A NEW METHOD FOR ISOLATION OF A RESTRICTION ENZYME FROM HEMOPHILUS PARAINFLUENZAE

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SUMMARY

A rapid procedure which gives high yields of the restriction enzyme Hpa I from Hemophilus parainfluenzae is described. The procedure effectively removes a second restriction enzyme Hpa II as well as exonucleolytic activity. The optimal ionic conditions for the enzyme are similar to those found for one of the enzymes isolated from Hemophilus influenzae. The enzyme is stable at 37° C for several hours but it is rapidly inactivated at 60° C. Patterns are presented which show the electrophoretic separation of the digestion products of two viral DNAs by this enzyme.

INTRODUCTION

The DNA restriction endonucleases which have been isolated from Hemophilus influenzae and Hemophilus parainfluenzae have been used to analyze the genome of SV40 virus and ØX bacteriophage (1, 2, 3). These enzymes, unlike the more complex restriction enzyme from E. coli B, produce unique, double stranded fragments of SV40 virus DNA and should be generally useful reagents for the localization of genetic function on any small DNA molecule. A recent article by Marx (4) has outlined the significance of these endonucleases for studying the function of DNA. The procedure, originally described by Smith and Wilcox (5) has been used by several laboratories to purify the Hemophilus enzymes (3, 6, 7). An alternate procedure, for the rapid isolation of the Hemophilus parainfluenzae restriction enzyme, Hpa I, from moderate size cell cultures, is described in this report, along with some properties of the enzyme.

MATERIALS AND METHODS

Hemophilus parainfluenzae was obtained from a strain provided by Dr. S. Goodgal, and was grown to late log phase in 10 liters of brain-heart infusion broth (purchased from Baltimore Biological Laboratories) supplemented with 2 µg/ml of NAD (Nutritional Biochemical Corp.). The cells were harvested by centrifugation and stored at -70°C.

All buffers were prepared at 24°C and all purification steps were carried out at 0°C. 6.5 g of Hemophilus parainfluenzae were broken by sonication in 40 ml of buffer A (2.0 mM dithiothreitol, 5% glycerol and 30 mM Tris-Cl, pH 8.4; fraction 1). Cell debris was removed by centrifugation at 10 000 x g for 30 minutes and the supernate (fraction 2) volume was increased to 47 ml with buffer A. 0.2 ml of 1 M $MgCl_2$ and 3 ml of 5 M NaCl was added to the supernate which was divided into four 12.5 ml fractions which were centrifuged in a Spinco Ti-60 rotor at 175 000 x g for 180 minutes. Proteins in the supernate (fraction 3, 48 ml) were precipitated by the addition of 16 g of $(NH_4)_2SO_4$. After centrifugation at 10 000 x g for 30 minutes, the precipitate was dissolved in buffer B (14 mM β-mercaptoethanol, 5% glycerol, 0.2 mM EDTA and 20 mM Tris-Cl, pH 8.4). This solution was diluted with buffer B to give a conductivity equal to a solution containing 0.16 M $(NH_4)_2SO_4$, 5% glycerol and 20 mM Tris-Cl, pH 8.4 (fraction 4). Nucleic acids were removed by passage through a DEAE-cellulose column (20 cm x 1.5 cm) (8), at 0.5 ml/minute. The flow through solution (fraction 5), which contained less than 1% nucleic acid as determined by ultraviolet absorbance at 260 nm and 280 nm (9), was dialyzed for 3 hours against buffer B with 50 mM NaCl. The pH of the dialyzed solution was lowered to 7.4 with 0.1 N HCl and fraction 5 was applied to a DEAE-cellulose column (0.9 cm x 7.9 cm) equilibrated with buffer C (50 mM NaCl, 14 mM β-mercaptoethanol, 5% glycerol, 0.2 mM EDTA and 20 mM Tris-Cl, pH 7.4). The column was run at 12 ml/hour and the flow through and wash protein solution (fraction 6) was diluted with an equal volume of buffer D

(14 mM β-mercaptoethanol, 5% glycerol, 0.2 mM EDTA and 20 mM sodium phosphate, pH 6.5) and applied at 10 ml/hour to a phosphocellulose column (0.6 cm x 8.5 cm) which was equilibrated with buffer D at pH 6.6. The enzyme binds to this column and it is removed by elution with buffer B with 0.5 M NaCl (fraction 7).

The enzyme was assayed using the techniques described by Smith and Wilcox (5) and DeFilippes (10). With the former method, DNA viscosity measurements were performed at 30°C in an Ostwald viscometer using E. coli DNA purchased from Calbiochem. 250 µg of DNA in 5 ml of buffer V (80 mM NaCl, 10 mM $MgCl_2$, 14 mM β-mercaptoethanol and 10 mM Tris-Cl, pH 7.8) were placed in the viscometer and a sample of the fraction containing enzyme was added to the solution with gentle mixing.

Specific viscosity measurements are plotted as described by Smith and Wilcox (5) and their definition of a unit of enzyme activity is adopted for this report. All measurements were made on a single lot of E. coli DNA which was prepared and used within one week.

In my hands, the procedure described above yields, in fraction 7, 3 to 5 times more total enzyme activity per gram of cells initially disrupted, than that found with previous procedures. This was true with three different preparations. The reasons may be that the procedure is rapid, especially in the early stages, and that nucleic acids are more completely removed by the DEAE-cellulose method (fraction 5) than they are by agarose gel chromatography (5) or by streptomycin precipitation. The high speed centrifugation step in the 300 mM NaCl, 4 mM $MgCl_2$ buffer, as well as the ammonium sulfate precipitation, are necessary in order to remove nucleic acids with relatively small DEAE-cellulose columns. If the high speed centrifugation is omitted prior to the ammonium sulfate precipitation step, then 5 to 10 times the amount of nucleic acid will pass through the DEAE-cellulose column; and the flow through solution, which contains the enzyme, will form a precipitate with storage at 0°C. If the ammonium

Table I

Enzyme Purification

Fraction No.	Volume (ml)	Total Protein (mg)	Specific Activity (units/mg)
2. Low-speed supernatant	40	341	0.10
3. High-speed supernatant	48	173	0.12
4. Ammonium Sulfate	16.2	125	0.16
5. DEAE-cellulose I	18.4	86.2	0.26
6. DEAE-cellulose II	58.0	16.8	0.92
7. Phosphocellulose	2.5	3.7	8.06

10 to 100 µl of a given fraction were added to the standard reaction solution. The amount added was adjusted so that the decrease in viscosity over a 10 minute period was approximately the same for most samples. Three viscosity determinations were used for each fraction tested. Enzyme yield has not been presented since the presence of a second restriction nuclease and a large amount of polynucleotide in fractions 2 through 4 would make this an unreliable figure.

sulfate step is omitted then a much larger DEAE-cellulose column is required to remove nucleic acids. The second DEAE-cellulose column removes an exonuclease which digests double stranded DNA. This exonuclease frequently contaminates the restriction enzyme preparation when it is purified by the earlier method using phosphocellulose chromatography. This step was suggested by the work of Gunther and Goodgal (11) who used these conditions to purify a similar exonuclease from Hemophilus influenzae. Others (12) have used this method to eliminate exonuclease activity from Hemophilus parainfluenzae restriction enzyme preparations. Both DEAE-cellulose column steps give good recovery of the restriction enzyme. The enzyme

solution is dialyzed to lower ionic strength at pH 8.4, and then diluted to lower pH values, since enzyme activity is lost during dialysis below pH 8.3. The slowest step is the concentration of the enzyme on the phosphocellulose column. Good recovery of the enzyme is achieved using 1.5 to 2.5 ml packed volume of phosphocellulose in a long, thin column. If the column is less then 4 cm long some enzyme will not be adsorbed. This last concentration step is always carried out immediately after fraction 6 is obtained. Table 1 presents a summary of the purification procedure using the viscometric assay to measure enzymatic activity. Fractions 2 through 5 are contaminated with another restriction enzyme, Hpa II (7), so that the assay does not specifically measure the activity of Hpa I in the early steps. The presence of the previously noted exonuclease does not markedly affect the viscosity measurements. A more important consideration, however, is that fractions 3 and 4 contain about one tenth as much nucleic acid as protein. The endogenous nucleic acid, which is introduced with the sample used to measure enzyme activity and which is not digested by the enzyme, gives spuriously high viscosity values. The specific activity of fraction 7 enzyme, in three different preparations, was at least 1.6 times that found with preparations made by the original procedure (10). The enzyme is stable for several months when stored in buffer B, at pH 8.4, with 50% glycerol at -20°C. It is stable for at least two weeks at 0°C. The entire purification procedure takes less than two days time if 10 grams of cells are processed.

Table II gives some properties of the enzyme found in fraction 7. These characteristics show it to be similar to the enzyme described by Smith and Wilcox, which was isolated from Hemophilus influenzae strain Rd. The enzyme is only slightly inhibited by sRNA. This is useful if one wishes to digest fragments produced by this enzyme with another restriction enzyme or an exonuclease from E. coli, since it is known that sRNA inhibits the endonuclease I activity which is found in E. coli (13). Also, single

Table 2

Effect of Ionic Concentration on Endonuclease Activity[a]

	$MgCl_2$	$MnCl_2$	$CaCl_2$	NaCl			Relative Activity
A	10			30			315
	10			40			375
	10			55			522
	10			80			1000
	10			130			267
	10			230			76
	10			330			13
B	0[b]			80			0
	1.2			80			466
	2.5			80			643
	5			80			730
	15			80			964
	20			80			931
		10		80			71
			10	80			6
	10	2		80			166
	10		2	80			551
C	10			80	Minus β-mercapto-ethanol		500
D	10			80		Plus sRNA 60µg/ml	909
	10			80		Plus ssDNA 50 µg/ml	936
E					pH		
	10			80	7.3		645
	10			80	7.5		714
	10			80	7.6		741
	10			80	7.7		769
	10			80	7.8		1000
	10			80	7.9		1000
	10			80	8.1		714

Relative activity is based on DNA viscosity measurements as described in Methods. a) Numbers give millimolar concentrations, b) Solution contains 0.4 mM Na_3 EDTA. A, B, C, and D were measured in 10 mM Tris-Cl buffer pH 7.8. E has 10 mM Tris-Cl buffer at the indicated pH. A, B, D, and E have 14 mM β-mercaptoethanol. D has 300 micrograms of soluble RNA (sRNA) from E. coli (purchased from Calbiochem) in the 5 ml solution in one case and; 250 µg of sonicated E. coli test DNA which has been alkali denatured to single stranded DNA (ssDNA) in the other case. Both solutions were tested with the usual DNA described in Methods.

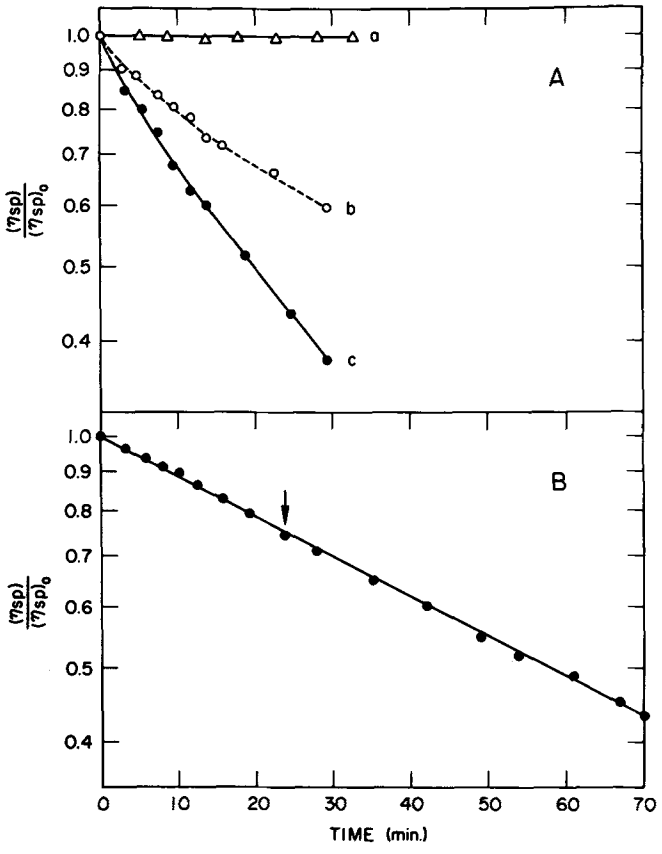

Figure 1. Heat stability of the enzyme.

A. 10 µl of enzyme from fraction 7 was diluted into 100 µl of buffer V and heated at a) 60°C, b) 55°C, and c) 48°C or 37°C for 10 minutes and then quickly cooled at 0°C. This sample was then added to 4.9 ml of standard test solution which in this case had a pH of 7.45.

B. 20 µl of enzyme from fraction 7 was mixed with 250 µg of test DNA in 4.8 ml of buffer V and incubated at 37°C for 3 hours. 250 µg of test DNA, in 0.25 ml of buffer V, was then added to this solution and the viscosity was measured. The arrow indicates at what point the flow time of this solution reached a value equal to the zero time value of the original solution.

stranded DNA, which has been sonicated to less than 300 000 daltons, seems to have little effect on enzyme activity, when used at the same concentration (50 µg/ml) as the test DNA. Using a more sensitive assay, which depends on the restriction enzyme breakage of supercoiled SV 40 DNA (10), it was found that a 20 fold excess of single stranded DNA from bacteriophage ØX174 could reduce the amount of SV 40 DNA I which was cleaved to less than one half that found with controls which did not contain single stranded DNA. These experiments were performed using 0.06 µg of SV 40 DNA I in 0.1 ml of buffer V. Non-saturating amounts of enzyme were used to digest the DNA at 37°C for 10 minutes. It does appear then that the enzyme can react with single stranded DNA, although it does not degrade it.

The enzyme found in fraction 7 is stable at 37°C. There is no change in the activity of the enzyme, using the standard viscometric assay, if it is incubated in buffer V at 37°C for 3 hours prior to the addition of the test DNA. The enzyme also remains active at 37°C in the presence of DNA. First the enzyme is incubated at 37°C for 3 hours with the standard test DNA in buffer V. This DNA is almost completely digested by the enzyme in this time, leaving large fragments of DNA in solution. When an equal amount of the E. coli DNA is added to this test solution the enzyme proceeds to digest the new DNA. Figure 1 B indicates a slower rate of digestion when compared with the rate found for the standard test. This is caused, for the most part, by the contribution of the previously digested DNA to the overall solution viscosity. When the enzyme is heated for 10 minutes in buffer V, at increasing temperatures, it retains some activity until a temperature of 60°C is reached. Figure 1A, shows typical relative viscosity curves for enzyme samples which had been heated at the indicated temperature.

Figure 2 shows limit digests of SV 40 DNA I and λ DNA (a gift from Dr. R. Wu) by the fraction 7 enzyme. The pattern shown for the SV 40 DNA digest is the same as that found for digests obtained with enzyme prepared by previous procedures (10). Generally 5 to 10 µg of DNA were

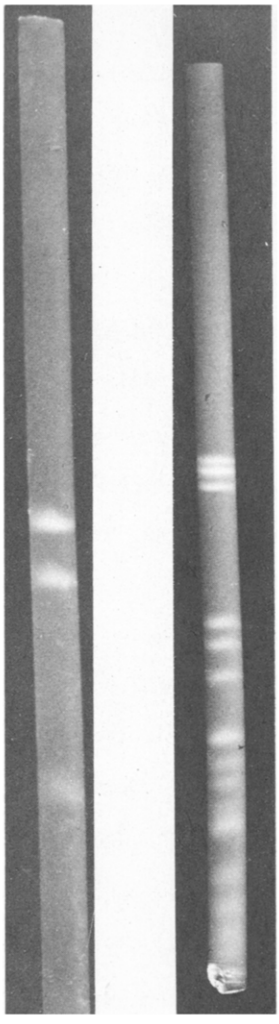

Figure 2

Agarose gel electrophoresis patterns of limit digests of viral DNA by fraction 7 restriction enzyme. DNA fragments were electrophoresed at 22 volts for 12 hours at 24°C on a 10 cm gel of 1.6% agarose. Electrophoresis buffer contained 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA and 0.2% sodium dodecyl sulfate, at pH 7.9. The gels were soaked in a 5 μg/ml ethidium bromide solution for 60 minutes and the ethidium stained DNA bands were photographed by their fluorescence when exposed to ultraviolet light. This technique was developed by Dr. H. Boyer (personal communication). The left gel pattern shows SV 40 DNA fragments and the right λ DNA fragments.

digested by an equal weight of fraction 7 enzyme for 2 hours at 37°C. The reaction mixture was then dialyzed against 1 liter of buffer which contained 12 mM Tris, 10 mM NaH_2PO_4, 2 mM EDTA, 0.2% sodium dodecyl sulfate and 20% glycerol, pH 7.9, for 3 hours at 24°C. Usually 1 to 5 µg of DNA was applied to each gel. When radioactive DNA labeled with [^{3}H] thymidine at a specific activity of 95 000 counts/min/µg was digested with fraction 7 enzyme for 5 hours at 37°C less than 0.2% of the counts were acid soluble at the end of the incubation.

The isolation procedure which has been presented allows the investigator to prepare enough enzyme to digest several hundred micrograms of DNA. The average yield of bacterial cells, grown to late log phase, is over 5 grams per liter of medium, so no extraordinary equipment such as a fermentor, is necessary for cell production. The fraction 7 enzyme, although it is not as pure as other preparations (14), contains no other nucleases attacking duplex DNA. Also, it is convenient to have a rapid preparative procedure where the final product is tested as quickly as possible. The ultimate test of a restriction enzyme preparation, as a reagent, is its ability to produce large, unique, duplex DNA fragments. This means that one must eventually look for unique fragments by gel electrophoresis, which is a time consuming process. The viscosity assay, which is the most useful during the early stages of purification, will not readily reveal the presence of contaminating nucleases which do not cause diplotomic breaks in DNA. Finally, the stability of the fraction 7 enzyme, when stored as described, adds to the usefulness of the present method.

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