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Graphical Abstract

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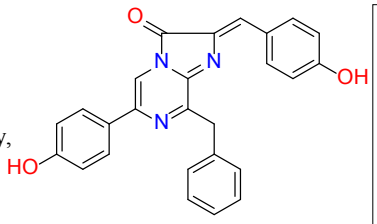
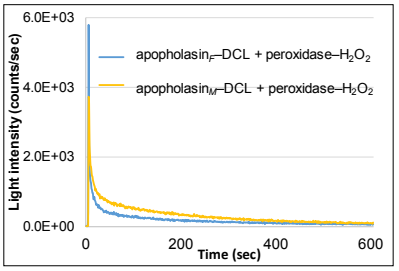


dehydrocoelenterazine



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ABSTRACT

Pholasin is a photoprotein derived from the glowing bivalve mollusk, *Pholas dactylus*. Even though the chemical structure of the prosthetic group (chromophore) responsible for the light emission character of the mollusk remains unknown, research has shown that the presence of dehydrocoelenterazine (DCL) increased light emission and that the dithiothreitol adduct of DCL was isolated from Pholasin®. To date, our research has been focused on activating apopholasin, the naturally occurring apoprotein of Pholasin®, using DCL. In the current study, the expression of recombinant apopholasin via a baculovirus–silkworm multigene expression system is reported. Additionally, the purification of apopholasin using a Flag®-affinity column, the activation of apopholasin using DCL, and the initiation of its luminescent character through the addition of a peroxidase–hydrogen peroxide mixture are reported. The proxidase–H_2O_2-dependent luminescence was observed from the recombinant apopholasin activated with DCL.

Introduction

Pholasin is a photoprotein isolated from the glowing bivalve mollusk, *Pholas dactylus*.[1,2] It is also commercially available from cultured *P. dactylus* sources[3] and is often used for the analysis of reactive oxygen species and antioxidants.[4] The luciferin–luciferase reaction is responsible for the bioluminescence seen in *P. dactylus*.[5] Since luciferase is a copper-containing peroxidase and pholasin is a type of luciferin, the oxidation of pholasin by luciferase in the presence of oxygen results in the emission of a blue light.[6] It is well-known that pholasin has a prosthetic group (chromophore) that is responsible for its light-emitting properties.[7] Despite this, the precise chemical structure of the above-mentioned chromophore is still a mystery. In 2008, it was discovered that the addition of dehydrocoelenterazine (DCL) to Pholasin® increased its light production, thus making DCL an active organic substance for Pholasin®.[8] Additionally, the isolation and identification of the dithiothreitol (DTT) adduct of DCL (DCL–DTT) were performed by mixing Pholasin® with DTT.[9] Since these results implied that there must be a DCL or DCL derivative of Pholasin®, it was theorized that the photoprotein's chromophore contained a DCL-related moiety and was similar to the chromophore of symplectin,[10] a photoprotein derived from the flying squid *Symplectoteuthis oualaniensis/Sthenoteuthis oualaniensis*[11] (Fig. 1). The chromophore of symplectin is produced by the binding of DCL to a cysteine residue in the apoprotein of symplectin (aposymplectin).[10g]

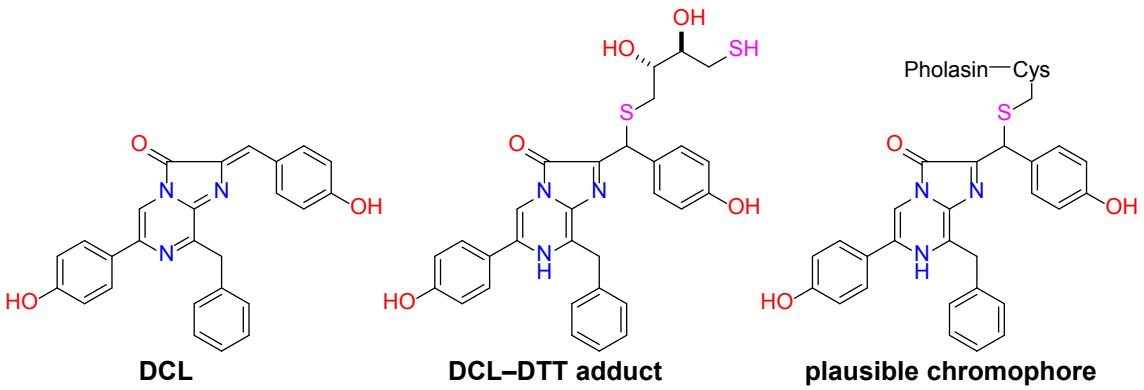
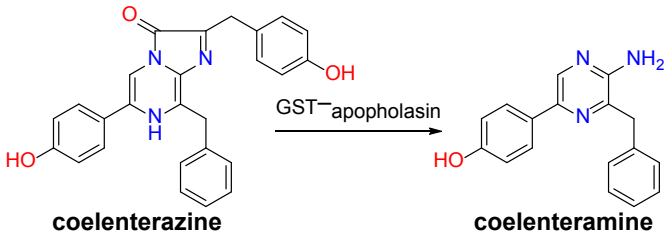


Fig. 1. Structures of dehydrocoelenterazine (DCL), the adduct of DCL with dithiothreitol (DCL–DTT adduct), and a plausible structure for the chromophore of pholasin.

As noted previously, efforts are underway to determine the exact chemical structure of the chromophore responsible for luminescence. In 2000, Campbell *et al.* reported a process for cloning and expressing apopholasin, the apoprotein of pholasin. Here, recombinant apopholasin emitted light when the apoprotein was mixed with a methanolic extract of *P. dactylus*.[12] Recently, Inoue *et al*. reported that a fusion protein consisting of glutathione *S*-transferase and apopholasin (GST–apopholasin) showed luciferase-like activity for the oxidation of coelenterazine into coelenteramine while emitting a blue light (Scheme 1).[13] In their system, DCL did not afford any light emission.



Scheme 1. Luciferase reaction catalyzed by GST–apopholasin.

Thus far, our research interest has been focused on activating the recombinant apopholasin using DCL as a possible substance for Pholasin®. Here, we report the expression of apopholasin, the preparation of active pholasin by incubation with DCL, and a method for initiating light emission of the apopholasin–DCL adduct complex (hereafter referred to as apopholasin–DCL to distinguish it from *Pholas*-derived Pholasin®).

Materials and Methods

Expression and purification of apopholasin

The expression and purification of apopholasin were induced in a baculovirus–silkworm multigene expression system produced by ProCube™ (Sysmex Corporation; http://procube.sysmex.co.jp/eng/).[14] Briefly, two kinds of genes were synthesized as templates for apopholasin, namely, a full-length pholasin gene (675 bp) and a matured pholasin gene (615 bp) from Eurofins Scientific (https://www.eurofins.com/) and Gene Script (https://www.genscript.com/). The full-length pholasin gene and the matured pholasin gene were obtained according to the protocol reported by Campbell *et al*.[12] The above-mentioned genes, which encoded for Met1–Trp225 (full-length) and Glu21–Trp225 (mature), were amplified via PCR using primers (forward: 5′-ATAAATAGATCTCCCATGGCTTGCATCGTGTTCG-3′ and reverse: 5′-CAGAACTTCCAGCCCCCAGAAGAACTCGCCAGTC-3′ designed for the full length apopholasin, forward: 5′-ATAAATAGATCTCCCATGGAGGAAGTCCAATGTGCCATGAAC-3′ and reverse: 5′-CAGAACTTCCAGCCCCCAGAAGAACTCGCCAGTCTCGCTC-3′ designed for the matured apopholasin) from synthesized gene templates with an optimized codon usage for insect. The PCR products were inserted into the pHS04 transfer vector (Sysmex Corporation) at *Sma*I site using the In-Fusion® HD cloning kit (Clontech). The pHS04 is a modified version of the vector pUC19 for *B. mori* nucleopolyhedrovirus (BmNPV) and designed to express a fusion protein containing histidine (His)-tag and Flag®-tag at the C-terminal fragment of apopholasin. These transfer vectors were co-transfected with BmNPV DNA into a *B. mori*-derived cell line (BmN). After seven days of incubation, the recombinant baculoviruses were injected into the body of silkworm pupae. The five pupae were harvested six days after infection, frozen, and then homogenized with PBS buffer (25 mL, pH 7.4) containing a protease inhibitor cocktail. The homogenized solutions were centrifuged to separate the supernatant from the precipitate using an ultracentrifuge (100,000 g) for 1 h at 4 °C. The supernatants containing the His-tag and Flag®-tag fusion apopholasin (full-length and mature) were applied to the DDDDK-tagged protein purification gel (0.35 mL) (MBL Co., Ltd.), and the fusion proteins were eluted with Flag® peptide containing PBS buffer (1.5 mL, pH 7.4). The eluted fractions were used to activate with DCL. The concentration of these apoproteins was estimated to be 0.1–0.2 mg/mL based on the absorbance levels seen at 280 nm using a NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific).

Activation of apopholasin using DCL and quantification of its luminescence

Apopholasin (100 µL) was mixed with DCL (2 µL, 2 mM in DMSO) that had been previously synthesized according to the reported methods.[15] The resulting solution was kept in an ice bath for 1 h to activate apopholasin. Once activated, apopholasin–DCL (10 µL) was placed in an ATTO AB-2200 luminometer, and a mixture of horseradish peroxidase (10 µL, 5 mg/mL in 10 mM of Na_2HPO_4 buffer, pH 8.5; TCI, lyophilized, powder, 260 U/mg) and hydrogen peroxide (200 µL, 5% in 10 mM of Na_2HPO_4 buffer, pH 8.5) was added. The subsequent light emission process was recorded for 10 min. This reaction was performed for both full-length and mature apopholasin. The luminometer was calibrated with luminol; 1 count was estimated to be 8.7×10^3 photons.[16]

Results and Discussion

Full-length apopholasin (apopholasin$_F$) consists of 225 amino acids with a signal peptide from Met1 to Gly20, whereas the matured apopholasin (apopholasin$_M$) corresponds to the region between Glu21 and Trp225. Since there are seven cysteine amino acids in apopholasin$_M$, it is likely that one of the cysteine amino acids might bind to DCL to form the pholasin chromophore. For purification using a DDDDK-tagged protein purification gel, Flag®-tag was placed at the C-terminal of the respective apopholasin (Fig. 2).

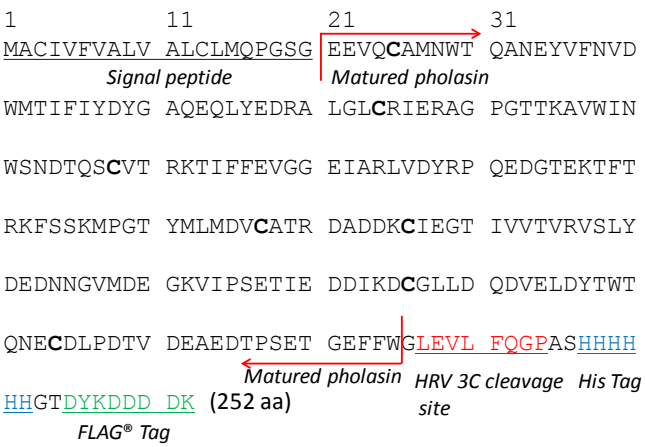


Fig. 2. Amino acid sequence for apopholasin with cleavage by HRV 3C protease at the histidine (His) tag and the Flag®-tag of the C-terminal. Apopholasin$_F$ has a signal peptide (Met1–Gly20). The seven cysteine amino acids in apopholasin$_M$ are highlighted in bold.

Additionally, the preparation of two nucleotides that had been optimized for insect allowed for expression in the baculovirus–silkworm multigene system (Figs. 3 and 4). The recombinant apopholasin$_F$ and apopholasin$_M$ were successfully expressed in the baculovirus–silkworm multigene system, and purification was conducted using a DDDDK-tagged protein purification gel. The recombinant apoholasins were eluted with the Flag® peptide containing a PBS buffer at pH 7.4 (Figs. 5 and 6). The purity of the apopholasins was estimated to be 78% for apopholasin$_F$ and 45% for apopholasin$_M$, and this was determined using a calibrated densitometer (Bio-Rad) based on the bands of the SDS–PAGE. From these results, we noticed that apopholasin$_F$ was more efficiently expressed than apopholasin$_M$ in the baculovirus–silkworm multigene system. Additionally, the purity of the apopholasin$_F$ fraction was much higher than that of the apopholasin$_M$ fraction.

```
1                16                31                36
ATGGCTTGCATCGTG TTCGTTGCTCTGGTT GCTCTGTGCTTGATG CAGCCAGGTTCTGGC
GAGGAAGTCCAATGT GCCATGAACTGGACA CAAGCCAATGAGTAC GTATTCAACGTGGAC
TGGATGACCATCTTC ATCTACGACTATGGA GCCCAAGAACAGCTC TACGAAGATCGTGCT
CTCGGTTTGTGTAGG ATTGAACGCGCTGGT CCTGGTACGACTAAA GCCGTGTGGATTAAC
TGGAGCAACGACACT CAGTCATGCGTGACC AGGAAAACCATCTTC TTCGAAGTTGGAGGA
GAGATTGCGAGACTA GTGGACTATCGACCT CAAGAGGACGGTACT GAGAAAACCTTTACG
CGCAAGTTTTCGTCC AAGATGCCAGGAACG TACATGCTCATGGAT GTCTGTGCAACACGC
GATGCAGACGACAAG TGCATAGAGGGTACA ATTGTCGTTACTGTC CGTGTGTCCCTTTAC
GACGAGGACAACAAT GGCGTCATGGATGAA GGCAAGGTAATCCCC AGTGAAACGATAGAG
GACGACATCAAGGAC TGTGGCTTGCTGGAC CAGGATGTGGAACTG GATTACACCTGGACC
CAGAATGAATGCGAT TTACCCGACACAGTA GATGAGGCGGAAGAT ACACCGAGCGAGACT
GGCGAGTTCTTCTGG GGGCTGGAAGTTCTG TTCCAGGGGCCCGCT AGCCATCATCATCAT
CATCATGGTACCGAC TACAAGGACGACGAT GACAAATAG
```

Fig. 3. Nucleotide sequence for apopholasin$_F$ optimized for insect with a signal peptide at the N-terminal (underlined), and cleavage sites were created by the HRV 3C protease (red) at the His-tag (blue) and Flag®-tag (green) of the C-terminal. The sequence for matured apopholasin, which starts from GAG to TGG, is highlighted with the red box.

```
1                16                31                36
ATGGAGGAAGTCCAA TGTGCCATGAACTGG ACACAAGCCAATGAG TACGTATTCAACGTG
GACTGGATGACCATC TTCATCTACGACTAT GGAGCCCAAGAACAG CTCTACGAAGATCGT
GCTCTCGGTTTGTGT AGGATTGAACGCGCT GGTCCTGGTACGACT AAAGCCGTGTGGATT
AACTGGAGCAACGAC ACTCAGTCATGCGTG ACCAGGAAAACCATC TTCTTCGAAGTTGGA
GGAGAGATTGCGAGA CTAGTGGACTATCGA CCTCAAGAGGACGGT ACTGAGAAAACCTTT
ACGCGCAAGTTTTCG TCCAAGATGCCAGGA ACGTACATGCTCATG GATGTCTGTGCAACA
CGCGATGCAGACGAC AAGTGCATAGAGGGT ACAATTGTCGTTACT GTCCGTGTGTCCCTT
TACGACGAGGACAAC AATGGCGTCATGGAT GAAGGCAAGGTAATC CCCAGTGAAACGATA
GAGGACGACATCAAG GACTGTGGCTTGCTG GACCAGGATGTGGAA CTGGATTACACCTGG
ACCCAGAATGAATGC GATTTACCCGACACA GTAGATGAGGCGGAA GATACACCGAGCGAG
ACTGGCGAGTTCTTC TGGGGGCTGGAAGTT CTGTTCCAGGGGCCC GCTAGCCATCATCAT
CATCATCATGGTACC GACTACAAGGACGAC GATGACAAATAG
```

Fig. 4. Nucleotide sequence for apopholasin$_M$ optimized for insect with cleavage by HRV 3C protease (red) at the His-tag (blue) and the Flag®-tag (green) of the C-terminal. The sequence for matured apopholasin, which starts from GAG to TGG, is highlighted with the red box.

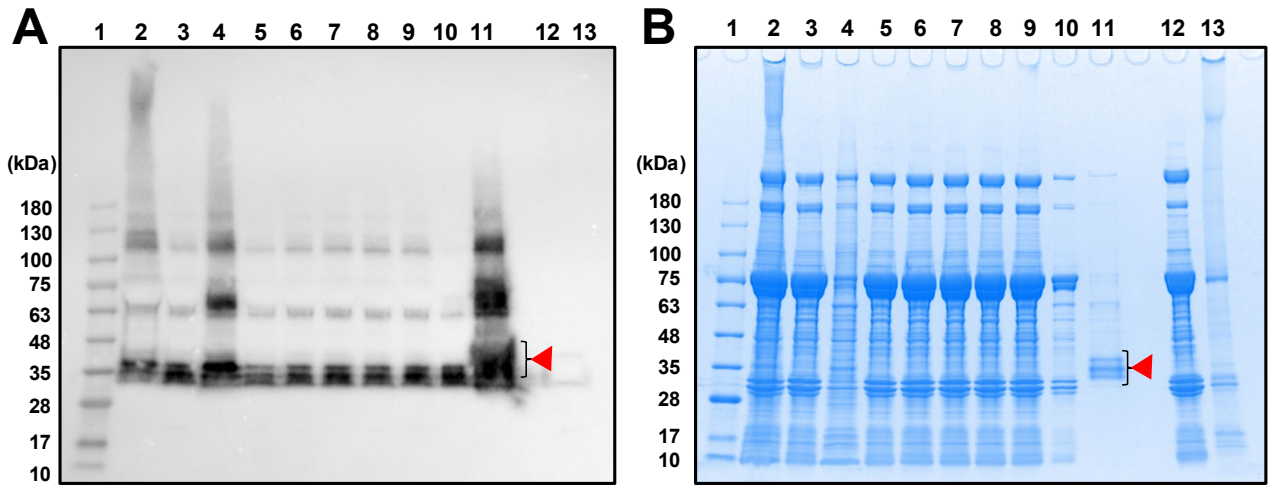


Fig. 5. SDS–PAGE for apopholasin$_F$, where the target apopholasin is indicated with the red mark. A) western blotting with monoclonal Anti-Flag® M2–Peroxidase (HRP) (Sigma-Aldrich). B) CBB stained; where Lane 1, marker for the molecular weight; Lane 2, homogenate of the pupae; Lane 3, supernatant fraction of the pupae homogenate; Lane 4, precipitate fraction of the pupae homogenate; Lane 5–9: the flow-through fractions from the resin; Lane 10, the washed fraction from the resin; Lane 11, the eluted fraction from the resin; Lane 12, negative control (supernatant fraction of the uninfected pupae homogenate); Lane 13, negative control (precipitate fraction of the uninfected pupae homogenate).

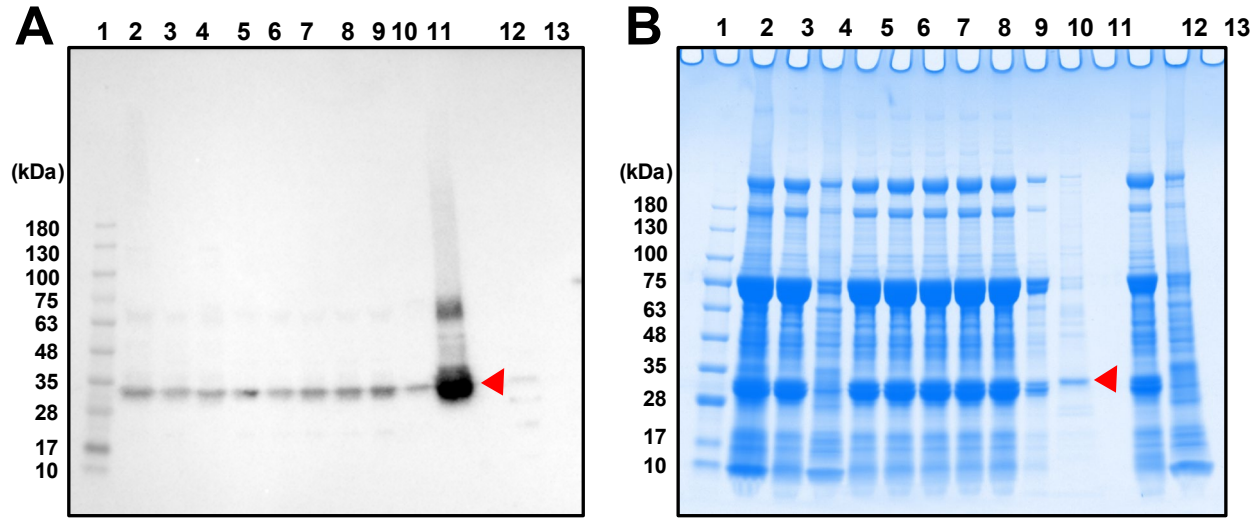


Fig. 6. SDS–PAGE of apopholasin$_M$ and target apopholasin was indicated with a red mark. A) western blotting with monoclonal Anti-Flag® M2–Peroxidase (HRP) (Sigma-Aldrich). B) CBB stained, where Lane 1, marker for the molecular weight; Lane 2, homogenate of the pupae; Lane 3, supernatant fraction of the pupae homogenate; Lane 4, precipitate fraction of the pupae homogenate; Lane 5–9: the flow-through fractions from the resin; Lane 10, the washed fraction from the resin; Lane 11, the eluted fraction from the resin; Lane 12, negative control (supernatant fraction of the uninfected pupae homogenate); Lane 13, negative control (precipitate fraction of the uninfected pupae homogenate).

The apopholasins were incubated with DCL for 1 h in an ice bath in order to prepare the respective apopholasin–DCL. This was done to see if DCL-based activation of the apopholasins was possible. First, a mixture of solutions A and B from a Pholasin®-based ABEL® kit was added to the respective apopholasin–DCL complex since, generally, the luminescence of Pholasin® is initiated by the addition of this A–B mixture from the ABEL® kit. However, since light emission was not observed at all, it was theorized that the conformation adopted by the apopholasins was not ideal for the relevant light emission processes. Then, the recombinant apopholasins had been treated with DTT or subjected to heating and annealing procedures in the presence or absence of DTT to obtain active apopholasins. After incubation with DCL, the results obtained remained unchanged, i.e., light emission was not observed. In trying to obtain active apopholasin–DCLs, we determined that the addition of a horseradish peroxidase–hydrogen peroxide mixture would lead to the activation of the apopholasin–DCL complexes (Fig. 7).

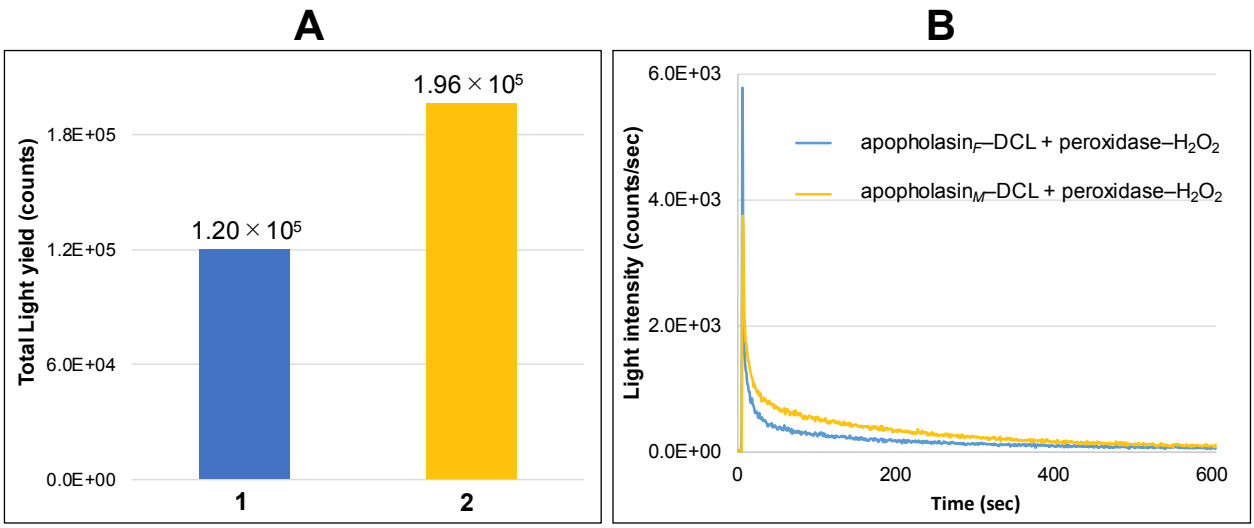


Fig. 7. Total light yields (A) and time-course of luminescence (B) for 10 min of apopholasin–DCLs. A: (1) apopholasin$_F$–DCL + peroxidase and H_2O_2. (2) apopholasin$_M$–DCL + peroxidase and H_2O_2. B: time-course of light emission for apopholasin$_F$–DCL (blue) and apopholasin$_M$–DCL (yellow). Each apopholasin–DCL was placed in a luminometer in order to quantify the amount of light emitted from the sample. After 5 s, a peroxidase–H_2O_2 mixture was quickly injected, and the resulting light emission was monitored for 10 min. The experiments were conducted in triplicate.

Both the DCL-incubated apopholasin$_F$ and apopholasin$_M$ emitted intense light when a peroxidase–H_2O_2 mixture was added, and the resulting luminescence was noted for 10 min before gradually decreasing. From these results, it was clear that luminescence was dependent on the presence of the apopholasins as DCL alone did not result in any light emission when the peroxidase–H_2O_2 mixture was added. This trend was also seen during the activation of commercially available Pholasin®. The luminescent profile of commercially available Pholasin® incubated with DCL resembled those of the apopholasin–DCLs (Fig. 8).

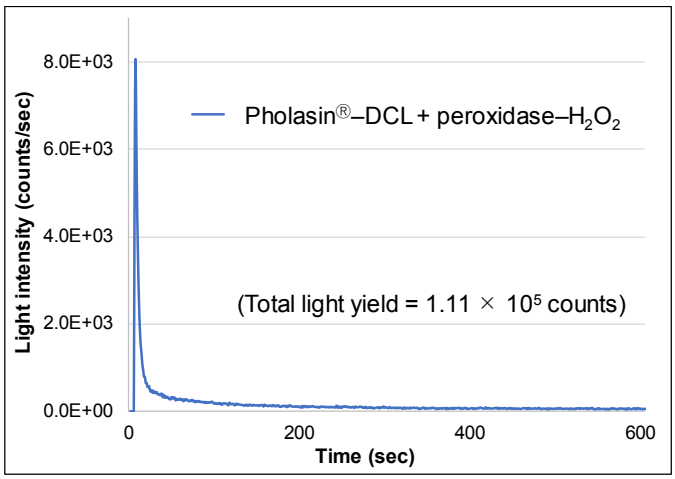


Fig. 8. Time-course of luminescence of Pholasin® after injection of the peroxidase–H_2O_2 mixture. DCL-incubated Pholasin® (10 µL) was placed in a luminometer to quantify the amount of light emitted from the sample. After 5 s, the peroxidase–H_2O_2 mixture (200 µL) was quickly injected, and the resulting light emission was monitored for 10 min. The experiments were conducted in triplicate.

From these results, it was theorized that luminescence in Pholasin® would be produced if similar activators were used, i.e., incubation with DCL and activation via an enzyme–peroxide mixture. The catalase–H_2O_2 mixture is known to cause luminescence in symplectin.[17] However, this mixture failed to trigger luminescence strongly in the apopholasin–DCL complex. Another peroxide, *tert*-butyl hydroperoxide (TBHP), was also tested, but light emission was poor even in the presence of peroxidase or catalase activators (Fig. 9). From these results, it was clear that a peroxidase–hydrogen peroxide mixture specifically initiated the luminescent character of apopholasin–DCL.

The luciferin–luciferase reaction in *Pholas dactylus* is known to be responsible for the mollusks' light emission characteristics since pholasin is a type of luciferin and luciferase is a copper-containing peroxidase.[6a] Reichl *et al*. reported that the oxidation of Pholasin® by compound I or II of horseradish peroxidase induced an intense light emission.[18] Thus, the use of a recombinant apopholasin and DCL was simply a reproduction of this biological system. However, a complete luminescent profile for the apopholasin–DCL complexes has not been determined, because a sufficient amount of apopholasins had not been obtained. The task of developing a complete luminescent profile for apopholasin–DCL is now underway as we are in the process of collecting protein starting material and cleaving the relevant His- and Flag®-tags using HRV 3C protease. Since Pholasin® is a glycoprotein,[6a,19] apopholasin$_F$ and apopholasin$_M$, as expressed in the baculovirus–silkworm multigene system, are also glycosylated. The precise structure of the sugar chain for both proteins is still unknown. Further analysis is needed to understand the relationship between the sugar chain's structure and the luminescent activities of the apoholasins.

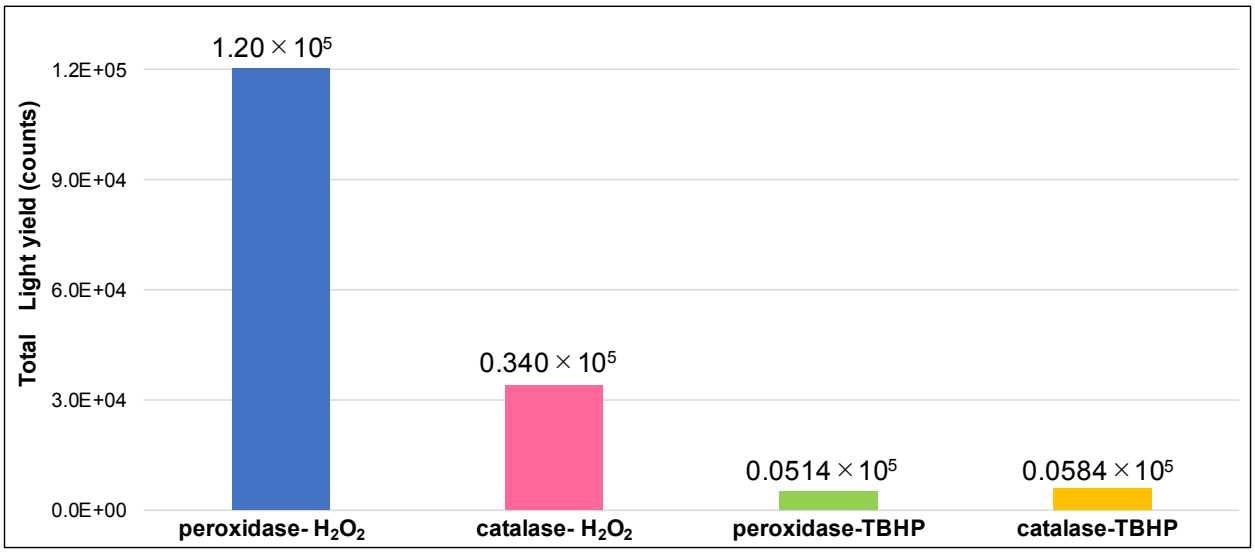


Fig. 9. Total light yields for 10 min of apopholasin$_F$–DCL: (1) apopholasin$_F$–DCL + peroxidase and H_2O_2, (2) apopholasin$_F$–DCL + catalase (5 mg/mL in PBS buffer, pH 8.5; Sigma-Aldrich, lyophilized powder from bovine liver, 2,000–5,000 U/mg) and H_2O_2, (3) apopholasin$_F$–DCL + peroxidase and TBHP (5%), (4) apopholasin$_F$–DCL + catalase (5 mg/mL in PBS buffer, pH 8.5) and TBHP (5%). The experiments were done in triplicate.

Conclusion

Recombinant apopholasins (full-length and mature) were successfully expressed using a baculovirus–silkworm multigene system. Here, the incubation of the recombinant apopholasins with DCL afforded active apopholasin–DCLs. The light emission properties of the apopholasin–DCLs were initiated by the addition of a horseradish peroxidase–hydrogen peroxide mixture. Despite this, TBHP and catalase did not strongly activate the apopholasin–DCLs. This peroxidase–H_2O_2-dependent luminescence of apopholasin–DCLs is the first example of the recombinant apopholasin activation with DCL.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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