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Use of immobilized triazine dyes in the purification of DNA topoisomerase I (Topo I) and terminal deoxynucleotidyl transferase (TdT) from calf thymus

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ABSTRACT

The interaction between TdT and Topo I, and twelve various triazine dyes immobilized on Sepharose CL-6B was studied. Yellow lightproof 2KT–Sepharose and Bordeaux 4ST–Sepharose were used to purify TdT and Topo I, respectively. The principal role of copper ions, complexed to the dye molecules, in the dye–protein interaction was evaluated.

INTRODUCTION

Terminal deoxynucleotidyl transferase (TdT) and DNA topoisomerase I (Topo I) from calf thymus gland are widely used in molecular biology. TdT is a unique DNA polymerase requiring no template and catalysing the addition of deoxynucleoside triphosphates at the 3′-end of poly- or oligodeoxynucleotide initiator and releasing inorganic pyrophosphate. This enzyme is generally used in genetic engineering for elongation of the DNA chains and for 3′-end labelling [1,2]. Topo I catalyses the relaxation of duplex DNA, knotting of single-stranded rings and intertwining of complementary single-stranded rings, and forms catenanes between one nicked and one closed circular duplex. This enzyme is employed in structural studies of DNA and in modelling of genetic processes [3].

In order to develop an efficient routine method for the purification of these proteins, a variety of adsorbents should be available. As the use of immobilized triazine dyes for this purpose has not previously been reported, we studied the interaction between these enzymes and adsorbents based on twelve triazine dyes, and two of them proved to be suitable for use in purifcation.

EXPERIMENTAL

Materials

All immobilized triazine dyes were kindly provided by the Laboratory of Adsorbent Synthesis and pBR 322 DNA by the Laboratory of Genetic Engineering of

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this Institute. Tris and EDTA were purchased from Serva (Heidelberg, Germany) and all other reagents from Merck (Darmstadt, Germany).

Preparation of initial extracts

A 500-g sample of thymus glands freed from debris and clotted blood were minced with a meat grinder and homogenized with an equal volume of 100 m*M* Tris–HCl (pH 6.8)–0.3 *M* KCl–2.5 m*M* K_3-EDTA–50 m*M* Na_2SO_3–1 m*M* PMSF–10% (v/v) ethylene glycol–14 m*M* 2-mercaptoethanol–10 m*M* KF–10 m*M* NAD five times for 30 s each in a Waring-type blender. After centrifugation at 30 000 *g* for 1 h, the supernatant was diluted with 50 m*M* Tris–HCl (pH 6.8) 2 m*M* K_3-EDTA–14 m*M* 2-mercaptoethanol–10% ethylene glycol to a volume of 1.4 l, and the 35–70% ammonium sulphate fraction precipitate was dissolved in 40 m*M* Tris–HCl (pH 8.0)–1.6 m*M* K_3-EDTA–14 m*M* 2-mercaptoethanol–10% ethylene glycol. This extract, dialysed overnight against the same buffer but containing glycerol instead of ethylene glycol, was applied to a Servacel DEAE-52 column (30 × 5 cm I.D.), and the flow-through fractions were collected. Further fractionation was done on a Bio-Rex 70 column (30 × 2.6 cm I.D.) in the same buffer. The column was developed with a 500-ml KCl gradient from 0 to 1 *M*. TdT activity eluted at 0.1–0.15 *M* KCl and Topo I at 0.18–0.22 *M* KCl. These preparations were used for further studies.

Screening procedure

A 0.15-ml aliquot of an appropriate adsorbent, equilibrated with 20 m*M* Tris–HCl (pH 8.0)–10 m*M* $MgCl_2$–0.1 m*M* K_3-EDTA–10 m*M* 2-mercaptoethanol–10% glycerol, and 0.5 ml of enzyme preparation dialysed against the same buffer, were thoroughly mixed in Eppendorf tubes. After incubation for 30 min in an ice-bath, the suspension was centrifuged for 30 s at 15 000 *g* and supernatants were collected for further analysis.

Enzyme assays

TdT activity was assayed according to Coleman [4] and Topo I as indicated by Ferro *et al.* [5].

Protein assay

A Bio-Rad Labs. protein assay kit was used to determine protein concentrations.

RESULTS

The supernatants after screening were subjected to analysis, and both protein concentration and enzyme activity were determined. The results are summarized in Table I. Two adsorbents were selected for further studies: Yellow Lightproof 2KT–Sepharose CL-6B and Bordeaux 4ST–Sepharose CL-6B (Fig. 1).

After optimization of the chromatographic conditions, the following parameters were obtained: Topo I, elution at 0.12–0.21 *M* KCl, purification 11.2-fold, yield 67%, specific activity (3–4) × 10^5 U/mg (Fig. 2); TdT, elution at 0.22–0.55 *M*, purification 10.5-fold, yield 80%, specific activity (5–7) × 10^4 U/mg (Fig. 3).

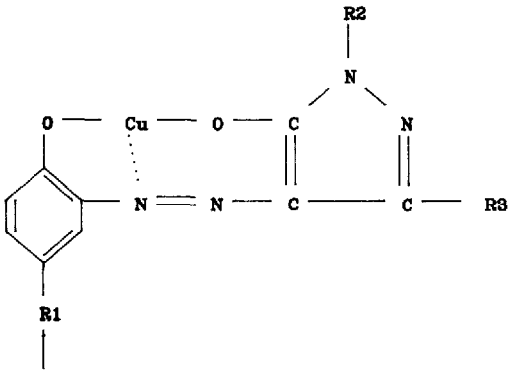
It should be noted that complexed Cu^{2+} ions are present in the molecules of both dyes thus selected, and that these ions play a crucial role in the affinity interaction. As

TABLE I

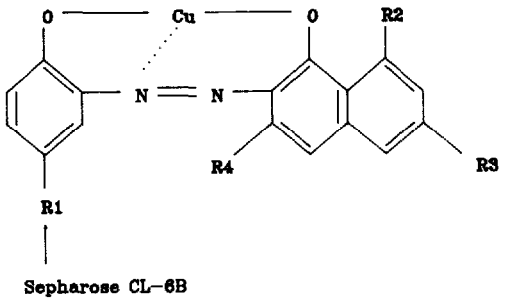
DATA ON DYE–ENZYME INTERACTIONS

Triazine dye immobilized on Sepharose CL-6B	TdT		Topo I	
	A[a]	B[b]	A[a]	B[b]
Red-Brown 2K	94.0	+	56.2	+
Scarlet 6S	68.2	+	42.5	+
Pharmacia Red	79.8	+	43.8	+
Pharmacia Blue	83.9	+	32.5	+
Orange 5K	77.5	+	62.4	+
Yellow Bright 5Z	81.6	+	34.7	+
Bordeaux 4ST	94.1	+	15.4	+
Scarlet 2Z	74.7	+	28.5	−
Golden 2KX	20.2	−	34.7	−
Active Orange KX	48.2	−	42.3	+
Scarlet 4ZT	19.7	−	51.2	−
Yellow Lightproof 2KT	24.6	+	19.5	−

[a] Fraction of protein bound (%).
[b] + = Target enzyme bound; − = target enzyme not bound.



(1)



(2)

Fig. 1. Essential part of the chelating ring structure: (1) Yellow Lightproof 2KT; (2) Bordeaux 4ST. R1–R4 = reactive groups.

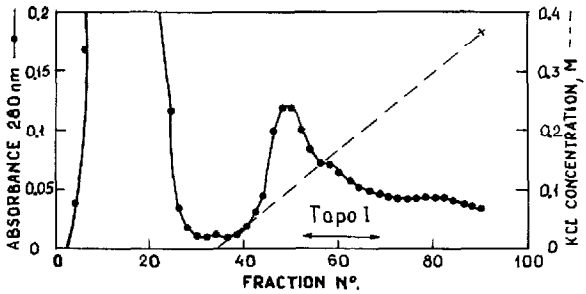


Fig. 2. Purification of Topo I on Bordeaux 4ST–Sepharose CL-6B. The 45-ml column was equilibrated with 20 m*M* Tris–HCl (pH 8.0)–10 m*M* $MgCl_2$–0.1 m*M* EDTA–10 m*M* 2-mercaptoethanol–10% (v/v) glycerol. KCl gradient from 0 to 0.4 *M*. Flow-rate, 15 ml/h.

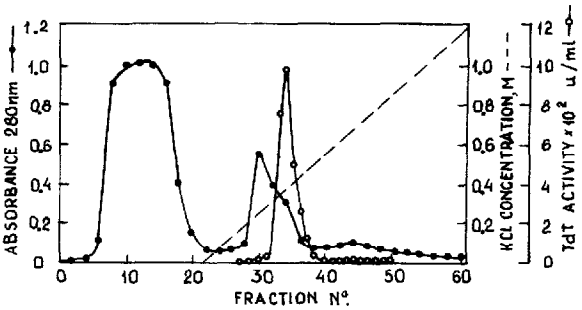


Fig. 3. Purification of TdT on Yellow Lightproof 2KT–Sepharose CL-6B. The 60-ml column was equilibrated with 20 m*M* Tris–HCl (pH 7.0)–2 m*M* $MgCl_2$–2 m*M* $ZnCl_2$–0.5 m*M* EDTA–14 m*M* 2-mercaptoethanol–10% (v/v) glycerol. KCl gradient from 0 to 1.2 *M*. Flow-rate, 20 ml/h.

TdT enzyme is sensitive to copper ions, we were forced to replace this ligand by zinc, and a Zn^{2+}/Mg^{2+}/EDTA ratio of 2:2:1 in the chromatographic buffer was found to be satisfactory, because in the absence of the transition metals we observed no interaction. The complete replacement of copper with zinc in the dye was accomplished by extensive washing of the adsorbent with several volumes of 100 m*M* EDTA (pH 5), deionized water and finally 15 m*M* $ZnCl_2$.

In contrast, Topo I is stable in the presence of copper complexed in the Bordeaux 4ST molecule. However, this enzyme showed only partial binding, but the addition of 10 m*M* $MgCl_2$ solved this problem. When the copper ions had been washed out with EDTA, the interaction, in contrast to that with TdT, was so strong that we were unable to elute the active enzyme under any conditions.

These results clearly demonstrate the pseudo-affinity nature of the interaction in both instances, *i.e.*, the interaction in the vicinity of active centre, and undoubtedly further studies are required to verify this conclusion.

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