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Cloning, Expression, Purification and Characterization of an Isotype of Clytin, a Calcium-Binding Photoprotein from the Luminous Hydromedusa *Clytia gregarium*

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The cDNA for an isotype of clytin, a calcium-binding photoprotein from the luminous jellyfish *Clytia gregarium*, was identified and named clytin-II. The histidine-tagged apoprotein of clytin-II expressed into the periplasmic space of *Escherichia coli* cells was isolated by nickel chelate affinity chromatography. Recombinant clytin-II regenerated from apoprotein by incubation with coelenterazine was purified. The yield of purified clytin-II was 13 mg from 2 l of cultured cells with purity >95%. The luminescence properties of clytin-II were characterized by comparison with clytin-I and aequorin, and semi-synthetic clytin-II was also prepared. The initial luminescence intensity of clytin-II triggered by Ca^{2+} was 4.5 times higher than that of clytin-I and aequorin, but the luminescence capacity was close to clytin-I and aequorin. Thus, clytin-II is a useful protein, showing high sensitivity in the signal-to-noise ratio of luminescence intensity.

Key words: aequorin, coelenterazine, luminescence spectrum, protein secretion, isotype clytin.

Abbreviations: Apoclytin, apoprotein of clytin; clytin-II, isotype of clytin (clytin-I); OmpA, the outer membrane protein A; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; Imax, maximum intensity; rlu, relative light units; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis.

Bioluminescence in the hydrozoan coelenterates involves a Ca^{2+}-binding photoprotein and a green fluorescent protein (*1*). The four luminescent hydromedusae including *Aequorea*, *Clytia* (formerly *Phialidium*), *Mitrocoma* (formerly *Halistaura*) and *Obelia* are known (*2*, *3*) and they all have Ca^{2+}-binding proteins: aequorin (*4–6*), clytin (=phialidin) (*7–9*), mitrocomin (=halistaurin) (*10–11*) and obelin (*12–16*). A Ca^{2+}-binding photoprotein is a non-covalent complex of 2-hydroperoxycoelenterazine and apophotoprotein (22–26 kDa protein) (*17–19*), and emits light by an inter-molecular reaction upon Ca^{2+} binding. After the Ca^{2+}-triggered luminescence reaction, apoprotein can be regenerated to photoprotein by incubation with coelenterazine and molecular oxygen in the presence of reducing reagents and Ca^{2+}-chelating reagents. The best-characterized photoprotein is aequorin (*1*) and has been used as a calcium indicator both *in vitro* and *in vivo* (*20*).

In 1993, the cDNA cloning for a photoprotein from *Clytia gregarium* was performed by the method of plaque hybridization with the apoaequorin cDNA as a probe. Eight positive clones were isolated and a clone with the shortest insert and Ca^{2+}-triggered luminescence activity was assigned to pCL41. After determining the nucleotide sequence of pCL41, a photoprotein from *C. gregarium* was identified and was named 'clytin'(clytin-I in this paper) (*9*). By comparing with aequorin, clytin-I consists of 189 amino acid residues in a single polypeptide chain with three EF-hand motifs characteristics for calcium binding, and it is a member of Ca^{2+}-binding photoproteins including aequorin, mitrocomin and obelin (*21*, *22*). Recently, recombinant clytin-I was highly purified from *Escherichia coli* cells using a histidine-tagged secretion vector, piP-H6-M(*23*), and characterized (*23*). In this paper, immunoblot analyses in luminous jellyfishes; *C. gregarium*, *Aequorea aequorea* and *Mitrocoma cellularia*, using anti-recombinant apoaequorin polyclonal antibody suggested that homologous proteins including isotype clytin are present in *C. gregarium*. After investigating the positive cDNA clones from *C. gregarium*, we identified two clones with higher luminescence intensity than clytin-I, which we term 'clytin-II'. Recombinant clytin-II was expressed, purified and characterized by comparing with clytin-I and aequorin.

MATERIALS AND METHODS

Materials—Specimens of *C. gregarium*, *M. cellularia* and *A. aequorea* were collected at Friday Harbor Laboratories, Friday Harbor, Washington, on 2 October 1991. Specimens of *C. gregarium* (~2 cm, diameter) and the outer margins of *M. cellularia* (5–7 cm, diameter) and *A. aequorea* (6–8 cm, diameter) were frozen in liquid nitrogen and stored at –80°C. The recombinant clytin-I and aequorin were prepared as previously reported (*23*, *24*). The IgG fraction of anti-recombinant apoaequorin serum was obtained as previously described (*25*). The sources of chemicals were as follows: ethylenediaminetetraacetic acid disodium salt (EDTA·2Na), $CaCl_2 \cdot 2H_2O$, 100 mM $CaCO_3$ standard solution, Tris(hydroxymethyl)aminomethane, (±)-dithiothreitol (DTT), 2-mercaptoethanol, imidazole,

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$NiSO_4 \cdot 6H_2O$, NH_4SO_4 (Wako Pure Chemicals, Osaka, Japan); chelate Sepharose Fast Flow, Q Sepharose Fast Flow, butyl Sepharose Fast Flow (Amersham Biosciences, Piscataway, NJ, USA); bovine serum albumin, Fraction V, (Seikagaku Co., Japan), *hcp*-coelenterazine (*hcp*-CTZ), *f*-coelenterazine (*f*-CTZ) and *n*-coelenterazine (*n*-CTZ) (Molecular Probe, Eugene, OR, USA); coelenterazine (CTZ), *h*-coelenterazine (*h*-CTZ) and *Bis*-coelenterazine (*Bis* -CTZ) (Chisso, Yokohama, Japan); *e*-coelenterazine (*e*-CTZ) and methoxycoelenterazine (*MeO*-CTZ) were kindly provided by Dr K. Teranishi (Mie Univ., Japan).

Protein Analysis—SDS–PAGE analysis was carried out under reducing conditions using a 12% separation gel, as described by Laemmli (*26*). Protein concentration was determined by the dye-binding method of Bradford (*27*) using a commercially available kit (Bio-Rad, Richmond, CA, USA) and bovine serum albumin as a standard (Pierce; Rockford, IL, USA).

Western Blot Analysis—Immunoblot analysis of luminous jellyfishes was performed using anti-recombinant apoaequorin antibody, as previously described (*25*). Briefly, the tissues of jellyfish were homogenized in 500 µl of Tris–HCl (pH 8.0) containing 10 mM EDTA on an ice bath. After centrifugation at 15 000 *g* for 5 min, the supernatant was dissolved in Laemmli's sample buffer and heated at 90°C for 3 min. The sample was applied on a 12% gel and was run at 25 mA for 2 h. The proteins were transferred electrophoretically onto nitrocellulose membranes (Bio-Rad, 0.45 µm) at 150 mA for 2 h and the membrane was used for blot analysis (*25*). Pre-adsorbed anti-recombinant apoaequorin IgG with the purified apoaequorin was prepared as follows: the mixture of 20 µl of anti-apoaequorin IgG fraction (20 µg protein) and 50 µg of recombinant apoaequorin was incubated in 500 µl of 1% casein in 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl and 0.05% Tween-20 (Bio-Rad, CA, USA) at 25°C for 2 h and used for immunoblot analysis.

DNA Sequence Analysis—The nucleotide sequence was determined with an Applied Biosystems model 310 DNA sequencer using a BigDye terminator v1.1 cycle sequencing kit.

Expression and Purification of Clytin-II from E. coli Cells—The procedures for expression and purification of clytin-II were similar to the case of clytin-I (*23*). The expression vector, piP-H-CL-II, is the bacterial secretion vector with the signal peptide sequence of the outer membrane protein A (OmpA) and six histidine residues under the control of lipoprotein promoter (lpp) and *lac* operator. Briefly, the *Sac*I/*Xho*I fragment of apoclytin-II, obtained from pCL31 by PCR procedure using primer sets (CLII-N-EL-SacI: 5′ g ggc GAG CTC GAT CCT GAT TTT GCA AAT, *Sac*I underlined; CLI-C-*Xho*I: 5′ cgg CTCGAG TTA AGG AAC AAA ATT GCC GTA, *Xho*I site underlined), was inserted to the *Sac*I/*Xho*I sites of piP-H6-M(*11*) [23] to give piP-H-CL-II (Supplementary data 1). The host *E. coli* strain WA802 (CGSG5610) with piP-H-CL-II was grown in 10 ml of Luria–Bertani medium containing ampicillin (50 µg/ml) at 30°C for 16 h. The seed culture was added to 400 ml of LB medium in 3 l of a Sakaguchi flask and was cultured for 16 h at 37°C. The bacterial cells harvested by centrifugation at 5 000 *g* for 5 min from 2 l culture were suspended in 200 ml of 50 mM Tris–HCl (pH 7.6) and then disrupted by sonication with a Branson (Danbury, CT, USA) model 250 sonifire in an ice bath. After centrifugation at 12 000 *g* for 10 min, the resultant supernatant (200 ml) was applied on a Ni-chelate column (2.5 × 6.5 cm), equilibrated with 50 mM Tris–HCl (pH 7.6). The fractions (40 ml) containing apoclytin-II, eluted with 0.1 M imidazole in 50 mM Tris–HCl (pH 7.6), was regenerated to clytin-II in 200 ml of 50 mM Tris–HCl (pH 7.6)-10 mM EDTA containing 10 mg of DTT and 2 mg of coelenterazine at 4°C for 16 h. The regenerated clytin-II was applied on a Q Sepharose Fast Flow column (Pharmacia; 2.5 × 6 cm), equilibrated with 20 mM Tris–HCl (pH 7.6)-10 mM EDTA ('Buffer A'), and the column was washed with 0.1 M NaCl in Buffer A (100 ml) until the absorbance at 280 nm decreased below 0.1. The adsorbed clytin-II on the gel was eluted with 0.4 M NaCl in Buffer A (50 ml). The active fraction was combined, adjusted to 2 M $(NH_4)_2SO_4$ and was applied on a column of butyl Sepharose 4 Fast Flow (Pharmacia; 1.5 × 6 cm), equilibrated with 10 mM Tris–HCl (pH 7.6)-2 mM EDTA containing 2 M $(NH_4)_2SO_4$ ('Buffer B'). The column was washed with Buffer B (35 ml) until the absorbance at 280 nm decreased below 0.02, and then clytin-II was eluted with 10 mM Tris–HCl (pH 7.6)-2 mM EDTA containing 1.2 M $(NH_4)_2SO_4$. Peak fractions (13 ml) with luminescence activity were combined and stored at –80°C.

Determination of Luminescence Activity of Clytin-II—The luminescence activity of clytin-II was determined as follows: After regenerating to clytin-II by incubation with 1 µl of coelenterazine (1 µg/µl in ethanol) in 1 ml of 30 mM Tris–HCl (pH 7.6)-10 mM EDTA containing 1 µl of 2-mercaptoethanol at 4°C for 2 h, the mixture was placed in a 96-well white plate (Nunc, Cat. No 236108). By injection with 100 µl of 50 mM $CaCl_2$ in 50 mM Tris–HCl (pH 7.6), the initial luminescence intensity, luminescence capacity and half decay time of semi-synthetic clytins were measured for 10 s with a Berthold technologies (Bad Wildbad, Germany) model Centro 960 luminometer equipped with a 9124B photomultiplier (Electron Tube Limited, UK). The maximum intensity (I_{max}) of 100 pg of the purified recombinant aequorin showed 8.9×10^4 rlu.

Measurement of Ca^{2+} Sensitivity of Clytin-II—Purified recombinant clytin-II was dissolved in 50 mM Tris–HCl (pH 7.6) containing 0.01 mM EDTA, 0.1% bovine albumin and 150 mM NaCl, and 10 µl of clytin-II (40 pg) was injected into 50 µl of various Ca^{2+} concentrations ($10^{-8.5}$ to 10^{-4} M) in 50 mM Tris–HCl (pH 7.6). The luminescence intensity was determined with a Centro 960 luminometer.

Measurements of Absorption and Bioluminescence Spectra of Clytin-II—The absorption spectrum of recombinant clytin-II was measured in 50 mM Tris–HCl (pH 7.6)-10 mM EDTA with a Jasco (Tokyo, Japan) V-560 spectrophotometer (band width 1.0 nm; response, medium; scan speed, 200 nm/min) at 22–25°C using a quartz cuvette (10-mm light path). Bioluminescence emission spectra of clytin-I, clytin-II and aequorin with Ca^{2+} were measured on a Jasco FP-6500 fluorescence spectrophotometer (emission band width, 10 nm; response, 0.5 s; sensitivity, medium; scan speed, 1 000 nm/min) at 22–25°C with the excitation light source turned off, as previously described (*28*).

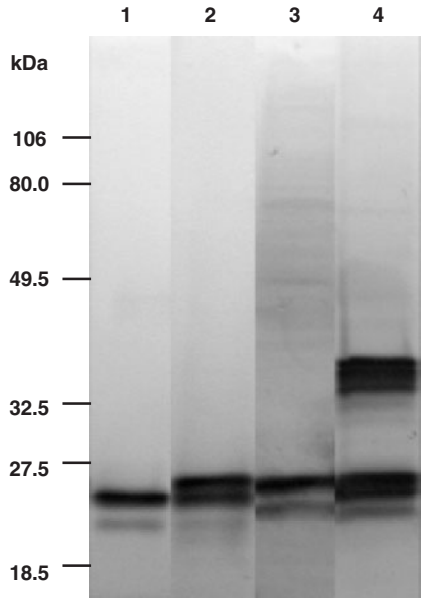

Fig. 1. **Immunoblot analysis of luminous jellyfishes using anti-apoaequorin antibody.** Lane 1, purified recombinant apoaequorin (10 ng protein); lane 2, 1 μl of extracts (500 μl) from half outer margin of a jellyfish *A. aequorea*; lane 3, 10 μl of extracts (500 μl) from outer margin of a jellyfish *M. cellularia*; lane 4, 40 μl of extracts (500 μl) from whole body of a jellyfish *C. gregarium*. The numbers on the left represent apparent values of the pre-stained molecular mass markers (Bio-Rad): phosphorylase b (106 kDa), bovine serum albumin (80.0 kDa), ovalbumin (49.5 kDa), carbonic anhydrase (32.5 kDa), soybean trypsin inhibitor (27.5 kDa) and lysozyme (18.5 kDa).

Mass Spectrometry—Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was measured on an AutoFLEX (Bruker Daltonics) in the positive reflector mode using sinapic acid (Invitrogen) as a matrix, as previously described (*29*).

RESULTS AND DISCUSSION

Immunoblot Analysis of Luminous Jellyfishes by Anti-Apoaequorin Antibody—Using anti-recombinant apoaequorin polyclonal antibody (*25*), the luminous hydromedusae *A. aequorea*, *C. gregarium* and *M. cellularia* were examined by immunoblot analysis. As shown in Fig. 1, the extracts of light organs of *A. aequorea* and *M. cellularia* from single jellyfish showed two bands around 25 kDa (lanes 2 and 3). Three independent specimens from *A. aequorea* and *M. cellularia* also showed similar blot patterns (data not shown). In *C. gregarium*, however, whole extracts of two independent specimens showed identical blot patterns with four bands at 33 to 37 kDa and three bands around 25 kDa (lane 4). To confirm the specific binding of anti-recombinant apoaequorin antibody in *C. gregarium*, *A. aequorea* and *M. cellularia*, the pre-adsorbed anti-apoaequorin by treatment with recombinant apoaequorin was used and did not react with these bands at all (data not shown). These results suggested that an independent specimen of *C. gregarium* has two protein species with low (~25 kDa) and high (33–37 kDa) molecular weights, having immunological reactivity with anti-aequorin antibody. In the earlier report by Levine and Ward (*7*), a high molecular weight protein with Ca^{2+}-triggered luminescence activity was not detected by the gel filtration of the extracts of *Phialidium* (=*Clytia*) using Bio-Gel P-150 (*7*). These results might be expected to indicate an isotype photoprotein or a homologous protein of Ca^{2+}-binding photoproteins with high molecular weight in *C. gregarium*. As previously reported, natural aequorin isolated from jellyfishes of *A. aequorea* shows the heterogeneity (*20*, *30*) and at least six kinds of isotype aequorin are identified (*30*). Thus, the presence of isotype clytins is expected in *C. gregarium* species. The isotype aequorins show the similar luminescence properties, but aequorin with high molecular weight was not found in isotype aequorins (*30*).

Identification of Isotype Clytin (Clytin-II)—From the result of immunoblot analysis in *C. gregarium*, we reinvestigated the cDNA clones. The eight positive clones from the cDNA library of *C. gregarium* were assigned as pCL11, 21, 31, 41, 51R, 61R, 71 and 81. Four clones including pCL11, 31, 41 and 81 showed the calcium-triggered luminescence activity, when the crude extract from the bacterial cells containing the cDNA clone was incubated with coelenterazine in the presence of 2-mercaptoethanol (*9*). The cDNA clone of pCL41 with shortest insert and Ca^{2+}-triggered luminescence activity was characterized, and we conclude that clytin is a member of calcium-binding photoprotein including aequorin, mitrocomin and obelin (*9*, *22*).

The restriction enzyme maps of eight cDNA clones were made (Fig. 2A) and the nucleotide sequences were determined. As the results, cDNA clones were classified into two groups (Fig. 2A). One group, termed 'Clytin-I (CL-I)', is the cDNA clones of pCL11, pCL41, pCL51R and pCL81, and the other group, termed 'Clytin-II (CL-II)', contains the cDNA clones of pCL21, pCL31 and pLC61R. Thus, clytin from pCL41 (*9*) is belonged to clytin-I. The clone pCL71 with 265 bp did not show significant homology with clytin. The construction of cDNA library from *C. gregarium* was performed without PCR procedures (*9*) and the differences of nucleotide sequences between clytin-I and clytin-II might not be artificial events by PCR. The alignments of amino acid sequences deduced form nucleotide sequences were shown in Fig. 2B. In the amino acid sequence of clytin-I and clytin-II from 1 to 189, the different amino acids within clytin-I and clytin-II were only 2 amino acid residues, respectively. On the other hand, the identical amino acids between clytin-I and clytin-II showed high similarity with 88.4%. For other photoproteins including aequorin, mitrocomin and obelin, the sequence identity of clytin-II was summarized in Table 1.

The clone of pCL61 for clytin-II showed the longest open reading frame and the putative initial methionines are present at positions, 29 and 39 upstream from the amino terminus of clytin-II (Fig. 2, methionine underlined). Two open reading frames consist of 219 and 228 amino acid residues with the calculated average mass values of 24853.0 and 26115.5, respectively. On SDS–PAGE under heat-denatured conditions, the purified recombinant aequorin migrates to around 25 kDa, showing a higher molecular size than the calculated average mass value of 21632.0 (*29*). However, in *C. gregarium*, immunoblot analysis showed homologous proteins with 33–37 kDa and

these are not likely to correspond to the longest open reading frames of pCL61 with the calculated average mass values of 24853.0 and 26115.5. Presumably, the 25 kDa bands of *C. gregarium* on the gel were the mixture of the truncated forms of intact clytin-I, clytin-II and 33–37 kDa proteins. Unfortunately, the cDNA clones for 33–37 kDa proteins were not identified in our cDNA library using pCL31 as a probe. The 33–37 kDa proteins might be a homologous protein of Ca^{2+}-binding photoproteins. As the genomic structure of clytin gene was not identified yet, the possibility of an alternative splicing to produce the isotype clytins still remained.

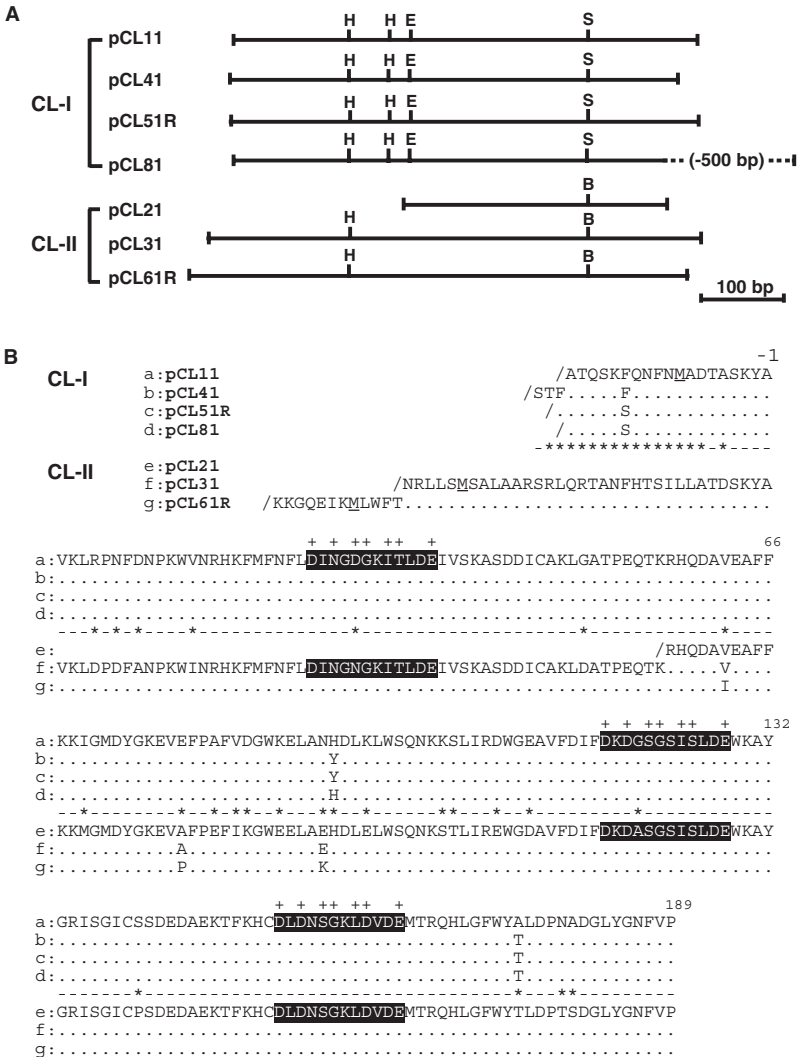

Fig. 2. **Identification of clytin isotype, clytin-II.** (A) Restriction enzyme map of cDNA clones for clytin-I (CL-I) and clytin-II (CL-II). Restriction enzymes are follows: H, *Hin*dIII; E, *Eco*RI; S, *Sal*I; B, *Bam*HI. (B) Amino acid sequences of cDNA clones for clytin-I and clytin-II. Dots (.) show the identical amino acid in clytin-I and clytin-II, respectively. Closed boxes indicate the EF-hand domain and plus symbols (+) are the conserved amino-acid residues for Ca^{2+}-binding. Asterisks (*) indicate the different amino acids between clytin-I and clytin-II. The sequences reported in this paper have been deposited in the GenBank database. The accession numbers are follows: pCL21, AB360784; pCL31, AB360785; pCL61R, AB360786; pCL11, AB360787; pCL41, L13247; pCL51R, AB360788; pCL81, AB360789.

Table 1. **Sequence identity of clytin-II (CL-II) with various photoproteins.**

Photoproteins[a] (clone)	Clytin-II (pCL31)	Clytin-I	Aequorin	Mitrocomin	Obelin
Clytin-I (pCL41)	88.4	–			
Aequorin (pAQ440)	61.9	64.0	–		
Mitrocomin (pMI17)	60.8	64.5	68.8	–	
Obelin (pET19-OG)	76.2	77.8	65.6	64.6	–
Obelin (pOL101)	76.7	77.8	68.8	64.0	86.2

[a]Amino acid sequences of photoprotein are obtained as follows: clyitn-II (pCL31, accession no AB360785) from *C. gregarium*, clytin-I (pCL41, L13247) from *C. gregarium*, aequorin (pAQ440, L29571) from *A. victoria*, mitrocomin (pMI-17, L31623) from *M. cellularia*, obelin (pET19-OG, AF394688) from *O. geniculata*, and obelin (pOL101, U07128) from *O. longissima*.

Expression and Purification of Recombinant Clytin-II from E. coli Cells—To characterize the properties of clytin-II, recombinant clytin-II was purified following the similar procedures for clytin-I (*23*). The apoclytin-II expression vector, piP-H-CL-II, has the OmpA signal peptide sequence, six-histidine tag sequence and apoclytin-II cDNA sequence from 3 to 189 amino acid sequences. The histidine-tagged apoclytin-II expressed into the periplasmic space of *E. coli* cells was purified by nickel chelate affinity chromatography and apoclytin-II was regenerated to clytin-II by incubation with coelenterazine in the presence of DTT. Recombinant clytin-II was further purified by Q Sepharose chromatography and butyl Sepharose chromatography. The yield of recombinant clytin-II was 13 mg from 2 l of cultured cells with purity >95% on SDS–PAGE analysis (Supplementary data 2 and 3). To confirm the correct cleavage of the OmpA signal peptide sequence in clytin-II, mass spectrum analysis was performed by MALDI-TOF-MS. The mass value of *m/z* 23193.5 for $[M+H]^+$ was observed and this value is in good agreement with the calculated average mass value of 23189.8. Thus, the OmpA signal peptide sequence of apoclytin-II was cleaved correctly and clytin-II consists of 202 amino acid residues including the additional amino acid sequence of Ala-Asn-Ser-His-His-His-His-His-His-Gly-Lys-Leu-His-Met-Glu at amino terminus (Supplementary Data 1).

Absorption and Bioluminescence Spectra of Recombinant Clytin-II—The absorption spectrum of clytin-II is shown in Fig. 3. The absorption maxima were found to be 283, 291 and 460 nm and the absorption values of 0.1% solution at 280 nm and 460 nm were 2.97 and 0.093, respectively. These values are close to that of 2.94 and 0.095 in clytin-I (*23*). The absorbance at 460 nm is derived from 2-hydroperoxycoelenterazine. The bioluminescence spectrum of clytin-II triggered by Ca^{2+} showed an emission peak at 470 nm with a full width at half maximum of 76 nm and is identical to that of clytin-I (Fig. 4). On the other hand, aequorin showed an emission peak at 460 nm with a full width at half maximum of 84 nm (Fig. 4). The absorption values of 0.1% aequorin solution at 280 nm and 460 nm were 2.97 and 0.098, respectively (*31*).

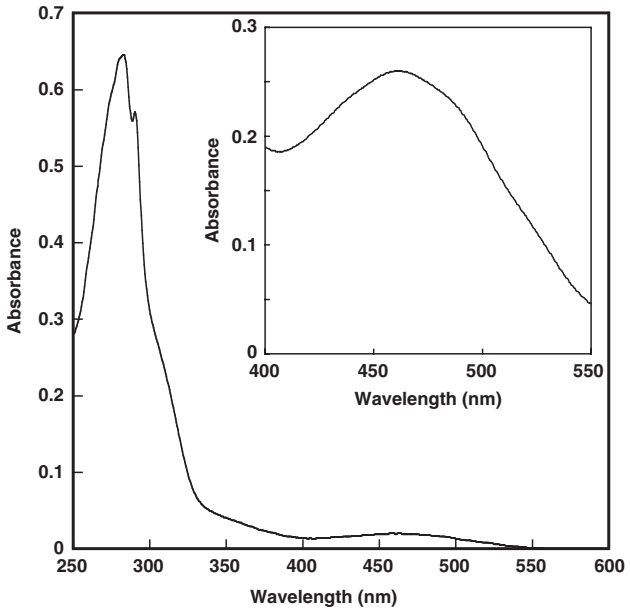

Fig. 3. **Absorption spectrum of recombinant clytin-II.** The protein concentration is 0.21 mg/ml. Inset: the protein concentration is 2.80 mg/ml.

Luminescence Pattern of Clytin-II Triggered by Ca^{2+}—During the determination of luminescence intensity for clytin-II by injection of Ca^{2+}, we found that the half-decay time of I_{max} for clytin-II was faster than that for clytin-I and aequorin. To compare their luminescence intensity, the protein amounts of clytin-I, clytin-II and aequorin were normalized using the absorbance value at 460 nm with of that 0.095, 0.093 and 0.098, respectively. As shown in Fig. 5, the normalized initial intensity of clytin-II was 4.5 times higher than that of clytin-I

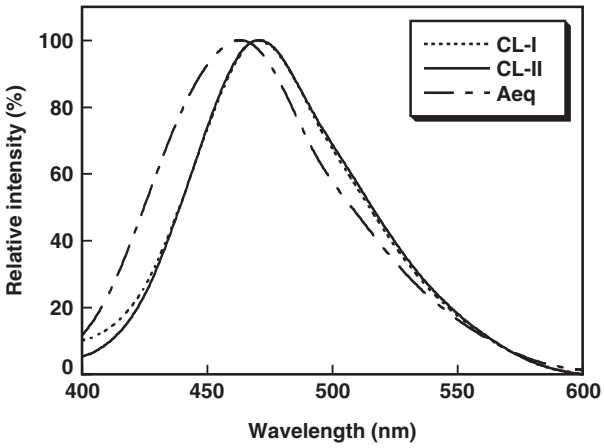

Fig. 4. **Bioluminescence emission spectra of recombinant clytin-II (CL-II), recombinant clytin-I (CL-I) and recombinant aequorin (Aeq).** The amount of photoprotein in 200 µl of 50 mM Tris–HCl (pH 7.6)-1 mM EDTA is as follows: clytin-II, 0.24 mg; clytin-I, 1.3 mg; aequorin, 0.58 mg.

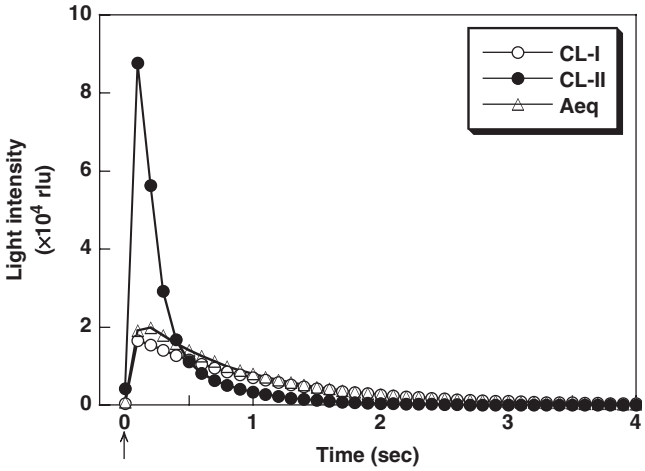

Fig. 5. **Ca^{2+}-triggered luminescence pattern of recombinant clytin-II (CL-II), recombinant clytin-I (CL-I) and recombinant aequorin (Aeq).** Arrow indicates the point of injection of calcium solution. The protein concentrations are determined using the absorbance value at 460 nm and the intensities are normalized by protein concentration.

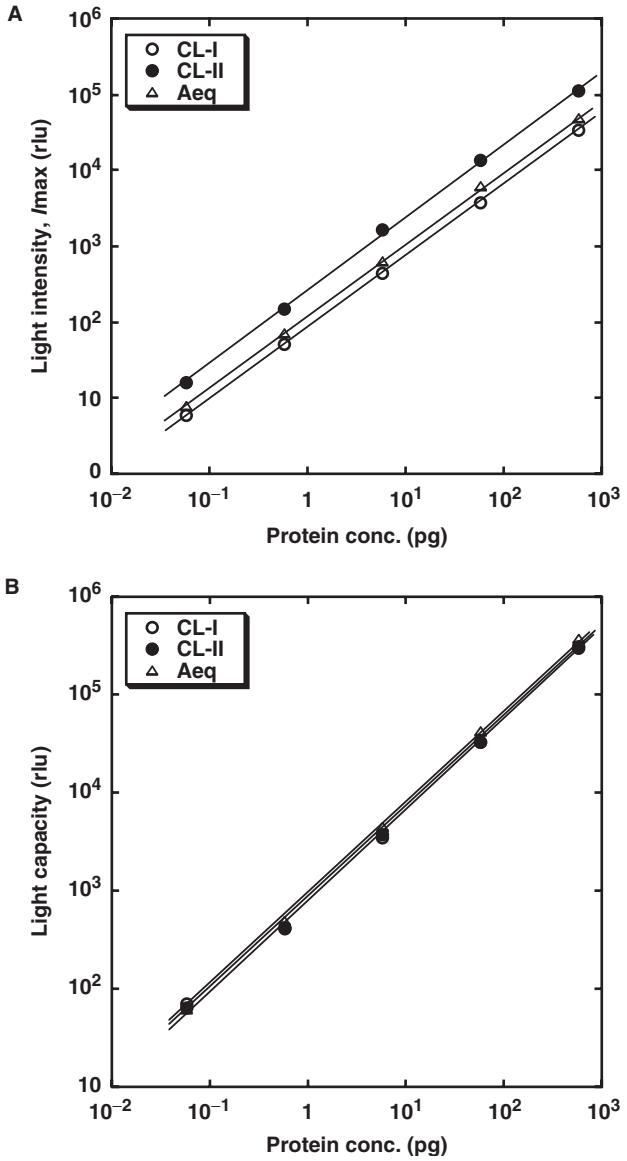

Fig. 6. **Initial light intensity and luminescence capacity of recombinant clytin-II (CL-II), recombinant clytin-I (CL-I) and recombinant aequorin (Aeq).** (A) Linearity of initial intensity in various protein concentrations. (B) Capacity of luminescence in various protein concentrations.

and aequorin. The half-decay times of I_{max} for clytin-I, clytin-II and aequorin were 0.87 s, 0.23 s and 0.90 s, respectively. Further, the linearity of luminescence intensity and the luminescence capacities of clytin-I, clytin-II and aequorin were determined (Fig. 6). It is of interest that the luminescence capacity of clytin-II was very similar to both clytin-I and aequorin. Thus, the signal-to-noise ratio of clytin-II is 4.5 times higher than that of clytin-I, and clytin-II might be a useful protein for detecting Ca^{2+}, even if the reason for relative high luminescence intensity of clytin-II is clear. It is necessary to determine the crystal structures of clytin-I and clytin-II for explaining the high luminescence intensity of clytin-II. On the other hand, the half-decay times

Table 2. **Semi-synthetic clytin-II prepared from apoclytin-II and coelenterazine analogues.**

Coelenterazine analogues	I_{max} (Int.)[a] (%)	Half decay time of I_{max} (s)
CTZ	100 (100)	0.23
h-CTZ	123.3 (70.2)	0.18
hcp-CTZ	ND[b]	ND [b]
cp-CTZ	ND[b]	ND [b]
f-CTZ	45.6 (41.6)	0.25
fcp-CTZ	6.1 (4.7)	0.18
n-CTZ	3.0 (33.6)	2.08
Bis-CTZ	0.0 (0.0)	–
MeO-CTZ	2.4 (1.9)	0.20
e-CTZ	56.7 (32.4)	0.18

[a]The assay procedures are as follows: apoclytin-II (1 μg) eluted from a nickel-chelate column is regenerated to semi-synthetic clytin-II by incubation with coelenterazine analogue (2 μg/2 μl of ethanol) in 1 ml of 30 mM Tris-HCl (pH 7.6)–10 mM EDTA containing 2-mercaptoethanol (1 μl) on ice bath for 6 h. The regenerated sample (1 μl) is assayed by injection with 100 μl of 50 mM $CaCl_2$ in 50 mM Tris–HCl (pH 7.6). The maximum intensity (I_{max}) and the luminescence capacity (Int.) of semi-synthetic aequorin were measured for 10 s in 0.1 s interval.

[b]Luminescence intensity is not determined by a Berthold 960B luminometer, due to faster Ca^{2+}-trigger luminescence reaction of semi-synthetic clytin-II.

of I_{max} using the crude extracts of recombinant obelin from *Obelia longissima* (*15*), was estimated to be 0.49 s (data not shown).

Semi-Synthetic Clytin-II—To characterize further luminescent properties of clytin-II, semi-synthetic clytin-II was prepared according to the similar procedures for semi-synthetic clytin-I (*23*). The maximum intensity, luminescence capacity and half-decay time of I_{max} for semi-synthetic clytin-II were determined (Table 2). The profile of luminescence intensity for semi-synthetic clytin-II was almost same to that of semi-synthetic clytin-I (*23*). However, the luminescence response with Ca^{2+} for *hcp*-clytin-II and *cp*-clytin-II was faster than that of clytin-II and the accurate luminescence intensity and luminescence capacity could not be determined by a luminometer.

Calcium Titration—The Ca^{2+} sensitivity of recombinant clytin-II was determined by injection of clytin-II into the standard Ca^{2+} solution of $10^{-8.5}$ to 10^{-4} M, and clytin-II could detect Ca^{2+} at the range of $10^{-6.5}$ to 10^{-5} M (Fig. 7). The sensitivity to Ca^{2+} was less than that of recombinant aequorin (*32*, *33*), but was similar to native clytin (phialidin) (*7*) and clytin-I (*23*). Thus, recombinant clytin-II is useful for measuring high concentration of Ca^{2+}.

Supplementary data are available at *JB* online.

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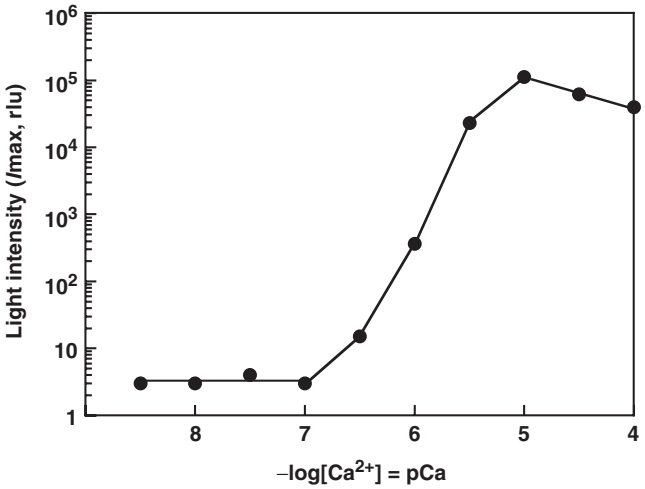

Fig. 7. **Relationship between Ca^{2+} concentration and the initial light intensity of recombinant clytin-II.**

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