

Accounts

Bioluminescence in the Limpet-Like Snail, *Latia neritoides*

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Latia neritoides is a small limpet-like snail that produces a bright green bioluminescence; it is found only in New Zealand streams. The light-emitting system is unique. Although *Latia* bioluminescence has been studied since 1880, its mechanism is unclear. Shimomura and Johnson clarified the elements of the mechanism, including the structures of luciferin and luciferase, in 1968. However, neither the emitter nor the mechanism of the excited state of luciferin has been determined. We studied molecular mechanisms to clarify the characteristics of luciferin and luciferase and to produce a new application for this system.

Bioluminescence, the production of light without heat by living organisms, has elicited the interest of scientists for a long time. The biological functions of bioluminescence are thought to involve defence against predators, assistance in predation, communication in reproduction, and metabolic and biochemical pathway by-product formation in organisms. Bioluminescence reveals a diversity of organisms from bacteria to fish, because the luciferins (substrates) of the various phylogenetically distant systems on unrelated chemical classes and the luciferases (enzyme) based on different gene family.[1] So, for example, a biologist researches the behavior, ecology, and taxonomy of bioluminescent organisms to reveal their diversity. A chemist determines the luciferin structure and analyses the chemical reaction to produce new chemiluminescent materials. A biochemist explores a new luciferase from organisms to understand the metabolic pathway and its mechanism, and a bioengineer develops a biotechnological tool by using information from earlier bioluminescence research. Research on many bioluminescent organisms has gradually revealed various aspects of this mysterious feature.[2–4] However, there is one bioluminescent system that has not been sufficiently clarified to date. Here, we account for the molecular mechanism of bioluminescence of the limpet-like snail, *Latia*.

1. Chemical Mechanism of Bioluminescence

Bioluminescence is cold light that is characterized by a wide range of colors:[6] it is blue in jellyfishes, dinoflagellates, and ostracods; blue-green in bacteria and the limpet-like snail, *Latia*; and green-red in fireflies and railroad worms (Table 1). The light emitted by bioluminescent substrates is identical to that emitted by chemiluminescent substrates when the energy level of the light emitter molecules falls from the excited state to the ground state.[5] The variety of colors in bioluminescence is attributed to differences in the structures of luciferase and

Table 1. Chemistry and Color of Bioluminescence in Different Organisms

Luminous organisms	Luciferin, Cofactor	Luciferase/kDa	Emission max./nm
Bacteria (*Photobacterium*, *Vibrio*)	$FMNH_2$, RCHO	80	495–500
Dinoflagellates (*Lingulodinium*, *Pyrocystis*)	Tetrapyrrole, H^+	130	475–483
Cnidarians (*Aequorea*, *Renilla*)	Coelenterazine, Ca^{2+}	25	460–490
Molluscs (*Latia*)	Enol formate	180	536
Crustacea (*Vargula*; *Cypridina*)	Imidazopyrazinone	70	465
Insects (*Photinus*; *Photuris*)	Benzothiazoline, Mg^{2+}, ATP	60	550–620

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$$\text{Luciferin} + O_2 \xrightarrow{\text{luciferase}} (P)^* \longrightarrow P + h\nu \qquad (1)$$

Scheme 1.

luciferin. Bioluminescent systems are not evolutionarily conserved; gene codings for luciferase proteins are not homologous and those for luciferins also differ.[3] It is important to note that all luciferases are oxygenases. They incorporate oxygen into a product molecule (P) in an electronically excited state $(P)^*$ via a dioxetane intermediate; the process is sufficiently energetic to result in the emission of a photon (Scheme 1). The luminescence maximum corresponds to the fluorescence emission maximum of oxyluciferin in the excited state. In general, bioluminescence reactions occur when luciferin is oxidized by molecular oxygen and the reaction is catalyzed by luciferase. Table 1 shows the structures of luciferin and its oxidized product, oxyluciferin, for bacteria, dinoflagellates, *Renilla*, ostracods, and the firefly. *Renilla* luciferin (coelenterazine) is structurally quite similar to ostracod *Vargula* (Cypridina) luciferin. It contains the imidazopyrazinone skeleton that is supposedly biosynthesized from three amino acids. In the light-emitting reaction, the luminescence maxima of firefly, *Renilla*, and ostracod bioluminescences are 540–630, 450–470, and 450–460 nm, respectively, which correspond to the fluorescence emission maxima of their oxyluciferins.[3] On the other hand, the molecular weights of luciferases are widely distributed (25–180 kDa) and their structures, which originate from the various phylogenetically distant systems, belong to different super families, viz. the calcium-binding protein, aequorin, in jellyfish and the adenylation enzyme in fireflies and railroad worms. Bioluminescence reactions are triggered by various cofactors, viz. the calcium ion in *Aequorea* jellyfish, the proton (H^+) in dinoflagellates, and ATP in fireflies and railroad worms.

The biological function of bioluminescence in fireflies is thought to be communication for mating, and their color differences are adapted to their visual systems.[6] Luciferin is converted to luciferyl adenylate, which is then oxidized by molecular oxygen and catalyzed by luciferase to yield light, oxyluciferin, CO_2, and AMP (Fig. 1). Various explanations for the chemical origin of color differences have been given. The first is based on differences in the ionic structure of the excited oxyluciferin; the first excited monoanion state deprotonates to form a dianion that emits yellow light (Fig. 1a).[5] A second explanation involves the polarity of the oxyluciferin binding site,[7] and a third contends that the conformation of oxyluciferin is so rigid that the first-excited singlet state induces a structural restriction on luciferase that changes the energy of electronic state transitions (Fig. 1b).[8] Luciferases have been cloned and analyzed from many fireflies and beetles. Luciferases consist of 543–550 amino acid residues and belong, in a molecular evolutionary sense, to the adenylate enzyme family.[3,9] The three-dimensional structure of *Photinus pyralis* luciferase has been determined, and an activation site and reaction mechanism have been proposed.[10,11] As a result of the acquisition of knowledge about the chemical reaction and the structure of luciferin and luciferase, the firefly bioluminescence system is now used widely as a reporter of gene expression and as a cellular marker.[12] Recently, we developed a tricolor reporter in vitro assay

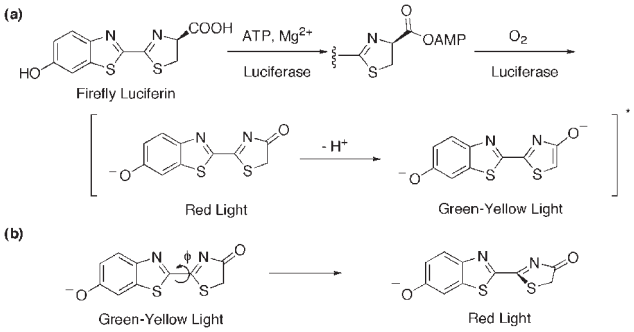


Fig. 1. Chemical mechanism of the bioluminescence reaction of firefly luciferin catalyzed by firefly luciferase. (a) Luciferin is converted into an adenylate in the presence of ATP, which is oxygenated in the presence of oxygen forming a peroxide intermediate by splitting off AMP. When the energy levels of the excited states fall to the ground states, a photon of light ($\lambda_{max} = 550$–560 nm) and red light ($\lambda_{max} = 615$ nm) is emitted. (b) According to an alternative mechanism proposed by McCapra, the conformation of oxyluciferin is so rigid that the first-excited singlet state suffers a structural restriction of luciferases that changes the energy of electronic state transitions.

system in which the expression of three genes can monitored simultaneously. This is accomplished by splitting the emissions from green, orange, and red beetle luciferases with a long-pass filter.[13] Thus, bioluminescence is a unique system that has great potential for new applications.

Most bioluminescence is derived from the final product of the luciferin–luciferase reaction. The luminescence reaction in bacteria is slightly different from other reactions, as it requires luciferase, molecular oxygen, reduced flavin ($FMNH_2$), and a long-chain aldehyde. Initially, the luciferase forms a complex with $FMNH_2$, which is subsequently oxidized to its peroxide. The complex of luciferase and peroxy $FMNH_2$ reacts with the long-chain aldehyde, and it is then broken down to 4a-hydroxy flavin with the emission of light and the production of acid. Finally, the hydroxy flavin is converted to FMN with the loss of H_2O. It is thought that the light emitter in bacterial luminescence is the luciferase-bound 4a-hydroxy flavin (Fig. 2a).[4]

One exception to these general reactions is found in dinoflagellates; their oxyluciferin has no potential for fluorescence;[14] the luminescence maximum ($\lambda_{max} = 475$ nm) corresponds to the fluorescence emission maximum of luciferin (Fig. 2b). On the other hand, fluorescence is not found in *Latia* luciferin or oxyluciferin. Shimomura and Johnson called for clarification of the bioluminescence system of *Latia* but, after 30 years, the basis of this emission remains unclear.[15–18] We have attempted to understand the bioluminescence mechanism by analysing the relationships between the function and the structure of luciferin. We also purified luciferase to identify the emission species and to develop new applications for future use.

2. Biology of *Latia*

The limpet-like snail *L. neritoides* lives in swift-running streams throughout the North Island of New Zealand and is the only bioluminescent creature known to live in fresh water.[18–21] Suter was the first to describe specimens and was sur-

prised to find "all the animals highly phosphorescent with a violet light" and commented that "this was intensified by a touch with a needle. The secreted mucus was also phosphorescent for some time".[22] Later, Bowden mixed hot water (70 °C) with an extract of crushed *Latia* and reported the luciferin–luciferase reaction on mixing a hot water (70 °C) extract of crushed *Latia* (after cooling) with cold water containing once-luminescent mucus.[23] We collected specimens of *Latia* during 1997–1999 from a running stream at Mt. Pirongia, New Zealand (Figs. 3a, b). These *Latia* have rather small oval seed-like shells and a body mass of 0.03–0.05 g. They are black and are not easily noticed because they cling to the underside of stones in the riverbed (Fig. 3c). A sample of about 100 g was obtained by three people over several days. We confirmed that *Latia* ejects a bright green luminescent mucus (Fig. 3d) that is similar to that of sea-fireflies after chemical, mechanical, or electrical stimulation, but we did not observe the phosphorescence that Suter reported.

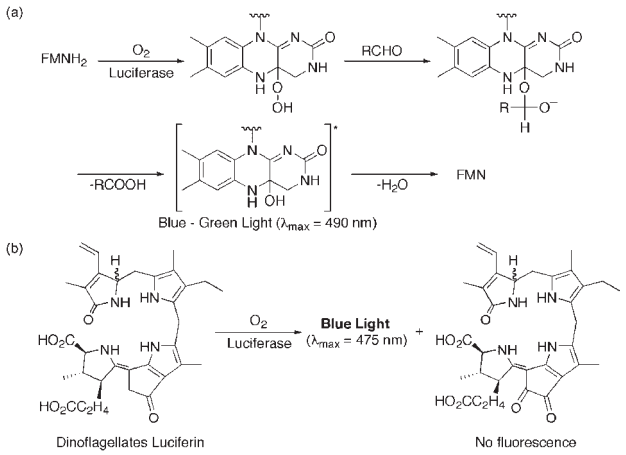
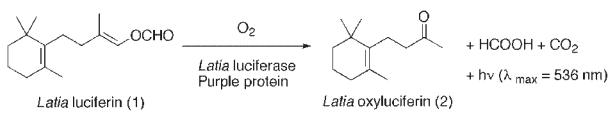


Fig. 2. Chemical mechanism of the bioluminescence reaction in bacteria and dinoflagellates. (a) A complex of bacterial luciferase and $FMNH_2$ is oxidized to peroxide; the complex of luciferase and peroxy $FMNH_2$ reacts with long-chain aldehyde, and is then broken down to 4a-hydroxy flavin with light emission. (b) The conversion of dinoflagellate luciferin to oxyluciferin is triggered by a proton and catalyzed by luciferase, and then produces light ($\lambda_{max} = 475$ nm).

3. Chemistry of *Latia* Luciferin

After Bowden,[23] Shimomura and Johnson isolated *Latia* luciferin, determined its structure, and proposed a chemical reaction (Scheme 2).[18] *Latia* luciferin is a colorless, non-fluorescent liquid and is a highly hydrophobic, non-polar solvent-soluble compound. *Latia* luciferin **1** emits a yellowish green light ($\lambda_{max} = 536$ nm) in the presence of *Latia* luciferase, the purple protein, and molecular oxygen, decomposing itself into compound **2** as an oxyluciferin, formic acid, and carbon dioxide. Interestingly, the luciferin and oxyluciferin in *Latia* are not fluorescent and have no absorption in the visible range of the spectrum, which indicates that both the mechanism and the emitter of *Latia* bioluminescence are different from those of other bioluminescent organisms. This luciferin was subsequently synthesized by Fracheboud et al. in 1969[24] and by Kishi et al. in 1970.[25]

To understand the molecular function of *Latia* luciferin, we prepared it and its analogues by a slight modification of the



Scheme 2.

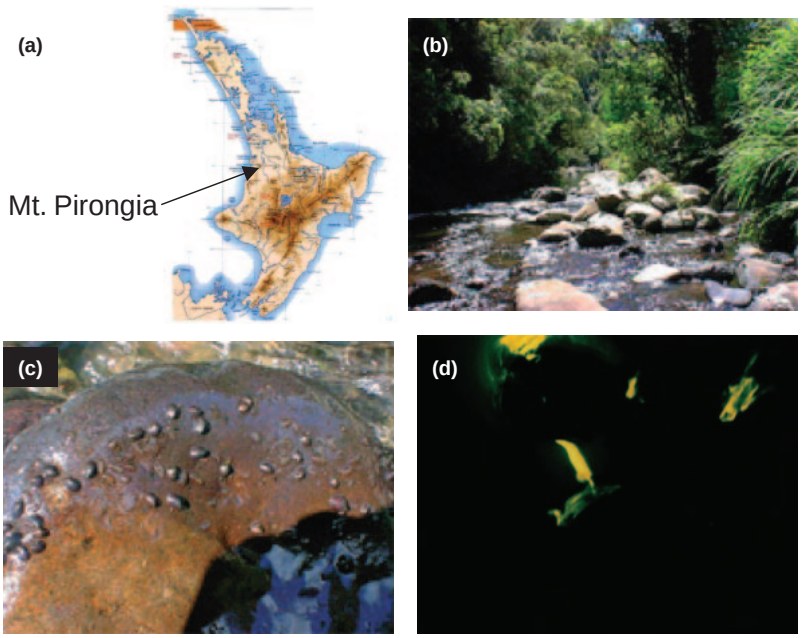


Fig. 3. (a) Location of the collecting point for *Latia neritoides* in the North Island of New Zealand. (b) View of the collecting point for *Latia neritoides* near Mt. Pirongia. (c) Living condition of *Latia neritoides* on the river. (d) Bioluminescence of *Latia neritoides*.

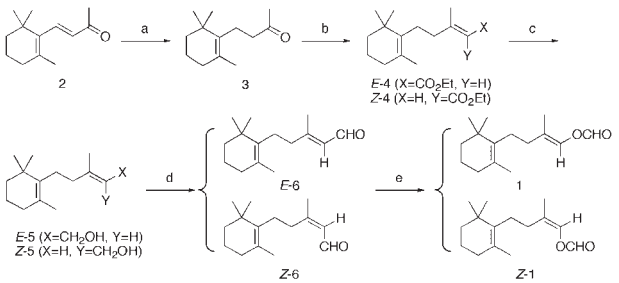
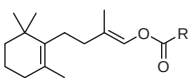


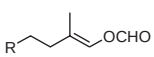
Fig. 4. Preparation of *Latia* luciferin (**1**) and its isomer (*Z*-**1**). Reagents and conditions: (a) Bu_3SnH, toluene, 95%; (b) $(EtO)_2P(O)CH_2CO_2Et$, LiHMDS, THF, reflux, 5 h, 76% for *E*-**4**, 9% for *Z*-**4** after separation; (c) DIBAH, toluene, 0 °C, 100% for *E*-**5**, 100% for *Z*-**5**; (d) MnO_2, CH_2Cl_2, rt, 2 h, 95% for *E*-**6**, 95% for *Z*-**6**; (e) SeO_2, 60% H_2O_2, *t*-amylalcohol, 0 °C, 45% for **1**, 25% for *Z*-**1**.

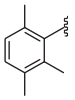
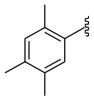
procedure reported by Kishi (Fig. 4).[26] Selective hydrogenation of β-ionone (**3**) was followed by Horner–Emmons olefination of the resulting ketone (**2**) (*Latia* oxyluciferin). The procedure afforded α,β-unsaturated esters *E*-**4** and *Z*-**4** after separation. Reduction of *E*-**4** with DIBAH, followed by the oxidation of the resulting allylic alcohol with MnO_2, gave the desired α,β-unsaturated aldehyde *E*-**6**. Baeyer–Villiger oxidation of *E*-**6** was achieved by using 60% hydrogen peroxide in *t*-pentyl alcohol in the presence of selenium(IV) oxide to give *Latia* luciferin (**1**). The corresponding geometrical isomer *Z*-**1** was prepared via *Z*-**4** by a similar procedure. Using a crude *Latia* luciferase solution, the *Z*-**1** regioisomer of *Latia* luciferin (**1**) had 60% of the activity of **1**, indicating that the recognition site of the enol ester moiety in luciferase has a certain degree of flexibility. On the other hand, α,β-unsaturated aldehyde *E*-**6**, which is supposed to be a biosynthetic precursor of **1**, and *Z*-**6** had no bioluminescence activity. The bioluminescence properties of *Latia* luciferin analogues clarify the relationship between function and the structure of luciferin and suggest the molecular mechanism. *Latia* luciferin benzoate analogues[27] and luciferin analogues having a methyl-substituted phenyl group instead of the natural 2,6,6-trimethylhexene ring[28] were prepared. Enol acetate *E*-**7** and its regioisomer *Z*-**7** showed 66% and 44% of the activity of **1**, respectively, indicating that the formyl (–CHO) group is not functionally essential. For the series of benzoate analogues, the luminescence peak intensities of *E*-**8** to *E*-**12** were 4.2–0.79%, but their total light activities for 2 h were 85–16% compared with **1**, suggesting that these analogues have a potential bioluminescence reaction (Table 2). The reason for the delay in the bioluminescence reaction is unclear, although it may be caused by factors such as their bulky structure, electronic state, and cleavage rate of the enol ester moiety, and the charge of the modified moiety (Fig. 5). Thus, *Latia* luciferase has an esterase activity and generates the enolate anion **13** (Fig. 12) from enol esters in the environment of the enzyme. On the other hand, the analogues *E*-**14**, *E*-**15**, and *E*-**16**, having methyl-substituted phenyl groups instead of the natural 2,6,6-trimethylcyclohexene ring system, showed luminescent activities, although they were less potent than that of the authentic *Latia* luciferin. The bioluminescence spectra of *E*-**14**, *E*-**15**, and *E*-**16** were identical to those of the authentic *Latia* luciferin, indicating that the 2,6,6-trimethylcyclohexene moiety of luciferin does not affect the bioluminescence spectra (Fig. 6). These results indicate that the number and the position of the methyl group(s) on the phenyl ring are important for substrate recognition in the *Latia* luciferase. In addition, the 2,6-dimethyl group on the phenyl ring may be in a good position, as with natural luciferin. Interestingly, no delayed emission in bioluminescence was observed for the luciferin analogues **14**–**16**, although they exhibited a lower emission activity. From these results, it is deduced that luminescence can be controlled by modification of the enol ester and 2,6,6-trimethylcyclohexene moieties of lu-

Table 2. Bioluminescence Activities of *Latia* Luciferin and its Analogues



						
1	*E*-**7**	*E*-**8**	*E*-**9**	*E*-**10**	*E*-**11**	*E*-**12**
Total Light/%						
100	66	85	71	69	28	16
Peak Intensity/%						
100	N.D.	4.2	3.2	2.9	1.1	0.79



					
1	*E*-**14**	*E*-**15**	*E*-**16**	*E*-**17**	*E*-**18**
Total Light/%					
100	32	7.7	1.4	—	—
Peak Intensity/%					
100	7.2	3.0	0.54	—	—

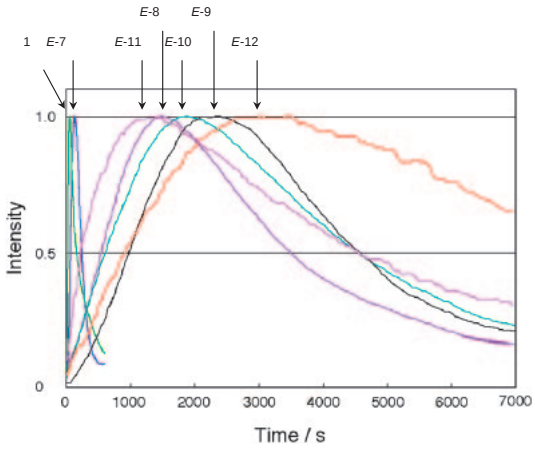


Fig. 5. Kinetics of the bioluminescence reaction of *Latia* luciferase and *Latia* luciferin and its analogues.

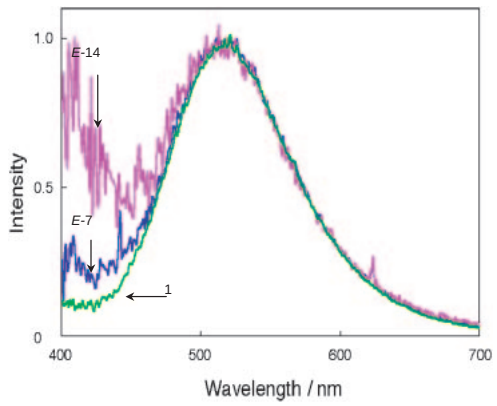


Fig. 6. Bioluminescence in vitro spectrum of purified *Latia* luciferase and synthetic *Latia* luciferin and its analogues. Bioluminescence spectra were obtained using an AB1850 spectrofluorometer (ATTO) equipped with a back-illumination-type attachment LN/CCD-512TKB (Princeton Instruments, CA). Width of the slit was 0.5 mm, and spectral resolution was 20 nm. The bioluminescent spectrum was collected for 10 s.

ciferin. The bioluminescence spectra of all luciferin analogues were the same as those of *Latia* luciferin, which strongly indicates that the light emitter cannot be luciferin or its analogues and is contained in *Latia* luciferase.

4. Biochemistry of *Latia* Luciferase

Shimomura and Johnson identified two proteins that participate in the light-emitting reaction and purified a 173 kDa protein considered to be luciferase (EC 1.14.99.21) and a purple protein of 39 kDa as cofactor.[17,18] Their purification procedure included DEAE-cellulose, Sephadex G-200, and Sephadex G-25 column chromatography. The purified luciferase is bioluminescent, with a molecular mass of 173 kDa as estimated by ultracentrifugation. We also purified *Latia* luciferase to clarify the structure. The enzyme responsible for the secretion of luciferase was predicted to be a glycoprotein. The enzyme was isolated from the extract in five steps including affinity chromatography for oligosaccharide chains. The active fraction that was first isolated by size-exclusion chromatography using

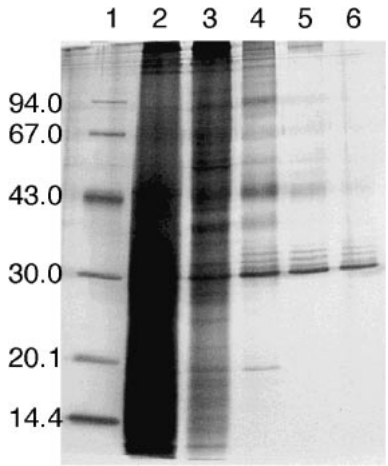


Fig. 7. Analysis of *Latia* luciferase by SDS-PAGE (12.5%) at various steps of the purification procedure. All samples were adjusted to the same bioluminescent activity (2×10^5 RLU). *Lane 1*, marker proteins; *Lane 2*, 33–60% $(NH_4)_2SO_4$ fractionation; *Lane 3*, HiPrep Sephacryl S-200; *Lane 4*, HiTrap Con A; *Lane 5*, Mono Q; and *Lane 6*, Superdex 200.

HiPrep Sephacryl S-200 was slightly yellow. This fraction had two fluorescence peaks (575 and 670 nm), and one of these (575 nm) could correspond to that of the purple protein.[17] The fluorescent elements did not bind to the Con A column during the next purification step for affinity chromatography. The active fraction did not show the fluorescent peaks at 575 nm or 670 nm and the specific activity of luciferase was increased, indicating that the enzyme and the purple protein are separable. These fractions were subjected to ion exchange FPLC using a Mono Q column. The elution pattern of the active fractions was widely distributed between samples containing 0.3 and 0.4 M NaCl, showing that the enzyme is heterogeneous. After the active fractions had been concentrated, the enzyme was purified to a single band on SDS-PAGE by silver staining using size-exclusion FPLC with two Superdex 200 columns (Fig. 7). The reaction between synthetic luciferin and the purified enzyme produced a greenish light ($\lambda_{max} =$ 536 nm), which is similar to the spectrum of luciferin and the crude extract in vitro, and therefore reconfirmed that the purified enzyme was *Latia* luciferase (Fig. 8). The relative molecular mass of *Latia* luciferase on a Superdex 200 size-exclusion column was about 180 kDa. Figure 7 and Figure 9a show SDS-PAGE patterns for each fraction from the ammonium sulfate precipitate to the purified enzyme, suggesting that the molecular mass of luciferase is 31 kDa. Furthermore, during MALDI-TOF analysis, the observed molecular mass was 31.6 kDa (Fig. 9b). These results suggest that *Latia* luciferase is a homo-oligomeric protein with 31.6 kDa subunits and a homohexamer of ca. 180 kDa corresponding to Shimomura's purification. On the other hand, the luciferase was completely bound to the Con A column without methyl α-D-glucopyranoside. When the luciferase was treated with PNGase F, the molecular mass of the resultant protein was 29–30 kDa on SDS-PAGE (Fig. 9a) and 29.1 kDa on MALDI-TOF (Fig. 9c), suggesting that *Latia* luciferase is an N-linked glycoprotein. The molecular mass of the carbohydrate chain was estimated to

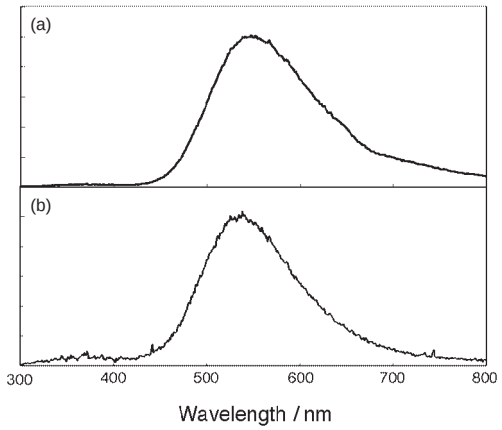


Fig. 8. (a) Bioluminescence in vivo spectrum of *Latia*. (b) Bioluminescence in vitro spectrum of synthetic *Latia* luciferin and purified *Latia* luciferase. Bioluminescence spectra were measured using an AB1850 spectrofluorometer (ATTO) equipped with a back-illumination-type attachment LN/CCD-512TKB (Princeton Instruments, CA). The width of the slit was 0.5 mm, and spectral resolution was 20 nm. The bioluminescent spectrum was collected for 10 s.

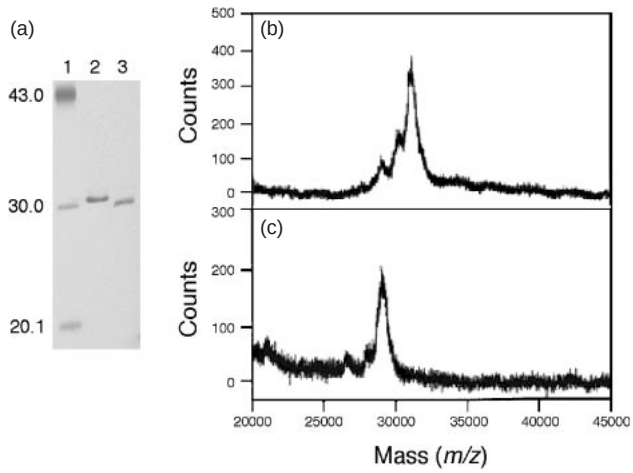


Fig. 9. The change of the molecular mass by the enzymatic digestion using endoglycosidase F (PNGase F). (a) Analysis by SDS-PAGE (12.5%). *Lane 1*, marker proteins; *Lane 2*, purified *Latia* luciferase; *Lane 3*, deglycosylated *Latia* luciferase. (b) Matrix-assisted laser desorption/ionization (MALDI) mass spectra for purified *Latia* luciferase and (c) deglycosylated *Latia* luciferase. An Applied Biosystem mass spectrometer was used to obtain MALDI mass spectra for determining molecular mass in a linear time of flight (TOF) mode. The nitrogen laser was set to deliver 337 nm wavelength pulses (5 ns) to the sample. Sinapinic acid was used as the matrix.

Table 3. Purification of Luciferase from *Latia neritoides*

Latia (39 g)	Total activity /RLU	Protein /mg	Specific activity /RLU·mg^{-1}	Yield /%	Fold /Times
1. $(NH_4)_2SO_4$ prep.	3.95×10^8	117	3.38×10^6	100	1
2. Sephacryl S200	2.63×10^8	52.2	5.04×10^6	67	1.5
3. Con A	2.30×10^8	6.60	3.49×10^7	58	10.3
4. Mono Q	2.19×10^8	2.00	1.10×10^7	55	32.5
5. Superdex 200	1.87×10^8	0.52	3.59×10^8	47	105

be about 2 kDa. In this procedure, for example, approximately 520 μg of the purified luciferase was obtained from 39 g (wet weight) of the *Latia* sample, and its activity was about 105-fold greater than that of the fraction from the 33–60% ammonium sulfate precipitation. This indicates that the purple protein could not have contributed to the apparent bioluminescence (Table 3). Shimomura, Johnson, and co-workers reported that, for a fixed amount of luciferin and purple protein, the rate of the light emitting reaction is essentially of the first order in luciferase, with the total amount of emitted light being independent of the luciferase concentration.[17] In contrast, the kinetics of light emission are complex for a fixed amount of luciferase and luciferin and a varying amount of purple protein, with the time lag being attributed to a slow interaction between purple protein, luciferin, and oxygen. The fluorescence properties of purple protein do not overlap with *Latia* bioluminescence. These results suggest that purple protein is not essential but may have some influence on the reaction.

We summarize the enzymatic properties of purified *Latia* luciferase in Table 4. The specific activity of our purified luciferase was 3.59×10^8 RLU/mg and its optimum pH was around pH 7.2. The K_m value of luciferase for luciferin was about 8.7 μM when calculated with a linear Lineweaver–Burk plot, indicating that the reaction between the purified enzyme and luciferin effectively produced a greenish light, and that the binding of luciferin to luciferase was highly specific. The activity of the active form of *Latia* luciferase was maintained above 90% when incubated for 12 h at 37 °C, whereas its activity when incubated at 50 °C for 10 min was only about 50%. This partially inactivated luciferase was reanalyzed using size-exclusion FPLC (Fig. 10). The elution pattern showed two

Table 4. Enzymatic Properties of *Latia* Luciferase

Processing Type	N-linked glycoprotein
Specific activity/RLU·mg^{-1}	3.59×10^8
Molecular mass	
Gel filtration	180 kDa
SDS-PAGE	31 kDa
MALDI-TOF	31.6 kDa
Isoelectric point	5.4–6.0
K_m	8.7 μM

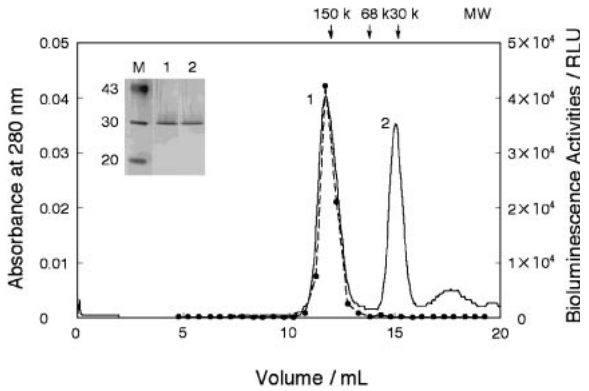


Fig. 10. Size-exclusion FPLC of partially denatured luciferase. The protein was detected by its absorbance at 280 nm (solid line) and bioluminescence activity (●). Inset: analysis of two peaks by SDS-PAGE (12.5%); *Lane 1*, marker proteins; *Lane 2*, 0.5 μg of peak 1; and *Lane 3*, 0.5 μg of peak 2.

peaks on Superdex 200: the upper peak fraction at around 180 kDa had bioluminescence activity, whereas a new peak fraction at around 30 kDa had no activity. Interestingly, each peak on SDS-PAGE analysis showed the same band at 31 kDa, which corresponds to the monomeric component of luciferase. These results suggest that the oligomeric form could be active, whereas dissociation of the enzyme causes inactivation of the bioluminescence reaction. However, it is unclear whether the monomeric component of the inactive peak had been modified. The well-known bacterial luciferase is a hetero-dimeric protein consisting of α and β subunits,[29] and *Oplophorus* luciferase is a hetero-oligomeric enzyme, consisting of 35 kDa and 19 kDa peptides.[30] In this series of luciferases, *Latia* luciferase is the first example of one in which the homo-oligomeric form is essential for the active enzyme.

5. Light Emitter of *Latia* Bioluminescence

The reaction between the synthetic *Latia* luciferin and the purified *Latia* luciferase produces a greenish light that coincides with the natural bioluminescence spectrum (Fig. 8). In this case, it does not require other proteins or cofactors, indicating that the luciferase contains a chromophore as the light emitter. The enzyme is colorless and does not have maximal absorption in the visible range (Fig. 11a). Shimomura and Johnson reported that the normally colorless, non-fluorescent luciferase fluoresced visibly in an alkaline or KCN solution.[18] The spectrum of this fluorescence is very similar to that of *Latia* bioluminescence and to the fluorescence of flavin adenine dinucleotide (FAD). This result indicates that *Latia* luciferase might be bound to a flavin group, which may be triggered by the bioluminescent reaction and accepted by the excited energy of oxyluciferin, although its fluorescence was quenched under the reduced state. We also detected the maximum fluorescent wavelength at about 535 nm but it was very weak, similar to the fluorescence of flavin (Fig. 11b), which supports the possibility that *Latia* luciferase has a tightly bound flavin group that constitutes the light emitter.[18] However, the FAD as a chromophore, or light-mediating species could not be identified. In other systems, accessory fluorescent proteins, such as GFP in coelenterates, receive the excitation energy of the oxyluciferin via energy transfer and emit it with their own fluorescence spectra. The intermediate state of luciferin has not been determined. In other cases such as in the bacterial reaction, the emitter is a reaction intermediate. Sometimes, as in the case of dinoflagellates, although the precise chemical identity of the emitter is unknown, it is accepted that it is a polypyrrole derivative of luciferin. Furthermore, although *Pholas* and Diptera luciferin has not been identified, the luciferases have been identified,[31,32] and *Pholas* luciferase has been cloned.[31] In contrast, there is, until now, no strong candidate for the role of emitter in the case of *Latia*.

The quantum yield of luciferin is very low, 0.003 per luciferin oxidized at 25 °C, but as much as 8 per purple protein molecule, which apparently recycles, indicating that the reaction efficiency is very low compared with the other bioluminescent reaction.[17] In general, the quantum yield of bioluminescence is very high: for example, 0.88 for firefly, 0.23 for aequorin, and 0.31 for ostracod.[33–35] The reason for the low efficiency in *Latia* bioluminescence is not clear but may relate to non-fluorescent properties of luciferin and oxyluciferin or to energy transfer to the bounded chromophore from excited oxyluciferin.

6. Remarks: Remaining Mystery

The proposed mechanism of *Latia* bioluminescence is summarized in Fig. 12. In the luciferin–luciferase reaction, *Latia* luciferin probably converts to the excited state of oxyluciferin via the dioxetanone structure; then its excited energy is transferred to the bounded chromophore on *Latia* luciferase, and bioluminescence is produced. The excited state of oxyluciferin

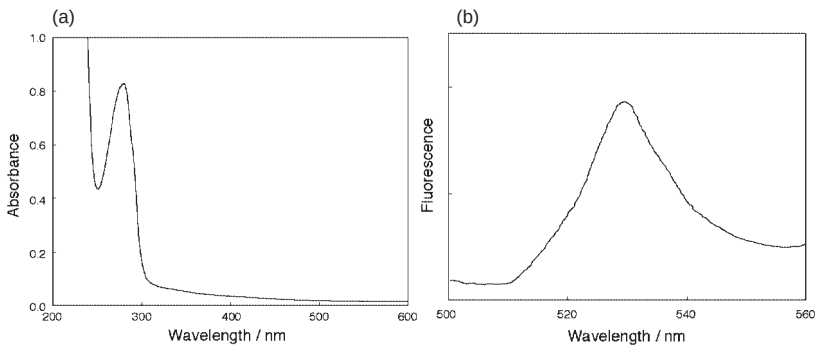


Fig. 11. (a) UV–vis absorption spectrum of *Latia* luciferase. (b) Fluorescent spectrum of *Latia* luciferase.

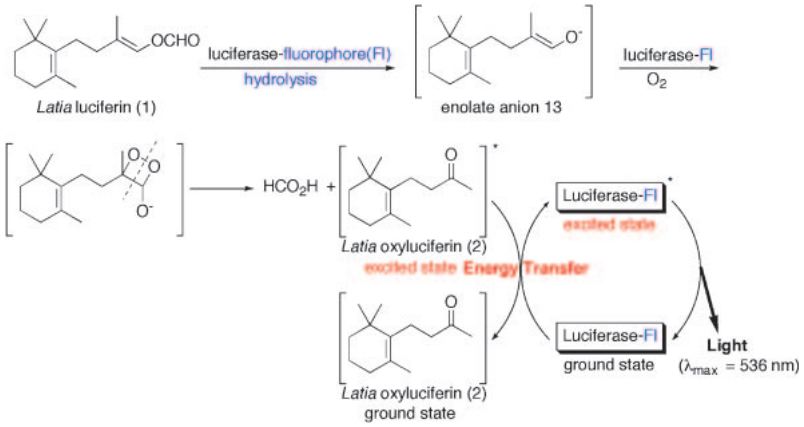


Fig. 12. Proposed chemical mechanism of the bioluminescence reaction of *Latia* luciferin catalyzed by luciferase. *Latia* luciferin in the luciferin–luciferase reaction probably converts to the excited stated of oxyluciferin via the dioxetanone structure; its excited energy is transferred to the bounded chromophore on *Latia* luciferase and bioluminescence is produced.

does not determine color but is an energy source for producing light. Therefore, the emitter remains a mystery. We think that it may be possible to control the bioluminescence reaction, including its color and lifetime, with recombinant proteins reconstructed from flavin derivates or luciferin analogues. This is one of the reasons that prompted us to reinvestigate the *Latia* system. In view of the ease of synthesis of luciferin and the greenish emission, we also hoped that a recombinant *Latia* luciferase might become a useful tool as a reporter of gene expression.

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References

1 E. N. Harvey, "Bioluminescence," Academic Press Inc., New York (1952).
2 L. J. Kricka, *Methods Enzymol.*, **305**, 333 (2000).
3 Y. Ohmiya, *Seikagaku*, **76**, 5 (2004).
4 J. W. Hastings, *Gene*, **173**, 5 (1996).
5 T. Wilson and J. W. Hastings, *Annu. Rev. Cell Dev. Biol.*, **14**, 197 (1998).
6 M. A. Branham and J. W. Wenzel, *Cladistics*, **19**, 1 (2003).
7 L. J. White, R. Rapport, H. H. Seliger, and T. A. Hopkins, *Bioorg. Chem.*, **1**, 92 (1971).
8 F. McCapra, D. J. Gilfoyle, D. W. Young, N. J. Church, and P. Spencer, "Bioluminescence and Chemiluminescence: Fundamental and Applied Aspects," ed by A. Campbell, L. J. Kricka, and P. E. Stanley, John Wiley and Sons, UK (1994), pp. 387–391.
9 V. R. Viviani, *Cell. Mol. Life. Sci.*, **59**, 1833 (2002).
10 E. Conti, N. P. Franks, and P. Brick, *Structure*, **4**, 287 (1996).
11 E. Conti, T. Stachelhaus, M. A. Marahiel, and P. Brick, *EMBO J.*, **16**, 4174 (1997).
12 K. V. Wood, Y. A. Lam, and W. D. McElroy, *J. Biolumin. Chemilumin.*, **4**, 289 (1989).
13 Y. Nakajima, M. Ikeda, T. Kimura, S. Honma, Y. Ohmiya, and K. Honma, *FEBS Lett.*, **565**, 122 (2004).
14 H. Nakamura, Y. Kishi, O. Shimomura, D. Morse, and J. W. Hastings, *J. Am. Chem. Soc.*, **111**, 7607 (1989).
15 O. Shimomura and F. H. Johnson, "Bioluminescence in Progress," ed by F. H. Johnson and Y. Haneda, Princeton Univ. Press, NJ (1966), pp. 391–404.
16 O. Shimomura and F. H. Johnson, *Biochemistry*, **7**, 1734 (1968).
17 O. Shimomura and F. H. Johnson, *Biochemistry*, **7**, 2574 (1968).
18 O. Shimomura and F. H. Johnson, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2086 (1972).
19 J. E. Gray, *Proc. Zool. Soc. London*, **17**, 164 (1849).
20 S. Moore and V. B. Meyer-Rochow, *Verh. Int. Ver. Theor. Angew. Limnol.*, **23**, 2189 (1988).
21 V. B. Meyer-Rochow and S. Moore, *Int. Revue ges. Hydrobiol.*, **73**, 21 (1988).
22 H. Suter, *Trans. N. Z. Inst.*, **23**, 93 (1890).
23 B. J. Bowden, *Biol. Bull.*, **99**, 373 (1950).
24 M. G. Fracheboud, O. Shimomura, R. K. Hill, and F. H. Johnson, *Tetrahedron Lett.*, **10**, 3951 (1969).
25 F. Nakatsubo, Y. Kishi, and T. Goto, *Tetrahedron Lett.*, **11**, 381 (1970).
26 S. Kojima, S. Maki, T. Hirano, Y. Ohmiya, and H. Niwa, *Tetrahedron Lett.*, **41**, 4409 (2000).
27 M. Nakamura, M. Masaki, S. Maki, R. Matsui, M. Hieda, M. Mamino, T. Hirano, Y. Ohmiya, and H. Niwa, *Tetrahedron Lett.*, **45**, 2203 (2004).
28 M. Nakamura, M. Mamino, M. Masaki, S. Maki, R. Matsui, S. Kojima, T. Hirano, Y. Ohmiya, and H. Niwa, *Tetrahedron Lett.*, **46**, 53 (2005).
29 J. W. Hastings, K. Weber, J. Friedland, A. Eberhard, G. W. Mitchell, and A. Gunsalus, *Biochemistry*, **12**, 4681 (1969).
30 S. Inouye, K. Watanabe, H. Nakamura, and O. Shimomura, *FEBS Lett.*, **481**, 19 (2000).
31 S. L. Dunstan, G. B. Sala-Newby, A. B. Fajardo, K. M. Taylor, and A. K. Campbell, *J. Biol. Chem.*, **275**, 9403 (2000).
32 V. R. Viviani, J. W. Hastings, and T. Wilson, *Photochem. Photobiol.*, **75**, 22 (2002).
33 H. H. Seliger and W. D. McElroy, *Arch. Biochem. Bio-*

phys., **88**, 136 (1960).
34 O. Shimomura and F. H. Johnson, *Nature*, **227**, 1356 (1970).
35 O. Shimomura and F. H. Johnson, *Photochem. Photobiol.*, **12**, 291 (1970).

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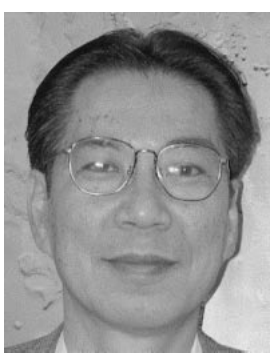

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