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Efficient conversion to Cypridina luciferin from Cypridina luciferyl sulfate, coupled with enzymatic sulfation of acetic acid

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ABSTRACT

In *Cypridina* (*Vargula*) *hilgendorfii,* Cypridina luciferin is converted from Cypridina luciferyl sulfate by a sulfotransferase with adenosine 3′, 5′-diphosphate (PAP), and is used for the luminescence reaction of Cypridina luciferase. We found that the luminescence activity of crude extracts of *C. hilgendorfii* was significantly stimulated by the addition of acetic acid. This stimulation may be explained by an efficient supply of PAP from 3′-phosphoadenosine 5′-phosphosulfate (PAPS) catalyzed by a sulfotransferase. Thus, acetic acid acts as a sulfate acceptor from PAPS, followed by forming acetyl sulfate and PAP. The structure of acetyl sulfate was identified using mass spectrometry and it spontaneously decomposed to acetic acid and free sulfate ion in aqueous solutions. This enzymatic conversion from Cypridina luciferyl sulfate to Cypridina luciferin could be coupled with acetic acid and PAPS by a sulfotransferase.

1. Introduction

The bioluminescence of *Cypridina hilgendorfii* (presently *Vargula hilgendorfii*) is produced by a luciferin-luciferase reaction [1]. Cypridina luciferin [2–4] is oxidized by Cypridina luciferase with molecular oxygen (O_2), yielding blue light (λ_{max} = 460 nm) accompanied by the formation of oxyluciferin and CO_2 (Fig. 1). Cypridina luciferin has an imidazopyrazinone structure, similar to that of coelenterazine [5]. It has been reported that Cypridina luciferin and coelenterazine are biosynthesized from free L-amino acids in living animals [6–8]. However, the biosynthetic pathways of these luciferins have not been elucidated to date.

Previously, we reported the isolation of Cypridina luciferin 3-enol sulfate (Cypridina luciferyl sulfate) from *C. hilgendorfii* as an acid-labile derivative of Cypridina luciferin [9]. Using crude extracts of *Cypridina* specimens, Cypridina luciferyl sulfate could be enzymatically converted to Cypridina luciferin in the presence of adenosine 3′, 5′-diphosphate (PAP) by a sulfotransferase. The reverse reaction also occurred in the presence of Cypridina luciferin and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) as a sulfate donor [9]. In general, sulfotransferase catalyzes the transfer of a sulfate group from PAPS to a substrate, producing a sulfated substrate and PAP [10]. It is known that hormones, neurotransmitters, drugs, and xenobiotic compounds act as substrates for sulfation [11,12].

Recently, we demonstrated that a sulfotransferase in *Cypridina* specimens could efficiently use coenzyme A (CoA), adenosine 5′-monophosphate (5′-AMP) and adenosine 3′-monophosphate (3′-AMP) as a sulfate acceptor from Cypridina luciferyl sulfate and produced Cypridina luciferin, in a similar manner to the case with PAP [13]. In the sea pansy *Renilla reniformis,* it has been reported that the conversion of coelenterazine to coelenterazine 3-enol sulfate is catalyzed by a sulfotransferase and this is reversible reaction [14–16]. Cypridina luciferyl sulfate and coelenterazine 3-enol sulfate might be the storage forms of Cypridina luciferin and coelenterazine, respectively. Another sulfate derivative of coelenterazine is coelenterazine disulfate, which is known as a luciferin from the firefly squid *Watasenia scintillans* [17]. These products might be enzymatically synthesized by sulfotransferases.

Abbreviations: PAP, adenosine 3′, 5′-diphosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; RP-HPLC, reversed-phase HPLC; RLU, relative light units; TFA, trifluoroacetic acid; LC/ESI-TOF-MS, liquid chromatography electrospray ionization time-of-flight mass spectrometry.

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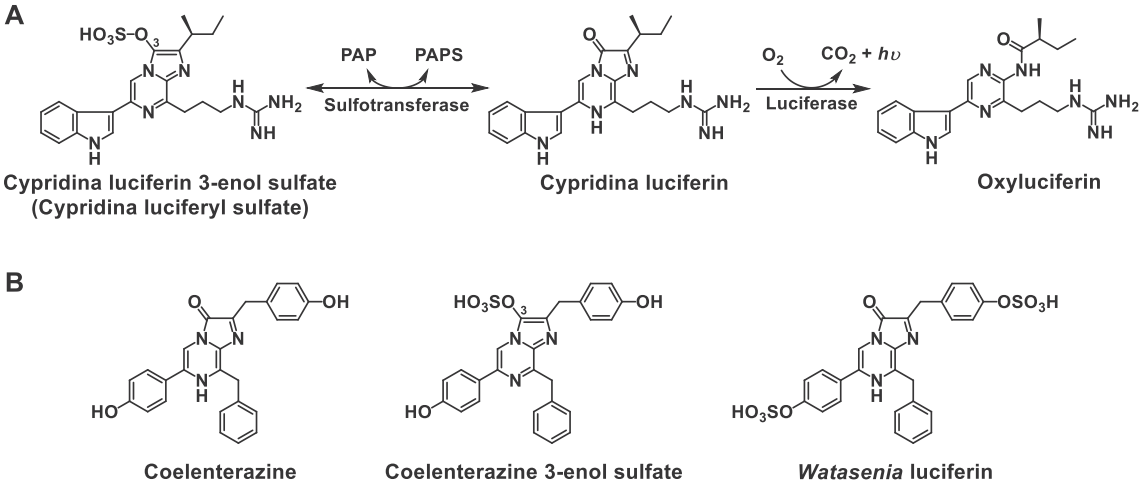

Fig. 1. Luminescence reaction of Cypridina luciferase with Cypridina luciferin and the chemical structures of coelenterazine and its sulfate derivatives.
A. Luminescence reaction in *C. hilgendorfii*. Cypridina luciferyl sulfate is converted to (*S*)-Cypridina luciferin by a sulfotransferase, and (*S*)-Cypridina luciferin is catalyzed by Cypridina luciferase to produce oxyluciferin, CO_2, and light. **B**. Chemical structures of coelenterazine, coelenterazine 3-enol sulfate and coelenterazine disulfate (*Watasenia* luciferin).

During studies on the biosynthetic pathway of Cypridina luciferin, we found that the addition of CoA to the reaction mixture of Cypridina luciferyl sulfate stimulated the luminescent activity. This is the first report that a sulfotransferase in *Cypridina* specimens can use CoA as a sulfate acceptor [13]. Interestingly, the crude extracts of *Cypridina* specimens with CoA in 0.1 M glycine-NaOH showed higher luminescence activity than that in 0.1 M Tris-HCl, sodium phosphate, and ammonium acetate [13]. To clarify the reason for less luminescence intensity with these assay buffers than that with glycine-NaOH, we examined the effect of free acids such as hydrochloric acid, phosphoric acid, and acetic acid on the luminescence activity of crude extracts. Unexpectedly, the addition of acetic acid and phosphoric acid to the crude extracts significantly stimulated the luminescence intensity. In this study, we investigated the luminescence stimulation with free acids using a reaction mixture containing Cypridina luciferyl sulfate, PAP, and crude extracts of *Cypridina* specimens, and found that the enzymatic formation of acetyl sulfate from acetic acid stimulates the luminescence activity in the Cypridina luciferin-luciferase reaction.

2. Materials and methods

2.1. Chemicals

Adenosine 3′, 5′-diphosphate (PAP) and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) were purchased from Sigma (St. Louis, MO). Acetic acid was obtained from Nacalai Tesque, Inc. (Kyoto, Japan). All other chemicals used in the study were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), Tokyo Chemical Industry Co. (Tokyo, Japan) or Nacalai Tesque, Inc. Cypridina luciferyl sulfate was purified from Cypridina specimens as previously reported [9]. *Cypridina* specimens were collected at Chita (Aichi, Japan) on August 4, 2011, and Naruto (Tokushima, Japan) on August 24, 2017, and stored at −80 °C before use.

2.2. Determination of luminescence activity

The luminescence activity of Cypridina luciferase was determined using an Atto (Tokyo, Japan) AB2270 luminometer (Ver.1.025) for 60 s in 0.1s intervals in a glass tube (Nippon Electric Glass Co., Shiga, Japan). The maximum luminescence intensity (I_{max}) in the absence of a cut filter (F0) was determined and is shown as relative light units (RLU). The mean value of I_{max} ($n = 3$) was calculated. In our assay conditions using an AB2270 luminometer, 1 RLU was estimated to be 1.2×10^3 photons/0.1 s, based on the I_{max} value of recombinant aequorin (JNC Corp., Japan) as a light standard, as previously described [18].

2.3. Preparation of crude extracts and G25-fraction of Cypridina specimens

The preparation methods for crude extracts and the G25-fraction of *Cypridina* specimens were essentially the same as previously described [9,13]. Briefly, the frozen *Cypridina* specimens were homogenized in 0.1 M glycine-NaOH (pH 8.5), and kept on ice for over 6 h to terminate the endogenous luminescence reaction. To remove sulfate derivatives and endogenous nucleotide derivatives such as PAP and PAPS from crude extracts, crude extracts were applied on a Sephadex G25 column (6 × 88 mm, a 5 mL disposable pipette, Falcon) and the void volume fractions were used as “G25-fraction” containing Cypridina luciferase and sulfotransferase(s).

2.4. Stimulation of luminescence intensity in four buffers by the addition of PAP to crude extracts of Cypridina specimens

Cypridina specimens (49.2 mg, wet weight) were homogenized in 300 μL of 0.1 M glycine-NaOH (pH 8.5) and were kept on ice for 6 h. Crude extracts (10 μL) were added to 990 μL of 0.1 M glycine-NaOH, Tris-HCl, ammonium acetate or sodium phosphate buffers (pH 7.5 or 8.5). After incubating at 22–25 °C for 1 min, 1.5 h, 3 h and 6 h, the luminescence activity in each buffer (100 μL) was determined by adding 1 μL of 1 mM PAP.

2.5. Preparation of 0.5 M acid solution at pH 5.0, 6.0, and 7.0

The acid solutions were prepared as follows, and were used by dilution with H_2O.

a) 0.5 M acetic acid solutions at pH 5.0, 6.0, and 7.0 were prepared by 0.5 M sodium acetate with 0.5 M acetic acid.
b) 0.5 M phosphoric acid solutions at pH 5.0, 6.0, and 7.0 were prepared with 0.5 M NaH_2PO_4 and 0.5 M Na_2HPO_4.
c) 0.5 M sulfuric acid solutions were prepared by 0.5 M Na_2SO_4 with 0.5 M H_2SO_4. The solution of 0.5 M Na_2SO_4 was adjusted

to the pH at pH 5.0, 6.0, and 7.0 with 0.5 M H_2SO_4 and filled up 10 mL with H_2O.

d) 0.5 M hydrochloric acid solutions were prepared from 1 M HCl (5 mL) by adjusting to the pH at pH 5.0, 6.0, and 7.0 with 1 M NaOH and filled up to 10 mL with H_2O.

2.6. Stimulation of luminescence intensity by the addition of acid solution to crude extracts of Cypridina specimens

Cypridina specimens (87.5 mg, wet weight) were homogenized in 300 μL of 0.1 M glycine-NaOH (pH 8.5) and used. The luminescence reaction was started by adding 5 μL of 0.2 M sulfuric acid, 0.2 M hydrochloric acid, 0.2 M phosphoric acid or 0.2 M acetic acid solution (pH 5.0, 6.0 or 7.0) to 100 μL of 0.5 M glycine-NaOH (pH 7.5) containing 5 μL of crude extracts and 1 μL of 1 mM PAP.

2.7. Stimulation of luminescence activity by the addition of acid solution to G25-fraction of Cypridina specimens

The G25-fraction of *Cypridina* specimens was obtained from the frozen specimens (196.5 mg, wet weight) in 200 μL of 0.1 M glycine-NaOH (pH 8.5). The luminescence reaction was started by adding 5 μL of 0.2 M acetic acid or 0.2 M phosphoric acid solution to 100 μL of 0.5 M glycine-NaOH (pH 7.5) containing Cypridina luciferyl sulfate (490 ng), 2 μL of G25-fraction and 1 μL of 1 mM PAP.

2.8. Effect of acetic acid and phosphoric acid on luminescence activity in the reaction mixture containing G25-fraction, Cypridina luciferyl sulfate, and PAP

In a reaction mixture containing 2 μL of G25-fraction, Cypridina luciferyl sulfate (490 ng), and 1 μL of 1 mM PAP in 100 μL of 0.5 M glycine-NaOH (pH 7.5), the luminescence reaction was started by adding 5 μL of 0.2 M acetic acid or 0.2 M phosphoric acid solution (pH 5.0) into the reaction mixture. The heat-denatured G25-fraction was prepared using a heating block (TAITEC Co., Saitama, Japan) at 100 °C for 10 min and cooled to room temperature.

2.9. Stimulation of luminescence activity in G25-fraction of Cypridina specimens by acetic acid at various pHs

The crude extracts of *Cypridina* specimens were prepared from the frozen specimens (205 mg, wet weight) by homogenizing them in 300 μL of 0.1 M glycine-NaOH (pH 8.5). The luminescence reaction was started by adding 5 μL of 0.2 M acetic acid solution to 100 μL of 0.5 M glycine-NaOH (pH 7.5) containing 2 μL of G25-fraction, Cypridina luciferyl sulfate (490 ng), and 1 μL of 1 mM PAP.

2.10. Identification of acetyl sulfate in the reaction product of acetic acid, PAP, Cypridina luciferyl sulfate, and G25-fraction by mass spectrometry

To identify the reaction product of acetic acid with G25-fraction, 2 μL of 0.5 M acetic acid solution (pH 5.0) was added to 30 μL of 0.5 M glycine-NaOH (pH 7.5) containing 10 μL of G25-fraction, Cypridina luciferyl sulfate (750 ng), and 1 μL of 1 mM PAP. After incubation for 15 min at 20–25 °C, the reaction mixture was extracted three times with $CHCl_3$ (300 μL) and evaporated *in vacuo*. The resultant residues were dissolved in 60 μL of CH_3CN, 20 μL of which was subjected to LC/ESI-TOF-MS. The acetyl sulfate produced was analyzed by the infusion method of mass spectrometry using the XEVO Q-TOF MS System (Waters Japan, Tokyo, Japan) in negative ion mode. HRMS(ESI): *m/z* calcd for $C_2H_3O_5S$ $[M - H]^-$ 138.9701, found 138.9700.

2.11. Chemical synthesis of acetyl sulfate as an authentic sample for mass spectrometry

Acetic acid (61.9 mg) was added to 10 mL of CH_2Cl_2 containing sulfur trioxide pyridine complex (824.8 mg, Tokyo Chemical Industry Co.) and trimethylamine (520.4 mg, Wako Pure Chemical Industries, Ltd.). After incubating for 1 h at 0 °C, the reaction mixture was evaporated to give crude residues (57.2 mg). The resultant residues were dissolved in CH_3CN and analyzed with ESI-TOF-MS without further purification.

HRMS (ESI): *m/z* calcd for $C_2H_3O_5S$ $[M - H]^-$ 138.9701, found 138.9701.

2.12. Enzymatic conversion of acetic acid to acetyl sulfate by using G25-fraction and PAPS

The reaction was started by adding 2 μL of 0.5 M acetic acid solution (pH 5.0) to 20 μL of 0.5 M glycine-NaOH (pH 7.5) containing 5 μL of G25-fraction and 2 μL of 0.1 mM PAPS. After incubation at 22–25 °C for 30 min, the reaction mixture was extracted three times with $CHCl_3$ (300 μL) and evaporated *in vacuo*. The resultant residues were dissolved in 60 μL of CH_3CN, 20 μL of which was analyzed with ESI-TOF-MS. HRMS (ESI): *m/z* calcd for $C_2H_3O_5S$ $[M - H]^-$ 138.9701, found 138.9699.

2.13. Stimulation of luminescence activity by adding various concentrations of acid chemicals to the reaction mixture containing G25-fraction, PAP and Cypridina luciferyl sulfate

Each acid solution of the chemicals was adjusted to pH 5.0 using 1 M NaOH. The reaction mixture contained 2 μL of G25-fraction, Cypridina luciferyl sulfate (490 ng), and 1 μL of 1 mM PAP in 100 μL of 0.5 M glycine-NaOH (pH 7.5). The stimulation of luminescence activity was examined by adding various concentrations of acid derivatives solution.

3. Results and discussion

3.1. Effects of buffers on luminescence intensity using crude extracts of Cypridina specimens

The crude extracts of *C. hilgendorfii* containing a sulfotransferase, Cypridina luciferase, Cypridina luciferyl sulfate and other cofactors were prepared by homogenizing the specimens in 0.1 M glycine-NaOH (pH 8.5), and were allowed to keep in an ice-bath for 6 h [13]. After disappearance of endogenous luminescence in crude extracts, crude extracts were added to each of 0.1 M glycine-NaOH (pH 7.5 and 8.5), 0.1 M Tris-HCl (pH 7.5 and 8.5), 0.1 M ammonium acetate (pH 7.5 and 8.5), and 0.1 M sodium phosphate (pH 7.5 and 8.5), respectively. After incubation for 1 min, 1.5 h, 3 h, and 6 h, luminescence activity was determined by the addition of PAP (Fig. 2A). As a result, the addition of crude extracts to 0.1 M ammonium acetate or 0.1 M sodium phosphate was less potent of luminescence activity than that to 0.1 M glycine-NaOH or 0.1 M Tris-HCl. However, it is unclear that ammonium acetate and sodium phosphate reduce the luminescence activity.

3.2. Effects of acid solutions on luminescence activity of the reaction mixture containing crude extracts of Cypridina specimens, Cypridina luciferyl sulfate, and PAP

Each 0.5 M solutions of sulfuric acid, hydrochloric acid, phosphoric acid, and acetic acid with pH 5.0, 6.0, and 7.0 were prepared as described in *Materials and Methods*. The stimulation of luminescence was examined by adding a respective acid solution at a

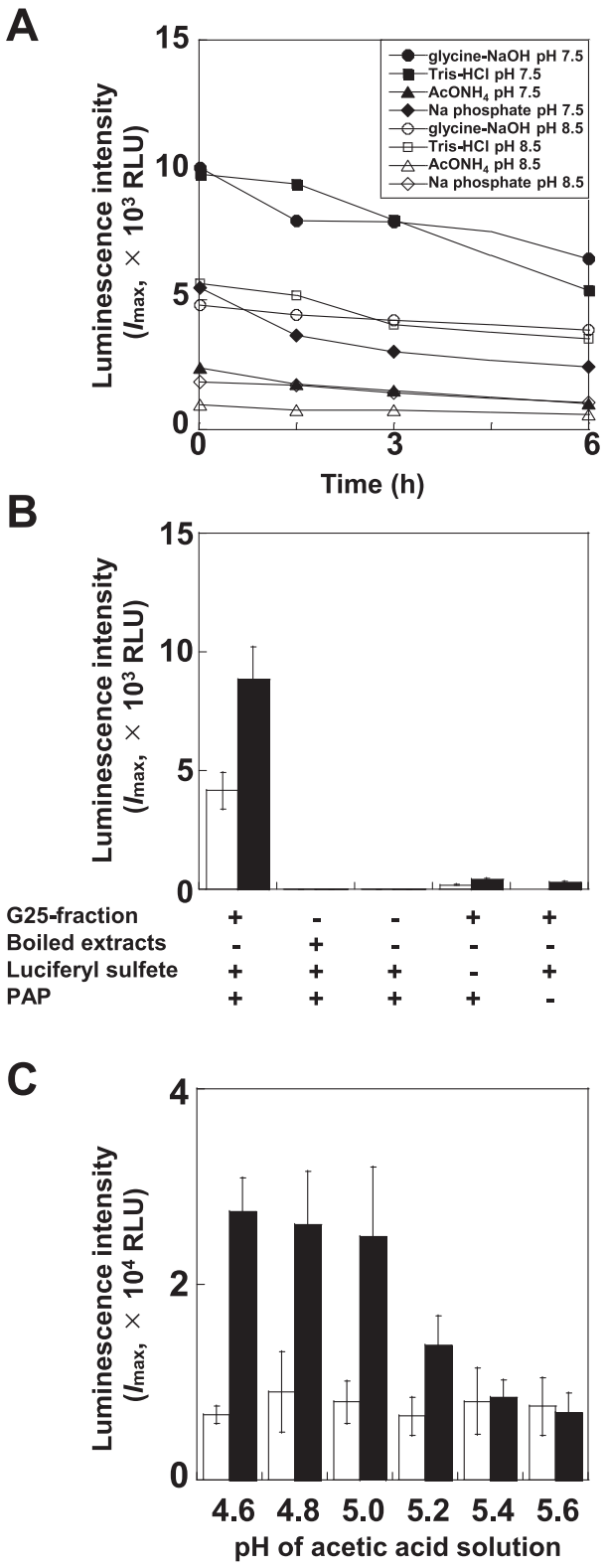

Fig. 2. Effects of assay conditions on luminescence activity using crude extracts of *Cypridina* specimens.
A. Effects of assay conditions on luminescence activity of the reaction mixture containing crude extracts of *Cypridina* specimens, Cypridina luciferyl sulfate, and PAP. **B**. Stimulation of luminescence activity by the addition of acetic acid solution to the reaction mixture containing G25-fraction and Cypridina luciferyl sulfate. Open and closed bars indicate the addition of PAP and acetic acid, respectively, to the reaction mixture containing G25-fraction and Cypridina luciferyl sulfate. **C.** Effects of acetic acid on luminescence activity at various pH conditions. Open and closed bars indicate the addition of PAP and acetic acid at various pHs (4.6–5.6) to the reaction mixture containing G25-fraction and Cypridina luciferyl sulfate.

final concentration of 10 mM to the reaction mixture containing crude extracts and PAP in 0.5 M glycine-NaOH (pH 7.5). To avoid the pH effect on the enzymatic reaction, a high concentration of 0.5 M glycine-NaOH (pH 7.5) was used. The solution of acetic acid and phosphoric acid (pH 5.0) significantly stimulated the luminescence activity, whereas sulfuric acid and hydrochloric acid solutions did not stimulate luminescence (Fig. S1). The same stimulations were observed by the addition of acetic acid solution prepared from acetic acid with NaOH and phosphoric acid solution from H_3PO_4 with NaOH (Fig. S2) at pH 5.0. These results suggested that the luminescence stimulation by acetic acid or phosphoric acid might be involved in the different conversion system from Cypridina luciferyl sulfate to Cypridina luciferin under these acidic conditions [9].

3.3. Effect of acetic acid on luminescence activity in the reaction mixture containing G25-fraction, Cypridina luciferyl sulfate, and PAP

The luminescence activity in the reaction mixture containing G25-fraction, Cypridina luciferyl sulfate, and PAP in 0.5 M glycine-NaOH (pH 7.5) was determined by addition of acetic acid solution (pH 5.0). In the absence of Cypridina luciferyl sulfate, PAP, or G25-fraction, the luminescence activity was not stimulated and was not also stimulated by the heat-denatured G25-fraction (Fig. 2B). These results suggested that the luminescence stimulation of G25-fraction by the addition of acetic acid solution was an enzymatic reaction.

3.4. Effects of various pHs in acetic acid solution on luminescence activity in the reaction mixture containing G25-fraction, Cypridina luciferyl sulfate, and PAP

Acetic acid solutions at various pHs were added to the reaction mixture containing G25-fraction, Cypridina luciferyl sulfate, and PAP, and the stimulation of luminescence activity was determined (Fig. 2C). The luminescence activity was stimulated from pH 5.4 to 5.0 of acetic acid solution. The portion of free acetic acid at pH 5.4 and 5.0 corresponds to 18% and 36%, respectively, which were calculated using the pKa value of 4.76 for acetic acid [19]. This result suggested that the free carboxylic form of acetic acid was essential for stimulating luminescence intensity, since it increases under strong acidic conditions at pH 4.6 to 5.0.

3.5. Mass spectral analysis of the sulfated product of acetic acid

The predicted product of acetyl sulfate in the reaction mixture containing G25-fraction, Cypridina luciferyl sulfate, and PAP with acetic acid in 0.5 M glycine-NaOH (pH 7.5) was directly analyzed with ESI-TOF-MS. However, the mass spectrum of acetyl sulfate (Fig. 3A–a) was not detected under our analytical conditions. As we expected that acetyl sulfate might be unstable in an aqueous solution, the reaction mixture was extracted with $CHCl_3$ and evaporated. The resultant residues were dissolved in CH_3CN and analyzed by ESI-TOF-MS. As a result, the mass spectrum of the reaction product showed a small peak of *m/z* 138.9700 ($[M - H]^-$) in negative ion mode (Fig. 3A–b). MS/MS spectral analysis of *m/z* 138.97 showed three major product ions: *m/z* 59, *m/z* 80 and *m/z* 97 (Fig. 3A–c), expected that *m/z* 138.97 shows the fragment ions with *m/z* 59 (acetate ion, $[CH_3CO_2]^-$), with *m/z* 80 ($[SO_3]^{-\bullet}$), and with *m/z* 97 ($[HSO_4]^-$).

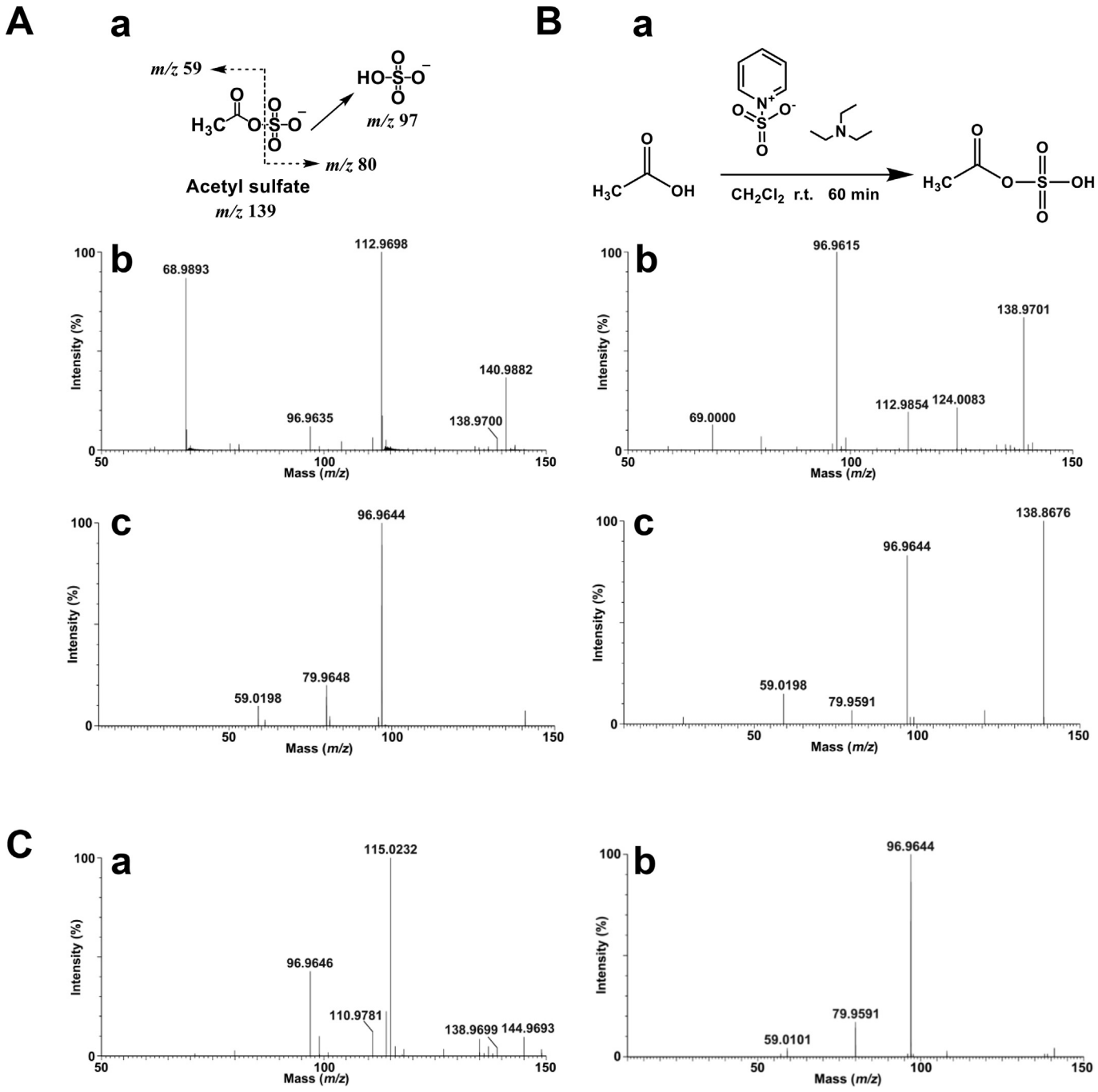

Fig. 3. Mass spectrometry of the reaction product from G25-fraction, Cypridina luciferyl sulfate, PAP, and acetic acid.
A. a, Predicted mass fragmentation of acetyl sulfate; **b**, Mass spectrum of the reaction product of G25-fraction, Cypridina luciferyl sulfate, PAP, and acetic acid; **c**, MSMS spectrum of *m/z* 139 in the reaction product of G25-fraction, Cypridina luciferyl sulfate, PAP, and acetic acid. **B. a**, Chemical synthesis of acetyl sulfate from acetic acid; **b**, Mass spectrum of chemically synthesized acetyl sulfate; **c**, Predicted mass fragmentation of acetyl sulfate. MS/MS spectrum of chemically synthesized acetyl sulfate. **C. a**, Mass spectrum of the reaction product of G25-fraction, PAPS, and acetic acid; **b,** MS/MS spectrum of *m/z* 139 in the reaction product of G25-fraction, PAPS, and acetic acid.

3.6. Chemical synthesis of acetyl sulfate

Acetyl sulfate as an authentic compound for mass spectrometry was chemically synthesized from acetic acid according to the similar procedures as previously reported [9,20]. Acetic acid was treated with sulfur trioxide pyridine complex in CH_2Cl_2 at 25 °C for 1 h to give acetyl sulfate (Fig. 3B–a). Acetyl sulfate dissolved in CH_3CN can be detected by mass spectrometry; however, the signal peaks of acetyl sulfate were not detected when the samples were dissolved in H_2O or methanol, indicating that acetyl sulfate is unstable in aprotic solvents and may be easily hydrolyzed to acetic acid and sulfate ion (SO_4^{2-}) in the aqueous solution. For this reason, the chemically synthesized acetyl sulfate was directly subjected to ESI-TOF-MS without further purification. The mass spectrum of acetyl sulfate showed a peak at *m/z* 138.9701 (Fig. 3B–b), and the MS/MS spectra of acetyl sulfate were identical to those of acetyl sulfate obtained from the reaction mixture containing G25-fraction, Cypridina luciferyl sulfate, PAP and acetic acid solution

in 0.5 M glycine-NaOH (pH 7.5) (Fig. 3B–c).

3.7. Enzymatic conversion of acetic acid to acetyl sulfate using G25-fraction and PAPS

To confirm the formation of acetyl sulfate by transferring the sulfate group of PAPS to acetic acid using G25-fraction of crude extracts, the acetic acid solution was incubated with G25-fraction and PAPS in 0.5 M glycine-NaOH (pH 7.5) and the reaction product was analyzed by MS and MS/MS spectrometry. The products showed an MS/MS spectrum identical to that of the chemically synthesized acetyl sulfate (Fig. 3C–a and b). These results suggest that acetic acid could be converted to acetyl sulfate by G25-fraction and PAPS, even though an enzyme such as a sulfotransferase was not isolated from *Cypridina* specimens.

3.8. Prediction of enzymatic cycling of Cypridina luciferyl sulfate to Cypridina luciferin coupled with acetic acid and PAPS

Based on our results, we propose an enzymatic recycling system for the sulfation of acetic acid using crude extracts of *Cypridina* specimens, as shown in Fig. 4. The sulfate group of Cypridina luciferyl sulfate was transferred to PAP to produce Cypridina luciferin and PAPS, followed by the transfer of the sulfate group of PAPS to acetic acid to give acetyl sulfate and PAP. Since acetyl sulfate was unstable in aqueous solutions, it was hydrolyzed to acetic acid and sulfate ion (SO_4^{2-}). Therefore, the conversion of Cypridina luciferyl sulfate to Cypridina luciferin by the coupled reaction of acetic acid and PAPS in crude extracts of *Cypridina* specimens might be irreversibly desulfation cycle.

3.9. Stimulation of luminescence activity by other acidic chemicals in the mixture containing G25-fraction, Cypridina luciferyl sulfate, and PAP

In the presence of phosphoric acid, the luminescence intensity of crude extracts of *Cypridina* specimens was stimulated more than that with acetic acid, and the stimulation with phosphoric acid was an enzymatic reaction, similar to that of acetic acid (Fig. S3). Thus, phosphoric acid may act as an efficient acceptor of the sulfate group from PAPS. The addition of phosphoric acid to crude extracts stimulated the luminescence activity in the presence of PAPS. The predicted product from phosphoric acid might be sulfophosphoric acid, but not detected by mass spectrometry. Presumably, sulfophosphoric acid is unstable in aqueous solutions. On the other hand, the addition of phosphoric acid at pH 7.0 to the reaction mixture of glycine-NaOH buffer containing crude extracts and PAP stimulated the luminescence activity (Fig. S1). This stimulation might be occurred in general phosphate buffer.

Further, the luminescence stimulation with other acid chemicals, and several carboxylic acids was examined (Fig. S4). Among several carboxyl acids, maleic acid, 3-methylcrotonic acid, and isopropylmalonic acid effectively stimulated the luminescence activity (Fig. S5). These compounds may be sulfated by the similar manner to that of acetic acid, even though the products of these sulfated compounds have not been identified, because of their instability or difficulty in purification. Presumably, these carboxylic acids or phosphoric acid may act as sulfate acceptors from PAPS by a sulfotransferase in the G25-fraction of *Cypridina* specimens.

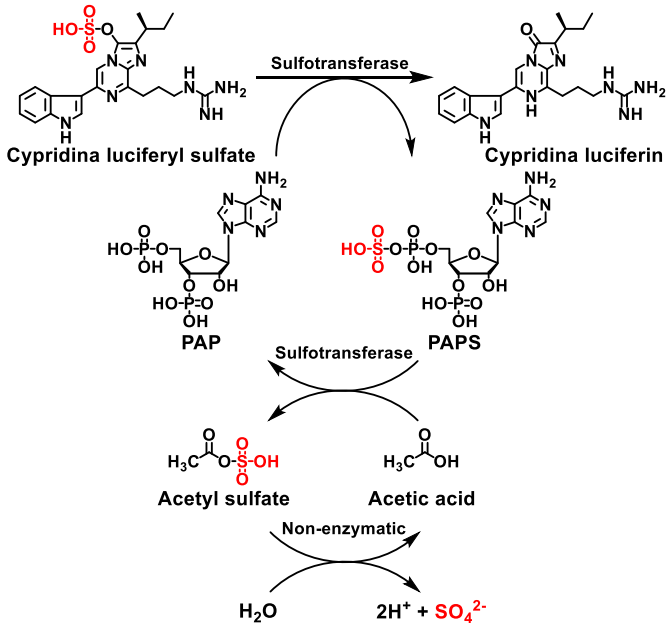

Fig. 4. Proposed mechanism of the luminescence stimulation by acetic acid in the reaction mixture containing G25-fraction, Cypridina luciferyl sulfate, and PAP.

4. Conclusion

The luminescence intensity of the reaction mixture containing Cypridina luciferyl sulfate, PAP, and crude extracts of *Cypridina* specimens was significantly stimulated by acetic acid. This luminescence stimulation may be explained by the enzymatic cycling as follows: the sulfate group of Cypridina luciferyl sulfate was transferred to PAP, giving PAPS and Cypridina luciferin, and then the sulfate group of PAPS was transferred to acetic acid to give acetyl sulfate and PAP. The resultant acetyl sulfate was decomposed into acetic acid and sulfate ion in water, and PAP was recycled to be reused as a sulfate acceptor from Cypridina luciferyl sulfate to produce Cypridina luciferin for the luciferase reaction.

Declaration of competing interest

The authors have declared no financial interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2020.05.167.

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