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博 士 論 文 の 要 約

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Study on the Biosynthesis of the Luminous Substrate in the Firefly Squid *Watasenia scintillans*
（ホタルイカ*Watasenia scintillans*における発光基質の生合成に関する研究）

Bioluminescence is found in various organisms, from microorganisms to invertebrates and even vertebrates, across terrestrial and marine environments. The mechanisms behind this luminescence have been applied in fields such as molecular biology, medicine, and agricultural sciences. However, the mechanisms of bioluminescence are diverse and vary across species, many of which remain to be elucidated. The firefly squid, *Watasenia scintillans,* emits a vibrant blue light from the photophores located on its arms, eyes, and skin. Generally, bioluminescence results from the oxidation of luciferin, a light-emitting substrate, in the presence of molecular oxygen (O_2) and the enzyme luciferase. Most luminous marine organisms utilize coelenterazine (CTZ) as their luciferin.

In contrast, the luciferin of *W. scintillans* is coelenterazine disulfate (CTZ 2,6-SO_3H), which can be synthesized by sulfating the two phenolic hydroxyl groups of CTZ. CTZ 2,6-SO_3H is oxidized to produce light, coelenteramide disulfate (CTMD 2,6-SO_3H), and CO_2 in the presence of O2, ATP, and Mg^{2+}. CTZ is produced by certain marine plankton and is widely distributed among their food webs. Therefore, it is believed that *W. scintillans* acquires CTZ, a potential precursor of CTZ 2,6-SO_3H, through dietary intake. However, CTZ 2,6-SO_3H has not been found in any known food sources, and no sulfotransferase (SULT) that converts CTZ into CTZ 2,6-SO_3H has yet been identified in *W. scintillans*. It remains unclear whether *W. scintillans* biosynthesizes CTZ 2,6-SO_3H from CTZ. Moreover, biosynthesis using CTZ derived solely from the diet is unlikely to meet the high demand for CTZ 2,6-SO_3H during continuous light emission. This raises the possibility that *W. scintillans* possesses a biosynthetic pathway that recycles the luminescent reaction product, CTMD 2,6-SO_3H, back into CTZ 2,6-SO_3H, although the detailed mechanism of this pathway remains elusive. Therefore, the aims of this study were as follows: (1) identifying SULT responsible for the sulfation of CTZ at its two phenolic hydroxyl groups in *W. scintillans*, and (2) investigating the biosynthetic pathway by which *W. scintillans* may convert CTMD 2,6-SO_3H back into CTZ 2,6-SO_3H.

A Novel Coelenterazine Sulfotransferase from the Squid *W. scintillans*

To investigate whether *W. scintillans* possesses SULT responsible for producing CTZ 2,6-SO_3H from CTZ, a putative SULT gene was identified in *W. scintillans* and successfully expressed in *Escherichia coli*. The recombinant protein was purified, and its sulfation activity was tested *in vitro* using CTZ and the sulfonate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Ultra-performance liquid chromatography with photodiode array and mass spectrometry (UPLC-PDA-MS) analysis of the incubated reaction products revealed PAPS-dependent formation of CTZ 2-benzyl monosulfate (CTZ 2-SO_3H), confirming the

enzyme functions as a SULT in *W. scintillans*. However, no CTZ 2,6-SO_3H was detected under the tested conditions, suggesting that additional enzyme(s) may be required to produce CTZ 2,6-SO_3H in *W. scintillans*. To further characterize the SULT in *W. scintillans*, its sulfation activity toward coelenteramine (CTM), a CTZ derivative having one phenolic hydroxyl group, was tested. As a result, CTM was not sulfated by the SULT in *W. scintillans*, suggesting a strict substrate specificity. Notably, comparative UPLC-PDA-MS analysis revealed that the recombinant putative SULT from *Acanthosepion pharaonis,* which exhibits significant homology with the SULT in *W. scintillans*, catalyzed the sulfation of both CTZ and CTM. These results highlight the functional divergence of the SULT in *W. scintillans*, suggesting its evolutionary specialization in the biosynthesis of light-emitting substrate.

Identification of Potential Intermediates in the Recycling Biosynthesis of CTZ 2,6-SO_3H in the Liver of *W. scintillans*

A previous study has reported significantly higher concentrations of CTZ present in the liver compared to the photophores, suggesting its potential involvement in luminous substrate biosynthesis. Therefore, to investigate the presence of a recycling system of the luminescent reaction product, CTMD 2,6-SO3H, for synthesizing CTZ 2,6-SO_3H in *W. scintillans*, compounds extracted from the liver, a non-luminous organ, were analyzed. Livers from lyophilized *W. scintillans* were carefully separated, finely ground, and homogenized in methanol. The homogenate was filtered, concentrated, and then analyzed by UPLC-PDA-MS. The analysis of UV spectra of detected peaks in the methanolic liver extract suggested the presence of CTM and CTM monosulfate (CTM-SO3H), which can be formed from CTMD 2,6-SO3H, in the liver of *W. scintillans*. Although the complete recycling process of CTZ 2,6-SO_3H remains under investigation, the presence of these potential intermediates in the non-luminous liver strongly suggests that liver plays a crucial role in the metabolism or recycling of CTMD 2,6-SO_3H, contributing to CTZ 2,6-SO_3H production. Further investigation of liver-specific intermediates and enzymes will be essential to elucidate the detailed mechanism.

This study identified a SULT that produced CTZ 2-SO_3H from CTZ for the first time in *W. scintillans,* along with two intermediate compounds detected in the liver, suggesting the presence of a biosynthetic pathway that recycles this luminescent reaction product into CTZ 2,6-SO_3H. These findings will advance our understanding of the biosynthetic pathway and highlight the liver's metabolic role in *W. scintillans*. These molecular insights could contribute to the development of novel bioluminescent tools utilizing the unique luciferin-luciferase system of *W. scintillans*, enabling more efficient coelenterazine-based reporters for applications ranging from sensitive biosensors and rapid pathogen detection to advanced bioimaging technologies in medical diagnostics.