A RAPID PROCEDURE FOR PURIFICATION OF EcoRI ENDONUCLEASE

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SUMMARY

A convenient and rapid procedure has been developed to purify restriction endonuclease Eco RI. The method involves sonication of cells at low ionic strength, precipitation of the endonuclease with Polymin P (a polyethyleneimine), elution of the enzyme from the Polymin P precipitate, ammonium sulfate precipitation and chromatography on phosphocellulose. The purified restriction endonuclease is free of exonuclease and other endonucleases.

INTRODUCTION

The restriction endonuclease EcoRI is widely used in the mapping analysis of procaryotic and eucaryotic DNAs as well as in the recombinant DNA cloning technology (1). The enzyme has been purified in many laboratories, using either the original procedure of Yoshimori (2) or by other recently published procedure (3-5). All of the above mentioned procedures are time-consuming and involve ultracentrifugation with several additional purification steps resulting in an overall low yield of purified enzyme.

In order to simplify the purification of this enzyme we looked for rapid and effective purification steps. Since Zillig et al. (6) used Polymin P (a polyethyleneimine) very efficiently in the purification of bacterial DNA-dependent RNA polymerase, we thought that this chemical could also be a good tool for the purification of other DNA-binding proteins.

We describe here the purification of the restriction

Abbreviation : SV40, Simian Virus 40

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endonuclease EcoRI using Polymin P. The procedure is very rapid and simple and does not require either ultracentrifugation, streptomycin sulfate precipitation, or the presence of detergents in buffers. The purification can be completed within two days giving, with a high yield, an EcoRI enzyme free of contaminating nuclease activities.

MATERIAL AND METHODS

E.coli RY13 cells were obtained from the Laboratoire de Chimie Bactérienne du CNRS, Marseille (France). ^{3}H-labeled SV40 DNA (specific activity 3×10^4 cpm/µg) was prepared as described elsewhere (7). Adenovirus-2 DNA was purified by a modification of the original method of Doerfler (8, 9). Polymin P was a kind gift of BASF (WHOZ Hauptlaboratorium B9, Hochschullieferung, 6700 Ludwigshafen/Rhein - Germany). The 10% solution was prepared as described by Burgess and Jendrisak (10). All buffers and solutions were autoclaved before use and prepared from double-distilled water and the highest grade chemicals available.

EcoRI endonuclease assay was carried out for one hour at 37°C in 20 µl (final volume) of a mixture containing 100 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 1 µg Adenovirus-2 DNA and various amounts of EcoRI enzyme. The reaction was stopped by addition of sodium dodecylsulfate (0.1%), sucrose (10%) and bromophenol blue (0.05%) (final concentrations). The whole reaction mixture was electrophoresed (1.5 volt/cm, 14 hours) through a 1.4% agarose (Seakem, MCI Biomedicals, Rockland, Maine, USA) slab gel (17 × 13 × 0.3 cm) in 40 mM Tris-HCl pH 7.8, 5 mM sodium-acetate, 10 mM EDTA. The gels were stained with ethidium bromide (0.5 µg/ml) in electrophoresis buffer and photographed using ultra-violet illumination. 1 unit of EcoRI endonuclease activity is able to fully digest (11) 1 µg Adenovirus-2 DNA in one hour. Assay for possible contaminating endonuclease activity was carried out with covalently closed circular SV40 DNA (Form I) which was incubated with EcoRI endonuclease and subsequently analysed on alkaline sucrose gradient (13). Contaminating exonuclease activity of the EcoRI enzyme preparation was measured by release of acid-soluble radioactivity from labeled ^{3}H-DNA of SV40 cleaved with restriction endonuclease Hind III prior to the exonuclease assay in order to increase the sensitivity of the assay. Parallelly we tested whether linear SV40 DNA Form I with EcoRI enzyme were able to recircularize after re-annealing at 50°C in 2 M NaCl solution and subsequent treatment with T_4 DNA ligase (12). Protein was determined according to Lowry et al. (14).

PURIFICATION PROCEDURE

All steps were carried out at 4°C unless otherwise specified. Centrifugations were run in a MSE 25 high speed centrifuge at 30.000 g.

1. Cell disruption. E. coli RY13 cells (50 g) were suspended in 150 ml of extraction buffer (20 mM potassium phosphate pH 7.0, 10 mM β-mercaptoethanol). The cell suspension was sonicated with

a Branson sonifier B30 (full power) using a large probe for a total time of 5 min. The temperature was monitored and not allowed to exceed 8°C. The sonicated cell suspension was centrifuged for 15 min. The pellet was discarded and the clear supernatant (180 ml) was adjusted to 0.2 m KCl by addition of solid KCl (Fraction I).

2. Polymin P precipitation. A 10% solution of Polymin P (pH 7.4) was added to Fraction I to a final concentration of 0.2% (20 µl/ml) while stirring. Stirring was continued for 10 min and the precipitate was collected by centrifugation (30 min). The pellet was homogenized with the help of a glass-teflon homogenizer in 100 ml of Buffer 1 (10 mM potassium-phosphate pH 7.4, 10 mM β-mercaptoethanol, 0.5 mM EDTA). The suspension was centrifuged for 10 min and the supernatant was discarded. The pellet was resuspended as above in 150 ml of Buffer A containing 0.6 M KCl and centrifuged as previously. 140 ml of a clear supernatant was obtained (Fraction II).

3. Ammonium sulfate precipitation. Endonuclease EcoRI was precipitated from Fraction II by adding solid ammonium sulfate (0.313 g/ml) while stirring. The pH was maintained at 7.4 by adding 1 M NaOH. Stirring was continued for an additional 30 min period. After centrifugation for 30 min the protein pellet was dissolved in 10 ml of Buffer B (20 mM potassium-phosphate pH 7.4, 1 mM EDTA, 5 mM β-mercaptoethanol, 10% glycerol). The enzyme solution was dialized against 2 liters of Buffer B containing 0.2 M KCl. After two hours the conductivity of dialyzed solution was measured and its conductivity was adjusted to that of Buffer B containing 0.2 M KCl.

4. Phosphocellulose column chromatography. The dialyzed fraction was applied (flow rate, one column volume/hour) to a 2.5 × 10 cm column of phosphocellulose (Whatman P11) carefully washed and equilibrated with Buffer C (20 mM potassium-phosphate pH 7.4, 0.5 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol) containing 0.2 M KCl. The column was washed with 50 ml of Buffer C containing 0.2 M KCl and eluted with a linear salt gradient (250 ml) from 0.2 to 0.9 M KCl in Buffer C. The active fractions eluted between 0.5 and 0.6 M KCl were pooled and dialyzed (4 hours) against 2 × 2 liters of Buffer C containing 0.2 M KCl. Autoclaved gelatin was added (0.5 mg/ml) and the dialysis was continued against 2 liters of Buffer C containing 0.2 M KCl and 50% (w/w) glycerol. The purified enzyme was stored at -20°C.

RESULTS AND DISCUSSION

The purification method described in this paper allows to purify restriction endonuclease EcoRI from E. coli RY13 strain in two days with high recovery (Table I).

Any method for cell disruption can probalby be used. In the course of this work three different procedures were tried : sonication at low ionic strength, grinding the cells with alumina or lysozyme-sodium deoxycholate lysis. However, in the two

TABLE I

SUMMARY OF PURIFICATION OF EcoRI ENDONUCLEASE[a]

Purification step	Volume (ml)	Total protein (mg)	Total activity units × 10^{-3}	Specific activity units/mg	Yield %
Fraction I	180	4100	3727	909	-
Fraction II	150	145	3317	22730	89
Fraction III	10	68	2340	34100	60
Fraction IV	35	10	2150	205000	55

Fractions I, II, III and IV are cell extract, Polymin P eluate, ammonium sulfate precipitate after dialysis and pooled fractions of phosphocellulose chromatography after dialysis, respectively (Material and Methods).

[a]from 50 g of cells

latter cases, an additional mild sonication was required in order to obtain sufficient precipitation of nucleic acids and EcoRI endonuclease with Polymin P. The optimal concentration of Polymin P during the precipitation was determined in an experiment similar to that described by Burgess and Jendrisak (10). Fig. 1 A shows that using more than 0.2% Polymin P is not necessary and should even be avoided, since it could result in an increase of the amount of nucleic acids and proteins in the pellet. The presence of 0.2 M KCl during the Polymin P precipitation does not affect the EcoRI enzyme precipitation and is required in order to prevent the precipitation in many proteins as well as some nucleic acids (data not shown).

The optimal conditions for extracting EcoRI endonuclease from the Polymin P pellet was determined as described by Burgess and Jendrisak (10). Eluting the Polymin P pellet with buffers containing more than 0.6 M KCl yielded only minor additional amount of EcoRI endonuclease (Fig. 1 B) and resulted in the elution of more protein and nucleic acids (the 280/260 nm absorbance ratio was 1.4 in 0.6 M KCl eluate and 0.9 in 0.9 M KCl eluate, respectively). Washing the Polymin P pellet with Buffer A before elution was useful in removing some protein and nucleic acids with negligible amount of EcoRI endonuclease activity.

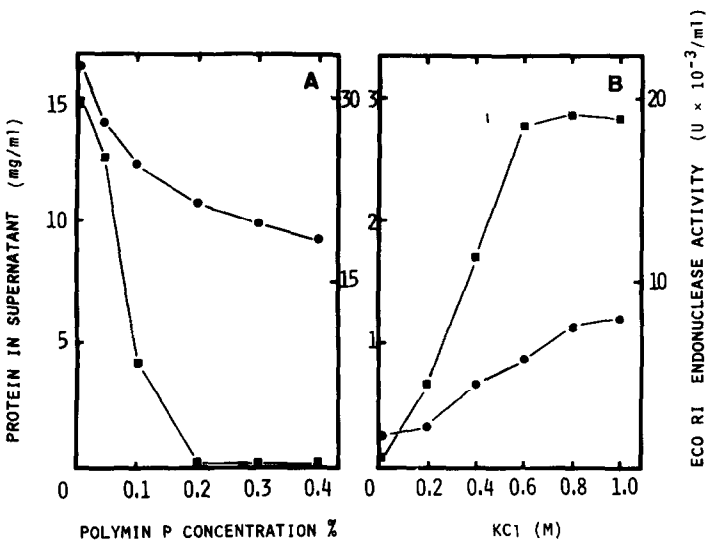

Fig. 1 :

A. Precipitation of EcoRI endonuclease and protein with Polymin P. Polymin P was added to cell extract (Material and Methods) at various concentrations as indicated. After centrifugation at 30.000 g for 15 min, aliquots of the supernatants were taken, diluted in Buffer A and the smallest quantitiy of enzyme which was required to fully digest 1 µg Adenovirus-2 DNA under standard conditions was determined. Samples were also taken for protein determination.

B. Effect of salt concentration on the elution of EcoRI endonuclease and protein from the Polymin P pellet. To 15 ml of cell extract Polymin P was added to a final concentration of 0.2% (Material and Methods). Aliquots (2.5 ml) were centrifuged and the pellets were homogenized in 2.5 ml of Buffer A containing various concentrations of KCl as indicated. After centrifugation the supernatant was assayed for EcoRI endonuclease activity and protein concentration (Material and Methods).

— ■ —, EcoRI restriction enzyme activity; — • —, protein concentration.

Quantitation of EcoRI endonuclease activity in the crude extract was not reliable due to the many interfering factors present at this early stage of purification. Assuming that total EcoRI endonuclease activity present in the cell could be precipitated with Polymin P at a concentration of 0.2% (Fig. 1 A) and could be quantitatively eluted with 1.0 M KCl (Fig. 1 B) the crude extract contained about 3.7×10^6 units of EcoRI enzyme per 50 g of cells (Table I). With these assumptions the purification achieved by Polymin P precipitation and elution with 0.6 M KCl was approximately 25 fold (Table I) with a yield of about 89%. Further purification by ammonium sulfate

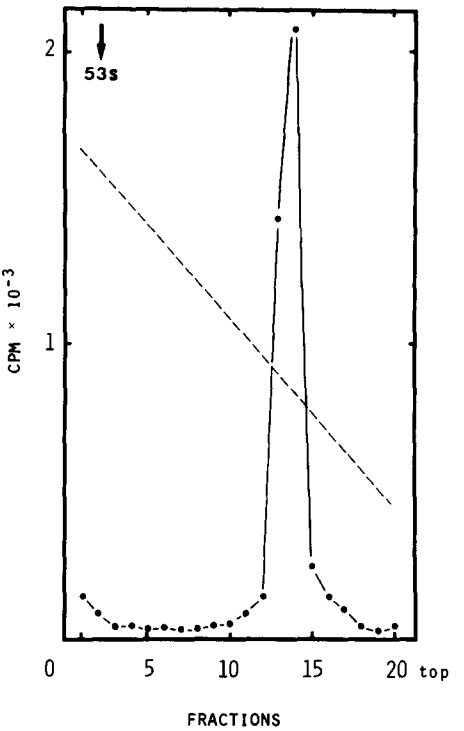

Fig. 2 :

Endonuclease test. 20 units of EcoRI endonuclease and 0.5 µg of ^{14}C-SV40 DNA (specific activity 2.7×10^4 cpm/µg) were incubated at 37°C for 3 hours in a total volume of 200 µl containing 100 mM Tris-HCl pH 7.8 and 10 mM $MgCl_2$. The reaction was stopped by addition of 20 mM EDTA and 0.3 N NaOH (final concentrations). The whole mixture was sedimented through an alkaline sucrose gradient (3.7 ml, 5-20%) in a Spinco SW 56 rotor at 50.000 rpm for 2 hours.

precipitation and phosphocellulose chromatography resulted in an enzyme preparation with a specific activity of about 20.500 units/mg protein and an overall recovery of about 55% (2.15×10^6 units/50 g of cells). It is difficult to validly compare the yield of the present purification and the specific activity of our purified enzyme with those of other methods previously described (2-5), since the enzyme activity was not expressed in the same units. However, the yield of our method appears to be at least 5 times higher than that achieved with previous methods.

The main concern about the enzyme purity of a restriction endonuclease enzyme preparation is its possible contamination

TABLE II

EXONUCLEASE TEST

Time	EcoRI enzyme (units)		
	0	30	150
	Acid-soluble radioactivity (cpm)		
0 h	38	-	-
2 h	44	41	50
4 h	41	49	85

^{3}H-SV40 DNA (specific activity = 30,000 cpm/µg) digested with Hind III restriction enzyme, phenol extracted and ethanol precipitated, was incubated at 37°C in 100 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$ with no, 30 and 150 units of purified EcoRI enzyme per µg of DNA. At the indicated time, aliquots corresponding to 0.3 µg of DNA were removed in triplicate and assayed for soluble radioactivity. The background (29 cpm) was not substracted from the mean value of the triplicates.

by exonuclease and other endonuclease activities. Our Eco RI endonuclease preparation was free of other double strand endonuclease activity, since SV40 DNA Form I cleaved by a 40 fold excess of purified EcoRI enzyme sedimented as a single sharp peak (16 S) in an alkaline sucrose gradient (9) (Fig. 2). In addition the enzyme was free of exonucleases as judged by the following criteria : 1) less than 0.5% of radioactivity was released from Hind III SV40 ^{3}H-DNA fragments during a 4 hour incubation period with 150 fold excess of purified enzyme (Table II); 2) linear SV40 DNA obtained by treating SV40 DNA Form I with a 20 fold excess of purified EcoRI enzyme was very efficiently converted to covalently closed circles (Form I DNA) upon treatment with T_4 ligase (result not shown).

In conclusion this new method of purification of EcoRI restriction enzyme provides a very convenient way to prepare large amounts of enzyme suitable for DNA mapping analysis and *in vitro* recombinant DNA studies. The possibility to use this technique for the purification of other restriction enzymes is being studied in our laboratory. We have already successfully applied it to purify EcoRII. However, the concentration of

Polymin P which gave sufficient precipitation of EcoRII endonuclease proved to be higher (0.3%) and the enzyme was eluted from the pellet at 0.4 M KCl. The 0.4 M KCl eluate contained some non-specific nucleases which were removed by chromatography on a DEAE cellulose column as described by Yoshimori (2).

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