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Firefly luciferase purification using polyethylene glycol and Dyematrex Orange A

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Abstract

Efficient measurement of adenosine triphosphate by bioluminescence depends on the quality of firefly luciferase used. A rapid purification of this enzyme is reported that permits removal of enzymes interfering in the bioluminescent reaction. The enzyme was extracted from firefly tails and precipitated with PEG 20 000, and the resulting pellet was subjected to chromatography on a Dyematrex gel (Orange A), which retains the interfering enzymes but does not bind luciferase. As shown by adenylate kinase activity determination and sodium dodecyl sulfate polyacrylamide gel electrophoretic examination of the resultant preparation, partial purification of luciferase was successful in giving a preparation without interfering enzymes.

1. Introduction

The production of light catalysed by firefly luciferase depends on the presence of luciferin, adenosine triphosphate (ATP), magnesium and molecular oxygen [1]. In the crude extract of firefly tails, the presence of adenylate kinase, nucleotide diphosphate kinase and other ATP-converting enzymes [2–6] can produce light with nucleotides other than ATP. It is therefore necessary to remove these interfering enzymes during purification of firefly luciferase.

Several methods of purification have been reported [7–18]. With some of these a crystalline firefly luciferase was obtained after ammonium sulfate precipitation of the crude extract of firefly tails [7,16,17], and with others the purified luciferase was obtained after binding of the enzyme on an immobilized ligand and a long specific elution step [13–15]. The aim of all of these methods was to obtain a firefly luciferase preparation free from interfering enzymes.

Recently, Amicon (Danvers, MA, USA) developed the dye ligand Dyematrex Orange A. This matrix gel is composed of a triazinyl dye of spherical shape, of diameter 50–150 μm, immobilized on cross-linked agarose, and is remarkable for the small number of proteins that have been found to bind to it.

Firefly luciferase contains a large number of hydrophobic amino acids [19–22], making it possible to use polyethylene glycol (PEG) [23] for precipitation of the enzyme. Here we report a rapid and convenient procedure, which consists of precipitation of firefly luciferase with PEG 20 000, followed by affinity chromatography on an Orange A gel, which binds many interfering enzymes of the bioluminescent reaction but not

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luciferase, which can therefore be obtained without a specific elution step. The capacity of the method to remove interfering enzymes was demonstrated by the determination of adenylate kinase and nucleoside diphosphate kinase as references in each step of purification. The quality of the luciferase preparation was demonstrated by the very low background of luciferin–luciferase mixture. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that following this chromatography, firefly luciferase had been partially purified.

2. Experimental

2.1. Instruments and materials

An Optocomp II luminometer (Tecan, Voisins le Bretonneux, France) was used for the measurements of luciferase activity. A PhastSystem (Pharmacia–LKB Biotechnology, Uppsala, Sweden) was used for SDS-PAGE.

Desiccated firefly tails were obtained from Sigma (St. Louis, MO, USA). Dyematrex Orange A gel was purchased from Amicon (Beverly, MA, USA). All products for electrophoresis were obtained from Pharmacia–LKB Biotechnology.

2.2. Purification

Firefly luciferase extraction

A 200-mg amount of desiccated firefly tails was pounded in Fontainebleau sand (particle size 150–210 μm) (Prolabo, Paris, France) after being extracted with 1 ml of extraction buffer [10 m*M* $Mg(CH_3COO)_2$–1.5 m*M* NaN_3–5 m*M* dithiothreitol (DTT)–2 m*M* EDTA–20 m*M* Tris (pH 7.75)]. After pounding, 14 ml of same buffer were used to rinse the mortar. The mixture of sand and subcellular organelles was centrifuged at 1400 *g* for 5 min at 4°C and the supernatant solution was removed and kept on ice. The pellet obtained was washed with 5 ml of the same buffer and recentrifuged using the same conditions. The second supernatant solution was added to the first and centrifuged at 10 000 *g* for 30 min at 4°C, and the crude extract solution was recovered.

Precipitation of firefly luciferase

The clear crude extract solution obtained after the third centrifugation was mixed with 18% (w/v) of PEG 20 000 and after homogenization was kept on ice for ca. 30 min, then centrifuged at 10 000 *g* for 30 min at 4°C. The precipitate was dissolved in 5 ml of elution buffer [10 m*M* $Mg(CH_3COO)_2$–1.5 m*M* NaN_3–5 m*M* DTT–2 m*M* EDTA–20 m*M* Tris (pH 7.75) containing 20% glycerol] [16].

Dyematrex gel media preparation

A 10-ml amount of Orange A gel was mixed with 20 ml of 0.5 *M* NaCl solution and this mixture was poured into an empty PD-10 column containing a Vyon filter in the upper outlet (Pharmacia, Uppsala, Sweden; 80 × 15 mm I.D.) which had been closed. After the bed had stabilized, a second Vyon filter was settled, giving a bed height of 35 mm. The Orange A gel column was regenerated with 8 *M* urea, washed with the extraction buffer and then equilibrated with the elution buffer before use. The outlet end of the column was connected to a peristaltic pump (Pharmacia) that regulated the flow-rate at 10 ml/h.

Chromatography of firefly luciferase

Orange A gel chromatography was carried out between 4 and 10°C. After PEG precipitation, 5 ml of extract were applied to the Dyematrex Orange A gel column and eluted with the elution buffer for the first collected fractions. Foaming occurred in some fractions. In a second step, 0.5 *M* NaCl was used to strip off the bound proteins from the Dyematrex Orange A column. The volume of each fraction collected was 1 ml. After each use, the column was washed with four volumes of extraction buffer and stored capped at 4–8°C and containing a solution of 0.02% NaN_3.

2.3. Enzyme and protein assays

All assays were conducted at room temperature (22 ± 2°C).

Luciferase assay

Luciferase activity was determined by measuring light production by means of an Optocomp II luminometer. The numbers of photons were integrated in 5 s. The reaction was started by manual injection of 10 μl of sample to 100 μl of luciferase activity reagent in a polystyrene tube. The luciferase activity reagent contained 10 μl of 10^{-8} *M* ATP and 20 μl of a standard solution of luciferin in 10 ml of 5 m*M* $MgSO_4$–0.5 m*M* EDTA–0.5 m*M* DTT–25 m*M* Tris (pH 7.75) reagent buffer. The difference between the signal of each sample and the background light gave the luciferase activity expressed in units/ml, since the light intensity was expressed in relative light units (RLU). All chromatographic fractions were assayed and peak fractions containing more than 40% of the maximum luciferase activity were pooled.

Adenylate kinase assay

Adenylate kinase activity was determined according to the procedure of Bergmeyer et al. [24] at 340 nm and 25°C, and expressed in U/l (μmol/min·l), and all fractions were assayed.

Nucleoside diphosphate kinase assay

Nucleoside diphosphate kinase activity was determined according to the procedure of Bergmeyer et al., except that CDP was used instead of dTDP, at 37°C [25]. Only the crude extract, the precipitate and pooled fractions were assayed.

Protein assay

Protein concentrations were determined by the bicinchoninic acid (BCA) method [26] with bovine serum albumin (BSA) as standard, and expressed in mg/ml.

SDS-Page

SDS-PAGE was performed with PhastSystem equipment using 20% polyacrylamide homogeneous gels (PhastGel homogeneous 20; Pharmacia) [26]. Each sample (crude extract and pooled fractions from chromatography) was mixed (3:2 and 2:1, respectively) with TD 2X solution containing 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, 6% (w/v) SDS and 25% (v/v) 0.5 *M* Trizma base (pH 6.8), and heated at 100°C for 2 min. A 4-μl volume of these treated samples was applied to the gel using a PhastGel applicator 6/4 μl (Pharmacia). Gels were run with SDS buffer strips (Pharmacia) at 250 V, 10 mA, 3 W, 15°C, 95 Vh. They were then fixed and stained with PhastGel Blue R (Pharmacia) according to the instructions of the manufacturer [27].

2.4. Background assay

The background was determined by measuring light production as in the luciferase activity assay. The reaction was started by injection of 10 μl of sterile injectable water instead of standard ATP in a polystyrene tube containing 100 μl of luciferin–luciferase mixture [360 μ*M* luciferin and 0.800 mg of protein (luciferase preparation) in 10 ml of 10 m*M* $Mg(CH_3COO)_2$–4 m*M* DTT–2 m*M* EDTA–0.1 g/l BSA–25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.75)]. Two luciferin–luciferase mixtures were prepared, for the preparation of luciferase precipitate and for pooled fractions. Prior to use, these mixtures were stored at room temperature and in the dark for several hours in order to reduce residual inherent light production.

After 12 h of incubation, assays were performed at intervals of 6 h for the two luciferin–luciferase mixtures.

3. Results

3.1. Purification

From 200 mg of firefly tails purified, 15 mg of total proteins were recovered in the pellet after precipitation with PEG 20 000 and 5 mg in the

Table 1
Firefly luciferase purification

Purification step	Total protein (mg)	Total activity[a] (units) · 10^5	Recovery (%)	Specific activity[a] (units/mg) · 10^4	Purification factor (-fold)	Adenylate kinase activity (U/l)	Nucleoside diphosphate kinase activity (U/l) · 10^3
Crude extract	134	1.892	100	0.140	1	1031	32.9
Precipitate after PEG	15	1.309	69	0.873	6	188	49.3
Pooled fractions from chromatography	5	1.804	95	3.608	26	3	0.39

Crude extract: 18 ml. Precipitate: 5 ml. Pooled fractions from chromatography: 3 ml.
[a] Luciferase activity is expressed in RLU/s · ml, where one unit = RLU/s.

pooled fractions after chromatography (Table 1). Fig. 1 shows that most of the proteins subjected to Orange A chromatography were washed off with the first non-specific eluent in the initial protein peak. A second protein peak corresponding to the few proteins bound to the Orange A appeared after elution with 0.5 *M* NaCl.

Firefly luciferase was purified 26-fold from the crude extract by these two purification steps.

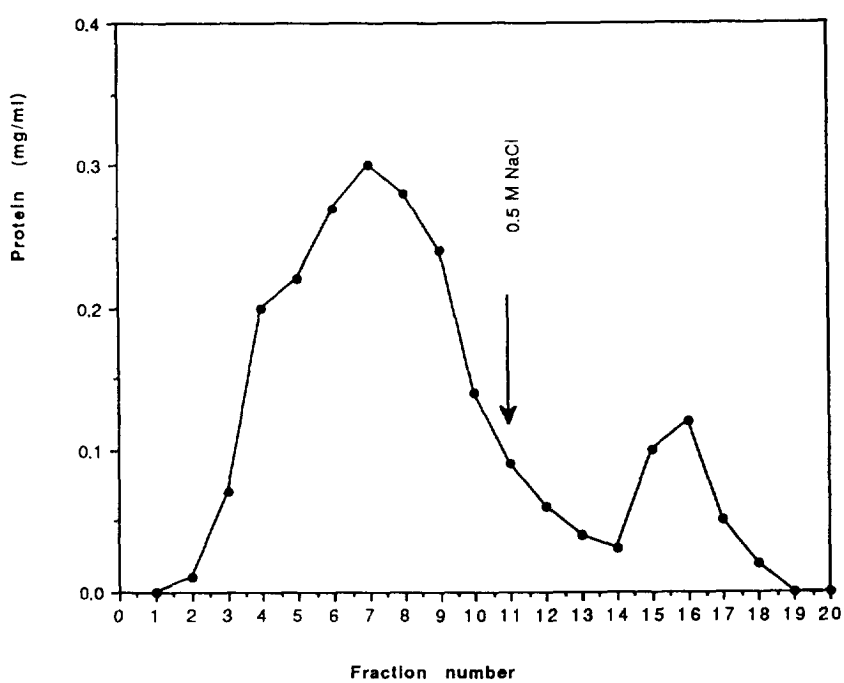

Fig. 1. Protein distribution. After the fractionation of firefly luciferase (see Fig. 2), the protein concentrations of all fractions were measured (see Experimental). Unbound and bound proteins were obtained by a non-specific eluent and 0.5 *M* NaCl, respectively (see arrow).

3.2. Enzyme activity assay

The use of 18% (w/v) of PEG 20 000 allowed the recovery of 69% of the total luciferase activity from the crude extract, with elimination of 82% of total adenylate kinase activity. The majority of the remaining adenylate kinase (18% of activity found in the crude extract) was retained by Orange A so that the final preparation had an adenylate kinase activity of only 0.3% of that of the crude extract (Table 1). Nevertheless, 18% of PEG 20 000 did not eliminate nucleoside diphosphate kinase, which was concentrated in the precipitate. As adenylate kinase, nucleoside diphosphate kinase that was present in the precipitate was retained by Orange A, and the final preparation had a nucleoside diphosphate kinase activity of 0.8% of that of the precipitate after PEG, and 1.2% of that of the crude extract (Table 1). The decrease in adenylate kinase activity, nucleoside diphosphate kinase activity and protein concentration in the pooled fractions after chromatography and the presence of glycerol [16] in the non-specific eluent buffer induced an increase in the luciferase activity, which rose to 95% of the luciferase activity of the crude extract. Fig. 2 shows one peak of luciferase activity obtained by the non-specific elution, and one peak of adenylate kinase activity obtained after the use of 0.5 *M* NaCl.

3.3. SDS-PAGE

Fig. 3 shows the results of SDS-PAGE of the final preparation in which partially purified luciferase was obtained. The molecular masses of proteins estimated from their mobility on SDS-PAGE were 66 000 for one subunit of luciferase [21,28] and 20 000 for adenylate kinase [24,29]. Most of the low-molecular-mass proteins (less than 30 000) present in the crude extract (lane 2)

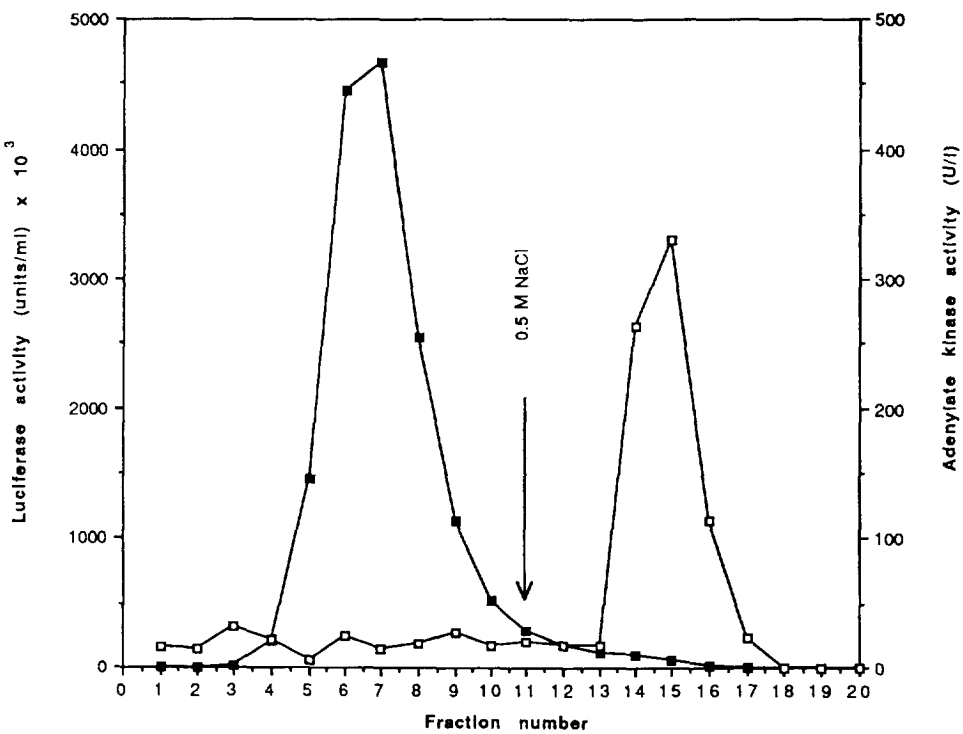

Fig. 2. Fractionation of firefly luciferase on Orange A column. The Orange A column and the conditions for the measurement of activities are described under Experimental. Fractions 6, 7, 8 were pooled (3 ml). The arrow indicates the beginning of elution with 0.5 *M* NaCl, which stripped off the bound proteins from the column, and then the appearance of the adenylate kinase activity in fractions 14, 15, 16 was observed. ■ = Luciferase activity; □ = adenylate kinase activity.

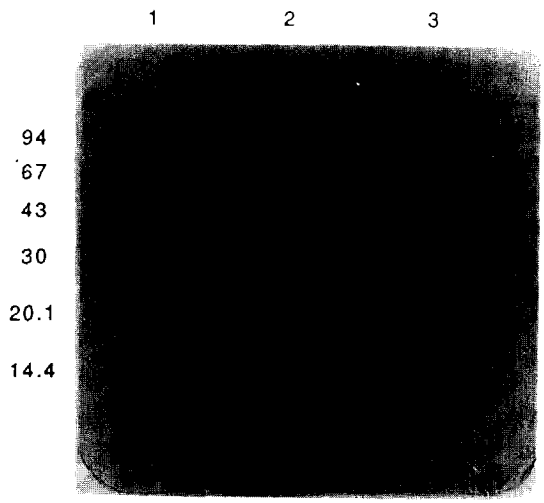

Fig. 3. SDS-PAGE of firefly luciferase in PhastGel 20% polyacrylamide homogeneous medium (Pharmacia). For the conditions of electrophoresis, see Experimental. The gels was run for 95 Vh, ca. 30 min. Staining: PhastGel Blue R (Coomassie Blue 350) (0.1% PhastGel Blue R solution in 30% methanol and 10% acetic acid in distilled water); destained in acetic acid–methnaol–water (1:3:6). Lanes: = 1 marker protein (M_r values $\times 10^{-3}$ are given); 2 = crude extract; 3 = pooled fractions from chromatography.

were not observed in the pooled fractions from chromatography, but another band of protein of M_r 40 000 was observed (lane 3).

3.4. Background assay

The blank value was high in the luciferase preparation before purification, and became much lower in the purified luciferase (pooled fractions). The light emitted by the purified luciferase represented 20% of the light emitted by the luciferase preparation before chromatography. Table 2 shows that the light emission and the stability of luciferase preparation depend on the presence of the contaminant enzymes.

4. Discussion

The method that we have developed, using PEG 20 000 and Orange A chromatography, permitted us to obtain a luciferase preparation with 95% of total activity, and free from interfering enzymes such as adenylate kinase and nucleoside diphosphate kinase. The activities of adenylate kinase and nucleoside diphosphate kinase after precipitation show that 18% PEG 20 000 is capable of excluding part of the adenylate kinase, but incapable of excluding nucleoside diphosphate kinase (Table 2). The presence of these interferences in the luciferase preparation was demonstrated by comparison of

Table 2
Background emission of luciferase preparations before and after chromatography on Orange A

Time (h)	Background (RLU per 5s)	
	Before chromatography[a]	After chromatography[b]
12	1676	307
	1640	287
	1616	306
18	1105	256
	1104	279
	1129	258
24	955	227
	962	215
	919	195

Each value represents the mean of three measurements, and the first measurement started 12 h after storage of luciferin–luciferase mixtures in the dark at room temperature.
[a] Luciferase preparation of precipitate after PEG.
[b] Final luciferase preparation (pooled fractions).

the backgrounds obtained with a non-purified and a purified luciferase preparation (Table 2). Moreover, it is possible to obtain a decrease in the background by dialysis before the chromatographic step, better than by chromatography without dialysis, because the presence of nucleosides is capable of interfering in the measurement of the activity of enzymes as nucleoside diphosphate kinase. The stability of the purified luciferase preparation was better than that of the non-purified luciferase preparation because of the absence of the interfering enzymes (Table 2).

In the purified luciferase preparation, another protein was observed and the nature of this contaminant protein was not established, but the nature and the role of this protein will be studied in future investigations.

Several methods have been reported for the purification of firefly luciferase. Most of these use ammonium sulfate [7,16,17] to precipitate the proteins present in the crude extract, whereas others use ammonium sulfate precipitation and classical affinity chromatography [10,12–15] with various ligands that bind luciferase. Both approaches have certain disadvantages, which are overcome by using our procedure. The first type of method requires a long dialysis and a protein concentration step. The use of PEG in our method is convenient as PEG does not need to be subsequently removed from the preparation. It is in fact possible to use PEG as an activator of luciferase activity [30], hence its presence in the precipitate is not a problem. The second type of method, classical affinity chromatography specific for luciferase, requires the use of ATP or luciferin to elute the bound enzyme [13–15], so that the fractions containing appreciable luciferase must be subjected to a long dialysis step, which is not required with our method.

Our method has the disadvantage that the final preparation obtained contains firefly luciferase and another protein (Fig. 3, lane 3). However, for the purpose of ATP measurement this luciferase preparation can be used under the same conditions as pure luciferase.

In conclusion, PEG precipitation followed by Orange A chromatography permits the removal of the enzymes, such as adenylate kinase, that interfere with the bioluminescent reaction used in the measurement of metabolites. The other contaminant enzymes, such as nucleoside diphosphate kinase, were retained by Orange A chromatography. Unlike other methods commonly used, this method is rapid and requires only a small amount of starting material (reagents and equipment), so that it could be used routinely in laboratories working with bioluminescence.

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