**

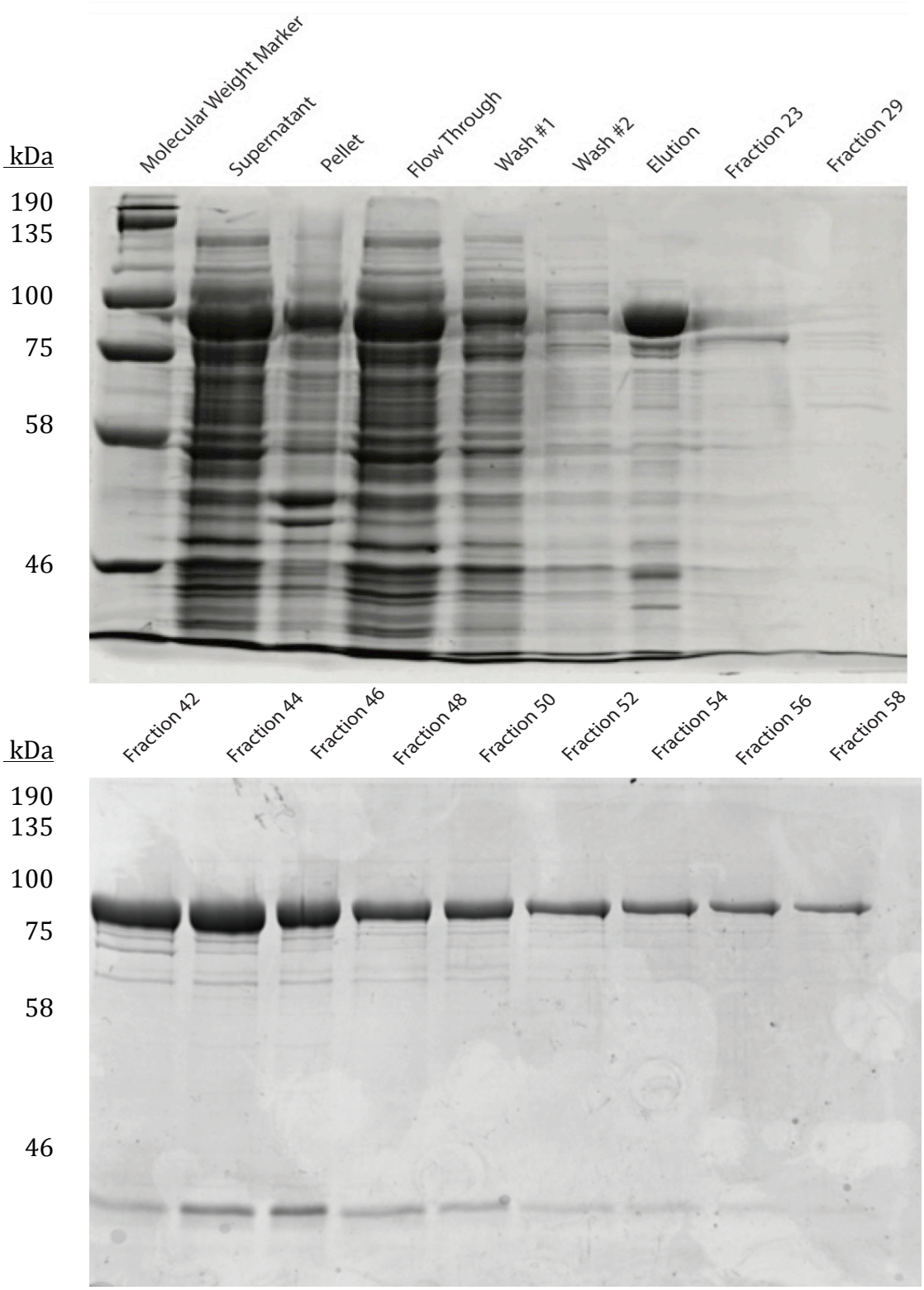


**Figure 8: 10% gel of Sec6 purification products**

Fractions 40-58 from the MonoQ 10/10 column were pooled and concentrated.

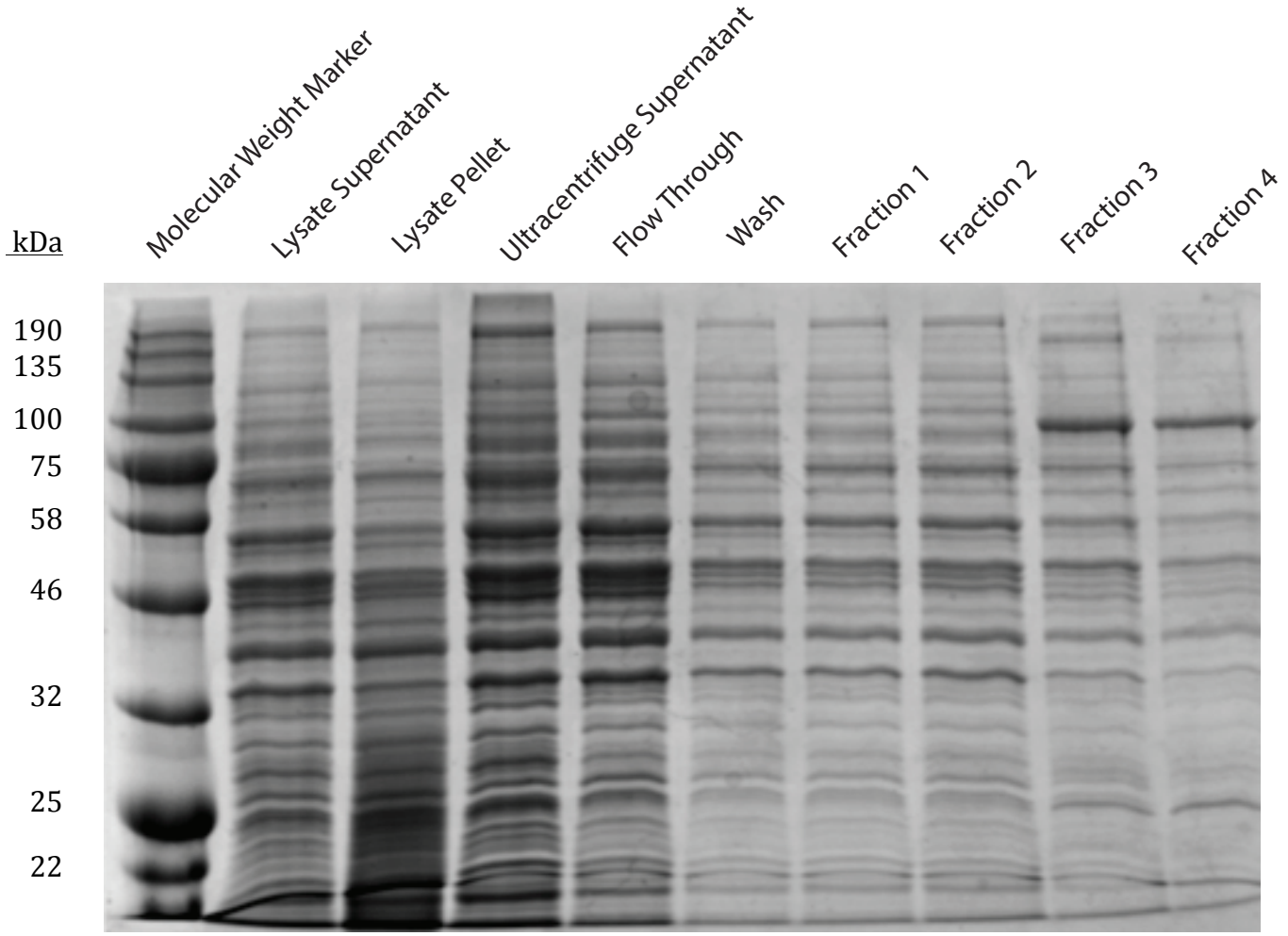


**Figure 9: 10% gel of Sec1 cell disrupter purification products**

Fractions 3 and 4 from the Ni bead BioRad column had the most distinct Sec1 bands on the gel, and Fractions 3-5 were pooled for future use.

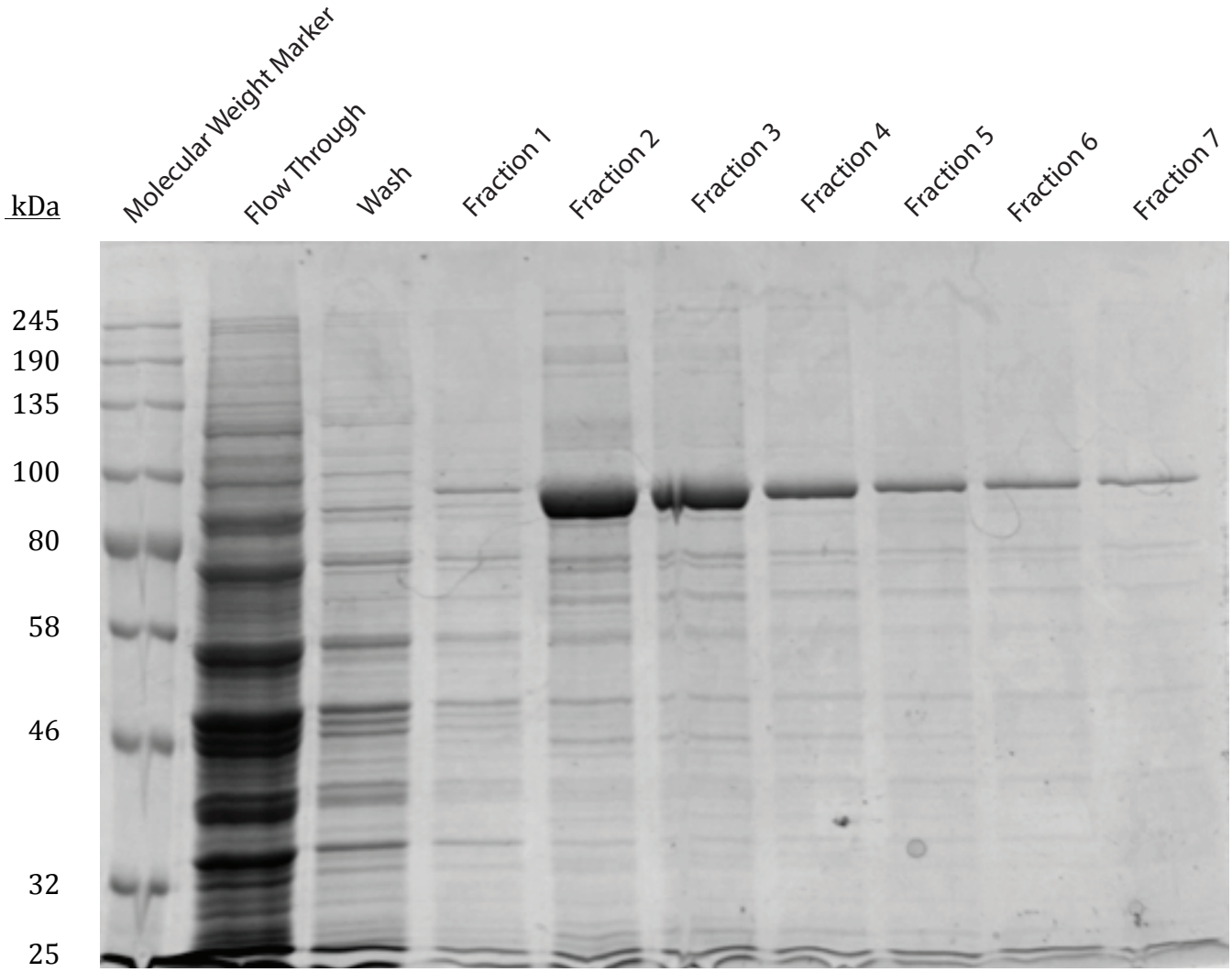


**Figure 10: 10% gel of Sec1 noodle-grind purification products**

The bands of Sec1 in fractions 2-7 from the Ni bead BioRad column are quite distinct and much larger than all other observed bands in the sample. Fractions 2-4 were the most concentrated and were pooled.

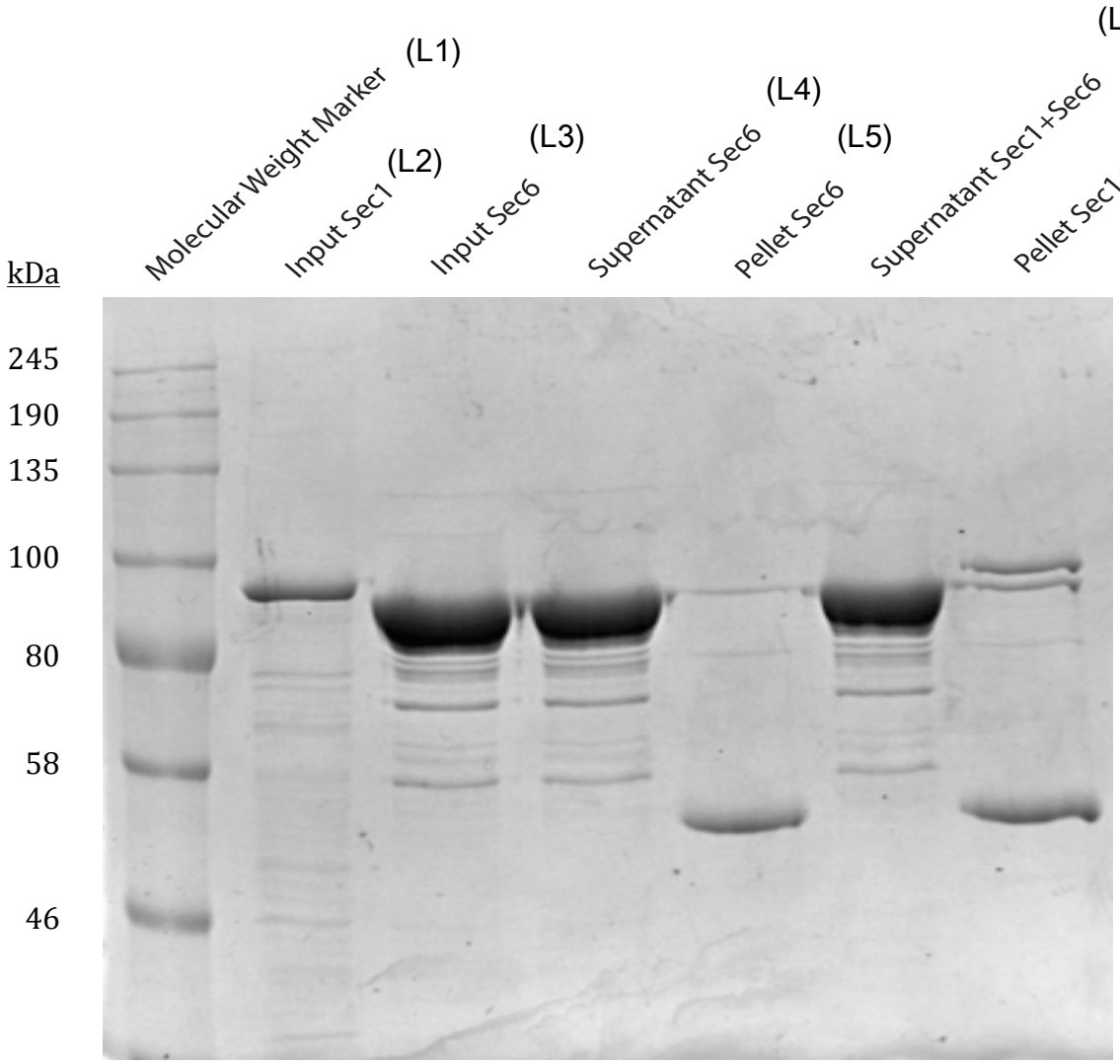


**Figure 11: 8% gel of a Sec1 and Sec6 binding assay with a 1:5 dilution of Sec6**

This gel was run to show that Sec1 and Sec6 interact. Lane 1 is the molecular weight marker, Lane 2 shows purified Sec1, Lane 3 shows purified Sec6, Lane 4 shows the supernatant of a binding reaction with no Sec1 (control), Lane 5 shows the pellet of a binding reaction with no Sec1 present (control), Lane 6 shows the supernatant of a binding reaction with Sec1 and Sec6, Lane 7 shows the pellet of a binding reaction with Sec1 and Sec6. The two bands in Lane 7 indicate that Sec1 and Sec6 bound to each other during the binding assay and dissociated when they were bead boiled. The large band that is low in the fifth and seventh lanes is the heavy chain of the antibody; the light chain has been run off the gel to better observe the separation of Sec1 and Sec6.

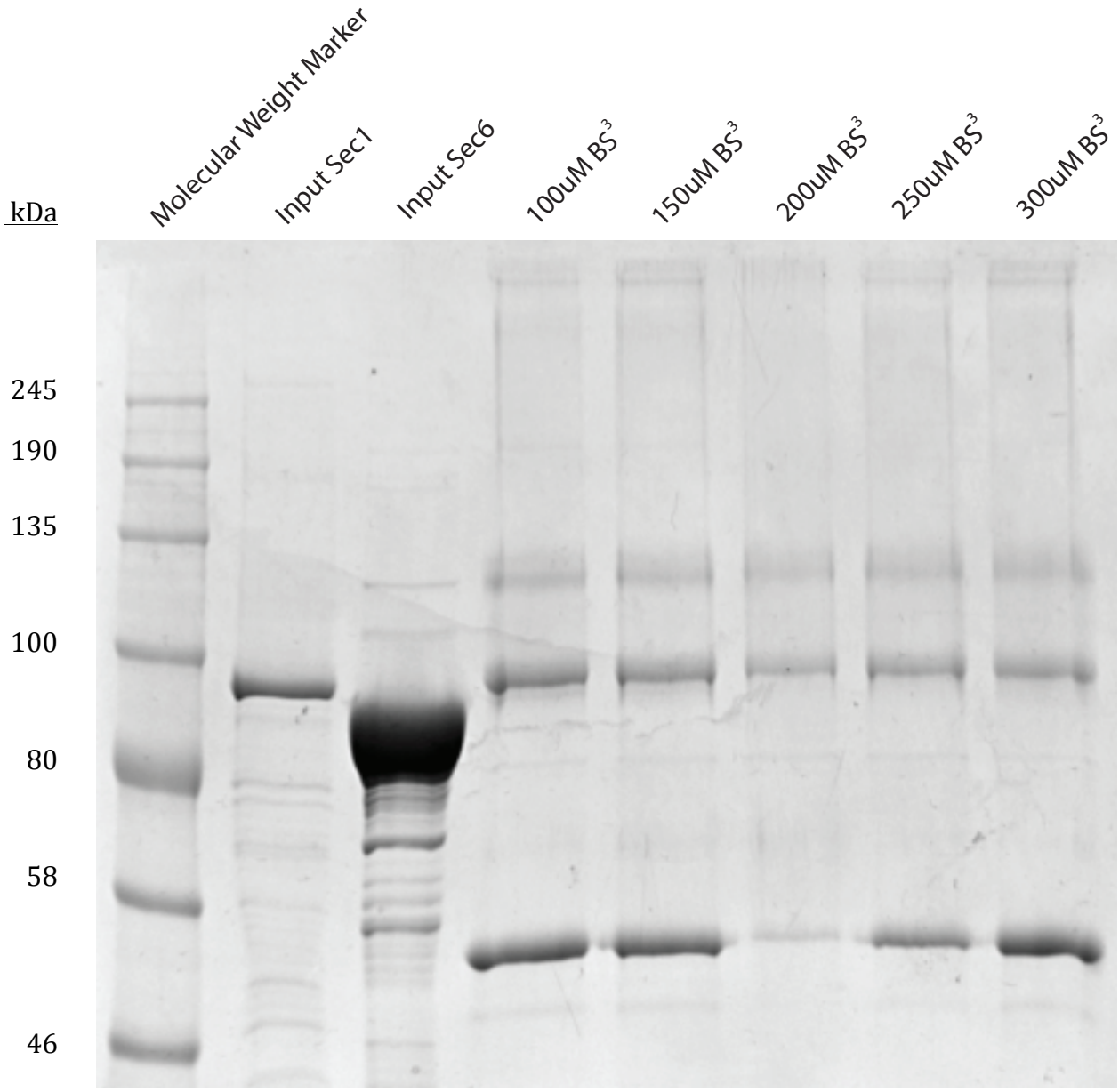


**Figure 12: 7% gel of a Sec1/Sec6 crosslinking experiment with 100-300µM concentrations $BS^3$**

The crosslinked protein is above the Sec1 band because it has a greater molecular weight. There is no apparent Sec6 band in the crosslinking experiments because there was excess Sec1, so all of the Sec6 bound to the Sec1 and unbound Sec6 was washed away. There was excess Sec1 that did not crosslink and that is why there is still a distinct Sec1 band still present. The lowest band in the crosslinked samples is the heavy chain of the antibody; the light chain was run off of the gel.