Bioorganic & Medicinal Chemistry Letters 18 (2008) 5657–5659

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Pholasin luminescence is enhanced by addition of dehydrocoelenterazine

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ARTICLE INFO

Article history:
Received 17 July 2008
Revised 22 August 2008
Accepted 23 August 2008
Available online 7 September 2008

Keywords:
Pholasin
Dehydrocoelenterazine
Prosthetic group
Chromophore
Luminescence

ABSTRACT

Pholasin is a bioluminescent photoprotein of *Pholas dactylus*. Pholasin is a commercially available photoprotein used to measure intracellular reactive oxygen species. Although extensive efforts have been carried out to determine the chemical structure of the prosthetic (chromophore) group, it still remains unclear to date. Herein, we report the enhancement of pholasin luminescence by the addition of dehydrocoelenterazine, which is organic substance of luminous squids' photoprotein.

Pholasin is a bioluminescent photoprotein of *Pholas dactylus*, which is a marine, rock-boring, bivalve mollusk. Since the discovery of its luciferin–luciferase reaction by Dubois,[1] many efforts have been done in order to analyze its bioluminescent mechanism.[2,3] Pholasin is now commercially available from Knight Scientific Ltd. (Plymouth, UK), and Pholasin® is the registered trade mark of Knight Scientific Ltd. Since pholasin luminescence is strongly activated by reactive oxygen species (ROS), pholasin has been used to measure the activities of intracellular peroxidase and myeloperoxidase.[4–8] Pholasin has a prosthetic group which exhibits luminescence and the prosthetic group covalently binds to protein. Müller et al. studied the prosthetic group of pholasin and reported that coelenterazine (**1**) and *Vargula* luciferin (**2**) could not activate pholasin luminescence.[9] They concluded that a flavin moiety (**3**) might be involved in the pholasin bioluminescence by using a fluorescent spectroscopy (Fig. 1). However, precise structure of the prosthetic group of pholasin was not determined and remained unclear. Due to the ambiguous structure of the prosthetic group, recombinant pholasin has not been activated as native pholasin[10].

As mentioned by Shimomura,[11] luminescence of photoproteins obtained from luminous squids (*Symplectoteuthis luminosa* and *Symplectoteuthis oualaniensis*), were also strongly activated by the addition of ROS prepared from the combination of hydrogen peroxide and catalase. These photoproteins also possessed a prosthetic group, which bounded to photoprotein. The chromophore was comprised of dehydrocoelenterazine (DCL) (**4**) (Fig. 1) and apophotoprotein. This similar mechanism in the activation of luminescence between pholasin and *Symplectoteuthis* luminescence prompted us to investigate whether DCL (**4**) had any effect on pholasin luminescence.

Our strategy was simple, thus we only investigated whether pholasin luminescence was enhanced by the addition of DCL (**4**) to pholasin solution.[12] We assayed for pholasin with and without DCL (**4**) by following the protocol of Pholasin® ABEL®-21 Kit (Scheme 1).[13] As shown in Graph 1, luminescence of pholasin with DCL (**4**) resulted in three times higher light intensity than that of pholasin without DCL (**4**). Both luminescence of pholasin with and without DCL (**4**) finished within 5 min, and total light yield of pholasin with DCL (**4**) gave four times higher light yield than that of pholasin without DCL (**4**) in 5 min (Graph 2). Without pholasin, DCL (**4**) did not yield any light in the buffer, thus DCL (**4**) required pholasin to emit light.

We then investigated optimum amount of DCL (**4**) required to enhance pholasin luminescence. Increase in amount of DCL (**4**) did not affect enhancement of pholasin luminescence (Graph 3). We also noticed that increase in volume of DMSO did not show any inhibitory effect for pholasin luminescence.

To verify whether 4.0 mM concentration is the optimal concentration of DCL (**4**) required, we investigated DCL (**4**) effect on pholasin luminescence below 4.0 mM. As shown in Graph 4, pholasin luminescence was dependent of the concentration of DCL (**4**). Decreasing concentration of DCL (**4**) from 4.0 mM caused the decrease in the total light yield of pholasin luminescence. Below 2.0 mM, DCL (**4**) did not show any enhancement of pholasin luminescence. Thus, we proved that luminescence of commercially available Pholasin® ABEL®-21 Kit (100 μL) was enhanced by addition of dehydrocoelenterazine (2.0 μL of 4.0 mM DMSO).

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doi:10.1016/j.bmcl.2008.08.113

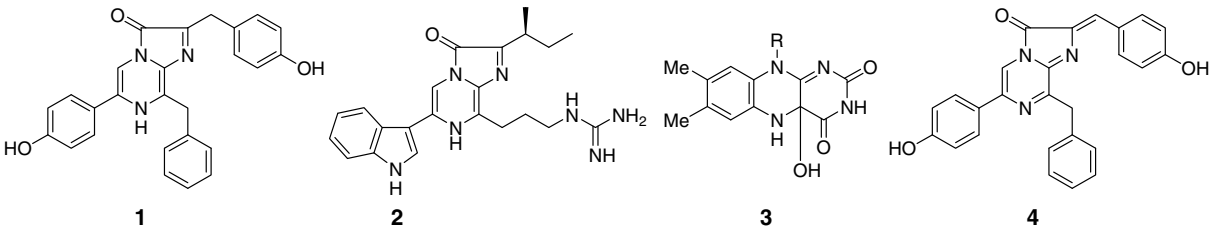
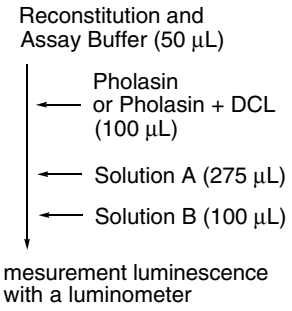
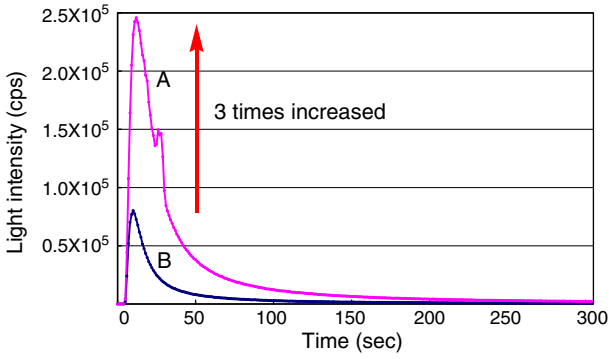


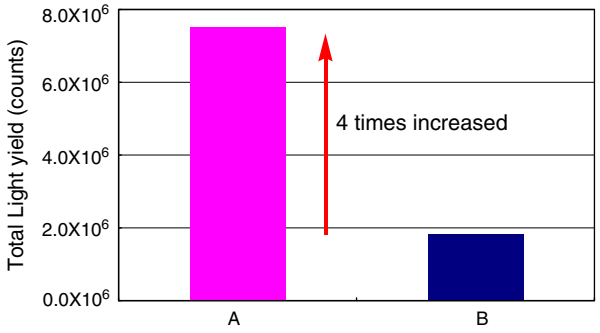
Figure 1. Structure of coelenterazine (**1**), *Vargula* luciferin (**2**), 4a-hydroxyflavin (**3**), and dehydrocoelenterazine (**4**).



Scheme 1. Assay for pholasin luminescence with and without DCL (**4**).

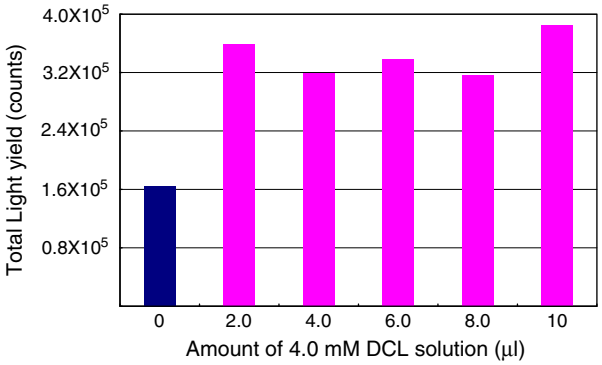


Graph 1. Time course of pholasin luminescence with DCL (A) and without DCL (B).

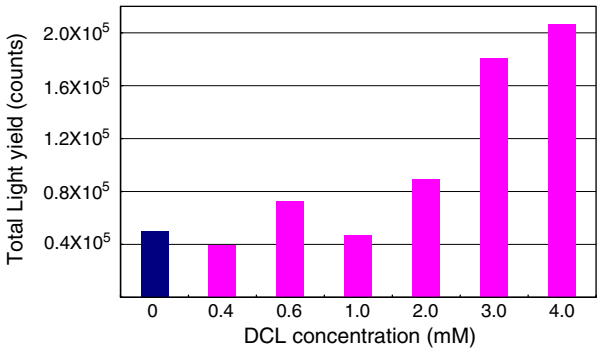


Graph 2. Total light yield of pholasin luminescence in 5 min with DCL (A) and without DCL (B).

During these studies, we noticed that the light intensity of pholasin luminescence weakened over time, although we followed the kit's manual by storing our pholasin solution in a refrigerator at 3 °C. Koppenol group reported that pholasin® solution could be used during 1 day[5], thus, we investigated the decrease in activity of pholasin for further days. As shown in Graphs 5 and 6, light intensity



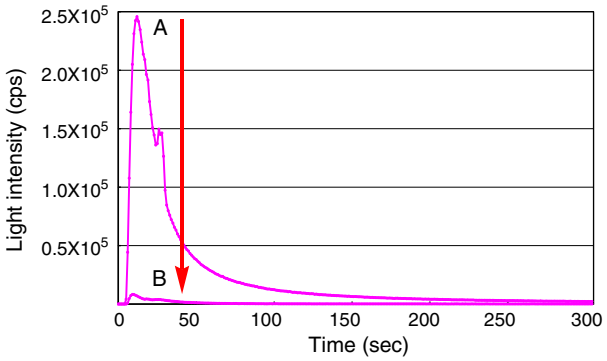
Graph 3. Total light yield of pholasin luminescence against amount of DCL (**4**) over 4.0 mM. Numbers on *X*-axis show amount of 4.0 mM DCL (**4**) in DMSO (μL) added to pholasin solution (100 μL).



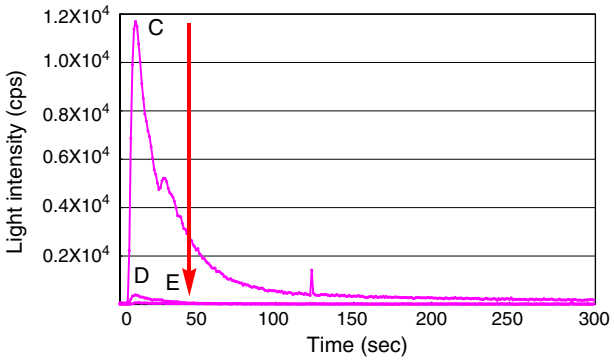
Graph 4. Concentration dependence of pholasin luminescence against DCL (**4**) below 4.0 mM.

of pholasin luminescence weakened after dissolving in reconstitution buffer. After 1 day, pholasin luminescence decreased by 5% of the freshly reconstitute pholasin. After 3 days, the luminescence was still enough for measurement. However, it was not easy to monitor time course luminescence after 6 days. We need to derive a condition to effectively store pholasin solutions without losing their luminescent activities in our further studies.

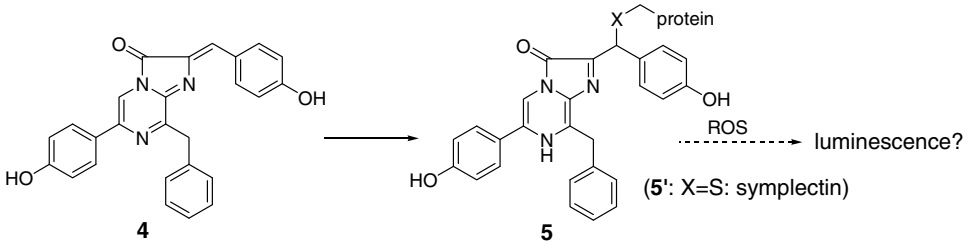
By addition of DCL (**4**) to pholasin, we observed the enhancement of pholasin luminescence. We confirmed that this luminescence was not caused by a mixture of the buffers and DCL (**4**) based on our controlled experiment. However, we could not conclude that DCL (**4**) is a true organic substance required to form a prosthetic group of pholasin. This is because there is a possibility that pholasin luminescence with DCL (**4**) might occur with different luminescent mechanism from its original mechanism. It is known that DCL (**4**) itself could not react with any ROS to show luminescence, since DCL (**4**) is oxidized product of coelenterazine (**1**).[14] In order to react with ROS, DCL (**4**) needs to be converted



Graph 5. Time course of pholasin luminescence in the presence of DCL (**4**) at the same day (A) when pholasin was reconstituted, and 1 day (B) after pholasin was reconstituted.



Graph 6. Time course of pholasin luminescence in the presence of DCL (**4**) at 2 days (C), 6 days (D), and 7 days (E) after pholasin was reconstituted. Peak max of light intensities of C and B of Graph 5 are almost the same value.



Scheme 2. Plausible chromophore (**5**) for pholasin luminescence with DCL (**4**).

to the reduced form like a chromophore (**5**), which is similar to the chromophore (**5′**) of symplectin (photoprotein of *Symplectoteuthis oualaniensis*)[15] (Scheme 2). We now postulate that DCL (**4**) might react with an amino acid residue of pholasin to form chromophore (**5**) to be luminescence.

Only photoproteins of *Symplectoteuthis* species are recognized as photoproteins that use DCL (**4**) as organic substance to form chromophore. If DCL (**4**) is the organic substance for pholasin, this might imply that pholasin is the second example of photoprotein, which shows luminescence by forming a chromophore with DCL (**4**). Pholasin could also use DCL (**4**) as an organic substance, since both photoproteins show strong luminescence in the presence of ROS.

Our finding that pholasin luminescence is enhanced by addition of DCL (**4**) could be useful as recombinant pholasin might be activated with addition of DCL (**4**). Increase in pholasin luminescence could be useful to measure intracellular ROS with higher sensitivity. We could observe three to four times dilute ROS with only a simple combination of pholasin and DCL (**4**).

In conclusion, we observed the enhancement of pholasin luminescence by simple addition of DCL (**4**). This could be useful to activate recombinant pholasin and to assay for ROS with higher sensitivity. Further investigation of this enhance mechanism of pholasin luminescence by addition of DCL (**4**) is underway.

Acknowledgments

We acknowledge the financial support to M.K. from Grant-in-Aid for Young Scientists (B) [19780087 (2007)] from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan. Authors are extremely grateful to Dr. Duanpen Lertpibulpanya for proofreading this manuscript.

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12. Pholasin® ABEL®-21 Kit (antioxidant test kit with pholasin for superoxide and other free radicals), supplied by Knight Scientific Ltd. (Plymouth, UK), was purchased from Funakoshi Co., Ltd. (Japan). The kit contains freeze-dried pholasin, reconstitution and assay buffer, solution A, and solution B (We could not obtain the detail of these buffer such as components, pH, and concentration of buffer, thus we just followed the procedure of the kit.). The pholasin solution was stored in a refrigerator at 3 °C after dissolving in the reconstitution and assay buffer (5 mL). DCL (**4**) was synthesized by using reported and improved method[14–16] and was dissolved in dimethylsulfoxide (DMSO) to make up a 4.0 mM solution. This solution was stored in a freezer at −20 °C.
13. To a solution of pholasin (100 μL) was added an aliquot (2 μL) of DCL (**4**) solution (4.0 mM in DMSO) cooled with ice water. The resultant solution was incubated at 4 °C for 30 min. Then, to the solution of pholasin with and without DCL (**4**), the solution A (275 μL) and the solution B (100 μL) from the kit[12] were added to initiate luminescence (the ratio of these buffers was slightly modified to save pholasin consumption in our experiments). The resulting luminescence was recorded on a luminometer (ATTO AB-2200 luminescencer) for 5 min at room temperature.
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