PURIFICATION OF *ARMILLARIA MELLEA* LUCIFERIN BY THIN-LAYER CHROMATOGRAPHY

THESIS

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CHAPTER I

INTRODUCTION

Bioluminescence is the emission of visible light by living organisms as the result of enzyme-catalyzed reactions.[1] Different species of organisms emit light in various regions of the visible spectrum.[2]

Bioluminescence was first observed *in vitro* when a hot aqueous extract of an organism was mixed with a cold aqueous extract that had been allowed to stand until the initial luminescence had disappeared. The hot extract was found to contain a substrate, luciferin, that was utilized by an enzyme, luciferase, contained in the cold extract.[3] Bioluminescence is, therefore, a chemical process with light as a product, and not a physical process that is dependent on prior absorption of light.

Many different kinds of organisms exhibit bioluminescence. Among plants, the phenomenon seems to be confined to certain bacteria, algae, and fungi, but thirty of the approximately eighty different classes of animals contain luminescent species.[4] Table 1 gives the discovery dates of the luciferin-luciferase reaction in some luminous organisms.

Cold light may be produced by either intracellular or extracellular reactions. The former mechanism occurs in

Table 1. Dates of Discovery of Luciferin-Luciferase Reactions of Some Luminous Organisms

Luminous organisms	Dates	Discoverers
Pyrophorus (elaterid beetle)	1885	Dubois
Pholas dactylus (clam)	1887	Dubois
Photinus (American firefly)	1916	Harvey
Luciola (Japanese firefly)	1917	Harvey
Cypridina (ostracod crustacean)	1917	Harvey
Pyrocypris (ostracod crustacean)	1921	Harvey
Odontosyllis (marine fireworm)	1931	Harvey
Systellaspis (shrimp)	1931	Harvey
Latia (fresh water limpet)	1950	Bowden
Achromobacter and *Photobacterium* (luminous bacteria)	1953	Strehler and McElroy
Heterocarpus (shrimp)	1955	Haneda
Gonyaulax (dinoflagellate)	1957	Hastings and Sweeney
Apogon and *Parapriacanthus* (fish)	1958	Handea and Johnson
Renilla (sea pansy)	1959	Cormier
Collybia, *Armillaria* and *Omphalia* (fungi)	1959	Airth and McElroy
Balanoglossus (marine euterpneust)	1963	Dure and Cormier
Octochaetus multiporus (earthworm)	1966	Johnson, Haneda and Shimomura
Hoplophorus (shrimp)	1966	Johnson, Haneda, Stachel and Shimomura

fireflies, bacteria, and fungi, whereas extracellular reactions are typical of many copepods and marine annelid worms. Extracellular luminescence requires that chemical components be released into the surrounding medium where the light reaction occurs.[4]

Organisms appear to produce light for different reasons. For example, the firefly uses bioluminescence to attract the opposite sex for mating purposes[5] and certain glowworms use light to attract their prey.[6]

Practical applications for the luminescent reactions of several organisms have been found. Bioluminescent proteins from the jellyfish Aequorea and the marine worm Chaetopterus are used to detect calcium or strontium ions and ferrous iron, respectively. The luciferin-luciferase reaction of fireflies is used as a sensitive analytical tool for the detection of adenosine triphosphate (a constituent of all living matter), and bacterial luminescence is used as an oxygen indicator.[7]

Presently there are several luciferins whose structures have been elucidated. The luciferins and luciferases isolated from different sources are different. The purpose of this research is the evaluation of various methods, particularly thin-layer chromatography, for the purification and isolation of luciferin from the fungus Armillaria mellea. The complete elucidation of the fungal luminescent system must ultimately depend on the isolation and purification of the luciferin and the other components involved.

CHAPTER II

LITERATURE REVIEW

A. Cypridina

The bioluminescent system first studied was that of the marine ostracod crustacean Cypridina hilgendorfi, a 3 mm long organism found in large numbers in Japanese coastal waters.[4] The luciferin-luciferase reaction for Cypridina was discovered in 1916 by E. N. Harvey,[3] who found that the organism expelled the enzyme and substrate into sea water where the reaction occurred. The only requirements for in vitro luminescence in the Cypridina system are luciferin, luciferase, and oxygen.[4] The luminescence has an emission peak in the blue region of the spectrum at 460 nm.[4]

Early attempts to isolate the luciferin led to a "purified" product which was not crystalline. Much of the difficulty encountered in purification was due to the small quantities of the oxygen-labile luciferin that was present. Luciferin comprises about 0.025% of solids in Cyridina.[8] Work with the luciferin has been facilitated, however, because dry specimens may be preserved for indefinite periods of time. The dried material will luminesce when moistened with water and is the starting point for most investigations on the luciferin and luciferase. However, more recent

procedures developed by Johnsen et al. have permitted an increase in yield of luciferin by starting with living Cypridina.[9] The first crystalline luciferin (hydrochloride form) was obtained in 1957 by chromatography of a derivative of luciferin.[10]

Many structures were proposed for the "purified" luciferin. The luciferin has been claimed to be a peptone, a phospholipid, a hydroquinone-like compound having a keto-hydroxy chain, a flavoprotein, and a pyridine nucleotide. However, fifty years of research in several laboratories culminated with the identification of the complete structure of the luciferin at Nogoya University in 1965. The total synthesis of Cypridina luciferin was achieved in 1966[11] and its structure is shown in Figure 1. Cypridina luciferin contains elements of the amino acids tryptamine, arginine, and isoleucine. No studies concerning biosynthetic pathways for the luciferin have been published.

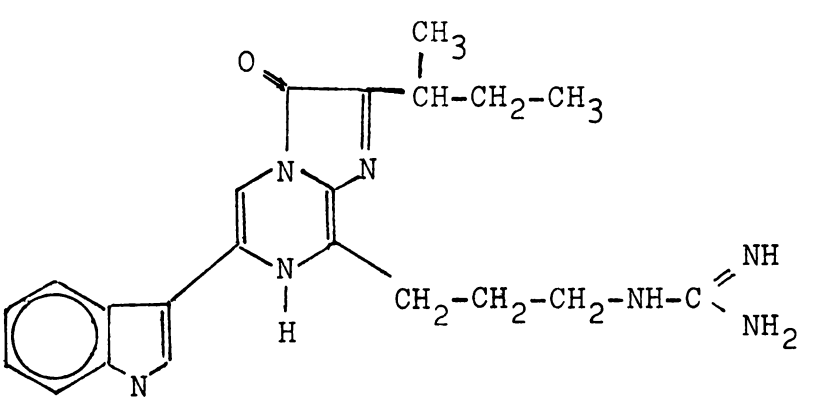


Figure 1. Structure of Cypridina hilgendorfi luciferin.[12]

B. Firefly (Lampyridae)

More is known about firefly luminescence than the luminescence of any other species. Extensive studies have been carried out on fireflies (Photinus pyralis, Photuris pennsylvanica, and other species), and large amounts of scientific literature have accumulated. The color of light (green to yellow) has received considerable attention.[13, 14] The luciferin from all firefly species examined has been found to be identical by chromatographic, spectroscopic, and fluorescence comparison.[15] The color differences among the different species are thought to be due to structural differences in the luciferases. Requirements for in vitro luminescence have been found to be adenosine triphosphate, Mg^{++}, firefly luciferin, luciferase, and oxygen.[16]

Crystalline luciferin from the firefly was first obtained by Bitler and McElroy in 1957.[17] The luciferin was stable in oxygen-free alkaline media, but readily oxidized in the presence of oxygen to dehydroluciferin, a compound also present in the firefly.[3] The structure of firefly luciferin was determined by White et al. and has been confirmed by synthesis.[18] The structure for the luciferin (Photinus pyralis) is shown in Figure 2.

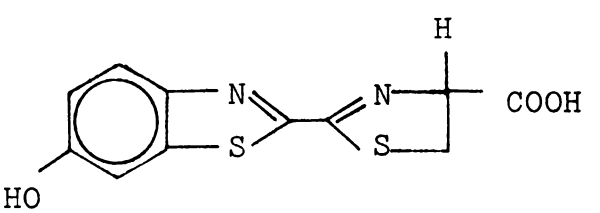
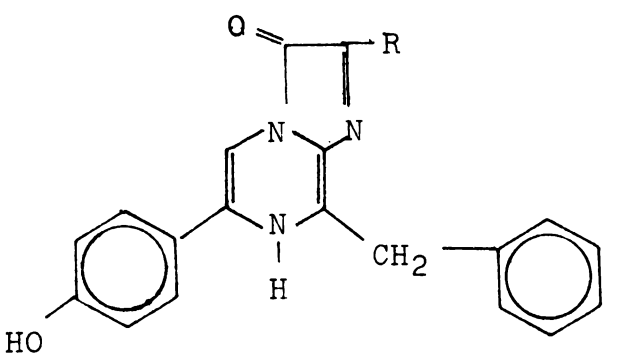


Figure 2. Structure of firefly luciferin.

C. Renilla

Renilla reniformis (sea pansy) is a small individual zooid that makes up part of a stalked kidney-shaped colony. When the colony is stimulated, a wave of luminescence spreads over the entire colony.[19] The luminescent response of Renilla has been used to study the elementary nerve system in this organism.[20]

Methods for preparing luminescent extracts from Renilla and some of the requirements for luminescence have been described by Cormier. In vitro luminescence requires an adenine-containing nucleotide, 3', 5'-diphosphoadenosine; an oxidizable substrate, Renilla luciferin; oxygen; and an enzyme fraction, Renilla luciferase.[21] Renilla luciferin was isolated (5 to 10 mg from 30,000 sea pansies) in chromatographically pure form by Hori and Cormier.[22] The luciferin was stable in basic solution, relatively heat stable, and resistant to oxidation. The structure of Renilla luciferin is shown in Figure 3.[23]



R is an unknown Alkyl group

Figure 3. Renilla luciferin.

D. Latia

The New Zealand limpet Latia neritoides, a fresh water snail, is the only luminescent animal that spends its entire life cycle in fresh water.[24] These black shelled limpets may be found clinging to stones in rivers and lakes throughout North Island of New Zealand.[25]

Luciferin from Latia was isolated by Shimomura et al. in 1966.[26] The luciferin is a colorless non-fluorescent oil, easily dissolved in hexane, and has a molecular weight of 236 as shown by mass spectroscopy. The proposed structure for Latia luciferin has been confirmed by synthesis and is shown in Figure 4.[27]

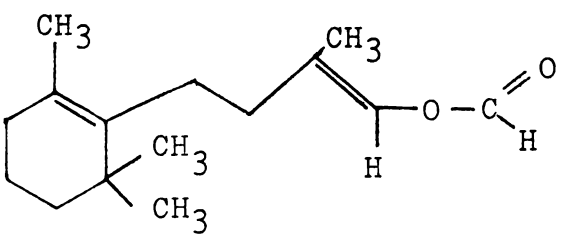


Figure 4. Latia luciferin

The only requirements for luminescence in the Latia system are luciferase, luciferin, and oxygen.[24] No evidence for any other activators or cofactors has been found.

E. Aequorea

An unusual luminescent system has been demonstrated for the jellyfish, Aequorea. Shimomura, Johnson, and Saiga were able partially to purify a luminescent substance from Aequorea.[28] The luminescent material, termed aequorin, was found to exhibit typical protein properties: it reacted with biuret and ninhydrin, was heat and acid labile, and was precipitated by ammonium sulfate. Aequorin has lately been further separated into protein and a substance designated AF-350 (from its ultraviolet absorption maximum at 350 nm).[29] The structure of AF-350 is shown in Figure 5.

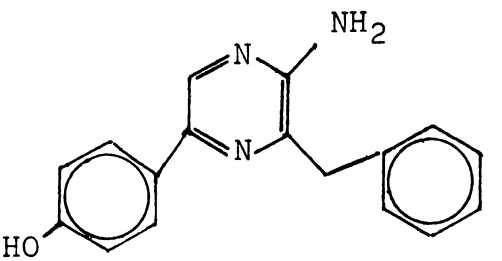


Figure 5. The structure of AF-350 (light-emitting moiety of Aequorin).[30]

Free oxygen is not required for luminescence. The only necessary components are the photoprotein, aequorin, and a trace of calcium or strontium ion.[3] The light blue emission has a half-life of one second at 20° C.[31]

F. Bacteria

Luminous bacteria (all marine) luminesce continuously with an intensity that is a function of the oxygen concentration. Despite intensive investigations it is not certain what substance is to be regarded as the bacterial luciferin.[3] In 1951 Shoup and Strehler found that certain powder preparations from Achromobacter fischeri were luminescent when suspended in water, and demonstrated that the rate limiting component for luminescence was reduced nicotinamide adenine dinucleotide, NADH.[1] Strehler and Cormier discovered in 1953 that an additional substance from hog kidney cortex greatly stimulated luminescence in bacteria. The factor was identified as the C_{16} straight-chain aldehyde, palmitaldehyde.[32] Subsequent studies have shown that a number of homologous straight-chain aldehydes (C_6 to C_{18}) support light emission in vitro.[1] During the studies on the aldehyde requirement, it was found that reduced flavin mononucleotide ($FMNH_2$) greatly stimulated luminescence and that the function of NADH was to reduce oxidized FMN.[1] Reduced flavin mononucleotide, an enzyme, oxygen, and a long-chain aliphatic aldehyde are now regarded as essential factors for bacterial luminescence.[3]

G. Fungi

The luminescence of fungi, like that of bacteria, is continuous *in vivo*. The most common luminescent species occur among the Basidiomycetes.[24] A widespread luminescent fungus is the wood destroyer *Armillaria mellea*, the most common cause of "luminous wood".[33]

The luciferin-luciferase reaction for fungi was demonstrated by Airth and McElroy in 1959.[34] The reaction mixture consisted of a hot-water extract from *A. mellea*, a cold-water extract from *Collybia velutipes*, and either NADH or reduced nicotinamide dinucleotide phosphate (NADPH).[34] The bacterial system components, a long-chain aldehyde, and $FMNH_2$, were tested for activity in the fungal system, but all results were negative.[24] Thus requirements for bacterial and fungal luminescence are not identical.

Airth's studies of *A. mellea* luciferin indicate that the substance is water soluble and insoluble in the organic solvents acetone, chloroform, benzene, and an ethyl ether-ethanol mixture (3:1 v/v).[35] The luciferin is also reported to be unstable at pH 8.5, and to be more readily destroyed at low temperatures in air than in nitrogen and hydrogen atmospheres.[35]

Kuwabara and Wassink have reported a purified active substance from the luminescent fungus *Omphalia flavida*.[36] Purification procedures involved solvent extraction, centrifugation, column chromatography and thin-layer chromatography. The active preparation was a brownish-orange crystalline

solid that showed three spots on thin-layer chromatograms. Luminescent activity was proportional to the concentration of the material in one of the spots. This spot exhibited blue fluorescence when excited at 320 nm. However, a sample of this isolated material has been found to be inactive as fungal luciferin in the assay of Airth and colleagues.[37] No further reports on the properties of this material have appeared since 1966.

Fungal luminescence is a two step reaction involving the reduction of fungal luciferin by NADH or NADPH and a soluble enzyme, and the subsequent light-emitting oxidation of reduced fungal luciferin by molecular oxygen and a particulate enzyme.[38]

H. Bioluminescent Reactions

The bioluminescent systems that have been studied in sufficient detail may be classified into different groups based upon the reaction type. The useful summary in Table 2 was first suggested by Cormier and Totter.[1, 24]

Table 2. Bioluminescent Reactions Classified According to Reaction Type.

Type reaction	Examples	Emission maxima, (nm)
A. Pyridine-nucleotide linked:		
(1) $NADH+H^+ + FMN \xrightarrow{\text{oxidase}} FMNH_2 + NAD^+$	Bacteria	475-505
$FMNH_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} \text{light}$		
(2) $NADH+H^+ + L \xrightarrow{\text{oxidase}} LH_2 + NAD^+$	Fungi	530
$LH_2 + O_2 \xrightarrow{\text{luciferase}} \text{light}$		
B. Adenine-nucleotide linked:		
(1) $LH_2 + ATP + O_2 \xrightarrow[Mg^{2+}]{\text{luciferase}} \text{light}$	Firefly	552-582
(2) $LH_2\text{-}O\text{-}SO_3^- + D.P.A. \xrightarrow[\text{kinase}]{\text{luciferyl}} LH_2 + PAPS$	Sea pansy	485
$LH_2 + O_2 \xrightarrow{\text{luciferase}} \text{light}$		
C. Simple enzyme-substrate systems:		
$LH_2 + O_2 \xrightarrow{\text{luciferase}} \text{light}$	Cypridina (crustacean)	460
	Apogon (fish)	460
	Parapriacanthus (fish)	460
	Pholas (clam)	480
	Gonyaulax (protozoan)	470
	Odontosyllis (annelid)	507
	Latia (limpet)	520
D. Peroxidation systems:		
$LH_2 + H_2O_2 \xrightarrow{\text{luciferase}} \text{light}$	Balanoglossus (acorn worm)	Blue
	Diplocardia (annelid)	500

Table 2--Continued

Type reaction	Examples	Emission maxima, (nm)
E. Activation of "precharged" systems:		
(1) precharged particle $\xrightarrow[O_2]{H^+}$ light	Gonyaulax (protozoan)	470
(2) precharged protein $\xrightarrow{Ca^{2+}}$ light	Aequorea (hydromedusid)	460
F. Unclassified systems:		
	Chaetopterus (annelid)	460
	Octochaetus (annelid)	Orange-yellow
	Hoplophorus (shrimp)	Blue

CHAPTER III

METHODS AND PROCEDURES

A. Growth of Fungi

Armillaria mellea was used exclusively in this study. Mycelial cultures were maintained on agar slants and useful amounts of fungi were grown by surface culture after serial transfer to 250 ml erlenmeyer flasks and then to 2800 ml fernbach flasks.

Slant medium consisted of 36 g of mycophil agar, 5 g of yeast extract, and enough deionized water to make a liter of solution. The mixture was adjusted to pH 5.0 with 10 N hydrochloric acid, and each 20 x 150 mm culture tube (slant) was filled with 7 ml of agar medium. Flask medium consisted of 85 g of mycophil broth, 8.5 g of mycophil agar, 8.5 g of yeast extract, and 1.2 l of deionized water. The medium was adjusted to pH 6.5 with sodium hydroxide. Ten milliliters of medium was added to the 250 ml flasks and each fernbach flask received 100 ml of medium. The slants and flasks were closed with cotton plugs or screw caps, autoclaved at 130° for 15 minutes, and cooled before inoculation with mycelia.

Sufficient quantities of *A. mellea* cultures were obtained by the following transfer and inoculation procedures. When large quantities of mycelia were desired, several

pieces of mycelium, with some adhering agar, were transferred from a slant culture to a 100 ml stainless steel homogenizing cup that contained 15 ml of sterile water. The transfer was effected with a knife that had been sterilized by flaming and cooled by plunging into sterile agar. The tough, fibrous mycelia were fragmented on a Lourdes (Model MM-1B) homogenizer at a low speed (powerstat setting of 40) for 15 minutes. This treatment gave good dispersal without breaking too many cells. Sterilized pipets were used to transfer approximately 5 ml of the blended mycelia into 250 ml erlenmeyer flasks that contained media.

After about a week the cultures in the small flasks were fully developed and luminous. These cultures then served as inoculant for the larger fernbach flasks. The contents of three small flasks were poured into 65 ml of sterile water contained in a stainless steel homogenizing cup and gently homogenized as described for the material from the slants. The homogenized cell suspension was then carefully transferred to an inoculum reservoir comprised of a sterile 250 ml erlenmeyer flask fitted with a cotton plug. Five milliliter portions of the inoculant were transferred with sterile wide-mouth pipettes to fernbach flasks that contained medium. The mouths of all flasks were flamed immediately after the cotton plugs were removed and again just prior to reinsertion of the plugs. Also, the principal axes of the open flasks were kept horizontal to the desk top to minimize airborne contamination. Care was exercised to

avoid wetting the cotton plugs. All transfers and inoculations were performed in a hood that had been sprayed and cleaned with 2% vestol solution. Growth in the flasks was uniform, and dense mats of mycelia covered the surface of the medium. Contamination, if present, was usually obvious. The growth from the three small flasks produced enough mycelia to inoculate eighteen large fernbach flasks. The greater the amount of inoculant, the more rapidly the cultures developed, the more compact were the mats, and the less likely that contaminating growths would appear.

B. Harvesting of Mycelia

Different procedures were used to harvest mycelial cultures depending upon whether the cultures were eventually intended as a luciferin or luciferase source. Culture mats of maximum luminescence were used for extracting luciferin, and aged mats that had lost all luminescence or were only faintly luminescent were used for luciferase extraction.

Procedure (A) for Luciferin

Powders prepared from mycellia harvested 14 to 17 days after the fernbach flasks were inoculated had the greatest amount of luciferin activity. Compact culture mats were removed from the fernbach flasks and the agar media was rinsed away with deionized water. The mats were then placed in a beaker containing boiling methanol. The hot methanol inactivated enzymes present that might contribute to the degradation of luciferin. After 3 to 5 minutes in the boiling methanol the mats were removed, the excess methanol blotted or allowed to evaporate, and then the mats were placed in a vacuum desiccator containing Drierite. The mats remained in the evacuated desiccator one or more days and were then ground in a mortar to a fine powder. The powders were placed in containers, stoppered, and stored in a refrigerator until used for the extraction of luciferin.

Procedure (B) for Luciferase

Cultures grown for four weeks in fernbach flasks were used for the preparation of powders to be used as a luciferase source. The mats were removed from the flasks and the

excess agar was rinsed off with deionized water. The mats were then placed on paper towels that were removed and replaced every few minutes over an hour period in order to effect a quick removal of excess moisture. The mats were then wrapped in paper towels and placed in a vacuum desiccator that contained Drierite. The mats were dried under vacuum until completely dry, then ground in a mortar to a fine powder. The powders were kept in small, air-tight jars in the refrigerator until utilized for the preparation of luciferase.

C. Assay Preparations and Procedures

Enzyme Preparation from Dried Fungal Powder

Fungal powder (0.7 g) prepared by harvest procedure B was added to 16 ml of partly frozen preparion buffer in a 100 ml homogenizing cup. The composition of preparation buffer is shown in Table 3. The cup was kept in an ice bath and the powder was homogenized at a powerstat setting of 50 for 1.5 minutes, allowed to cool for about a minute, then homogenized at a setting of 90 for 0.5 minutes. The homogenized suspension was transferred to an ice cold centrifuge tube and centrifuged at 1300 x g for 10 minutes at 3° C. The supernatant that was removed from the pellet by pipet contained the enzyme and was sufficient material for about 20 assays. This crude preparation continuously lost activity and could not be stored overnight at any temperature without complete loss of activity. The enzyme therefore had to be prepared fresh for each assay. Preparation of active enzyme, if not a black art, was such an exact science that one never had confidence in a preparation until it was actually used in the assay. A source of difficulty was undoubtedly the fact that the active crude enzyme preparation had to consist of two enzymes of very different properties--one soluble and the other membrance bound.

Assay Mixture and Procedure

The reaction mixture for the assay consisted of 0.2 ml of Tween solution, 1.0 ml of assay buffer, 0.4 ml of NADH solution, 1.0 ml of luciferin solution, and 0.4 ml of

Table 3. Preparation Buffer Composition[a]

Molarity	Constituent	Weight (g)[b]
0.05 M	KH_2PO_4	6.8 g
0.05 M	K_2HPO_4	8.7 g
0.32 M	Sucrose	109.4 g
0.005 M	$Na_2H_2EDTA \cdot 2H_2O$	1.86 g

[a]The pH was adjusted to 7.5
[b]Weights given are for one liter of solution

Table 4. Assay Buffer Composition[a]

Molarity	Constituent	Weight (g)[b]
0.15 M	NaH_2PO_4	20.7 g
0.15 M	Na_2HPO_4	21.3 g
0.01 M	Na_2H_2EDTA	3.72 g

[a]The pH was adjusted to 7.5
[b]Weights given are for one liter of solution

luciferase solution, for a total of 3.0 ml of solution. The composition of assay buffer is shown in Table 4. Tween-80, a detergent that apparently aids the solution of luciferin in water, was prepared as a 2% (v/v) solution by dissolving 0.2 ml of Tween in 10 ml of deionized water. The cofactor in the luminescent reaction, NADH, was prepared as a 10% solution in assay buffer and was prepared as a fresh solution in the estimated amounts needed for the number of samples of a particular assay session. A fresh 0.1 M solution of dithiothreitol (DTT) was also prepared for each assay by dissolving 15 mg of DTT in 1.0 ml of water. This stock solution was used to prepare 10^{-3} M concentrations of DTT in the assay and preparation buffers just before use. The enzyme solution was used in the assay in the form and concentrations as it was prepared. The individual solutions were kept cold and the appropriate amounts were added with calibrated pipettes or syringes to a 12 x 75 mm assay tube. Luciferin preparations could be assayed directly or after dilution if they were in aqueous solutions. Solutions of luciferin in organic solvents had to be evaporated, usually with nitrogen, and the residue extracted with hot water and then cooled before assay. This transfer was necessary because the enzymes were inactive in the presence of organic solvents.

All the assay constituents except luciferase were added to the assay tube and the tube was placed in the sample holder of a light-tight box that also housed a photomultiplier

tube. The luciferase solution was then added with a syringe and the mixture stirred. The intensity of light emission was recorded. A standard hot-water extract prepared by extracting 200 mg of luciferin powder with 10 ml of hot water was always assayed to determine whether the luciferase was active. Control assays were made by substituting pure water for the luciferin solution to determine whether luciferin was present in the luciferase preparation. If luciferin activity of the luciferase preparation was high, the luciferase solution was allowed to stand briefly (about 10 minutes) until the activity had subsided.

D. Apparatus

The instrument used to measure light from the assay tube was a photomultiplier photometer (Model 150-200B) designed by Dr. Edward F. MacNichols Jr. at Johns Hopkins University. The photometer was coupled to a Sanford d.c. preamplifier (Model 150-1300) and a Sanford recorder (Model 150-100A). A simple diagram of the instrument is shown in Figure 6.

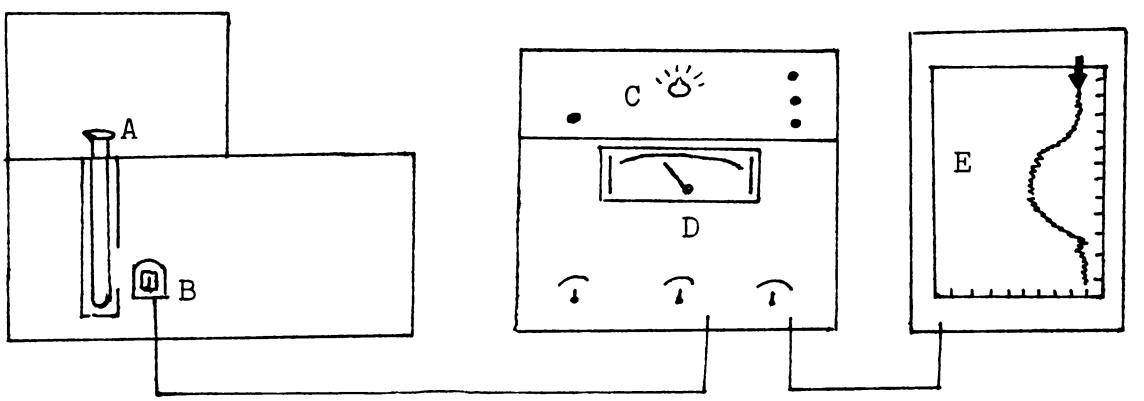


Figure 6. A simple diagram of the arrangement of the light measuring apparatus. A. sample holder. B. photomultiplier tube. C. preamplifier. D. photometer. E. recorder

The phototube was not calibrated for absolute numbers of light quanta, so relative light emission was measured. A light unit was arbitrarily set as a 0.5 cm pen deflection on the chart with a constant sensitivity setting on the instrument. Light units for a reaction were determined by subtracting the units of light produced before the luciferase was added (x) from the light units produced at the maximum light intensity (a) of the luciferin-luciferase reaction.

This difference (a-x) multiplied by 20 (an instrument sensitivity attenuation factor) was recorded as light units in a table for each assay. An example of a luciferin-luciferase reaction is shown in Figure 7 in which the total light units is equal to 160. The figure also shows the usual time required for the crude enzyme to produce maximum light intensity. The objective of the assay was to measure luciferin activity in terms of light units emitted. A measurement of the absolute concentration of luciferin was not possible since the molecular weight of the luciferin was not known.

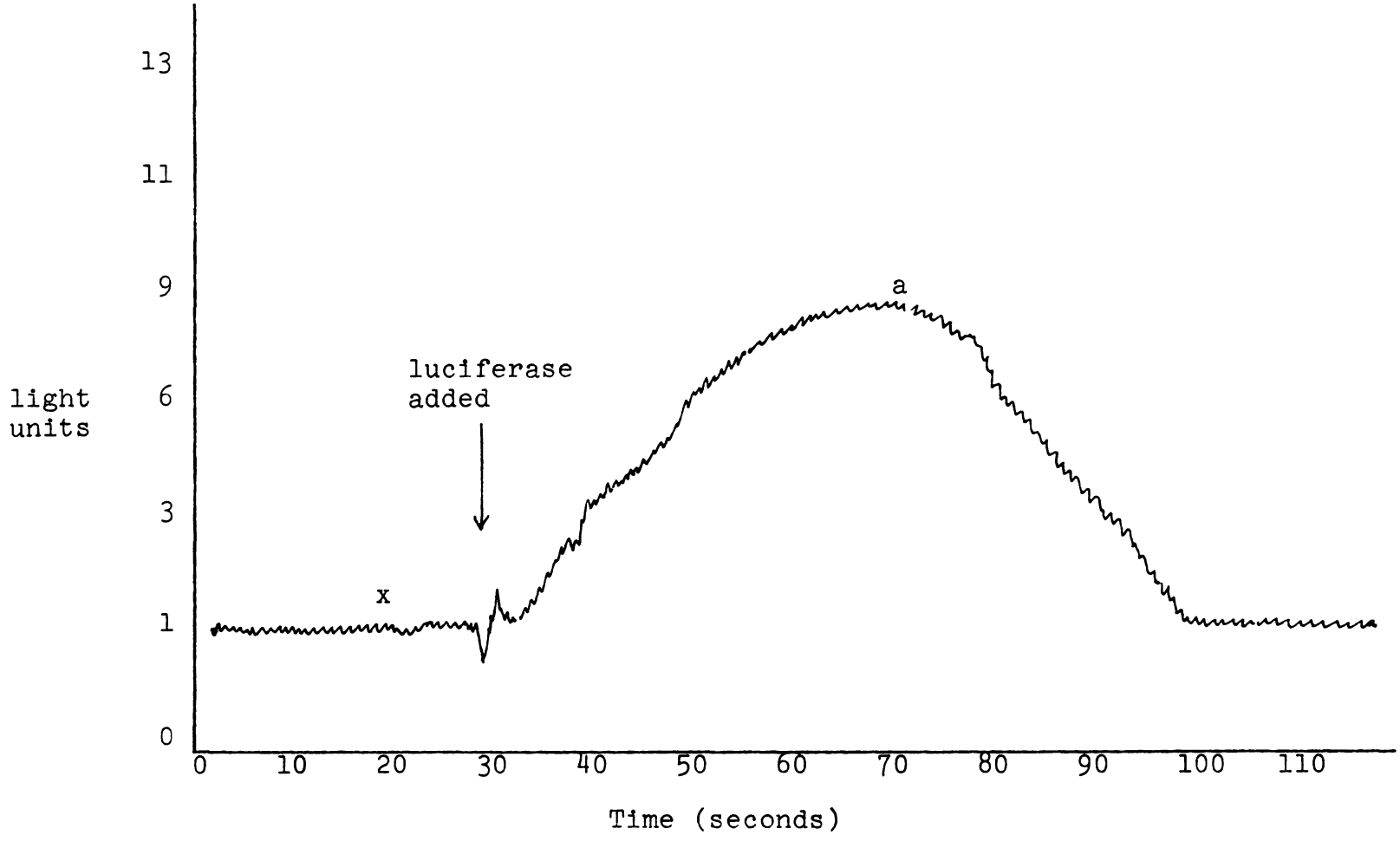


Figure 7. Graphic record of a luciferin-luciferase reaction.

E. Extraction and Purification of Luciferin

Extraction Procedure

Luciferin from dried mycelial powders was extracted with boiling water. About 100 ml of water was used for the extraction of 1 g of powder. The hot mixture was stirred for a few minutes, partially cooled, then centrifuged at 8000 x g for 10 minutes. The supernatant was removed from the residue, which was again extracted with 100 ml of boiling water and centrifuged as before. Both extracts were combined, cooled to 5-10° C, and adjusted to pH 3 with hydrochloric acid. The acidified hot-water extract was then extracted twice with ethyl acetate. Because emulsions usually formed, a relatively large amount of ethyl acetate had to be used. Typically, an ethyl acetate volume three times that of the aqueous volume was required to effect clean separations. Assay of the aqueous solution before and after ethyl acetate extraction, as well as the ethyl acetate solution itself, showed the luciferin to be quantitatively extracted into the ethyl acetate. The ethyl acetate solution was dried with Drierite.

Columns

Further purification of luciferin in the ethyl acetate extract was accomplished by the use of column chromatography. The ethyl acetate solution was first passed through a 1 x 4 cm cellulose column and then through a 1 x 18 cm Dowex-50 cation exchange column in the sodium form. Luciferin activity in solution after this treatment was stable in

contrast to the disappearance of all luciferin activity within 24 hours if kept in the original aqueous solution. The ethyl acetate solution was then concentrated to a volume of approximately 0.5 ml with a rotary evaporator at room temperature, applied to a 2 x 50 cm LH-20 Sephadex column (particle size: 10-25 μ), and eluted with methanol. The effluent from the Sephadex column was continuously monitored by an ultraviolet absorbance meter and 10 ml fractions were collected by use of an Isco Model 820 fraction collector. After the first experiment in which all the tubes from the column were assayed, it became possible to predict the luciferin-active region of the eluant from the ultraviolet record. Luciferin did not absorb at the monitoring wavelength used (240 nm), but characteristically associated compounds did. Those fractions which were thought to have activity (from the absorbance record) were assayed and the most active fractions were pooled and evaporated to dryness. The residue was then dissolved in deionized water and extracted with benzene. The benzene was discarded because preliminary assay showed no luciferin activity in the benzene, although there was considerable solid material on evaporation. The fraction was then adjusted to pH 3 with hydrochloric acid and extracted with ethyl acetate. The ethyl acetate was then concentrated to approximately 0.5 ml and fractionated a second time on a smaller LH-20 Sephadex column. The most active fraction, located by assay, was used as a source of purified luciferin for thin-layer chromatography

evaluation. Purification of A. mellea luciferin by ion exchange, cellulose and Sephadex columns is described in greater detail by Mr. Warren DuBose III, in a Master of Science thesis presented to the graduate council at Southwest Texas State University, April 1973.

Thin-layer Chromatography

Further purification of active luciferin fractions obtained from the Sephadex column was achieved by thin-layer chromatography. The Sephadex fractions were concentrated to volumes of 0.5 ml or less and applied to cellulose coated plates. Silica gel and alumina absorbents were investigated, but the recovery of luciferin activity from anything but cellulose was poor. Microcrystalline cellulose (particle size: 19μ) was used exclusively. Cellulose of this type is required for the preparation of thick layers of cellulose without binder. A slurry of 45 g of powdered cellulose and 220 ml of distilled water was prepared with a mechanical blender and poured into a Camag spreading device. The spreader was used to deposit a layer of cellulose 1.0 mm thick on flat, smooth glass plates (20 x 20 cm and 5 x 20 cm). The plates were allowed to dry and stored at room temperature until used. Luciferin samples were applied by touching the tip of a filled capillary tube to the dried adsorbent on the plate. A Desaga "spotting template" was used as a guide in applying the sample in a straight thin band across the plate 1.5 cm above one edge. The plates were developed in an ascending manner in closed glass containers. When the solvent

front reached a height 10 cm above the origin, the plate was removed, air dried, and examined under ultraviolet light. The cellulose from predetermined regions of the plate was quantitatively scraped from the plate with a spatula into labeled test tubes containing methanol. Aliquots were removed from the filtered methanol solutions and assayed for luciferin activity. The active fractions were then often pooled, concentrated, and rechromatographed.

Several partition-chromatography type solvent systems were investigated for their ability to move luciferin into a useful separation range on the plate. Three developing systems studied were (a) n-amyl alcohol: acetic acid: water (10:6:5 v/v), (b) ethyl acetate: isopropyl alcohol: water (65:7:28 v/v), and (c) 2% formic acid. Assay of the fractions from these developing systems indicated that the activity remained at the origin in system (c) and near the solvent front in systems (a) and (b). Two other solvent systems were more useful. Chromatograms were developed in n-butyl alcohol: acetic acid: water (2:1:1 v/v) and the upper phase of n-butyl alcohol: acetic acid: water (4:1:5 v/v). Assay results of the M series (2:1:1) and N series (4:1:5) are shown in Table 5. Most of the activity appeared in the upper part of the chromatogram (R_f 0.5 to R_f 0.9). Because n-butyl alcohol: acetic acid: water (2:1:1 v/v) was easier to handle, it was chosen for subsequent thin-layer work.

Table 5. Assay Results of M[a] and N[b] Series

M Series		N Series	
R_f	Activity (light units)	R_f	Activity (light units)
0 - 0.3	8	0 - 0.3	6
0.3 - 0.5	8	0.3 - 0.5	8
0.5 - 0.6	16	0.5 - 0.7	24
0.6 - 0.9	24	0.7 - 0.9	18
0.9 - 1.0	8	0.9 - 1.0	2

[a]Solvent system: n-butanol - acetic acid - water (2:1:1)

[b]Solvent system: n-butanol - acetic acid - water (4:1:5)

Some effort was devoted to a search for a chemical spray reagent that could be used to detect luciferin on the developed chromatograph plates and thus avoid repeated enzyme assays. It was further hoped that functional group information might be obtained from colors developed with certain sprays in regions that could be correlated with luciferin activity. Among the sprays used were 2,4-dinitophenylhydrazine, dimethylaminobenzaldehyde, ninhydrin, formaldehyde in aqueous sulfuric acid, and iodine. No spots were visible with any of the sprays. Evidence of material on the developed plates was provided by fluorescent bands that were visible when the plates were irradiated with ultraviolet light (254 and 350 nm). Blue and yellow bands were nearly always present in the luciferin-active portion of the plate, but exclusive association of luciferin with one or the other of these bands has not been accomplished.

CHAPTER IV

SUMMARY AND RESULTS

Methods described in this thesis represent a contribution to a complete purification procedure for fungal luciferin. The techniques of solvent partition, cellulose and cation exchange resin filtration, and fractionation on LH-20 Sephadex columns are applicable to luciferin mixtures, and produce essentially no loss in luciferin activity. Thin-layer chromatography can be used to refine the purity of luciferin samples from these columns.

The actual amounts of material completely processed have been too small to assess the purity of the final product or to gain good spectroscopic data. However, preliminary spectra have been obtained from a luciferin sample extracted from fungal powder with hot water, partitioned into ethyl acetate, passed through cellulose and cation exchange columns, fractionated on LH-20 Sephadex, and chromatographed three times on cellulose thin-layer plates. The material that was rechromatographed was obtained from the 1 cm wide zone of highest activity of a chromatogram that previously had been developed for a distance of 10 cm.

The infrared spectrum had medium intensity bands at 3400 and 3150 cm^{-1} in addition to strong bands at 1570 and

1375 cm^{-1}. Maximum absorption (methanol solution) in the ultraviolet was at 206 nm with a shoulder at 225 nm and a slight rise at 385 nm. Principal high mass ions in the mass spectrum were at 389, 573, and 620 mass units. Each of these ions was at the center of a cluster of 5 to 7 masses spaced two mass units apart. With a 60 megacycle proton magnetic reasonance spectometer and a micro sample tube (solvents: deuterated acetone and deuterated dimethyl sulfoxide) the only hydrogen that could be detected was at 2.0 and 2.2 parts per million below tetramethylsilane.

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