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Photoprotein of Luminous Squid, *Symplectoteuthis oualaniensis* and Reconstruction of the Luminous System

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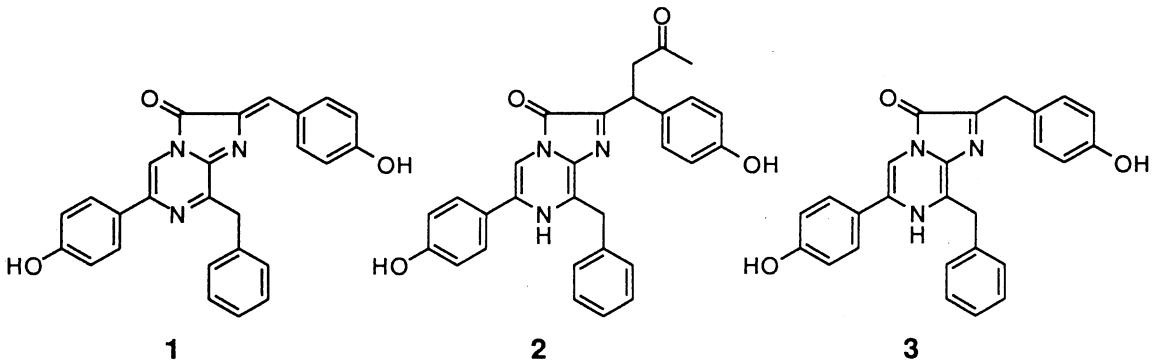
The photoprotein in the luminous squid, *Symplectoteuthis oualaniensis,* was extracted in high salt solutions at low temperatures. It was partially purified to show bioluminescence when warmed to room temperature. To the resulting protein solution after this luminescence, three solutions containing dehydrocoelenterazine, its DTT adduct and GSH adduct were added to observe immediate light, respectively. These recorded the first reconstruction of the luminous system in this squid. Coelenterazine, however, gave only weak light in 2 h after addition.

In our previous report[1] of luminous squid, *Symplectoteuthis oualaniensis* L[2] (Japanese name, Tobi-Ika), we have isolated the chromophore from the photogenic organs and identified dehydrocoelenterazine (**1**)[3] and its acetone adduct (**2**).[1] The chromophore is related to coelenterazine (**3**),[4] which is known in various marine bioluminescent organisms.[5] It was postulated that **1** should be equivalent to luciferin in this luminescent system and that **1** was assumed to be stored in a form of conjugate adduct.[1] We have bent our recent efforts for the extraction of the protein to study molecular-molecular interaction between the chromophore and the photoprotein concerned. Tsuji et al. had reported about the luminescence of *S. oualaniensis* that (i) monovalent cations (sodium or potassium ions etc.) were necessary for luminescence with the homogenate suspension of photogenic organ and (ii) the essential light-emitting components were membrane bound.[6] In this communication, we describe successful extraction of the photoprotein and the first observation of reconstruction of the luminous system,[7] which led us to conclude that dehydrocoelenterazine (**1**) is the luminous principle.



1 **2** **3**

Photogenic organs were removed from the frozen squids, *S. oualaniensis* and homogenized in an ice-bath

with Tris-HCl [50 mL at pH 7.6 containing 0.25 M sucrose, 1 mM dithiothreitol (DTT) and 1 mM EDTA] under argon atmosphere. The homogenate was centrifuged (10^4 g, 5 min), and the precipitate was suspended in the same buffer and centrifuged once again. The precipitate was dissolved in the above buffer that additionally contained 1 M KCl (40 mL), and the mixture was centrifuged (1.5×10^4 g, 20 min). (These were operated in a cold room at 4 °C to avoid luminescence.) This supernatant was separated and then further centrifuged at 10^5 g for 60 min at 4 °C, and the resultant supernatant *(solution A)* was used for the following experiments.

Gel filtration chromatographies of *solution A* with Biogel A-0.5m (Bio-rad, limiting molecular weight, 5×10^5 dalton) and Cellurofine GLC-300m (Seikagaku Kogyo, 10^5 dalton, eluted with above Tris-HCl buffer at pH 7.6 containing 1 M KCl) gave fractions, whose luminescence activity was monitored by diluting 10 times with this buffer at room temperature, respectively. Luminescence was observed only in the void fractions, and no smaller molecular fraction exhibited any light. The results suggested the luminescent substance is a photoprotein.[7]

Solution A was allowed to warm to room temperature to give light (luminescence spectrum max at 480 nm), while luminescence profile was monitored. The fluorescence spectra in 7, 14, 21, 28, 35 42 and 90 min are shown in Fig. 1, where the arrow at 530 nm means decreasing luminous material, but the arrows at 430 nm means first decreasing and then increasing due to a secondary product (possibly coelenteramine, *vide infra*). The differential spectrum between those of 7 min and 14 min is illustrated in Fig. 2 (solid line), which showed the peak at around 500 nm. This may be close to the corresponding fluorescence spectrum in protein-bound form, but it was different from the spectrum of coelenterazine in aq. MeOH (Fig. 2 dashed line) due to different environment. No coelenteramide (a possible oxidation product)[1] or only very little coelenteramine[8] was obtained in 20 min. Coelenteramine (Fluorescence max at 434 nm), however, became extractable at least in 2 h after the luminescence (Fig. 1). These suggested enzymatic hydrolysis of the primary product with a little delay of luminescence to form coelenteramine.

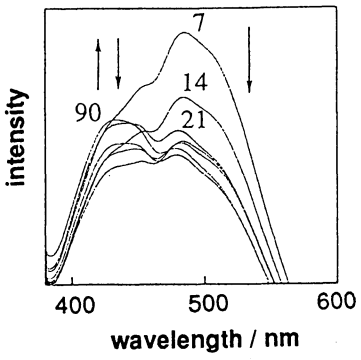


Fig. 1. Change of fluorescence.

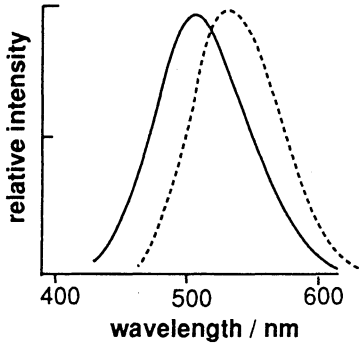


Fig. 2. Differential spectrum (and **3**).

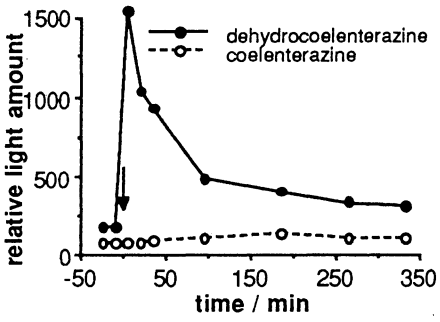


Fig. 3. Luminescence profile.

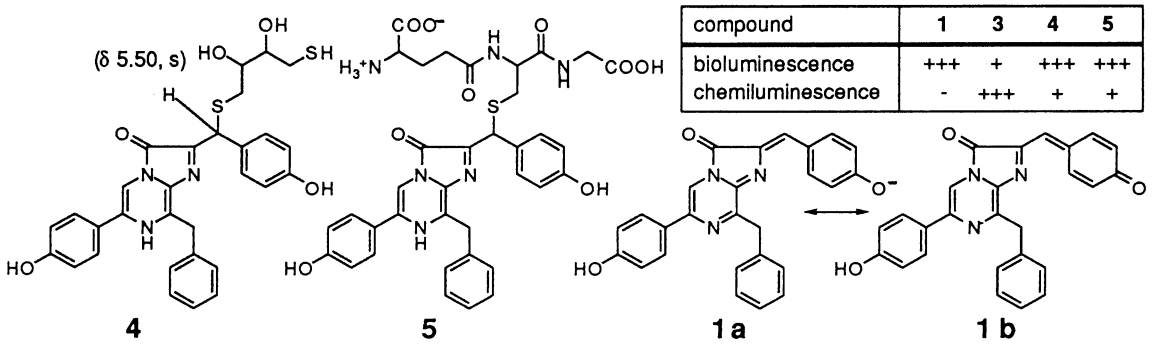
A part of *solution A* (1.1 mL) was allowed to warm to room temperature, while bioluminescence was observed (spectrum centered at 480 nm). When the luminescence became ca. 10% of the original intensity (usually after 1.5 h), this solution *(solution B)* was cooled down to 0 °C and then devided to 200 μL into 5 vials. To each of *solution B* in a vial placed in a chamber of photometer, dehydrocoelenterazine (**1**, 10 μL of 0.20 mg/mL MeOH) was added to record luminescence that was plotted against time (Fig. 3). The maximum light appeared immediately after the addition of **1** and the max intensity was about 50% of the original *solution A*. On the other hand, coelenterazine **3** (added instead of **1**) did not emit light at all in the beginning but showed very weak luminescence for 2-3 h (dotted line in Fig. 3).[9] The total light amount induced with **1** was larger

than 50 times of that with **3**. These experiments suggested that coelenterazine **3** cannot directly be involved in the luminescence system.[10] The DTT and glutathione (GSH) adducts **4** and **5** (*vide infra*) gave immediate luminescence after addition to *solution B* as similar pattern as **1** (solid line in Fig. 3). Incidentally, no light was detected by the addition of acetone adduct **2**.

In the chemiluminescence of acetone adduct **2** in DMSO containing 1.0% NaOH (0.25 M), the luminescence profile (intensity) was identical to that of coelenterazine **3**. Both the intensities of the light with DTT-adduct **4** and GSH-adduct **5**, on the other hand, were relatively weaker and slower than that induced with coelenterazine **3**. The results of luminescence with the 4 compounds are summarized in Table 1.[11]

Table 1

compound	1	3	4	5
bioluminescence	+++	+	+++	+++
chemiluminescence	-	+++	+	+



In our previous work,[1] we have postulated that dehydrocoelenterazine (**1**) might receive conjugate addition, in the bioluminescence process, at the β carbon of α-β unsaturated carbonyl system. We have reported DTT adduct **4** which was deduced through FAB mass spectrometric analysis and ^{1}H NMR (δ 5.50, s) showed that DTT connects with dehydrocoelenterazine at 2α carbon through sulfur atom.[12] Recently we reinvestigated the chemical nature of **1** using UV spectrophotometry and both bio- and chemi-luminescence activities; thus, **1** was mixed with DTT in MeOH or GSH in aq MeOH to give **4** or **5**. Then the various buffered aq MeOH solutions were added to **4** or **5** and the absorbance was recorded. Figure 4 illustrates those spectra of **1**, **3**, **4** and **5** observed at pH 6.3, 6.8, 7.7, 8.2, 8.8 and 9.2, respectively. No chemiluminescence was observed in aq MeOH.

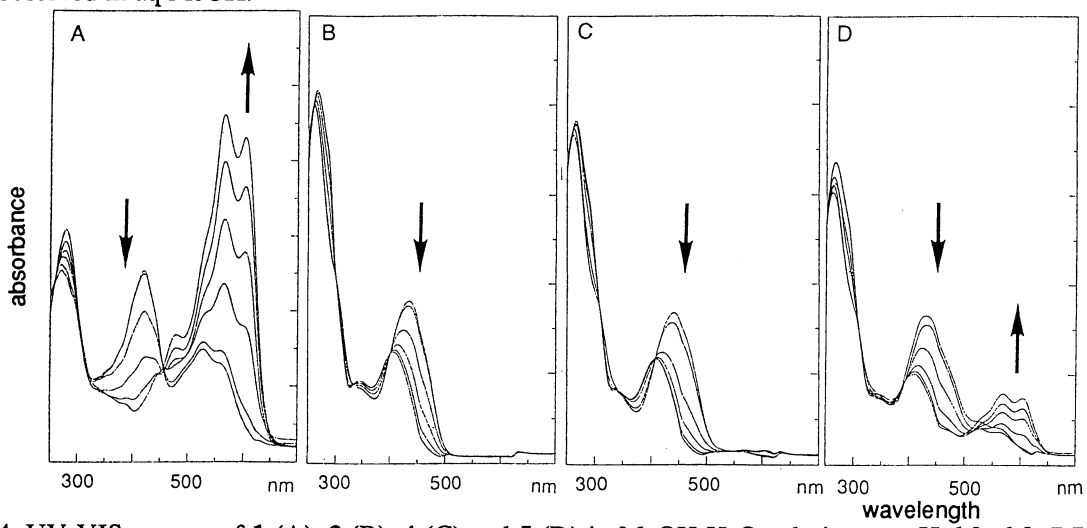


Fig. 4. UV-VIS spectra of **1** (A), **3** (B), **4** (C) and **5** (D) in MeOH-H2O solutions at pH 6.3, 6.8, 7.7, 8.2, 8.8 and 9.2. The arrows indicate the increasing pH, respectively.

Coelenterazine (**3**, Fig. 4-B) and DTT adduct **4** (Fig. 4-C) showed very similar equilibrium; thus, decreasing of the peak intensity at 440 nm and shifting of the maximum to 400 nm with increasing pH (given by arrows). The GSH adduct (**5**) partly exhibited the similar nature around 400-440 nm, while it partly resembled to show blue color (~600 nm) in high pH (Fig. 4-D). Dehydrocoelenterazine (**1**) did show a dramatic change of the red color into blue color (Fig. 4-A), that might correspond to the dissociation of the phenol to have the contribution of **1a** or **1b**. The GSH adduct (**5**) did show immediate bioluminescence (*vide supra*), but it did exhibit very weak chemiluminescence in DMF or DMSO (Table 1). The latter was due to dissociation into blue dehydrocoelenterazine (**1**) at higher pH, which was also proved by recovering the red **1** by addition of acid.

These studies on luminous squid afforded the first observation of pseudo L-L reaction with the photoprotein after it consumed the storing luciferin equivalent.

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10) This result led us to speculate the coelenterazine might be involved due to time lag to reach to the active site, or it may be oxidized to dehydrocoelenterazine in the enzyme system.
11) In this table, "+++" indicates relative luminescent activity (50%-100%) in bioluminescence or chemiluminescence; "+" indicates 1-10% activity and "-" means no or less than 1% activity, respectively.
12) ^{1}H NMR of **4** (CD_3OD, 600 MHz) δ 2.52* (2H, dd, J = 9.2, 6.5), 2.87 (1H, brdd, J = 12.5, 9.0), 3.02 (1H, brd, J = 12.5), 3.49 (1H, m), 3.55* (1H, dt, J = 12.5, 6.3), 4.42* (1H, d, J = 13.5), 4.49 (1H, d, J = 13.5), 5.50 (1H, s), 6.74* (2H, d, J = 8.0), 6.87 (2H, d, J = 8.0), 7.23* (1H, m), 7.29* (2H, m), 7.43 (2H, brd, J = 8.0), 7.45 (2H, d, J = 8.0), 7.50 (3H, m). * These signals were accompanied by similarly splitted peaks at 0.002-0.005 ppm down field due to the corresponding diastereoisomer.

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