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Lighting up the Ocean: Cloning, expression, and characterization of comb jelly photoproteins

Introduction

Many luminous organisms are found living in the ocean (Haddock et al. 2010). Bioluminescence, the ability for an organism to create its own light, has been found in numerous phyla such as Cnidaria, Annelida, and Ctenophora. Organisms in the ocean use bioluminescence for a variety of reasons such as a defense, offense, or reproduction (Figure 1). Bioluminescent light can also vary in color, which is determined by the maximum emission wavelength. One species of ctenophore mainly found in the deep ocean, *Beroe abyssicola,* has a mean species' wavelength of 491 nm, however another deep ocean ctenophore species, *Aulacoctena acuminata,* has a mean wavelength of 458 nm (Haddock et al. 1999).

To produce bioluminescence, generally it is the oxidation of a light-emitting molecule, luciferin, with a catalyzing enzyme, either luciferase or photoprotein. Coelenterazine is a type of luciferin that is most found in cnidarians and ctenophores. A photoprotein is a specialized luciferase that comes prebound with a luciferin and oxygen molecule. Photoproteins contain three EF-hand domains and the loop region of these domains binds to calcium or magnesium ions, Ca^{2+} or Mg^{2+}, which causes a conformational change in the protein (Rowe et al. 2007). The excited oxidized coelenterazine intermediates return to the ground state that releases the photons. Aequorin was the first photoprotein that was discovered, isolated, and characterized and eventually cloned from the hydromedusa *Aequorea victoria* (Shimomura et al. 1962). Not only do hydromedusae also have photoproteins but so do radiolarians. In the genus *Thalassicola,* they have calcium-activated photoproteins and use coelenterazine to produce bioluminescent light (Latz et al. 1991).

Ctenophores use coelenterazine along with photoproteins. Ctenophore photoproteins have many similarities to hydromedusan photoproteins, however ctenophore photoproteins are extremely sensitive to light (Ward et al. 1976). Most ctenophore photoproteins do not have cysteine residues and therefore do not need a reducing agent for regeneration. According to Burakova and Vysotski et al. 2025, a discovery was found in the ctenophore Beroe, photoprotein berovin, that determined position 106 influences the color of bioluminescence. In two of the transcripts, if there was an alanine in that position, it would emit a more blueish color, while a serine would emit a more greenish color.

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Eurhamphaea vexilligera (*E. vex*) is a bioluminescent ctenophore found mainly in shallow topical waters that produces a blue-green light. While most ctenophores have internal bioluminescence, *E. vex* is unique in that it also secretes a luminous exudate that persists for several minutes after being released at night. *E. vex* can also secrete orange ink which can act as a distraction in the daytime. The pathway of how *E. vex* produces this persistent bioluminescence is not understood, and the functional characteristics of its photoproteins have not been studied. Given the unique characteristics of *E. vex* secreted bioluminescence, we sought to isolate its photoproteins and determine their biochemical properties.

After obtaining a transcriptome from *E. vex*, we searched for photoprotein sequences using known ctenophore photoproteins and compared their amino acid sequences. At the amino acid sequence position 106, there was a serine (Protein S) vs an alanine (Protein A). We expect that the differences in the amino acids at position will show a difference in the color of bioluminescences. Along with position 106, protein A has three cysteine residues while protein S has no cysteine residues. Cysteine residues are essential to an accelerated regeneration time of the bioluminescent proteins (Markova et al. 2012; Kurose et al. 1989). If the cysteine residues are within about 2.03 to 2.05 Å, a disulfide bond will form which could affect the structure of the protein. Based on the AlphaFold Protein Structure model, Protein A has about 12.6 to 24.9 Å which might not make any disulfide bonds intramolecularly but could potentially form bonds intermolecularly (Supplemental Figure 3), which could affect the protein's regeneration efficiency and bioluminescence activity. To test this, we added the reducing agent, βME or Beta-Mercaptoethanol, which will break the covalent disulfide bonds. We hypothesize that the analysis of amino acid sequences, the replacement of the alanine for the serine will shift its light emission spectrum towards longer wavelengths and the cysteine residues will allow a more efficient regeneration of the apophotoprotein.

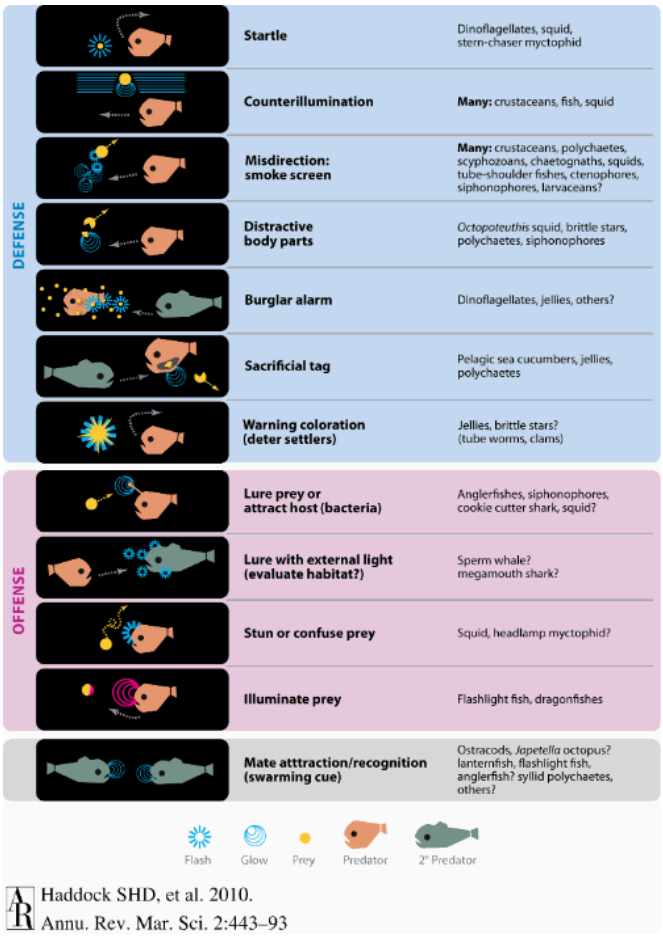


Figure 1. Description of the roles bioluminescence plays in different organisms.

Materials and Methods

gBlock DNA sequences and primer design

A PacBio long-read transcriptome was previously obtained from an *E. vex* specimen collected in Kona, Hawai'i by bluewater diving. Photoprotein isoform sequences were identified with BLAST searches using known ctenophore photoproteins. The candidate sequences were then aligned with the reference sequences along with related non-luminous calcium-binding proteins to determine which represent plausible photoproteins. We then inspected the residue identities at position 106 to select isoforms for expression cloning. We chose two candidates; transcript #587113 (Protein A) and transcript #297645 (Protein S). Protein A has an alanine at position 106 and 3 cysteine residues, and Protein S has a serine at position 106 and no cysteine residues (Figure 2). The sequences were codon-optimized for expression in *E. coli* BL21(DE3),

and restriction sites were added for ligation into the pET28a vector. Oligonucleotides and synthetic gBlock DNA sequences were purchased from Integrated DNA Technologies (IDT).

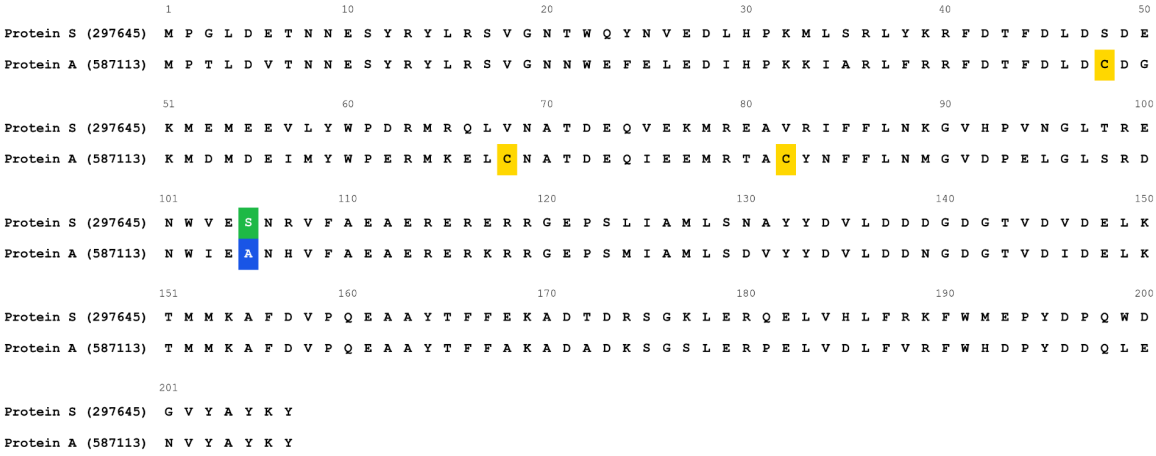


Figure 2. Amino acid sequence for both Protein A and Protein S aligned with multi-color to show amino acids of interest.

Restriction digest of vector and insert

In 1.5 mL DNA Lobind tubes, we added 6µl nuclease-free water, 2 µl 10x enzyme buffer, 10 µl 100ng/µl pET28a, 1 µl Ndel, and 1 µlXhol for the vector and 8 µl nuclease-free water, 2 µl 10x enzyme buffer, 8 µl insert DNA, 1 µl Ndel and 1 µl Xhol for the insert. We vortexed and spun, incubated at 37°C for 10 minutes, and heated enzymes at 80C for 5 minutes. We loaded the entire product of inserts and vector on 1% gel and ran at 90V for 60 min and cut out bands and followed Qiagen Gel Extraction. Then eluted DNA in 30 µl buffer EB and measured DNA concentration with the Qubit following the protocol for Qubit Assay concentration. Qubit concentrations (Supplemental table 1.) were used to figure out ligation calculations.

Ligation reactions

Using the Qubit concentrations, we calculated the reaction volumes. We combined 13.42 µl nuclease-free water, 2 µl 10x enzyme buffer, 4.38 µl of the empty vector, and 0.2 µl of ligase in 1.5 ml DNA Lobind tube. In another tube, the vector and protein S was combined with 9.96 µl nuclease-free water, 2 µl enzyme buffer, 4.38 µl of vector, 3.46 µl of S, and 0.2 µl of ligase. Last tube contained vector and protein A, 5.2 µl of water, 2 µl of buffer, 4.38 µl of vector, 8.22 µl of A, and 0.2 µl of ligase. We then flicked to mix and spun down, incubated at room temperature for an hour and stored at -20°C.

Vector x insert/vector size x 3 = insert amount in µl

Bacterial Transformation of plasmid into DH5a cloning strain

We thawed the DH5a for 15 minutes and added 5 µl of plasmid to the 50 µl of insert. We flicked the tubes to mix and placed them on ice for 30 minutes. Heat shocked at 42°C for 30 seconds and returned to ice for 2 minutes. We added 250 µl of SOC medium and placed it in the incubator for an hour with shaking at 225 rpm. We removed the tubes to make dilution of 1:10 and 1:100. Then spread on plates and place in the incubator for 16 hours. We removed the transformation plates and inoculated cultures with 2 ml liquid LB. Used a sterile loop to pick up the colony and swirled it in the LB culture tube for each of the cultures. Then placed those tubes in the incubator at 37°C with shaking for 16 hours. We followed the Qiagen Miniprep protocol and eluted plasmid DNA in 30 µl of water. Then measured the DNA concentration with the Qubit dsDNA assay (Supplemental table 2). Then we amplified the DNA for PCR using each primer pair (T7F/R and T7F/internal R). We added 1 µl of 10ng/µl plasmid DNA to each tube, flicked to mix, spun, and placed in the thermocycler. We prepared 100 ml 1% agarose gel at 100V for 45 minutes. Insert sequences were verified by colony PCR and Sanger sequencing with T7 universal primers and an internal insert-specific reverse primer.

Bacterial Transformation into BL21 (DE3) expression strain

We removed the express strain and thawed for 15 minutes and added 1 µl of plasmid with insert to 50 µl of cells. We flicked to mix and placed on ice for 30 minutes. We heat shocked at 42C for 30 seconds and returned to ice for 2 minutes. We added 250 µl of SOC media to each tube and placed it in the incubator at 37°C for an hour with shaking and made 1:10 and 1:100 dilutions. We plated and incubated for 16 hours. We used A5 and S1 because it was the closest to 10 ng/µl. We made glycerol stocks and amplified samples and ran another gel.

Protein expression induction of BL21 (DE3) clones containing plasmid

We added 2 ml of overnight culture to 200 ml LB kan and took an initial OD_{600} measurement. We incubated culture at 37°C with shaking at 225 rpm and took 1 ml every 30 minutes to measure the OD. Before induction, we removed 1 ml of culture and spun down at 6800 xg for 5 minutes and resuspended in 150 µl and added 150 µl 2x loading buffer and 60 µl DTT, boiled at 98°C for 5 minutes and stored at -20°C. Once the culture reached an OD600 of ~ 0.7m we induced by adding 1 ml 0.1 M IPTG and incubated at 37°C for 6 hours. Then spun at 4700 xg for 30 minutes and removed supernatant and stored the pellets at -80°C.

Protein extraction and NI-NTA purification

We prepared a lysis buffer to 10 ml equilibration buffer, added 10 mg lysozyme in one tube. We resuspended each pellet directly in a 5 ml ice cold lysis buffer and incubated it on ice for 30 minutes. Then sonicated on ice using microtip 40% amplitude. Split each lysate into 6x 1.5 ml microfuge tubes and centrifuge at 15,000 xg 4°C for 30 minutes and transfer and combine supernatant to 5 ml tube. We resuspened one pellet per protein in 833 µl equilibration buffer and saved 16 µl for SDS-PAGE. We continued with the column equilibration spinning at 700 xg for 2 minutes to remove the storage buffer. We did some additional imidazole washes like 3 20 mM imidazole washes, 2 40 mM, and then 3 elutions with 250 mM, which we then combined and desalted using Amicon 10 kDa MWCO cut-off spin filters. We proceeded with SDS-PAGE run at 185V for 50 minutes and stained with Coomassie blue overnight and destained with water for 7 hours (Supplemental Figure 2). To get the concentration of protein we ran a Qubit Protein Assay (Supplemental table 3)

Comparison to live E. vex spectrum

We received E. vex from the Monterey Bay Aquarium collected by blue-water diving in Kona, Hawai'i. We also took bioluminescence spectra from the E. vex to compare it to our recombinant proteins.

Regeneration of recombinant ctenophore apophotoprotein and luminescence assays

Apophotoprotein was added to a final concentration of 0.5 µM in a 1 mL reaction in regeneration buffer (50 mM Tris-HCl, NaCl (either 100 mM, 450 mM, or 1 M), 5 mM EDTA.) along with a molar excess of synthetic coelenterazine (5 µM; Nanolight Technologies). This was placed on the rotator and incubated at 4°C with rotation for 16 hours. Bioluminescence was measured with a luminometer and spectral measurements. To test the cysteine residues theory, we added 2 µl of BME, a reducing agent. We tested the photoproteins at 4 different ph levels, 7.4, 8, 9, and 10. For this assay, we used 450 mM of NaCl and varying pH levels of regeneration buffer. Samples were placed on a rotator for 16 hours at 4°C and measurements were taken. For the salt concentration assay, we tested either 100 mM (low salt), 450 mM (normal salt), or 1M (high salt) of NaCl with a pH level of 9. Samples were placed on a rotator for 16 hours at 4°C and measurements were taken. For the time assay, we tested regeneration time taking measurements at hour 4, 8, and 16 with a regeneration buffer at pH 9 and 450mM of NaCl placed at 4°C for incubation. Lastly, our light inactivation assay, where we placed the samples in 15, 30 and 60 minutes of light using

450mM of NaCl and pH 8. Samples were placed on a rotator for 16 hours at 4°C and measurements were taken.

Measurements and statistical analysis

Used a Tecan Infinite M200 plate reader to measure the RLU for each of the experiments to test the best condition of proteins. Regenerated ctenophore photoprotein using measurements above but used different buffers for each experiment. All graphs were analyzed using R.

Results

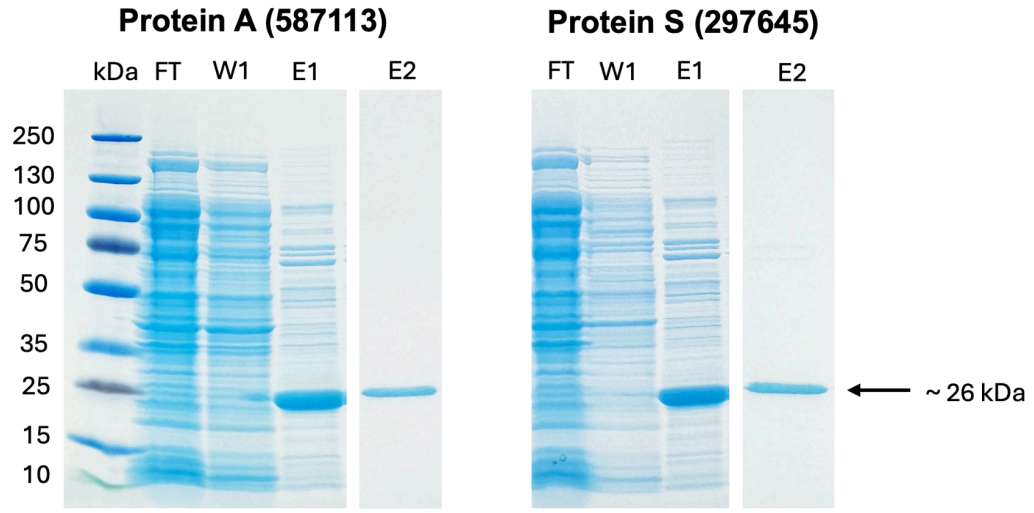


Figure 4. SDS-PAGE gel for both proteins. FT = flowthrough, W1 = 20 mM imidazole wash, E1 = elution after 20 mM imidazole washes, E2 = elution after 40 mM imidazole washes.

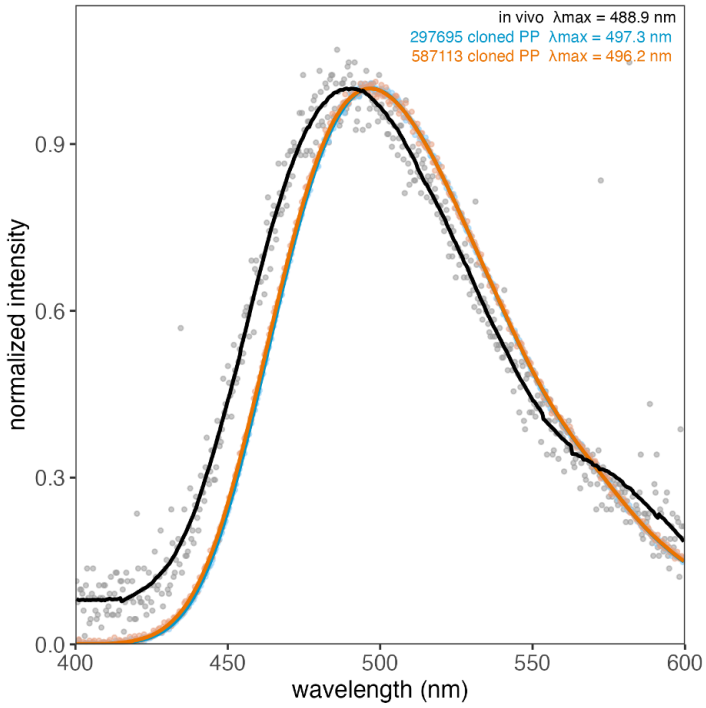


Figure 5. Shows a comparison spectrum of the live *E vex*, the cloned photoprotein A and S.

We were able to achieve good purity for both isoforms after the chromatography step (Figure 4). We found that Protein A and Protein S did not exhibit significantly different wavelengths. The wavelengths were similar with a λmax of 497.3 nm for Protein S and 496.2 nm for Protein A (Figure 5). Although, the *in vivo* λmax was 488.9 nm. We found that there was no significant difference in the spectra between using βME, the reducing agent, and no βME for each of the proteins (Figure 6).

When measuring the relative light unit (RLU) area under the curve, Protein A had a more intense light than Protein S overall. But Protein S had a brighter light without βME than with. There was no difference with and without BME for Protein A (Figure 7). For the pH level assay, we found that for both proteins the light intensity was at its highest using pH 8. Protein A, did slightly better at pH 7.4 than pH 10 while Protein S at pH 7.4 did the worst overall (Figure 8). The salt concentration assay showed both proteins had a higher intensity of light at high salt concentrations (1M NaCl) (Figure 9). The regeneration time assay showed that hour 4 had the strongest intensity for both proteins (Figure 10). Lastly, the light inactivation assay showed that samples left in with light experienced a loss in activity than the ones left in the dark (Figure 11).

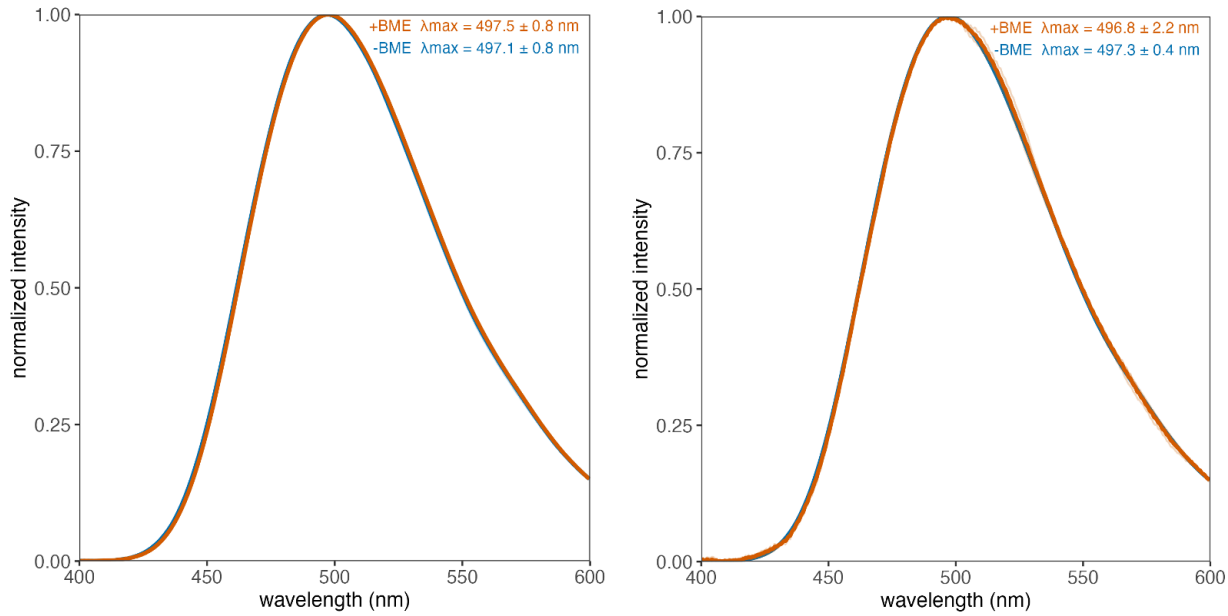


Figure 6. Spectra of Protein A (left) and Protein S (right) with and without BME.

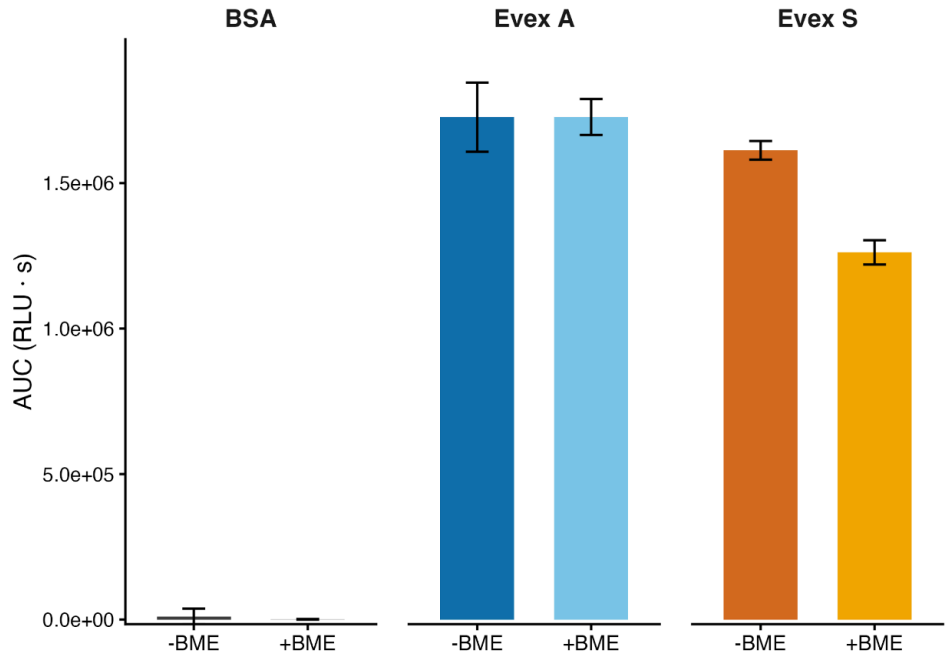


Figure 7. Area under the curve for both proteins with and without BME. BSA is the negative control.

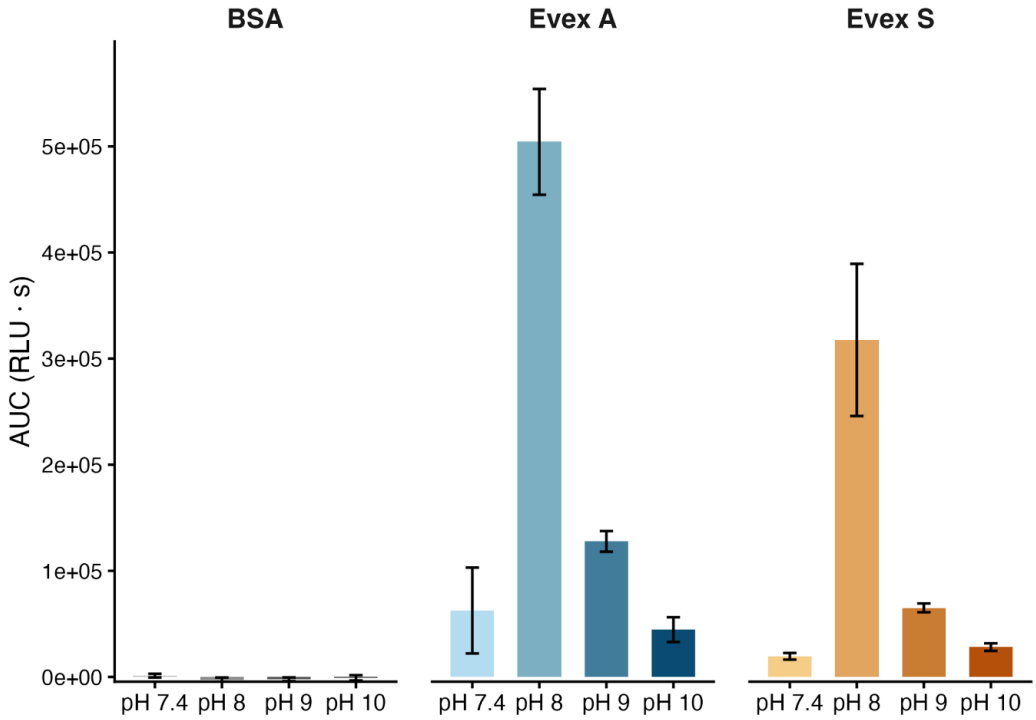


Figure 8. Area under the curve for both proteins with the different pH level regeneration buffers (pH 7.4, pH 8. pH 9, and pH 10).

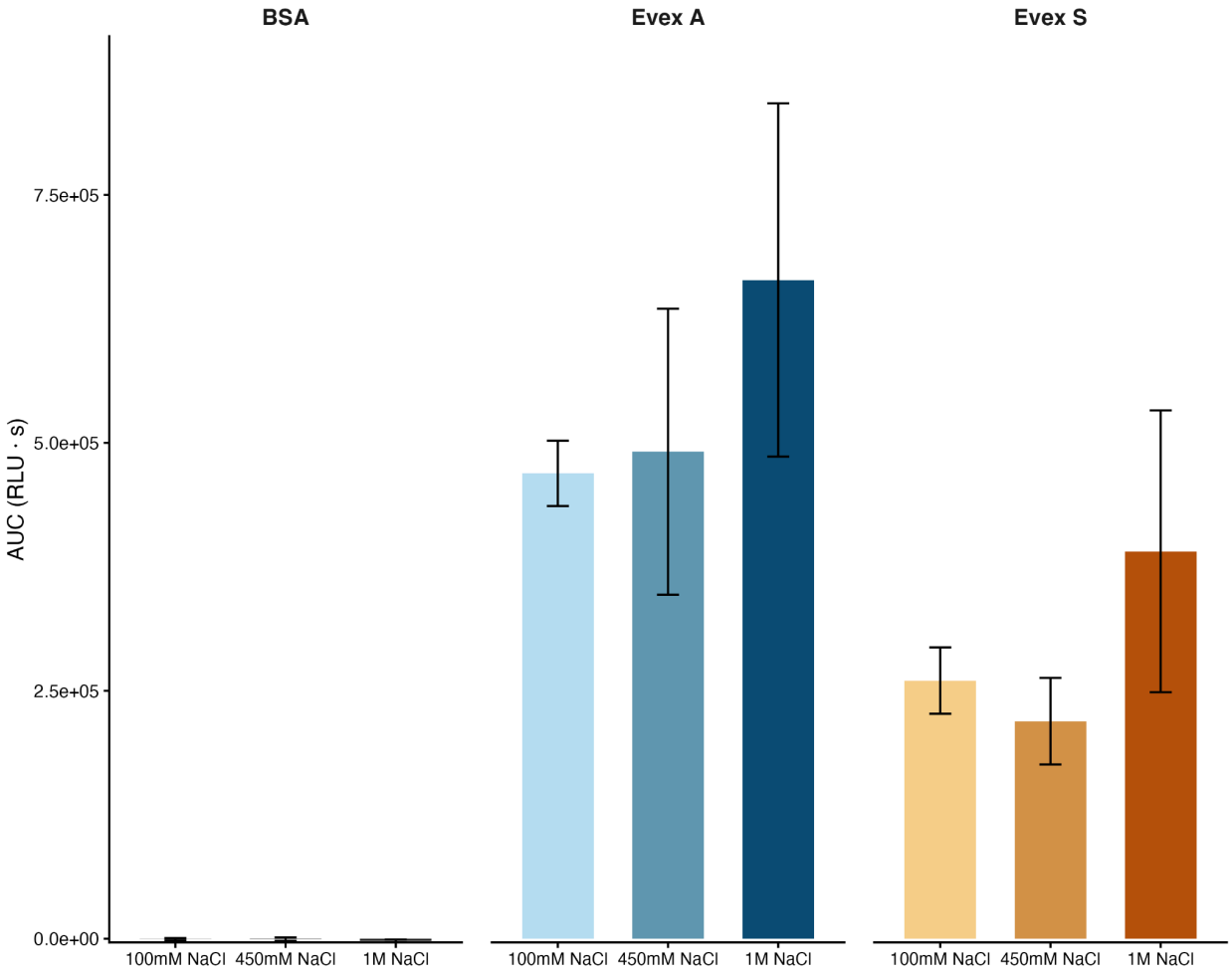


Figure 9. Area under the curve for both proteins with different salt concentration regeneration buffers at 100mM NaCl, 450 mM NaCl, and 1M NaCl.

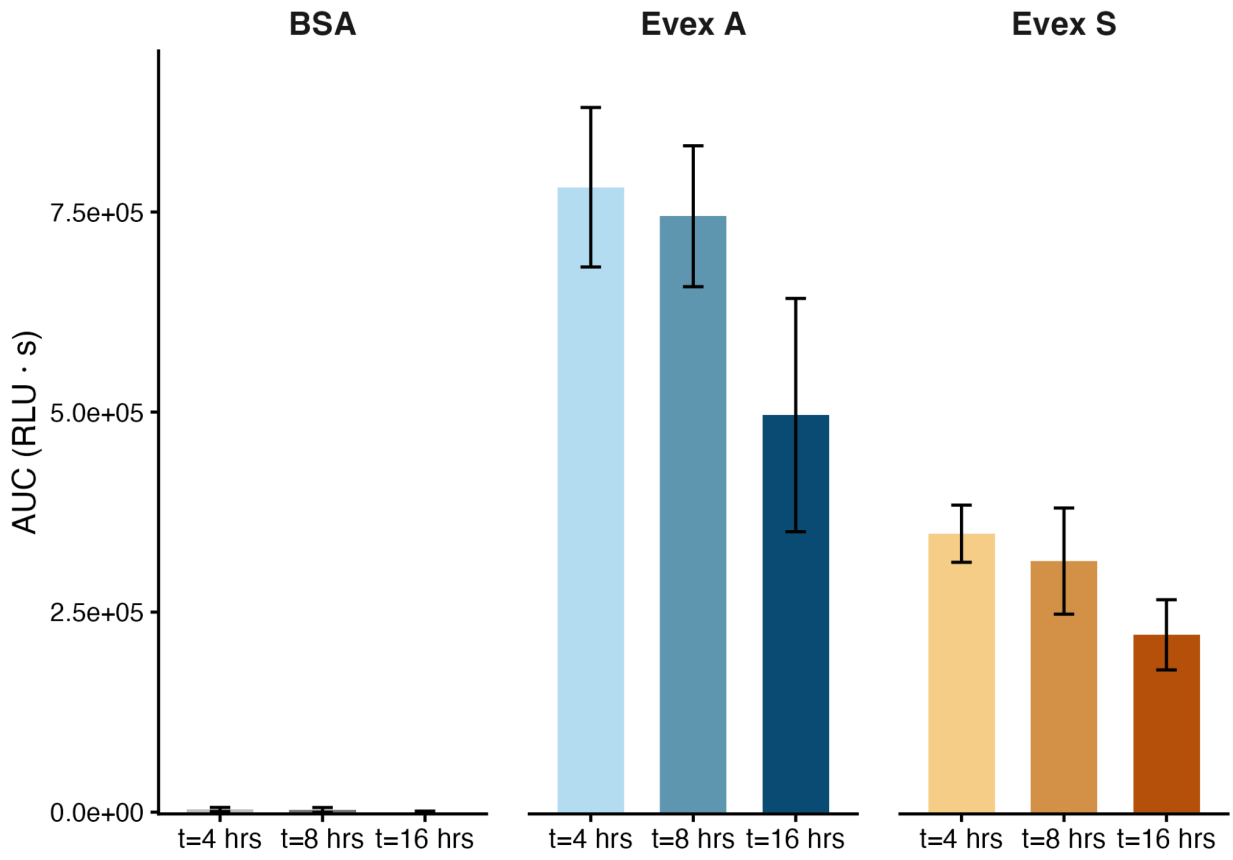


Figure 10. Area under the curve for both proteins with different regenerate times at hour 4, hour 8, and hour 16.

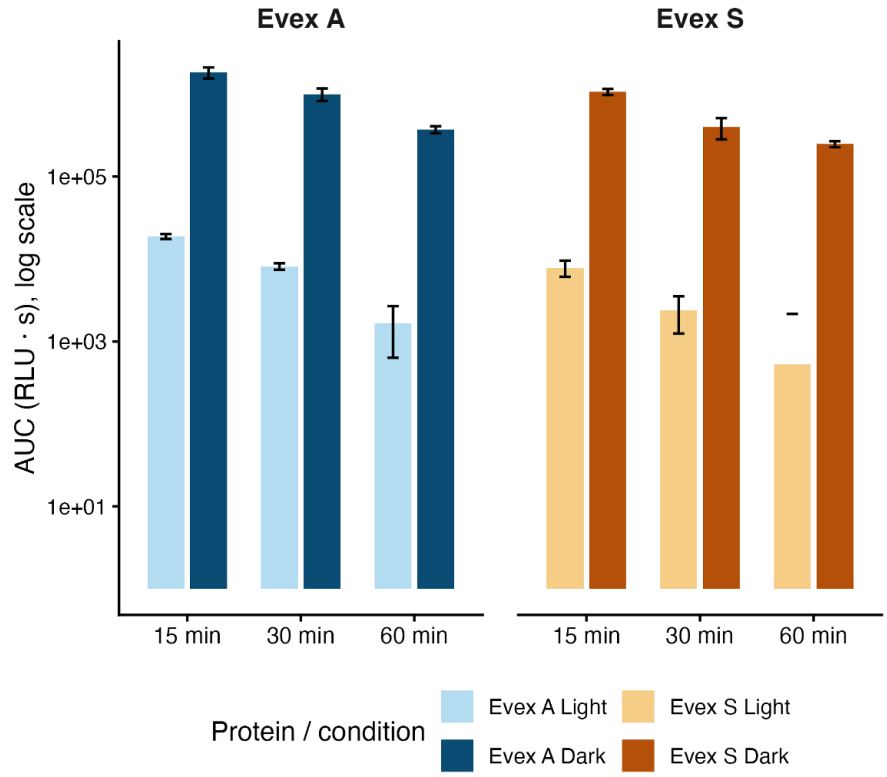


Figure 11. Area under the curve, log scale, of *E. vex* samples in the light vs the dark at different times.

Discussion

Overall, Protein A had the brightest bioluminescent light compared to Protein S throughout all of the experiments. This suggests that the differences between the two proteins could potentially be from other factors such as the coelenterazine binding to the protein, or the folding of the recombinant protein. Another possibility could be that Protein S is a little more unstable and degrades faster than Protein A. One way that we could test this is through another assay where we could incubate the proteins at the same temperature with differing times and measure their activity.

Another notable finding was the redshift observed in the cloned photoprotein to the light produced by the live organism. This shift indicates that the recombinant protein does not perfectly reproduce the spectral properties of the light produced *in vivo*. Some possible explanations include differences in post-translational modification, the protein

microenvironment. This is important to note because using recombinant photoproteins as a proxy to understand the light emission of photoproteins could be beneficial to know for the next study. For are other possible explanations for the redshift such as how the coelenterazine may bind with the isoform or possible dimers formed in the recombinant proteins (S. V. Markova and Vysotski 2015).

The buffer and environmental conditions that were used for each experiment had a substantial light intensity and this effect was not uniform across the photoproteins. Seawater, at approximately pH 8, tended to produce the best results overall, but the optimal pH depends on the specific photoprotein being tested. For example, the photoprotein berovin from the ctenophore bathocyroe performed considerably better at pH 9 (Svetlana V. Markova et al. 2012). This is another example that the optimal conditions in a controlled environment do not correspond to the conditions these animals experience naturally. In some cases, we observed brighter light emissions at salt concentrations and pH levels outside the animal's natural range.

Two factors related to reagent handling had a clear effect on the measured brightness which was the freshness of the coelenterazine and the amount of time and storage conditions before the experiment (ice vs refrigeration). Coelenterazine is prone to oxidizing on its own, so for some experiments the coelenterazine used was a fresh batch while for others it was not (Webb et al. 2010). A fresh batch produced brighter luminescence than an older one. Longer times in the ice rather than refrigerated, reduced the regenerative capacity of the photoprotein. This is reflected in the hour 16 measurement, which was lower than the other timepoint by an order of magnitude.

Some adjustments to the assays could have strengthened the results. Using the same batch of coelentrazine across all trials would have made it possible to separate the effect of reagent degradations from the experimental variable. Throughout all of the optimal conditions, more frequent sampling between the surrounding "optimal" points would help determine whether this represents a true reflection of the optimal conditions. Testing whether ice vs refrigeration had an effect on the loss of proteins rather than observing this could strengthen the results as well. Lastly, using *in vivo E. vex* in these different optimal conditions could also be a great comparison to the isoform photoproteins instead of just using the live *E. vex* to measure the spectrum of their bioluminescence.

These results are important beyond the optimal assays because photoproteins are used to understand how these animals interact with their environment. Understanding their communication in the dark ocean is essential to learning more about these animals. When we

learn and understand how sensitive light output is to pH, salinity, and handling time tell us that these bioluminescent systems are likely to be affected by a changing ocean. As conditions like ocean pH and temperature shift, organisms who use signaling and defense mechanisms depend on photoproteins being able to work. Characterizing the range is necessary to understanding how these animals might respond to environmental changes and learning what it takes to protect them.

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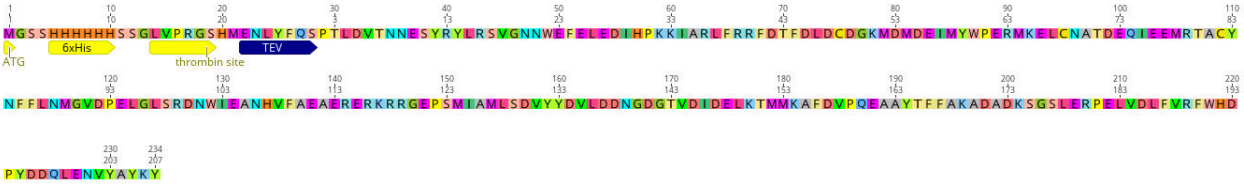
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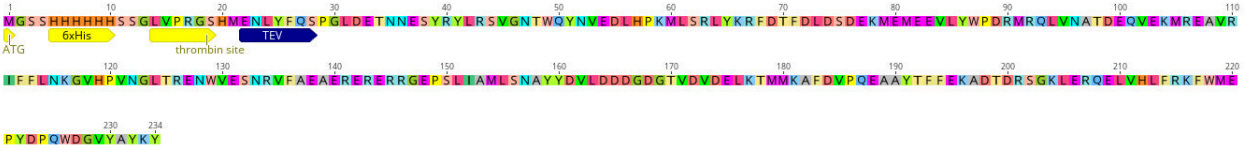
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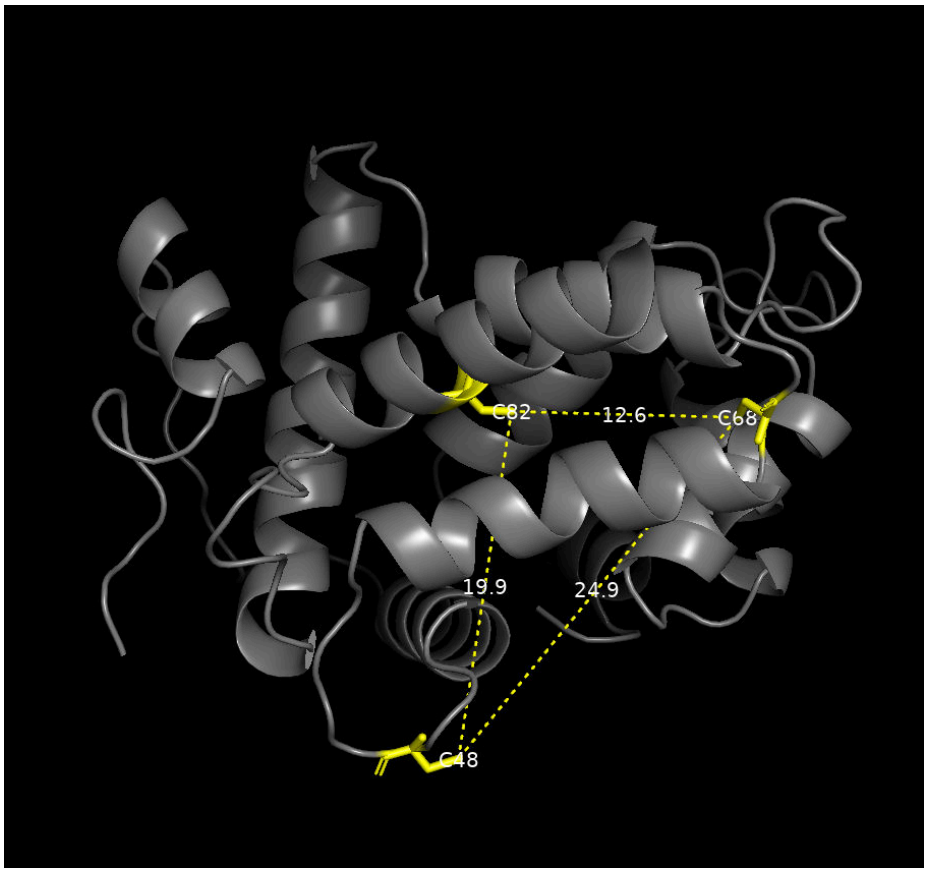
Supplemental Information

Supplemental Figure 1. The amino acid sequence of Protein A.



Supplemental Figure 2. The amino acid sequence of Protein S.





Supplemental Figure 3. The protein structure of Protein A showing the cysteine residues and their proximity to each other.

Vector	11.4 ng/µl
Protein A	2.21 ng/µl
Protein S	5.24 ng/µl

Supplemental Table 1. Qubit concentrations for restriction digest of the vector and the inserts.

Sample number	Concentration (ng/µl)
A1	16.1

A2	8.56
A3	14.5
A4	9.60
A5	10.2
A6	8.87
EV1	12.7
EV2	9.83
EV3	11.6
EV4	12.2
EV5	5.60
S1	12.4

Supplemental Table 2. Qubit concentrations from cloning strain.

Sample	Concentration (ug/µl)
Protein A	1.42
Protein S	2.28

Supplemental Table 3. Qubit concentrations from expression strain.