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A general method for the purification of restriction enzymes

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ABSTRACT

An abbreviated procedure has been developed for the purification of restriction endonucleases. This procedure uses chromatography on phosphocellulose and hydroxylapatite and results in enzymes of sufficient purity to permit their use in the sequencing, molecular cloning, and physical mapping of DNA.

INTRODUCTION

The widespread use of restriction endonucleases has revolutionized DNA biochemistry (3, 4). We have devised a simple procedure for the preparation of several such enzymes sufficiently free from other nucleases and phosphatases to permit their use in sequencing, molecular cloning, and physical mapping of DNA. The following enzymes have been purified by this procedure: AluI, BamI, BglI, BglII, EcoRI, EcoRII, HaeII, and HaeIII, HgaI, HincII, HsuI (isoschizomer of HindIII), PstI, SalI, TaqI, HpaI and HpaII.

BACTERIAL STRAINS AND GROWTH CONDITIONS

Strain sources, culture conditions and yields of cells are summarized in Table 1. Haemophilus strains were grown in a New Brunswick 14 liter microferm in the following medium: 37 g Brain Heart Infusion (BBL), 0.2 ml stock solution of nicotinamide adenine dinucleotide (NAD), and 10 ml stock solution of Hemin per liter. The NAD stock (10 mg/ml) was filter-sterilized and stored at -20°C. The Hemin stock (0.1% in 4% triethanolamine) was sterilized at 65°C for 15 min and stored at 4°C. A 500 ml culture, inoculated with a single colony and grown 16 hours, was used to inoculate 12 liters of medium in the microferm. This was grown 5-7 hours at 37°C with 11 l/min aeration and 3-500 rpm mixing. Increase in cell density was monitored on a Klett-Summerson photometer with a green filter, and the cells were harvested by centrifugation when the cell density stopped increasing (approximately 400-600 Klett units).

Streptomyces albus was grown in a New Brunswick microferm in Zubay's medium, which contains 28.9 g K_2HPO_4, 5.6 g KH_2PO_4, 10 g yeast extract, 10 mg

Table 1

Enzyme	Strain	Source of Strain	[a]Fementor/Medium	Yield
AluI	Arthrobacter luteus	R. J. Roberts	Hi density/SLBH	280 g
BamI	Bacillus amyloliquifaciensH	G. Wilson	Hi density/SLBH	55 g
BglI, BglII	Bacillus globigii	G. Wilson	Hi density/SLBH	60 g
EcoRI	Escherichia coli pMB4	H. W. Boyer	Hi density/SLBH	250 g
EcoRII	Escherichia coli RY22	H. W. Boyer	Hi density/SLBH	250 g
HaeII, HaeIII	Haemophilus aegypticus	R. J. Roberts	Microferm/BHI	55 g
Hga	Haemophilus gallinarium	R. J. Roberts	Microferm/BHI	60 g
HincII	Haemophilus influenza C	R. J. Roberts	Microferm/BHI	47 g
HpaI, HpaII	Haemophilus parainfluenza	H. W. Boyer	Microferm/BHI	60 g
Hsu	Haemophilus suis	R. J. Roberts	Microferm/BHI	25 g
SalI	Streptomyces albus	——	Microferm/Zubay's	—
Taq	Thermus aquaticus	R. J. Roberts	Microferm/Castenholz	22 g

[a]Details of growth conditions and compositions of the media are given in the text. Strains grown in the Labline hi density fermentor could also be grown in Zubay's medium in the New Brunswick microferm or other comparable fermentation units.

vitamin B_1 per liter plus 1% glucose (autoclaved separately). 11 liters of medium was inoculated with 1 liter of culture (started from a single colony and grown 36 hr at 37°C). This was grown 36 hr at 37°C.

Thermus aquaticus was grown in a New Brunswick microferm in Castenholz TYE medium (Bergey's manual). This medium was prepared by mixing 100 ml sterile 1% TYE, 1 ml Nitsch's trace element solution, 50 ml Castenholz salts A, 50 ml Castenholz salts B and 800 ml distilled water. 1% TYE is 10 g tryptone, 10 g yeast extract plus 1 liter distilled water. Nitsche's trace elements contains 0.5 ml H_2SO_4, 2.2 g $MnSO_4$, 0.5 g $ZnSO_4$, 0.5 g H_3PO_3, 0.016 $CuSO_4$, 0.025 g Na_2MoO_2, 0.046 g $CaCl_2 \cdot 6H_2O$ per liter. Castenholz salts A contains 2.0 g nitriloacetic acid, 20.0 ml of 0.03% $FeCl_3$, 1.2 g $CaSO_4 \cdot 2H_2O$, 2.1 g KNO_3 per liter and Castenholz salts B contains 2.0 g $MgSO_4 \cdot 7H_2O$, 0.16 g NaCl, 14.0 g $NaNO_3$, 2.2 g Na_2HPO_4 g per liter. The optimum temperature for growth of Thermus aquaticus is 70°C; however the maximum temperature achieved by our microferm was 63°C. 11 liters of medium was inoculated with 1 liter of

Table 2

Enzyme	Sonication: Lysozyme	Sonication: Total Time	Assay: [a]Buffer	Assay: [b]# Sites in Substrate	Assay: [c]Crude Extract	Assay: P.C. Column Eluate	Assay: Time
AluI	100µg/ml 30'	4'	666	10	—	2 µl	30'
BamI	100µg/ml 30'	12'	TM	1	—		
BglI	100µg/ml 15'	3'	666	1	—	2 µl	60'
BglII	100µg/ml 15'	3'	666	λDNA 5	—		
EcoRI	—	4'	TM + 0.1M NaCl	1	0.05µl	.05µl	10'
EcoRII	—	4'	TM	5	2 µl	2 µl	30'
HaeII	—	4'	666	2	—	1 µl	30'
HaeIII			666	pMB8 >12	—	1 µl	30'
Hga	—	4'	666	pKB252 5	8 µl	4 µl	20'
HincII	—	4'	666 + 0.05M NaCl	2	—	—	—
HpaI	—	4'	666 +	pMB9 >10	—	2 µl	15'
HpaII	—	4'	0.05M NaCl		—	2 µl	15'
Hsu	—	4'	666 + 0.05M NaCl	1	—	1 µl	15'
Pst	100µg/ml 15'	2'	TM	1	—	2 µl	30'
SalI	—	5'	666 + 0.15M NaCl	1	—	1 µl	60'
Taq	—	5'	666	pBR345 1	—	—	—

[a]TM buffer is tris-HCl 0.1M, $MgCl_2$ 5mM, pH 7.5; 666 buffer is tris-HCl 6mM, $MgCl_2$ 6mM, βME 6mM, pH 7.5.

[b]Substrate DNA was pBR322 except where noted otherwise.

[c]More careful adjustment of amount of extract and time of incubation might render specific restriction patterns visible in some of these strains.

culture grown overnight at 70°C and this was grown 6 hr at 63°C.

Other strains were grown in a high density fermentor (Lab-line Instruments) to late log phase in SLBH medium (11 g tryptone, 22.5 g yeast extract, 51 ml 1M K_2HPO_4 and 15.7 ml 1M KH_2PO_4 per liter plus 0.4% glycerol).

ENZYME ASSAYS

Enzymes were assayed by electrophoresis of digested DNA on a 1% agarose gel Tris-borate buffer (10.8 g tris base, 0.93 g Na_2EDTA and 5.5 g boric acid per liter) for 1-1/4 hr at 16 V/cm (2).

Any defined DNA with a convenient number of restriction sites can be used as a substrate. We have generally used pBR322 (1) except as noted in Table 2.

The DNA for EcoRII assays was prepared from E. coli strain GM31 which is dcm⁻ (lacking DNA cytosine methylase). The buffers used for assaying the various enzymes are listed in Table 2. Because of interference by other nucleases, we were unable to assay some of the enzymes until after phosphocellulose chromatography (Table 2). For all except EcoRI, 0.5-2.0 µl of extract or phosphocellulose column eluate is incubated in 30 µl containing 0.2 µg DNA at 37°C (60°C for Taq) for 15-60 min. EcoRI is coded for by a multicopy plasmid and therefore is present at a much higher concentration than the other enzymes; crude extract and phosphocellulose eluate were diluted approximately 20 fold. These variations are summarized in Table 2. The amounts of enzyme to add and time of incubation are approximate and are included only to serve as a guide.

PREPARATION OF CELL EXTRACT

The following purification is standardized to 50 g of cells. Frozen cell pellets were thawed and suspended in 200 ml of 10mM K_2HPO_4-KH_2PO_4 pH 7.0, 7mM mercaptoethanol, 1mM EDTA (extract buffer) containing 25 µg/ml phenylmethyl sulfonylfluoride (PMSF), 1mM NaN_3 and 0.4M NaCl. Several of the enzymes have been successfully prepared without NaN_3 and the proteolytic inhibitor (PMSF); however these are generally added as a precaution.

The cell suspension was sonicated with a Branson model W-350 set at maximum output using a 1 inch horn. Sonication was carried out in 30"-90" pulses with the cell suspension stirring on ice; the temperature was monitored and kept below 10°C. Several of the strains were resistant to sonication. These were stirred on ice with the addition of 100 µg/ml lysozyme for 15-30 minutes before sonication. (See Table 2) Completeness of sonication was monitored by following increase in absorbance at 280 of centrifuged aliquots after periods of sonication. In the case of EcoRI, release of enzyme activity closely paralleled release of A_{280} material.

The sonicated cell suspension was centrifuged for 1 hour at 100,000 X g (Beckman type 35 rotor). The supernatant was decanted and the conductivity was diluted to the equivalent of 0.2M NaCl or 0.1M NaCl, depending on the strain (see Table 3) and the pH was adjusted to 7.0. This extract was applied directly to a column of phosphocellulose.

PHOSPHOCELLULOSE CHROMATOGRAPHY

Careful preparation of phosphocellulose is essential to the success of this purification method. 125 g of Whatman P11 was suspended in 4 liters of 0.2N HCl diluted 1:1 with 95% ETOH and stirred gently for 30 min at room temperature. The slurry was allowed to settle and the supernatant aspirated or decanted to remove fines and other particulate matter. The resin was

Table 3

Enzyme	Phosphocellulose Chromatography			Hydroxylapatite Chromatography		[b]Yield	Used for DNA Sequencing
	Molarity NaCl for Loading Enzyme	Molarity NaCl in Gradient	Molarity NaCl Where Enzyme Elutes	Molarity of Potassium Phosphate Gradient	[a]Elution	Units of Enzyme from 50g of cells	
AluI	0.2	0.2 - 1.0	0.35	.01 - .25	.10	4,000	+
BamI	0.2	0.2 - 0.6	0.35	.01 - .25	.08	60,000	+
BglI	0.2	0.2 - 0.8	0.3	.01 - .5	.3	-	NT
BglII			0.5	.01 - .4	.27	-	NT
EcoRI	0.2	0.2 - 0.8	0.6	.01 - .500	.20	1,000,000	+
EcoRII	0.2	0.2 - 0.6	0.4	.01 - .25	.04	15,000	+
HaeII	0.2	0.2 - 1.0	0.4	.01 - .50	.25	60,000	+
HaeIII			0.9		.25	180,000	+
Hga	0.2	0.2 - 1.0	0.4	—	—	6,000	NT
HincII	0.1	0.1 - 0.6	0.35	.01 - .25	.06	—	NT
HpaI	0.2	0.2 - 1.0	0.45	.01 - .50	.30	500	NT
HpaII			0.30	.01 - .50	.20	25,000	NT
HsuI	0.2	0.2 - 0.6	0.3	.01 - .25	—	60,000	+
Pst	0.2	0.2 - 0.6	0.35	.01 - .40	.20	30,000	NT
SalI	0.2	0.2 - 0.8	0.4	—	—	25,000	NT
Taq	0.2	0.2 - 0.8	0.4	.01 - .25	.12	8,000	+

[a]The elution positions are approximate.
[b]A unit is defined as the amount of enzyme required to digest 1 µg of DNA to completion in 1 hr at 37°C (or 60°C for Taq). These are all minimum estimates.

collected by vacuum filtration and washed 2 to 3 times by suspension in 4 liters of distilled water. The pH was adjusted to near neutral with 1M NaOH and the resin was suspended in 4 liter 0.1N NaOH, stirred for 30 min at room temperature, collected by filtration, and suspended in 4 liters of 1mM EDTA. It was stirred for 30 min at room temperature and washed 3 times with distilled water. The pH was adjusted to near neutral with 1M HCl and the resin was suspended in extract buffer plus 0.2M NaCl. The pH was carefully adjusted to pH 7.0 before pouring a column, since many column volumes of dilute buffer are required for equilibration of the pH in a packed column. 125 g of dry phosphocellulose provides enough resin for a 500 ml packed column bed volume. If this preparation is scaled up, or if the starting resin is very dirty (phosphocellulose lots vary), the volumes or the number of the acid-ethanol and NaOH washes should be increased. Fines should be removed by allowing the resin to settle and aspirating or decanting the supernatant after several of the washes. The resin should be stirred gently and not too long so that fines are not generated, and it should not be allowed to stand at the extremes of pH for more than two hours.

A phosphocellulose column of 150-200 ml bed volume (2.5 X 35 cm) is sufficient for 50 g cell. It should be washed with several volumes of extract buffer plus NaCl (see Table 3) prior to loading. The extract was loaded directly onto the column and the column washed with several column volumes of extract buffer plus NaCl (see Table 3 for NaCl conc.) or until the A_{280} has decreased to near zero. The column was developed with a linear gradient of NaCl in extract buffer, 1-1.5 liter in volume (approximately 7 times the column volume). The NaCl concentrations for the individual enzymes are given in Table 3 together with the approximate NaCl concentration at which the enzyme elutes. Ten ml fractions were collected and assayed for endonuclease activity. Peak fractions were pooled and subjected to hydroxylapatite chromatography.

HYDROXYLAPATITE CHROMATOGRAPHY

A 20 ml bed volume column of hydroxylapatite (Clarkson Chemical Co.) was packed and equilibrated with extract buffer plus 0.2M NaCl. The phosphocellulose pool was applied directly to this column and the column was washed with about 100 ml of extract buffer plus 0.2M NaCl. Enzymes were eluted with a 300 ml gradient of potassium phosphate pH 7.0 containing 1mM EDTA, 7mM βME, 1mM NaN_3 and 0.2M NaCl. (Table 3 lists potassium phosphate concentrations for the individual enzymes.) 4 ml fractions were collected and assayed for enzyme activity.

Peak fractions were pooled and dialyzed against storage buffer (20mM $K_2HPO_4 \cdot KH_2PO_4$ pH7, 0.2M NaCl, 1mM EDTA, 7mM βME, 1 mM NaN_3 diluted 1:1 with glycerol). This dialysis concentrated the enzyme about three fold. Enzymes in this buffer were stable for many months at -20°C and working aliquots are also kept at -20°C. A more concentrated enzyme preparation can be obtained by dialyzing the pooled fractions from the hydroxylapatite column against solid PEG 6000 prior to dialysis against storage buffer.

Three enzymes, SalI, EcoRII, and HgaI, did not bind well to hydroxylapatite. Approximately 70% of the EcoRII, 90% of the HgaI, and all of the SalI activity were found in the flow through from this column; however, in the cases of EcoRII and SalI, most of the contaminating activities bound, and this was still an efficient purification step. Flow-through fractions were concentrated by dialysis versus solid polyethylene glycol (PEG6000) followed by dialysis against storage buffer.

BIOREX 70 CHROMATOGRAPHY

After phosphocellulose and hydroxylapatite chromatography, HgaI still contained a large amount of exonuclease. The hydroxylapatite flow-through was concentrated, dialyzed against EB, and further purified by chromatography on Biorex 70 (Biorad).

A 20 ml bed volume column was equilibrated with EB and the dialyzed hydroxylapatite pool applied to the column. The column was washed with EB and developed with a gradient of 0 to 0.5 M NaCl in EB. Much of the exonuclease was present in the flow-through, while the Hga eluted at approximately 0.35 M NaCl. EcoRII and HpaII were also subjected to Biorex 70 chromatography. As in the case of HgaI, exonuclease was present in the flow-through, and the endonuclease eluted at 0.35 - 0.4 M NaCl.

In our laboratory, all of the enzymes except HgaI, EcoRII, and HpaII have been used routinely after phosphocellulose and hydroxylapatite chromatography. If further purification is required, however, Biorex 70 may generally be an excellent step, since for the enzymes tested the major contaminants were present in the flow-through, and the enzyme eluted at a relatively high NaCl concentration.

DISCUSSION

This procedure is a simplification of a previously published method for EcoRI purification (2). All enzymes listed bind to phosphocellulose at NaCl concentrations high enough to dissociate the enzyme from the nucleic acids in the crude extract. This permits direct loading of the crude extract on the phosphocellulose column; nucleic acids do not bind to the column and are

eliminated in the flow-through and wash. Streptomycin and ammonium sulfate precipitations are eliminated and dialysis steps are reduced. This shortens the purification procedure and has the additional benefit that some enzymes (i.e. Hsu and SalI) are more stable and are obtained in higher yield than when prepared by more lengthy procedures. The approximate yield of the enzymes obtained from 50 g of cells is listed in Table 3.

Digestion of DNA with all of the enzymes produces fragments which yield clear gel patterns. Except in the case of Hga, which has not been tested, the fragments serve as substrates for DNA ligase, and ligation results in regeneration of the restriction sites. By these criteria the enzymes are sufficiently free of non-specific nucleases and phosphatases to be useful as tools in physical mapping and cloning. All of those tested (Table 3) are also pure enough to be used in DNA sequencing.

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