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Discovery and Characterization of Novel Fluorescent Proteins and Chromoproteins from the Intertidal Sea Anemone *Anthopleura dowii* Verrill 1869

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Abstract

After analyzing the published transcriptome of the sea anemone *Anthopleura dowii* Verrill, 1869, we identified three putative fluorescent proteins whose features were unknown. Expression of the synthetic genes encoding these proteins in *E. coli* revealed them to be yellow fluorescent (adYFP), green fluorescent (adGFP), and dark blue nonfluorescent (chromoprotein adCP). Expression experiments involving *E. coli* cultures at 30°C and 37°C, followed by electrophoretic analysis and high performance liquid chromatography purification, revealed differential expression/solubility of the three proteins: adYFP was highly soluble when expressed at 30°C and poorly soluble at 37°C, adGFP was partially soluble only at 30°C, and adCP was only soluble at 37°C, with no protein detected in either soluble form or as inclusion bodies at 30°C. These results contribute to explaining the color polymorphism between sea anemones harboring the same genetic information, in response to different environmental conditions. The discovery of these fluorescent variants and chromoproteins may enable their use, through engineering, as genetic reporters, environmental sensors, or tools for cellular visualization in the field of biotechnology.

1 Introduction

In 1962, the green fluorescent protein (GFP) was isolated for the first time from the hydromedusa *Aequorea victoria* [1]. GFP is a protein composed of 238 amino acid residues, whose chromophore forms spontaneously from its own amino acid sequence, which explains its autonomous fluorescence and its subsequent biotechnological value [2–4]. It only requires oxygen to autofluoresce [5]. In bioluminescent cnidarians that emit flashes of green light, GFP functions as an energy transfer acceptor, shifting the wavelength of emitted blue light within the luminescent system [6, 7]. This role has been documented not only in *Aequorea* and *Obelia* but also in luminous anthozoans such as *Renilla* and related taxa [8–11]. It was long believed that fluorescent proteins were present only in marine bioluminescent organisms [12]. However, in 1999, Sergei Lukyanov *et al*. demonstrated that these proteins were also present in non-bioluminescent organisms such as corals and sea anemones [13]. Here, it is important to mention that two years earlier, Joerg Widennman filed a patent application on two fluorescent proteins isolated from the sea anemone *Anemonia sulcata* [14, 15] and four years earlier, in 1995, Sophie Dove *et al.* reported the isolation and characterization of some pink and blue GFP-like chromoproteins from five corals collected at the Great Barrier Reef [16]. That same year, Charles Mazel reported the *in situ* fluorescence of many unknown GFP-like proteins found in 12 corals collected at Biscayne National Park in Florida [17]. Interestingly, in some cases, spectroscopic analysis performed on separate specimens of a single species indicated clear differences in emission spectra.

For more than 3 decades, fluorescent proteins have been essential molecular tools facilitating the study of many cellular processes, including gene expression, protein localization, protein mobility, protein interactions, and protein fate [18–20]. Many fluorescent proteins with diverse properties have been cloned from organisms across three phyla of multicellular animals, namely, Cnidaria, Arthropoda, and Chordata, particularly the first of these. Cnidarian fluorescent proteins, especially those from anthozoans

such as corals and sea anemones, are thought to have evolved from an ancestral green fluorescent protein and later diversified in both color and function [21, 22]. The proteins within this group are associated with body coloration, light-related biology, and various physiological processes, while certain ecological functions have been proposed but remain under debate [22–24].

The GFP-like fluorescent protein superfamily is widely distributed in Metazoa. The hallmark of these proteins is a β-barrel structure within which the chromophore responsible for fluorescence is post-translationally formed and embedded [25, 26]. Phylogenetic studies suggest that an ancestral gene of this superfamily was already present in the common ancestor of Cnidaria and Bilateria [27–29]. In anthozoans, the ancestral protein subsequently underwent broad molecular and spectral diversification, including numerous independent transitions in color and repeated convergent evolution of red fluorescent proteins and purple–blue chromoproteins [30, 31]. In corals, three main paralogous lineages have been identified: two generating diverse fluorescent colors and one remaining as non-fluorescent chromoproteins [10, 32, 33].

Fluorescent proteins have a significant impact on sea anemones due to the wide variation in color of the fluorescence they emit. In intertidal anemones, it has been found that allelic variation in a single fluorescent protein–coding gene can generate color polymorphisms by affecting both the intensity of protein fluorescence and the level of protein expression [22]. These variants also differ in antioxidant capacity, and experimental work has shown that fluorescent proteins can protect living cells against oxidative stress. This provides strong evidence that a single fluorescent protein can contribute both to visible coloration and to cellular protection [21, 22, 33, 34].

In sea anemones of the genus *Anthopleura*, the level of fluorescent proteins changes with ambient light conditions, and fluorescence persists even after prolonged darkness [21]. Meanwhile, in *Nematostella vectensis*, fluorescence is spatially and temporally regulated during development rather than randomly distributed, suggesting that some fluorescent proteins may exhibit tissue-specific expression patterns [35]. This means that, in sea anemones, fluorescence is not merely decorative but developmentally and physiologically important.

It has been shown that sea anemones possess fluorescent proteins with a diverse array of colors, such as cyan, green, yellow, orange and red; even engineered color-shifting variants of DsRed fluorescent protein have been obtained, suggesting parallel outcomes between natural and laboratory evolution [36, 37]. Meanwhile, in the blue chromoprotein cjBlue in *Cnidopus japonicus*, it has been demonstrated how small structural changes in the chromophore environment can markedly alter optical properties, since even a single amino acid substitution can shift the observed color [38]. Likewise, it has been found that the deep sea anemone *Cribrinopsis japonica* possesses a tentacular GFP that is not associated with bioluminescence, however, it is involved in behavioral responses to blue light and remains stable under harsh physical conditions, suggesting its functional adaptation in deep-sea environments [39, 40].

In anthozoans, including sea anemones, fluorescent proteins are involved in coloration and also in physiological and ecological processes, particularly responses to light, symbiosis-related stress, and

antioxidant protection. However, other biological functions, such as communication, camouflage, prey attraction, and protection against predators, have hardly been studied [24].

Nine years ago, the transcriptome of a specimen of the sea anemone *Anthopleura dowii* Verrill, 1869, collected at the rocky intertidal zone of El Sauzal, Baja California, Mexico, was sequenced to search for possible venom components in its tentacles [41]. The results revealed that its venom is made up of toxins that act on voltage-regulated Na^+ and K^+ channels, cytotoxins, and a diverse group of hydrolases [42]. Because a large body of evidence has found that a single sea anemone specimen may express various fluorescent proteins at the same time [31] [29, 33, 43] once that transcriptomic sequence was released, we searched for possible GFP-like proteins by comparing it with the amino acid sequence of the red fluorescent protein eqFP578 found in *Entacma quadricolor*, another sea anemone [44]. This analysis identified three putative fluorescent proteins. Here, we describe their characterization by cloning their synthetic genes into *E. coli*. Surprisingly, we found temperature-dependent differential folding of the three proteins, which may explain the different phenotypes observed in organisms of the same species [17, 22], in addition to the effect of light as previously reported by other researchers [21, 43]. The results of this research contribute to the study of fluorescent proteins from sea anemones, deepen our understanding of animal adaptation to environmental stress, aid the development of bioimaging tools, and should facilitate the characterization of novel proteins from unexplored species, particularly those from extreme habitats.

2 Results and discussion

2.1 Sequence analysis of three putative fluorescent proteins from *A. dowii*

Based on transcriptomic analysis of the sea anemone *A. dowii* Verrill, 1869, we identified three putative fluorescent proteins and named them adFP1, adFP2, and adFP3 because their properties were initially unknown. Sequence alignment revealed that adFP1 shares 55.4% identity with adFP2 and 71.5% with adFP3, while adFP2 and adFP3 share 57.0% identity. The differences in sequence identity among these putative fluorescent proteins suggest that they could be phenotypically distinct. Interestingly, the reference protein eqFP578 exhibits 62.4% sequence identity with adFP1, 61.4% with adFP2, and 60.1% with adFP3. In contrast, the average sequence identity of these three proteins with the original GFP from *Aequorea victoria* is 18.1% which can be explained by the evolutionary distance between jellyfish and sea anemones, although both belong to the phylum Cnidaria.

A BLAST search using the NCBI database identified the closest homolog to adFP1 as a green fluorescent protein from the deep-sea anemone *Cribrinopsis japonica*, which shares 69% sequence identity with this protein, designated cjFP510 [39]. For adFP2, high sequence identity (89%) was observed with the far-red fluorescent protein AQ143, which was engineered from the parental blue chromoprotein aeCP597 cloned from the sea anemone *Actinia equina* (beadlet sea anemone) [45]. The chromoprotein aeCP597 displays an absorption maximum at 597 nm, which determines the coloration of the sea anemone basal disk. A

BLAST search using adFP3 as a query revealed 75.4% sequence identity with the green fluorescent protein asFP499 found in the sea anemone *Anemonia sulcata* var. *rufescens* [46].

2.2 Gene synthesis and cloning

Genes encoding the three adFP proteins were designed based on their amino acid sequences. For adFP1 and adFP3, each gene was manually constructed by alternating the two most frequently used *Escherichia coli* codons for each amino acid, beginning at the amino-terminal end. In contrast, adFP2 was designed using SnapGene software (from Dotmatics, available at snapgene.com), which selected only the 20 preferred *E. coli* codons for the entire gene. The corresponding proteins and designed genes are presented in Fig. 1S. This design strategy was expected to result in the highest expression of adFP2 in *E. coli*.

The adFP1 and adFP3 genes were assembled in a single PCR step using the simplified gene synthesis approach [47] as previously demonstrated for the monomeric red fluorescent protein mKate2 [48]. This method used a diluted mixture of 24 partially overlapping oligonucleotides (sequences shown in Tables S1 and S3) that served as templates and were extended by the outermost oligonucleotides listed in the same tables. The outermost oligonucleotides also incorporated *NdeI* and *BamHI* restriction sites to facilitate cloning [48]. This assembly strategy required the chemical synthesis of 24 oligonucleotides, each averaging 58 nucleotides in length, resulting in a total of 1,392 nucleotides to fully cover both the sense and antisense strands of each gene.

To reduce the cost of gene synthesis, the adFP2 gene was assembled from eight designed oligonucleotides, each averaging 110 bases in length (Table S2), with complementarity restricted to their terminal regions [49]. These oligonucleotides totaled 880 bases, representing a reduction of 512 bases compared with the initial approach. However, a one-pot PCR assembly using all oligonucleotides simultaneously did not yield a complete gene in a single PCR step, even under varied PCR conditions, as was successful for adFP1 and adFP3. Consequently, a stepwise assembly strategy was adopted, involving four successive PCR reactions that began with the innermost oligonucleotides and concluded with the outermost. While the first strategy was faster and simpler, it was somewhat more expensive due to the higher cost of oligonucleotides.

The three synthetic genes were cloned into the constitutive plasmid pJOQ using the *NdeI/BamHI* restriction sites [50]. Following transformation, *E. coli* cells expressing adFP1 exhibited yellow fluorescence, adFP2 appeared gray and non-fluorescent, and adFP3 displayed green fluorescence, as observed in the pellets of cultures grown at 30°C and 37°C (Fig. 2S). These results suggested temperature-dependent differential expression of the three genes, as evidenced by variations in color and fluorescence intensity.

Cells were resuspended in buffer and lysed by sonication to analyze the protein content of the soluble and insoluble fractions using denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After lysis, adFP1 yielded a yellow fluorescent supernatant, adFP3 produced a green

fluorescent supernatant, and adFP2 resulted in an indigo non-fluorescent supernatant, in contrast to the gray color observed in the pellet. The insoluble heterologous protein fraction for each variant was colorless. On the basis of these findings, adFP1, adFP2, and adFP3 were named *A. dowii* yellow fluorescent protein (adYFP), *A. dowii* chromoprotein (adCP), and *A. dowii* green fluorescent protein (adGFP), respectively. Notably, adCP and adGFP exhibited the same color as their closest homologs identified by BLAST analysis, whereas adYFP was yellow rather than green, differing from its closest homolog, cjFP510.

SDS-PAGE analysis of the soluble and insoluble fractions qualitatively confirmed differential folding and solubility of the three proteins at both temperatures, as shown in Fig. 2. At 30°C, adYFP was predominantly present in the soluble fraction and minimally in the insoluble fraction. Meanwhile, at 37°C, adYFP was mainly detected in the insoluble fraction. A similar pattern was observed for adGFP: At 30°C, it was present in both fractions in comparable amounts, while at 37°C, it was detected only in the insoluble fraction. In contrast, adCP was found exclusively in the soluble fraction at 37°C. In terms of an explanation for these findings, in the literature it is indicated that inclusion bodies form when the translation rate exceeds the folding rate of the protein, as observed for adYFP and adGFP [51]. However, the absence of inclusion bodies for adCP under both temperature conditions suggests that the translation rate may have been slower than the folding rate, or the intermediate mRNA was highly unstable, despite the use of preferred *E. coli* codons. Thus, utilizing host-preferred codons does not universally ensure the optimal expression of recombinant genes [52]. Further experiments are required to determine the cause of the poor expression.

Gene expression is defined as the production of an observable phenotype by a gene, typically through the synthesis of a protein. However, most gene expression studies report only mRNA data, and the term “gene expression” is frequently used interchangeably with “mRNA measurement,” rather than direct quantification of the target protein [53]. Various research groups have reported that there is no general linear correlation between mRNA and protein production, as post-transcriptional regulation may contribute more significantly to changes in protein levels than to changes in mRNA levels [54, 55]. Consequently, factors such as mRNA stability, translation rate, protein folding, and protein half-life are critical post-transcriptional determinants of protein abundance [53, 56].

To measure gene expression, the ideal approach is to quantify the final total amount of the target protein. For recombinant proteins, this process is facilitated by adding a polyhistidine tag, which enables separation from other cellular proteins using immobilized affinity chromatography (IMAC). Separation can be achieved through batch purification or with a chromatograph that employs a controlled imidazole gradient for improved separation and real-time monitoring of the elution process via UV detection.

2.3 Expression, purification, and quantification of GFP-like proteins

GFP-like proteins were purified by IMAC, as described in the methods section. For this process, three Ni-NTA columns were connected in tandem, as illustrated in the inserts in Fig. 3. The chromatograms in

Fig. 3 indicate that most housekeeping proteins from *E. coli* were removed within the first 15 min of the run. At approximately 19 min, a fraction of *E. coli* proteins with moderate affinity for the stationary phase was eluted. Finally, the GFP-like proteins derivatized with a polyhistidine tag were eluted at approximately 24 min, with an average of 0.3 M of imidazole. The polyhistidine tag was incorporated during the cloning process. The final peak was collected for the subsequent characterization of the purified target proteins, and the elution buffer was immediately exchanged for PBS via ultrafiltration to remove imidazole. This purification step also confirmed that adYFP and adGFP exhibited yellow and green fluorescence, respectively, whereas adCP in the solution was indigo and non-fluorescent, as shown in the left panel of Fig. 4.

SDS-PAGE analysis indicated that the purified proteins were > 95% pure, as shown in lanes 2–4 in the right panel of Fig. 4. Under denaturing conditions, the proteins migrated as 27 kDa species, consistent with EGFP as a reference and in agreement with their expected molecular weight [57]. In contrast, size-exclusion chromatography demonstrated that the proteins formed tetramers, a property commonly observed in fluorescent proteins from corals and sea anemones (Fig. 3S) [58].

Quantitative analysis of the pure proteins by the BCA method (Table 1) corroborated qualitative observations based on pellet color intensity and SDS-PAGE band intensity. Specifically, adYFP expression was higher at 30°C than at 37°C (53 mg/L versus 11 mg/L, respectively). adGFP was produced at 30°C (24 mg/L), with only trace amounts detected at 37°C. In contrast, adCP was produced at 37°C (3.5 mg/L) but not at 30°C.

Protein folding and solubility are influenced by amino acid sequence and temperature, which may account for the color polymorphism observed in *Anthopleura* spp [22]. It is likely that adYFP and adGFP are highly expressed in *A. dowii* during winter, whereas adCP expression predominates in spring and summer when temperatures are elevated. The concurrent expression of these three proteins likely produces a range of color variations in the organism. Consequently, *A. dowii* may exhibit multiple phenotypes from a single genotype, representing a clear example of polyphenism. This phenomenon is attributed to the simultaneous presence and expression of several GFP-like genes within the same genome.

2.4 Physicochemical characterization

UV-Vis absorption analysis of the pure proteins revealed that adYFP displayed maximum absorption at 520 nm, whereas adCP did so at 590 nm and adGFP at 480 nm (Fig. 5). Excitation of these proteins at their maximum absorbance produced maximum emission peaks at 530 nm for adYFP and 495 nm for adGFP. adCP was non-fluorescent even at high concentrations. These fluorescence characteristics strongly suggest that adYFP and adGFP possess GFP-like chromophores [25, 26, 59]. Furthermore, sodium hydroxide treatment produced an absorption peak at 446 nm, which is characteristic of denatured GFP and facilitates determination of the extinction coefficient for GFP-like proteins [59]. In contrast, adCP was intensely colored but non-fluorescent, indicating the presence of a DsRed-like

chromophore, likely in a *trans* configuration and a non-planar arrangement, consistent with observations in most chromoproteins [60, 61].

NaOH denaturation was used to determine the extinction coefficients, yielding values of 100,628 M^{-1} cm^{-1} for adYFP, 83,462 M^{-1} cm^{-1} for adCP, and 82,511 M^{-1} cm^{-1} for adGFP. These values are higher than that of the reference protein EGFP (53,806 M^{-1} cm^{-1}) under identical conditions. The quantum yield for adYFP was 0.568, while that for adGFP was 0.663, with EGFP serving as the reference [62, 63]. Consequently, the brightness of adYFP (57.1) and adGFP (54.7) surpassed that of the classical reference protein EGFP (32.3). (Brightness is defined as the product of the extinction coefficient and quantum yield, divided by 1000.)

Table 1
Physicochemical properties of the GFP-like proteins cloned from the sea anemone *A. dowii*.

Protein	MW[a] (KDa)	Color	Yield of Pure proteins in cultures (mg/L)[b]	λ_{abs} (nm)	λ_{Ex} (nm)	λ_{Em} (nm)	□ (M^{-1} cm^{-1})	φ	Oligomeric state
adYFP	26.72	yellow	30°C: 53.1 37°C: 11.3	518	518	530	100,628	0.568	tetramer
adCP	27.22	indigo	30°C: 0 37°C: 3.5	589	NA	NA	83,462	NA[c]	tetramer
adGFP	26.55	green	30°C: 24.2 37°C: 0	480	480	495	82,511	0.663	tetramer
EGFP	28.05	green	37°C: 85.2	490	490	507	53,806	0.60	monomer

*MW: molecular weight in Kilodaltons, considering the C-terminal Tag -SGGSHHHHHH.

[b]After growing 24 h at the indicated temperature and storing the pellet for 72h at 4°C.

[c]NA: Not applicable

2.5 Proposed structure of adYFP

Although adGFP and adYFP both contain the GFP-type chromophore, their differing colors can be explained by the structural characteristics of the yellow fluorescent proteins 1YFP [64] and phiYFP [65]. 1YFP was engineered from the original GFP protein through the mutations T203Y, S65G, V68L, and S72A, with the first of these responsible for the red shift in emission from 510 nm to 528 nm [26]. Crystallographic analysis of 1YFP (1YFP pdb) demonstrated that the phenol group of Y203 is stacked over the GFP chromophore of the protein, reducing its excitation and emission energy toward longer

wavelengths via electronic interactions of the conjugated π orbitals of both aromatic systems. [64]. A similar effect was observed in the crystal structure of the natural yellow fluorescent protein phiYFP (4HE4 pdb), cloned from the marine jellyfish *Phialidium*, where Y203 is also stacked over its chromophore. This result aligns with expectations, as GFP and phiGFP share more than 50% amino acid sequence identity due to their common jellyfish origin. The structural basis for the color of adYFP was elucidated through its structure as predicted by alphafold3 [66], which revealed that the phenol group of Tyr195 is positioned over the chromophore formed by Ser63, Tyr64, and Gly65, as shown in Fig. 6. The artificial creation of 1YFP from GFP and the identification of the natural yellow fluorescent proteins phiYFP and adYFP, all of which feature an aromatic amino acid stacked on the chromophore, suggest an interconversion between green and yellow fluorescent proteins over millions of years of natural evolution. In contrast, in adGFP, the equivalent amino acid positioned over the chromophore is the isobutyl group of Leu194, which cannot interact with the aromatic π system.

While this research was in progress, Deheyn *et al.* reported the yellow fluorescent protein AsYFP, which was isolated from the sea anemone *Anthopleura sola* collected at Hopkins Marine Station, Monterey Bay, California [22], approximately 800 km from El Sauzal, Baja California. This protein differs from adYFP by only two amino acids: D7E and Q150H. The protein was identified using classical methods, including isolation from the animal extract and partial characterization by amino acid sequencing; then, the gene was amplified by generating cDNA and cloned for heterologous expression. The study also demonstrated that different fluorescent phenotypes in sea anemones may result from genetic polymorphism; specifically, the single mutation G153D in asYFP produced a slightly more fluorescent protein, termed Neon AsYFP, which led to more fluorescent organisms. In contrast to this approach, the present research identified fluorescent proteins of additional colors by utilizing a transcriptome as a source of potential fluorescent proteins.

2.6 Protein engineering

Preliminary approaches to improve the expression of the three genes by error-prone PCR gave good results only for the protein adYFP. In the first mutagenesis cycle, several variants more brilliant than the parental protein emerged. All of these variants contained the mutation L60P, in addition to other mutations located along the protein sequence. After HPLC purification, the variant adYFP-L60P was recovered at 525 mg/L from culture, 10-fold higher than the level of the parental protein adYFP. Therefore, L60P seems to be a folding-related mutation similar to F64L, which improved the folding of avGFP at 37°C and was one of the key mutations that gave rise to EGFP, in addition to S65T [57]. The expression of the other two proteins, adGFP and adCP, was also improved, but following a different strategy, namely, the addition of the GFP-terminal MASKEEDN sequence at their amino end, as previously used in our laboratory to improve the expression of two other proteins [67, 68]. This strategy improved adCP expression by 15-fold, whereas adFP3 expression was improved 4-fold. However, detailed information on these improvements will be published elsewhere, as well as the effect of the I122R mutation, which simplified the oligomerization state of the three proteins.

The results of this research contribute to the study of fluorescent proteins from sea anemones, deepen our understanding of animal adaptation to environmental stress, aid the development of bioimaging tools, and should facilitate the characterization of novel proteins from unexplored species, particularly those from extreme habitats.

3 Conclusion

In this work, we synthesized and cloned three genes that encode GFP-like proteins, identified in the transcriptome of the Pacific sea anemone *Anthopleura dowii* Verrill, 1869. Two of these proteins were fluorescent, yellow and green, whereas the third was a dark blue, non-fluorescent chromoprotein. The use of a single transcriptome sample collected under specific conditions probably prevented us from identifying more GFP-like proteins associated with the variety of colors described by Barragán *et al* in live specimens [69]. They described colors such as green, orange, and pink around the mouth; orange, brown, gray, or purple radial lines from the base of the tentacles to the mouth; and gray, purple, or orange along the tentacles. Consequently, proteins associated with some of the reported colors remain to be identified. A more comprehensive analysis would require genome sequencing rather than transcriptome sequencing.

Differences in solubility of the three proteins between two temperatures were observed, suggesting temperature-dependent limitations in protein folding. Further investigation is required to determine whether these differences reflect phenotypic variation observed in live animals under different environmental conditions or are due to codon usage in *E. coli* expression systems. Additionally, light regulation of fluorescent proteins may contribute to these visual changes, as previously proposed.

This study expands the known diversity of GFP-like proteins by functionally characterizing three novel chromatic variants in *A. dowii*, establishing a direct link between the physicochemical properties of these molecules and visual polymorphism in their natural environment.

4 Methods

4.1 Assembly and cloning of synthetic genes

The putative fluorescent proteins adFP1, adFP2, and adFP3 decoded from the transcriptome of the sea anemone *Anthopleura dowii* Verrill, 1862, and the genes designed to express them in *E. coli* are shown in Fig. 1S.

The genes for adFP1 and adFP3 were assembled as previously reported for mKate2, in a single PCR step, using a set of 24 oligonucleotides (shown in Supplementary Tables 1S and 3S, respectively) to cover both DNA strands, the sense and antisense. Briefly, a mixture template was prepared by taking 5 µL of each oligonucleotide at 10 µM and making up the mixture to 500 µL with Milli-Q water. Five microliters of this mixture was used as a template in an ordinary 100 µL PCR reaction, performed with 5

µL of each of the outermost oligonucleotides at 10 µM (shown in the same tables) and the Vent DNA polymerase to assemble both genes independently.

The gene for adFP2 was assembled via four consecutive PCR reactions, using the Vent DNA polymerase. In the first PCR reaction, the innermost oligonucleotides PrFw1 and PrFw-1 were mixed in equimolar amounts (100 nmol each, Supplementary Table 2S) and amplified through 25 PCR cycles. For the second PCR reaction, 2 µL of PCR1 was used as a template and amplified with the primers PrFw2 and PrRv-2 in 25 cycles. For the third PCR reaction, 2 µL of PCR2 was used as a template and amplified with the primers PrFw3 and PrFw-3 in 25 cycles. For the last PCR reaction, 2 µL of PCR3 was used as a template and amplified with the outermost primers PrFw4 and PrFw-4 in 25 cycles. It is important to mention that all oligonucleotides for the assembly of the adFP2 gene were purified by gel electrophoresis, whereas those for the genes adFP1 and adFP3 were used in crude form because of their shorter length.

Since the outermost primers introduced the restriction sites NdeI (catatg) and BamHI (ggatcc) at the 5' and 3' ends of the synthetic constructs, respectively, the digested inserts were cloned into the pJOQ plasmid previously digested with the same pair of restriction enzymes. The cloning process yielded the plasmids pJOQ-adFP1, pJOQ-adFP2, and pJOQ-adFP3. Note that pJOQ was constructed by Dr. Joel Osuna using parts of the plasmids pET28 and trc99A to constitutively express genes under the control of the strong trc promoter. pJOQ contains the kanamycin resistance gene and any gene cloned within the NdeI/BamHI restriction sites will express the target protein appended with a carboxyl-terminal polyhistidine tag -SGGSHHHHHH, for purification purposes. The correctly assembled genes were selected by DNA sequencing.

4.2 Expression and purification

Each of the plasmids pJOQ-adFP1 (pJOQ-adYFP), pJOQ-adFP2 (pJOQ-adCP), and pJOQ-adFP3 (pJOQ-adGFP) was used to transform chemically competent *E. coli* MC1061 cells, which were spread on Petri dishes and incubated at 37°C for 24 h. The next day, four cultures of 250 mL of LB supplemented with kanamycin were inoculated with one colony expressing pJOQ-adYFP, another four with pJOQ-adGFP, and four more with pJOQ-adCP. Cultures of each variant were incubated at 30°C and at 37°C for 24 h, with shaking at 200 rpm, in duplicate. The biomass of each culture was recovered by centrifugation and stored at 4°C for 3 days to complete the maturation process. Then, it was resuspended in 12 mL of PBS and subjected to sonication to release the proteins from the cells. A total of 0.5 mL of the sonicated solution was withdrawn and saved for analysis by SDS-PAGE. The rest of the sonicated material was centrifuged for 10 min at 15,000 rpm and the supernatant was purified by HPLC, starting with 100% of buffer A for 12 min, and then changing to 100% of buffer B in 20 min, at a flow rate of 0.9 mL/min (buffer A: 0.1 M phosphate buffer, pH 7.4, containing 0.25 M NaCl; buffer B: 0.5 M imidazole in buffer A). We have observed that this gradual change in imidazole concentration allows better separation of recombinant proteins bearing the polyhistidine tag. The separation was achieved on three Ni-NTA columns of 1 mL (HisTrap HP from Cytiva) connected in tandem, as shown in Fig. 2. Detection was performed at 280 nm. We collected only the fractions that contained the expected protein, eluted at approximately 24 min. The elution buffer was immediately replaced with PBS buffer by ultrafiltration,

using Amicon Ultra-4 centrifugal filters. The purity of the three proteins was analyzed by SDS-PAGE in denaturing conditions, and their concentrations were determined by the BCA method, following the manufacturer's instructions.

4.3 Qualitative expression by SDS-PAGE

The sonicated sample withdrawn in the purification step was centrifuged at 15,000 rpm for 10 min. The supernatant was transferred to another clean tube to constitute the crude soluble fraction. The pellet was washed twice with 1 mL of PBS to remove traces of the soluble fraction and then resuspended in 0.5 mL of solubilization solution for inclusion bodies (ThermoScientific, Cat. # 78115), constituting the "insoluble" fraction. A total of 8 µL of each sample (soluble and insoluble fractions) was mixed with 4 µL of denaturing loading buffer and 8 µL of running buffer, and then boiled for 5 min before being loaded on the denaturing 12% SDS-PAGE gel. Cultures grown at 30°C and 37°C were analyzed.

4.4 Spectroscopic characterization

The absorbance of each purified protein was determined on an Implen microspectrophotometer, from 250 nm to 700 nm, analyzing 2 µL of sample at a concentration of 2 mg/mL in PBS.

4.5 Fluorescence characterization

The excitation and emission spectra of the pure mutant proteins were recorded on an LS-55 fluorescence spectrometer (PerkinElmer). The emission spectrum of each protein was obtained by fixing the excitation wavelength at the maximum absorbance wavelength obtained by UV/Vis spectrophotometry and recording the emission in the range "excitation wavelength + 10 nm" up to 750 nm using 5-nm emission and excitation slits. The excitation spectra were recorded in a similar mode by fixing the emission wavelength at 10 nm above its maximum and recording excitation from 350 nm to the maximum emission wavelength.

4.6 Extinction coefficient (ε) and quantum yield (φ)

The extinction coefficient of each functional protein was determined spectrophotometrically by measuring the absorbance of triplicate 5 µM solutions in 1· PBS at the maximum absorption wavelength and by measuring the absorbance at 446 nm for triplicate solutions in 1 M NaOH [62], taking into consideration that the chromophores of adYFP and adGFP must be very similar to the chromophore of avGFP, whose extinction coefficient in the alkali-denatured state is 44,000 M^{-1} cm^{-1} at 446 nm [70]. The extinction coefficient of adCP was determined by the same method because, during alkali treatment, the RFP-type chromophore is converted into a GFP-type chromophore due to hydrolysis of the N-acylimine bond found in its structure [71].

Quantum yield was determined by measuring the absorbance of six duplicate dilutions of each protein at the maximum absorption wavelength [63]. Next, the fluorescence of each dilution was recorded at the maximum emission wavelength, with excitation at the maximum absorbance wavelength. The plot of absorbance versus fluorescence produced a linear graph with a particular slope. As a standard to

determine the quantum yield (φ) of the mutants, we determined the relationship between absorbance (488 nm) and fluorescence (507 nm) for pure EGFP, whose reported φ value is 0.60 [72]. Dividing the sample's slope by EGFP's slope and multiplying by 0.60 gives the sample's quantum yield.

Declarations

Competing Interests

The authors have no competing interests to declare that are relevant to the content of this article.

Author Contribution

ARS assembled and characterized the gene adFP1 (adYFP). EBP designed and assembled the gene adFP2 (adCP). PLF assembled and characterized the gene adFP3 (adGFP) and repeated most of the experiments described in the present research. JAY purified all plasmids and sequenced them by the Sanger method. GSR and CRA revised and corrected the manuscript critically. PG conceived and conducted the project, analyzed all the results and wrote the report.

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Figures

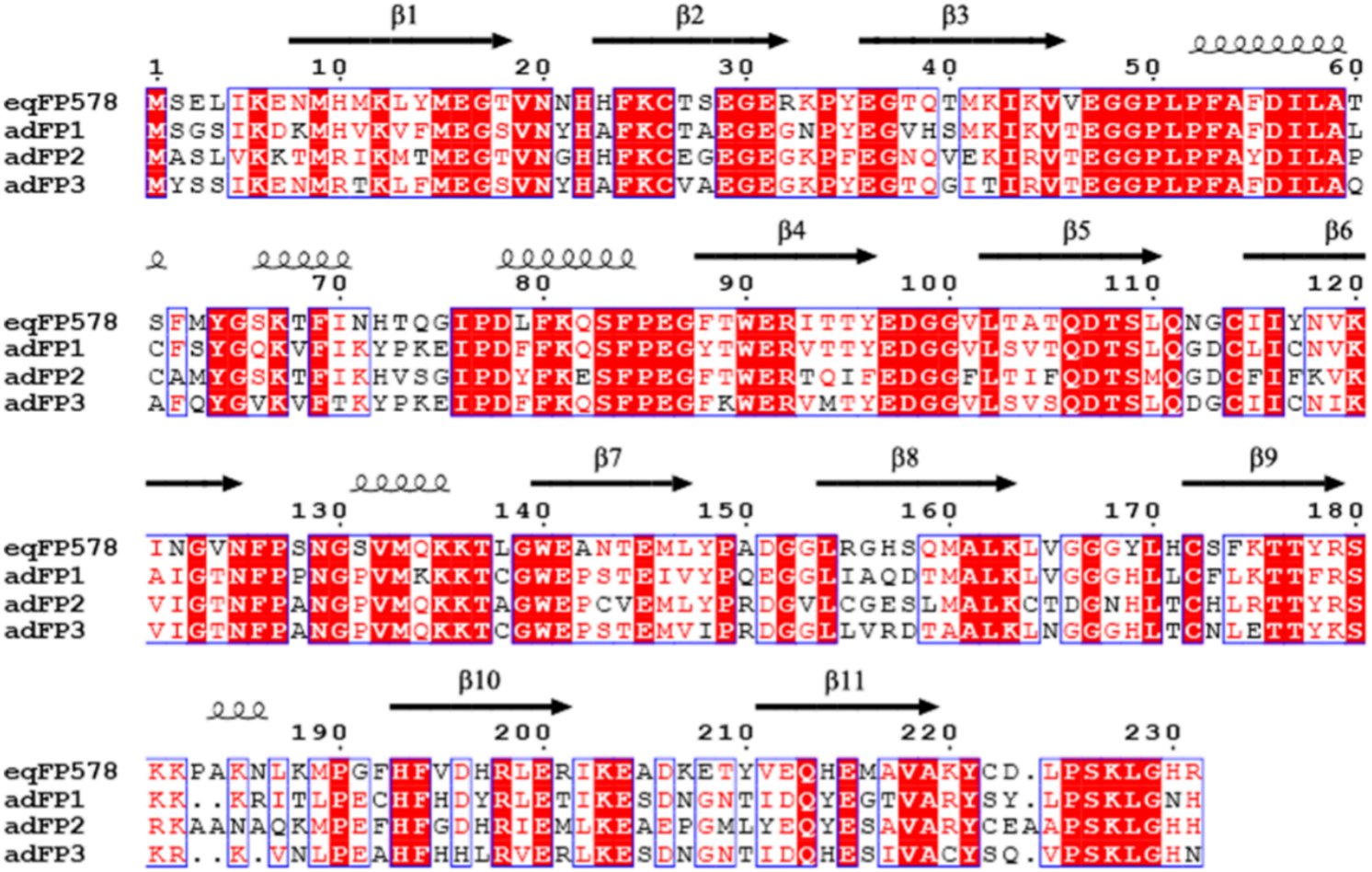


Figure 1

Amino acid alignment of three putative fluorescent proteins found in the transcriptome of the sea anemone *A. dowii*Verrill, 1869, using the protein eqFP578 from the sea anemone *Entacma quadricolor* as a reference. adFP1, adFP2, and adFP3 are composed of 229, 232, and 228 amino acids, respectively. The chromophore-forming amino acids are indicated by downward-pointing arrowheads. Here, it is important to note that the first amino acid of the chromophore-forming triad differs among the three putative FPs. Horizontal arrows indicate the residues that form the 11 β-strands surrounding the chromophore. α-Helices are indicated with coils. Figure created with the web server Espript 3.2

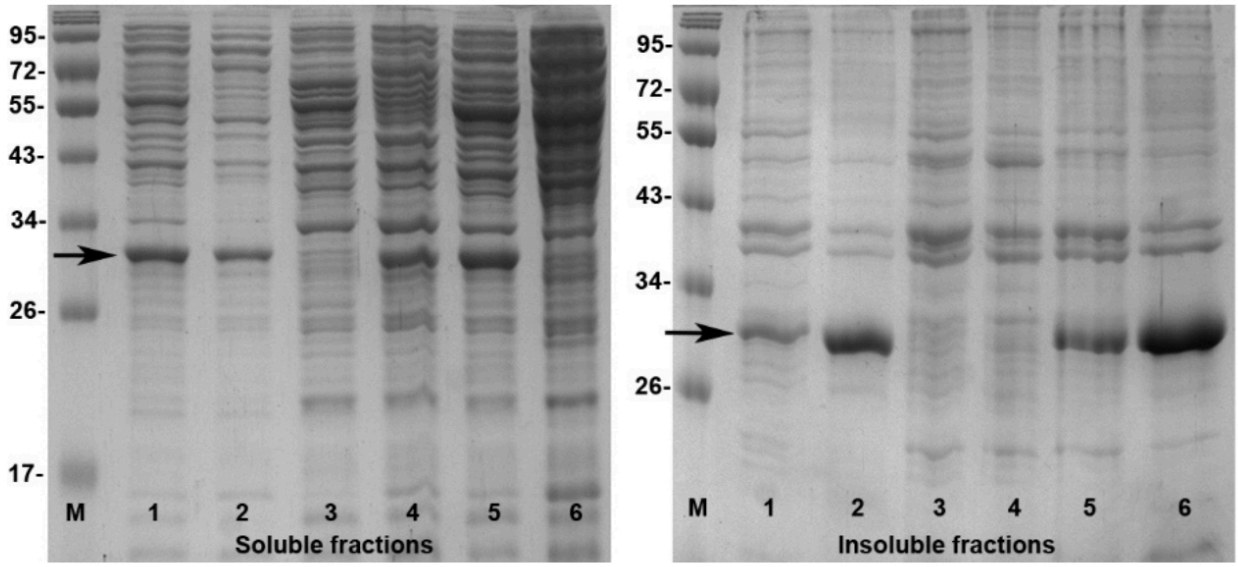


Figure 2

SDS-PAGE analysis of the soluble and insoluble fractions of each crude protein expressed at 30 °C and 37 °C. The left panel displays soluble fractions, while the right panel shows insoluble ones. M indicates the protein ladder; lanes 1 to 6 correspond to adYFP/30 °C, adYFP/37 °C, adCP/30 °C, adCP/37 °C, adGFP/30 °C, and adGFP/37 °C, respectively. The expected heterologous proteins are indicated by black arrows

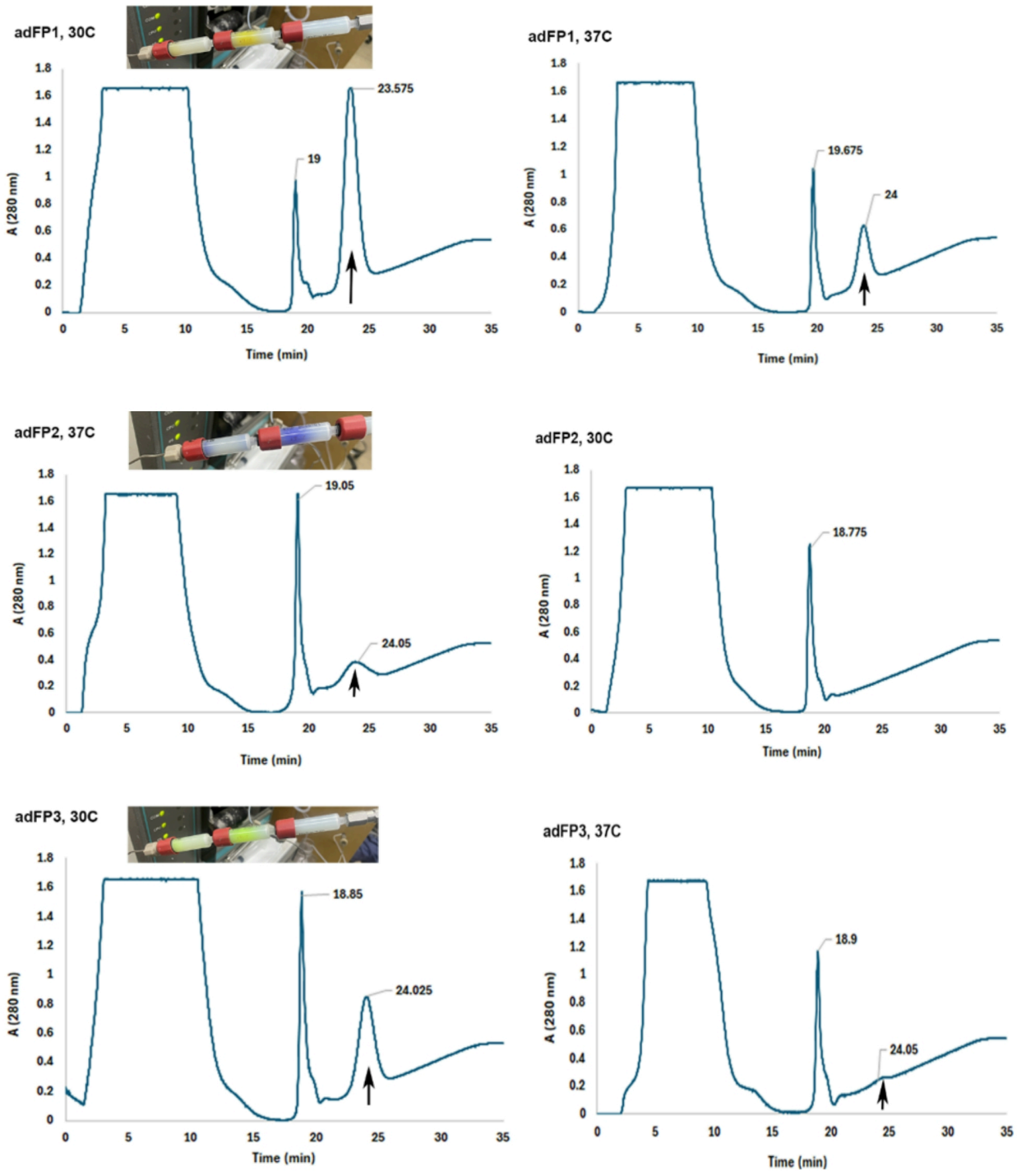


Figure 3

HPLC purification of the proteins adYFP (adFP1), adCP (adFP2), and adGFP (adFP3) was performed at 30 °C and 37 °C. Purification was performed by immobilized metal affinity chromatography using three 1 mL Ni-NTA columns connected in tandem. Black arrows indicate the expected heterologous proteins, which clearly differ in the levels at which they are produced at the two temperatures

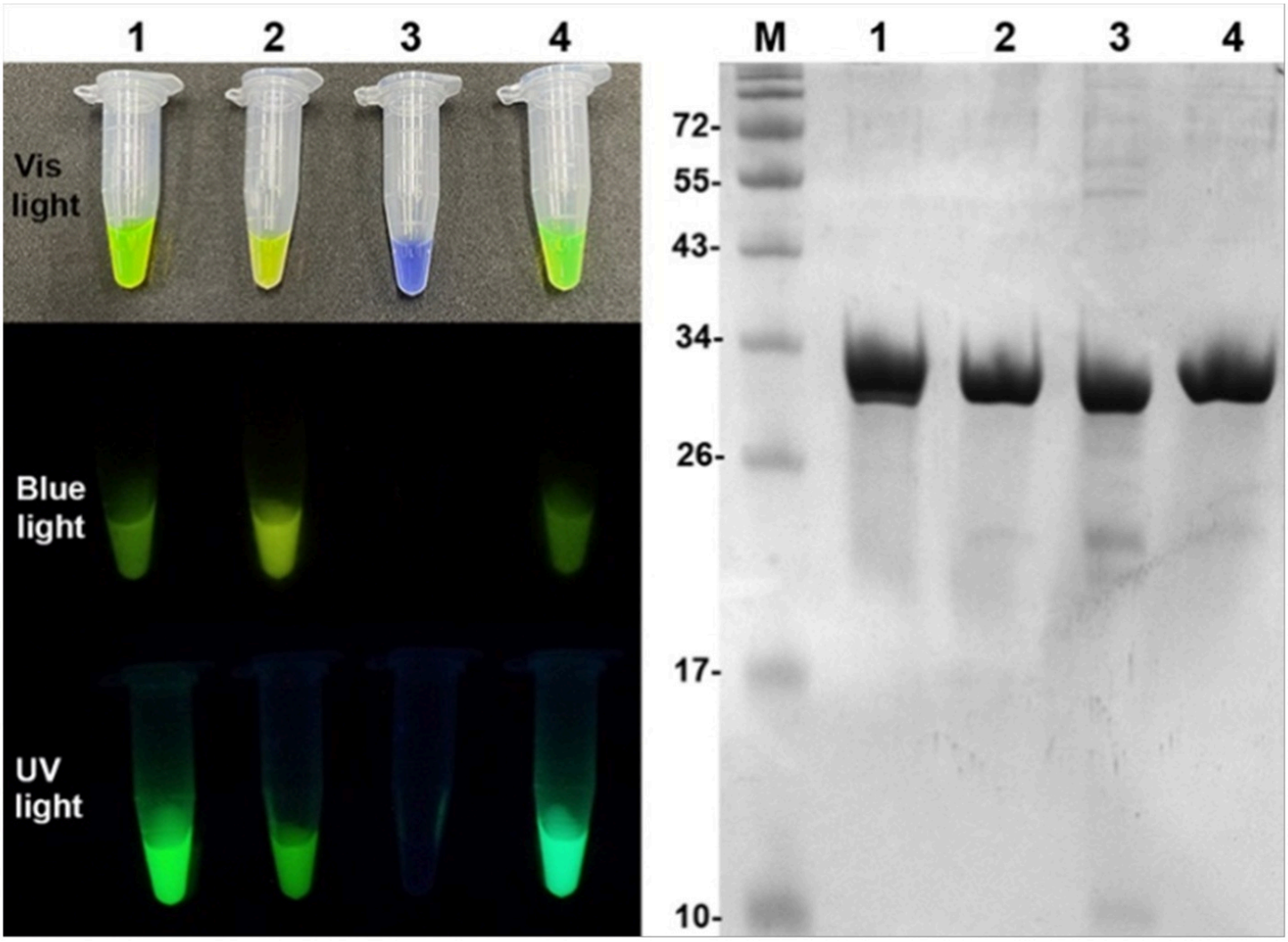


Figure 4

Analysis of purified proteins. The left panel displays purified proteins visualized with white, blue, and ultraviolet light at a concentration of 3 mg/mL. The right panel presents SDS-PAGE analysis of the pure protein. Gel stained using Coomassie blue. Lane 1: EGFP; Lane 2: adYFP; Lane 3: adCP; Lane 4: adGFP; M: protein ladder

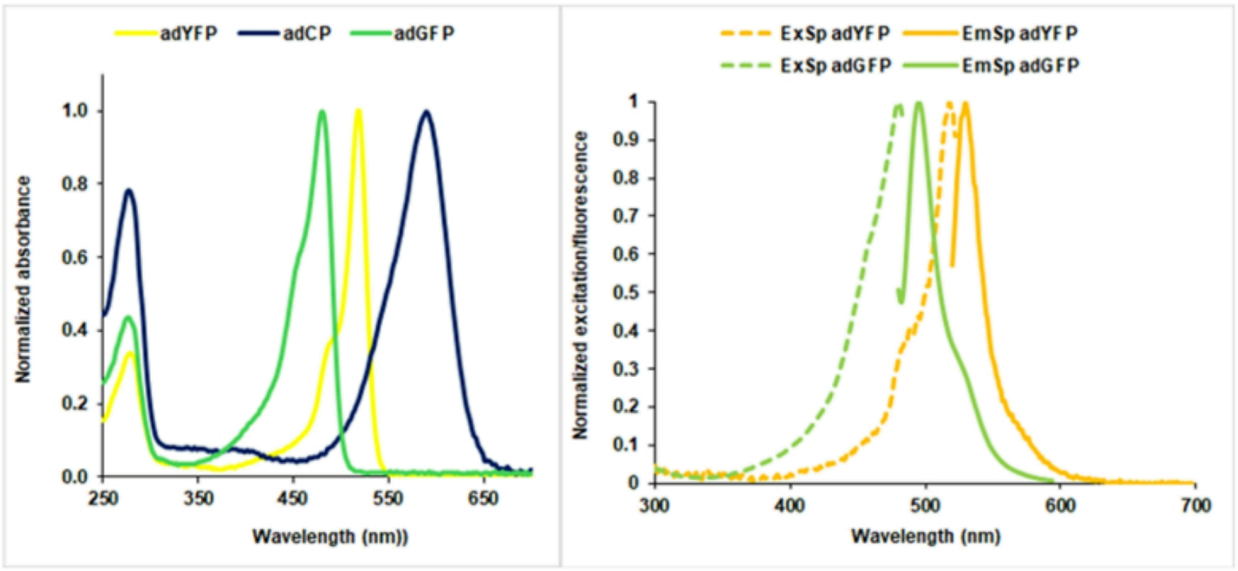


Figure 5

Spectroscopic analysis of the GFP-like proteins encoded in the sea anemone *A. dowii*. Left panel: absorbance spectra. Right panel: excitation and emission spectra. ExSp: excitation spectrum, EmSp: emission spectrum

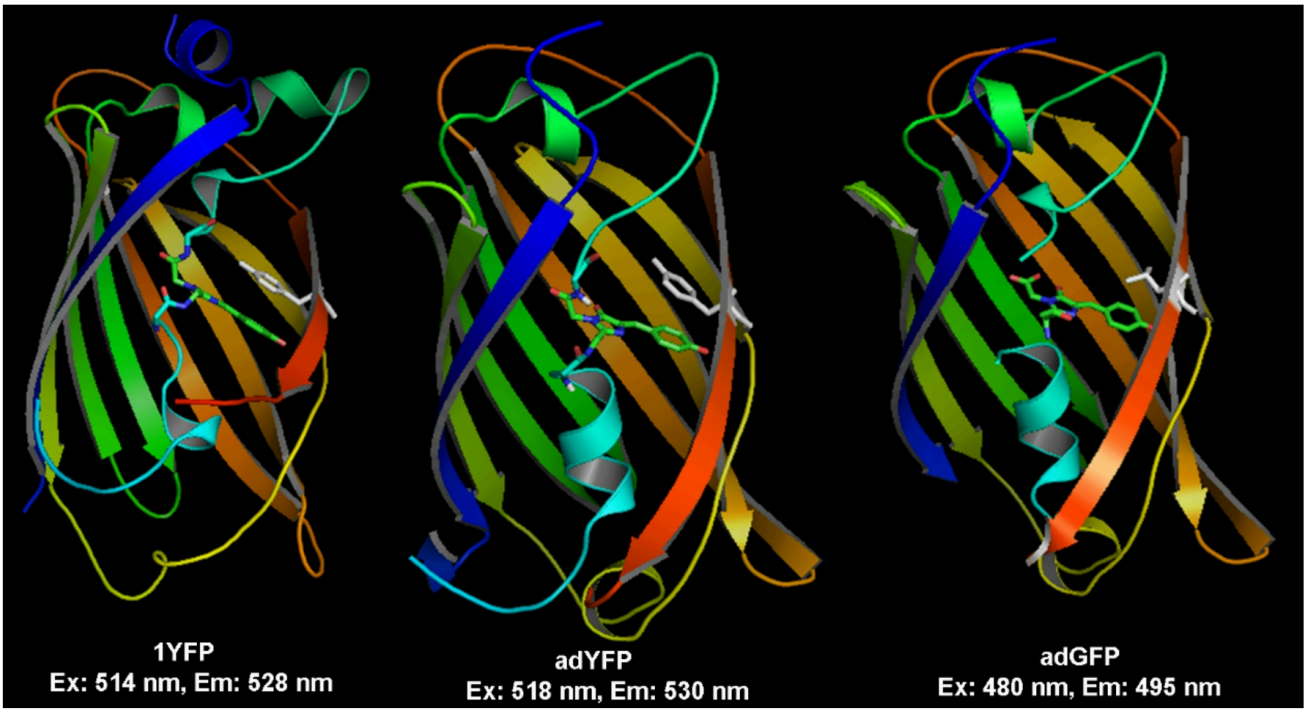


Figure 6

Proposed structure of the fluorescent proteins adYFP and adGFP, compared with the crystal structure of 1YFP (1YFP pdb). The amino acid stacked over the chromophore of each fluorescent protein is shown in white: Tyr203 in 1YFP, Tyr195 in adYFP, and Leu194 in adGFP. β-Strands 2, 3, and 11 were removed from the structures to show the stacking effect within the β-barrel structures

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