KOR. JOUR. MICROBIOL. March, 1985, p. 13-19 Vol. 23, No. 1

Purification and Characterization of Acc I Endonuclease

Sun Chul Kang and O. Joon Yoo
Department of Biological Science and Engineering
Korea Advanced Institute of Science and Technology Seoul, Korea

Acc I endonuclease의 정제와 효소적 특성에 관한연구

강선철 · 유욱준
(한국과학기술원 생물공학과)

Acc I endonuclease has been isolated from 300g (wet weight) cells of *Acinetobacter calcoaceticus*. The cells were broken by using French press at 20,000 p.s.i.. After ammonium sulfate fractionation, the enzyme was further purified by heparin agarose, DEAE-sephades, Affi.-gel Blue, phosphocellulose, and hydroxylapatite column chromatography. The purified Acc I endonuclease has a single polypeptide species and its subunit molecular weight was 45,000 ± 1,000 daltons as judged by 10% SDS-polyacrylamide gel electrophoresis. The isolated enzyme was essentially free of contaminating nucleases as judged by homochromatography by using a ^{32}P-labeled oligonucleotide. The enzyme showed maximum activity at pH values between 8.0 and 11.0, and in the presence of $MgCl_2$. Acc I endonuclease was maximally active in the absence of NaCl and was completely inhibited at 200 mM NaCl.

The type II restriction endonucleases, which cleave DNA at defined nucleotide seqences, are extenively used in the structural analysis of DNA molecules, and for the construction of recombinant DNA species *in vitro*. However, the detailed biöchemical investigation of these enzymes has not kept pace with their widespread uses. Although the slow progess in the study of restriction enzymes may in part be due to their great usefulness as research tools (where purification to homogeneity is rarely required), it is also attributable to the low content of these enzymes in the bacteria from which are derived. Thus, to obtain homogeneous restriction endonucleases for detailed structural and mechanistic studies, it is necessary to develop the procedures for large scale purification of the enzymes. Restriction endonuclease Acc I, II and III activities were first detected in strains of *Acinetobacter calcoaceticus*, ATCC 23055, by Roberts R. J. and his co-workers (unpublished personal communication, 1982). They also indentified that Acc I endonuclease recognizes the hexanucleotide sequence 5′-GT$\binom{A}{C}\binom{G}{T}$
cuts at the site indicated by the arrow. In this report we describe a procedure for the purification of Acc I endonuclease and the characterization of its enzymatic properties.

MATERIALS AND METHODS

Materials

The strain of *Acinetobacter calcoaceticus*, ATCC 23055, was provided by Dr. S. H. Kim. Streptomycin sulfate, eletrophoresis grade agarose, heparin, phosphocellulose, Coomassie brilliant blue (G-250) were from Sigma Chemical Co. Sepharose 6B and DEAE-sephadex were from Pharmacia Fine Chemicals. Cyanogen bromide

was from Wako Pure Chemical Co. Phosphocellulose was precycled and equilibrated according to the procedure of Green *et al.* (1978). λ-bacteriophage DNA was from New England Biolabs. Affi.-gel Blue and hydroxylapatite, were from BioRad Laboratories. S-adenosyl-L-(methyl-^3H)-methionine (7.8 Ci/mmole) was from New England Nuclear. All other chemicals used during isolation procedures are reagent grade. Hpa II methylase was isolated in our laboratory. Heparin agarose was prepared by the procedure of Davision *et al.* (1979).

Cluture of cells

A. calcoaceticus was grown in selective medium (per liter; 10g of glucose, 0.6g of KNO_3, 0.45g of Na_2HPO_4, 0.1g of $MgSO_4$, $7H_2O$ 0.2g of $CaCl_2$. $7H_2O$, 10mg of FeSo4. $7H_2O$, 3g of yeast extract) at 30°C with rapid aeration (1968). Media were supplemented with 0.2% glucose, 0.2% casamino acid, and 10 μg/ml thiamine in 10 liter culture. Culture pH was maintained at 7.5 by the stepwise addition of Tris-HCl (pH 7.5). Cells were harvested in late exponential phase (A_{600} = 8) and adjusted to 50% (w/w) glycerol. The cell paste was stored at −20°C until direct use is necessary.

Enzyme assays

Endonucleases that had been partially purified by column chromatography were assayed by incubating 1μl of each enzyme sample with 0.25μg DNA in a total volume of 10μl. Reaction mixture contained 6mM Tris-HCl (pH 7.5), 10 mM NaCl, 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 100μl/ml BSA. After incubation at 37°C for 30min, the sample was subjected to electrophoresis on a 1% agarose gels in TAE buffer. One unit of restriction endonuclease is defined as the amount of an enzyme required to produce a complete digestion of 1.0μg λ DNA in 60 minutes in a total reaction volume of 50μl at 37°C.

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 vertical gels (16 x 18 x 0.15cm). The protein bands were visualized by staining with 0.25% Coomassie brilliant blue in methanol: acetic acid: H_2O (5:1:5) for 2 to 3 hours and destaining in 7.5% acetic acid, 5% methanol for 5 to 20 hours.

Protein determination

The concentration proteins were determined according to the method of Marion, M. Bradford (1976).

Catalytic properties of Acc I edonuclease

(^3H)-labeled DNA was used as a substrate. (^3H)-labeled λ DNA was prepared by Hpa II methylase and ^3H-AdoMet. The labeled DNA was submitted to the enzyme digestion and the undigested DNA band was eluted from the agarose gels by the procedure of Girvitz *et al.* (1979). The cpm of the eluted (^3H)-labeled DNA was counted with 5ml of toluene based scintillation fluid. The relative activity was expressed as percent of cpm compared to the maximum activity.

Homochromatography

Homochromatography was carried out according to the procedure of Jay *et al.* Crude yeast RNA (30g) was partially dissolved in 300ml of water. Urea (420g in 100ml of water) was added, and the mixture was kept at 37°C until all components were in solution. The solution was then neutralized with IN KOH. Addition of IN KOH was continued until the appropriate concentration of KOH was obtained (50mM KOH for segments 6 to 20 nucleotides in length and 100mM KOH for shorter segments). The solution was then incubated at 65°C for 16 to 20 hours. The oligonucleotide samples were spotted on DEAE-cellulose plates. Homochromatography was performed at 60°C. The reaction products were located by autoradiography.

RESULTS

Purification of Acc I endonuclease

A summary of the purification of restriction endonuclease Acc I from 300g cells of *A. calcoaceticus* is presented in Table 1. All subsequent steps were performed at 0 to 4°C. 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, 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.

Step 1: Preparation of the crude extracts.;300g of *A. calcoaceticus* cells were suspended in 900ml of buffer A with gentle stirring overnight at 4 °C. The suspended cell paste was disrupted by French press (20,000 p.s.i.) and sonicator equipped with a macrotip. Temperature was maintained at 8 °C or less during the disruption steps. The extract was clarified by centrifugation at 35,000 rpm in Type 42.1 rotor (Beckman) for 60 minutes. The supernatant was diluted with buffer A to yield on A_{260} = 60 (Fraction I, 1,030ml).

Step 2: Streptomycin sulfate fractionation.; 260ml of freshly prepared 10% (w/v) streptomycin sulfate was added to Fraction I with gentle stirring over a period of 30 minutes and after an additional stirring for 1 hour, the precipitant was removed by centrifugation. The supernatant had an A_{280} value of 24 (Fraction II).

Step 3: Ammonium sulfate fractionation.;Powdered ammonium sulfate (760g) was added to Fraction II with gentle stirring over a period of 60 minutes to 80% saturation. After stirring overnight, the precipitate was collected by centrifugation for 20 minutes. Pellet was resuspended in 150ml of buffer B, and dialyzed against buffer B for 12 hours with three buffer changes (Fraction III, 185ml).

Step 4: Heparin agarose column chromatography.; Fraction III was applied to a heparin agarose colum (95 x 2.0cm) equilibrated with buffer B. The column was washed with buffer B and then eluted with a 1,300ml linear gradient of

Fig. 1. Heparin agarose colum chromatography. The reaction conditions are described in Methods. Fraction numbers are indicated on the top of the gel. A: λ DNA

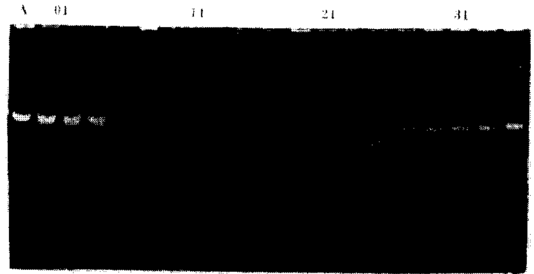

Fig. 2. DEAE-sephadex A-50 column chromatography. The reaction conditions are described in Methods. Fraction numbers are indicated on the top of the gel. A: λ DNA, o: washed out

NaCl(0.0 - 1.0 M) in buffer B. Fractions containing Acc I endonuclease activity (Fig. 1), which eluted at 0.3M Nacl, were collected and dialyzed against buffer B (Fraction IV, 300ml). Acc II endonuclease activity was also appeared at late fractions (Fig. 1).

Step 5: DEAE-Sephadex A-50 column chromatography.; Fraction IV was applied to a DEAE-sephadex A-50 column (21 x 1.8cm). After washing, the enzyme was eluted with a 200ml linear gradient of Nacl (0.0-0.8 M) in buffer B. Active fractions (Fig. 2), which eluted at 0.3M NaCl. were pooled and dialyzed against buffer B (Fraction V, 60ml).

Step 6: Affi.-gel Blue column chromatography. Fraction V was applied to a Affi.-gle Blue column (10 x 1.7cm). The column was then developed with a 100ml linear gradient of NaCl (0.0M - 1.0M) in buffer B. The fractions contain-

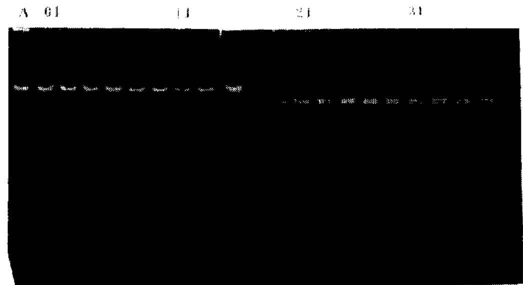

Fig. 3. Affi.-gel Blue column chromatography. The reaction conditions are described in Methods. Fraction numbers are indicated on the top of the gel. A: λ DNA, o: washed out

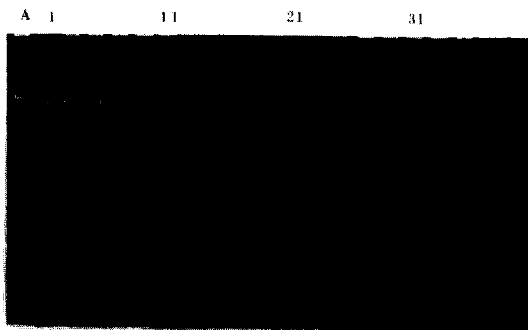

Fig. 4. Phosphocellulose column chromatography. The reaction conditions are described in Methods. Fraction numbers are indicated on the top of the gel. A: λ DNA

ing Acc I endonuclease activity (Fig. 3), which eluted at 0.4 M Nacl, were pooled (Fraction VI, 38ml).

Step 7: Phosphocellulose column chromatography.; Fraction VI was submitted to purification on phosphocellulose column (5.0 x 1.7cm). Enzyme activity (Fig. 4) was eluted with a 40ml linear gradient of NaCl (0.0 - 1.0M) in buffer B (Fraction VII, 5ml).

Step 8: Hydroxylapatite column chromatography.; Fraction VII was dialyzed against buffer C and then applied to a hydroxylapatite column (3.0 x 1.2cm). Linear gradient of phosphate (0.02 -1.0M) was used to elute the Acc I endonuclease activity. Active fractions (Fig. 5) were combined and concentrated by dialyses against buffer B containing 50% glycerol and stored at −20 °C (Fraction VIII, 1ml). Fraction VIII did not lose any

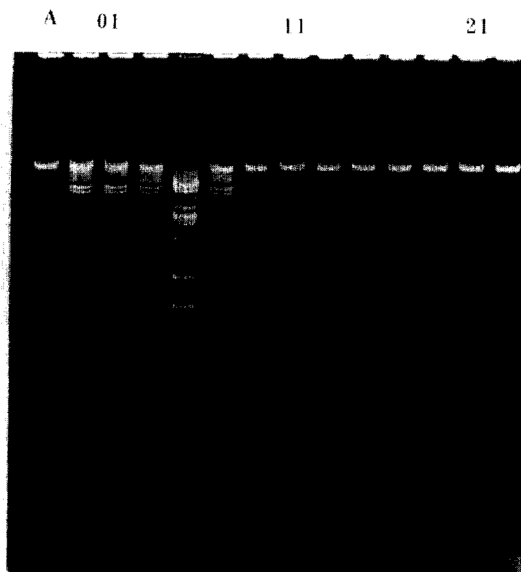

Fig. 5. Hydroxylapatite colum chromatography. The reaction conditions are described in Methods. Fraction numbers are indicated on the top of the gel. A: λ DNA, o:washed out

detectable amount of enzyme activity over a period of at least 6 months. 0.28mg of Acc I endonuclease was finally obtained from 300g of *A. calcoaceticus* cells. The isolated enzyme was essentially free from contaminating endonuclease, exonuclease and phosphatase activities as judged by homochromatography by using a ^{32}P-labeled single stranded oligonucleotide as a substrate (Fig. 6). SDS-polyacrylamide gel electrophoresis (Fig. 7B) showed that the isolated enzyme was

Table 1. The progress of the purification from 300g cells of *Acinetobacter calcoaceticus.*

	Total proteins (mg)	Total units	Specific activity (units/mg protein)	Percent recovery(%)	Purifica-fold
1) Crude extract	1.29×10^6	–	–	–	–
2) Streptomycin sulfate	1.32×10^4	–	45	–	1
3) Ammonium sulfate	3.06×10^3	–	200	–	4
4) Heparin-Agarose	360	6.0×10^5	1.7×10^3	100	38
5) DEAE-Sephadex A-50	99.4	2.8×10^5	2.8×10^3	47	62
6) Aff. Gel Blue	52.4	1.6×10^5	3.0×10^3	27	67
7) Phosphocellulose P-11	8.65	6×10^4	7.0×10^3	10	156
8) Hydroxyl-Apatite	0.28	3×10^4	1.1×10^5	5	2444

a. Protein concentrations are determined by the method of Marion, M. Bradford (27),

Fig. 6. Homochromatography.
The experimental procedure is described in "Methods". The oligonucleotide 5'-^{32}P-dCTCCTCCATCCATGG-3' was used for this experiment.
land a: Oligonucleotide,
land b: Oligonucleotide + Acc I endonuclease (1 hour incubation at 37 °Cwith 10 units of the purified enzyme).
lane c: Oligonucleotide + Calf intestinal phosphatase (1 hour incubation at 37 °C with 20 units of the enzyme)

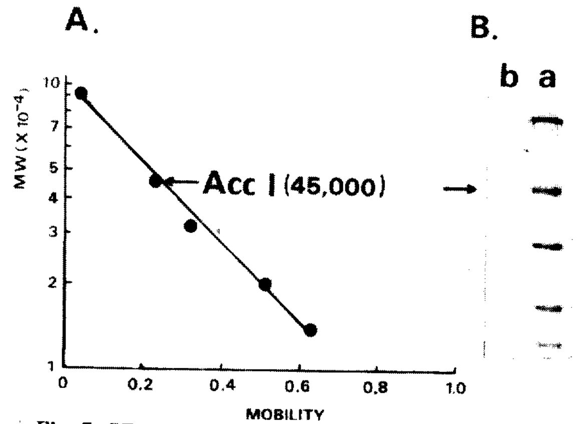

Fig. 7. SDS-polyacrylamide gel electrophoresis of Fraction VIII and standard proteins.
A: The subunit molecular weight of Acc I endonuclease
B: Lane a: standard proteins (phosphorylase B; 92,500, ovalbumin ;45,000, carbonic anhydrase; 31,000, soybean trypsin inhibitor;21,500, lysozyme; 14,400)
Lane b: Acc I endonuclease from Fraction VIII

nearly in homogeneous form.

The subunit molecular weight.

To determine the subunit size of the enzyme, 10% polyacrylamide gel electrophoresis was carried out in the presence of 0.1% SDS. Fraction VIII showed a single major band, as shown in Fig. 7B, with a mobility of 0.24 relative to bromophenol blue. When compared with standard proteins of known molecular weight the subunit molecular weight of the enzyme appeared to be 45,000 ± 1,000 daltons.

Catalytic properties

Acc I endonuclease maintained its maximum activity in broad pH range (between pH 8.0 and 11.0) (Fig. 8). The enzyme required magnsium ion (5 to 20 mM) for its activity and was maximally active at the NaCl concentration of 50mM (Fig. 9). But the enzyme activity was dramatically reduced when the NaCl concentration was higher than 150mM (Fig. 9).

DISCUSSIONS

0.28mg of Acc I endonuclease was isolated from 300g (wet weight) of *Acinetobacter calcoaceticus* cells. During the purification steps the protein was analyzed by SDS-polyacrylamide gel electrophoresis. The enzyme units were also determined at the same time. The Acc I endonuclease activities after heparin agarose and DEAE-sephadex columans showed unusual elution patterns. As shown in Fig. 1 and Fig. 2, the middle (fractions from 33 to 39 of Fig. 1 and fractions from 5 to 19 of Fig. 2) of the Acc I endonuclease activity fractions did not show any restrction DNA fragments. A possible reason for the result is that DNA binding proteins or nonspecific nucleases were eluted at the same fractions with Acc I endonuclease. However, the enzyme was separated from those activities after Affi. - gel Blue column chromatography (Fig. 3). The isolated enzyme showed single protein band on a 10% polyacrylamide gel (Fig. 7) and was

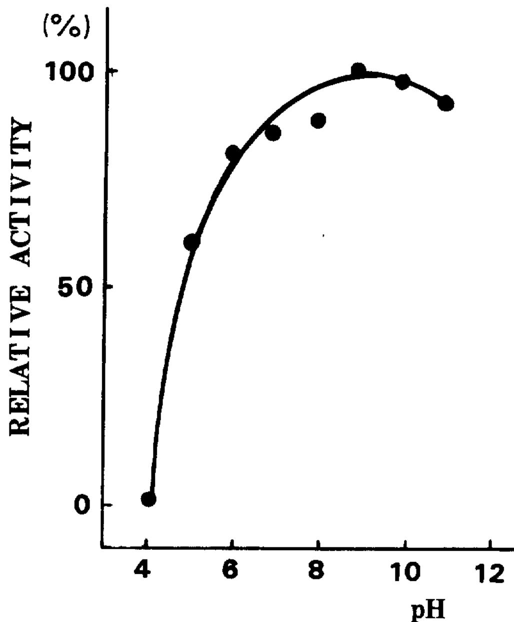

Fig. 8. pH depencence: Each reaction mixtures (10μl) are consisted of standard reaction condition (10mM NaCl, 6mM $MgCl_2$, 6 mM 2-mercaptoethanol, 10μg/ml BSA, 0.75μg of (^{3}H)-labeled DNA, Acc I endonuclease (0.5 unit/μ.' and 10 mM buffer). Used buffers were citric acid/NaOH, pH 4, sodium phosphate/NaOH, pH 5,6, and 7, Tris-HCl, pH 8 and 9, and glycine/NaOH pH 10, 11 and 12. Incubation was carried out for 30 minutes at 37 °C.

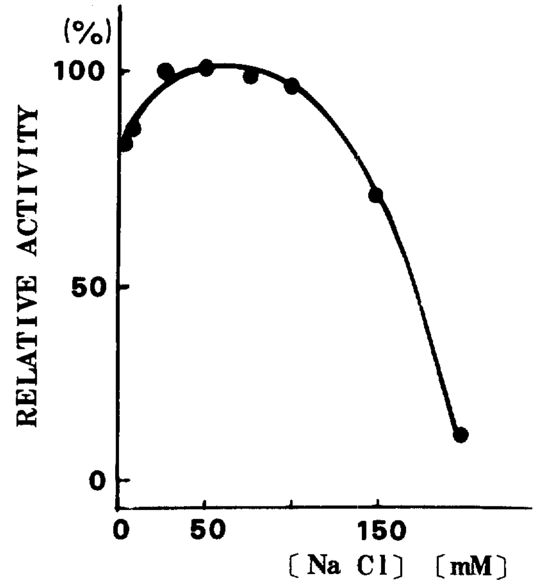

Fig. 9. Nacl effect on Acc I endonuclease activity. The reactions (10μl) were performed in the standard reaction condition, except that various NaCl concentrations were used. Incubation was carried out for 30 minutes at 37 °C.

essentially free of contamination endonuclease, exonuclease and phosphatase activities (Fig. 6). 1% agarose gel electrophoresis after prolonged (overnight) incubation of the enzyme with λ-DNA, showed again that the enzyme is free of contaminating nucleases. Furthermore, the ligation-recutting experiment also showed that the enzyme is free of phosphatase and nonspecific nuclease activities. This enzyme is stable for at least 6 months when it stored at -20 °C. The specific activity of the enzyme was 1.1 x 10^5 units/mg protein, which is ten times higher than Bam HI endonuclease (Leonard *et al.*, 1978) and lower than Bst I endonuclease (Catherine *et al.*, 1979). The subunit size of the enzyme, as determined by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate, was calculated to 45,000 ± 1,000 daltons (Fig. 7). Although the active form of Acc I endonuclease was not being determined, it was assumed that the enzyme reacts as a dimer in solution because all studied type II endonucleases actively function as dimer. The dependences of Acc I endonuclease activity on pH, salt and cofactors, were carefully deterined by using the readioactive (^{3}H-labled DNA as a substrate. Like all other restriction endonucleases, AccI endonuclease requires magnesium chloride as an essential cofactor. The enzyme activity was maximally active in the absence of NaCl and was completely inhibited at 200 mM NaCl. Sulfhydryl group compound (2-mercaptoethanol) was not required for its activity indicating that the cysteine resideue of Acc I endonuclease is not doing any important role at the active center. During the purification of Acc I endonuclease, Acc II endonuclease activity and unidentified methylase activity also appeared. The finding of Acc I methylase will be an encouraging result since the interaction of restriction endonuclease and its cognate methylase with DNA affords a potent opportunity for investigation of sequence-specific DNA-protein interactions.

적 요

제한효소 Acc I 을 정제하고그 효소적 특성을 연구하였다. 300g (wet weight) 의 *Acinetobacter calcoaceticus* 로부터 얻은 crude extract를 sample로 하여 ammonium sulfate fractionation을 거쳐 Heparin-agarose, DEAE-sephadex, Affi.-gel Blue, phosphocellulose, hydroxylapatite의 순서로 chromatography를 수행한 결과 0.28mg의 Acc I 제한효소를 얻었다. 효소의 specific activity는 mg당 1.1×10^{5}unit이었다. 정제된 Acc I 제한효소는 10% SDS-polyacrylamide gel electrophoresis에서 한개의 band로 나타났으며 그 분자량은 45,000±1,000이었다. 이 효소는 $MgCl_2$존재하에, pH 8.0에서 11.0사이에서 최대의 활성을 보였다. NaCl은 이 효소의 활성에는 필요하지 않았으나 150mM 이상에서는 급격한 효소 활성의 감소가 있었다.

REFERENCES

1. Baumann, P., M. Doudoroff, and R.Y. Stanier, 1968. A study of the *Moraxella* group II. Oxidative-negative species (Genus *acinetobacter*). *J. Bacteriol.* **95**, 1520-1541.
2. Catherine, M. Clarke and Brian S. Hartley, 1979. Purification, properties and specificity of the restriction endonuclease from *Bacillus stearothermophilus*. *Biochem. J.* **177**, 49-62.
3. Davision, B. L., Leighton, T., and Rabinowitz, J.C., 1979. Purification of *Bacillus subtilis* RNA polymerase with heparinagarose. *J. Biol. Chem.* **254**, 9220-9226.
4. Girvitz, S. C., Bacchetti, S., Rainbow, A. J., and Graham, F. L., 1980. A rapid and efficient procedure for the purification of DNA from agarose gels. *Anal. Biochem.* **106**, 492-496.
5. Green, P. J., Heyneker, H. L., Bolivar, F., Rodrigue, R. L., Betlach, M. C., Covanukias, A. A., Bachman, K. Russel, D. J. Tart, R., and Boyer, H. W., 1978. A general method for the purification of restriction enzymes. *Nucl. Acids Res.* **5**, 2373-2380.
6. Jay, E., Bambara, R., Padmamabhan, R., and Wu, R., 1974. DNA sequence analysis; a general, simple and rapid method for sequencing large oligodeoxyribonucleotide fragments by mapping. *Nucl. Acids Res.* **1**, 331-353.
7. Leonard, A. Smith and Jack, G. Chirikjian 1978. Purification and characterization of the sequence-specific endonuclease Bam HI. *J. Biol. Chem.* **254**, 1003-1006.
8. Marion, M. Bradford, 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
9. Weber, K., and Osborn, M., 1969. The reliability of molecular weight determinations by dodecyl sulfate-polycrylamide gel electrophoresis. *J. Biol. Chem.* **244**, 4406-4412.

(Received, January 25, 1985)