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Fractionation of Extracts of Firefly Tails by Gel Filtration

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Extracts of firefly tails were fractionated on Sephadex gels resulting in an improved analytical method for adenosine triphosphate (ATP). On low porosity gels luciferin, dehydroluciferin, and ATP were separated, and enzyme preparations with low content of ATP (blank value) and other low molecular weight substances were obtained. On high porosity gels the luciferase was well separated from adenylate kinase and partly separated from nucleoside diphosphate kinase. Enzyme preparations with highly increased specificity and low blank value were thereby obtained. In addition assay methods are described for the enzymes and substrates using the luciferin-luciferase analysis of ATP.

The ATP-dependent luminescence of extracts of firefly tails is caused by the presence of the enzyme luciferase and the substrate luciferin (review, *e.g.*, Ref. 1). Both of these compounds have been isolated from the extracts in crystalline form,[2,3] and the substrate has furthermore been obtained by chemical synthesis.[4] Present in the crude extracts are two enzymes, adenylate kinase (E.C. 2.7.4.3.) and nucleoside diphosphate kinase (E.C. 2.7.4.6.) which catalyze the formation of ATP from other nucleotides. As a consequence there is a lack of specificity in the enzymatic analysis of ATP with crude extracts of firefly tails. The crystalline enzyme can be used for a highly specific analysis, but the isolation requires very large amounts of fireflies. Ammonium sulphate fractionation of rather small volumes of extract has been used for partial purification.[1,5]

The present study was undertaken with the purpose of developing a method for fractionating extracts of firefly tails in quantities used for analysis of ATP. Two different preparations were obtained by gel filtration. One of the preparations was as unspecific as the crude enzyme, but contained much less ATP and other low molecular weight substances. The other preparation possessed a highly increased specificity with respect to adenosine diphosphate (ADP) and a somewhat increased specificity with respect to nucleoside triphosphates. Analysis of ATP with these preparations is described elsewhere.[6]

EXPERIMENTAL

Column chromatography

The columns were made of perspex tubes with end pieces of vyon filter (Porous Plastic Ltd., Dagenham Doch, England) (*cf.* Ref. 7). They were packed [7] with Sephadex gel prepared as described by the manufacturer (Pharmacia, Uppsala, Sweden). The concentrated enzyme extracts (about 50 mg desiccated firefly tails per ml 0.1 M glycyl-glycine with 1 mM EDTA, pH 7.7) (*cf.* Ref. 6) were pipetted onto the top of the column which was covered with filter paper in order to stabilize the gel surface. The flow through the column was regulated with a "Mini flow pump" (LKB, Stockholm, Sweden) and the fractions (about 3 ml) were collected with a time operated fraction collector. The gel filtration was carried out at 4°. A glycine-arsenate buffer (50 mM glycine, 10 mM Na_2HAsO_4, 1 mM EDTA, pH 7.7) was used as eluent.

Assay procedures

Luciferase. The luciferase activity was determined with the instrument used for the ATP analysis.[6] 800 μl of a solution of 5 mM $MgSO_4$, 8 nM ATP and luciferin in glycine-arsenate buffer were injected into the cuvette. The concentration of luciferin isolated by gel filtration corresponded to 0.5 mg firefly tails per ml. The volume of the solution to be assayed was between 10 and 100 μl. A flash of the same appearance as an ATP flash [6] would result and the flash height was proportional to the enzyme concentration. For determining the luciferase activity in the concentrated enzyme extract, it was necessary to dilute the sample 100 times with buffer in order to avoid interference from the luciferin present in the extract.

Luciferin. The relative concentration of luciferin was determined in the same way as the luciferase activity. Into the cuvette containing 10—100 μl of the solution to be assayed were injected 800 μl of a reaction mixture containing partially purified luciferase (corresponding to 0.5 mg firefly tails per ml), 5 mM $MgSO_4$, 8 nM ATP in glycine-arsenate buffer. The flash height was proportional to the amount of luciferin present. The concentrated crude extract was diluted 100 times for the assay.

Adenylate kinase. The adenylate kinase activity was determined by measuring the amount of ATP formed by incubating 3—15 μl of the solution to be assayed with 100 μl buffer-substrate solution (0.1 M glycyl-glycine, 10 mM $MgSO_4$, 80 μM ADP (purified as described), pH 7.7). The incubation (30—180 sec at room temperature) was performed in a cuvette for the ATP analysis and the ATP determined directly without prior deproteinization. Below 9×10^{-10} moles the amount of ATP formed was directly proportional to the amount of enzyme present. It was necessary to use partially purified luciferase for the analysis of ATP because of the interference of the adenylate kinase present in the crude enzyme.

Nucleoside diphosphate kinase. The nucleoside diphosphate kinase activity was determined in the same way as the activity of the adenylate kinase, except that ATP was analyzed in an aliquot (10 μl) of the incubation mixture. 5—15 μl of the solution to be assayed was incubated for a total period of 60 sec with 200 μl of the buffer-substrate solution (0.1 M glycyl-glycine, 10 mM $MgSO_4$, 50 μM ADP (purified), 50 μM inosine triphosphate, pH 7.7). Below 8 μM the concentration of ATP was proportional to the amount of enzyme present. The assay was sensitive for adenylate kinase, which, however, could be determined by omitting the inosine triphosphate from the reaction mixture.

Inorganic pyrophosphatase. Inorganic pyrophosphatase (E.C. 3.6.1.1.) activity was assayed by the method of Heppel.[8]

ATP. ATP was determined by the method previously described.[6]

Protein. Protein was determined by a biuret method with bovine serum albumin as standard.[9]

Table 1. K_d-values obtained by elution with glycine-arsenate buffer.

Compound	Sephadex gel type		
	G-10	G-25	G-100
ATP	0.0	0.6	1.0
Luciferin	30	5.4	1.4
Dehydroluciferin	60	10	1.7

Materials

Desiccated firefly tails (Stock No. FFT) were purchased from Sigma Chemical Compound, St. Louis, Mo., USA. Nucleotides were obtained from C.F. Boehringer & Soehne, Mannheim, Germany, or Sigma Chemical Company. ADP was purified by ion exchange chromatography by a method of Munch-Petersen and Neuhard.[10] Other chemicals were analytical grade. Deionized water was used throughout.

RESULTS

Fractionation on gels of low porosity. The data of Table 1 clearly indicate that luciferin and dehydroluciferin were strongly adsorbed to the gel matrix and that this adsorption was most pronounced for the low porosity gels. The reduced and oxidized forms of luciferin could be separated because of the difference in adsorption properties. The fluorescence spectra (excitation 366 mμ) coincided with those published by McElroy.[11] The purity of luciferin is reflected in the ratio between the 327 mμ and 263 mμ absorbance, being 4.5

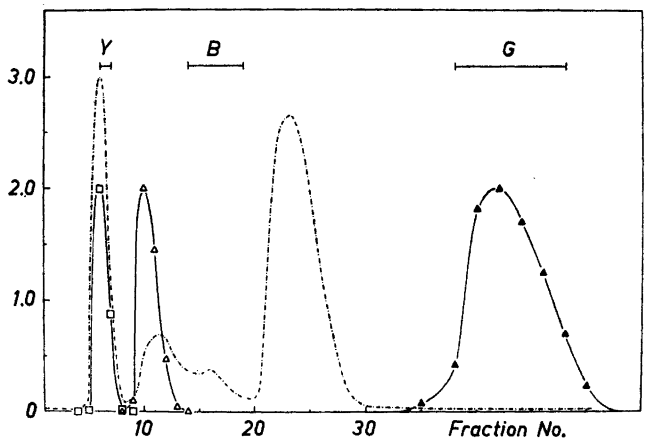

Fig. 1. Fractionation of extract of firefly tails on Sephadex G-25 (20—80 μ). Column diameter, 1.6 cm; height, 36 cm. A 1.1 ml extract (70 mg dried tails) containing 2mM ATP was applied onto the column which was eluted with glycine-arsenate buffer (10 ml/h, 1 fraction collected in 30 min). The fluorescent fractions (excitation 366 mμ) are indicated by the bars (B, blue; G, green; Y, yellow). The dashed curve represents absorbance (cm^{-1}) at 280 mμ (left hand scale). The assayed peaks are normalized: □, luciferase; △, ATP; ▲, luciferin.

for the crystalline compound.[11] Values of about 2 were obtained by this fractionation. The adsorption of luciferin and dehydroluciferin was highly decreased if water was used as eluent. For instance the K_d-value for luciferin on Sephadex G-10 was lowered from 30 to 0.7. Similar effects have been observed for other aromatic compounds with an acidic group, and have been explained as the result of adsorption and ion exclusion.[12]

Luciferase and the other proteins of the extract would be excluded completely from the low porosity gels, and the most favourable gel for removing the ATP from the extract was therefore G-25 (*cf.* Table 1). A chromatogram is shown in Fig. 1. Four routine preparations the eluting buffer was replaced by deionized water when the blue fluorescent compound had emerged from the column. Other conditions for the gel filtration can be taken from the legend for the figure. The maximal amount of extract fractionated on the column described was 4 ml equivalent to 200 mg firefly tails. The position of the various compounds could be established by their fluorescence (luciferase, weak yellow; luciferin, green; dehydroluciferin, yellow). Assays were thereby avoided.

Fractionation on gels of high porosity. Fig. 2 shows a chromatogram of an extract of firefly tails. As mentioned above an attempt was made to isolate luciferase free of nucleoside diphosphate kinase and adenylate kinase. The K_d-values of the three enzymes differed rather little, and the optimum conditions therefore had to be obtained with respect to choice of gel type and number of theoretical plates of the column. Increasing the porosity of the gel caused the two first enzymes to move towards the adenylate kinase peak. With Sephadex G-75 the number of theoretical plates was less critical for the separation of adenylate kinase and with Sephadex G-150 the separation of luciferase and adenylate kinase was hardly achieved. The separation of

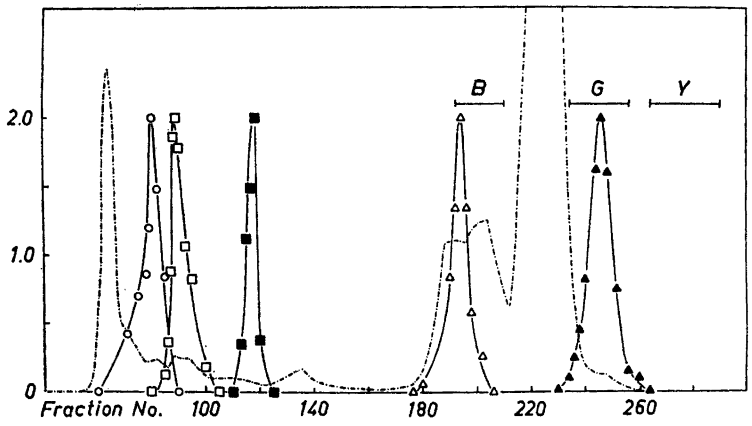

Fig. 2. Fractionation of extract of firefly tails on Sephadex G-100 (40–47 μ). Column diameter, 1.9 cm; height, 93 cm. A 3.0 ml extract (215 mg dried tails) was applied onto the column which was eluted with glycine-arsenate buffer (3 ml/h, 1 fraction collected in 30 min). The fluorescent fractions (excitation 366 mμ) are indicated by the bars (B, blue; G, green; Y, yellow). The dashed curve represents the absorbance (cm^{-1}) at 280 mμ (left hand scale). The assayed peaks are normalized: ○, nucleoside diphosphate kinase; □, luciferase; ■ adenylate kinase; △, ATP; ▲, luciferin.

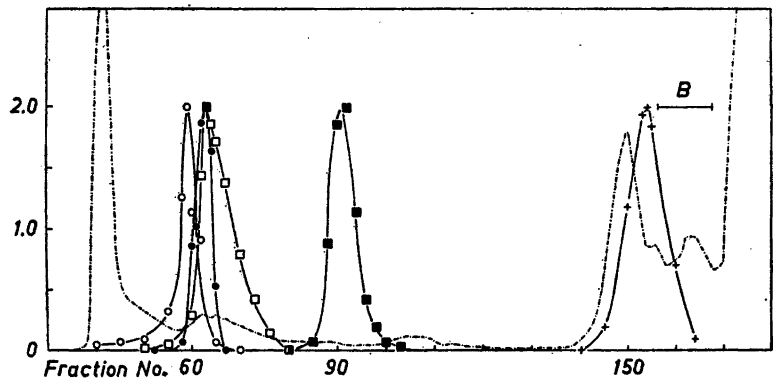

Fig. 3. Fractionation of extract of firefly tails on Sephadex G-10 (40—120 μ) and G-100 (40—47 μ). G-10 column: Diameter, 2.4 cm; height, 5 cm. G-100 column: Diameter, 2.4 cm; height, 106 cm. A 5.0 ml extract (270 mg dried tails) was applied to the G-10 column and elution performed as described (6 ml/h, 1 fraction collected in 30 min). The dashed curve represents absorbance (cm^{-1}) at 280 mμ (left hand scale). The fluorescent fractions (excitation 366 mμ) are indicated by the bar (B, blue). The assayed peaks are normalized: ○, nucleoside diphosphate kinase; ●, inorganic pyrophosphatase; □, luciferase; ■, adenylate kinase; +, [^{3}H] water.

nucleoside diphosphate kinase and luciferase was equally good on Sephadex G-100 and G-150 and somewhat poorer on Sephadex G-75. In order to obtain the separation shown in Fig. 2, it was necessary to operate the column with about 800 theoretical plates. The dry gel was sieved through a set of U.S. standard sieves, and the flow rate kept at about 1 ml/$cm^2 \times$h. The maximal capacity of the column amounted to the extract of about 100 mg desiccated firefly tails per cm^2 applied in a volume less than 1 % of the volume of the column.

As seen from Fig. 2, ATP, luciferin, and dehydroluciferin were separated on the G-100 column. The elution of luciferin required, however, about 5 days and a combination of two different gel columns was therefore used (Fig. 3). A very short column of G-10 was connected to the long G-100 column and the extract was applied on the short column. The two columns were eluted until the blue fluorescent compound had entered the G-100 column (about 50 ml eluent for the conditions described). At this time all ATP would be in the G-100 column, while the luciferin, well separated from the dehydroluciferin, had not yet reached the bottom of the G-10 column. This was disconnected from the long column, the elution of which was continued normally while the short column was eluted with deionized water. The separation of luciferin and dehydroluciferin was not impaired seriously by this elution.

The recovery of luciferin was 90—105 % (5 expts.). The yield of luciferase in the experiment depicted in Fig. 3 appears in Table 2. Other experiments gave values ranging from 40—50 % yield. The purified luciferase was stable for months when stored at —25°.

Table 2. Purification of luciferase by gel filtration.

Step	Volume ml	Units[a]/ml	Total units [a]	Protein mg/ml	Specific activity units [a]/mg
Crude extract	5	5 000	25 000	29	172
85 % of luciferase peak	33	370	12 200	0.5	740

[a] Arbitrary unit.

DISCUSSION

The elution pattern of substances absorbing at 280 mμ appeared to be similar for all the firefly extracts tried (*cf.* Figs. 1, 2, and 3), and it can be used to estimate roughly the position of the fractions of interest. The fluorescent fractions were also useful for this purpose.

As indicated in Fig. 1 luciferase eluted from the short column showed yellow fluorescence, probably due to the presence of dehydroluciferin which is known to be strongly bound to the enzyme.[11] On gels of high porosity the same effect was observed. The fluorescence of the luciferase band would decrease gradually as it moved down the column. The passage of the extract through the G-10 column before the G-100 column affected the shape of the luciferase peak (Figs. 2 and 3). An asymmetrical peak with a steeper leading edge was obtained. The shape of the other peaks was not changed significantly. The change of the luciferase peak might be caused by the removal of luciferin and dehydroluciferin, for instance by affecting the association of the two monomeric units of the enzyme.[13]

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