Lantern shark *Etmopterus* use coelenterazine as substrate for their luciferin-luciferase bioluminescence system

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Abstract

The lantern shark, *Etmopterus*, are a group of deep-sea bioluminescent species. They emit blue light mainly from ventral body surface, and the major biological function is considered to be a counterillumination. In this study, we detected both coelenterazine and coelenterazine-specific luciferase activity in the ventral photophore tissue. These results suggested that the lantern shark use coelenterazine as a substrate for the luciferin-luciferase reaction of their bioluminescence.

Introduction

The bioluminescence in cartilaginous fish is known in some squaliform species of the families Dalatiidae, Etmopteridae, and Somniosidae (Straube et al., 2015; Duchatelet et al., 2021). They have numerous tiny photophores mainly through the ventral body surface, and its biological function is considered to be counterillumination, a strategy for camouflage from predators to cloak their silhouette by environmental downwelling light (Straube et al., 2010; Claes et al., 2014). Some of the species also possess photophores on dorsal surface, fins and dorsal spines, and these light emission is suggested as a function of aposematism or intraspecific communication (Claes and Mallefet, 2009; Duchatelet et al., 2019).

Coelenterazine is a compound used as luciferin or chromophore of photoprotein in various marine luminous organisms beyond the phyla; Radiozoa, Cercozoa, Porifera, Ctenophora, Cnidaria, Chaetognatha, Mollusca, Arthropoda, and Chordata (Haddock et al., 2010; Martini et al., 2020); some genes of the coelenterazine-dependent luciferase or photoprotein have been isolated from ctenophores, *Aequorea* jelly, *Renilla* sea pansy, *Pholas* bivalves, *Sthenoteuthis* squid, *Oplophorus* shrimps and copepods, and used for application bio-tools (Shimomura, 2006; Krasitskaya et al., 2020). In vertebrates, bony fishes in over 400 species of Stomiiformes and 250 species of Myctophiformes are considered use coelenterazine-dependent bioluminescence systems (Shimomura et al., 1980; Campbell & Herring, 1990; Mallefet & Shimomura, 1995; Thompson & Rees, 1995), but any of their luciferases have not been identified. Some luminous species in Pempheridae, Apogonidae, and Batrachoididae, which inhabit in shallow-water use cypridinid luciferin (Shimomura, 2006), but any of their luciferase

was not identified, except for the case in *Parapriacanthus*, which use cypridinid luciferase itself obtained from ostracod prey (Bessho-Uehara et al., 2020).

In this study, we focused on the bioluminescence system of the deep-sea shark of the genus *Etmopterus* (Squaliformes, Etmopteridae). *Etmopterus* is one of the most diversified group of sharks, in which currently about 40 species are recognized in the world, and they are probably all bioluminescent (Straube et al., 2010; Vásquez et al., 2015). It has been reported that their bioluminescence systems are intrinsic (not symbiotic) (Renwart et al., 2014; Duchatelet et al., 2019), but the reaction mechanism has not been elucidated. Based on our biochemical and chemical analyses, we concluded that etmopterid shark use coelenterazine as luciferin for their luciferin-luciferase bioluminescence system.

Results

Tissue distribution of the luminescence activities in three *Etmopterus* species. Luminescence activities of various tissues to coelenterazine were examined using three *Etmopterus* species; *E. molleri*, *E. pusillus*, *E. brachyurus*. The result showed that luminescence activity per protein content was predominant in ventral skins for three species (Figure 1). Of note, Specimens 1 and 2 (Figure 1A and 1B) are the same species morphologically of *E. molleri*, but they have small nucleotide differences between their *COI* sequences.

Detection of coelenterazine as an active fraction. The crude methanol extract of ventral skin in *E. molleri* was separated by octa decyl silyl (ODS) column, and the luminescence activities of the fractions for crude buffer extract were examined. The luminescence activity was detected as single peak (Figure 2A), and the retention time of the active fractions matches to that of authentic coelenterazine (Figure 2B). The liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry (LC-ESI-MS/MS) analysis of the active fraction showed the molecular and fragment ion patterns (Figure 2C), which exactly agree with those of coelenterazine (Oba et al., 2004) (Figure 2D). These results suggest that crude methanol extract contain a compound having luciferin activity to intrinsic luciferase, and the intrinsic luciferin is coelenterazine.

Luminescence active fraction is coelenterazine specific enzyme. The crude buffer extract of ventral skin in *E. molleri* was separated by gel-filtration column, and the luminescence activities for both crude methanol extract and authentic coelenterazine were examined. The results showed that proteins in the crude buffer extract were separated (Figure 3A), and luminescence activity for crude methanol extract was detected as single peak (Figure 3B), with the retention time of the activity match to that for authentic coelenterazine (Figure 3C). These results suggest that crude buffer extract contain a protein having luciferase activity to intrinsic luciferin, and the luciferase activity is coelenterazine specific.

Luminescence spectrum and pH dependence of the luciferase in *E. molleri*. The pH dependence of the luminescence activity was measured for the active fractions by fast protein liquid chromatography (FPLC) analysis of crude buffer extract with authentic coelenterazine. The results showed the optimum pH around 7.0. The luminescence spectrum at pH 7.0 was measured. The result showed an asymmetric single-peaked spectrum with an extended tail into the longer wavelength region, which is typical for those in the most bioluminescence organisms. The peak was at 460 nm, which corresponds to blue in human vision.

Heat and SDS susceptibility. The crude buffer extract from ventral skin of *E. molleri* was treated by heat or SDS to test the enzyme susceptibility. The results showed that the luminescence activity was dramatically lost by the treatments. These results suggest that the luminescence activity in the buffer extract is proteinous and denatured under unphysiological conditions like usual enzymes.

Discussion

We prepared both crude extract of luciferin and luciferase from three *Etmopterus* species, *E. molleri*, *E. pusillus*, and *E. brachyurus*, and their luminescence activities to coelenterazine were consistently predominant in ventral skin than other tissues (dorsal skin, muscle, or eye) for all three species. These results are consistent to the *in vivo* observation that the dorsal light emission is much higher than ventral light emission in *Etmopterus* species (Duchatelet et al., 2019). Thereafter, we studied on the luminescence mechanism using ventral tissue of *E. molleri*, because this species was collected most abundantly at Suruga Bay.

Determination of the luciferin molecule and luciferase protein is the most essential step to understand the bioluminescence mechanism of luminous organism concerned. In this regard, Nobel laureate Osamu Shimomura wrote in his book that "in studying bioluminescence reactions, it is crucially important to use the purified forms of the components necessary for light emission" not to lead to erroneous interpretations. In the present study, alternatively, we examined reciprocal analyses of the luciferin and luciferase using chromatographically separated fractions of luciferase and luciferin, respectively. By these analyses, we confirmed that the luciferase activity in buffer extract is coelenterazine, and also the luciferin activity in methanol extract is specific to coelenterazine-dependent luciferase. Mass spectrometry confirmed that the active fraction in methanol extract is chemically identical to coelenterazine. The enzymatic activity was denatured both by heat and SDS treatments. Optimum pH of the luminescence activity is near physiological condition, which is in contrast to the heterologous chemiluminescence activity of human albumin with coelenterazine at high alkaline pH (Vassel et al., 2012), suggesting the luminescence activity in shark skin extract is characteristic of a natural enzyme. The molecular size of the luciferase estimated by gel-filtration was 78-92 kDa. By further chromatographic separations, the luciferase protein can be purified.

Luminescence spectrum of the luciferase fraction with coelenterazine was peaked at 460 nm under neutral pH condition. *In vivo* spontaneous luminescence spectra of the etmopterid shark species were reported; 476 nm in *Etmopterus splendidus*, 477 nm in *E. molleri*, and 486 nm in *E. spinax* (Claes et al., 2014). The reason of the difference between our *in vitro* and former *in vivo* results are uncertain, but might be genetically differences of the specimens or some effect on colored pigment or reflector in photophores.

These our conclusion is in contrast to the previous report by Renwart and Mallefet, which suggested no free-form of coelenterazine and no coelenterazine-dependent luciferase activities in the photophore tissue of *Etmopterus spinax* (Renwart and Mallefet, 2013). But our present findings that lantern shark use coelenterazine for bioluminescence might be reasonable when considering the lantern shark dwelling in marine twilight zone and being at the higher on the food chain. It has been suggested that coelenterazine is biosynthesized in luminous copepod and comb jelly (Oba et al., 2009; Bessho-Uehara et al., 2020), and other luminous organisms generally obtained it from the diet, mainly copepods or other coelenterazine bearers (Oba & Schultz, 2014). During the dissection of the specimens, we found various animals in the stomach contents of *E. molleri*, such as myctophid lanternfishes and unknown crustaceans. In *E. spinax* stomach, various coelenterazine-dependent animals, such as *Oplophorus* and *Pasiphaea* shrimps, *Maurolicus* fish (Stomiiformes) and unidentified myctophid fishes, were detected (Neiva et al., 2006; Renwart & Mallefet, 2013). It has also been known that some non-luminous fishes accumulated coelenterazine (Shimomura, 1987). We think that *Etmopterus* lantern sharks obtained coelenterazine from dietary fishes and crustaceans (they probably also obtained coelenterazine from their diet such as copepods) for bioluminescence. In *E. spinax*, *in vivo* luminescence from ventral photophores was also detected in the late embryonic stage (Duchatelet et al., 2019), suggesting that coelenterazine will be vertically transferred from mother's body.

Bioluminescence origin of lantern shark is considered to be dated at Cretaceous Period (Straube et al., 2015), which is coincidental with the origin of other coelenterazine-dependent fishes, Stomiiformes and Myctophiformes (Davis et al., 2016). Determination of luciferase gene and coelenterazine-transport system of lantern shark, as well as stomiiform and myctophiform fishes, might be helpful to understand the bioluminescence diversification occurred simultaneously in multiple fish lineages at Cretaceous deep-sea.

Materials

Shark samples. *Etmopterus* specimens were bycaught with a trawl at Suruga Bay, Japan in 2019 and 2020, and frozen immediately at -20 ˚C by Tropical & Marine Co. (Japan). Species name was identified by DNA analysis and morphological characteristics. For DNA analysis, the partial region of cytochrome *c* oxidase subunit I gene (*COI*) was amplified and sequenced. In brief, total DNA was extracted from muscle of the body using Lysis Buffer for PCR (Takara, Japan) and Proteinase K (Takara). Polymerase chain reaction (PCR) was performed using the

primer set Fish-F1 and Fish-R2 (Ward et al., 2005) using Tks Gflex DNA polymerase (Takara, Japan). The PCR product was directly sequenced at Macrogen Inc. (Macrogen Japan), and the sequence was deposited in the GenBank/ENA/DDBJ database.

Tissue distribution analysis. Each tissue (ventral skin, dorsal skin, dorsal body muscle, and eye ball) was dissected from defrosted single specimen. About 0.5 g of each tissue sample was homogenized in 500 μL of cold extraction buffer (20 mM Tris-HCl, 10 mM EDTA, pH7.4) by plastic pestle, and centrifuged at 7,197 × *g* for 30 min. A 90 μL of the extraction buffer containing 1.18 μM of synthetic coelenterazine (JNC Corporation, Japan) was injected into 30 μL aliquot of supernatant, and the luminescence activity was measured for 2 min using a 96-well luminometer Centro LB960 (Berthold, Germany). The accumulated value (RLU, relative light unit) was normalized by the protein concentration of the extract, which was measured using Protein Assay Kit (Bio-Rad, Hercules, CA).

Extraction and HPLC separation of luciferin. Dissected ventral skin was homogenized in cold methanol (ml/wet weight g) containing 1:100 volume of 1M dithiothreitol using a homogenizer Ultra-Turrax T25 (Janke & Kunkel, Germany), and centrifuged at 7,197 × *g* for 30 min. The supernatant was collected as crude methanol extract. The crude methanol extract was desalted and concentrated by MonoSpin C18 revered-phase column (GL Science, Japan) and separated by high performance liquid chromatography (HPLC) using SEC System Prominence 501 (Shimadzu, Japan) with a column Cadenza CD-C18 (2.0×75 mm, Imtakt, Kyoto, Japan). The fluorescence was detected at excitation= 435 nm and emission= 530 nm using fluorescence detector RF-10AXL (Shimadzu). The mobile phase was aqueous/methanol solution containing 0.1% formic acid, and the linear gradient of methanol was from 25% to 95% (2% per min). The flow rate was 0.2 mL/min. Fractions were collected every 1 min.

Extraction and FPLC separation of luciferase. Dissected ventral skin was homogenized in cold buffer (20 mM Tris-HCl, 10 mM EDTA, pH7.4) (1 ml/ wet weight g) using a homogenizer Ultra-Turrax T25, and centrifuged at 7,197 × *g* for 30 min. The supernatant was collected as crude buffer extract. The crude buffer extract was filtrated using a membrane filter Millex-SV 5.0 μm (Merck, Germany) and separated by FPLC using AKTA Prime Plus (Cytiva, Sweden). The column for gel filtration was HiLoad 16/600 Superdex 200 prep grade (Cytiva) at flow rate of 1.0 mL/min; mobile phase, 20 mM Bis-Tris-HCl, 150 mM NaCl, pH 7.4. Fractions were collected every 3 min.

Bioluminescence assay of HPLC fractions. A 30 μL of crude buffer extract with 265 μL of 20 mM Tris-HCl (pH7.2) was mixed with 5 μL aliquot of each HPLC fraction, and the luminescence activity was measured for 5 min using a 96-well luminometer Centro LB960.

Bioluminescence assay of FPLC fractions. A 100 μL of 1/300 diluted crude methanol extract or 1.18 μM coelenterazine μL in 20 mM Tris-HCl (pH7.2) was injected into 50 μL aliquot of each FPLC fraction, and the luminescence activity was measured for 5 min using a 96-well luminometer Centro LB960.

Spectral measurement. *In vitro* bioluminescence spectrum was measured using active fraction of FPLC purification. The active fraction was concentrated using 50 kDa cutoff filter Amicon Ultra-4 (Merck, Germany), and of 20 μL was mixed with 2 μL of 5.9 mM coelenterazine and 278 μL of 20 mM Bis-Tris-HCl (pH 7.0). The luminescence spectrum was measured using a fluorescence spectrophotometer FP-777W (Jasco, Tokyo, Japan) with excitation light source turned off. The obtained raw spectrum was smoothed using a binomial method.

Mass spectrometry. LC-ESI-MS/MS analyses was performed under positive mode with nitrogen as the collision gas (collision energy, 30 V) using API 4000 (AB SCIEX, Framingham, MA) connected to an LC800 HPLC system (GL Sciences, Japan) with a column Cadenza CD-C18 (Imtakt). The mobile phase was aqueous/methanol solution containing 0.1% formic acid, and the linear gradient of methanol was from 25% to 95% (2% per min). The flow rate was 0.2 mL/min. For the product ion scan analysis, an aliquot (5 μL) of HPLC fraction was applied and m/z= 424.0, corresponding to the calculated $[M+H]^+$ mass value of coelenterazine, was monitored.

Heat and SDS treatment. The crude buffer extract was heat treated using heat block MG-1200 (Eyela, Japan) at 98 ˚C for 15 min, or mixed with sodium dodecyl sulfate (SDS) (final concentration, 2%) at room temperature (18 ˚C) for 5 min. A 90 μL of 1.18 μM authentic coelenterazine was injected into 10 μL aliquot of heat or SDS treated extract, and the luminescence activity was measured for 1 min using a 96-well luminometer Centro LB960.

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Author contributions

G.M., D.Y., and Y.O. conceived the idea of the study. Y.O. planned the study methodology. G.M., D.Y., J.P. and H.E. performed the experiments and species identifications. G.M. and D.Y. performed data analysis. G.M., D.Y., J.P., H.E. and Y.O. wrote the manuscript.

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Competing interests

The authors declare no competing interests.

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Figure legends

Figure 1. Tissue distributions of the luminescence activity with coelenterazine in *Etmopterus* spp. (A) *Etmopterus molleri* specimen 1, (B) *E. molleri* specimen 2 (C) *Etmopterus pusillus*, (D) *Etmopterus brachyurus*. V, ventral skin; D, dorsal skin; M, muscle; E, eye. The values are mean of triplicate measurements and shown as percentage to those in ventral skin.

Figure 2. Luminescence activity and LC-ESI-MS/MS analyses of the crude methanol extract of ventral skin in *E. molleri* (A, C) and authentic coelenterazine and (B, D). (A, B) Lines represent fluorescence emission; bars represent the luminescence activity of the fraction. Asterisks represent the fraction used for the LC-ESI-MS/MS. Panels C and D show the results of the product scan analysis for the asterisked fraction of A and B, respectively.

Figure 3. FPLC analyses of the crude buffer extract of ventral skin in *E molleri*. (A) UV detection at 280 nm. (B) Luminescence activity of the fraction with crude methanol extract. (C) Luminescence activity of the fraction with authentic coelenterazine.

Figure 4. Enzymatic characteristics of the FPLC purified luciferase with coelenterazine. (A) pH-dependent activity profiles. For pH adjustment, Bis-Tris-HCl, Tris-HCl, and Glycine-NaOH buffers were used for pH 6-7, pH 7-8.5, and pH8.5-9.5, respectively. (B) Luminescence spectrum under pH 7.0. (C) Luminescence activity after the treatment at 18 ˚C for 15 min (Control) or 98 ˚C for 15 min (Heat). (D) Luminescence activity after the treatment without (Control) and with 2% SDS (SDS) at 18 ˚C for 5min.

Figure 1

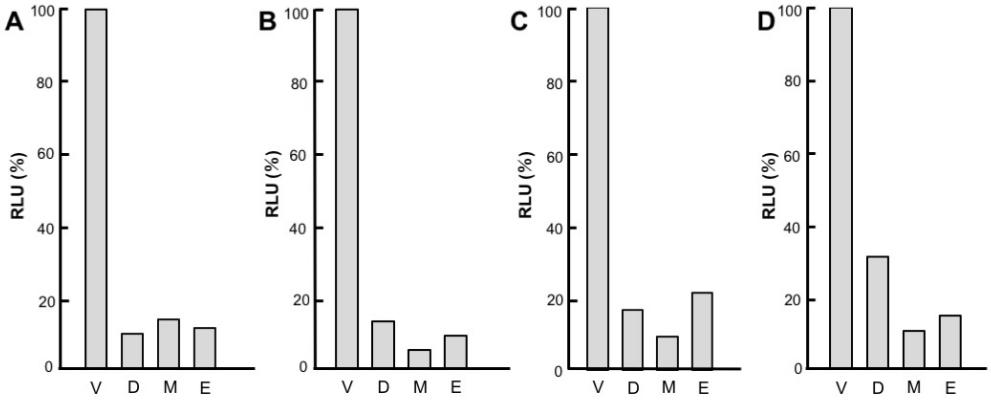

Figure 2

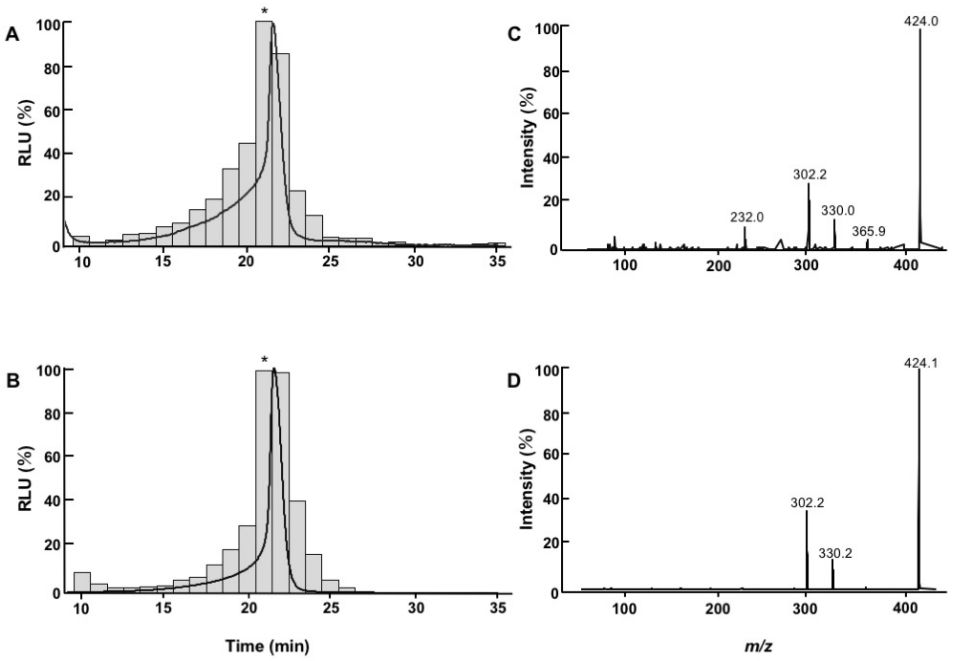

Figure 3

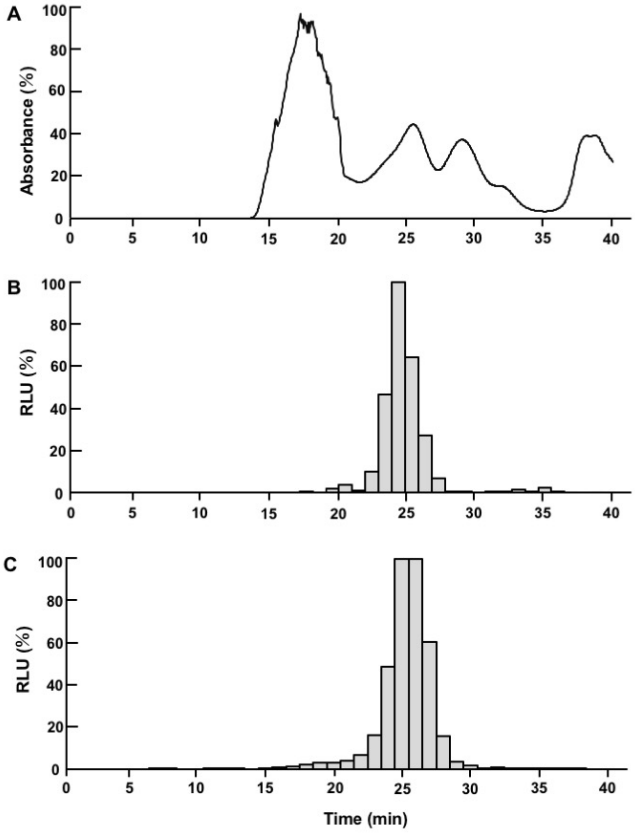

Figure4