SEPARATION OF FIREFLY LUCIFERASE USING AN ANION EXCHANGER

M. BÉNY and M. DOLIVO
Institut de Physiologie de l'Université, CH-1011 Lausanne, Switzerland

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1. Introduction

The determination of ATP by means of the Luciferin-luciferase reaction is highly sensitive and specific for ATP [1].

However, the crude extract of commercial 'Firefly Tails', commonly used contains besides the luciferin-luciferase system, enzymes which decreases the specificity of this extract: for example ATP is formed from ADP in presence of enzymes such as nucleoside diphosphokinase (EC 2.7.4.6.) or adenylate kinase (EC 2.7.4.3.) [2,3].

The aim of this work was to obtain a pure luciferin-luciferase system, free of contaminating enzymes, with a minimum of manipulations, in order to avoid the loss of too much luciferase. Gel filtration has already been used for this purpose [4,5] but it requires very long columns and a slow speed of elution to obtain a satisfactory, though not complete separation.

In order to improve the separation and decrease the height of the columns, we decided to fractionate these enzymes, not on the basis of their molecular weights which are very similar, but on the basis of their charges using the anion exchanger DEAE-Sephadex.

2. Materials and methods

2.1. *ATP Assay*

The light emission of the luciferin-luciferase reaction has been measured in a scintillation counter (TRICARB, 3340, Packard), with the method of F. Mir [6], adapted to a volume of 2.5 ml: ATP was kept in 0.5 ml solution, at pH 11, in scintillation vials at −60°C. One hour before the assay these vials were put in the scintillation counter at 4°C. The enzymatic reaction was initiated by the injection of 2 ml of a solution containing luciferin and luciferase at a concentration of 0.2 mg/ml of firefly tails in 0.1 M arseniate buffer, 1 mM EDTA and 20 mM $MgSO_4$, at pH 7.4. Five seconds after the injection, the counts were measured for 2.4 sec. The total count is proportional to the ATP concentration.

2.2. *Assays for the purification of luciferase*

The activity of this enzyme has been tested by means of the bioluminescence reaction, as for the ATP assay, but using 0.8 μM of ATP and 1 μM of luciferin.

A standard solution of firefly tails (FLE, Sigma) kept in aliquots at −60°C, was used for the standard curve, the results are expressed in μg of the FLE.

2.2.1. Luciferin and dehydroluciferin

Luciferin was determined by its absorbance at 327 nm (pH 7.0) the ratio of the absorbance at 327 nm/348 nm was used to determine the contamination through dehydroluciferin, which is a competitive inhibitor of the luciferin [1].

2.2.2. Inorganic pyrophosphatase (PPase)

The inorganic phosphate produced by the enzymatic hydrolyse of PP_i has been determined by a modified colorimetric method after Tono [7].

2.2.3. Adenylate kinase (Ak), nucleoside diphosphokinase (NDPK), GTP-Adenylate kinase (GTP-Ak) and guanylate kinase (Gk)

All these enzymes, in presence of suitable substrates, produce ADP which has been measured by the decrease of the absorbance at 340 nm (oxidation of NADH to NAD^+) using the enzymatic coupling of the reaction pyruvate kinase-lactate dehydrogenase as modified from Agarwal method [8].

2.2.4. Proteins

They have been determined by the Lowry method [9], as modified by F. Mir [6].

2.3. *Purification*

All operations have been carried out at 4°C.

2.3.1. Extraction

1 g of dessicated firefly tails (FFT of Sigma) has been frozen with liquid nitrogen in a mortar and finely grinded. The powder was washed three times with 25 ml cold acetone (−20°C) and dried.

9 ml of buffer (TES 0.05 M, pH 7.5, 1 mM EDTA, 15 mM $MgSO_4$) were added to the powder in a centrifuge tube. The extract was allowed to stand for 15 min at 18°C in order to solubilize the luciferase.

After a short centrifugation (10 min at 3000 × *g*) the pellet was extracted a second time with 5 ml of buffer and centrifugated for 10 min at 12 000 × *g*. The combined supernatants were centrifugated again 30 min at 20 000 × *g* and filtered on glass wool to eliminate a thin lipid suspension (see below under 3.4).

2.3.2. Luciferin separation by gel filtration on Sephadex G-10

A short column of Sephadex G-10 (Pharmacia, Sweden) (fig.1) did allow to separate the luciferin and eliminate the small molecular weight contaminants (ATP, etc) [8]. This column was connected by its lower end to a DEAE-Sephadex column (cf. 1.3) which is itself bound to a fraction collector (LKB-7000) by means of a peristaltic pump (PERPEX, LKB), which regulated the speed of the whole flow (16 ml/h).

The extract was introduced on the Sephadex G-10 column and washed with the buffer solution (TES 0.05 M, pH 7.5, 1 mM EDTA, 10 mM $MgSO_4$). When the proteins reached the top of the second column as indicated by a yellow fluorescence, both columns were disconnected.

Three hundred milliliters more of the same buffer were passed through the G-10 column and discarded. Then luciferin was eluted very quickly with water, the column being connected to the collector: luciferin is eluted first and dehydroluciferin later (fig.1). All the fractions containing luciferin (about 20 ml) were mixed together and adjusted to pH 9.0. Aliquots were then stored frozen at −60°C.

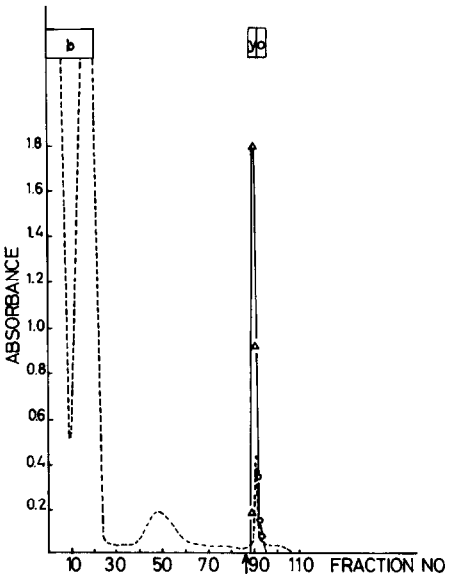

Fig.1. Gel filtration on Sephadex G-10. Column diameter, 2.6 cm; height, 7 cm. Flow: 16 ml/h; one fraction collected in 20 min. (- - -) A_{280}; (- △ -) A_{327}, $A_{327}/A_{348} > 1$ = Luciferin; (- ○ -) A_{348}, $A_{348}/A_{327} > 1$ =Dehydroluciferin. Fluorescence of the fractions is indicated by the bars (b=blue, y=yellow, o=orange).

2.3.3. Luciferase separation by DEAE-Sephadex chromatography

After the two columns had been disconnected, the same buffer (TES 50 mM, pH 7.5, 1 mM KEDTA, 10 mM $MgSO_4$) was passed through the DEAE-Sephadex. NDPk was eluted in the initial fraction (fig.2), the washing was continued until all the Ak was eluted (about 100 fractions) (fig.2).

Luciferase was then eluted using a 600 ml linear gradient from 10 mM – 40 mM $MgSO_4$ in the initial buffer. The fractions showing an enzymatic activity were mixed (fractions 120 – 200 in fig.2) and aliquots stored frozen at −60°C. The peak of activity was eluted at 23 mM $MgSO_4$. The chromatography was stopped at this point.

Between two preparations, the gel was kept in the initial buffer with 0.002% chlorhexidine.

The ionic strength of the initial buffer which is mostly due to the $MgSO_4$ is the way by which a good separation of the luciferase from the Ak is obtained.

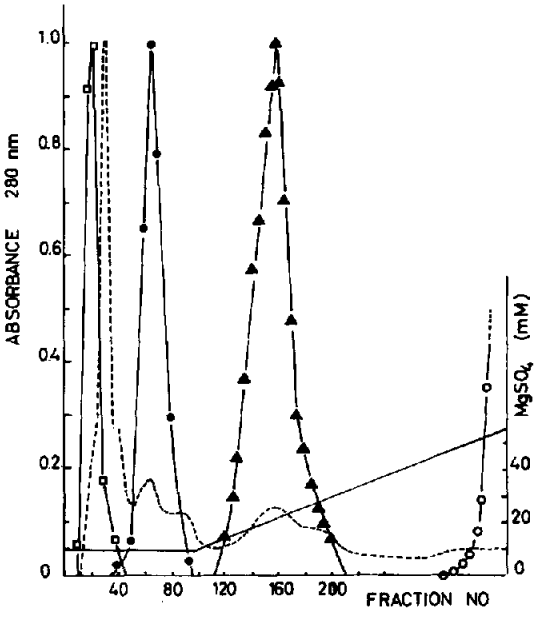

Fig.2. Exchange on DEAE-Sephadex. Column diameter, 2.1 cm; height 27 cm. Flow 16 ml/h. One fraction collected in 20 min. (- - -) A_{280} (left hand scale). The assayed peaks are normalized: (- ▲ -) Luciferase; (- ● -) Adenylate kinase; (- □ -) Nucleoside diphosphokinase; (- ○ -) Inorganic pyrophosphatase. Right ordinate: $MgSO_4$ gradient.

3. Results

3.1. *ATP Assay in presence of other nucleotides*

In order to test our purified extract, we have added various nucleotides in the standard assay solution. These nucleotides are the substrates for the enzymes to be eliminated by our purification.

3.2. *ADP*

The Ak influence was completely supressed by the purification.

3.3. *GTP + ADP*

NDPK activity was high in the crude extract but this activity had disappeared in the purified extract (fig.3).

3.4. *GTP + AMP*

ATP is produced by the system GTP-Ak (EC 2.7.4.10) + GK (EC 2.7.4.8) (Eq. fig.4): in the crude extract, their large influence on ATP assays

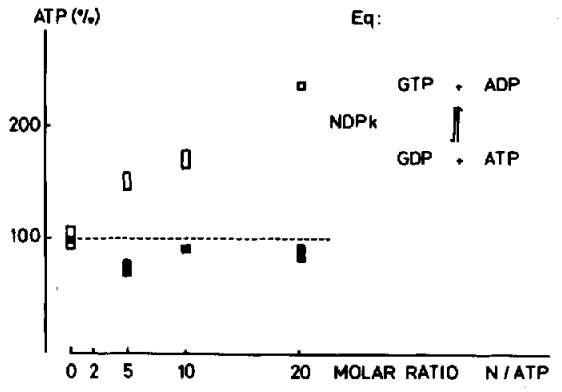

Fig.3. Influence of GTP + ADP. N=(GTP)=(ADP) The bars represent the mean ± SEM (N=3) of ATP measured (% of ATP alone): black bars for purified extracts, white bars for crude extracts.

comes from their very fast kinetics, for example: the transformation of AMP to ATP is completed before the beginning of the counting (5 s) in the ATP assay.

These enzymes are bound to a lipidic suspension: the treatment of the crude extract, with acetone and its centrifugation at high speed, followed by a filtration on glass wool (cf. 1.1), has been successful in purifying the extract from these enzymes (fig.4): no influence of these enzymes has been noticeable after this treatment.

3.5. *AMP*

We also checked that our purified extract did not

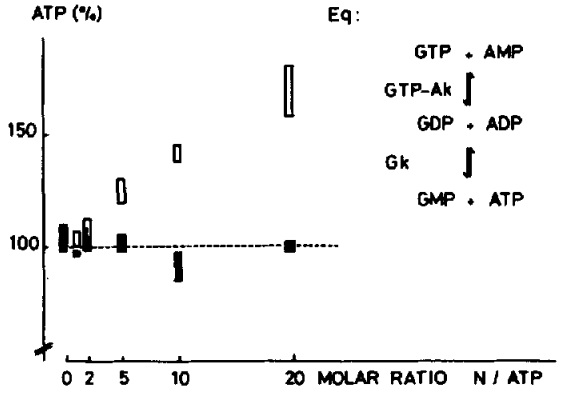

Fig.4. Influence of GTP + AMP N=(GTP)=(AMP) Further explanations see fig.3.

show an increase in ATP in presence of AMP and other trinucleotides as UTP and GTP in high concentrations, namely AMP/ATP, UTP/ATP or CTP/ATP = 40 (molar ratio).

3.6. *Yield*

As compared to a crude extract stored during the same time at 4°C it appears that the recovery of luciferase is a total one thanks to the complete separation of the enzymes. The yield is only affected by the decrease of activity at 4°C.

Attempts to improve the stability during storage by addition of dithiotreitol, calcium or sodium azide to the buffer, or by the use of other buffers (HEPES arseniate or glycine) did not succeed.

4. Conclusion

The use of a DEAE-Sephadex for the separation of luciferase has a clear benefit:

In contrast to the gel filtration [4] no enzymes (NDPK, Ak, PPase) are superimposed on the luciferase during the elution. The simple and reproducible separation needed only a small amount of gel (3 g DEAE-Sephadex for 1 g FFT). The size of the sample solution can be as large as 14 ml.

The constancy of the elution of the luciferase in respect to the concentration gradient (peak always at 23 mM $MgSO_4$) allows to avoid specific assays for the mixing of the fractions.

Acknowledgement

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