

Green fluorescent proteins as natural antioxidants: A study on *Protopalycha variabilis* (Duerden, 1898) and *Zoanthus sociatus* (Ellis, 1768)¹

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ABSTRACT - The green fluorescent protein (GFP) superfamily encompasses a class of fluorescent and non-fluorescent proteins extensively studied in biochemistry and cell biology. Despite significant research, the physiological roles of GFPs remain poorly understood. In particular, whether soft corals harbor GFPs with structural and functional properties comparable to those described in other cnidarians, and whether these proteins may contribute to photoprotection, remains largely unexplored. In this study, two novel green fluorescent proteins (GFPs) were isolated from the soft corals *Protopalycha variabilis* and *Zoanthus sociatus*, named PvarGFP and ZsocGFP, respectively. Purification was achieved through a combination of hydrophobic interaction, ion exchange, and size-exclusion chromatography. Both proteins were identified as monomers of approximately 27 kDa under denaturing conditions, regardless of the presence or absence of reducing agents. However, size-exclusion chromatography revealed that PvarGFP adopts a dimeric conformation, while ZsocGFP forms a tetramer. Amino acid sequencing indicated that PvarGFP shares high similarity with GFPs from other cnidarian species, whereas ZsocGFP is more closely related to a red fluorescent protein (RFP) from *Zoanthus* sp. Circular dichroism analysis showed that PvarGFP's secondary structure is predominantly β -conformation, with exceptional thermal stability, maintaining its structure up to 70 °C. Additionally, PvarGFP exhibited strong antioxidant activity, effectively scavenging free radicals and inhibiting β -carotene oxidation. These findings suggest a potential role for PvarGFP as a natural antioxidant, contributing to the coral's defense against oxidative stress.

Keywords: Cnidarian. Photoprotection. Marine biomolecules.

INTRODUCTION

The green fluorescent protein (GFP) superfamily encompasses a class of fluorescent and non-fluorescent proteins with an approximate molecular weight of 25 kDa. The GFP structure is characterized by a β -barrel composed of 11 β -sheets and a single internal α -helix, which houses a chromophore formed through post-translational modifications. The chromophore in fluorescent GFPs is encoded within the amino acid sequence and spontaneously forms in the presence of molecular oxygen via a molecular rearrangement of the polypeptide chain (Day; Davidson, 2014; Zimmer, 2002).

The jellyfish *Aequoria victoria* GFP (AvGFP) has been extensively studied and widely utilized in biochemistry and cell biology. It has gained recognition as a powerful tool for tracking gene expression and protein localization in intact cells, serving as a genetically encoded fluorescent marker and enabling exploration of protein-protein interactions (Okino *et al.*, 2018; Pereira *et al.*, 2018). Furthermore, AvGFP supports a diverse range of biotechnological applications including protein fusion, whole-organism imaging, and use as transcriptional reporters (Chan; kwong; szeto, 2022; Duan *et al.*, 2018; Ktajima *et al.*, 2018; Lassance *et al.*, 2018).

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Despite the wealth of research on GFPs, their physiological roles remain poorly understood. Some studies suggest that GFPs may play roles in photosynthesis, functioning as photoprotective agents by dissipating excess radiation, transferring unabsorbed radiation back to zooxanthellae, or participating in optical communication. Additionally, GFPs might aid in prey attraction, symbiont recognition, or reproductive partner identification through positive phototropism (Anter; Hussein; Mohammed, 2023; Ben-Zvi *et al.*, 2022; Dizon *et al.*, 2021; Poding *et al.*, 2023). In particular, it is still unknown whether soft corals harbor GFPs with structural and functional features comparable to those described in other cnidarians, and whether these proteins may contribute to photoprotection and oxidative stress defense. Addressing these questions is essential for better understanding of the ecological and physiological significance of GFPs in marine invertebrates.

In this study, we report the identification and purification of native GFPs from the soft corals *Protopalythoa variabilis* and *Zoanthus sociatus*. Our findings reveal that the GFP isolated from *Protopalythoa variabilis* exhibits *in vitro* antioxidant activity, providing new insights into the potential functions of GFPs in relation to their host organisms.

MATERIAL AND METHODS

1. Animal collection

Protopalythoa variabilis Duerden, 1898 and *Zoanthus sociatus* specimens were collected from Paracuru Beach (03°24'36"S, 39°01'50"W), Ceará, Brazil. Fresh samples were transported in a thermal box and stored at -20 °C. All collections were authorized through SISBIO (*Sistema de Autorização e Informação em Biodiversidade*, ID: 33913-8) and SISGEN (*Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado*, ID: AC14AF9).

2. Purification of GFPs

Purification of GFPs was performed using a classical multi-step chromatographic approach combining hydrophobic interaction, ion-exchange, and size-exclusion chromatography, as commonly employed for GFP isolation and protein purification.

Samples for protein extraction were freeze-dried and ground into a fine powder using a coffee mill. The powder was extracted using 25 mM sodium acetate buffer (pH 5.5) containing 1 M ammonium sulfate (SAB/(NH₄)₂SO₄) at a 1:10 (w/v) ratio. The mixture was homogenized with gentle stirring for 1 h at room temperature, then centrifuged at 5,000 × g for 20 min at 4 °C. The resulting supernatant (crude extract) was collected for further purification.

The crude extract (50 mL) was loaded onto a phenyl-Sepharose column (1.0 × 10.0 cm), pre-equilibrated with SAB/(NH₄)₂SO₄, at a flow rate of 1 mL·min⁻¹. Fractions of 2 mL were manually collected. Unbound fractions (P1) were washed with the equilibration buffer, while adsorbed fractions (P2) were eluted with 25 mM sodium acetate buffer (pH 5.5).

P2 fractions were pooled, dialyzed against distilled water, freeze-dried, and solubilized in 50 mM Tris-HCl buffer (pH 7.6, TB). The dialyzed P2 was subjected to ion exchange chromatography (IEX) using a DEAE-Sepharose column (1.0 × 8.0 cm) pre-equilibrated with TB. Unbound fractions (D1) were washed with TB, while adsorbed fractions (D2) were eluted with TB containing 2 M NaCl. D1 fractions were pooled, dialyzed, and freeze-dried.

The final purification step of size-exclusion chromatography (SEC) was performed using a BioSuite 250 HR SEC column (0.78 × 30 cm, 5 µm particle size, Waters Corp., MA, USA) coupled to an H-Class UPLC system (Waters). The D1 fraction was solubilized in TB containing 150 mM NaCl. After centrifugation, D2 was subjected to gel filtration chromatography. The column was pre-equilibrated with TB containing 0.5 M NaCl at a flow rate of 0.5 mL·min⁻¹. All purification steps were monitored at 280 nm and 450 nm. The isolated GFPs from *P. variabilis* and *Z. sociatus* were designated PvarGFP and ZsocGFP, respectively.

3. Molecular mass and homogeneity evaluation of GFPs

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis) was conducted according to Laemmli (1970) with and without 2-mercaptoethanol. A low-molecular-weight SDS marker kit (GE Healthcare Life Sciences) was used as the standard.

The native molecular mass of the GFPs was estimated via gel filtration chromatography using a BioSuite SEC column coupled to the Acquity UPLC system. The column was pre-equilibrated with TB containing 0.5 M NaCl and calibrated with the following molecular weight standards: conalbumin (75,000 Da), ovalbumin (45,000 Da), carbonic anhydrase (29,000 Da), ribonuclease A (13,700 Da), and aprotinin (6,500 Da).

The average molecular mass of PvarGFP was determined by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization – Time of Flight) mass spectrometry using an Autoflex III mass spectrometer (Bruker Daltonics, Germany). The matrix solution contained 10 mg·mL⁻¹ α-cyano-4-hydroxycinnamic acid (CHCA) in acetonitrile:water:TFA (50:47:3% v/v/v). Spectra were acquired in linear positive mode and processed using FlexAnalysis 3.4 software (Bruker).

4. Amino acid sequencing

A bottom-up mass spectrometry approach was used to elucidate the amino acid sequences of the GFPs. SDS-PAGE

was performed as described in Section 2.3. After staining, protein bands were excised, dehydrated with acetonitrile, and subjected to reduction and carbamidomethylation, as described by Shevchenko *et al.* (2006).

Carbamidomethylated PvarGFP (CA-PvarGFP) was digested using trypsin (Promega, WI), chymotrypsin (Sigma-Aldrich, MO), pepsin (Sigma), and Glu-C (Roche, Switzerland). Trypsin and chymotrypsin digestions were performed in 25 mM ammonium bicarbonate at a 1:50 (w/w, enzyme/substrate) ratio. Glu-C digestion was conducted in 25 mM ammonium bicarbonate at a 1:200 (w/w, enzyme/substrate) ratio. Pepsin digestion was performed in 100 mM HCl at a 1:50 (w/w, enzyme/substrate) ratio. All digestions were performed at 37 °C for 18 h. CA-ZsocGFP was digested with trypsin following the same protocol.

Digestion reactions were stopped with 2 µL of 2% formic acid (FA). Peptides were extracted following Shevchenko *et al.* (2006). Two microliters of peptide solution were loaded onto a C-18 nanocolumn (0.075 mm × 100 mm) coupled to a nanoAcquity system. LC-MS/MS analyses were performed using a hybrid Synapt HDMS mass spectrometer (Waters Corp., Milford, MA, USA), as described by CARNEIRO *et al.* (2017).

5. Circular dichroism

Circular dichroism (CD) spectra were recorded using a Jasco J-815 spectropolarimeter (Jasco International Co., Tokyo, Japan) equipped with a temperature-controlled Peltier device, following the protocol of Carneiro *et al.* (2017). Thermodynamic parameters for PvarGFP folding and unfolding were calculated by monitoring changes in ellipticity at 230 nm as a function of temperature (Miles; Janes; Wallace, 2021)

6. Antioxidant assay

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed following BLOIS (1958). β-Carotene oxidation inhibition was measured as described by Chew *et al.* (2008), and ferrous ion chelation activity was determined following Wang; Jónsdóttir e Olafsdóttir (2009).

PvarGFP was solubilized at four concentrations (12.5, 25, 50, and 100 µg·mL⁻¹). All assays were conducted in triplicate using a Biochrom Asys UVM340 microplate reader (Cambridge, UK).

RESULTS AND DISCUSSION

1. Purification of the GFPs

In this study, two novel GFPs from the soft corals *Protopalpythoa variabilis* and *Zoanthus sociatus* were

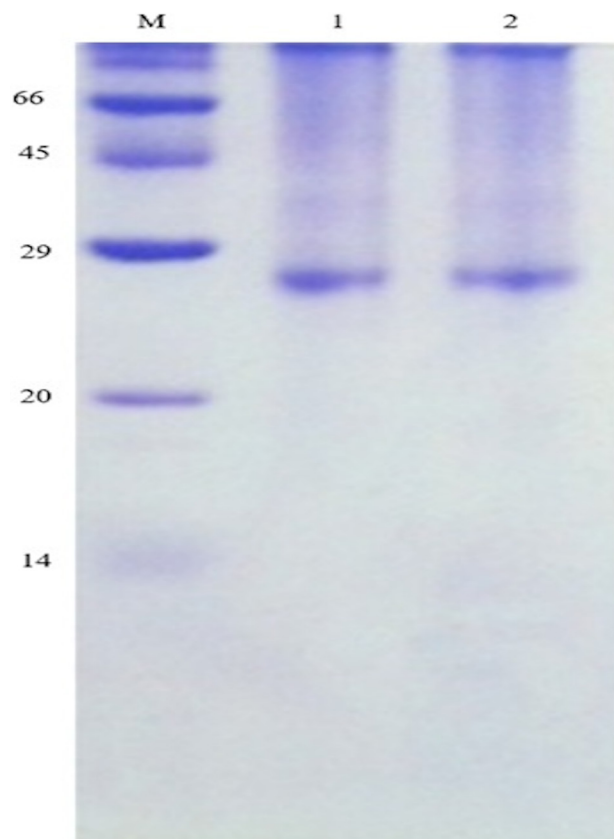
successfully isolated. ZsocGFP was characterized to a lesser extent compared to PvarGFP due to challenges in animal collection and the low protein yield.

GFPs were adsorbed onto the phenyl-Sepharose matrix in the initial purification step and subsequently eluted with sodium acetate buffer. GFPs were recovered in the unbound fraction during the IEX step. Finally, size exclusion chromatography (SEC) separated GFPs from all remaining contaminants.

2. Molecular mass

PvarGFP and ZsocGFP were identified as monomeric proteins with a molecular mass of 27 kDa under denaturing conditions, as determined by SDS-PAGE in the presence and absence of 2-mercaptoethanol (Figure 1).

Figure 1 - SDS-PAGE analysis of purified green fluorescent proteins (GFPs) isolated from the soft corals *Protopalpythoa variabilis* and *Zoanthus sociatus*. Proteins were analyzed under denaturing conditions to assess purity and apparent molecular mass. Lane M: low-molecular-weight protein marker; Lane 1: PvarGFP; Lane 2: ZsocGFP. Both proteins exhibited a major band at approximately 27 kDa



Similar to other fluorescent proteins, PvarGFP and ZsocGFP exhibited the ability to form oligomers (Zimmer, 2002). The molecular masses of native PvarGFP and ZsocGFP, as determined by SEC, were 47 kDa and 109 kDa, respectively, indicating dimeric and tetrameric conformations. Most GFPs are stable tetramers that resist destabilization except by irreversible denaturation. This oligomeric state may contribute to their proposed function as photoprotective shields (Anter; Husseiny; Mohammed, 2023).

MALDI-TOF mass spectrometry of PvarGFP revealed two singly charged ions, corresponding to isoforms designated as PvarGFP-1 and PvarGFP-2. Despite the presence of isoforms, no significant heterogeneity was observed in the amino acid sequence of PvarGFP (See section amino acid sequencing). The average molecular masses were calculated as $25,662 \pm 5$ Da for PvarGFP-1 and $26,579 \pm 5$ Da for PvarGFP-2.

3. Amino acid sequencing

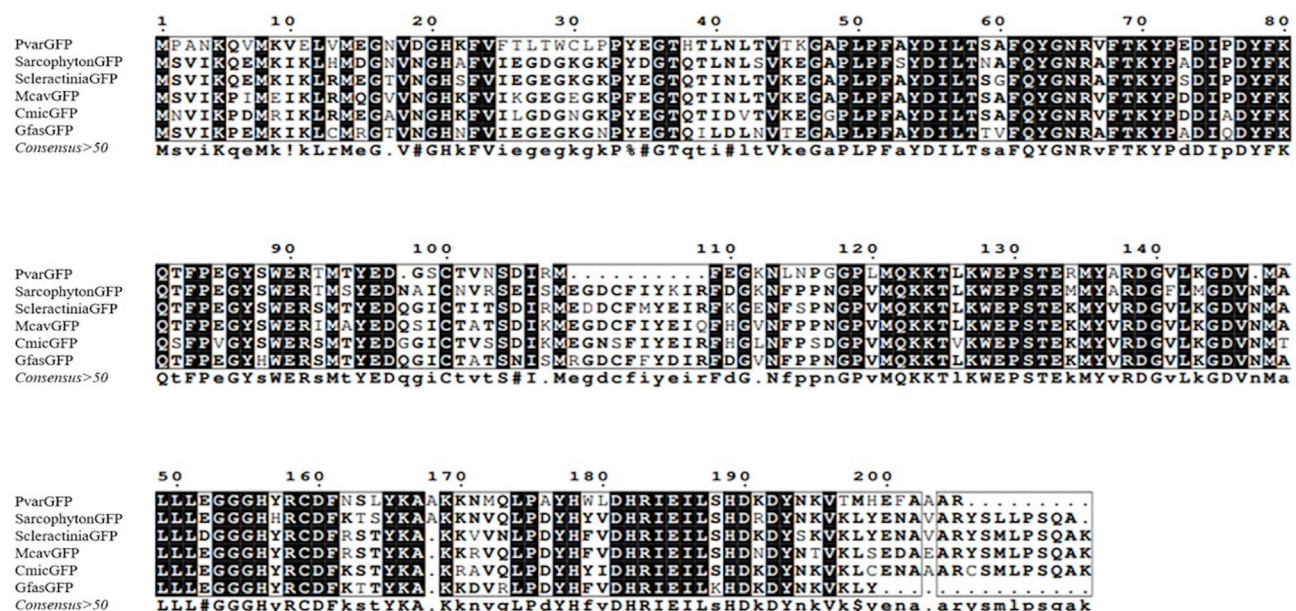
Amino acid sequencing confirmed that both PvarGFP and ZsocGFP are typical members of the GFP family. MS/MS (Mass spectrometry in tandem) analysis identified 89% of the amino acid sequence of PvarGFP. Overlapping peptides from various enzymatic digestions yielded a partial primary structure, which demonstrated high sequence identity with other fluorescent proteins (Figure 2). PvarGFP

demonstrated sequence similarity to GFPs from *Scleractinia* sp. [ACV52374.1] (77%), *Montastraea cavernosa* [AAK62982.2] (77%), *Sarcophyton* sp. [ACD13195.1] (76%), *Cyphastrea microphthalma* [CBI12485.1] (76%), and *Galaxea fascicularis* [BAD52001.1] (70%).

GFP-like proteins typically consist of 200 – 250 amino acids and lack glycosylation or phosphorylation sites, which is also a characteristic observed in PvarGFP (Anter; Husseiny; Mohammed, 2023). Sequence alignments revealed conserved regions, including critical residues for chromophore formation and post-translational modification. The chromophore of PvarGFP was sequenced from peptides T20, P2, and Q10, and it shares the tripeptide chromophore-forming sequence $^{63}\text{QYG}^{65}$ with GFPs from *Sarcophyton* sp., *Scleractinia* sp., and *M. cavernosa* (Nwafor *et al.*, 2021).

Distinct chromophore sequences differentiate PvarGFP from other GFPs, such as SYG in avGFP and NYG in *Zoanthus* sp. fluorescent protein. Notably, Y64 and G65 residues are highly conserved among GFPs, along with R91 and Q222, which play essential roles in chromophore formation. Arginine interacts with the carbonyl group of Y66, stabilizing the cyclization intermediate, while glutamine facilitates hydrogen removal from the α -carbon of tyrosine, aiding post-translational modification (Nwafor *et al.*, 2021; Seo; Kim; Kim, 2024).

Figure 2 - Multiple sequence alignment of PvarGFP and representative GFPs from cnidarian species (*Sarcophyton* sp. GFP [ACD13195.1], *Scleractinia* sp. [ACV52374.1], *Montastraea cavernosa* (McavGFP) [AAK62982.2], *Cyphastrea microphthalma* (CmicGFP) [CBI12485.1], and *Galaxea fascicularis* (GfasGFP) [BAD52001.1]). Conserved amino acid residues are highlighted in black



In turn, approximately 40% of the amino acid sequence for ZsocGFP was determined using MS/MS. The peptides identified (Table 1) showed similarity to sequences from other fluorescent proteins, including the red fluorescent protein from *Zoanthus* sp. [ACD03134.1]. This sequence similarity suggests that ZsocGFP may belong to a closely related subgroup within the GFP-like protein family, highlighting the structural and evolutionary diversity of fluorescent proteins in zoantharians. Although the partial sequence coverage limits a more comprehensive structural analysis, the conserved peptide regions support its classification as a canonical GFP-like protein and reinforce the occurrence of diversified fluorescent protein lineages within soft corals.

4. Circular Dichroism

The CD spectrum of native PvarGFP exhibited a single minimum at 210 nm, indicating a predominance of β -conformation in its secondary structure (Figure 3A). Analysis using the CONTIN prediction method (Sreerama; Woody, 2000) suggested a secondary structure composition of 7% α -helix, 61% β -sheet/ β -turn, and 32% coil.

PvarGFP demonstrated relative thermostability, retaining its secondary structure at temperatures up to 70 °C. The melting temperature (T_m) was estimated to be 77 °C (Fig. 3B).

5. Antioxidant activity

The biological functions of GFPs remain enigmatic. Their stability under diverse conditions suggests potential roles in adapting to extreme environments, such as

Table 1 - Peptide fragments obtained from tryptic digestion of ZsocGFP and identified by LC–MS/MS analysis. The detected peptides were used for partial amino acid sequence determination and showed similarity to fluorescent proteins from cnidarian species

Peptide Name	<i>m/z</i>	Sequence	Mass		Δ (Da)
			Observed	Calculated	
T1	588.28	HGLTKEMTMK	1174.54	1174.58	0.04
T1'	596.26	HGLTKEMoxiTMK	1190.51	1190.58	0.07
T2	479.75	EMTMKYR	957.49	957.44	-0.05
T3	1045.79	QAINLCVVEGGPLPFAEDILSAAFNYGNR	3134.35	3143.54	0.19
T4	771.37	FYGVNFPADGPVMK	1540.73	1540.74	0.01
T4'	779.37	FYGVNFPADGPVMoxiK	1556.73	1556.73	0.00
T4''	562.61	FYGVNFPADGPVMoxiKK	1684.80	1684.82	0.02
T5	569.81	GDVSMYLLK	1137.60	1137.61	0.01
T6	665.36	LRCQFDTVYK	1328.71	1328.65	-0.06

M^{oxi}- oxidized methionine

Figure 3 - CD spectra of PvarGFP. (A) Far-UV CD spectra (190 - 240 nm) of PvarGFP. The cuvette path length was 1.0 cm; protein concentration was 0.1 mg/mL in phosphate buffer, 50 mM, pH 7. (B) Determination of PvarGFP T_m from changes in CD as a function of temperature. PvarGFP solubilized in phosphate buffer, pH 7, in different temperatures

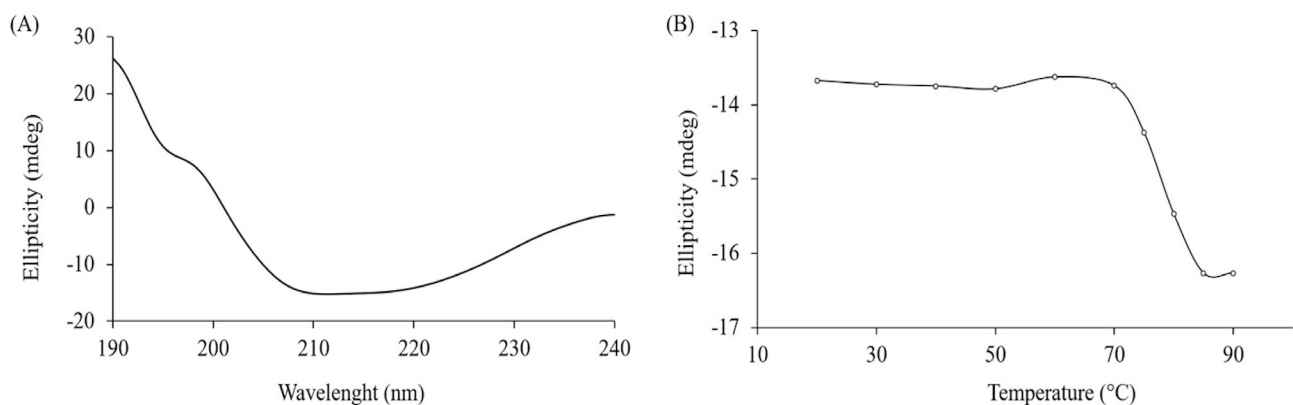
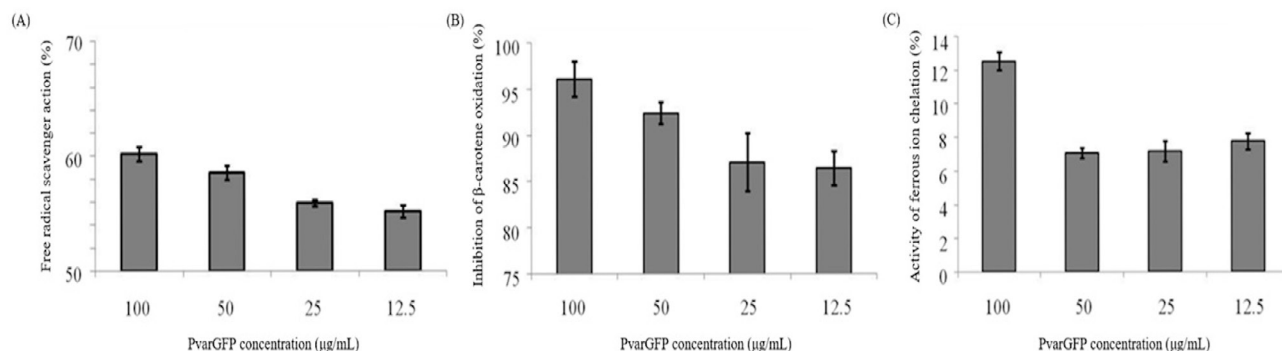


Figure 4 - Antioxidant activity of PvarGFP. (A) Free radical scavenger action (DPPH-method); (B) β -carotene bleaching assay; (C) Ferrous ion chelating activity. PvarGFP was assayed in four concentrations: 100, 50, 25 and 12.5 $\mu\text{g/mL}$



thermal and luminous stress or pathogenic infections (Palmer; Modi; Mydlarz, 2009). GFPs might also act as supplementary antioxidants to the endogenous enzyme systems, including peroxidases, superoxide dismutase, and catalase (Levy *et al.*, 2006; Mydlarz; Harvell, 2007).

PvarGFP displayed significant free radical scavenging activity and strong inhibition of β -carotene oxidation, as assessed by the DPPH and β -carotene assays, respectively. In the DPPH assay, PvarGFP exhibited activity levels of 60.23%, 58.59%, 55.96%, and 55.17% at concentrations of 100 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, and 12.5 $\mu\text{g/mL}$, respectively (Figure 4A).

In the β -carotene assay, PvarGFP demonstrated inhibition levels of 96.13%, 92.44%, 87.08%, and 86.43% at concentrations of 100 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, and 12.5 $\mu\text{g/mL}$, respectively. Both activities exhibited a dose-dependent response (Figure 4B).

Conversely, PvarGFP showed low ferrous ion chelation activity, with values of 7.1%, 7.16%, 7.76%, and 12.1% at concentrations of 12.5 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, and 100 $\mu\text{g/mL}$, respectively (Figure 4C).

Similar antioxidant activities have been observed in other GFP family proteins, such as AvGFP, which demonstrates peroxide radical scavenging and superoxide dismutase-like activity (Bou-Abdallah; Chasteen; Lesser, 2006). Moreover, studies have suggested the involvement of GFPs in immune responses and oxidative stress mitigation, as reported in crude extracts and purified proteins from various coral species (Palmer; Modi; Mydlarz, 2009).

Corals, including *P. variabilis*, are frequently exposed to stressors such as high temperatures, extreme pH, and reactive oxygen species (ROS). These conditions are often exacerbated by symbiont activity and can lead to oxidative stress and coral

bleaching (Bou-Abdallah; Chasteen; Lesser, 2006). In this context, identification of GFP-like proteins in both species reinforces the hypothesis that these molecules may participate in protective physiological responses. While the antioxidant activity experimentally demonstrated for PvarGFP provides direct evidence of such a role, the structural conservation and sequence similarity observed for ZsocGFP suggest that related protective functions may also occur in *Z. sociatus*. Together, these findings expand the current knowledge on GFP diversity in soft corals and support their potential involvement in oxidative and environmental stress adaptation.

CONCLUSION

This study demonstrates that soft corals harbor canonical GFP-like proteins with structural and biochemical features conserved among cnidarian fluorescent proteins. The identification of PvarGFP and ZsocGFP expands the current diversity of known coral GFPs and confirms that these proteins retain characteristic oligomeric organization, conserved chromophore-related residues, and remarkable thermal stability. Furthermore, the antioxidant activity observed for PvarGFP supports the hypothesis that GFPs may play a physiological role beyond fluorescence, acting as supplementary photoprotective and oxidative stress-defense molecules. These findings provide new insights into the adaptive significance of GFPs in soft corals and reinforce their potential ecological and biotechnological relevance.

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DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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