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X-ray crystallographic studies of a fluorescent protein Akane1 from *Scleronephthya gracillimum*

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Fluorescent proteins (FPs) are popular tools used to monitor the localization of a biomolecule and its interaction *(in vivo* or *in vitro)*. Changes in fluorescence intensity caused by environmental elements (e.g. protons or metals) form the basis for the development of pH indicators or metal biosensors. FPs are constantly being engineered or discovered for efficient and extended applications. In this study, we report the expression, purification, characterization, crystallization, and preliminary X-ray diffraction studies of a fluorescent protein Akane1 from *Scleronephthya gracillimum*. This protein exhibited a dimeric state in solution. According to the spectral analysis, Akane1 showed excitation and emission maxima peaks at 501 and 510 nm, respectively. The crystals of Akane1 were obtained under a reservoir solution containing 100 mM Tris-HCl (pH 7.0), 200 mM calcium acetate, and 20% (w/v) PEG 3000. The crystals of Akane1 diffracted to 1.93 Å and belonged to the space group $P2_1$, with unit cell parameters of a = 56.02 Å, b = 126.67 Å, c = 62.03 Å, and β = 107.52°. The initial phase of Akane1 was obtained by the molecular replacement method.

INTRODUCTION

Fluorescent proteins are versatile optical probes in molecular cell biology that enable the monitoring of localization of target proteins, oligomeric states, protein-protein interaction, and fluorescence resonance energy transfer (FRET) (Gerdes and Kaether, 1996; Misteli and Spector, 1997; Tsien, 1998; Zimmer, 2002). They are also used to detect the gene expression of membrane proteins fused with fluorescent proteins using high fluorescence sensitivity (Kawate and Gouaux, 2006). As fluorescence intensity varies with the concentration of protons or the transition metal ions, fluorescent proteins have also been proposed to be used in pH indicators or metal biosensors (Bae et al., 2017, 2018; Kim et al., 2016).

The GFP-like fluorescent protein has a β-barrel structure, and the tripeptide forms a chromophore through post-translation modifications—folding, cyclization, dehydration, and aerial oxidation (Tsien, 1998; Zimmer, 2002). The peptide possesses the inherent spectroscopic properties of the fluorescent protein, mainly determined by the chromophore and the amino acids of the hydrogen bonding network in vicinity of the chromophore (Remington, 2011). Based on the information obtained from spectral and structural analysis, enhanced FPs are being developed by mutating the major amino acids of existing FPs (Heim and Tsien, 1996). Studies to explore and characterize new fluorescent proteins are in progress, providing expanded opportunities for various applications.

Scleronephthya gracillimum (previously named *Alcyonium gracillimum*) is an azooxanthellate coral typically found in Korea, Japan, and Taiwan (Seo et al., 1995). This species is biochemically and pharmacologically important, as it contains bioactive steroids exhibiting cytotoxic and antiviral activities (Seo et al., 1995). It is also known to cause allergic disease in fishermen (Kato et al., 2017). Kato et al. isolated an allergenic protein from *S. gracillimum* using ELISA and immunoblotting (Kato et al., 2017). This novel allergen was further analyzed by in-gel digestion and LC-ESI-/MS/MS. The spot components containing the QSFPEGFSWER sequence from 2D-PAGE was matched to that of GFP-like protein in *Lobophyllia hemprichii* and *Scleractinia* sp. Kato et al. reported an allergenic protein that corresponds to a GFP like protein (named as Akane) (Kato et al., 2017). This protein exhibits fluorescence emission in the red and green regions of spectra at 628 nm and 508 nm, respectively. Homologous genes of the akane gene from *S. gracillimum* were found in the protein database UniProt (www.uniprot.org) (Figure 1). Even though the genetic sequence of akane1 has been determined, the spectral and structural analysis of the protein has not been performed.

In this study, we first report the protein expression, purification, and preliminary X-ray crystallographic analysis of the Akane1 protein from *S. gracillimum*. We present the results of fluorescence emission and excitation and initial phasing obtained using the molecular replacement method.

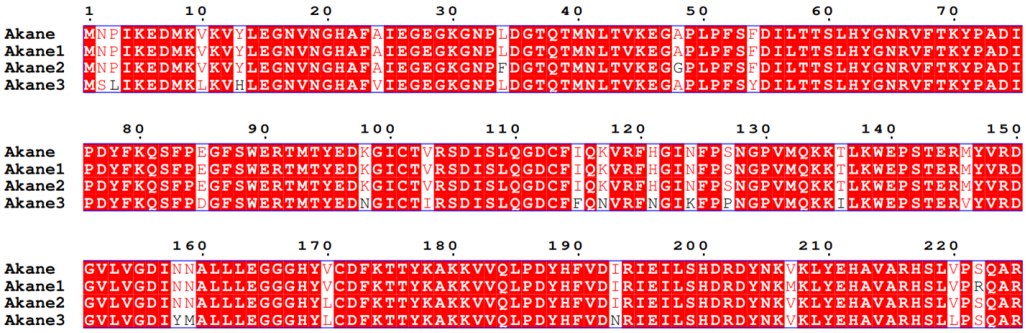

FIGURE 1 | Sequence alignment of Akane (A0A1L7NRM8), Akane1(A0A1L7NRM5), Akane2 (A0A1L7NRN7), and Akane3 (A0A1L7NRN4) of the Akane family from *S. gracillimum*. The conserved sequences are highlighted in red.

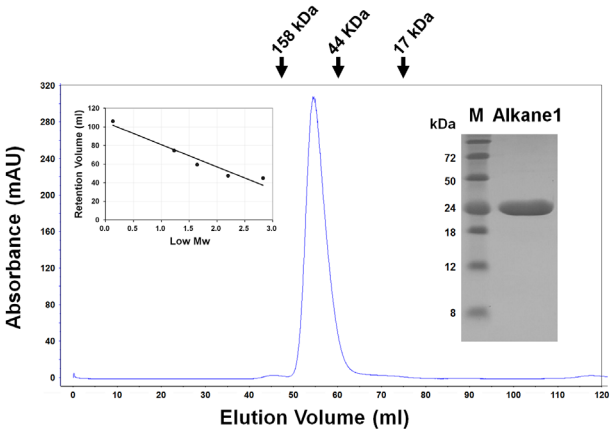

FIGURE 2 | Profile of gel filtration chromatography of Akane1. The molecular weights of the protein standards are indicated by arrows at the top. The purity of Akane1 is > 99% as observed by Coomassie blue-stained SDS–PAGE.

RESULTS AND DISCUSSION

Akane1 was cloned into pET-28a vector and was heterologously expressed in *E. coli* BL21 (DE3) cells. Akane1 was purified to homogeneity through a two-step purification method comprising His-affinity and size-exclusion chromatography. The yield of recombinant Akane1 was 8.5 mg/liter. Akane1 was eluted in a single peak upon gel filtration analysis, showing that the protein was dimeric state in solution (Figure 2). The protein purity of Akane1 was > 99%, according to the SDS-PAGE results (Figure 2). Akane1 is yellow-colored in the visible spectrum, whereas it exhibits green light when exposed to blue LED at a wavelength of 470 nm (Figure 3A). Fluorescence spectra showed excitation and emission maxima peaks at 501 nm and 510 nm, respectively (Figure 3B). Initially, Akane1 crystals were obtained with sitting-drop vapor diffusion method under a reservoir solution containing 0.1 M Tris-HCl (pH 7.0), 0.2 M calcium acetate, and 20% (w/v) PEG 3000. Optimization was performed by scaling up protein and reservoir solutions. The improved crystals were obtained with hanging-drop vapor diffusion method (Figure 4). The crystals grown using this optimized condition diffracted to

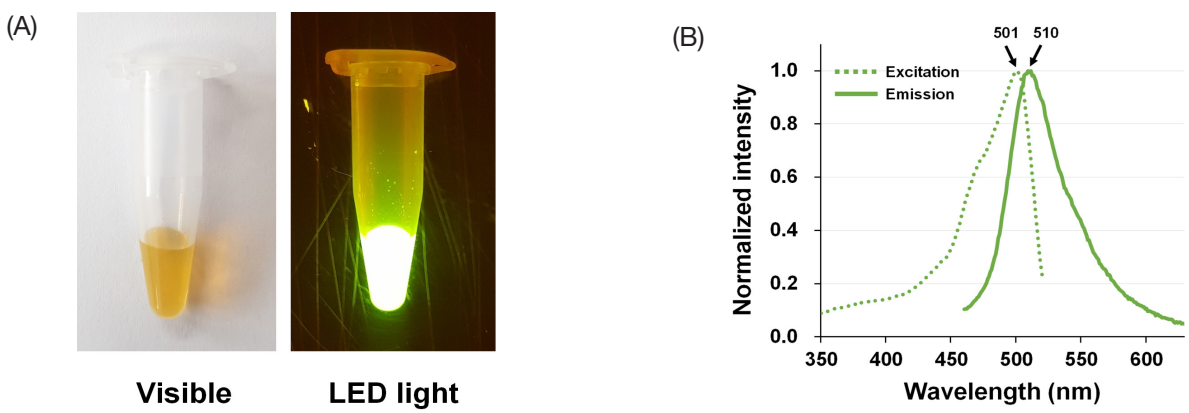

FIGURE 3 | Spectral analysis of Akane1. (A) Purified Akane1 on visible and LED light. (B) Fluorescence excitation and emission spectra for purified Akane1 protein in 10 mM Tris-HCl (pH 8.0) and 200 mM NaCl.

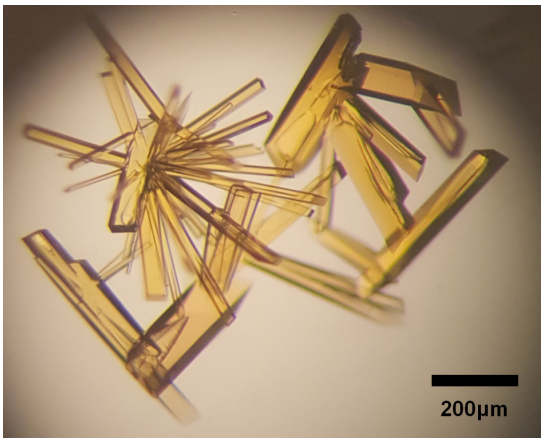

FIGURE 4 | Crystals of Akane1. The approximate dimensions of Akane1 crystals of diffraction quality grown in 0.1 M Tris-HCl (pH 7.0), 0.2 M calcium acetate, and 20% (w/v) PEG 3000.

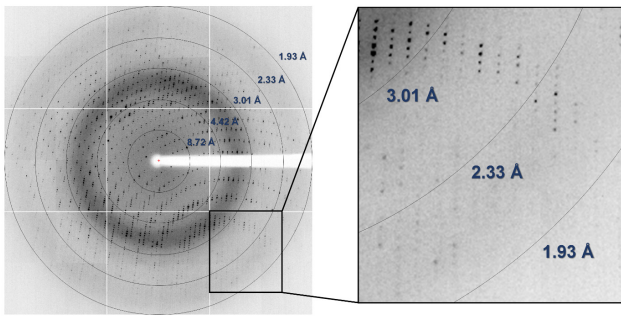

FIGURE 5 | A typical diffraction pattern of an Akane1 crystal. The exposure time was 1 s, crystal-to-detector distance was 280 mm, and oscillation range per frame was 1°. The diffraction image was collected on a Pilatus 6M detector. An enlarged image is shown on the right.

1.93 Å resolution and a data set was subsequently collected from a single crystal (Figure 5). The crystals belonged to space group $P2_1$, with unit-cell parameters a = 56.02 Å, b = 126.67 Å, c = 62.03 Å, and β = 107.52°. We assumed the presence of 4 molecules per asymmetric unit, which yielded a Matthews coefficient of 2.06 $Å^3$/Da and a solvent content of 40.25% (Matthews, 1968). Complete data-collection statistics are given in Table 3. Initial molecular replacement was performed using the crystal structure of photoswitchable green fluorescent Dronpa (PDB code 2GX0) (Nam et al., 2007) as the search model, which has the sequence identity of 69.8%. A clear solution was found with Top LLG and TFZ values of 5066.638 and 51.5, respectively. Initial R_{work} and R_{free} values were found to be 27.44 and 31.97, respectively. Dronpa is present as monomer in solution, with maximum of excitation and emission are 480 nm and 518 nm, respectively. Therefore, the monomer structures of Akane1 and Dronpa are similar, but they have different physical and spectral properties. The structure refinement and spectral analysis of Akane1 are under progress.

TABLE 1 | Protein expression information

Protein	Akane1
Expression vector	pET28a
Expression host	*E. coli* BL21 (DE3)
Protein sequence of the construct	(MGSSHHHHHHSSGLVPR↓GSHM) MNPIKEDMKVKVYLEGNVNGHAFAIEGEGKG NPLDGTQTMNLTVKEGAPLPFSFDILTTSLHY GNRVFTKYPADIPDYFKQSFPEGFSWERTMT YEDKGICTVRSDISLQGDCFIQKVRFHGINFP SNGPVMQKKTLKWEPSTERMYVRDGVLVG DINNALLLEGGGHYVCDFKTTYKAKKVVQLP DYHFVDIRIEILSHDRDYNKMKLYEHAVARH SLVPRQAR

*N-terminal expression tag is indicated by a bracket.
*Thrombin cleavage site is indicated by an underscore.

METHODS

Cloning and protein expression

The coding sequence of akane1 (UniProt accession number: A0A1L7NRM5) from *S. gracillimum* was synthesized and cloned into the pET28a vector using NdeI and XhoI restriction enzymes (Table 1). This plasmid encodes the complete sequence of Akane1 along with the sequence MGSSHHHHHHSSGLVPRGSHM at the N-terminal (thrombin cleavage site is indicated by an underscore). Akane1 was transformed into *Escherichia coli* BL21 (DE3) for protein expression. An overnight culture from a single colony was used to inoculate Luria-Bertani (LB) broth supplemented with 50 mg/ml kanamycin. Cells were incubated at 37°C with shaking until they reached an optical density of 0.6–0.8 at 600 nm. Recombinant protein expression was induced with 1 mM isopropyl-β-d-1-thiogalactopyranoside (IPTG). The cultures were grown at 18°C for another 18 h and cells were harvested by centrifugation at 3500 rpm for 20 min.

Protein Purification

The cells were resuspended in 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, and 5 mM β-mercaptoethanol. Cells were disrupted by sonication and cell debris were removed by centrifugation at 13,000 rpm for 1 h. The sample was loaded onto the HisTrap column (GE Healthcare) equilibrated with buffer consisting of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 5 mM β-mercaptoethanol. The target protein was eluted with buffer made up of 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM β-mercaptoethanol, and 500 mM imidazole (concentrations ranging from 20 to 500 mM). The eluted protein was concentrated to the volume of 2 ml, and thrombin (Haematologic Technologies Bovine alpha thrombin) was added to cleave the N-terminal expression tag at 4°C for 16 h. The uncleaved Akane1 protein was removed by passing the sample through a HisTrap column. After concentrating protein using a centricon (Millipore, cutoff 10 kDa) at 3500 rpm at 4°C, the supernatant was loaded onto a HiLoad 16/600 Superdex 75 pg (GE Healthcare) equilibrated with protein crystallization buffer containing 20 mM Tris–HCl (pH 8.0) and 150 mM NaCl. The purified proteins were analyzed by SDS–PAGE with Coomassie staining. The protein concentration was measured by UV absorption at 280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). The fractions were pooled and concentrated to 32 mg/ml for crystallization screening.

Spectral analysis

Excitation and emission fluorescence spectra of 50 μM Akane1 in 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl were recorded at 25°C. The excitation spectrum was determined by monitoring fluorescence emission at 500 nm

while scanning the excitation light between 350 and 525 nm. The emission spectrum was determined by monitoring fluorescence emission between 460 and 650 nm while exciting the sample at 450 nm. Fluorescence and absorbance spectra data were recorded using a Synergy H1 microplate reader (Biotek).

Crystallization

Initial crystal screening was performed with commercially available crystallization kits from Crystal screen (Hampton research) and Wizard™ Classic (Molecular Dimensions) (Table 2). The process was carried out at 22°C using the sitting-drop vapor-diffusion technique on MRC2 well crystallization plate (Swissci) UVP (Hampton Research). During screening, 0.5 µl protein solution was mixed with 0.5 µl precipitant solution and equilibrated against 500 µl precipitant solution. Akane1 crystals grew under four conditions, with the largest crystals observed in drops containing Wizard Classic condition No. F6 containing 100 mM Tris-HCl (pH 7.0), 200 mM calcium acetate, and 20% (w/v) PEG 3000. Crystal optimization was performed at 22°C using a hanging-drop vapor-diffusion method on a VDX plate with sealant (Hampton research) (Table 2). Protein solution (1 µl) was mixed with 1 µl precipitant solution and equilibrated against 500 µl precipitant solution containing 0.1 M Tris-HCl (pH 7.0), 0.2 M calcium acetate, and 20% (w/v) PEG 3000. Crystals grew to the maximal dimensions of 300 x 100 x 100 µm within a day.

TABLE 2 | Crystallization conditions for Akane1

	Crystal screen	Crystal optimization
Methodz	Hanging drop vapor diffusion	Sitting drop vapor diffusion
Plate type	96-well plate	VDX 24-well plate
Protein concentration (mg/ml)	32	32
Reservoir solution	0.1 M Tris-HCl (pH 7.0), 0.2M calcium acetate, and 20% (w/v) PEG 3000	
Volume and ratio of drop (µl)	0.5 + 0.5	1.0 +1.0
Volume of reservoir (µl)	100	500

TABLE 3 I Data collection and statistics

Crystal	Akane1
Space group	$P2_1$
Cell dimension	
a, b, c (Å)	57.02, 126.67, 62.03
α, β, γ (°)	90.00, 107.52, 90.00
Resolution (Å)	50.00-1.93 (1.96-1.93)
No. of observations	351032
No. of unique reflections	59497
Redundancy	5.9 (4.9)
Data completeness (%)	93.7 (95.4)
Average I/σ (I)	32.11 (3.52)
R_{sym} (%)	0.068 (0.386)
R_{meas}	0.074 (0.431)
R_{pim}	0.029 (0.187)
CC*	0.960

*values in parentheses are for the highest resolution shell.

Data collection and processing

The X-ray diffraction data were collected on the beamline BL-11C at Pohang Accelerator Laboratory (PAL), Korea (Park et al., 2017). Crystals were flash-cooled in nitrogen at 100 K after a brief period of soaking in reservoir solution with 25% (v/v) glycerol. Wavelength of the X-rays used was 0.9795 Å. A total of 360 frames of data were collected. Processing of diffraction images and scaling of integrated intensities were performed using HKL2000 (Otwinowski and Minor, 1997). The data-collection statistics are summarized in Table 3. Molecular replacement was performed using Phaser-MR (Phenix GUI) (Adams et al., 2010) with the structure of photoswitchable green fluorescent Dronpa (PDB code: 2GX0) (Nam et al., 2007) as the search model.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEDGEMENTS

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