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1985

Yoon, H. S., Suh, H., Han, M. H., & Yoo, O. J. (1985). Purification and characterization of Alu I methylase. Korean Biochemistry Journal., 18(1), 82-87.

<https://hdl.handle.net/10356/95467>

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Purification and Characterization of Alu I Methylase

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Abstract

Alu I methylase has been isolated from 300g (wet weight) cells of *Arthrobacter luteus*. After ammonium sulfate fractionation, the protein which has methylase activity was purified through phosphocellulose, DEAE-cellulose, Heparin agarose, and Hydroxylapatite column chromatography. The methylated DNA by the purified methylase was resistant against Alu I endonuclease. The purified Alu I methylase was essentially homogeneous as judged by 10% SDS-polyacrylamide gel electrophoresis, and the apparent subunit molecular weight was $56,000 \pm 1,000$. The specific activity of the enzyme was 1.32×10^5 units per mg protein.

Since the discovery by Smith (1970), Type II restriction and modification enzymes have been invaluable both as tools for the study of gene structure and as models for sequence specific DNA-protein interactions. In particular, comparison of a Type II endonuclease with its cognate methylase may reveal valuable information on two distinct proteins that interact with the same nucleotide sequence.

In 1976, Roberts *et al.* (1976) reported that Alu I endonuclease from *Arthrobacter luteus* recognizes base sequence 5'-d (AG[↓]CT)-3' with the cleavage site indicated by the arrow, but there was no report about Alu I methylase activity.

In this paper, a procedure for the purification of Alu I methylase is described. The subunit molecular weight and the specificity of the enzyme are also presented.

Materials and Methods

Materials

The strain of *Arthrobacter luteus* was provided by Dr. B. G. Min (Bethesda Research Laboratories). Ammonium sulfate and reagent grade glycerol were purchased from Kanto Chemical Co. (Japan). Streptomycin sulfate, electrophoresis grade agarose, phosphocellulose, DEAE-cellulose, heparin, and hydroxylapatite were from Sigma Chemical Co. (St. Louis, Miss., USA). Sepharose 4B was from Pharmacia Fine Chemicals

(Uppsala, Sweden). Cyanogen Bromide was from Wako Pure Chemical Co. (Japan). Phosphocellulose was precycled and equilibrated according to the procedure of Green *et al.*, (1978). Sodium dodecyl sulfate, acrylamide, N,N'-methylene-bisacrylamide, ammonium persulfate, TEMED (N,N,N',N'-tetramethylethylenediamine), coomassie brilliant blue (R-250), amido black 10B, and bromophenol blue were from BioRad Laboratories (Richmond, Calif., USA).

S-adenosyl-L- [methyl-³H] -methionine (15 Ci/mmol) was from New England Nuclear (Boston, Mass., USA). S-adenosylmethionine was from Sigma. All other chemicals used during isolation and DNA sequencing procedures are reagent grade. pBR 322 DNA was isolated by the procedure of Clewell and Helinski (1972). Alu I endonuclease and Hinc II endonuclease were purchased from Bethesda Research Laboratories. Heparin agarose was prepared by the procedure of Davidson *et al.* (1979).

Culture of cells

Arthrobacter luteus ATCC 21606 was grown in 20 liter culture of LB broth (per liter; 10 g of bactotryptone, 5 g of yeast extract, 10 g of NaCl) supplemented with 0.2% glucose, 0.2% casamino acid, and 10 µg/ml thiamine in a Microferm fermentor (New Brunswick Scientific Co.) at 37°C. Culture pH was maintained at 7.5 by addition of NaOH. Cells were harvested in late exponential phase ($A_{260}=9$) and adjusted to 50% (w/w) glycerol. The cell paste was stored at -20°C.

Enzyme assays

The assay for methylase activity measures transfer of [³H] -methyl groups from [³H] AdoMet to pBR 322 DNA or Col EI DNA. Reaction mixture (25 µl) contained 50 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, 50 µg/ml BSA, 10 mM 2-mercaptoethanol, 0.8 µM [³H] AdoMet, 40 µg/ml of DNA, and the indicated amount of enzyme. After incubation of 15 to 60 minutes at 37°C, 10 µl samples were spotted on 1 cm² of Whatman DE81 paper and washed five times with a large excess of 0.2 M NH₄HCO₃ solution, twice with ethanol, twice with diethyl ether, and dried for 30 minutes at 60°C.

The [³H] CPM was counted with 5 ml of toluene based scintillant (PPO 4g, POPOP 50 mg in 1 l of toluene) by using Beckman scintillation counter (LS-3110 Series). Under these assay conditions, the apparent specific activity of [³H]AdoMet was 9,800 CPM per pmole. One unit of methylase activity transfers 1 pmole of [³H] -methyl group from [³H]AdoMet to pBR 322 DNA in 1 hour at 37°C which binds to DE81 paper.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of 0.1% SDS was carried out according to the method of Weber and Osborn (1969) on 10% acrylamide slab gels (16 × 18 × 0.15 cm). The protein bands were visualized by staining with 0.25% coomassie brilliant blue in methanol: acetic acid: H₂O (5 : 1 : 5) for 2 to 3 hours and destaining in 7.5% acetic acid, 5% methanol for 5 to 20 hours.

Agarose gel electrophoresis

Electrophoresis of DNA was performed on 1% agarose horizontal gels ($8 \times 9 \times 0.5\text{cm}$) in TAE buffer (0.04 M Tris-acetate, 2 mM EDTA, pH 8.2). DNA was visualized by staining with 1 g/ ml of ethidium bromide for 30 minutes and the stained gels were photographed on a short-wave ultraviolet light with Polaroid Type 667 film.

Protein determination

The concentrations of proteins were determined according to the method of Schaffner and Weissman (1973).

Results

Purification of Alu I methylase

A summary of the purification of Alu I methylase from 300g of *Arthrobacter luteus* is presented in Table 1. All steps were performed at 0 to 4°C. Unless indicated otherwise, centrifugation was at $12,000 \times g$ for 30 minutes in GSA rotor. Buffers used during the purification procedure were as follows: Buffer A; 50 mM Tris-HCl, pH 8.0, 10 mM 2-mercaptoethanol, 5% (w/v) glycerol, and 0.1 mM EDTA, Buffer B; 20 mM sodium phosphate, pH 7.8, 20 mM 2-mercaptoethanol, 10% (w/v) glycerol, 0.1 mM EDTA, and 20 mM NaCl, Buffer C; 20 mM potassium phosphate, pH 7.8, 0.1 mM EDTA, 20 mM 2-mercaptoethanol, 10% (w/v) glycerol, and 10 mM KCl.

Preparation of the cell extracts

300g of *Arthrobacter luteus* cells were suspended in 900 ml of buffer A with gentle stirring overnight at 4°C. The suspended cell paste was passed twice through French Press at 12,000 p.s.i. and then was sonicated in 100 ml portions using ArtekSonicator equipped with a macrotip. Temperature was maintained at 5°C or less with an ice bath. The extract was clarified by centrifugation at 35,000 rpm in Type 42.1 rotor (Beckman) for 60 minutes. The supernatant was diluted with 800 ml of buffer A to yield an A_{260} of 60 (Fraction I, 2,000 ml).

Streptomycin sulfate fractionation

500 ml of freshly prepared 6% (w/v) streptomycin sulfate was added to fraction I with gentle stirring over a period of 45 minutes and after an additional stirring for 30 minutes, the precipitate was removed by centrifugation. The supernatant had an A_{280} of 20 and a A_{280}/A_{260} ratio of 0.95 (fraction II, 2,482 ml).

Ammonium sulfate fractionation

Powdered ammonium sulfate (1,005 g) was added to fraction II with gentle stirring over a period of 60 minutes to 65% saturation. After stirring overnight, the precipitate was

collected by centrifugation, resuspended in 200 ml of buffer B, and dialyzed against 10 volumes of buffer B for 12 hours with three buffer changes (fraction III, 308 ml).

Phosphocellulose column chromatography

Fraction III was applied at 30ml/hour to a phosphocellulose column (3×30 cm) equilibrated with buffer B. The column was washed with 400 ml of buffer B and then eluted with 1,000 ml linear gradient of NaCl (0.02-1.0 M) in buffer B. 1 μ l from every fractions was used for methylase assay.

Alu I methylase activity eluted between 0.26 M and 0.38 M NaCl (Fig. 1). The pooled fractions were dialyzed against 10 volumes of buffer B for 12 hours with two buffer changes (fraction IV, 92 ml).

DEAE-cellulose column chromatography

Fraction IV was applied at 35 ml/hour to a DEAE-cellulose column (20 ml disposable plastic syringe) equilibrated with buffer B. The column was washed with 40 ml of buffer B and then eluted with 140 ml linear gradient of NaCl (0.02-0.60 M) in buffer B. Alu I methylase activity eluted between 0.14 M and 0.28 M NaCl (Fig. 2). The fractions containing Alu I methylase activity were pooled and dialyzed against 10 volumes of buffer B for 6 hours (fraction V, 45 ml).

Heparin-agarose column chromatography

Fraction V was applied at 18 ml/hour to a heparin-agarose column (1.4 × 15cm) equilibrated with buffer B. The column was washed with 30 ml of buffer B and then eluted with 120 ml linear gradient of NaCl (0.02-1.0 M) in buffer B. Alu I methylase activity eluted between 0.30 M and 0.40 M NaCl (Fig. 3). The fractions containing this activity were pooled and dialyzed against 10 volumes of buffer C for 6 hours (fraction VI, 17 ml).

Hydroxylapatite column chromatography

Fraction VI was applied at 2 ml/hour to hydroxylapatite column (2 ml disposable plastic syringe) equilibrated with buffer C. The column was washed with 4 ml of buffer C and then eluted with 30 ml linear gradient of potassium phosphate, pH 7.8 (0.02-0.5 M) in buffer C. Alu I methylase activity eluted between 0.08 M and 0.14 M potassium phosphate, pH 7.8 (Fig. 4). The fractions containing this activity were pooled and dialyzed against buffer B supplemented with 50% (w/v) glycerol (fraction VII, 1.6 ml).

Fraction VII lost little activity (<3%) over a period of at least 5 months at -20°C and was free of nonspecific methylase activity since no methylation was observed with pBR 322 DNA cleaved by Alu I endonuclease (Table 2). 0.88 mg of Alu I methylase was isolated from 300 g of *Arthrobacter luteus* cells. Fraction, VII was nearly homogeneous as judged by 10% SDS polyacrylamide gel electrophoresis (Fig. 5B). The specific activity of fraction VII was 1.32×10^5 units/mg.

The molecular weight of Alu I methylase

10% polyacrylamide gel electrophoresis containing 0.1% SDS was carried out to determine the subunit size of the enzyme. Fraction VII showed a single major protein band as shown at Fig. 5B, lane a, with a mobility of 0.24 relative to bromophenol blue. When compared with the proteins of known molecular weight, the mobility yields an apparent molecular weight of $56,000 \pm 1,000$ (Fig. 5A).

The specificity of Alu I methylase

Since the isolation of Alu I methylase had not been reported, it was important to determine that Alu I endonuclease and Alu I methylase recognize the same specific sequence of DNA strand. As a substrate, pBR 322 DNA, which contains 16 Alu I sites, was used. pBR 322 DNA methylated by Alu I methylase was not cleaved by Alu I endonuclease but was cleaved by Hinc II endonuclease (Fig. 6). Furthermore, pBR 322 DNA cleaved by Alu I endonuclease was not methylated by Alu I methylase (Table 2). These results clearly showed that Alu I endonuclease and Alu I methylase recognize the same DNA sequence on pBR 322 DNA.

Discussion

The purification steps summarized at Table 1 for 300 g of *Arthrobacter luteus* cells were carried out twice under the same conditions. At each step of purification, protein was analyzed by SDS-polyacrylamide gel electrophoresis. Total of 1.16×10^5 units (0.88 mg) of Alu I methylase was recovered after hydroxylapatite column chromatography which is equivalent to 11% of the methylase activity detected from the crude extract.

The Alu I methylase thus isolated is free of contaminating exonuclease and nonspecific methylase activities as judged by gel electrophoresis and scintillation counter after prolonged incubation with the enzyme. This enzyme lost little activity (<3%) over a period of at least 5 months at -20°C . The specific activity of the enzyme is 1.32×10^5 units/mg which is higher compared to the specific activity of Eco RI methylase (Rubin *et al.*, 1977) and lower compared to that of Hpa I methylase (Yoo *et al.*, 1982).

The subunit size of the enzyme, as determined by polyacrylamide gel electrophoresis in the presence of 0.1% SDS, was calculated to $56,000 \pm 1,000$ (Fig. 5). The molecular weight of the native form of Alu I methylase will have to be determined by further sedimentation equilibrium analysis to examine whether Alu I methylase exists as a monomer in solution.

pBR 322 DNA methylated by Alu I methylase was resistant to the cleavage of Alu I endonuclease, and pBR 322 DNA cleaved by Alu I endonuclease, was not methylated by Alu I methylase (Table 2). It is clear from these results that the purified methylase recognizes the same base sequence as Alu I endonuclease on the DNA.

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List of Tables

- | | |
|---------|---|
| Table 1 | Purification of Alu I methylase from 300g of <i>Arthrobacter luteus</i> . |
| Table 2 | pBR 322 DNA cleaved by Alu I endonuclease is not methylated by Alu I methylase. |

List of Figures

- Fig. 1 Phosphocellulose chromatography. The reaction conditions are described in Methods.
- Fig. 2 DEAE Sephadex A-50 chromatography. The reaction conditions are described in Methods.
- Fig. 3 Heparin Agarose chromatography. The reaction conditions are described in Methods.
- Fig. 4 Hydroxylapatite chromatography. The reaction conditions are described in Methods.
- Fig. 5 Molecular weight and homogeneity determination of Alu I methylase. (A) the molecular weight of the reduced and denatured enzyme with respect to the standard proteins(phosphorylase B; 92,500, BSA; 66,200, ovalbumin; 45,000, carbonic anhydrase; 31,000, soybean trypsin inhibitor; 21,500, lysozyme; 14,400) (B) SDS-polyacrylamide gel electrophoresis of Fraction VII and standard proteins listed above. Lane a, Alu I methylase; lane b, standard proteins.
- Fig. 6 Determination of specificity of Alu I methylase using pBR 322 DNA. 1 μ g of pBR 322 DNA was methylated under standard assay conditions using 120 units of Alu I methylase in 25 μ l reaction mixture. The methylated DNA was then treated with Alu I endonuclease or Hinc II endonuclease as indicated. a, no enzyme treatment; b, 120 units of Alu I methylase treatment followed by 25 units of Alu I endonuclease digestion; c, 120 units of Alu I methylase treatment followed by 25 units of Hinc II endonuclease digestion; d, Alu I endonuclease digestion; e. Hinc II endonuclease digestion.

	Total protein (mg) ^a	Total units	Specific activity (units/mg protein)	% Recovery	Purification fold
I - Crude extract	29,500	1.05×10^6	35.5	100	1
II - Streptomycin sulfate	20,000	8.23×10^5	41.2	80	1.2
III - Ammonium sulfate	7,850	7.24×10^5	92.2	69	2.6
IV - Phosphocellulose	93	4.31×10^5	4,620	41	130
V - DEAE-cellulose	52	3.25×10^5	6,250	31	176
VI - Heparin-agarose	4.76	2.42×10^5	50,800	23	1,430
VII - Hydroxylapatite	0.88	1.16×10^5	131,800	11	3,710

a. Protein concentration determined by the method of Schaffner and Weissman(1973).

Table 1

Reaction ^a	Alu I endonuclease	Alu I methylase	³ H CPM ^b
A	—	—	49
B	+	+	58
C	—	+	12,170

- a. Each reaction contained 1 μg of pBR 322 and 0.8 μM [³H] AdoMet in 25 $\mu\ell$ buffer (15 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 50 mM NaCl, 0.1 mM EDTA, 11 mM 2-mercaptoethanol, 30 $\mu\text{g}/\text{ml}$ BSA) and incubated at 37°C. Reaction A was incubated without either enzyme. Reaction B treated first with 9 units of Alu I endonuclease was incubated for 3 hours, and then treated with 120 units of Alu I methylase for 30 minutes. Reaction C was treated only with 120 units of Alu I methylase for 30 minutes. b. Radioactivity in 5 $\mu\ell$ of reaction mixture on DE 81 paper was counted. The methylase assay is described in “Methods.”

Table 2

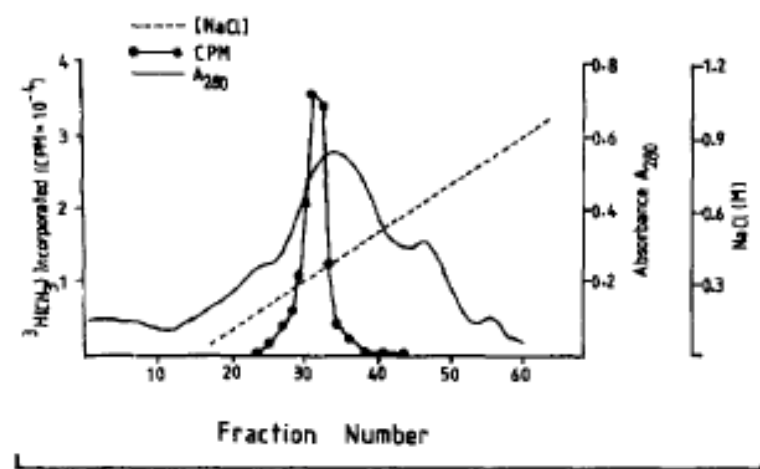


Fig. 1

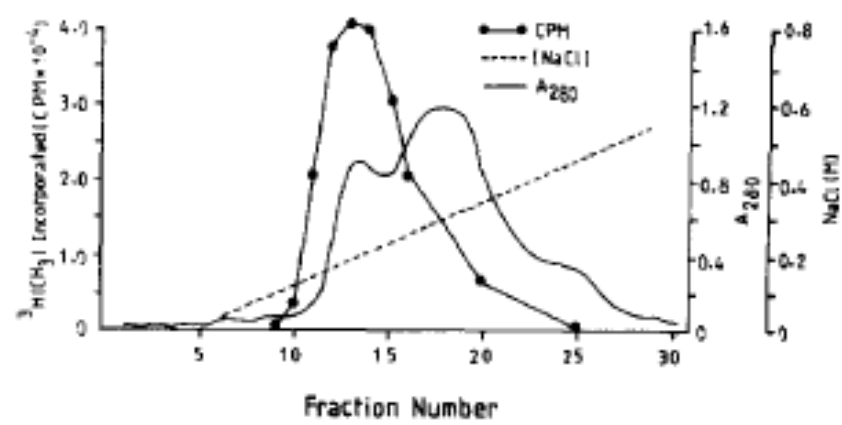


Fig. 2

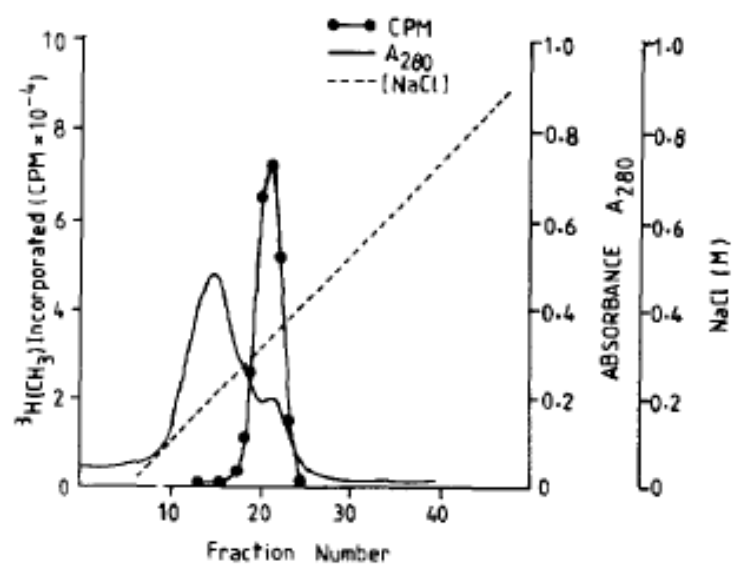


Fig. 3

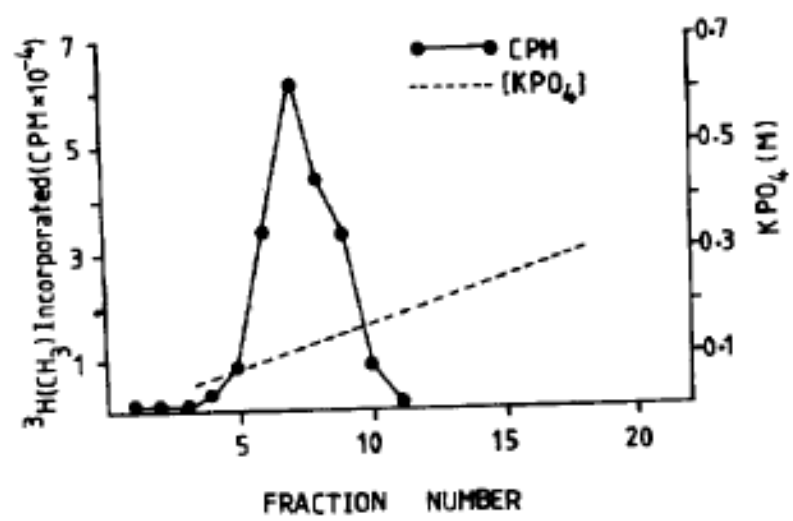


Fig. 4

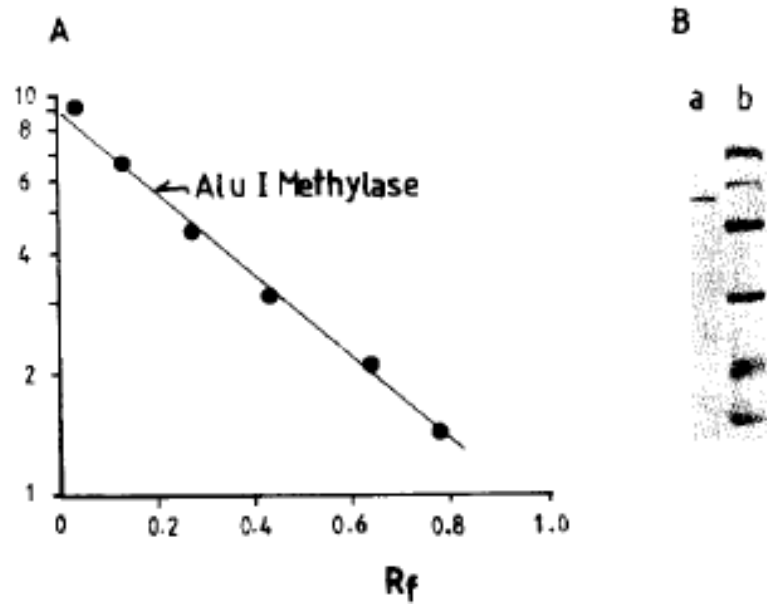


Fig. 5

A B C D E

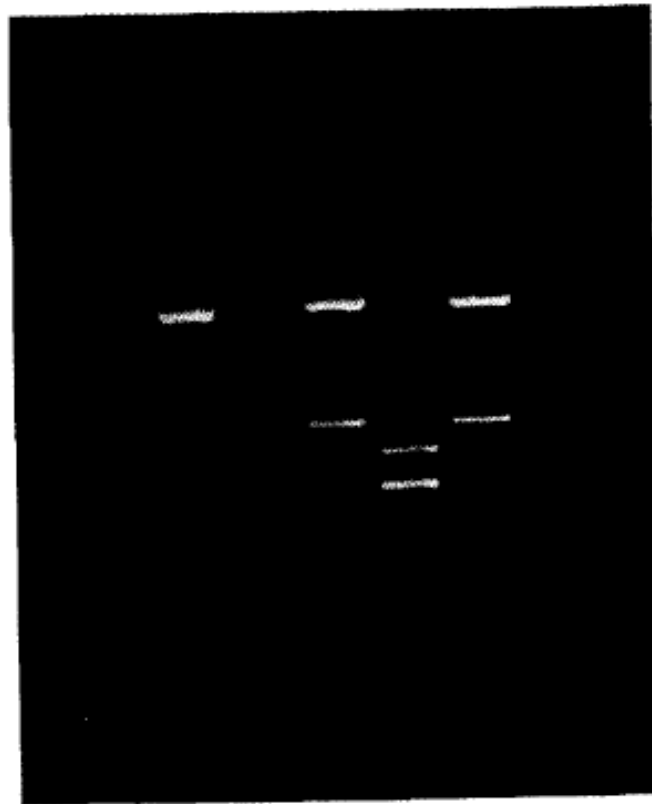


Fig. 6

초록 : Alu I methylase의 정제와 특성

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Alu I methylase를 순수 정제하였다. 300g (wet weight)의 *Arthrobacter luteus*에서 얻은 crude extract로부터 ammonium sulfate fractionation을 거친 후 phosphocellulose, DEAE-cellulose, Heparin-agarose, Hydroxylapatite등의 chromatography과정을 통하여 0.88mg의 Alu I methylase를 얻었다. Specific activity는 mg당 1.32×10^5 unit이었다. 이 methylase에 의하여 methylation된 DNA는 Alu I endonuclease에 의하여 절단되지 않았으며, 그 methylation site는 5'-d(AG[↓]CT)-3'임이 확인되었다. 정제된 Alu I methylase는 10% SDS-polyacrylamide gel electrophoresis에서 한개의 major band로 나타났으며 그 분자량은 $56,000 \pm 1,000$ 이었다.