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Extraction and purification of a luminiferous substance from the luminous mushroom *Mycena chlorophos*

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Bioluminescence has attracted considerable attention in the area of biophysics, primarily because the phenomenon can fundamentally be interpreted as the conversion of chemical to light energy. Although the molecular mechanisms underlying luminescence have been studied extensively in fireflies and bacteria, few studies have been undertaken in luminous fungi. This relative lack of information is likely due to the absence of a common and species-specific reaction-type in the luminous fungi examined to date. We recently succeeded in extracting, for the first time, a luminiferous substance from the fungus *Mycena chlorophos*. The substance was purified and characterized according to its chemical and optical properties. It is hoped that this information will facilitate the clarification of a novel molecular mechanism in fungal bioluminescence systems.

Key words: Biological conversion of chemical energy into light energy, Luminiferous fungi, Luciferin-luciferase (L-L) reaction, High-performance liquid chromatography (HPLC), Active oxygen

In addition to biochemical and genetic engineering studies, bioluminescence has attracted considerable attention in the area of biophysics, particularly since this phenomenon could potentially be applied to the development of light sources that generate relatively little heat. Bioluminescence is essentially a dynamic energy conversion system in which chemical energy is efficiently converted into light energy through a series of intracellular metabolic pathways[1]. However, unlike photosynthesis, where light energy is converted to chemical energy, the energy transfer in bioluminescent systems occurs in the opposite direction. Although the molecular mechanisms of luminescence have been examined extensively in fireflies[2,3], relatively little research has been conducted on luminescence in mushrooms[4]. This apparent lack of research is likely due to the fact that, although all luminous mushroom species emit yellow-green light at around 525 nm[5], the mechanisms employed by mushrooms to produce light vary between species. Specifically, in some taxa, the luminescent reaction is enzymatic and uses luciferin catalyzed by luciferase (L-L-type), while in other taxa the reaction is non-enzymatic (NE-type) and is initiated by the active oxygen[5]; a common and species-specific reaction-type has not been reported in any of the luminous mushrooms investigated to date. For example, Nakanishi *et al.* identified illudin S (lampterol) as the substance responsible for luminescence in *Omphalotus japonicus*[6], while Goto *et al.* concluded that lampteroflavin was the luminescent substance in the same species[7]. Furthermore, neither group was able to determine whether the reaction was of the L-L- or NE-type. In another study, Shimomura characterized four analogues of the methylamine-activation product of the fatty acid ester of panal as being the light-emitting substance in *Panellus stipticus*, and showed that this ester was involved in reactions of the NE-type[8].

Here we describe the extraction and purification of a luminiferous substance from a cryopreserved (−80°C) *Mycena chlorophos* fruiting body obtained from large-scale culture. We also measured the fluorescence spectrum of the sub-

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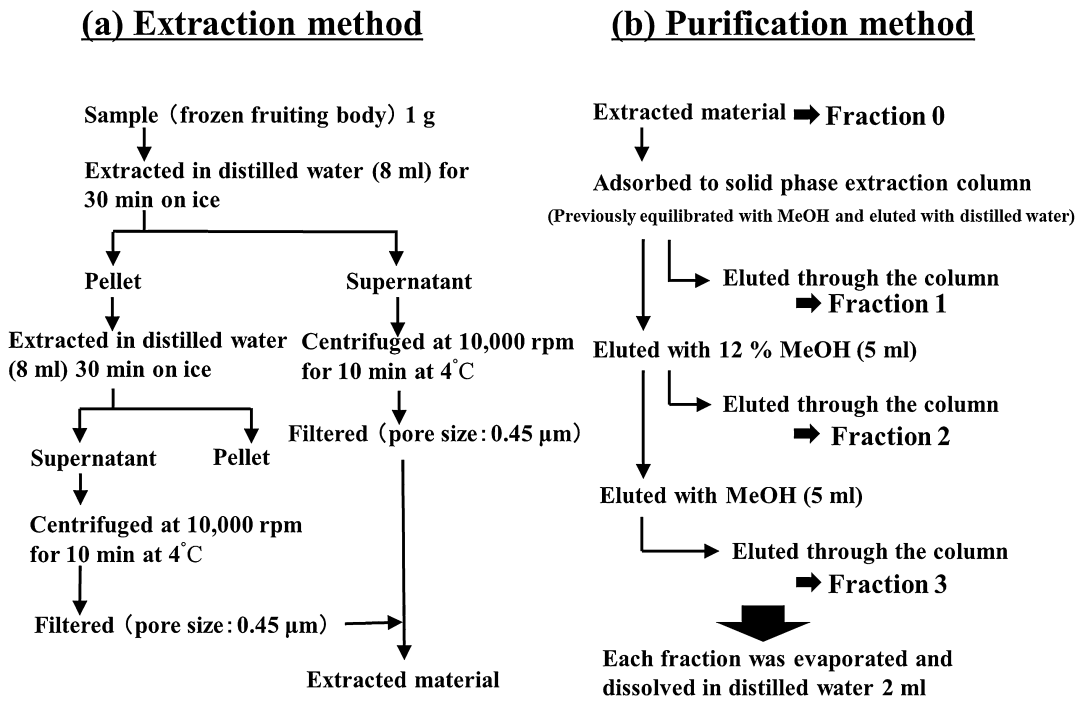

Figure 1 (a) Extraction and (b) purification methods used for obtaining the luminescent substance from a cryopreserved fruiting body sample of *M. chlorophos*.

stance to determine whether the *in vitro* bioluminescent reaction could be attributed to the extracted substance.

Materials and Methods

We cultured and harvested the fruiting body of the luminous mushroom using the following method. Hyphae of *M. chlorophos* were inoculated into media consisting of either sawdust or stalks of the *Birouyashi* palm. The media, which were supplemented with rice bran to promote elongation of mycelia, were maintained in an incubator for approximately one month at room temperature. The mature media were then placed on a bed of *Mizugoke*, a genus of sphagnum, and incubation was continued for another month in a dark-room at 23–25°C and a relative humidity of 95–100%. As a result, the dikaryotic *M. chlorophos* mycelia in the media formed a fruiting body. The fruiting body was then harvested and frozen as light emission from the body decreases after as few as 3 days following basidiocarp formation.

The extraction procedure for the luminiferous substance is outlined in Figure 1 (a). Briefly, a cryopreserved sample of fruiting body (1 g) was soaked in 8 ml distilled water at 0°C for 30 min. The resulting extract, which contained the luminiferous substance, was then centrifuged at 10,000 rpm for 10 minutes at 4°C using a high-speed microcentrifuge. After collection of the supernatant, 8 ml of distilled water was added to the pellet, which was then left to stand for an additional 30 min before being centrifuged again. As before, the supernatant was collected and combined with the supernatant collected after the initial centrifugation step. This supernatant (total 16 ml) was then lyophilized and reconstituted in 2 ml distilled water to produce the extract (fraction 0) for analysis by high-performance liquid chromatography (HPLC; model L-7420, Hitachi Inc., Japan). Figure 1(b) shows the purification method applied to the extracted sample. A solid phase extraction column (Bond Elut C-18, Varian, Palo Alto, CA, USA) was used to purify the sample. Fraction 0 was adsorbed onto the solid phase of the extraction medium, followed by elution with 5 ml of 12% MeOH and then 5 ml of 100% MeOH. The eluted fractions were labeled fractions 1, 2, and 3, respectively. Each fraction was then added to 2 ml distilled water and analyzed by HPLC. The fluorescence spectra of the three fractions were also measured to confirm extraction of the luminiferous substance. Based on the assumption that the structure of luminiferous substrate might be similar to both lampteroflavin and riboflavin (Fig. 2), HPLC-grade riboflavin (Wako Ltd., Japan) was used as a control.

Results and Discussion

HPLC analysis of fraction 0 detected the majority of components within approximately 10 minutes, with a single peak detected after approximately 21 minutes (Fig. 3). The peak of fraction 3 had an elution time that was similar to that of riboflavin. In addition, like the riboflavin standard, fraction 3 had a slight shoulder peak despite having been purified, implying that there was some deterioration in col-

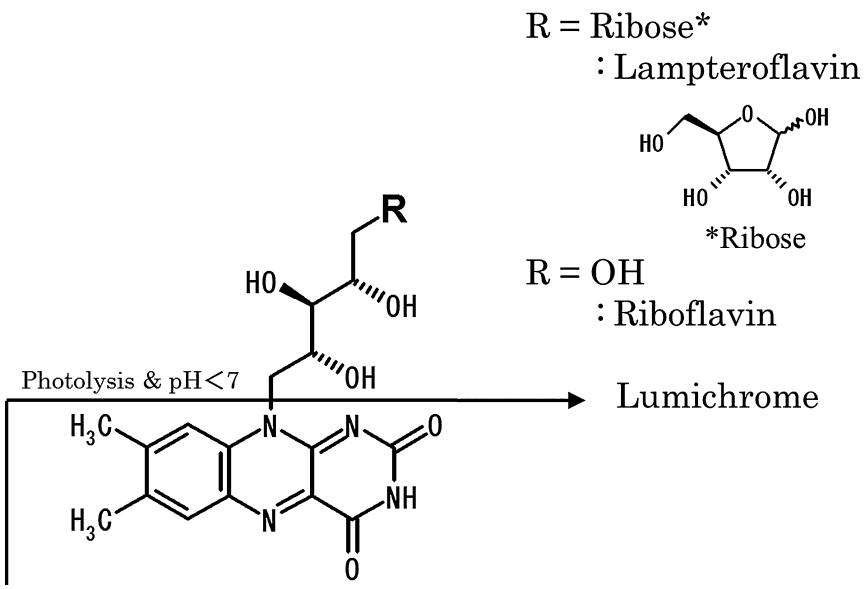

Figure 2 Chemical structure of lumichrome in flavin derivatives.

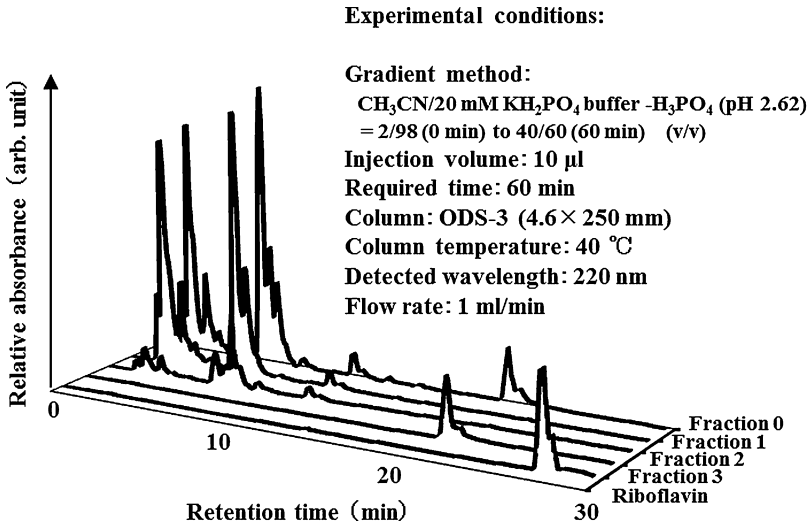

Figure 3 HPLC spectra of fractions 1, 2, 3 and riboflavin purified using a solid-phase extraction column.

umn resolution. The data obtained from the HPLC analysis is, therefore, shown without any modification.

While fractions 1 and 2 lacked any clear fluorescence peaks, fraction 3 and riboflavin both exhibited peaks at 523 nm and 528 nm, respectively (Fig. 4). As in *S. stipticus*[5], where the fluorescence spectrum almost overlapped with the bioluminescence spectrum, we propose that the single component included in fraction 3 may have been a flavonoid molecule and that it was responsible for the observed luminescence in *M. chlorophos*. Furthermore, although excitation in the region of 220 nm could not be confirmed, close correspondence was observed between the excitation spectrum of purified fraction 3 and the absorption spectrum of riboflavin (Fig. 5). The missing peak in the 220 nm region of the excitation spectrum could be attributed to a decrease in the intensity of the xenon lamp used to measure the excitation spectra at wavelengths below 250 nm, which limited measurement sensitivity. We therefore concluded that the excitation spectrum of fraction 3 would be the same as the absorption spectrum of riboflavin in this spectral region (i.e. <250 nm).

To verify whether the luminescence reaction in *M. chlorophos* is of the L-L-type, we intend to screen fruiting body tissue fragments for luciferase and its cofactors. Alternately, it may be possible to verify whether the reaction is of the NE-type by determining whether luminescence occurs when fraction 3 is added to an active oxygen-generating system, such as xanthine-xanthine oxidase. Furthermore, once the

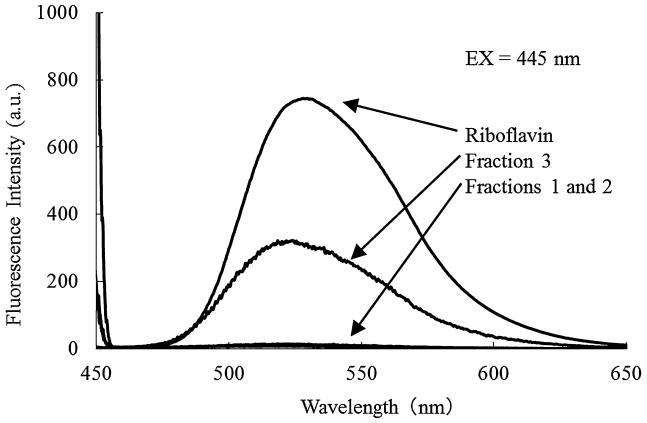

Figure 4 Fluorescence spectra of fractions 1, 2, 3 and riboflavin (2.7×10^{-5} mol/L).

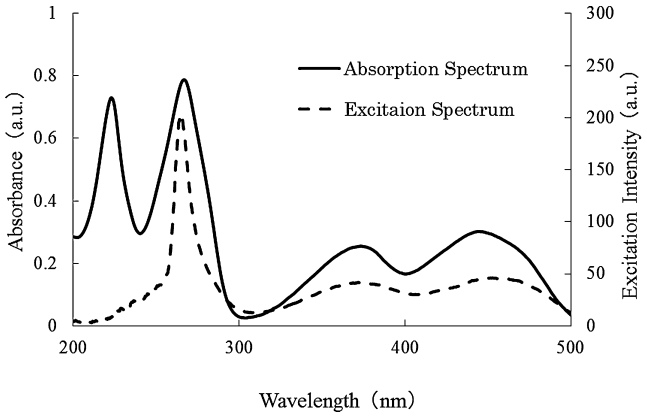

Figure 5 Comparison of riboflavin absorption spectrum (left vertical axis) and fraction 3 excitation spectrum (right vertical axis). While the concentration of riboflavin is the same as that shown in Figure 4, that of fraction 3 is not shown due difficulties associated with measuring the very small quantity of fraction 3 obtained. The intensity of the excitation spectrum was measured at 525 nm.

molecular structure of the luminiferous substance has been determined, quantum-chemical methods could then be applied to accurately clarify the elementary processes associated with the chemiexcitation pathway in fungal bioluminescence in the future.

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