

CRYSTALLINE FIREFLY LUCIFERASE

by

ARDA A. GREEN AND W. D. MCELROY**

The McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Md. (U.S.A.)

The production of light in firefly (*Photinus pyralis*) extracts depends upon the presence of oxygen, luciferin, adenosine triphosphate (ATP) and Mg^{++} . This oxidative reaction, which is catalyzed by the enzyme, luciferase, excites the luciferin molecule, which subsequently decomposes to emit a quantum of light[†]. The mechanism of excitation and the nature of the transient in the reaction are unknown. The present report describes the further purification and crystallization of firefly luciferase. Some of the physico-chemical and enzymic properties of this crystalline preparation are also reported.

PREPARATION

The live fireflies are dehydrated in a continuous vacuum over calcium chloride for 24 hours. The dried lanterns are removed by hand and stored in the deep freeze. They may be kept in this state for at least two years without significant loss of activity. Thirty grams of the dried lanterns, from about 6,000 fireflies, are processed at once.

An acetone powder is made after first grinding the dried lanterns in a mortar and pestle with about two grams of sand. One hundred ml of cold acetone are then added, mixed thoroughly and the suspension filtered on a Buchner funnel. The powder is then washed twice more with 200 ml aliquots of cold acetone and air dried. After drying in a desiccator, the material may be stored in the deep freeze for months or used immediately. All subsequent processes are carried out in the cold; solutions are kept in an ice bath and centrifuging done in a refrigerated centrifuge.

The acetone powder is ground thoroughly in a mortar and then extracted with 100 ml of $10^{-3}M$ versene plus 7 ml N NaOH. This brings the pH to about 7.8 as measured in the glass electrode standardized at room temperature with the solution cold. The pH of the extract would be about 8 at room temperature. The enzyme is kept in $10^{-3}M$ versene at all times.

The first extract is centrifuged at 3,000 r.p.m. for 10 minutes, and the residue is extracted twice with 60 ml water plus 1 ml N NaOH. The combined extracts are then centrifuged at 20,000 g in the refrigerated Servall for thirty minutes. The residue is discarded, and the enzyme (ca. 1000 light units/mg) is removed from the supernatant by adsorption on calcium phosphate gel.

Two calcium phosphate gel adsorptions are used. In the first, three parts of

[†] Supported in part by a grant from the National Science Foundation.

^{**} Contribution No. 133 of the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland.

References p. 176.

enzyme are mixed with two parts of gel containing 16.5 mg $(\text{Ca}_3\text{PO}_4)_2$ per ml. The gel at pH 7.5, is measured and then centrifuged, the supernatant is discarded. The gel is then thoroughly mixed with the extract and recentrifuged. The gel is next washed with 50 ml $10^{-3}M$ versene, pH 8, and recentrifuged. This gel absorbs about 15% of the total recoverable activity in relatively impure form. It is eluted with 20% saturated $(\text{NH}_4)_2\text{SO}_4$, but kept separate. The combined supernatants from the first gel are treated as above with 2.2 times their volume of gel. The remaining luciferase is absorbed. The supernatant contains most of the pyrophosphatase and luciferin present in the original extract. Luciferase is eluted from the gel with 20% saturated (0.82 M) $(\text{NH}_4)_2\text{SO}_4$ in $10^{-3}M$ versene at pH 7.9. Three elutions of 50 ml each are made from the first gel and three elutions of 100 ml each from the second gel.

Ammonium sulfate fractionation

The first two elutions of the second gel contain about one-half of the eluted protein and three-quarters of the luciferase activity. They are immediately fractionated with ammonium sulfate. The pH is adjusted with 1 N NaOH to approximately 7.7-7.9. Solid ammonium sulfate is added with stirring. Fractions are taken at 40, 50, 60 and 70% saturated ammonium sulfate. Percent saturation means percent of 4.1 M . The precipitates are separated by centrifuging at 20,000 g for fifteen minutes and dissolved in $10^{-3}M$ versene at pH 7.9. The results of the fractionation procedure are given in Table I.

TABLE I

AMMONIUM SULFATE PRECIPITATION OF THE ELUATES FROM THE CALCIUM PHOSPHATE GELS

Fraction	$(\text{NH}_4)_2\text{SO}_4$ M/l	Light units	Light units per mg protein	% Total light units	% Total protein
Combined elutions from 1st gel	2.87	1,060,000	1,400	13.5	32
Combined 1st and 2nd elutions from 2nd gel	1.64	110,000	400	1.4	12
	2.05	520,000	2,500	6.6	8
	2.46	4,200,000	8,100	53.0	22
	2.87	1,000,000	4,300	12.7	10
3rd elution from 2nd gel	2.87	1,000,000	2,700	12.7	16

The light units are arbitrary and are measured on a specially designed instrument². The reaction mixture consists of enzyme, 0.1 ml 0.1 M Mg SO_4 , 0.2 ml standard luciferin solution, 0.2 ml ATP, 1 mg per ml, and 0.05 M glycine pH 7.9 to make the final volume to 2.5 ml. The reaction is started by adding the ATP. This is layered gently on top of the reaction mixture in a small test tube in front of the photocell, then mixed as rapidly as possible in the dark with a stirring rod. The initial flash of light reaches its peak in less than two seconds. This "flash-height" is used as units of enzyme. Proteins were measured by the method of Lowry *et al.*³, using crystalline bovine serum albumin as the standard.

This preparation of the enzyme differs from that published previously⁴ chiefly in that an acetone powder is made. This step denatures some contaminating protein and also removes some inhibiting substances so that the crude extract is presumably more active. Thus, the best material obtained by ammonium sulfate precipitation after

References p. 176.

elution from the second calcium phosphate gel is only eight times as active per milligrams of protein as the crude extract. In comparison, a similar fraction in the original procedure was thirty times as active. It is impossible to compare the two methods directly since a different light-measuring instrument is now in use.

Under the above conditions, most of the enzyme is recovered from the gels. Further fractionation with $(\text{NH}_4)_2\text{SO}_4$ results in no loss of activity. All of the enzyme could be brought to a purity level where crystallization is possible. As a result of the first $(\text{NH}_4)_2\text{SO}_4$ fractionation, 50% of the enzyme is in a crystallizable state, but is usually refractionated once. Crystals have been obtained from material with a specific activity of 5,000 light units per mg protein, but it is better to start with material of about twice that level of purity.

Crystallization of the enzyme depends upon the fact that it is a euglobulin, *i.e.*, a water-insoluble protein. Crystallization takes place upon dialysis against a solution of low ionic strength. The dialyzing solution contains $10^{-3}M$ versene, $0.01M$ NaCl and $0.002M$ Na_2HPO_4 , the final pH being adjusted to 7.2-7.4. After dialysis overnight, the crystals are centrifuged in the cold and dissolved in $0.4M$ $(\text{NH}_4)_2\text{SO}_4$ and $0.001M$ versene at pH 7.8. Recrystallization can be effected by redialyzing against more of the same dialyzing solution. If the dialysis is too speedy, the enzyme may not crystallize. Some of the best crystals we have obtained formed in the presence of $0.15M$ NaCl and $0.02M$ phosphate buffer in solutions prepared for electrophoresis. The crystals are needles or rods as can be seen in Fig. 1. Frequently, small bundles or rosettes of needles are also seen. A protocol of one series of crystallizations is given in

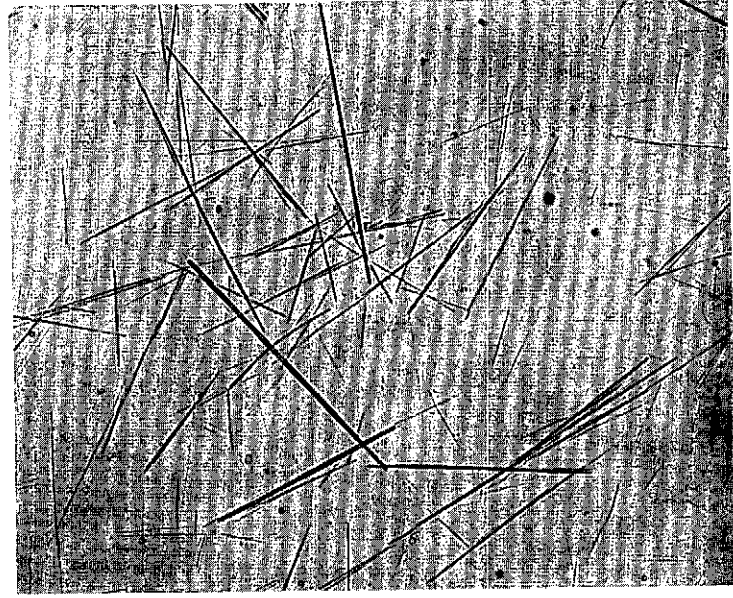


Fig. 1. Crystalline firefly luciferase. Magnification 100.

References p. 176.

Table II. The specific activity has increased to about thirty times that of the original extract from the acetone powder. Three different preparations have been brought to this level of purity. The crystals are homogeneous upon ultracentrifugation and electrophoresis (see below). Furthermore, the contaminating enzyme, pyrophosphatase has been reduced from an activity level in the crude extract of 1 mg of P liberated in 15 min per mg protein to zero.

TABLE II
PURIFICATION OF LUCIFERASE BY REPEATED CRYSTALLIZATION

	Total units	Percent protein	Units per mg protein
Original solution	$21 \cdot 10^6$	100	8,500
Crystals I	$18.3 \cdot 10^6$	34	20,000
Crystals II	$16 \cdot 10^6$	26	23,000
Crystals III	$14 \cdot 10^6$	20	27,000

Stability of the crystalline enzyme

Although the ammonium sulfate fractions are stable in the frozen state at a neutral pH, the crystalline enzyme, even the presence of $(\text{NH}_4)_2\text{SO}_4$, is unstable when frozen and thawed. Cysteine or glutathione does not protect. In fact, enzyme kept in the refrigerator for 24 hours in the presence of 0.03 *M* cysteine becomes almost completely inactive. Versene, on the other hand, protects the enzyme from inactivation. The present procedure is to precipitate the dissolved crystals with $(\text{NH}_4)_2\text{SO}_4$ (2.4 *M*) in the presence of 0.001 *M* versene at a pH between 7.5 and 8.0 and store at 4° C. To prepare for use, a portion of the suspension is centrifuged, and the precipitate then dissolved in versene at pH 7.9. This solution is stable for days at 4° C, if the protein concentration is about 10 mg per ml.

PHYSICO-CHEMICAL CHARACTERISTICS

Electrophoretic analysis was carried out on the Perkin-Elmer Electrophoresis Apparatus using the 2 ml cell and a 0.02 *M* phosphate buffer in 0.15 *M* NaCl. The material is apparently homogeneous as is shown in Fig. 2. The isoelectric point is between pH 6.2 and 6.3, as determined by extrapolation of the mobility curve in Fig. 3. At pH 6.43, the lowest pH used, the enzyme crystallized in the electrophoresis cell so that it was impossible to make determinations nearer the isoelectric point.

The sedimentation constant was determined on the Spinco analytical ultracentrifuge: S_{25}^0 (0.15 *M* NaCl) = 5.6. Diffusion constants were not determined, but the above value is consistent with a molecular weight of about 100,000. The pictures showed a single symmetrical peak.

The ultraviolet absorption curve is shown in Fig. 4. The position of the maximum does not change in alkaline solution, so presumably there is no tyrosine present. The slight rise at 258 *mμ* may be due to luciferin since the protein solution fluoresces slightly. However, the light produced with no luciferin added is 0.004% of that produced under the same conditions with optimal amounts of added luciferin.

One mg of protein per ml in the 1 cm Beckman cell gives an optical density

reading of 0.75 at $278\text{ m}\mu$. This cannot be used as a measure of protein concentration in the impure solutions on account of the large amounts of contaminating material absorbing in this range.

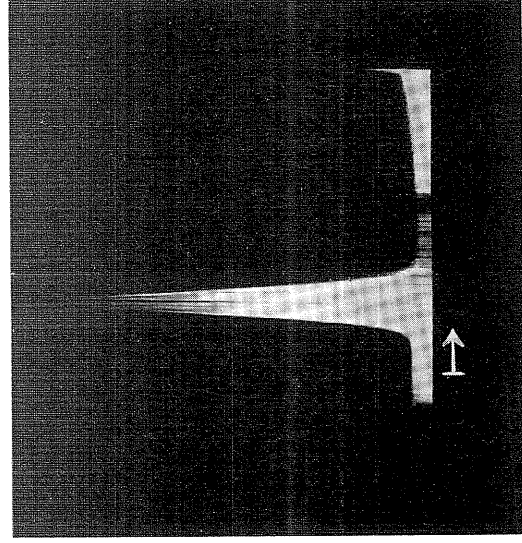


Fig. 2. Electrophoretic pattern of crystalline luciferase, pH 7.13 in 0.15 M NaCl and 0.02 M phosphate after 3 hours.

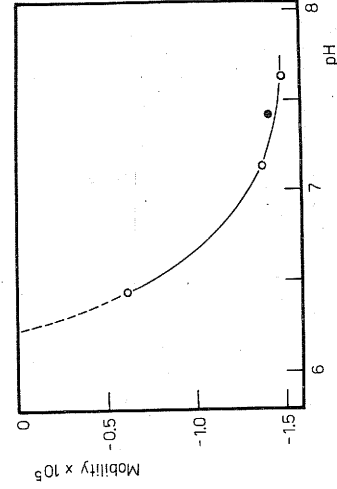


Fig. 3. Electrophoretic mobility of crystalline luciferase in 0.02 M phosphate buffers in 0.15 M NaCl.

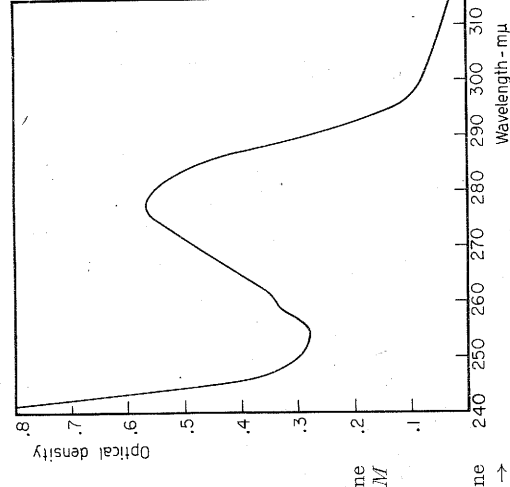


Fig. 4. Ultraviolet absorption curve of crystalline luciferase.

ENZYMIC PROPERTIES

Purification of the enzyme by crystallization has made few changes in the traditional enzymic characterisations which were reported previously⁴.

The temperature optimum is 23°C . The decreased activity at higher temperatures is due to a reversible inactivation of the enzyme. The irreversible inactivation at 35°C in the reaction mixture can be prevented by adding 0.4 M $(\text{NH}_4)_2\text{SO}_4$.

References p. 176.

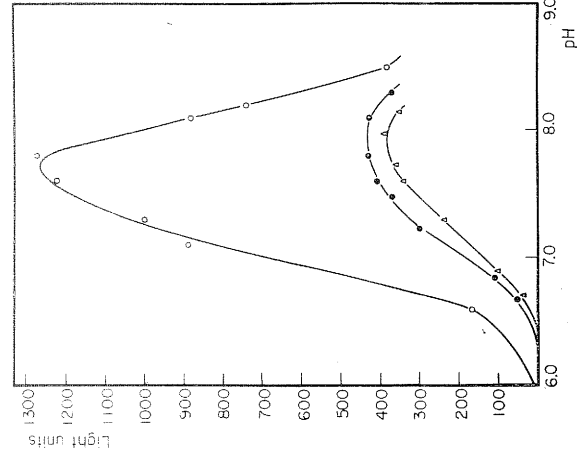


Fig. 5. Effect of pH on luciferase activity in 0.04 *M* glycine, O; 0.08 *M* sodium phosphate, ●; and in 0.08 *M* potassium phosphate, △. The same concentration of enzyme was used in all determinations.

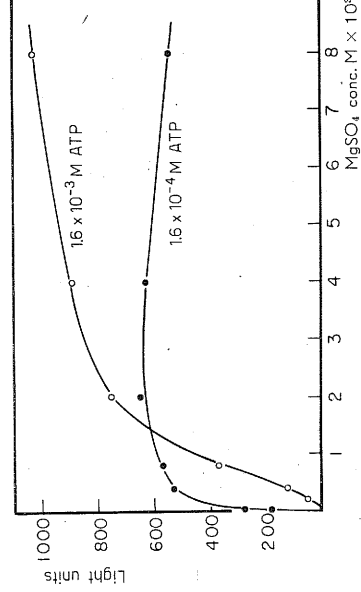


Fig. 6. Effect of Mg^{++} concentration on luciferase activity at two differing concentrations of ATP.

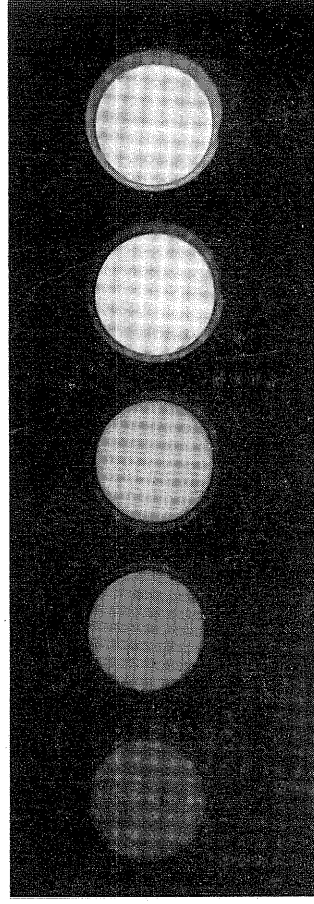


Fig. 7. The effect of ATP on the production of light by crystalline luciferase. All reagents were kept constant except for the ATP concentrations which were 0.08, 0.2, 0.4, 1.2, and $2.0 \cdot 10^{-4}$ *M*, increasing from left to right.

References p. 176.

The pH maximum in glycine or phosphate buffer is about 7.8. However, sodium and potassium phosphate buffers inhibit markedly, as can be seen in Fig. 5.

Increasing amounts of luciferin result in a typical substrate concentration curve with, perhaps, some inhibition in the higher ranges. Presentation of the data awaits a purer luciferin which can be accurately measured.

The interrelationship between Mg^{++} concentration and ATP is shown in Fig. 6. It is evident that at low ATP concentration, high concentrations of Mg^{++} inhibit, and at low Mg^{++} concentrations, high ATP inhibits. Our routine assays are performed in $4 \cdot 10^{-3}M$ $MgSO_4$.

The effect of ATP on light production is shown in Fig. 7 and 8. Fig. 7 is a photograph of the actual light emitted which shows the dependence of the light intensity on the amount of ATP. The ATP concentrations are 0.08, 0.2, 0.4, 1.2 and $2 \cdot 10^{-4}M$. In Fig. 8, the regular determinations have been carried out at two luciferin concentrations, one five times the other. The curves drawn through the points are theoretical ones according to the Michaelis-Menton equation. Investigations on the mechanism of the reaction leading to light emission are in progress and these results will be published elsewhere.

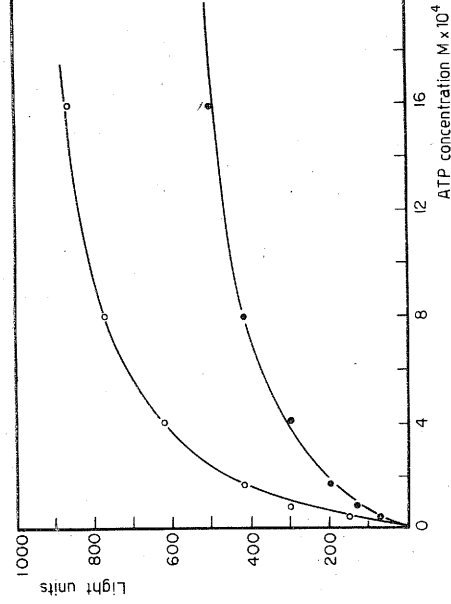


Fig. 8. The effect of ATP concentration on the production of light. In curve A the luciferin concentration gives maximum activity and in curve B the concentration is $1/5$ as high. The Michaelis-Menton constants for curves A and B are 2.3 and $3.5 \cdot 10^{-4}M$ ATP respectively. The corresponding values for V_{max} are 1000 and 600 light units.

We should like to thank Miss HILDA BECK for her capable assistance.

SUMMARY

1. The method for purification and crystallization of firefly luciferase is described.
2. The crystalline enzyme is apparently homogeneous as evidenced by ultracentrifugation and electrophoretic determinations. The molecular weight is probably around 100,000 and the isoelectric point between pH 6.2 and 6.3.
3. The effect of temperature, pH and the concentration of luciferin, Mg^{++} and ATP is described.

REFERENCES

- 1 E. N. HARVEY, *Bioluminescence*, Academic Press, Inc., New York, 1952.
- 2 J. W. HASTINGS, W. D. McELROY and J. COULOMBRE, *J. Cell. Comp. Physiol.*, 42 (1953) 137.
- 3 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
- 4 W. D. McELROY and J. W. HASTINGS in F. H. JOHNSON, *Bioluminescence of Biological Systems*, American Association for the Advancement of Science, Washington, D.C., 1955. p. 161.

Received November 15th, 1955