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Identification and characterization of a fluorescent flagellar protein from the brown alga Scytosiphon lomentaria (Scytosiphonales, Phaeophyceae): A flavoprotein homologous to Old Yellow Enzyme

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Identification and characterization of a fluorescent flagellar protein from the brown alga *Scytosiphon lomentaria* (Scytosiphonales, Phaeophyceae): A flavoprotein homologous to Old Yellow Enzyme

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The posterior flagellum of the zoospore of the brown alga *Scytosiphon lomentaria* exhibits bright green autofluorescence. To identify the fluorescent flagellar substance(s), we isolated flagella from zoospores and partially purified a flavoprotein by anion-exchange and gel-filtration chromatography. Spectrofluorometric and chromatographic analyses showed that the flavoprotein had an apparent molecular mass of 41 kDa and a non-covalently bound flavin mononucleotide as a chromophore. Based on partial amino acid sequences of the protein, a cDNA of the 41-kDa flavoprotein was cloned and sequenced. The deduced amino acid sequence of the cDNA was homologous to that of the Old Yellow Enzyme family distributed in proteobacteria, yeasts and vascular plants.

Key words: Brown algae, flagella, flavin mononucleotide, flavoprotein, Old Yellow Enzyme, *Scytosiphon lomentaria*

Introduction

Algal flagella principally act as locomotive and sensory organelles. Zoospores of heterokont algae, such as the brown algae, have two flagella, namely the anterior and posterior flagella (Inouye, 1993). The anterior flagellum with mastigonemes generates a driving force with a reversed direction while the posterior flagellum performs bending movements to change the swimming direction, allowing chemotactic and phototactic responses (Geller & Müller, 1981; Kawai *et al*., 1990). Thus, the shapes and functions of the two flagella are totally different from each other, although the so-called '9 + 2' microtubular structure is common to both (Bouck, 1971; Moestrup, 1982). This suggests the existence of specific proteins related to the differentiated functions of the flagella. However, there have been few biochemical analyses of the flagella of heterokont algae, since a method for their large-scale isolation has not been established.

In some brown algae, the posterior flagellum exhibits bright green autofluorescence under a fluorescence microscope, while the anterior flagellum does not (Müller *et al*., 1987; Coleman, 1988; Kawai, 1988). The fluorescence spectra of zoospore suspensions (Müller *et al*., 1987; Kawai, 1988) and the microscopic fluorescence spectra of isolated posterior flagella (Kawai *et al*., 1996) suggest that flavin- and pterin-like substances are localized there. These substances are thought to be involved in the phototactic response of zoospores (Kawai *et al*., 1996). The swelling of the posterior flagellum is considered to be a photoreceptive site for phototaxis (Kreimer *et al*., 1991; Kreimer, 1994), and the fluorescence in the swelling is brighter than in other segments of the flagellum. In this study, we performed a large-scale isolation of flagella from the zoospores of a brown alga,

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Scytosiphon lomentaria, and partially purified a flavoprotein from the flagellar preparation as a candidate for the fluorescence substance(s).

Materials and methods

Isolation of flagella

Scytosiphon lomentaria (Lyngbye) Link (Phaeophyceae) was collected from February to May of 2000–2003, at Charatsunai, Muroran, Hokkaido, Japan (42°19′N, 140°59′E) and also at Esaki, Hokutan-cho, Awaji Island, Hyogo, Japan (34°36′N, 134°59′E).

The gametophytes were washed with filtered seawater, and then placed on several sheets of paper and maintained in darkness at about 10°C. After several days, the gametophytes were immersed in cold filtered seawater on an ice-cooled tray and placed in sunlight. Zoospores were liberated from gametangia of the gametophytes within 10 min of exposure to sunlight irradiation.

The liberated zoospores were filtered through Miracloth (Calbiochem, La Jolla, USA) and collected by centrifugation at 1,300 *g* for 5 min (CR-21G; Hitachi, Tokyo, Japan). The zoospores were suspended in isolation buffer [30 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Dojindo, Kumamoto, Japan)-NaOH, pH 7.0, 5 mM $MgSO_4$, 5 mM ethylene glycol bis(*β*-aminoethylether)-*N*,*N*,*N*,*N*-tetraacetic acid (Dojindo), 25 mM KCl, 1 M sorbitol] and shaken vigorously in a VIX-100 VIAL MIXER (Taitec, Saitama, Japan) twice for 5 min in order to detach the flagella from the zoospores. After removal of the cell debris by low-speed centrifugation (2,500 *g* for 5 min, 3 times, CF-15R; Hitachi), the detached flagella were collected by high-speed centrifugation (11,900 *g*, 60 min, CT-13R; Hitachi), and the flagellar pellet was immediately frozen in liquid nitrogen and stored at −80°C. All the steps for flagellar fractionation were performed at 4°C. About 1 ml of flagellar pellet was obtained from 3 kg (wet weight) of gametophytes of *S. lomentaria*.

Purification of flavoprotein

Frozen flagella (ca. 1 ml) were re-suspended in 3 ml extraction buffer (40 mM 1,3-bis[tris (hydroxymethyl)methylamino]propane(ICN Biomedicals, Aurora, USA)-HCl, pH 6.5) containing the Complete™ protease inhibitor (Roche Diagnostics, Mannheim, Germany) at 4°C and centrifuged at 11,900 *g* for 60 min (CT-13R; Hitachi), followed by ultra-centrifugation at 150,000 *g* for 30 min (CS120GX; Hitachi) to obtain a cleared extract. The following manipulations were performed under dim red light ($< 0.2\,\mu mol\,m^{-2}\,s^{-1}$) produced by a fluorescent tube (FL15W; Matsushita, Osaka, Japan) covered with an acrylic filter (Acrylite 102; Mitsubishi Rayon, Tokyo, Japan).

After concentration by ultra-filtration (Biomax 5; Millipore, Bedford, USA), the extract was analysed by liquid chromatography following Iseki *et al.* (2002). The extract (0.5 ml) was applied to an anion-exchange column (4.6 × 50 mm, POROS HQ/M; Perseptive Biosystems, Framingham, USA) equilibrated with starting buffer (40 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane-HCl, pH 6.5) at 4°C and eluted with a linear gradient of NaCl (0–0.3 M in the starting buffer). The eluate was collected into 78 fractions (0.5 ml each) at a flow rate of 2.5 ml/min. A 0.05 ml aliquot from each fraction was diluted to 0.2 ml with the starting buffer and used for spectrofluorometry after de-naturation by heat-treatment (95°C for 5 min) using a thermostatic aluminum bath (ALB-120; Iwaki, Tokyo, Japan). A 0.1 ml aliquot of each fraction was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Fractions with a higher fluorescence at 525 nm (fraction nos. 43–45) were combined and concentrated (final volume: 0.2 ml) by ultra-filtration with Biomax 5, and then separated by gel-filtration chromatography. The combined fraction (0.15 ml) was applied to a gel-filtration column (10 × 300 mm, Superose 6HR10/30; Amersham Pharmacia, Freiburg, Germany) equilibrated with an elution buffer (40 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane-HCl, pH 6.5, 150 mM NaCl) at 4°C. The elutate was collected into 55 fractions (0.5 ml each) at a flow rate of 0.2 ml/min. A 0.2 ml aliquot of each fraction was heated at 95°C and used for fluorometric analysis, while the remaining 0.3 ml aliquot of each fraction was used for SDS-PAGE. Gel Filtration Standard 6 (Bio-Rad, Hercules, USA) were used as molecular weight markers for the gel-filtration chromatography. The protein concentration was determined by the Bradford micro-assay method (Bradford, 1976) using a Protein Assay Kit II (Bio-Rad).

Microscopy and spectrofluorometry

Zoospores and isolated flagella were observed under bright-field microscopy in the differential-interference and epifluorescence modes (BX50-FLI; Olympus, Tokyo, Japan) and imaged with a digital camera (AXIO-CAM and AXIO-VISION systems; Carl Zeiss, Hallbergmoos, Germany). The fluorescence spectra of zoospores, isolated flagella, crude extracts and chromatographed fractions were measured with Hitachi F4500 and Hitachi 850 spectrofluorometers.

SDS-PAGE

Protein samples were dissolved in SDS sample buffer [50 mM tris(hydroxymethyl)aminomethane-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 6% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue]. SDS-PAGE was performed according to the method of Laemmli (1970) using a Mini-Protean III System (Bio-Rad). Polyacrylamide gels (10% or 12%) were electrophoresed at 20 mA, and then stained with Coomassie Brilliant Blue R-250 (ICN Biomedicals) or a Silver Stain II Kit (Wako, Osaka, Japan). The molecular mass was estimated using Precision Standards (Bio-Rad).

Thin-layer chromatography

The flavoprotein-rich fraction obtained by anion-exchange chromatography was concentrated by ultrafiltration (Biomax 5) for chromophore analysis. The concentrate was heated at 95°C for 5 min to release the chromophore(s) of the flavoprotein. After removal of the denatured proteins by ultrafiltration, the filtrate was evaporated by vacuum aspiration in the dark. The residue was dissolved in methanol, applied to a Silicagel 70 thin-layer chromatography (TLC) plate (40 × 80 mm; Wako), and developed with *n*-butanol/acetic acid/water (4:1:5). The flavin spots were detected by fluorescence using an ultraviolet transilluminator (DT-35LMP; ATTO, Tokyo, Japan). Riboflavin (Wako), flavin mononucleotide (FMN; Sigma Chemical, St. Louis, USA) and flavin adenine dinucleotide (FAD; Sigma) were used as standards.

Protein sequencing

Since the *N*-terminal amino acid of the 41-kDa flavoprotein was blocked, internal amino acid sequences were analysed after digestion according to Cleveland *et al.* (1977). The flavoprotein-rich fraction obtained by anion-exchange chromatography was separated by SDS-PAGE (in a 12% polyacrylamide gel), and the 41-kDa band (ca. 5 μg) was excised from the gel. The protein band (5 × 6 × 0.75 mm) was applied to a well of an 18% polyacrylamide gel, and overlaid with 64 ng of *Staphylococcus aureus* V8 protease (Wako) in 0.5 × SDS sample buffer. After digestion at 20°C for 40 min, the fragmented peptides were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P; Amersham Pharmacia). Six peptide bands (26, 27, 28, 32, 35 and 38 kDa: 10–150 pmol) stained with Coomassie Brilliant Blue were subjected to *N*-terminal amino acid sequencing using a gas-phase protein sequencer (Procise 494; Applied Biosystems, Foster City, USA). As a result, the amino acid sequences of two internal regions of the 41-kDa flavoprotein were determined: ATQVSTDGQGYXLTPGVFT from the 32–38 kDa fragments and SYQPDGKAP PAPSAIACPDGEWFTMEGPKPFPVPRE from the 26–28 kDa fragments.

PCR-based cloning and sequencing

Four degenerate primers (41degeA: 5′-TAYCAR CCNGAYGGNAARGC-3′; 41degeB: 5′-CCYT CCATNGTRAACCAYTC-3′; 41degeC: 5′-GARW SNTAYCARCCNGAYGG-3′; and 41degeD: 5′-AANGGYTTNGGNCCYTCCA T-3′) were designed according to the internal amino acid sequences of the 41-kDa flavoprotein. Total RNA was isolated from phototactically collected zoospores using an RNeasy Plant mini Kit (Qiagen, Hilden, Germany). First-strand cDNAs were synthesized from the total RNA using the reverse transcriptases M-MLV (Invitrogen, Carlsbad, USA) or ReverTra Ace (Toyobo, Osaka, Japan) and the Oligo dT-3 Sites Adaptor Primer (Takara, Otsu, Japan). The target sequence in the cDNAs was amplified by LA Taq DNA polymerase (Takara) with a set of degenerate primers (41degeC and 41degeD) via 30 polymerase chain reaction (PCR) cycles (denaturation at 96°C for 20 s, annealing at 53°C for 30 s and extension at 72°C for 40 s) using a GeneAmp 9700 (Applied Biosystems). Next, using the PCR products as a template, the target cDNA was further amplified by LA Taq DNA polymerase with another set of primers (41degeA and 41degeB) via 30 PCR cycles (denaturation at 96°C for 20 s, annealing at 57°C for 30 s and extension at 72°C for 40 s). The second PCR product (77 bp) was gel-purified using a QIAquick Gel Extraction Kit (Qiagen) and cloned into a pGEM-T Easy vector (Promega, Madison, USA). The cloned PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 310 (Applied Biosystems).

A specific forward primer (41GS1: 5′-TCCCTCGGCAATCGCATG-3′) for 3′-rapid amplification of cDNA ends (RACE) was designed according to the sequence of the second PCR product (77 bp). 3′-RACE was performed using Ex Taq DNA Polymerase (Takara) with the 41GS1 primer and the 3sites Adaptor Primer (3′-Full RACE Core Set; Takara). The PCR products (1.4–1.5 kbp) of the 3′-RACE were cloned into a pGEM-T Easy vector and sequenced using an ABI PRISM 3100. To extend the cDNA of the 41-kDa flavoprotein to the 5′-end, specific reverse primers (41GS2: 5′-GAATTGCTCGACGCAG TTTTTGATCT-3′; 41GS3: 5′-TTTGATCTCCT CCACCGTCATCTC-3′; and 41GS4: 5′-GCCC TTCCATCGTAAAGCAC-3′) were designed according to the sequences of the 3′-RACE products. The 5′-portion of the cDNA was amplified by thermal asymmetric interlaced (TAIL)-PCR (Liu *et al.*, 1995) using an arbitrary degenerate primer (5′-WGTGNAGWANCA NAGA-3′), and the PCR products (0.6–0.7 kbp) were cloned and sequenced as described above. To obtain the complete coding sequence of the cDNA for the 41-kDa flavoprotein, we used specific forward and reverse primers (41GS5: 5′-AGCGGCATTGAACTACCCGAG-3′ and 41GS6: 5′-ACGCGTTCAAAGCCAGTCCTC-3′, respectively) derived from the untranslated regions of the 3′-RACE and TAIL-PCR products. The target cDNA was amplified by Pyrobest DNA Polymerase (Takara) with the primer set (41GS5 and 41GS6) via 30 PCR cycles (denaturation at 96°C for 20 s, annealing at 69°C for 30 s and extension at 72°C for 90 s). The PCR products (1.3 kbp) were purified with a QIAquick Gel Extraction Kit, and sequenced directly using a BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3100. To confirm the accuracy of the sequence, the PCR amplifications and direct sequencing of the PCR products were repeated four times.

The sequence of the cDNA encoding the 41-kDa protein has been submitted to the DDBJ/GenBank/EMBL databank under Accession Number AB188118. Sequence analysis against databases was performed using the NCBI BLAST server and alignment of the 41-kDa protein was performed with the CLUSTAL W program (Thompson *et al.*, 1994).

Results

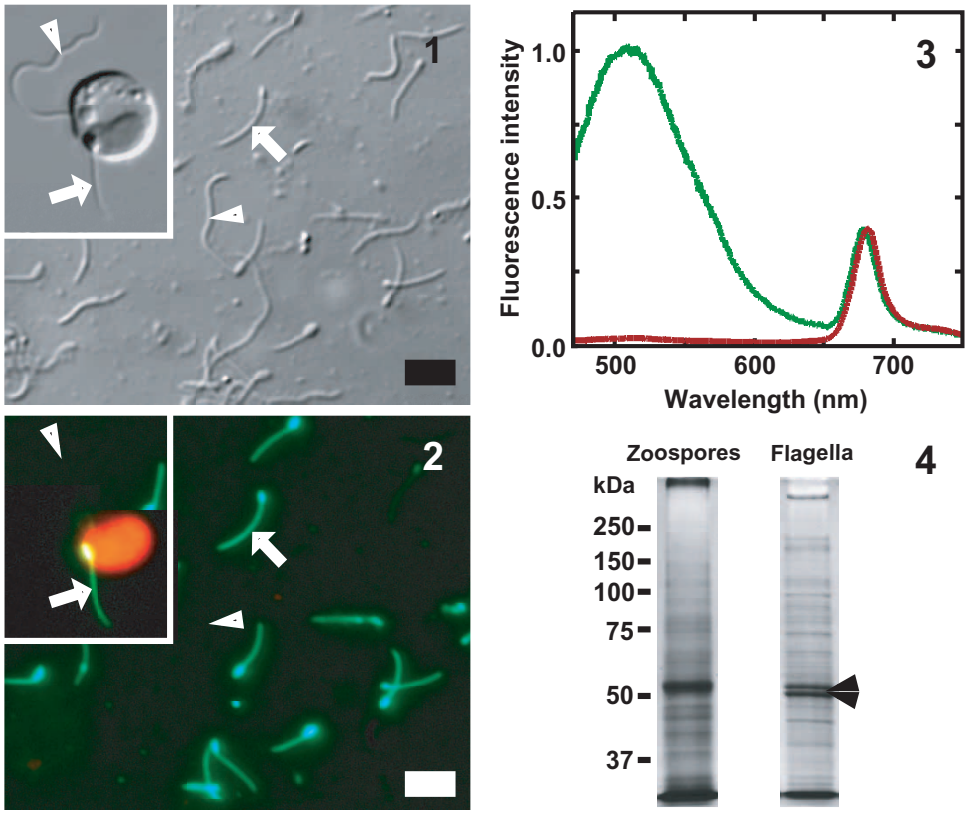
Isolated flagella

Figs 1–3 show microphotographs and fluorescence spectra of freshly isolated flagella from zoospores of *S. lomentaria*. The flagellar preparation contained both posterior and anterior flagella which were easily discriminated by their differential-interference (Fig. 1) and fluorescence (Fig. 2) images. The isolated flagella had the same size and shape as flagella of fresh zoospores indicating that the flagellar preparation was fully intact (Fig. 1). The isolated posterior flagella also retained the proximal swelling (Fig. 1).

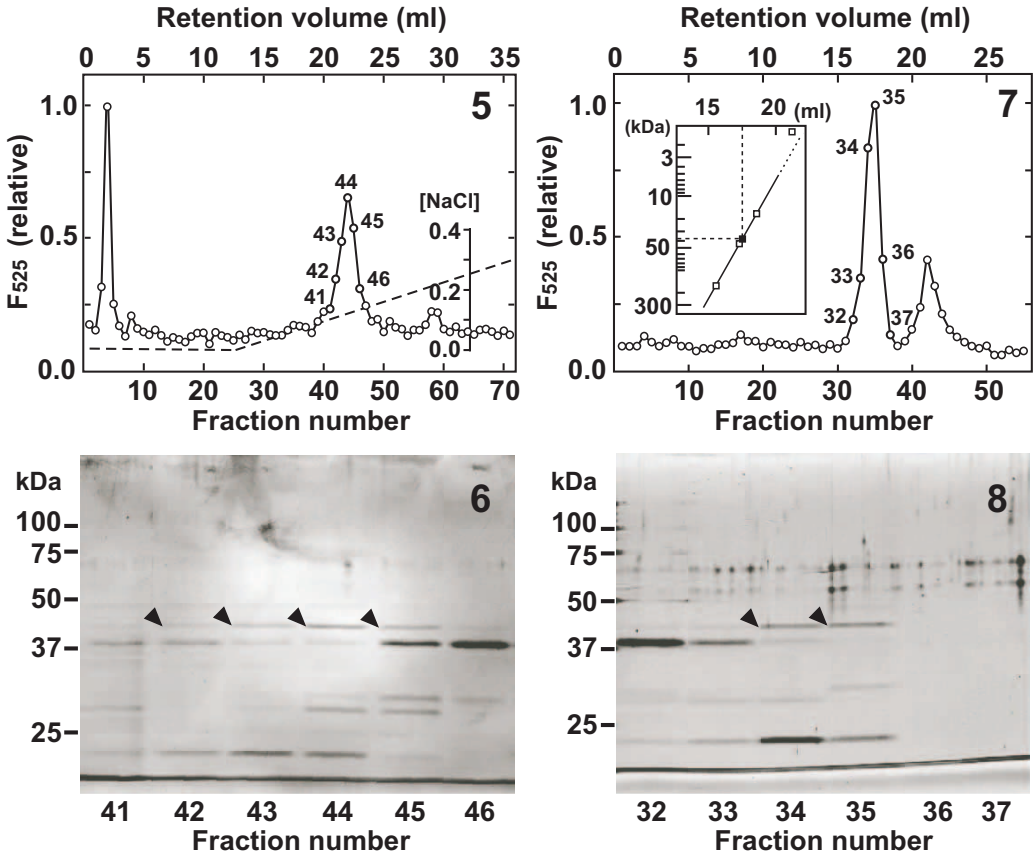
The flagellar preparation was almost free of cell debris such as thylakoid fragments (Figs 1, 2). The fluorescence spectra of the isolated flagella showed about 50-fold enhanced green fluorescence (around 530 nm) relative to the red fluorescence of chlorophyll *a* (around 680 nm), indicating that the flagellar preparation contained little contamination (Fig. 3). Furthermore, SDS-PAGE revealed that 52- and 50-kDa bands, corresponding to α- and β-tubulin, respectively, could be distinguished in the flagellar fraction (Fig. 4), but not in the intact zoospores. A broad band around 52 kDa in the intact zoospores was mainly ribulose-bisphosphate carboxylase/oxygenase, as evaluated by Western blotting analysis (data not shown, cf. Nagasato *et al.*, 2003). The fluorescence spectra confirmed that flavin-like substances were present in the posterior flagella, as previously reported (Müller *et al.*, 1987; Kawai, 1988; Kawai *et al.*, 1996).

Purification and characterization of flavoprotein

To isolate the fluorescent substance(s), including flavoprotein, from the posterior flagella, we tried to solubilize the flagellar fraction with detergents, such as *n*-dodecyl-β-D-maltoside. However, a viscous substance appeared during treatment with these detergents, which disturbed the subsequent purification. Therefore, we adopted a freeze-thaw treatment in the extraction buffer without detergent to solubilize flavoprotein, although the



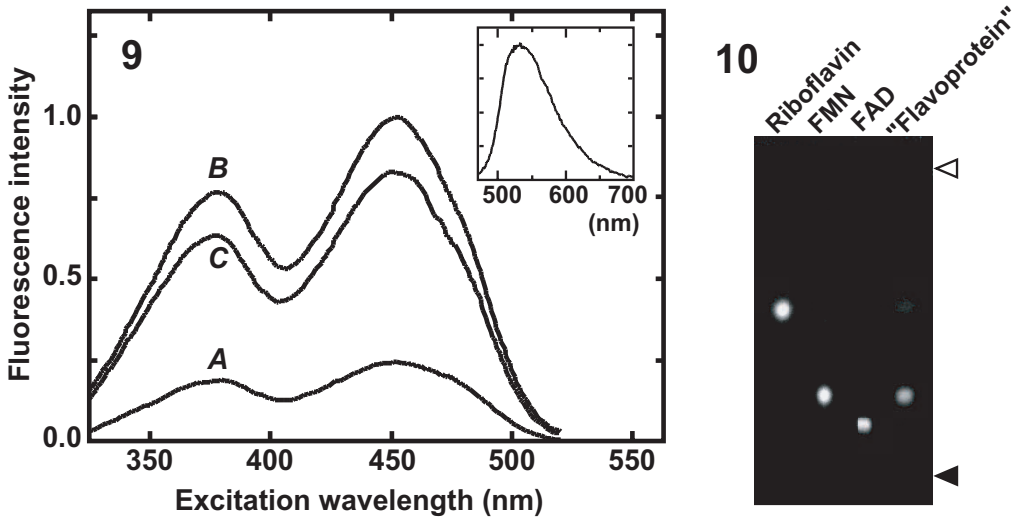
Figs 1–4. Light micrographs and fluorescence spectra of isolated flagella of *Scytosiphon lomentaria* and photographs of SDS-PAGE gels. Figs 1, 2. Differential-interference image (Fig. 1) and fluorescent image (Fig. 2) of isolated flagella under blue-violet light excitation of the same preparation. Insets show intact zoospore. The arrows indicate posterior flagella, while the arrowheads indicate anterior flagella. Scale bars represent 5 µm. Fig. 3. Fluorescence emission spectra of isolated flagella (green) and intact zoospores (red) under excitation at 440 nm. The fluorescence intensity was normalized to the fluorescence maxima of chlorophyll *a* (around 680 nm). Fig. 4. SDS-PAGE of intact zoospores (Zoospores) and isolated flagella (Flagella). The gels were stained with Coomassie Brilliant Blue. The arrowheads indicate α- and β-tubulin.



Figs 5–8. Graphs of elution profiles and photographs of SDS-PAGE gels of relevant elution fractions. Fig. 5. Anion-exchange chromatogram profile of a crude flagellar extract (130 µg protein). Open circles show the elution profile of the 525 nm fluorescence excited by 370 nm light (F_{525}), while the broken line shows the linear gradient of eluted NaCl (0–0.3 M). Fig. 6. SDS-PAGE of fractions 41–46, shown in Fig. 5. 0.1 ml aliquots of each fraction were used and the gel was stained with Coomassie Brilliant Blue. Arrowheads indicate the 41-kDa band. Fig. 7. Gel-filtration chromatogram profile of the combined fraction (3.6 µg protein), fractions 43–45 from the anion-exchange chromatography. Open circles show the elution profile of the 525 nm fluorescence excited by 370 nm light (F_{525}). A calibration curve (inset) was derived using the following molecular mass standards (open squares): bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B_{12} (1.4 kDa). The position of the flavoprotein is shown by a closed square. Fig. 8. SDS-PAGE of fractions 32–37, shown in Fig. 7. 0.2 ml aliquots of each fraction were used and the gel was silver-stained. Arrowheads indicate the 41-kDa band.

extraction yield was rather low (ca. 5%). The crude flavoprotein-containing extract was subjected to a combination of anion-exchange and gel-filtration chromatographies. In the anion-exchange chromatography, flavoprotein was eluted at 0.1 M NaCl (fractions 43–45, Fig. 5) as determined by the fluorescence at 525 nm. Strong fluorescence in the flow-through (fraction 4) was derived from free flavin(s) (Fig. 5). The fractions containing flavoprotein (fractions 43–45) were further purified by gel-filtration chromatography (Fig. 7). The main elution peak (fractions 34–35) came from flavoprotein and a minor peak (fractions 42–43) from free flavins. The apparent molecular mass of the flavoprotein in fraction 35 was about 40 kDa (Fig. 7, inset). SDS-PAGE analysis of the fractions around the fluorescence peaks in the anion-exchange and gel-filtration chromatographies revealed several bands, e.g., 23 kDa, 27 kDa, 38 kDa and 41 kDa (Figs 6, 8). However, comparison of the elution profiles between the fluorescence and the protein bands indicated that the 41-kDa band was most likely to be the target flavoprotein (cf. Figs 5, 6, and Figs 7, 8). The results of the gel-filtration chromatography and SDS-PAGE further demonstrated that the 41-kDa flavoprotein existed in a monomeric state in solution.

The fluorescence emission spectra of the 41-kDa flavoprotein-rich fraction after gel-filtration chromatography (fraction 35) were measured with excitation at 440 nm (Fig. 9, inset) and 370 nm (data not shown). The two spectra were almost identical and had a single emission maximum at 534 nm. The excitation spectrum of the same fraction had excitation maxima at 380 nm and 451 nm (Fig. 9, curve *A*). These spectral characteristics were attributed to flavin(s) in the 41-kDa flavoprotein. The fluorescence intensity of the denatured sample was about 4-fold higher than



Figs 9, 10. Identification of the chromophore of the 41-kDa flavoprotein. Fig. 9. Fluorescence excitation spectra of the 41-kDa flavoprotein at 550 nm emission. The native flavoprotein at pH 6.5 (*A*) was denatured by heat-treatment at 95°C (*B*), and then titrated to pH 3 with 20 μmol citric acid (*C*). The fluorescence emission spectrum (inset) of the native flavoprotein was measured at 440 nm excitation. Fig. 10. TLC analysis of the chromophore of the 41-kDa flavoprotein ('Flavoprotein'). As standards, 14 pmol of riboflavin, FMN and FAD were used. The starting points and solvent front in the development are indicated by closed and open arrowheads, respectively. The R_f values of riboflavin, FMN and FAD were 0.54, 0.26 and 0.16, respectively.

that of the native sample (Fig. 9, curves *A*, *B*), indicating that the flavin chromophore was non-covalently bound to the protein moiety through chromophore-protein interaction in the native protein. At pH 3, the fluorescence intensity of the flavin(s) released from the 41-kDa flavoprotein was slightly lower than that at pH 6.5 (Fig. 9, curves *B*, *C*), indicating that FAD was not involved in the flavoprotein. Among the typical flavins, only FAD produces greater fluorescence at acidic pH than at neutral pH, while FMN and riboflavin give slightly weaker fluorescence at acidic pH (Bessey *et al.*, 1949).

TLC analysis of the flavin(s) released from the 41-kDa flavoprotein revealed one major spot and one trace spot (Fig. 10). The R_f value of the major spot (0.25) was almost identical to that of FMN (0.26) (Fig. 10), indicating that the chromophore of the 41-kDa flavoprotein was FMN.

Identification of the flavoprotein

Since the *N*-terminal amino acid of the 41-kDa flavoprotein was blocked, peptides of the 41-kDa flavoprotein after V8 protease digestion were used for its identification. From the *N*-terminal amino acid sequences of these six peptides, the amino acid sequences of two internal regions of the 41-kDa flavoprotein were obtained (cf. Fig. 11). Based on these internal sequences, degenerate primers were designed and the partial nucleotide sequence of the cDNA encoding the 41-kDa flavoprotein was determined. We then extended the cDNA to the 3′- and 5′-ends using RACE and TAIL-PCR, respectively, and determined the full coding sequence (1,107 bp) of the cDNA. The deduced amino acid sequence of the cDNA was composed of 368 amino acids with a calculated molecular weight of 40,748 (Fig. 11), which coincides with the apparent molecular mass of 41 kDa obtained by SDS-PAGE. We also detected a DNA fragment encoding the 41-kDa flavoprotein in genomic DNAs isolated from axenic gametophytes of *S. lomentaria* by PCR (data not shown). This excluded the possibility that the cDNA may have been obtained from bacterial contamination. The 41-kDa flavoprotein showed close sequence similarities with isoforms/homologues of Old Yellow Enzyme (OYE), such as morphinone reductase (MorB, identity: 44%) in proteobacteria (French & Bruce, 1995), 12-oxophytodienoate reductase 1 (OPR1, 43%) in land plants (Biesgen & Weiler, 1999), and Old Yellow Enzyme 1 (OYE1, 33%) in yeast (Saito *et al.*, 1991) (Fig. 11).

Discussion

In this study, we purified a fluorescent flagellar substance from the brown alga *S. lomentaria* and identified it as a flavoprotein with a molecular mass of 41 kDa. 4′,5′-Cyclic FMN (riboflavin-4′,5′-cyclic phosphate) was previously identified in mature thalli of *S. lomentaria* and proposed as a

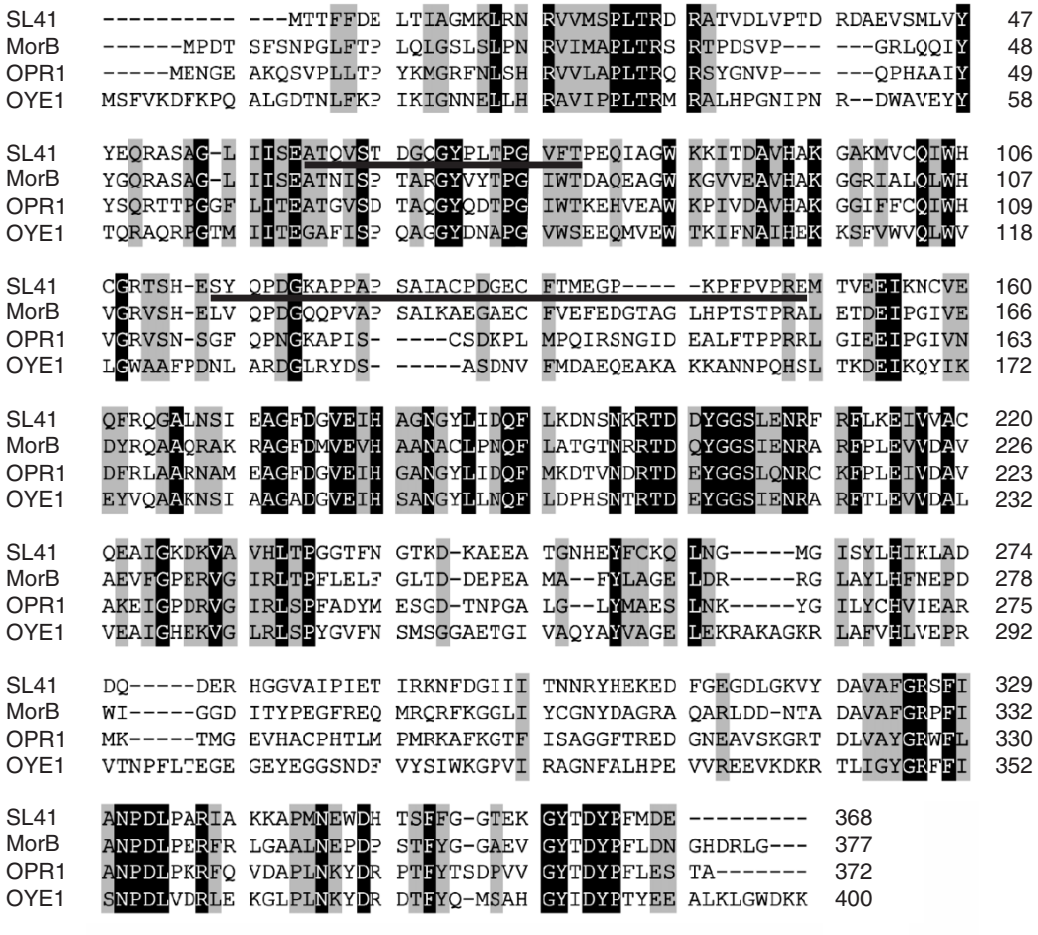


Fig. 11. Alignment of the deduced amino acid sequences of the 41-kDa flavoprotein and members of the Old Yellow Enzyme family. The deduced amino acid sequence of the 41-kDa flavoprotein from *S. lomentaria* (SL41; Accession Number AB188118) was aligned with those of morphinone reductase from *Pseudomonas putida* (MorB; AAC43569), 12-oxyphytodienoate reductase 1 from *Arabidopsis thaliana* (OPR1; AAC78440) and Old Yellow Enzyme 1 from *Saccharomyces pastorianus* (OYE1; Q02899). Identical and similar amino acid residues are shown on black and grey backgrounds, respectively. The underlined sequences in SL41 (amino acids 61–79 and 114–149) are identical to the results of the protein sequencing.

candidate for the green fluorescent substance of posterior flagella (Yamano *et al*., 1996). However, the chromophore of the 41-kDa flavoprotein, which was a more plausible fluorescent flagellar substance, was FMN. We also detected a pterin-like substance(s) with a fluorescence emission maximum at around 460 nm (cf. Kawai *et al*., 1996) in the flagellar extracts. In the gel-filtration chromatography of the flagellar extract, a pterin-like substance was eluted at less than 5 kDa, indicating that this chromophore was not bound to a protein (data not shown).

From the deduced amino acid sequence, the 41-kDa flavoprotein is a new member of the OYE family. OYE was the first flavoprotein discovered in brewer's bottom yeast (Warburg & Christian, 1933). The protein contains FMN as a cofactor that participates in general redox reactions (Williams & Bruce, 2002). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH) serves as the physiological reductant for the enzyme-bound flavin (Williams & Bruce, 2002), while quinones, Fe^{3+}, cytochrome *c* and ferricyanide have been reported as natural and artificial oxidants (Vaz *et al*., 1995; Williams & Bruce, 2002). Although extensive biochemical and spectroscopic characterization has been reported, the true physiological role and natural substrates of most members of the OYE family have remained obscure (Williams & Bruce, 2002). One exception is 12-oxophytodienoate reductase, a member of the OYE family in vascular plants, which catalyses the intermediate step in jasmonic acid biosynthesis (Schaller *et al*., 2000).

We identified the 41-kDa flavoprotein of brown algae as a new member of the OYE family. The green fluorescent substance in the posterior flagella has been reported to be correlated with the phototactic ability of zoospores (Müller *et al*.,

1987; Kawai, 1988, 1992). Furthermore, the action spectra of the phototactic responses of the brown algae *Ectocarpus siliculosus* and *Pseudochorda gracilis* (Kawai *et al*., 1990, 1991) suggested that the flavin-like substance(s) was involved as a blue light sensor in phototaxis. Recently, a novel flavoprotein was identified as the green fluorescent substance in the flagellar swelling of *Euglena gracilis* (Iseki *et al*., 2002). This FAD-containing flavoprotein functions as the photoreceptor for the photophobic response. The 41-kDa flavoprotein of brown algae is very interesting in terms of green fluorescent substance(s) in posterior flagella and the phototactic ability of zoospores. On the other hand, the flavoprotein may be involved in the metabolic or signalling pathway of the brown algal pheromone(s) (Pohnert & Boland, 2002), since some members of the OYE family catalyse the reductive step of oxylipin metabolism (Williams & Bruce, 2002). Extensive studies are needed to elucidate the physiological and molecular functions of the flavoprotein of posterior flagella.

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