INHIBITION OF CHICK EMBRYO DNA LIGASE BY dATP : ITS USE IN ENZYME PURIFICATION

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SUMMARY

Highly purified chick embryo DNA ligase (EC.6.5.1.1) obtained in our laboratory using classical methods, mainly column chromatographies shows a bimodal pH activity and an high affinity inhibition by dATP. A single step passage of crude extract containing DNA ligase through an anion exchange resin (Dowex AG_1X_2) saturated with dATP allows an important purification of the enzyme retained on the column at pH 7.5 and eluted at pH 8.6. Specific activity of the purified enzyme preparation is more than 600 fold higher than that of the crude extract. Analysis of the eluant by polyacrylamide gel electrophoresis shows a main protein containing the enzyme activity.

We have recently showed that highly purified DNA ligase from chick embryo using five different steps of column chromatography has two optimal pH activities (respectively 7.5 and 8.6) and is strongly inhibited by dATP (1). This inhibition occurs only at pH 7.5 with a K_I of high affinity ($K_I=2.10^{-6}M$). Since nucleotides are strongly retained on anion exchange, it was interesting to examine whether dATP saturated Dowex AG_1X_2 would be able to show some affinity for the DNA ligase present in crude extracts from chick embryos. In this paper, we report a simple method which leads in a single step to more than 600 fold purification of this enzyme.

METHODS

DNA ligase activity was assayed by the method of Weiss et al. (2) as described under Table I. The nicked ^{32}P DNA substrate was prepared according to Weiss et al. (3) with the following modifications: the pancreatic deoxyribonuclease was allowed to react at 25° for 40 min and was inactivated by a 30 min heating at 65° followed by a 12 hrs dialysis against 50 mM Tris-HCl buffer (pH 8) at 4°. Alcaline phosphatase used for the obtention of 5'-OH was inactivated by addition of sodium dodecylsulfate to a final concentration of 1%. The incubation mixture was maintained at 4° for 1 hr and centrifuged

TABLE 1. PURIFICATION OF A DNA LIGASE USING DOWEX SATURATED WITH dATP

fraction	total enzyme activity (units)	total protein (mg)	specific activity (units/mg)	yield
Crude extract	103,4	1100	0,094	100
Eluate pH 8.6 from Dowex dATP	74,1	1,20	61,0	71

The assay for polynucleotide ligase measures the conversion of 5' ^{32}P phosphomonoesters in DNA containing single-strand breaks to an acido-insoluble form after incubation with alcaline phosphatase. The reaction mixture (1 ml) contained 10 ug 5'^{32}P phosphoryl DNA (equivalent of 50,000 cpm at day zero), 0,1 mM ATP , 60 mM Tris-HCl ,pH 7.5, 6.6 mM $MgCl_2$, 10 mM dithiothreitol and 50 mM NaCl. 5.10^{-5} to 5.10^{-3} enzyme units were added in the standard assay after dilution in 0.05 M Tris-HCl buffer pH 7.6 containing 0.01 M mercaptoethanol and 0.5 mg of bovine serum albumin. After 20 min incubation at 37° the mixture was precipitated by TCA, redissolved and incubated with alcaline phosphatase as described by Weiss et al. (2).

20 min at 27 000 xg. Supernatant was then dialyzed 12 hrs against 50 mM Tris HCl buffer pH 8 before labelling of the -OH termini by ^{32}P.

The results are expressed in units. One unit is defined as the amount of enzyme catalyzing the conversion of 1 mμmole of ^{32}P phosphomonoester into a phosphatase resistant form in 20 min. Proteins estimations were performed using the procedure of Lowry et al. (4). Analysis of the purified enzyme was carried out on polyacrylamide gels electrophoresis according to the method of Weber and Osborne (5).

Dowex AG_1X_2 (Cl-form) was purchased from Bio-Rad. This column, after equilibration with 50 mM Tris-HCl buffer pH 7.5, was saturated with dATP (0.8 meq/ml of resin bed). The saturation of the column was checked with a solution containing ^{3}H dATP. This step was followed by a 30 ml 50 mM Tris-HCl buffer (pH 7.5) washing.

RESULTS AND DISCUSSION

Twenty five grams of nine days old chick embryos carefully minced in buffer containing 20% glycerol, 50 mM Tris-HCl pH 7.6, 0.1 mM EDTA were

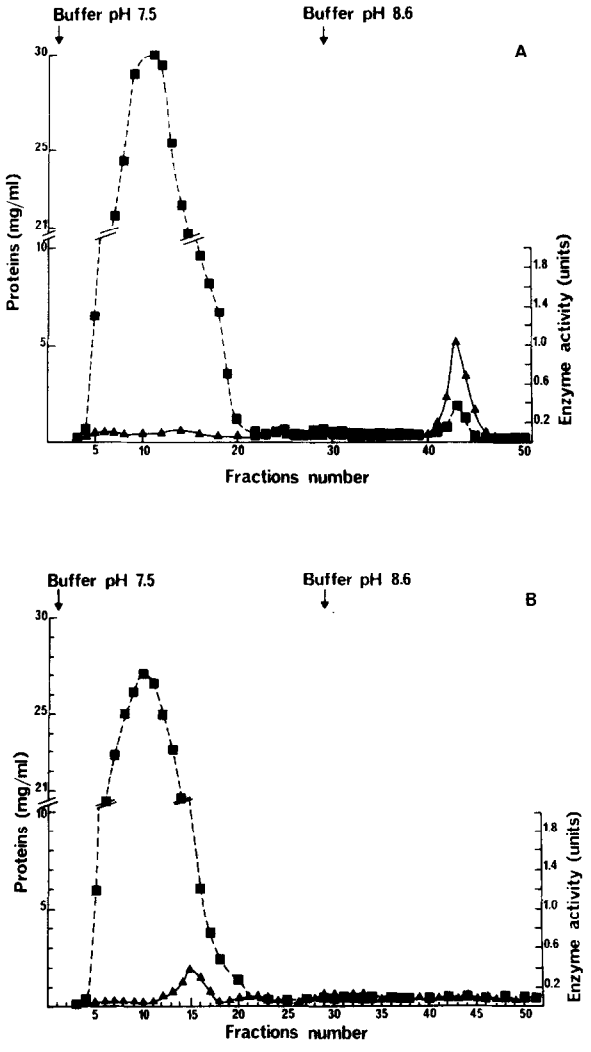

Figure 1. A- Dowex dATP affinity chromatography of chick embryos crude extracts. After saturation of the Dowex column (0.7 x 6.5 cm) with dATP, the protein extract (equivalent of 1100mg) was adsorbed. Fractions of 1 ml were collected and 10 ul used to determine the enzyme activity (▲---▲). Absorbance at 280 nm is also recorded (....).
B - dATP was adsorbed on the column and proteins were passed through (equivalent of 1.75 g).

ground in a Potter homogenizer (4 strokes at 1 200 rev/min). The extract was filtered through cheesecloth followed by a 1,000 xg centrifugation for 10 min. The supernatant after recentrifugation at 30,000 xg for 30 min was applied on a 0.7 x 6.5 cm column saturated with dATP. The flow rate was kept

approximately at 0.5 ml/min. The column was then washed with 30 ml 50 mM Tris-HCl buffer pH 7.5 until the absorbance at 280 nm of the effluent was less than 0.002. The enzyme was then eluted by 50 mM Tris-HCl at pH 8.6. The DNA ligase activity of the eluent is shown in Figure 1_A. More than 95% of the proteins were not retained on the column at pH 7.5 and the enzyme was eluted at pH 8.6 as a single peak. Figure 1_B depicts a control experiment showing the non-retention of the enzyme on an identical column without dATP.

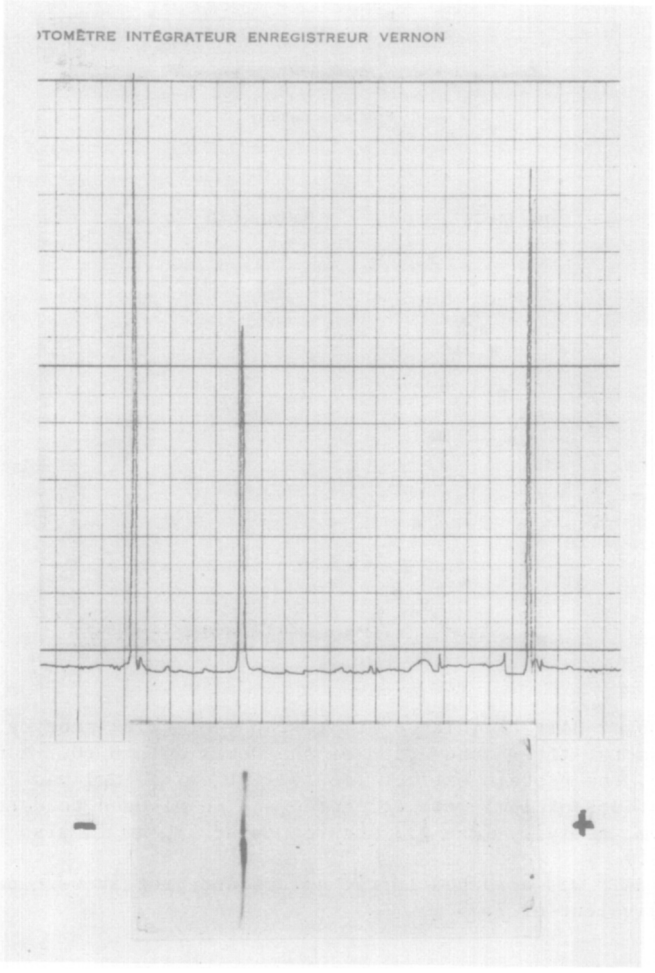

Figure 2. Analysis of Dowex dATP purified DNA ligase by gel electrophoresis. Forty ug of protein from the concentrated eluate were applied on a 10% acrylamide gel and subjected to electrophoresis at pH 8.7 and 2.5 mA per gel for 2 hrs. Migration was from pole - to pole +. The absorbance of the stained gel was recorded with a Vernon spectrophotometer recorder.

The pooled eluate containing DNA ligase was concentrated by dialysis against 50 mM Tris-HCl buffer pH 7.5 containing 50% glycerol and the purified enzyme was stored at -20%.

The specific activity of the DNA ligase present in the extract was 0.094 units/mg of protein. The total amount of the activity applied to the column was 103,4 units. The recovery of enzymatic activity from the column was 71%, with a 640 fold purification. The eluted enzyme has a specific activity of 61.0 units/mg protein (see Table 1). This specific activity is comparable to that obtained by methods requiring several steps. When 40 µg of the preparation were analyzed by polyacrylamide gel electrophoresis after Coomassie blue staining, one strong band was visible. When the gel was sliced and checked after elution for DNA ligase, all the activity was found to be associated with the main band. The enzyme purified by this method shows a molecular weight (165 000) and a bimodal pH dependance (7.5 and 8.6) in good agreement with that purified by several chromatographic steps though with a smaller specific activity.

It was unexpected that a strong anion exchange resin saturated with dATP shows affinity to DNA ligase. Indeed the dATP which is retained by its phosphate moiety probably does not maintain its normal structure in these conditions. Further work is needed to explain the mechanism of this retention. Practically, the method is very convenient to obtain in a single step highly purified preparations of chick embryo DNA ligase.

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