Preparation and properties of insolubilized restriction endonucleases

Yan-Hwa Lee, Robert W. Blakesley, Leonard A. Smith and Jack G. Chirikjian

Department of Biochemistry, Georgetown University Medical Center, Georgetown University, Washington, DC, USA

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ABSTRACT

Type II restriction endonucleases Bam HI and Eco RI were covalently coupled to Sepharose. These insolubilized enzymes generated fragment patterns for several viral DNAs identical to those produced by the respective free enzymes. Conditions for optimal activity were similar for both bound and unbound forms of the enzymes. Insolubilization improved thermal stability of Bam HI and Eco RI. The bound enzyme can be recovered from reaction mixtures and reused several times. Upon storage at 4°C, coupled endonucleases remained stable for several months.

INTRODUCTION

The type II restriction endonucleases have become valuable reagents for research in molecular biology. This is primarily due to their unique property of cleaving DNAs at a limited number of specific sites (1-3). These enzymes were employed for physical DNA mapping, for elucidation of cDNA, and for plasmid construction and gene cloning (3-5). Presently, over 100 such restriction endonucleases isolated from a variety of bacterial sources are known. Most of these enzymes recognize and cleave DNA at specific sequences, which are usually in the form of a palindrome. In several instances, enzymes from different sources share a common recognition site. More detailed information on these enzymes can be found in a recent comprehensive review (6).

Examination of the purification procedures for several type II restriction endonucleases indicates that these enzymes are potentially membrane bound (7). In as much as our effort is directed toward the understanding of the biochemistry of these enzymes, we wish to investigate the effect of insolubilization on the activities of these enzymes.
This paper describes the coupling and characterization of two restriction endonucleases, Bam HI and Eco RI, covalently bound to Sepharose. The insolubilized forms of these enzymes appear to retain their enzymatic specificity, and to be stable to storage at 4°C for at least six months. Among the several practical advantages of the new form of these endonucleases are the rapid removal of protein from the reaction mixture, and the potential for preparation of chromatographic columns with enzymatic activity.

MATERIALS AND METHODS

Enzymes: Eco RI and Bam HI, restriction endonucleases from Escherichia coli RY13 and Bacillus amyloliquefaciens H, respectively, were obtained from Bethesda Research Laboratories, Inc., or prepared in our laboratory using modified published procedures (8,9). When prepared in our laboratory, cells (10 g) were suspended in 50 mM phosphate (K+) pH 7.5, 0.5 mM EDTA and 5 mM 2-mercaptoethanol, then broken by sonication. The supernatant from a 2 hour, 100,000 x g centrifugation of the sonicate was fractionated on a phosphocellulose column equilibrated with 20 mM phosphate (K+) pH 7.5, 0.1 mM EDTA, 5 mM 2-mercaptoethanol and 10% glycerol (column buffer). The adsorbed endonuclease activity was eluted upon application of a gradient of 0 to 1 M KCl in column buffer. Peak fractions containing restriction activity were pooled and freed of contaminating nuclease by fractionation on an hydroxylapatite column, using 0 to 1 M KCl gradient elution in column buffer. Either the active fraction from this step or the commercial preparation was used for coupling experiments of Eco RI and Bam HI to Sepharose.

CNBr-activated Sepharose: In order to activate the Sepharose 4B (Pharmacia) 100 g was suspended in 100 ml of distilled water. The pH was monitored continuously throughout the reaction. To the suspended Sepharose was added 100 mg of finely divided solid cyanogen bromide (CNBr). The pH was adjusted to 11 by addition of 8 N NaOH with vigorous stirring. After 10 min at 20°C, 200 ml of cold 1 M phosphate (K+) buffer (pH 7.2) was added. The suspension was washed by filtration on a scinttered glass funnel with 20-30 volumes of cold buffer. Activated Sepharose was stored at 4°C in an equal volume of buffer and routinely used.
Enzyme Insolubilization: The enzymes were insolubilized by combining approximately 2000 units of Eco RI or Bam HI in 0.5 ml of buffer containing 10 mM phosphate (K+) pH 7.2, 0.5 mM EDTA, and 50% glycerol with 0.5 ml of packed CNBr-activated Sepharose. The resulting slurry was adjusted to 0.15 M phosphate (K+) (pH 7.5), then gently mixed "end over end" at 4°C for 16 hrs. Residual reactive groups in the Sepharose were blocked by suspension of the gel slurry in 0.1 M Tris-HCl (pH 7.5) for 2 hrs at 4°C. The excess uncoupled endonuclease was removed from the gel by centrifugation, resuspension of the gel in 0.5 M NaCl, 25 mM Tris-HCl (pH 7.5) 10% glycerol, and 1 mM dithiothreitol, followed by recentrifugation. This washing procedure was repeated four more times. Finally, the enzyme coupled gel was equilibrated with 50 mM Tris-HCl (pH 7.5), 20% glycerol, and 1 mM dithiothreitol by washing five times in this buffer. The final gel slurry was stored at 4°C.

Removal of any noncovalently-bound endonuclease was indicated by the following (Fig. 1): 1) no restriction endonuclease activity was detected in any of the supernatants derived from the gel washing procedure described; and 2) a parallel experiment was performed in which Sepharose replaced the CNBr-activated Sepharose in the coupling reaction. In this case, when the product was subjected to the washing procedures described above, restriction endonuclease activity was found in the wash, but no activity was associated with the recovered Sepharose.

The efficiency of coupling Bam HI and Eco RI to Sepharose as measured by activity was approximately 50-90%. We define the efficiency simply as the ratio of the total units of enzyme recovered in the matrix bound form to the total units of activity input into the insolubilization reaction. Thus, this measurement is based upon the activity bound to Sepharose, but without correcting for any possible rate differences between bound and free enzyme.

Enzyme Assay: Assay for Eco RI endonuclease activity was carried out in a reaction mixture (40 μl) containing 0.4 to 2 μg λ, adenovirus or SV40 DNA, 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 2 mM 2-mercaptoethanol, 50 mM NaCl, and 100 μg/ml auto-
Fig. 1. Examination of coupling of Bam HI and Eco RI to Sepharose. Phage lambda DNA (1 μg) was incubated in either Bam HI (lanes 1 and 2) or Eco RI (lanes 3-6) reaction mixture with an appropriate enzyme fraction, then the products displayed on a 1.4% agarose slab gel as described in the text. Additions to the reactions were: Lane 1, none; lane 2, 1 unit (30 μl) of insolubilized Bam HI; lane 3, 5 μl of high salt wash of insolubilized Eco RI; lane 4, 30 μl (packed volume) from coupling reaction of Eco RI (30 μl) and unactivated Sepharose; lane 5, 1 unit (30 μl) insolubilized Eco RI; and lane 6, none.

claved gelatin. Both reaction mixtures were incubated for 1 hr at 37°C. Reactions containing matrix-bound enzyme were then centrifuged and the supernatants removed. To these supernatants or to reaction mixtures containing unbound enzyme was added one-fourth volume of a solution containing 50% glycerol and 0.02% bromophenol blue.

Adenovirus and λ DNA fragments were separated on a 1.4% agarose slab gel (10 cm x 12 cm) at 100 volts for 3 hrs following procedures previously described (10,11). SV40 DNA digests were fractionated on a 1.2% agarose slab gel at 200 volts for 2 hrs using Tris-borate EDTA buffer (90 mM Tris-borate, pH 8.3, 2.5 mM Na₂EDTA) (12). The gels were stained with ethidium bromide (1 μg/ml) for 10 min and photographed during illumination with a shortwave ultraviolet light.

A unit of enzyme activity is defined as that amount of enzyme
required to completely digest 1 μg of λ DNA at 37°C in 60 min. Routinely, enzymatic activity is measured by serial dilution of the enzyme into the linear range to obtain the minimum amount of enzyme necessary to obtain complete digest. Specific activity is defined as units per gram of enzyme-coupled Sepharose. The specific activity obtained for insolubilized Bam HI and Eco RI was 3,000 and 11,000 units/g, respectively.

RESULTS
In order to demonstrate that coupling of restriction endonucleases to Sepharose did not alter their catalytic properties three standard DNAs were used as substrates. The fragmentation pattern of λ, adenovirus or SV40 DNA produced by incubation with the insolubilized enzymes was identical to that produced with free enzymes (Figure 2). Each panel of Figure 2 is a gel electrophoretogram of three reactions: viral DNA alone; viral DNA incubated with soluble enzyme; and viral DNA incubated with insoluble enzyme. Phage λ DNA digestion with Bam HI (panel A) or Eco RI (panel B) produced the expected pattern of six fragments. Adenovirus DNA was converted to four fragments by Bam HI (panel C) and six fragments by Eco RI (panel D). Both enzymes converted SV40 DNA from superhelical component I to linear component III (panels E and F). In all cases, when insoluble replaced soluble enzyme in the reaction, no difference in the patterns of DNA fragments could be detected.

These results indicate that both linear and superhelical DNAs could be effectively digested by insolubilized enzymes. In addition, when appropriate incubation conditions were imposed on the Eco RI-coupled Sepharose, the Eco RI* pattern (13) also was obtained. Thus, coupling via the cyanogen bromide linkage apparently does not interfere with critical elements of the active site of these enzymes. This is particularly important since stereochemical hinderances upon coupling of enzymes are well documented in other systems (14,15).

A study of the effect of heat on the activities of the soluble and insoluble enzymes demonstrated that coupling enhanced the thermal stability by at least 10°C for each enzyme (Table 1). Exposure of stock solutions of the insolubilized Bam HI to 65°C for 5 min resulted in little loss of activity, while the soluble
Fig. 2. Comparison of viral DNA digests of soluble and insoluble Bam HI or Eco RI. Panels, A, C and E are reactions with Bam HI and panels B, D and F are reactions with Eco RI, each assayed as described in the text. The substrates were phage lambda DNA (panels A and B), adenovirus type II DNA (panels C and D) and SV40 component I DNA (panels E and F). In all panels, lane 1 was one unit of soluble enzyme, lane 2 was one unit of insolubilized enzyme, and lane 3 was no enzyme added to the reaction mixture.
form treated for 5 min at 55°C lost nearly all activity. In fact, treatment of soluble Bam HI at 60°C for only 2 min caused complete inactivation, whereas the activity of bound Bam HI remained stable to incubation at 60°C for 2 min. Free Eco RI, when incubated at 40°C, lost all activity, whereas Eco RI bound to Sepharose lost only 20% of its activity when treated at 50°C. Although one cannot generate from this data reaction temperature optima for either enzyme, the results clearly demonstrate an improved thermal stability upon insolubilization.

Matrix-bound enzyme, lyophilized to dryness from solutions containing 1% (w/v) dextran T70, retained greater than 90% of its activity. Such lyophilized enzyme samples could be stored at room temperature without substantial loss of activity for at least one week.

In order to further assess kinetic activity of Bam HI and Eco RI upon insolubilization, a time course assay for each enzyme was performed. Aliquots of each reaction were removed at various times, then analyzed by agarose gel electrophoresis. As controls, soluble Bam HI and Eco RI were treated in an identical manner. As shown in Figure 3, results for the matrix bound form of each enzyme

| Enzyme          | Heat Treatment | % Activity *
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<tr>
<td>Bam HI (soluble)</td>
<td>none</td>
<td>100</td>
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<td></td>
<td>45°C, 5 min.</td>
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<td>55°C, 5 min.</td>
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<td>60°C, 2 min.</td>
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<td>Bam HI (insoluble)</td>
<td>none</td>
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<td></td>
<td>60°C, 2 min</td>
<td>90-100</td>
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<tr>
<td></td>
<td>65°C, 5 min</td>
<td>80-90</td>
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<tr>
<td>Eco RI (soluble)</td>
<td>none</td>
<td>100</td>
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<td>40°C, 5 min</td>
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<tr>
<td>Eco RI (insoluble)</td>
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<td>40°C, 5 min</td>
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<td>45°C, 5 min</td>
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<td>50°C, 5 min</td>
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*Activity based on the minimum amount of enzyme from a series of dilutions necessary to completely digest 1 ug of lambda DNA with appearance of expected fragment pattern.
Fig. 3. Comparison of time course assays for soluble and insoluble forms of Bam HI and Eco RI. Panels A and B are reactions with matrix bound and free Bam HI, respectively. Panels C and D are reactions with matrix bound and free Eco RI, respectively. Each assay mixture containing 1 μg of lambda DNA was treated as described in the text. In each panel the time of incubation was as follows: Lane 5, 10 min; lane 4, 20 min; lane 3, 30 min; lane 2, 45 min; lane 1, 60 min.

was similar to that of its respective free form. Thus, no apparent change in the time course of DNA degradation was observed upon insolubilization of the enzyme.

DISCUSSION

Restriction endonucleases are interesting enzymes due to their properties of site-specific recognition and digestion of DNAs. Their action is apparently dictated by both the primary and
secondary structures of the substrate DNA (16). Although several such enzymes were shown to cleave specific sequences within synthetic oligonucleotides, it is unclear what additional effect the tertiary structure of large, naturally-occurring DNAs contributes to the recognition and catalytic processes. Inspite of this uncertainty, the unique properties of restriction endonucleases make them potential models for studying protein-nucleic acid interactions.

One of our major aims for the past several years was to elucidate details of the recognition and subsequent catalysis of DNA by type II restriction endonucleases. In as much as there is evidence implying that these endonucleases are membrane bound, one approach to studying them is to determine what effect insolubilization (i.e., mimicking membrane binding) has on their activity. Thus, in each set of experiments reported herein a comparison is made between the results obtained from enzymes bound to Sepharose and those from the respective soluble forms of the enzyme treated in a similar manner.

Although several options for assessing enzyme activity were considered, some of which were potentially more precise, we chose to base our unit of activity on the ability of an enzyme to digest λ DNA to completion. This decision was dictated by: a) the difficulty of obtaining synthetic oligonucleotide substrates; b) the possibility that there are kinetic differences between λ DNA and an oligonucleotide substrate due to tertiary structural requirements by the enzyme; and, c) the definition found in the current literature where such enzymes are used as tools for molecular biology.

Using this definition and obtaining unit quantitation by serial dilution of the enzyme as described, the efficiency of coupling was determined to be better than 90% for Bam HI and Eco RI. The coupling efficiency is the ratio of the total units of enzyme recovered bound to Sepharose to the total units of initial activity. The lack of detectable activity in the supernatant after insolubilization (Fig. 1) and the presence of excess activated Sepharose in the binding reaction supports the idea that the majority of the enzyme molecules were bound to the matrix. One cannot totally exclude from our results small changes in enzymatic activity upon binding. However, since at least 90% of the
activity can be accounted for, it is unlikely that such changes occurred.

Although we have reported coupling of two restriction endonucleases, the method appears to be widely applicable. Following the procedures described herein, two other type II restriction endonucleases, Hpa I and Taq I, have been insolubilized. Since these enzymes were not characterized to the same extent as Bam HI and Eco RI, no data was presented here. We are unable at this time to pinpoint the amino acid residue(s) participating in the covalent bonding of these enzymes; however, it is not unreasonable to assume that the linkage is through amino residues. Coupling the enzymes through linkers was considered, but two such derivatives, 6-amino-hexanoic acid and 1,6-diaminohexane, showed no distinct advantage over the direct cyanogen bromide coupling described herein.

Matrix binding of restriction endonucleases does not change the catalytic activity observable by analyzing digestion patterns of several DNAs. Since both linear and superhelical DNAs could be effectively digested (Fig. 2), it is reasonable to assume that no major stereochemical hindrances were induced upon coupling. Interestingly, coupling did provide an increased thermal stability to the enzymatic activity. Ongoing experiments indicate that matrix bound Bam HI and Eco RI are both stable for at least six months and can be reused at least five times without any loss of activity. Such increased stability also makes this form of these enzymes attractive for study of DNA structures at high temperatures.

Matrix bound restriction endonucleases offer several practical advantages over their soluble counterparts. The insoluble enzymes can be either packed in the form of a column or rapidly pelleted from solutions by centrifugation. These procedures allow quick and complete removal of restriction endonucleases from reaction mixtures. Such recovered enzymes are reusable several times over, permitting cleavage of large amounts of DNA with relatively few units of enzyme. Quick and complete removal of enzymes also avoids the inhibition frequently observed upon subsequent digestion by a second endonuclease. In addition, no phenol extraction is required, resulting in increased recovery of digestion products and eliminating tedious and time-consuming manipulations. These procedural advantages together with
enhanced stability clearly make the insoluble form of these enzymes valuable reagents.

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REFERENCES
