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Note

Purification of luciferase by affinity elution chromatography on Blue Dextran columns

Comparison of Sepharose and silica as support matrices

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The popularity and significance of the luciferase-catalysed bioluminescence in the firefly is well documented. Its remarkable specificity and sensitivity towards adenosine triphosphate (ATP) has induced us to work out methods for the purification of luciferase. The essence of the reaction is the production of light from luciferin in the presence of ATP, Mg^{2+} and molecular oxygen as shown in eqns. 1 and 2

$$LH_2 + ATP + E \rightarrow E \cdot LH_2 \cdot AMP + PP \quad (1)$$

$$E \cdot LH_2 \cdot AMP + O_2 \rightarrow E + P + CO_2 \quad (2)$$

where LH_2 = luciferin, E = enzyme, $E \cdot LH_2 \cdot AMP$ = the luciferyl adenylate complex, P = the product oxyluciferin and PP = the pyrophosphate.

Several partial and complete luciferase purifications have been reported[1–3]. However, some of them require lengthy chromatographic procedures while others do not yield complete separation. This communication describes an improved separation technique for luciferase.

Recent literature on nucleotide-dependent enzymes deals with their purification by affinity elution chromatography on Blue Dextran Sepharose, a semi-specific high-molecular-weight compound substituted with the mono-chlorotriazinyl dye Cibachron Blue F3GA which can mimic a polynucleotide, presumably due to an attraction of the blue chromophore to a nucleotide-binding site of the enzyme. It has been reported by Thompson *et al.*[4] that Blue Dextran linked to Sepharose may be used as an affinity chromatographic medium for many proteins which bind dinucleotides and ATP.

EXPERIMENTAL

Cyanogen bromide-activated Sepharose 4B was purchased from Pharmacia, firefly lantern, Blue Dextran and ATP from Sigma and DTT from E. Merck. All other chemicals were of reagent grade.

Sample preparation

Extraction of firefly abdomen was carried out according to the method of

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Antonik[5] with slight modifications. A 1-ml volume of dithiothreitol (DTT) (195 mg/ml) was added per 100 mg of firefly abdomen at the end of the centrifugation. This was necessary for the stability of the crude extract. The preparation was dialysed against 0.02 *M* Tris–acetate, pH 7.8 with 1 m*M* EDTA for 24 h with three changes of the buffer.

Luciferase assay

Luciferase activity was estimated by PICO ATP apparatus (Jobin Yvon, France). The reaction mixture containing 50 µl of 10^5 pg/ml ATP in 0.01 *M* MOPS (3-N-morpholinopropanesulphonic acid), pH 7.4 containing 10 m*M* $MgSO_4$, 50 µl of luciferin (0.168 µg/ml) and 50 µl of luciferase sample was introduced in a special cuvette in the PICO ATP apparatus at 18–20°C and the intensity of light in millivolts during the first 2 sec of emission was recorded by a photomultiplier. The unit of activity is the maximum intensity of light recorded in millivolts per picogram ATP per milligram protein at 562 nm.

Chromatography

Blue Dextran was coupled to oxiran silica by the procedure of Chang *et al.*[6]. Blue Dextran Sepharose was prepared by the method of Ryan and Vestling[7]. The efficiency of the coupling reaction was controlled by measuring the differential absorbance in the visible region of the coupled gel suspended in 87% glycerine. A 0.3-ml aliquot of the dialysed extract was injected into the column of Blue Dextran coupled to Sepharose or silica and the elution was started with 0.01 *M* MOPS containing 20 m*M* $MgSO_4$ and 1 m*M* DTT and continued until no protein was detected in the fractions. Then the eluent was changed to 0.5 m*M* ATP in 0.01 *M* MOPS containing 20 m*M* $MgSO_4$ and 1 m*M* DTT to desorb the enzyme. The ballast proteins were stripped by eluting with a 3 *M* KCl solution. After each use, the columns were washed with four volumes of 3 *M* KCl and then exhaustively equilibrated with the starting eluent, 0.01 *M* MOPS, pH 7.4 containing 20 m*M* $MgSO_4$ and 1 m*M* DTT, before the start of the next cycle. Under these conditions no loss of the blue chromophore was observed. The same resin can be successfully used for more than six elutions.

Dialysis of the fractions

The fractions (1.0 ml) were pooled into groups of three and dialysed against 0.02 *M* Tris–acetate, pH 7.8 with 1 m*M* EDTA for 16–19 h. All operations were carried out at 4°C. Each pooled fraction was tested for luciferase activity and protein concentration. The absorbance at 278 nm was corrected against ATP in MOPS.

Sodium dodecyl sulphate (SDS)-gel electrophoresis and isoelectric focusing

The homogeneity of the preparation of purified luciferase was checked by SDS-gel electrophoresis in 7.5% polyacrylamide gel, pH 8.0–9.0. Isoelectric focusing was carried out in the range pH 3.5–10.0 with 8 *M* urea on 360 µm thick polyacrylamide gels freshly prepared on a cellophane support according to Gorg *et al.*[8].

RESULTS

A comparison of chromatography on Blue Dextran coupled to Sepharose and silica is shown in Table I. The elution profiles are shown in Figs. 1 and 2.

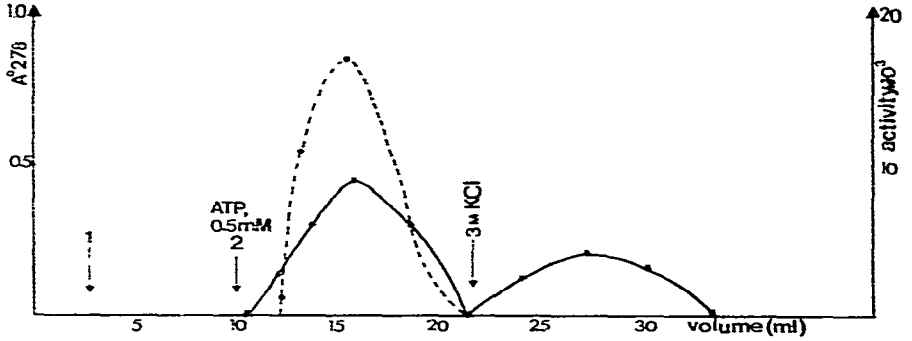

Fig. 1. Elution profile of luciferase on Blue Dextran coupled to Sepharose. A column of Blue Dextran Sepharose equilibrated with 0.01 *M* MOPS containing 20 m*M* $MgSO_4$ and 1 m*M* DTT, pH 7.4 was loaded with 0.3 ml of crude luciferase. The eluent was changed to 0.01 *M* MOPS containing 20 m*M* $MgSO_4$, 1 m*M* DTT and 0.5 m*M* ATP. Column fractions (1 ml) were assayed for enzyme activity and protein concentration (see Experimental). ——, Absorbance at 278 nm; – – –, luciferase activity.

It is evident that Blue Dextran coupled to silica is more favourable for luciferase purification since we were able to recover about 300% of the initial activity compared to about 180% in the case of Sepharose Blue Dextran. The purification in the two cases is 61-fold and 51-fold respectively for silica- and Sepharose-based derivatives (Table I). This method is superior to that of Lundin *et al.*[9], who used $(NH_4)_2SO_4$ precipitation followed by isoelectric focusing but obtained only a 8.1-fold purified enzyme with 65% of the initial activity. We found good evidence for the homogeneity of our purified sample on Blue Dextran Sepharose and silica by SDS-gel electrophoresis. Isoelectric focusing showed the presence of one band at a pH of about 5.0. The purified fractions did not show the presence of other contaminating enzymes present in the extract.

DISCUSSION

Blue Dextran is known to complex with a wide range of proteins because it is specific for a super-secondary structure called the dinucleotide fold. This structure forms the ATP-binding site in phosphoglycerate kinase[10] and NAD-binding sites in lactate dehydrogenase and many other enzymes[11].

In our studies, the nucleotide ATP which has the highest affinity for luciferase

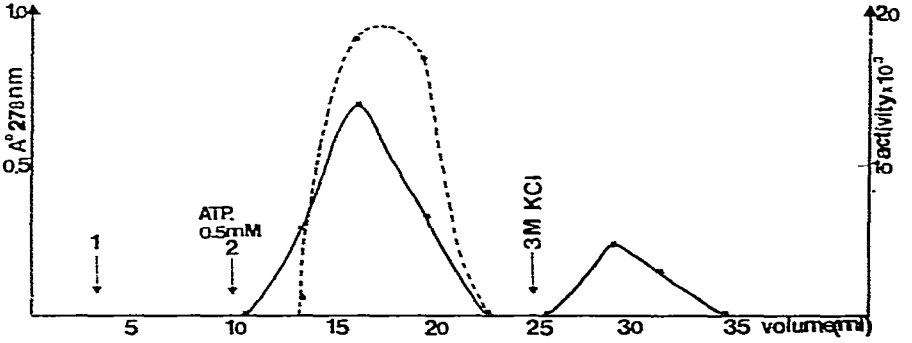

Fig. 2. Chromatogram of luciferase purified on Blue Dextran silica. Conditions and curves as for Blue Dextran Sepharose.

TABLE I

PURIFICATION OF LUCIFERASE ON BLUE DEXTRAN COUPLED TO SEPHAROSE AND SILICA

Purification steps	*Total protein*		*Total activity*		*Specific activity*	*Purification factor*
	Absolute value (mg)	*% Initial*	*Absolute value*	*% Initial*		
1 Crude extract	25.6	100	243,000	100	9346	0
2 Dialysed extract	1.5	5.8	247,300	102	164,900	17.0
3 Purified sample from Blue Dextran sepharose	0.96	3.7	460,100	180	479,300	51.0
4 Purified fraction from Blue Dextran silica	1.3	5.2	742,800	300	571,400	61.0

was most effective in eluting the bound enzyme. The optimum concentration of ATP was 0.5 m*M*. By contrast, NaCl at a concentration at least 200 times that of the specific ligand ATP was required to desorb the enzyme from the column. Thus ligand specificity for luciferase was made use of to distinguish binding to the dinucleotide fold from non-specific ionic binding. In our case, the relative affinity of Blue Dextran and ATP for the same active site of luciferase must have facilitated the selective elution of the enzyme from the presumably large number of proteins and other compounds in the firefly abdomen homogenate which are bound to the affinity column by non-specific interactions.

In a previous comparison of metal–chelate adsorbents based on Sepharose and silica, for the separation of nucleotides, a higher capacity was reported for silica-based adsorbent[12]. We find the same behaviour in this case, as the Blue Dextran silica column has a higher enzyme-binding capacity than the corresponding Sepharose-based adsorbent. The affinity status of these Blue Dextran columns remains controversial. Many authors have recently pointed out that these are in fact pseudo-affinity interactions. However it is too premature to draw any conclusion on the details of the interaction or to state the mode of inhibition of luciferase with respect to Blue Dextran and ATP. In the presence of ATP, the equilibrium between free enzyme and enzyme bound to Blue Dextran could be shifted towards free enzyme. We confidently predict the evidence of a dinucleotide fold in firefly luciferase.

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