PURIFICATION OF T4 RNA LIGASE BY 2',5'-ADP SEPHAROSE CHROMATOGRAPHY

M. SUGIURA+, M. SUZUKI+, E. OHTSUKA, S. NISHIKAWA, H. UEMURA and M. IKEHARA
+*National Institute of Genetics, Mishima 411* and *Faculty of Pharmaceutical Sciences, Osaka University, Suita 565, Japan*

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1. Introduction

RNA ligase was discovered in T4 infected *Escherichia coli* cells [1]. This enzyme catalyzes the intramolecular joining of 5'-P and 3'-OH termini of polynucleotides and the intermolecular joining of various combinations of oligo- and polynucleotides at their 5'-P and 3'-OH termini [2–8]. RNA ligase also catalyzes the addition of dinucleoside pyrophosphates [9] and mononucleotides [10,11] to oligonucleotide acceptors. Nucleoside 3',5'-diphosphates are the shortest donor substrates and their 3'-phosphates are essential for the reaction because nucleoside 5'-phosphates and nucleoside 2',5'-diphosphates are unable to serve as donors [9–11]. We found that 2',5'-ADP inhibited the RNA ligase reaction.

The procedures for purification of RNA ligase have been improved by several groups [2,3,5,12,13]. However, it was sometimes difficult to remove a trace of nuclease activity from RNA ligase preparations. Recently, we found that RNA ligase bound to 2',5'-ADP Sepharose in the presence of Mg^{2+} but not in its absence, and we introduced the 2',5'-ADP Sepharose chromatography at the last step of our purification procedure. The enzyme so obtained had no detectable RNase activity and was suitable for the synthesis of oligoribonucleotides with defined sequences.

2. Materials and methods

2.1. *Strains and reagents*

Phage T4 *am*N82 × E1140, a double amber mutant on genes 44 and 62, was given to us by Dr T. Minagawa. *E. coli* S296 *(met, trp, rna, rnb)* which is low in RNase I and II activities [14] was obtained from Dr M. Kuwano. 2',5'-ADP and 2',5'-ADP Sepharose 4B were purchased from Sigma and Pharmacia, respectively. [5'-^{32}P]poly(A)$_{20-30}$ and [^{32}P]pC–A–A–U were prepared as in [6].

2.2. *Preparation of RNA ligase*

RNA ligase was purified from *E coli* S296 infected with T4 *am*N82 × E1140 by the simplified procedure [12] where the ammonium sulfate fractionation (step 3) was replaced by a simple precipitation of proteins with 50% saturated ammonium sulfate and the second DEAE-cellulose chromatography (step 7) was omitted. This preparation (Sephadex fraction) was not yet homogeneous.

2.3 *Assays and characterization*

RNA ligase was assayed by the method based on the poly(A) cyclization reaction [1] as in [6]. The reaction mixtures (25–50 μl) contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 μg/ml bovine serum albumin, 100 μM ATP, 2 μM [5'-^{32}P]poly(A)$_{20-30}$ and RNA ligase (< 0.01 unit). Incubations were at 37°C for 30 min. RNase activity was monitored in reaction mixtures (10 μl) containing 50 mM Hepes–NaOH (pH 8.3), 10 mM $MgCl_2$, 10 mM dithiothreitol, 10 μg/ml bovine serum albumin, 15 μM ATP, 1.5–3.0 μM [^{32}P]pC–A–A–U and 0.5–1 unit RNA ligase. After incubation at 37°C, the reaction mixtures were subjected to homochromatography as in [7]. Electrophoresis on a 5% acrylamide gel containing 0.1% SDS was performed as in [15]. The gel was stained with Coomassie brilliant blue R250.

3. Results and discussion

3.1. *2',5'-ADP Sepharose chromatography*

2',5'-ADP Sepharose 4B was swollen overnight in 0.1 M potassium phosphate buffer (pH 7.0) and washed by decantation with over 100 ml/g dry power of the same buffer as suggested by the Company. The washed gel was packed into a plastic syringe and washed with 2 bed volumes of 10 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol and 5% glycerol (TDG) containing 1 M KCl and 10 mM EDTA. The column was then equilibrated with 10 bed volumes of TDG containing 5 mM $MgCl_2$. The RNA ligase preparation was dialyzed overnight against TDG containing 5 mM $MgCl_2$ and was loaded slowly on the column (2–3 drops/min). The column was washed with 3 bed volumes of TDG containing 5 mM $MgCl_2$ and then eluted with 2 bed volumes of TDG. Plastic syringes and tubes were used in order to minimize the loss of RNA ligase due to its adhesion on glass wall.

A typical chromatographic pattern is shown in fig.1. As seen in fig.1, RNA ligase bound to 2',5'-ADP Sepharose 4B in the presence of Mg^{2+} and was released when Mg^{2+} was removed by simple washing

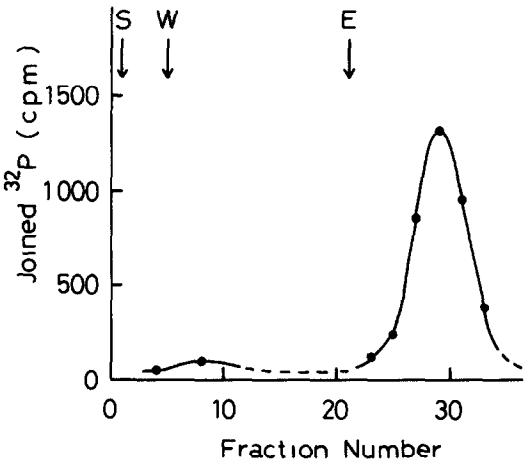

Fig.1. Chromatography of RNA ligase on 2',5'-ADP Sepharose 4B The Sephadex fraction (0.5 mg protein in 6 ml) was applied to a column (7 ml in a 10 ml syringe) (arrow S). The column was washed with 21 ml TDG + 5 mM $MgCl_2$ (arrow W) and RNA ligase was eluted with 14 ml TDG (arrow E). Fractions, 1.4 ml, from tubes 1–20 and fractions, 0 8 ml, starting with tube 21 were collected

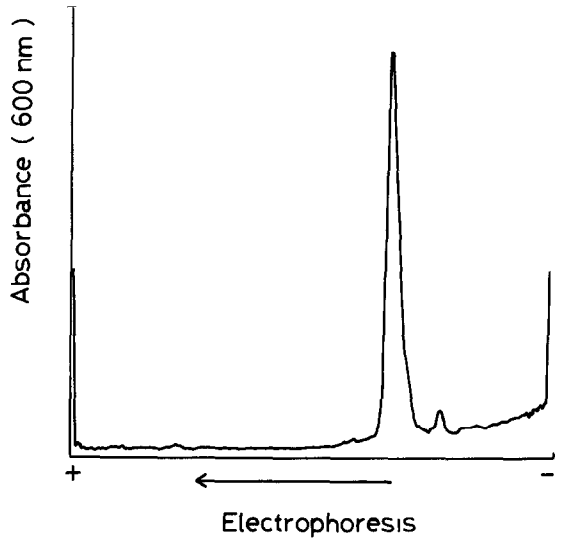

Fig.2. Polyacrylamide gel electrophoresis of RNA ligase The scan is of 3 μg RNA ligase purified by 2',5'-ADP Sepharose 4B.

of the column with the buffer without Mg^{2+}. The active fractions were pooled, concentrated in a dialysis bag covered with solid polyethylene glycol 6000 and dialyzed against 20 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol, 0.1 mM EDTA, 0.1 M KCl and 5% glycerol. The purified preparation was stable and stored at 0°C. The recovery was 50–80% and the spec. act. ~1000 units/mg protein was usually obtained.

The columns can be used repeatedly after washing.

3.2. *Characterization of the purified enzyme*

The purified RNA ligase preparation gave one major band on SDS–acrylamide gel electrophoresis and was estimated to be over 80% pure (fig.2).

Highly labeled [^{32}P]pC–A–A–U was incubated with the purified enzyme under the conditions of oligonucleotide joining and subjected to homochromatography. This assay was sensitive enough to detect the release of a single nucleotide from the substrate. As shown in fig.3, there was no detectable hydrolysis with the purified enzyme fraction and the joined products were obtained. On the other hand, the degradation products, [^{32}P]pC–A–A was seen in the reaction with the Sephadex fraction and the [^{32}P]pC–A–A increased with incubation period.

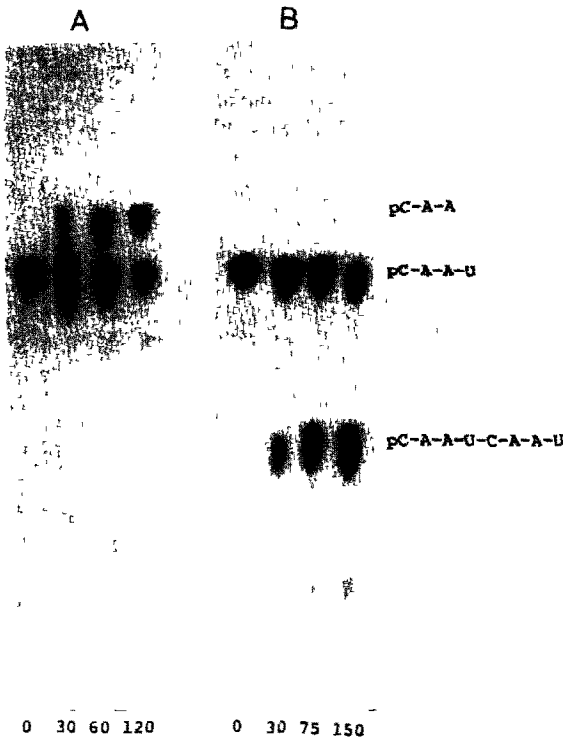

Fig.3. Homochromatography of the reaction mixtures containing [^{32}P]pC–A–A–U and RNA ligase before (A) or after (B) chromatography on 2′,5′-ADP Sepharose 4B

The RNA ligase purified by 2′,5′-ADP Sepharose 4B chromatography has no detectable RNase and is suitable for the joining of short oligoribonucleotides.

3.3. *Inhibition by 2′,5′-ADP*

2′,5′-ADP Sepharose 4B has been developed as a group-specific adsorbent for the fractionation of NADP$^+$-dependent enzymes [16]. RNA ligase adsorbed to the Sepharose could not be eluted by NADP$^+$, indicating that its affinity differed from that of NADP$^+$-dependent enzymes.

The effect of 2′,5′-ADP on poly(A) cyclization by RNA ligase was examined. As shown in fig.4, 2′,5′-ADP inhibited the RNA ligase activity On the other hand, RNA ligase utilizes 3′,5′-ADP as a donor substrate [10]. These facts together suggest that RNA ligase molecules bind to immobilized 2′,5′-ADP, a donor substrate analog, at their donor sites in the presence of Mg^{2+}.

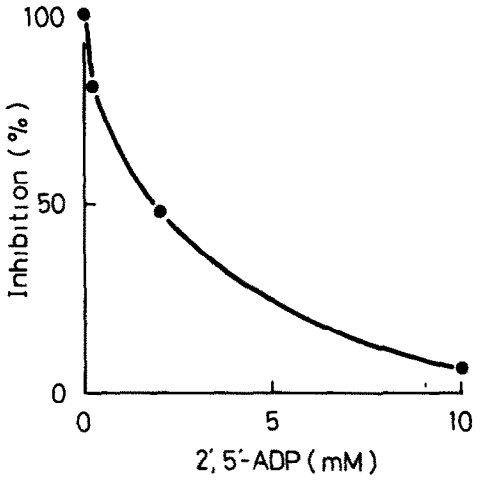

Fig.4. Effect of 2′,5′-ADP on poly(A) cyclization reaction of RNA ligase.

Acknowledgement

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