UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of the light producing system from the luminous brittlestar *Ophiopsila californica* (Echinodermata)

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Marine Biology

by

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2017

The Thesis of Leeann Alferness is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2017

DEDICATION

This thesis is dedicated to my mother Karen Oberkamper for unconditional support throughout my journey in pursuing my passion of marine biology as well as my father Phil Alferness for his scientific guidance and encouraging me to challenge myself with a focus in biochemistry. I would also like to dedicate my thesis to classmate and roommate Kimberly Chang for who I am forever grateful for our friendship and ability to help each other though this journey together. Lastly I would like to dedicate this work to my advisor Dimitri Deheyn and the Deheyn lab for their continued support through this challenging project.

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ACKNOWLEDGEMENTS

I would like express my deep gratitude to my advisor, Dimitri Deheyn, PhD for guiding me though this challenging project. Your positive attitude and never ending ideas allowed me to continue searching for answers even when it looked like a dead end. This experience you have given me allows me to go forward in the world of science with confidence, knowing how to working though difficult projects.

I would also like to recognize my in-lab mentor Evelien De Meulenaere, PhD for her help in learning new lab techniques and endless brainstorming sessions. I have learned so much from you these past two years and I don't think this project would have been possible without your support. I would also like to thank you for introducing me to the wonderful world of vendor shows, I can't imagine ever paying for a pen again.

I would also like to thank my committee members Assistant Professor Deirdre Lyons and Michael Latz, PhD for your support and willingness to be a part of my master's thesis project.

In addition to those above, I would like to thank Phil Zerofski for collecting all of the experimental animals needed for this research. To Professor Mark Hildebrand, thank you for allowing me to you your ultra-centrifuge that was necessary to complete this work. I would also like to thank Phil Alferness for facilitating MS analysis.

Finally, I want to give a huge thank you to all of my lab mates in the Deheyn lab both past and present. I especially want to thank Kimi Chang for her constant support and going through this process with me, as well as Michael Allen for is ability to fix anything in the lab when in breaks in the middle of an experiment. Being a part of this lab has taught me so much and anyone that has passed though even if only for a summer has had

an impact on my wonderful experience at Scripps Institution of Oceanography and I will miss you all dearly!

ABSTRACT OF THE THESIS

Characterization of the light producing system from the luminous brittlestar *Ophiopsila californica* (Echinodermata)

by

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Master of Science in Marine Biology

University of California, San Diego, 2017

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Light producing proteins (photoproteins) are attractive molecules to study because of their potential applications in biotechnology and biomedicine. Photoproteins have been identified in several different phyla, however a photoprotein from the phylum Echinodermata has yet to be characterized. The goal in this work is to extract the calcium-dependent photoprotein from brittlestar *Ophiopsila californica* for purification and identification. The photoprotein was successfully extracted while maintaining activity. Three chromatographic techniques were used to separate the functional

photoprotein from other constituents in the brittlestar extracts. The light producing fractions from each of these chromatography methods were analyzed using gel electrophoresis in attempt to identify a band that correlates with the amount of light production when comparing multiple fractions. A ~45 kDa band showed this positive correlation throughout each purification step. This band was characterized by ESI-QTOF-MS but did not result in the identification of a new photoprotein. Similar methods were attempted on isolated photocytes (light producing cells). However nearly all light producing activity was lost when attempting to extract the photoprotein from the cell. This indicated that the photoprotein relies on an intact membrane in order to function. This leads to the idea that the activation of this photoprotein could be different than the one previously proposed.

Executive Summary

Brittlestar *Ophiopsila californica* (a starfish cousin) produces bright flashes of green light when mechanically stimulated. Aside from the fact that the light production is reported to be calcium-dependent and involves a photoprotein system (thus not a traditional luciferin-luciferase system), no information is available on the identity of the photoprotein itself. The goal in this work was to extract the active photoprotein in solution, and to concentrate and purify it before moving into its sequencing and identification. The light production was used as a traceable marker, of the functional photoprotein throughout this process. By grinding brittlestars in calcium-free buffer, the photoprotein was extracted while maintain its ability to produce light upon appropriate excitation. Anion exchange (DEAE) chromatography was then used for initial separation of the functional photoprotein from the other constituents in the brittlestar extracts. Elution fractions were monitored for light production by adding calcium. Light production in certain fractions confirmed the presence of functional photoprotein. These fractions were then analyzed using gel electrophoresis in an effort to correlate light production with a specific protein band across different fractions. A ~45 kDa band appeared to correlate well with the amount of light production found across different elution fractions. Two additional chromatography methods were used to further purify the sample as well as confirm such protein abundance-luminescence activity correlation. The correlation of the ~45 kDa band and light production did decrease with further purification, however its presence was always noticed in every light producing fraction. This band was characterized by ESI-QTOF-MS, the results of which were inconclusive in terms of identifying a new photoprotein. To increase sample purity and concentration of

the photoprotein, a similar research approach was also performed on isolated and concentrated photocytes (light producing cells). Upon cell lysis and detergent extraction nearly all light-producing activity was lost from full photocytes. This supports the hypothesis that the photoprotein has a transmembranous domain and is unable to function outside of the cell membrane. Further experiments to characterize the activation of the photoprotein with in the cells will be reported and discussed.

Introduction

1. Bioluminescence from the Ocean

Bioluminescence is the production and emission of visible light from a chemical reaction in living organisms. Bioluminescence is relatively rare when considering the total number of species in which it is found. In the marine environment, it is extremely diverse in its occurrence, with bioluminescent species represented in almost every major phylum, from small unicellular eukaryotes to large vertebrates. The light is most often produced by the organism itself, although it is produced by some symbiotic bacteria as well (*1*). Bioluminescence is typically associated with the deep sea, because of fantastic and iconic images of luminous creatures released by the media from this extreme environment. However, many bioluminescent organisms can be found in intermediate depths as well as the shallow intertidal, but then mainly active at night.

Bioluminescence has a broad range of evolutionary functions for marine organisms that fall into two categories; being representative of a detoxification processes and/or being involved in visual communication. Evidence suggests that bioluminescence evolved first as a detoxification mechanism, defending against oxidative stress by scavenging reactive oxygen species (ROS), with the light being a by-product of antioxidative functions (*2, 3*). The requirement of oxygen before or during a bioluminescent reaction will be discussed in detail below. Organisms also use their ability to produce light for both interspecific and intraspecific communication. Organisms have been found to use bioluminescence to defend themselves against predators, as well as use it to help find/lure prey. It has also been shown that animals use their light production to communicate to members of their own species, for example attracting a

mate. Although these functions of bioluminescence are well accepted it is difficult to identify definitively, without an anthropocentric view, how any certain species uses their bioluminescence, thus more thorough research is needed in the area.

The study of light producing organisms has a wide source of applications from tracking molecules in biomedicine to commercial products such as light producing reporters of gene activity or diseases, or bioluminescent tools/toys such as the glow stick. It is estimated that bioluminescence in marine organisms has evolved separately at least 40 different times (*1*). Each evolutionary opportunity represents a different chemical mechanism, and of these 40 occurrences only 3-5 bioluminescent chemistries are well known today. This indicates great potential for the study of these light producing organisms with many undiscovered applications still to be revealed.

2. Bioluminescence in Brittlestars (Ophiuroids)

Brittlestars (Ophiuroidea, Echinodermata), close relatives of the sea stars, are characterized by a round central disk that is offset by five arms, with some departing from this generalized shape, having up to ten arms. They are known for many distinct features that make them very evolutionarily successful. These include: regeneration of body parts, cryptic pigmentation and camouflage, and unique reproductive abilities (*4*). In addition, the ability to produce visible light is widespread and relatively abundant in this class, with about 35% of 222 species tested being bioluminescent, out of a total of 2,064 total described species (*5*). Brittlestars are known to use their light producing abilities in several ways to defend themselves against predators. The main defense mechanism occurs in two steps. The first is to produce intense light to blind or startle the predator that initiated a physical contact. If the predator continues its attack the brittlestar can then

go through self-autonomy of an arm that continues to produce light to distract and lure away the predator. The bioluminescence is then terminated in the main body, allowing the brittlestar to escape undetected (*6-8*). Brittlestars are also known to coordinate light production within dense populations of their own species, a mechanism referred to as the *burglar alarm,* making the attacker more visible to its own predator (*9, 10*). Additionally, evidence suggests that light production can serve as an aposematic warning: the presence of a bright signal used as a warning to predators in unpalatable species (*6, 10*).

The species of interest in this work is the luminous brittlestar *Ophiopsila californica* (Figure 1). *O. californica* is one of the larger brittlestar species easily collected off the coast of California (arm length <200 mm, disk diameter <15 mm). It has bright orange arms that produce intense green luminescence. *O. californica* can be found off the coast of San Diego where they reside in sub-tidal sandy environments.

3. Chemistry of Bioluminescence

Light in most bioluminescent organisms is produced by a chemical reaction in which two separate chemicals are required, known as the luciferin-luciferase reaction. One is the substrate, generically referred to as luciferin that produces the light, and the other is the enzyme commonly referred to as luciferase that catalyzes the oxidation of luciferin. The binding of the luciferase makes luciferin substrate negatively charged and easily oxygenated (*11*). The luciferin molecule utilizes a structure known as a chromophore, which is usually an aromatic system of alternating single and double carbon-carbon bonds. When oxidized, electrons in the chromophore go from a ground state to an excited state and subsequently release energy as photons (Figure 2A) (*12*).

In some cases, however, like in *O. californica,* the luciferin and the catalyzing

protein (apoprotein) are bound together with oxygen to form a single unit known as a photoprotein (*13, 14*). At this point the reaction is incomplete and "on hold". The photoprotein can then be triggered to produce light with the addition of a particular cofactor ion, typically calcium. When the cofactor binds to the photoprotein the chromophore facilitates an increase in the energy state of electrons and light is produced by the relaxation of the electrons back to their ground state (Figure 2B). This reaction can occur in the presence or absence of oxygen which provides a great advantage to organisms that are regularly exposed to hypoxia (*13*). Luciferins are relatively highly conserved among phyla with four major luciferins being responsible for most light production in marine species. Conversely, luciferase and photoproteins are distinctive and resultant from many evolutionary lineages (*1*). The high diversity of these proteins postulate the potential for many different applications for light producing proteins in biotechnology and biomedicine and call for the continued exploration and discovery of new light producing molecules.

4. Light Production form *Ophiopsila californica*

As stated previously, a photoprotein is responsible for the light production in *Ophiopsila californica*, with the cofactor thought to be calcium (*15*). However, the photoprotein (chromophore as well as apoprotein entity) still remains unknown to this day. A distinguishing feature of *O. californica* is that the light production process may either be highly associated with the radial nerve cord or even be of neural origin. This is demonstrated by the propagation of the neuro-influx along the radial nerve cord being almost equal to the propagation of the luminesces along the arm (*15*). Many studies on bioluminescence of brittlestars have been done on a closely related species *Amphipholis*

squamata. Studies on *A. squamata* show that upon light production, there is a dynamic movement of membranes and merging of vesicles inside the photocytes (light producing cells) (*16*). This movement is thought to play a role in optimizing the efficiency of the light production, by maximizing the surface to volume ratio, and it is likely that a similar process happens in *O. californica* as well. Indeed, from the literature, it appears that both *A. squamata* and *O. californica* share large similarities in the light production process, in terms of neuro-control, spectral emission and dependence to calcium (*15*).

After the emission of light, the photoprotein in *O. californica* becomes fluorescent (*15*). Bioluminescence shows a wide spectrum, peaking at 495-515 nm, whereas when excited at 380 nm the fluorescence peak is slightly shifted at 518-526 nm (Figure 3). This observation had also been reported earlier and indicates an allosteric change (*15*). As discussed above, the chromophore is an integrated part of the photoprotein. However, it is unknown whether the chromophore remains associated with the photoprotein when fluorescence occurs. Though it is unlikely that the chromophore remains attached to the apoprotein, (based on known photoprotein chemistry) it should remain in the same sample/fraction as the photoprotein as long as the light production is stimulated after separation. This would allow measuring the photoprotein presence based on fluorescence if indeed the same chromophore is responsible for the bioluminescence and fluorescence. This ability would be very beneficial since bioluminescence can be transient and easily lost, while fluorescence is much more stable and reproducible.

5. Project Prospects and Goals

Describing an unidentified photoprotein is an attractive topic because of the potential applications in biotechnology and biomedicine. Its possible neural origin

indicates high potential in the development of neuro-toxicity assays. Very few studies have been done on the bioluminescent system of *O. californica*, or brittlestars in general. This would be the first light producing protein ever identified for an Echinoderm and it could have different properties than the few known photoproteins, primarily from the Cnidarian phylum. This could expand the use of light producing proteins in many fields.

The objective in this work was to extract (while maintaining its activity), purify and identify the photoprotein from *Ophiopsila californica*. In pursuit of this goal three platforms of different starting materials were used: frozen brittlestar arms, fresh arms, and isolated photocytes (light producing cells). Initial extraction and purification assays were done on frozen arms. It was important to use frozen arms to see if the photoprotein could still be activated after a prolonged period in the freezer, which would be essential for potential future research and manufacturing. Using fresh arms was implemented when a higher starting amount of active photoprotein was needed throughout the purification process. This process consisted of three orthogonal methods of chromatography; anion exchange followed by hydrophobic interaction and size exclusion. Chromatography fractions were tested for light production and run on electrophoresis gels. Protein bands that have a high correlation between light production and band size were then sent for sequencing, and molecular tools could be used to characterize the possible photoprotein. Lastly, intact photocytes were extracted/isolated to provide a higher concentration of photoprotein for purification. This acts as an initial purification step by separating out all other proteins from other cell types. This work is separated into two sections: 1) Research originated from full arms (Frozen and Fresh) and 2) Research originated from isolated photocytes, followed by a comprehensive discussion and conclusions.

Materials and Methods

The challenging nature of this project required the use of many different techniques using different platforms of samples; full arms (frozen and fresh) and isolated photocytes (light producing cells). A schematic of the sample platforms used for each procedure is represented in Figure 4. Overall, these techniques are aimed at extracting and purifying the active photoprotein for identification.

1. Research Originated from Full Arms (Frozen and Fresh)

1.1 Live specimen Collection

O. californica brittlestars were collected via SCUBA, using a small gardening shovel to dig the specimens out of the sediment (in this process, some arms would be lost by autotomy or by sectioning with the shovel, and these lost arms were also collected and preserved for further investigation). Separated arm samples were frozen at -80^{o}C prior to sample preparation. Surviving brittlestars, showing a full disk with at least a few arms, were kept alive in Marine Biology Research Division experimental aquarium at Scripps Institution of Oceanography (UC San Diego) until fresh samples were needed.

1.2 Arm Collection from Live Specimen

The live brittlestars were placed into 3.5% $MgCl_2$ for anesthesia. Once the brittlestar stopped moving (time varies between animal, but ranged from 10-20 min), no more than 1-3 arms were cut off close to the disk to ensure survival of the animal and regeneration of the lost arms. The brittlestar was then placed back in the aquarium tank and recovered within 15 minutes. Note: brittlestar should not remain in $MgCl_2$ solution for more than 30 minutes, after which anesthesia becomes lethal.

1.3 Sample Preparation

After weighing, the frozen or fresh arm was homogenized using a chilled mortar and pestle in 3-4 mL 50 mM TRIS-HCl in MilliQ water, pH 8.1 (referred to as the extraction buffer), per 0.5 g of arm. This calcium-free buffer was selected so no external calcium is provided that could cause light production prior to extraction and purification. A 3-mL aliquot of slurry was transferred to two 1.5 mL Eppendorf tubes and centrifuged at 9,000 RPM for two minutes using Beckman Microfuge®18 Centrifuge. The supernatant from both tubes were combined and represent the starting material for the first of two chromatography steps.

1.4 Measuring Luminescence

All the extraction and purification efforts were evaluated using ability of the photoprotein to produce light. This marker was assessed at many steps throughout purification in order to track the presence of active/functional photoprotein. Luminescence was tested for using a kinematic assay via the Sirius single-tube luminometer from Berthold Detection Systems. The luminometer was programed to inject v:v 50 µL of 400 mM $CaCl_2$ after 10 s and 100 µL of 10% H_2O_2 after 40 s. In general the typical response curve results in a sharp increase in signal, the signal reaching an apex, followed by a tailing decrease in intensity (Figure 5). The area under this "peak" after the $CaCl_2$ injection should be proportional to the concentration of photoprotein in the sample (*13*). The area under the peak after the 10% H_2O_2 would indicate the chromophore, pigment, and other proteins capable of oxidation and production of light. This assay shows which fractions contain the most photoprotein and therefore be used for further purification.

1.5 DEAE Ion Exchange Chromatography

Anion exchange chromatography was used for the first purification step of the supernatant. A diethylaminoethyl cellulose (DEAE) chromatography column was prepared by mixing DEAE resin (Sigma Aldrich, 45-165 μm) in Milli-Q water allowing resin beads to expand. Resin solution was then transferred into a 10-mL plastic gravity-feed chromatography column (BIORAD). Final bed volume was 0.8-1.0 mL, which is sufficient for up to 0.5 g arm sample. Using gravity elution, the column was then equilibrated with 20 mL of TRIS-HCl extraction buffer. The supernatant was pipetted onto the column and the flowthrough was then collected. After the flowthrough was collected three NaCl step gradient elutions were performed. Aliquots of 50 mM, 200 mM, and 400 mM NaCl in 50 mM TRIS-HCl were added consecutively to the column in 3-5 bed volume quantities. The eluates were collected in 1-mL fractions and each fraction tested for luminescence and then frozen at -80^{o} C. Light producing fractions from multiple DEAE chromatographic runs were pooled together and concentrated for further purification.

1.6 Ammonium Sulfate Precipitation

In an effort to concentrate the photoprotein in the collected fractions, an ammonium sulfate precipitation procedure was optimized. Optimization showed that 75% ammonium sulfate saturation provided the best yield. Ammonium sulfate was weighed out according to the volume of the combined fractions and slowly added to the sample over two minutes. The sample was then stirred on ice for 15 min, followed by a centrifugation at 15,000 RFC for 15 min. The supernatant was then removed and tested for light production to determine if the photoprotein had precipitated. After precipitation

was confirmed, the pellet was then resuspended in the desired volume of 50 mM TRIS-HCl buffer and tested for light production.

1.7 Hydrophobic Interaction Chromatography

Hydrophobic interaction chromatography (HIC) was then used as a secondary purification step. A 1-mL HiTrap™ Phenyl HP column on an ÄKTA Purifier FPLC system from GE Healthcare Life Sciences was prepared by washing with five column volumes (CV) of elution buffer: 50 mM TRIS-HCl, pH 8.1. This was followed by a second run of ten CV washes with the start buffer: 50 mM TRIS-HCl, 1.5 mM ammonium sulfate, pH 8.1. Fractions (500-1,000 μL) of concentrated DEAE fractions were then loaded onto the column followed by a wash with the start buffer until UV trace returns to baseline. Next, a gradient elution from 100% start buffer to 100% elution buffer over 15 min, at a flow rate of 1 mL/min was started. The eluate was collected in 1-mL fractions and each fraction tested for luminescence.

1.8 Size Exclusion Chromatography

Size exclusion chromatography was also used as an additional secondary purification step. A Superdex 200 10/300 GL column on an ÄKTA Purifier FPLC system from GE Healthcare Life Sciences was prepared by equilibrating ten CV of 50 mM TRIS-HCl, 200 mM NaCl, pH 8.1 buffer. After which, 700 μL of sample (concentrated DEAE fractions) was loaded onto the column. The column ran at a flow rate of 1 mL/min until no additional UV peaks were being detected. The eluate was collected in 1–mL fractions and tested for luminescence.

1.9 Total Protein Measurement

200 µL of each sample was added to a 96-well plate and the absorbance at 280 nm was taken using Molecular Devices Spectramax® i3 Spectrophotometer. This provides a bulk estimate of the total protein concentration in each sample. This allows the calculation of the specific activity of each elution fraction. The specific activity in this case is the ratio of photoprotein activity (Calcium peak integral, RLU) versus the measure of the total protein present ($A_{280\ nm}$).

1.10 Gel Electrophoresis

Two types of gels were utilized: Native gels which do not denature proteins and SDS- PAGE gels that do denature proteins.

Native Gel: 6% polyacrylamide native gel was cast using 30% acrylamide/BIS solution (BioRad). Aliquots (16-µL) of DEAE elution fractions were then loaded on to the gel and run for 1 hour and 40 minutes at 100V. The gel was imaged for fluorescence using an excitation wavelength of 365 nm.

SDS-PAGE Gel: Elution fractions were mixed with SDS loading dye (4 µl 4X SDS loading dye + 12 µL sample). They were then loaded, along with Benchmark prestained ladder onto a 4–20% precast polyacrylamide gel from BIO-RAD. The gel ran for 40 minutes at 200 V and was then stained with InstantBlue™ (Expedeon) Coomassie protein stain. Poor resolution of the BIO-RAD gels and InstantBlue™ resulted in switching to all subsequent SDS-PAGE to Blot™ 4-12% Bis-Tris Plus gels from Invitrogen by Thermo Fisher Scientific. A more sensitive stain, SYPRO® Ruby Protein Gel Stain from BIO-RAD, was also employed.

1.11 SDS-PAGE Gel Band Identification by ESI-QTOF-MS

Relevant SDS-PAGE gel bands were excised and subjected to the in-gel tryptic digestion protocol from Shevchenko, 2007. Digests were subjected to RP-HPLC separation using an Agilent 1290 UPLC system with a Zorbax C18, 50 x 2.1 mm, 1.8 μm particle-size column. Mobile phases were A) 0.1 % formic acid in water, and B) 0.1% formic acid in acetonitrile, using a multistep linear gradient from 1% B to 50% B in 36 minutes.

Tandem mass spectrometry was conducted using an Agilent 6550 electrospray ionization quadrupole-time-of-flight mass spectrometer (ESI-QTOF-MS). The system was operated in the positive ion mode; capillary voltage 4500V; gas temp 200˚C; fragmentor 175V; nozzle voltage 1000V; drying gas 14 L/minute. Collision induced dissociation (nitrogen as the collision gas) was used to fragment peptide ions into the characteristic B/Y ion series. Scan rate was set at 2 scans per second with up to five precursor ions per scan selected for fragmentation.

The resulting tandem mass spectra were subjected to an extensive protein database search using MASCOT MS/MS Ion Search (Matrix Science) which uses a probabilistic scoring algorithm for protein identification based on the precursor mass in addition to the mass and abundance of the B/Y ion series. The protein database used for MASCOT searches was NCBInr (Nation Center for Biotechnology Information non-redundant).

1.12 Data Processing and Analysis

Raw data outputs recorded in RLU/s, measured every 0.2 s, from the luminometer were exported from the luminometer microprocessor operating software to Microsoft

Excel. Each elution fraction was then analyzed using a scatter plot of RLU/s vs time. The integral of the 30 s calcium peak was then calculated for each elution fraction to find the area under the curve in order to take into account both the intensity as well as the duration of the light production. All figure error bars represent standard error of the mean (SEM) and all data are reported mean ± SEM. At this stage, only descriptive statistics were done because not enough replicate experiments were always possible.

2. Research Originated from Isolated Photocytes

The goal of the following two procedures is to dissociate and isolate the photocytes based on methods established in Deheyn *et al.* 2000.

2.1. Cell Dissociation

A fresh *O. californica* brittlestar arm was collected according to the procedure in section 1.1. The arm was then dissected in roughly 1-2 mm segments and placed in a tube with 3 mL of 0.5% protease (P5147 SIGMA) dissolved in artificial sea water (ASW). The arms segments were then incubated at 28˚C for 35 min with light agitation. Next, the segments were left to settle at the bottom of the tube and the supernatant, containing the dissociated cells, was removed and centrifuged for 5 min at 2,000 RPM. The supernatant was then removed and the cell pellet resuspended in ASW. This was repeated 3 times total, with an end volume of 1.1 mL of dissociated cells in ASW.

2.2. Photocyte Isolation

In order to separate cell types, a 63% Percoll® gradient with ultra-centrifugation was used. The Percoll® solution was diluted to 63% with ASW and osmolarity was adjusted using NaCl to that equal of the 100% ASW to prevent cells lysis. The ultra-centrifuge tube was filled with 10.6 mL of 63% Percoll® solution and 1.1 mL of the

dissociated cells. The mixture of Percoll® and dissociated cells was then centrifuged for 30 min at 20,500 RMP (Beckman L8–70 ultracentrifuge, rotor SW 41). This resulted in visible layers of cells that were tested for luminescence. The band with the most light production (photocyte layer) was then used for further experiments. Note: luminomerter kinetics assay (section 1.4) was modified for testing luminescence of photocytes. The new assay injects 400 mM $CaCl_2$ at 30 s and continues measurements until 60 s.

2.3 Photocyte Lysis

In an attempt to extract the active photoprotein from the photocytes, cells were lysed using four different methods. In all methods cells (600 µL aliquots) were tested for their initial luminescence then lysed. Contents were then centrifuged at 9,000 rpm for 2 min. The supernatant was then tested for light production as well as the pellet that was resuspended in 600 µL Ca-Free ASW.

1) Physical sheering: Cells were pumped in and out of a 27-gauge needle 20 times.

2) Sonication: Cells were sonicated while on ice for 3 minutes.

3*) Bead Beating:* Cell were put in 2ml tube with 1.4 mm beads placed inside a 7-mL tube filled with ice water to keep the sample from heating. Sample was homogenized for 3 minutes with Bead Mill Homogenizer by Bertin.

4) Homogenization: Cells were frozen with liquid nitrogen and homogenized with a mortar and pestle.

2.4 Detergent Extraction

In an effort to extract the photoprotein from the membrane into solution, four different detergents were used: octyl β-D-glucopyranoside (OBG), Triton X-100, sodium deoxycholate (SDO) and Tween 20. Photocyte extracts (400 µL) were tested for light

production and then spun down at 2,000 RPM for 5 min. The supernatant was removed and the cells were resuspended in 400 µL of the corresponding detergent in a wide range of concentrations (detergents dissolved in Ca-Free ASW). The concentration range was chosen based on the critical micelle concentration (CMC) of each detergent. The cells were incubated in the detergent for 5 min and centrifuged for 2 min at 9,000 RPM. The supernatant was then tested for light production as well as the pellet that was resuspended in 400 µL Ca-Free ASW.

2.5 Calcium Ionophore Treatments

A dose-response experiment with concentrations ranging from 10^{-4} through 10^{-10} M of calcium ionophore A23187 was carried out to determine the concentration required. After completion, a concentration of 1 µM was chosen for further experiments. The background luminescence of the photocytes was measured for 30 s followed by an injection of 1µM of A23187 after which the luminescence was measured for 30 s. At 60 s an aliquot of 400 mM $CaCl_2$ in ASW was injected and measured for 30 s.

Results

1. Research Originated from Full Arms (Frozen and Fresh)

1.1 Photoprotein Extraction (Frozen)

Extraction of the photoprotein in calcium-free 50 mM TRIS-HCl buffer was successful based on the ability of samples, homogenized in this buffer, to produce light with the addition of calcium as well as H_2O_2 (Figure 5). The H_2O_2 peak is consistently much higher than the calcium peak because H_2O_2 oxidizes the chromophore and possible other aromatic compounds and even pigments. Unlike the calcium it is not selective for the photoprotein exclusively. Light production upon the addition of calcium allowed the use of chromatography to begin isolating the photoprotein.

1.2 DEAE Chromatography Optimization (Frozen)

Diethylaminoethyl (DEAE) ion exchange chromatography was used for the first purification step of the unidentified photoprotein. The efficacy of this separation was evaluated using three separate analytical techniques; luminometer kinetics assay, relative specific activity, and gel electrophoresis. Each of these three techniques was employed to determine the elution profile of the photoprotein within the various fractions that were collected.

Luminometer Kinetics Assay:

Eluate fractions were measured for relative luminescence (RLU/s) as discussed above. The first 1-mL elution fractions collected after each step-increase in NaCl concentration had some ability to produce light upon calcium addition, the most being the 200 mM NaCl fraction (Figure 6). For each change in NaCl concentration the first of the collected fractions show a greater amount of light production. Therefore, only the first

elution fractions are represented in this figure. This is further illustrated with each of the 200 mM NaCl fractions (Figure 7). This figure shows the highest amount of light is produced in the first elution fraction and then decreases with each corresponding fraction. The average calcium and hydrogen peroxide peak integral value over time (RLU) for the different elution fractions over the course of 13 replicate DEAE chromatographic runs show that the highest luminescence was obtained in the first 200 mM NaCl fraction (Figure 8). The fractions with the highest RLU values were kept for later purification steps and identification techniques.

Specific Activity of DEAE Elution Fractions:

Absorbance at 280 nm ($A_{280\ nm}$) was measured to give an estimate of the total amount of protein in each elution fraction. This is then compared to the calcium peak integral luminescence value to get the relative specific activity of each fraction (integral RLU divided $A_{280\ nm}$). Specific activity is a measure of photoprotein activity versus the total protein in each fraction. The first 200 mM fraction showed the highest specific activity (Table 1, Figure 9).

Evidence of Photoprotein through Gel Electrophoresis:

Elution fractions were run under native conditions (non-denaturing) in an effort to visualize the photoprotein in its active, light-producing form. The native gel was imaged under UV light (excitation 365 nm) showing two fluorescent bands in the supernatant and 200 mM NaCl fractions (Figure 10). The fractions were also run using SDS-PAGE electrophoresis (denaturing conditions). The gel was stained with InstantBlue™ and imaged (Figure 11). There is a band found in the supernatant and the 200 mM NaCl fraction that is not found in the other samples (boxed in red in Figure 11).

The supernatant has multiple bands that can be seen coming off in the 0 mM and 50 mM NaCl fractions that are not present in the 200 mM NaCl elution fraction.

1.3 Photoprotein Investigation: Three Orthogonal Chromatography Methods (Fresh)

DEAE chromatography followed by two additional orthogonal methods were used in attempt to further purify the sample. This was done to correlate protein bands on the electrophoresis gels to light production in the resulting fractions.

Additional DEAE Chromatography:

After optimization of SDS-PAGE techniques, fractions from several additional DEAE chromatographic runs were imaged. Initial results indicated a good correlation between the calcium peak integral value for each fraction and the intensity of a ~45 kDa protein band on the electrophoresis gel (Figure 12). The experiment was repeated with additional NaCl elution steps. In the resulting gel, the correlation between light production and protein band intensity was not as strong. The 100 mM NaCl fraction produced an equal amount of light as the 400 mM NaCl fraction, but had no ~45 kDa protein band (Figure 13).

Ammonium Sulfate Precipitation:

Due to a gel's limited sample load volume, several concentration methods were tried, to increase the amount of potential photoprotein to be seen on a gel. The ammonium sulfate precipitation proved to be the most effective with the lowest sample loss, compared to the spin-column concentrator and vacuum concentration, in which almost all activity was lost. The precipitated pellet, re-suspended in 1:10 the original volume resulting in a 7-fold increase in light, indicating a 70% recovery of activity. After concentration, it was determined that additional chromatography methods were needed

for isolation.

Size Exclusion Chromatography:

The first orthogonal purification method, size exclusion chromatography (SEC), was used as a potential method for further purification of DEAE chromatography fractions. SEC was done on combined, active fractions from the DEAE column. Activity corresponded very well with a large protein peak that came off about half way through the chromatographic run (Figure 14). Activity was well maintained throughout purification, shown in luminometer kinetics assay, yet the SDS-PAGE gel showed purification to be insufficient (Figure 15). Additional SEC was performed with a longer column (Superdex 200 26/600 GL). Results indicated good separation, however there was a severe loss in photoprotein activity (results not shown). In both cases the ~45 kDa was still present in high amounts.

Hydrophobic Interaction Chromatography:

Hydrophobic interaction chromatography (HIC) was also used on combined, active DEAE fractions. Activity corresponded to a large protein peak that came off in the center of the elution gradient, indicating that the protein did bind to the column and did not come of in the wash (Figure 16). There was a significant loss in activity with the HIC column, however the results of the SDS-PAGE showed a highly purified ~45 kDa band (Figure 17).

Correlation Between Light Production and 45 kDa Band:

The correlation between the light production of each fraction and the intensity of the 45 kDa gel band was analyzed for each chromatographic method (Figure 18). Even though the strength of the correlation varies, it remains positive throughout each

technique.

1.4 SDS-PAGE Gel Band Identification by ESI-QTOF-MS (Fresh)

In-gel tryptic digestion, followed by tandem ESI-QTOF-MS analysis, was conducted on the 45 kDa band described above. Results were strongly suggestive that the primary component of this band is actin, from a family of globular multi-functional proteins that form microfilaments, is associated with the cytoskeleton, and found in almost all eukaryotic cells. The Mascot search engine identified 24 unique peptides that contributed to sequence homology across 13 unique proteins (Tables 2, 3). The greatest degree of homology was found in beta-actin from *Azumapecten farreri* (NCBInr ascension gi|60103646242, kDa) a marine scallop, with seven identified peptides representing 24 percent sequence coverage. Similar homology was found with several other aquatic organisms.

1.5 Additional Attempts to Identify the Photoprotein (Fresh)

Several additional methods were used in attempt to identify the photoprotein. Active fractions were run on SDS-PAGE gels before and after the addition of calcium to see if there is a shift in size of a band that could represent the shift from photoprotein to apoprotein. No such shift was seen in any fraction. The non-luminescent disk extracts were also run as a comparison to the light producing arms to eliminate any bands that were seen in both. The dissection of the disk to avoid the stomach provided very little material and proved insufficient to yield any protein on gel. Excitation/emission spectra analysis was also completed on the chromatography fractions in attempt to provide a new activity assay based on the more stable fluorescence. However, the fluorescence intensity did not correspond directly with the bioluminescence activity and had to be abandoned.

2. Research Originated from Photocytes

2.1 Photocytes Extraction and Isolation

Photocytes were successfully extracted and isolated while maintaining a high amount of activity as demonstrated by the light produced after the addition of calcium. Isolation via Percoll® gradient separated cells into two layers, with photocytes enriching the top layer. The photocyte layer produced a significantly larger amount of light after the injection of calcium (Figure 20). The photocyte layer had up to 800 times more light production than the bottom layer (N = 5). Cells from the photocyte layer under a light microscope revealed mostly photocytes with some muscle cell contaminates as described in Deheyn *et al.* 2000. Isolated photocytes were found to have up to 500 times the amount of light compared to the extracts from the full arms.

2.2 Photocyte Lysis

Photocytes were lysed via each of the four different lysis methods. No method resulted in the extraction of active photoprotein into solution. With all lysing methods tested, the activity present in the supernatant was no greater than 1% of the original activity. Every method resulted in a various amount in of loss of activity in the resuspended pellet compared to the control, indicating various levels of cell disruption (Figure 21). The results are represented as the integral of the calcium response peak of the remaining activity after lysis, divided by the integral of the calcium response peak before lysis.

2.3 Detergent Extraction

Cells were treated with octyl β-D-glucopyranoside (OBG) detergent in attempt to extract the active photoprotein. At low concentrations of OBG the light production

remained in the pellet after centrifugation. With an increase in concentration of the detergent, less light is produced in the pellet, however this lost activity doesn't appear in the supernatant (Figure 22). Data representation is the same as photocyte lysis. Similar results were found with three other detergents: Triton X-100, sodium deoxycholate (SDO), and Tween 20. No detergent tested could successfully put the active photoprotein into solution.

2.4 Calcium Ionophore Treatments

The injection of the calcium ionophore A13287 at 30 s triggered an intense peak of light. In addition, the photocytes that were injected with A13287 followed by a calcium injection at 60 s had a ten-fold higher light production peak compared to the sample that was injected with the Ca-Free ASW control (Figure 23).

Discussion

This work consists of an attempt to purify and characterize the light producing protein from brittlestar *Ophiopisla californica* as well as an investigation into how the photoprotein is activated within the photocyte. The results from this study indicate the best approach to continue in the attempt to identify, sequence, and eventually manufacture this new photoprotein.

1. Photoprotein still active after long-term freezer storage

The extracts from arms frozen for up to 24 months at -80° C could be activated to produce light with the addition of calcium as well as H_2O_2. As with most proteins there was some loss in activity compared to the fresh extracts. Nevertheless, the photoprotein's ability to be reactivated after long-term freezer storage is very attractive for future research and manufacturing possibilities.

2. Fluorescence is not a suitable activity assay to track the active photoprotein

Preliminary experiments using *O. californica* extracts showed that the activity of the photoprotein was unstable, with light production decreasing dramatically when working on ice at the bench. Excitation/emission spectra of the chromatography fractions was done to see if the fluorescence intensity coincided with the bioluminescence, in which case an assay based on the more stable fluorescence could be developed. The results showed no correlation with fluorescence intensity and light production at any wavelength. This indicates that either the chromophore responsible for the bioluminescence and the chromophore responsible for the fluorescence are two different entities, or that the phenomena of being fluorescent after light production occurs cannot

be replicated *in vitro*. As a result, a suitable activity assay based on florescence could not be developed to track photoprotein activity.

3. Chromatographic techniques reveal association between photoprotein and actin

DEAE chromatography fractions run on SDS-PAGE gels revealed a high correlation with the light production of each fraction and the intensity of a 45 kDa protein band. After subsequent purification with hydrophobic interaction and size exclusion chromatography, this correlation waivered. Nevertheless, the 45 kDa band appeared in fractions with high amounts of luminescence throughout every chromatography step.

Even though the correlation with light production and the intensity of this band varied, there were still several reasons to believe this band could still be the photoprotein. It is possible the protein band is made up of both active and inactive protein which would make it difficult to correlate to light production because this only represents active fractions. An additional reason being that previous research done by Osamu Shimomura (1986) suggested the size of the protein being ~45 kDa based on preliminary gel filtration chromatography (*14*).

In-gel tryptic digestion, followed by tandem ESI-QTOF-MS analysis strongly suggested that the primary component of this band is actin. Highest degrees of sequence homology match mostly marine invertebrates, indicating that this was actin from the brittlestar itself and not a contamination. The failure of the Mascot database search to return an identity confirmation for actin from *Ophiopsila californica* was not unexpected as this species is not represented in the NCBInr database.

Even though there was no evidence of the photoprotein in the ~45 kDa protein band, identifying actin allows several hypotheses to be made. The first is that the

photoprotein co-elutes with actin and is also present in the ~45 kDa band. It is likely that this protein evolved separately from known photoproteins in the NCBInr database and therefore it would be difficult to match the peptide based on homology searches. This would be supported by the preliminary work done by Shimomura indicating the size of the protein to be 45 kDa (*14*). The possibility that the photoprotein is highly associated with actin is also interesting because of the finding vesicles inside the photocytes of brittlestars change shape and contract during light production to maximize the light emitted by increasing the surface-to-volume ratio (*16*). This movement of vesicles and organelles is known to be facilitated by actin. This will be tested in upcoming experiments by adding actin in inhibitors to the photocytes to see if it effects light production. It is also possible that there is still not enough photoprotein to be visualized on a gel. Because there is no way to know how much light is produced by a certain amount of photoprotein, it is hard to determine if this is the case. Testing this would be extremely risky because we were relying on a small number of animals in our lab and supplies could be depleted at the expense of further experiments.

4. Activity of photoprotein is highly dependent on membrane integrity

When cells were triggered with calcium they produced up to 500 times the amount of light as the full arm extract. This points to a process within the cell that maximizes light production in the photoprotein. It was also determined that when the cells were lysed or a detergent was used, almost all activity was lost. This is indicated by the loss of activity in the pellet after lysis, or adding a detergent never appearing in the supernatant. No lysis method or detergent could successfully put the active photoprotein into solution. This suggests that the photoprotein activity is almost completely lost when

it is no longer in a cell with an intact membrane and that mere association with the membrane is not enough to activate the protein to produce light. This indicates that the photoprotein relies on something else in addition to, or instead of the traditional cofactor ion. It is conceivable that the photoprotein depends on a potential gradient or flux of calcium ions through a membrane to activate the protein to produce light. The association with a membrane is also supported by the binding of the protein to the hydrophobic resin with hydrophobic interaction chromatography. Future experiments will include the construction of a synthetic liposome around the photoprotein to see if it is possible to regenerate light production.

5. Calcium ionophore treatments could indicate calcium storage vesicles

When the calcium ionophore was injected into the calcium-free treated sample it caused, to our surprise, a large peak in light production similar to the injection of calcium. This had not been reported before in the literature related to luminous brittlestars. It could indicate a storage vesicle that contains a large amount of calcium that gets released when the calcium ionophore is added to the system, thus activating the photoprotein. Due to such a high and fast response when the calcium ionophore is added, it is possible that the photocytes contain a type of calcium storage vesicle that only releases the calcium when triggered to do so (as opposed to the release it “unspecifically” when exposed to Ca-free medium), such as the activation of a calcium release channel. Since the propagation of the neuro-influx along the radial nerve cord shows almost no delay to the propagation of the luminesces along the arm, it is possible that the photoprotein is highly associated with this channel within a membrane. Future work to explore this hypothesis would be to test known activation and inhabitation molecules of

studied calcium release channels to see if they influence the light production in the photocytes. The signal transduction pathways and secondary messengers that are involved with the activation of these calcium release channels can also be investigated.

6. Proposed model of light production mechanism: A starting point for future work

Based on previous studies and results from this work, a model of a possible light production mechanism in brittlestar *O. californica* is proposed here (Figure 24). Based on the finding that intracellular calcium release can activate the photoprotein, pathways that lead to the triggering of intracellular calcium release channels were researched. In sea urchin embryos, it was shown that a secondary messenger, cyclic ADP ribose (cADPR) was effective in mobilizing intracellular calcium stores (Figure 24A). cADPR stimulates the calcium-induced calcium release and activation of ryanodine receptors, a group of calcium release channels shown to release intracellular calcium (Figure 24B) (*19*). The results of this study indicate that the photoprotein is membrane associated and is likely triggered by a flux of calcium ions or potential gradient. It is proposed in this model that a ryanodine receptor could be responsible for this flux, stimulating light production (Figure 24C). Previous studies on the light production show, with the use of inhibitors, that cyclic adenosine monophosphate (cAMP) is also involved in the light production (Figure 24D) (*20*). cAMP could work with this model in several ways. One is that it is cAMP pathway may regulate the synthesis of cADPR (Figure 24E) (*19*). It is also known that the cAMP pathway results in the phosphorylation of L-type calcium channels. It has been proposed that an influx in calcium ions play a role in the stimulation of cADPR production as well (Figure 24F) (*21*). It is also possible that the L-type channels are responsible for bring in calcium to refuel this calcium storage vesicle (Figure 24G).

An additional pathway of intercellular release that could play a role in the light production is the secondary messenger NAADP, also known to mobilize intercellular calcium. It is known to act on two pore calcium release channels (Figure 24H). This could also provide the calcium ion flux to stimulate light production. Mush less is known about this pathway, however the literature postulates that NAADP could also be regulated by cAMP (Figure 24I) (*19*).

There are still many possibilities of what the exact mechanism of light production is in the animal, however the proposed model represents a good starting point for further studies of this system based known results from this work and previous studies. Future work will be focused on the use of known activators/inhibitors of the channels and messengers proposed here. Work will also be done in attempt to visualize the cellular localization of calcium, with use of a dye, and see how it moves after stimulation of light production to support the flux of internal activating the photoprotein. Going forward, knowing how the photoprotein is activated within the photocytes will allow the optimization of new techniques to fully extract, purify, and identify this novel photoprotein.

Conclusions:

This study demonstrates the complex nature of the light production system in *Ophiopsila californica*. The photoprotein appears to rely on several processes that allow light production to occur. The most evident from this study is the requirement of an intact membrane, demonstrated by lysis and use of a detergent inhibiting almost all activity. Chromatography results also indicate that the photoprotein is highly associated with actin. This supports the notion that photocytes in brittlestars change shape and contract during light production to maximize light emission that has been observed on a closely related species. Furthermore, the activation of light production upon the addition of the calcium ionophore could indicate the presence of calcium storage vesicles that rely on a particular mechanism to active the release of intracellular calcium. If these postulations are proven correct with the future experiments discussed above, it is possible that they all work together to facilitate light production.

To this day, there has been no photoprotein identified from an Echinoderm. Almost all other well-studied photoproteins are from the phylum Cnidaria. Despite the extreme complexity of the light production system, studying the photoprotein and how it is activated is extremely attractive because of the different properties it may possess compared to the other known and well-characterized photoproteins. It is known that the spectrum of bioluminescence is slightly shifted compared the most studied photoproteins with a range of 495-515 nm (*15*) compared to aequorin (465 nm max), obelin (475-495 nm), and clytin (470 nm max) (*22-24*). This shift in spectrum could provide differential probing using two different photoproteins. It is also the only known photoprotein whose light production is dependent upon association with the cell membrane. This property

may provide a selective advantage in the probing of membrane related processes. In addition, its possible neural origin indicates high potential in the development of neuro-toxicity assays. These characteristics, as well as ones yet to be identified through future analysis and characterization of the photoprotein, present the opportunity to expand the use and applications of light producing proteins in fields such as biotechnology and biomedicine.

Appendix

Figure 1. Brittlestar *Ophiopsila californica* under white light (L) and with arms producing bioluminescence (R). The disk is about 2 cm in diameter, and the arms as long as 20 cm.

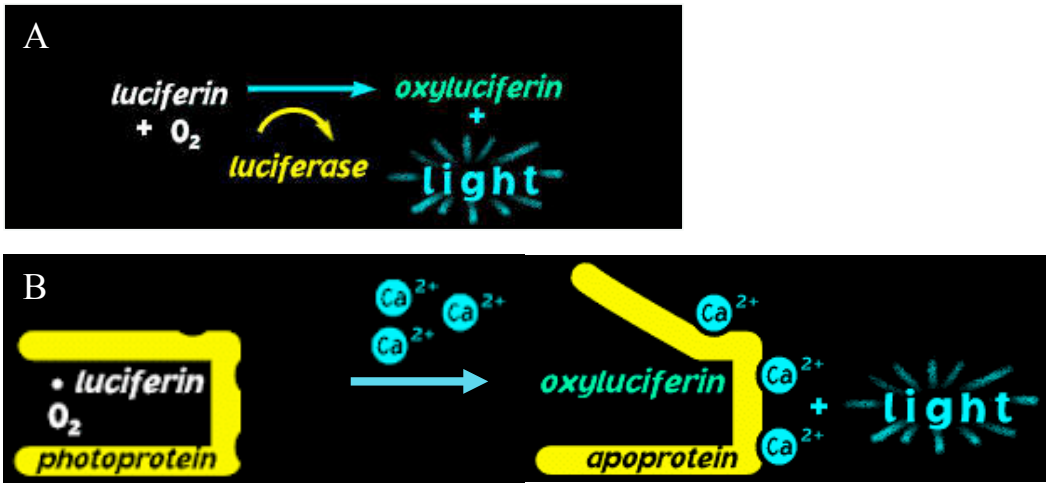

Figure 2. The two classic light production mechanisms (A) Luciferin-Luciferase reaction (B) Photoprotein reaction.

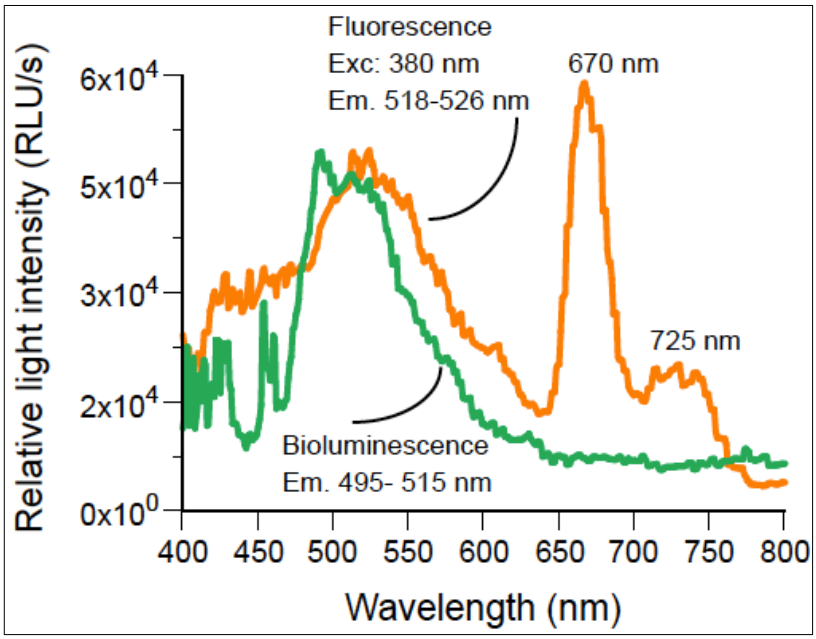

Figure 3. Spectrum of bioluminescence, and fluorescence (exc. 380 nm) from *O. californica*; courtesy of Deheyn 2009.

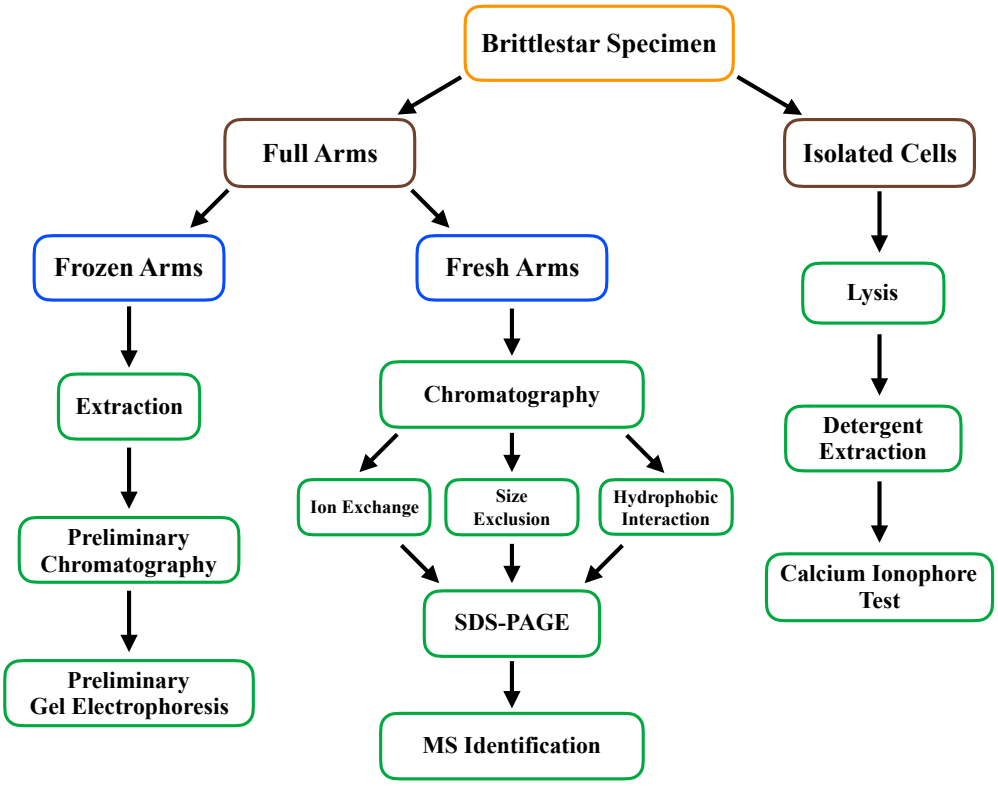

Figure 4. Schematic flow for experiments done under each of the three sample platforms: Full arms (frozen and fresh) and isolated cells.

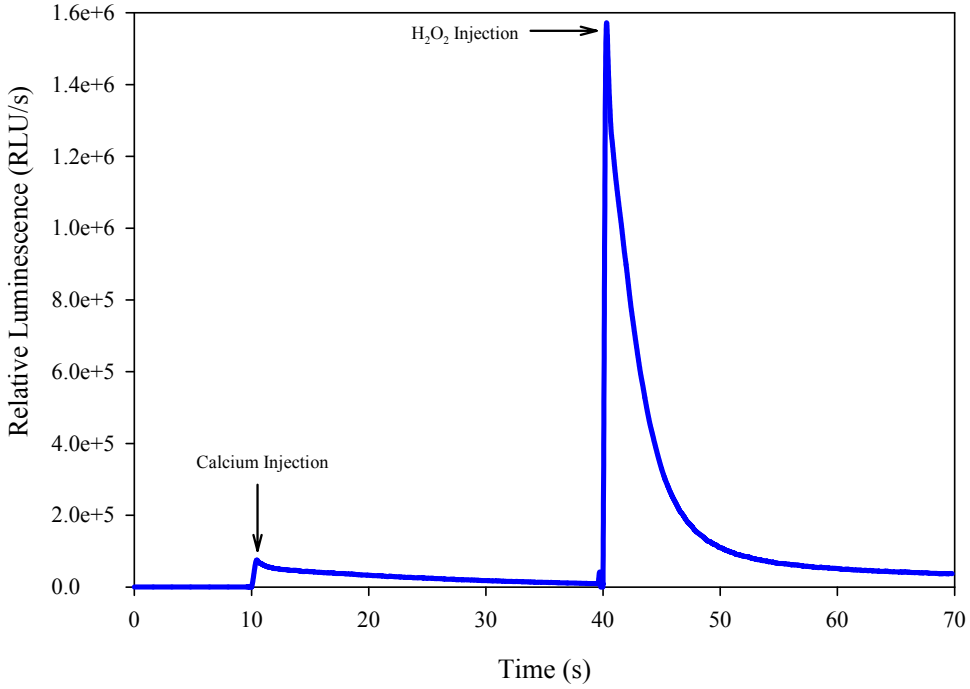

Figure 5. Example of light response peaks of extract from *O. californica.* The first peak is after the injection (10 s) of 400mM $CaCl_2$ and the second is after the injection (40 s) of H_2O_2.

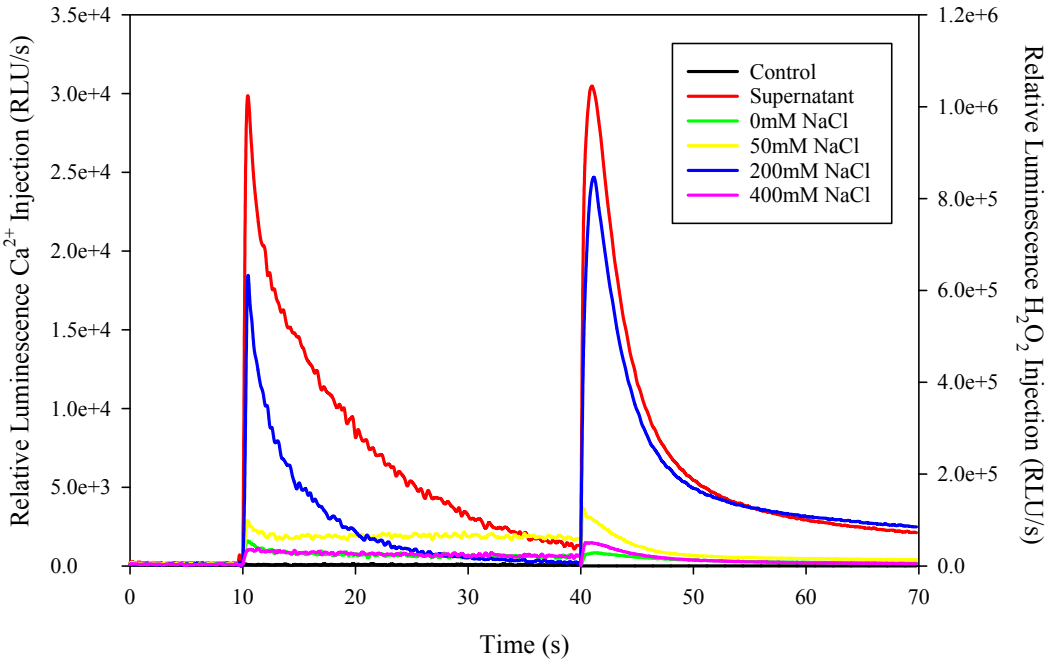

Figure 6. Kinetics of bioluminescence (RLU/s) of the NaCl fractions from initial DEAE purification. The first peak is after the injection (10 s) of 400mM $CaCl_2$ and the second is after the injection (40 s) of H_2O_2, shown on a separate axis.

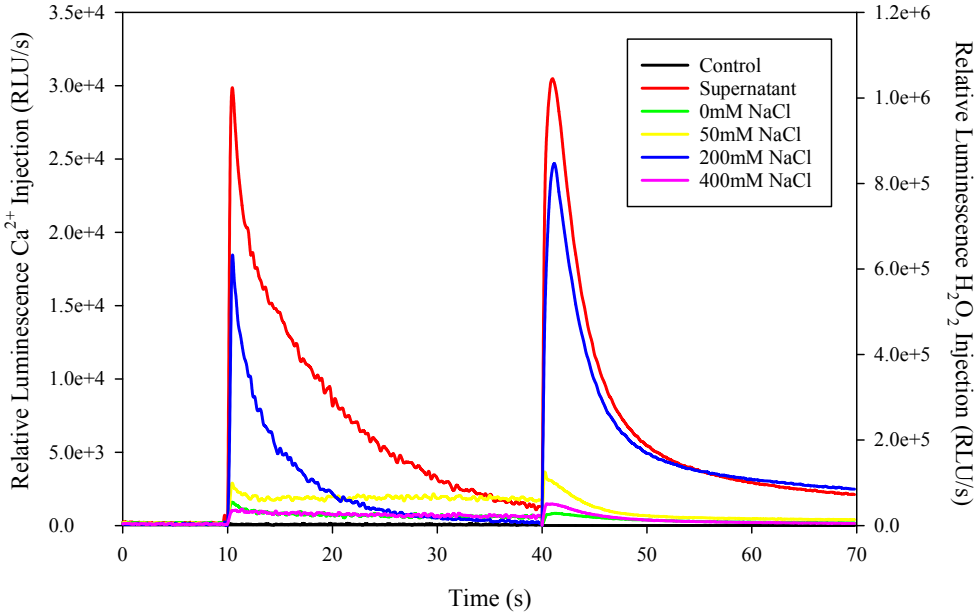

Figure 7. Kinetics of Bioluminescence (RLU/s) of the three 200 mM NaCl fractions from the initial DEAE purification compared to the supernatant. The first peak is after the injection (10 s) of 400 mM $CaCl_2$ and the second is after the injection (40 s) of H_2O_2, shown on a separate axis.

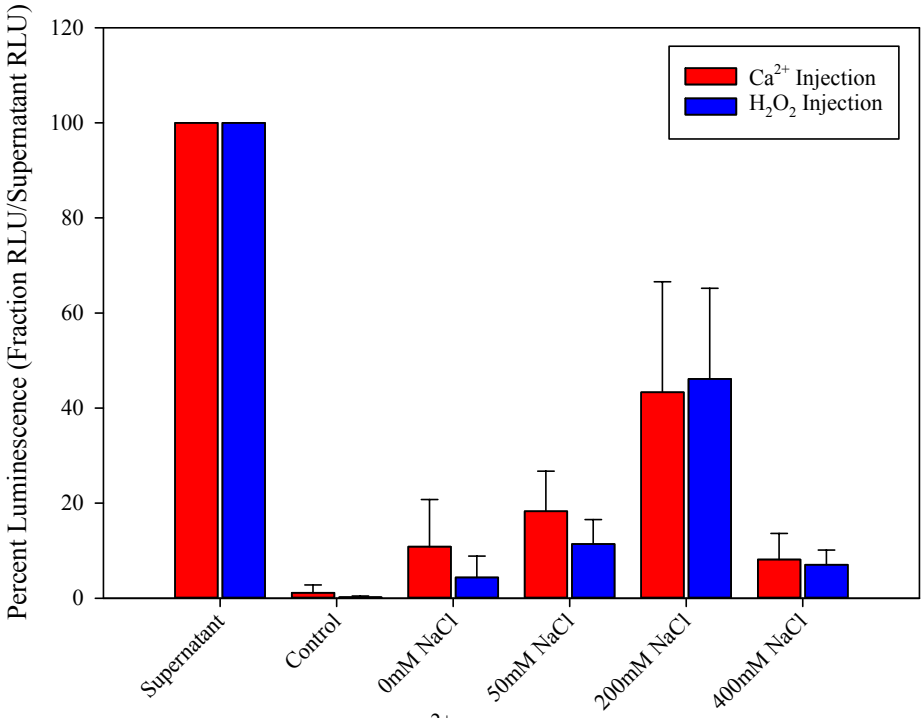

Figure 8. Integration of the Ca^{2+} and H_2O_2-induced bioluminescent peaks of the different NaCl elution fractions (n=13). The integration accounts for the intensity of the light over the duration of luminescence.

Table 1. Relative Specific Activity of DEAE Chromatography Fractions

Elution	Calcium Peak Integral RLU	Absorbance at 280 nm	Relative Specific Activity
Supernatant	2.36E+06	3.024	7.81E+05
Void	4.03E+04	0.209	1.92E+05
50mM NaCl (1)	3.36E+05	1.2865	2.61E+05
50mM NaCl (2)	2.22E+05	0.294	7.54E+05
50mM NaCl (3)	1.74E+05	0.130	1.34E+06
50mM NaCl (4)	1.16E+05	0.093	1.24E+06
200mM NaCl (1)	1.23E+06	0.403	3.05E+06
200mM NaCl (2)	4.62E+05	0.483	9.56E+05
200mM NaCl (3)	1.48E+05	0.270	5.50E+05
400mM NaCl (1)	1.94E+05	0.613	3.16E+05
400mM NaCl (2)	6.50E+04	0.369	1.76E+05

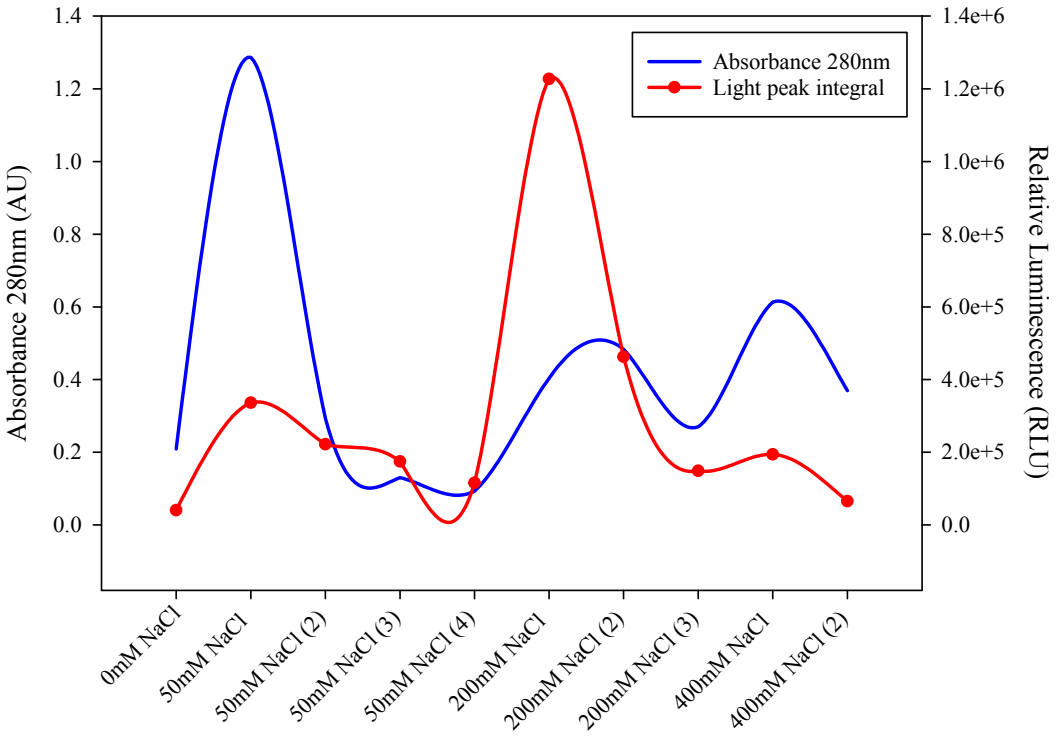

Figure 9. Chromatogram of DEAE column with absorbance at 280 nm (relative protein concentration) overlaid with the integration of the Ca^{2+}-induced bioluminescent peaks in each fraction. The 200mM fraction shows the highest light production to total protein ratio.

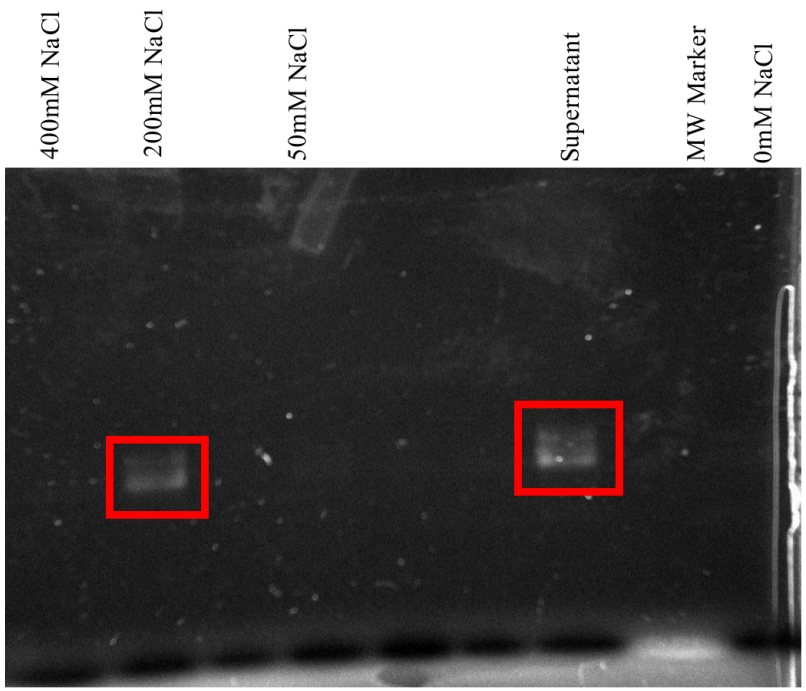

Figure 10. Native Gel Electrophoresis of DEAE Fractions. There are two fluorescent bands found in the supernatant and 200mM NaCl sample (boxed in red); an indication of the presence of the photoprotein in both of these samples.

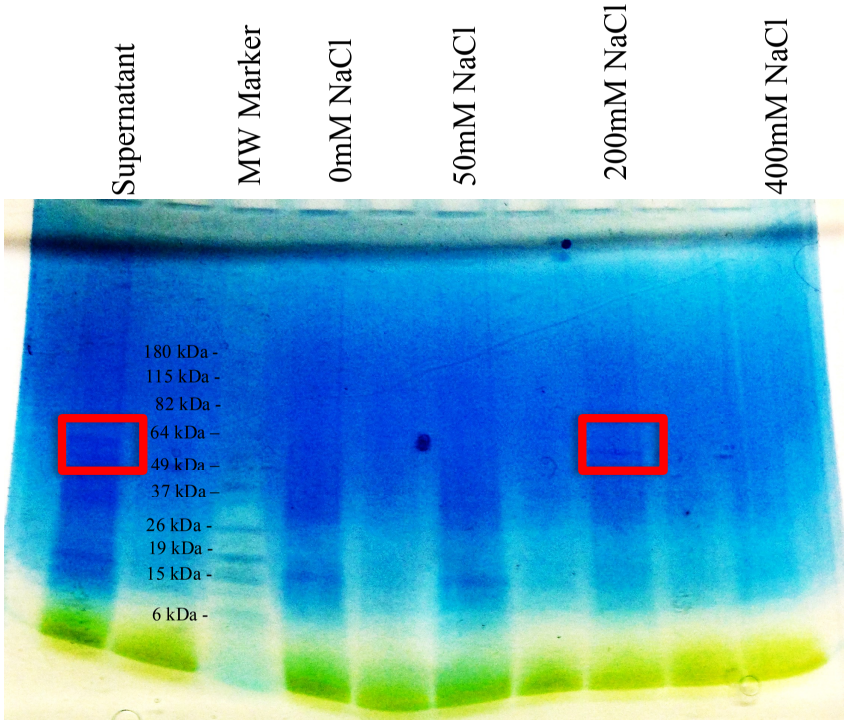

Figure 11. SDS-PAGE Gel Electrophoresis of DEAE Fractions. Shows the DEAE elution fractions run on an SDS-PAGE (4-20%) gel. There are bands of similar weights (~55 kDa) that appear in the supernatant and 200mM NaCl samples (boxed in red).

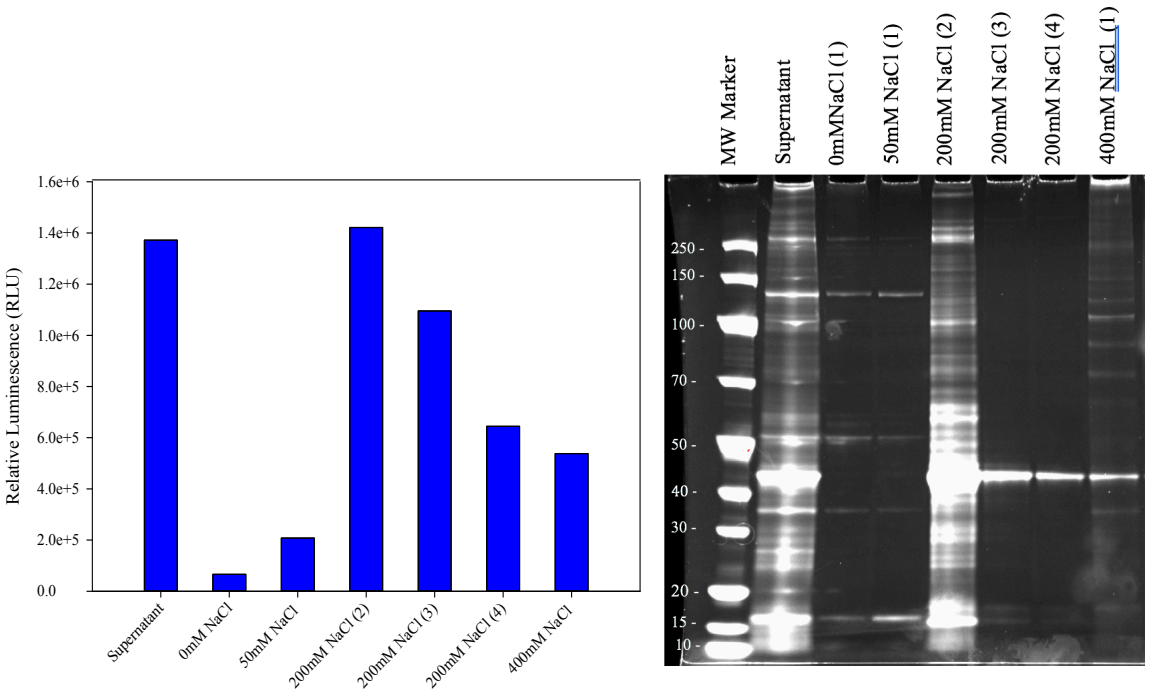

Figure 12. Comparison between light production of DEAE fractions and intensity of the 45 kDa protein band. Right: Integration of the Ca^{2+}-induced bioluminescent peaks of the different NaCl elution fractions**.** Left: SDS-PAGE gel of the same fractions.

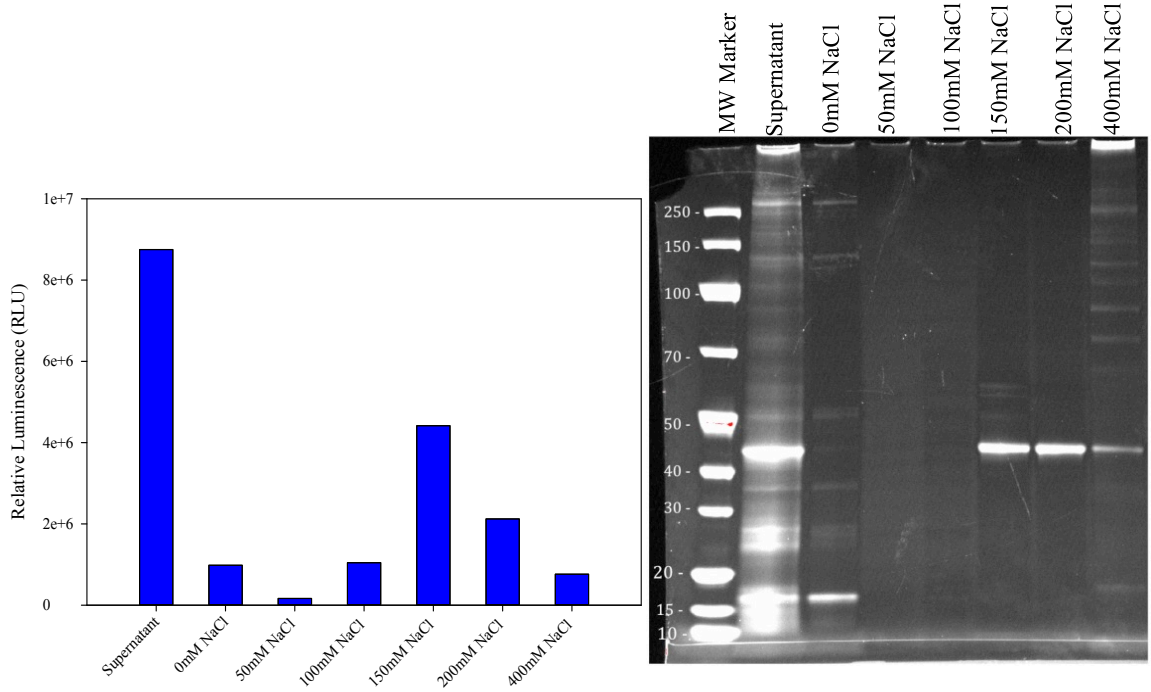

Figure 13. Comparison between light production of DEAE fractions with two additional step gradients and intensity of the 45 kDa band. Right: Integration of the Ca^{2+}-induced bioluminescent peaks of the different NaCl elution fractions. Left: SDS-PAGE gel of the same fractions.

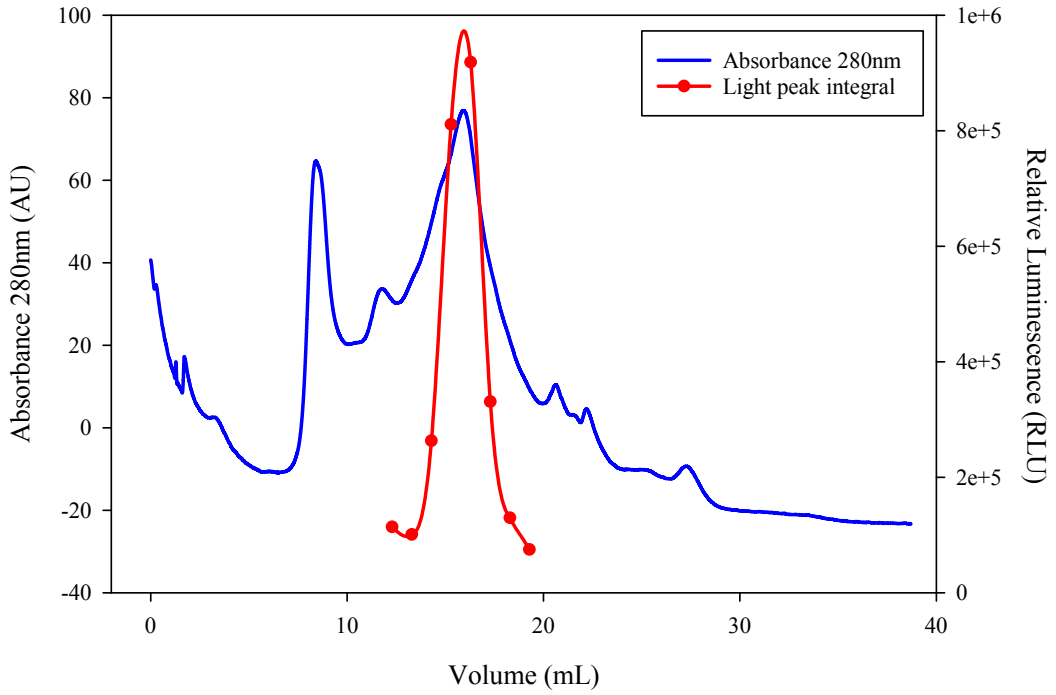

Figure 14. Chromatogram of SEC column with absorbance at 280 nm overlaid with the integration of the Ca^{2+}-induced bioluminescent peaks of each fraction. Light production corresponds well with large protein peak about half way through the elution.

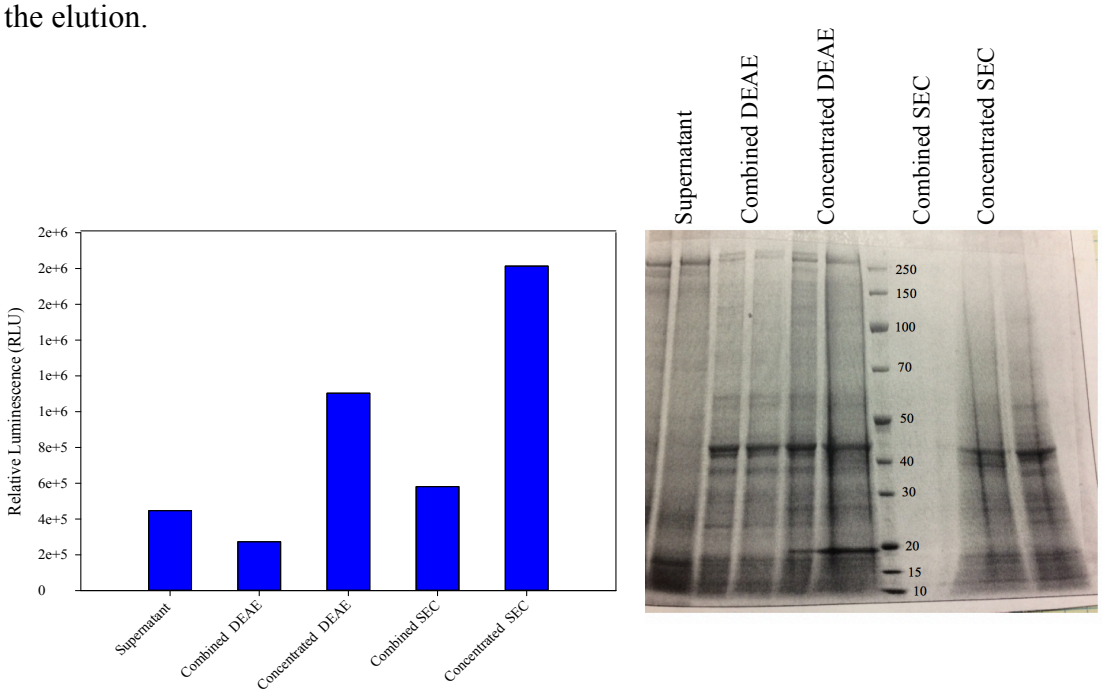

Figure 15. Comparison of combined/concentrated elution fractions from the DEAE and SEC chromatographic runs to the intensity of the bands on the gel. Right: Integration of the Ca-induced bioluminescent peaks of the fractions**.** Left: SDS-PAGE gel of the same fractions.

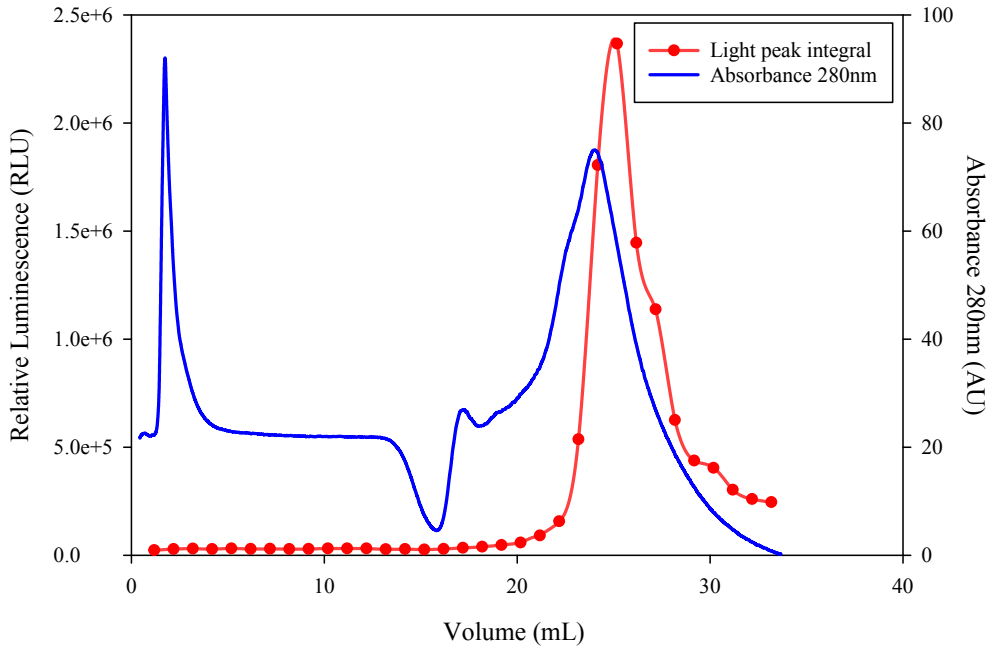

Figure 16. Chromatogram of HIC column with absorbance at 280 nm (relative protein concentration) overlaid with the light production in each fraction. Light production corresponds highly with large protein peak in the center of the elution gradient.

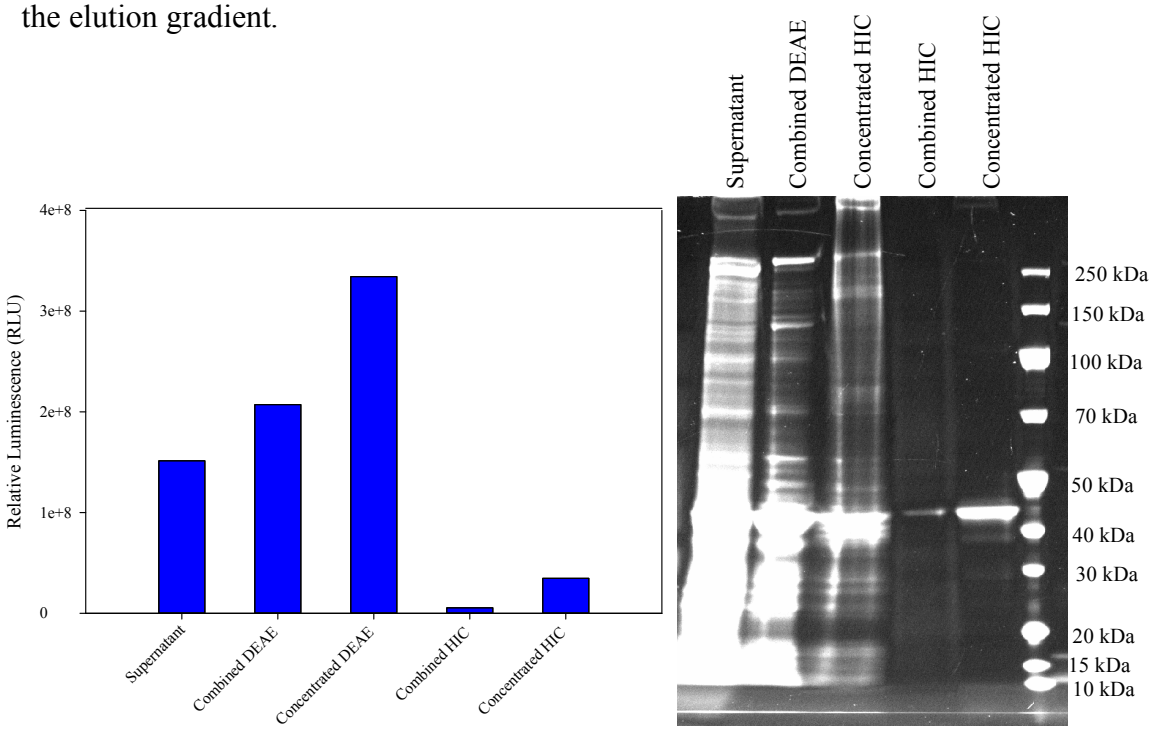

Figure 17. Comparison of the combined/concentrated elution fractions form the DEAE and HIC chromatographic runs and the intensity of the 45 kDa band. Right: Integration of the Ca-induced bioluminescent peaks of the fractions**.** Left: SDS-PAGE gel of the same fractions.

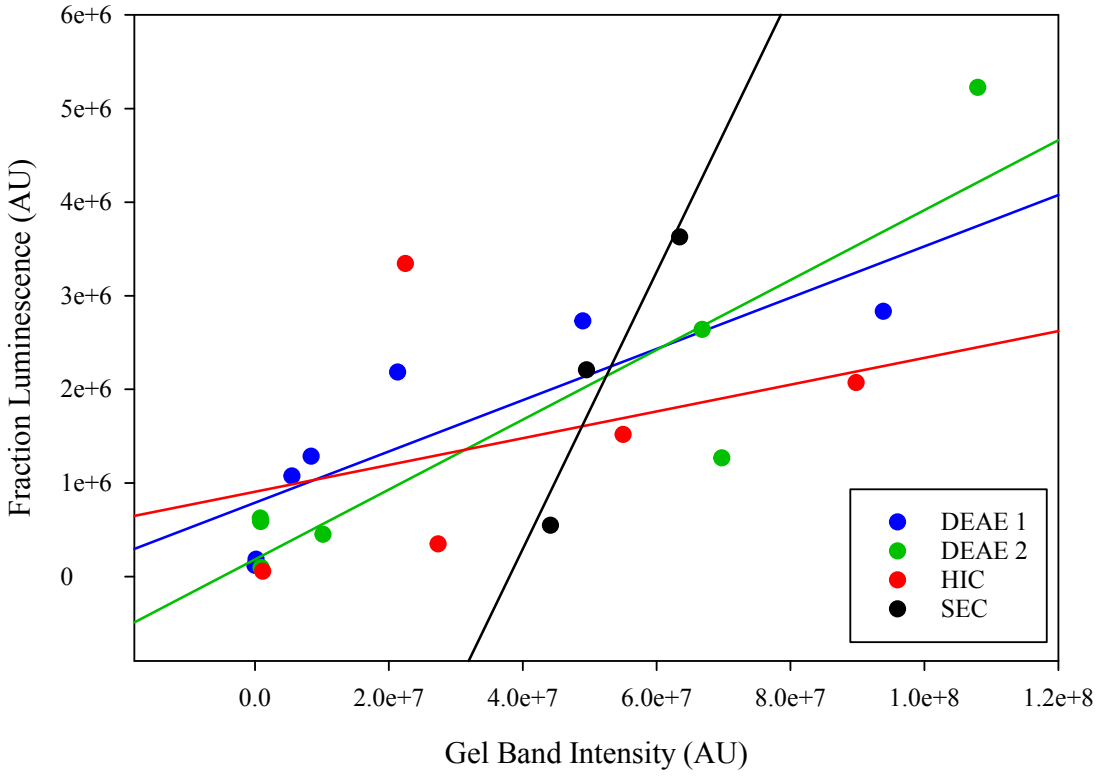

Figure 18. Correlation between fraction luminescence and the intensity of the 45 kDa gel band from the corresponding fraction. Data is expressed relative to a standard for better visualization. The strength of this correlation varies significantly, however it remains positive throughout every purification method.

Table 2. Mascot Search Results: Proteins with Significant Sequence Homology to Protein Found in 45 kDa SDS-PAGE Gel Band

MASCOT Search Results - Sequence Homology (p value = 0.05)								
Family	Member	Database	Accession	Score	Mass (kDa)	Num. of significant matches	Num. of significant sequences	Description: Protein and Species
1	1	NCBInr	gi|601036462	308	42047	24	7	beta-actin [Azumapecten farreri]
1	2	NCBInr	gi|158635327	260	42077	21	7	actin [Haliotis diversicolor]
1	3	NCBInr	gi|224305	259	41827	20	7	actin
1	4	NCBInr	gi|115918029	258	42059	22	8	PREDICTED: actin-15B [Strongylocentrotus purpuratus]
1	5	NCBInr	gi|171190503	256	15576	11	4	actin, partial [Uwebraunia musae]
1	6	NCBInr	gi|47551039	252	42024	20	7	actin, cytoskeletal 3A [Strongylocentrotus purpuratus]
1	7	NCBInr	gi|261286856	223	41167	19	6	beta actin, partial [Anguilla japonica]
1	8	NCBInr	gi|475669071	176	46063	12	4	Actin, gamma [Fusarium oxysporum f. sp. cubense race 4]
1	9	NCBInr	gi|187968791	158	25956	13	3	actin [Hurius sp. CJV-2008]
1	10	NCBInr	gi|6470281	148	38181	13	5	beta-actin, partial [Anolis carolinensis]
1	11	NCBInr	gi|21703190	135	18329	10	3	cytoplasmic actin [Boltenia villosa]
1	12	NCBInr	gi|449274743	130	40068	7	4	Actin, alpha cardiac muscle 1, partial [Columba livia]
1	13	NCBInr	gi|480318236	122	22461	10	3	actin, partial [Servaea vestita]

Table 3. Mascot Search Results: Identification of Individual Peptides found in 45 kDa SDS-PAGE Gel Band and their Contribution to Sequence Homology in Actin Family of Proteins

Sequence Homology for 13 Highest Scoring Proteins

Query	Observed (precursor)	Mr(expt)	Mr(calc)	ppm error	Score	Expect	Protein ID 1	2	3	4	5	6	7	8	9	10	11	12	13	Peptide
239	351.1659	700.3172	700.314	4.61	39	0.03					■					■				-.AGDDAPR.A
749	453.2108	904.407	904.4039	3.48	39	0.024												■		-.GFAGDDAPR.A
956	488.7297	975.4449	975.441	3.98	70	0.0011	■	■	■	■		■	■	■						K.AGFAGDDAPR.A
1283	533.7384	1065.4622	1065.4583	3.63	47	0.0011							■							-.VVDNGSGMCK.A
1468	566.7687	1131.5229	1131.5197	2.89	45	0.0085	■	■	■	■		■	■		■	■	■		■	R.GYSFTTTAER.E
1574	586.3249	1170.6353	1170.6318	2.96	58	0.048				■										K.EITALAPPTMK.I
1575	586.3249	1170.6353	1170.6496	-12.2	50	0.043				■										K.EITALAPXTVK
1722	599.7667	1197.5188	1197.515	3.24	42	0.0029	■	■	■	■	■	■	■			■		■		K.DSYVGDEAQSK.R
1750	600.2585	1198.5024	1198.499	2.83	29	0.0088								■						K.DSYVGDEAESK.R
2041	452.5422	1354.6047	1354.6001	3.42	32	0.0038								■						K.DSYVGDEAESKR.G
2078	686.3428	1370.6711	1370.6678	2.41	78	1.30E-06					■									R.TTGIVLDSGDGVTH.-
2195	724.4206	1446.8266	1446.8194	4.97	36	0.0049											■			-.VAIQAVLSLYASGR.T
2282	758.8568	1515.6989	1515.6954	2.36	72	0.0023	■	■	■	■		■	■	■						K.QEYDESGPSIVHR.K
2287	506.2411	1515.7016	1515.6954	4.09	77	0.00077	■	■	■	■		■	■	■						K.QEYDESGPSLVHR.K
2478	892.4835	1782.9525	1782.9516	0.52	51	0.0001									■					-.PEEHPVLLtEAPiNPK
2487	895.9533	1789.8921	1789.8846	4.15	89	6.60E-05	■	■	■	■		■			■	■	■	■	■	K.SYELPDGQVITIGNER
2639	977.538	1953.0614	1953.0571	2.19	45	0.036	■	■	■	■	■	■	■			■				R.vAPEEHPViITEAPlNPK
2653	652.3564	1954.0472	1954.0411	3.13	15	0.042						■								R.VAPEEHPVLLTEAPIDPK
2777	704.7061	2111.0963	2111.1085	-5.76	24	0.016													■	-.TTPAMYVAIQAVLSLYASGR.T
2813	1101.0326	2200.0506	2200.047	1.64	57	0.023				■										K.DLYANTVLSGGSTMYPGIADR.M
3065	840.0747	2517.2022	2517.1944	3.08	25	0.0079		■												K.LAYVALDFEQEMQTAASSSSLEK.S
3075	840.729	2519.1651	2519.1559	3.63	57	0.0026	■													K.LCYVALDFENEMATAAASSSLEK.S
3078	1260.5906	2519.1666	2519.1559	4.23	75	0.00089	■													K.LCYVALDFENEMATAAASSSLEK.S
3110	1268.5836	2535.1527	2535.1509	0.72	62	0.012			■									■		K.LCYVALDFENEMaTAASSSSIEK

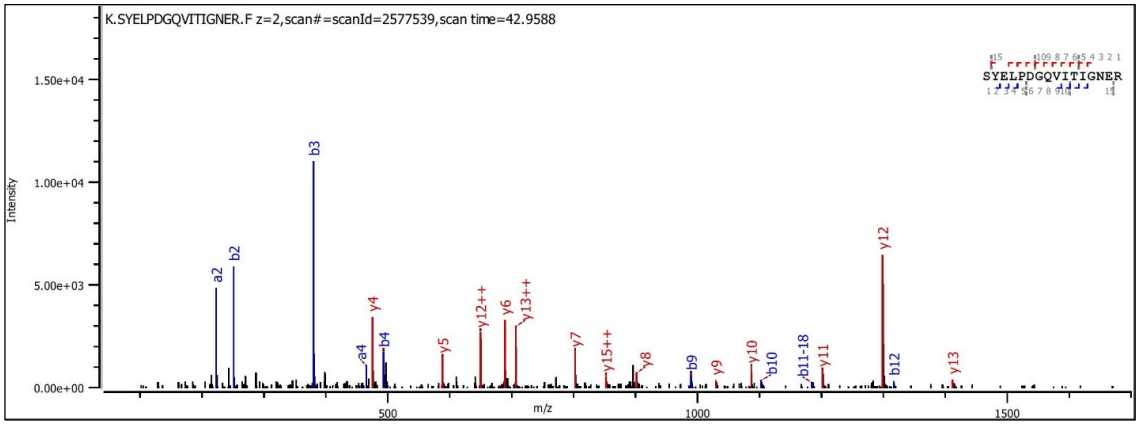

Figure 19. Representative tandem mass spectrum from peptide SYELPDGQVITIGNER found in 10 of 13 actin sequences that share homology with the 45kDa gel band.

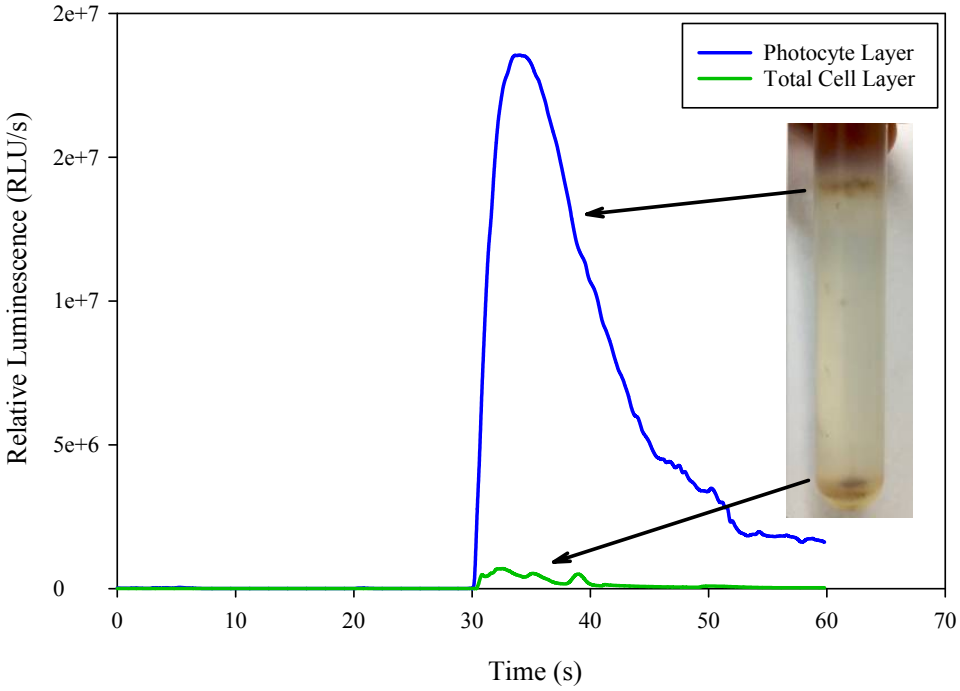

Figure 20. Kinetics of bioluminescence (RLU/s) of the top photocyte layer compared to the total cell bottom layer produced by the 63% Percoll® gradient.

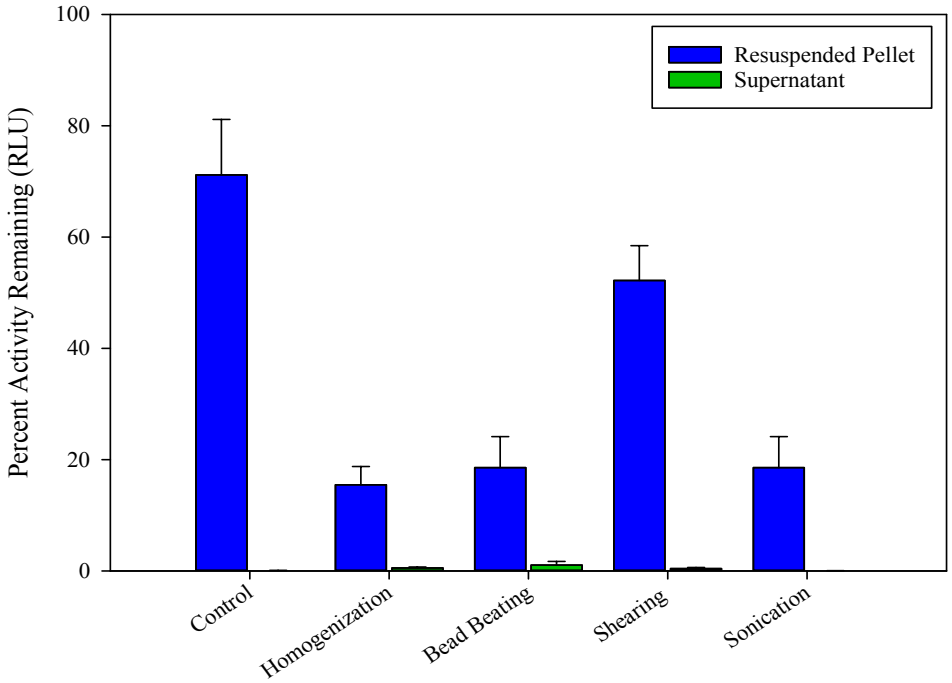

Figure 21. Effect of different lysis protocols on light production represented as the percent remaining compared to the original light production peak integral after the injection of calcium (N=3). This shows that with every lysis method almost no activity goes into solution.

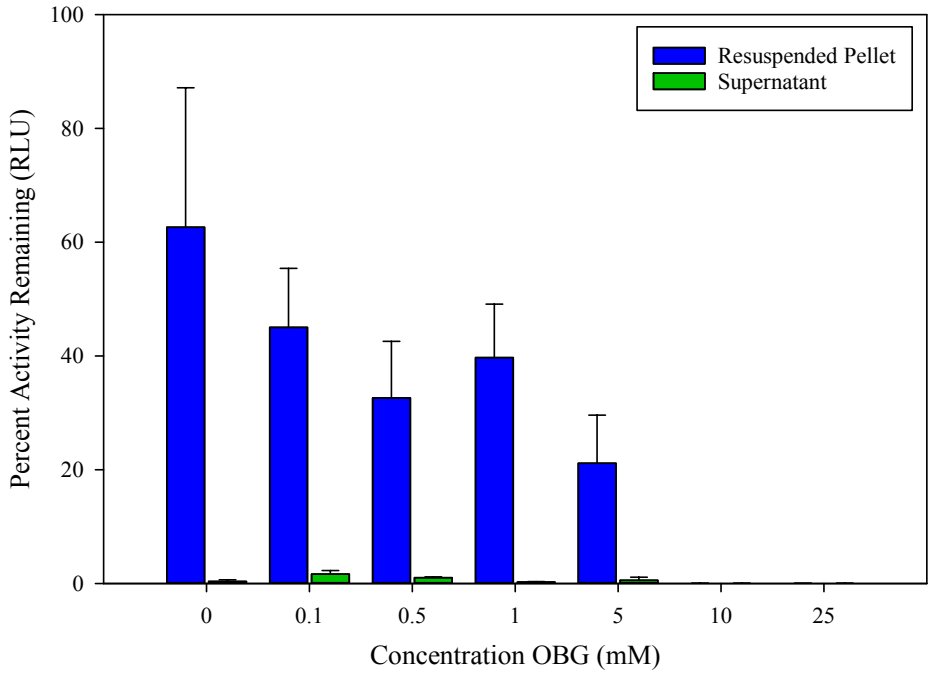

Figure 22. Effect of OBG on light production represented as the percent remaining compared to original light production peak integral after the injection of calcium (N=2). This shows that at every concentration almost no activity goes into solution.

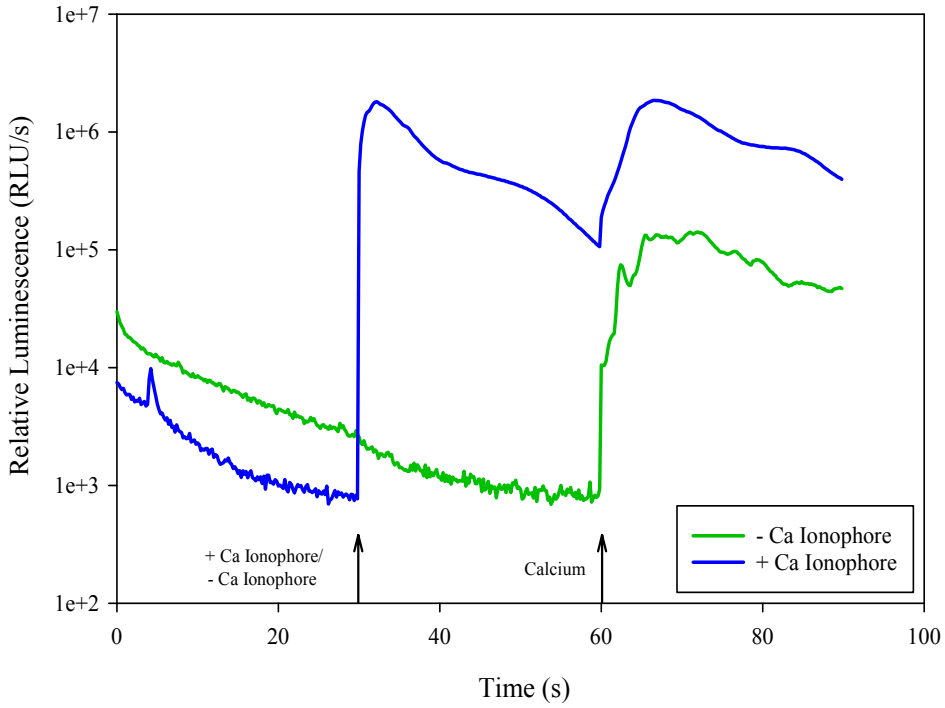

Figure 23. Effects of Calcium Ionophore A12387 on the light production of the photocytes.

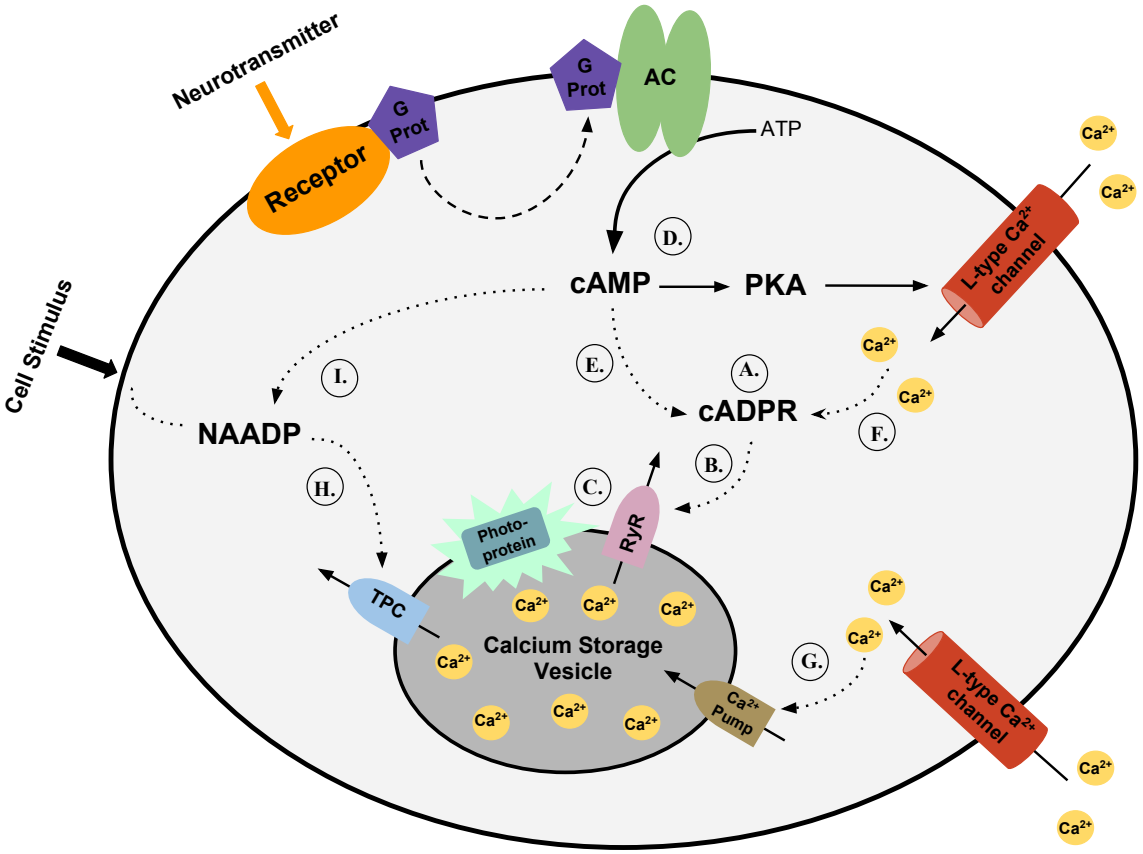

Figure 24. Proposed mechanism of light production in *O. californica.* Secondary messenger cADPR know to facilitate mobilization of intercellular calcium (A). cADPR activates ryanodine receptor (RyR) to release calcium (B). Flux of calcium out of the membrane could possibly trigger light production in the photoprotein (C). aCMP is shown to play a role in light production (D). cAMP is shown to facilitate the synthesis of cADPR (E). Calcium ion flux can stimulate cADPR (F). Calcium ion flux could also refuel the storage vesicle (G). Secondary messenger NAADP activates two pore calcium channels and could also cause a flux of calcium ions, thus activating the photoprotein (H). It is proposed that cAMP also plays a role in the regulation of NAADH (I).

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