0273–2289/86/1301–0029$02.00

Effect of Glutaraldehyde on the Activity of Some DNA Restriction Endonucleases

JOHN OLSZEWSKI AND BRUCE P. WASSERMAN*

Department of Food Science, New Jersey Agricultural Experiment Station, Cook College, Rutgers University, New Brunswick, NJ 08903

Received January 2, 1986; Accepted February 18, 1986

ABSTRACT

The effect of the bifunctional crosslinking reagent glutaraldehyde on the activity of the restriction enzymes Bam HI, *Hin*d III, *Eco*RI, and Tthlll I was investigated. The four enzymes exhibited differential sensitivity to inactivation. Tthlll I was the most sensitive, with activity losses occurring at levels of 0.0025% and above. *Hin*d III was the most stable of the four and remained fully active at concentrations as high as 0.075%. Addition of BSA to incubation mixtures generally had a stabilizing effect. Implications of these results for the design of glutaraldehyde-based methods for the immobilization of restriction endonucleases are discussed.

Index Entries: Glutaraldehyde, effect of on DNA restriction endonucleases; restriction endonucleases; glutaraldehyde-based methods, for immobilization of restriction endonucleases; restriction enzymes.

INTRODUCTION

Restriction endonucleases have become widely utilized for structural analysis of DNA and the construction of genomic maps, libraries, and cloning vectors (*1,2*). Current methodologies for endonuclease utilization are time consuming and involve several steps (*3*). Restriction enzyme utilization could be facilitated by development of immobilized endo-

*Author to whom all correspondence and reprint requests should be addressed.

nuclease catalysts. These would permit removal of the enzyme by centrifugation and possible reuse.

Several methods for the immobilization of restriction enzymes (*4–6*) and a ligase (*7*) on organic matrices have been reported; however, little work has been done with inorganic supports. One of the most commonly used methods for coupling enzymes to inorganic surfaces involves activation of the surface with primary amino groups followed by covalent binding of the enzyme with the bifunctional crosslinking reagent glutaraldehyde (*8–11*). To apply this method to restriction endonucleases, it is necessary to ascertain the effects of glutaraldehyde on restriction endonuclease activity. The objective of this research was to evaluate the stability of four restriction endonucleases exposed to glutaraldehyde. Upper-limit glutaraldehyde tolerance limits for the enzymes BamHI, *Hin*d III, *Eco*RI, and Tthlll I are defined.

MATERIALS AND METHODS

Materials

The restriction enzymes *Eco*RI, BamHI, Tthlll I, *Hin*d III, and the molecular weight marker lambda DNA digested with *Hin*d III were purchased from either International Biotechnologies Inc., New Haven, CT or Bethesda Research Laboratories. Enzymes from either source behaved identically on exposure to glutaraldehyde. The molecular weights of the lambda fragments are 23.1, 9.4, 6.5, 4.3, 2.3, and 2.0 kbase. The plasmid pBR322 was purchased from Bethesda Research Laboratories, Gaithersburg, MD. This preparation was found to consist of 90% supercoiled concatenated dimer and 10% multimers. In some experiments, pBR322 in the form of 95% supercoiled monomer, donated by Mr. Mike Yablonsky of the Dept. of Microbiology and Biochemistry, Rutgers University, NJ, was used. Glutaraldehyde (50% w/w aqueous solution) was a product of Kodak Laboratory Chemicals, Rochester, NY. Crystalline bovine serum albumin (BSA) was obtained from Pentex Inc., Kankakee, IL. All buffers and solutions were prepared with sterile distilled–deionized water. Photographs were taken with Polaroid positive/negative 4 × 5 film type 55.

Effect of Buffers on Restriction Enzyme Activity

The restriction enzymes Bam-HI (10 U), *Eco*RI (15 U), *Hin*d III (6 U), and Tthlll I (10 U), in a vol of 10 μL, were incubated individually for 15 min on ice with 20 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.8, 20 m*M* *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.8, or 20 m*M* sodium phosphate buffer, pH 8.0. Aliquots containing 2 U of activity were used for restriction digests.

Glutaraldehyde Incubations

Incubations were conducted in 10-μL reaction mixtures containing 2 m*M* MOPS buffer, pH 7.8, glutaraldehyde at levels ranging between 0 and 0.5% (w/v), and the enzymes at levels of 1 U/μL. In some experiments, BSA was added to a final concentration of 0.1% (w/v). In all incubations, the restriction enzymes were added last. The mixtures were incubated on ice for 15 min and reactions stopped by the addition of 1 μL of 100 m*M* Tris(hydroxymethyl)aminomethane hydrochloride (Tris) buffer, pH 8.0. Four-microliter aliquots were taken for restriction digests.

Enzyme Assay

Restriction digests for *Eco*RI and BamHI contained 0.5 μg of pBR322, 4 U of enzyme, 100 m*M* NaCl, 10 m*M* $MgCl_2$, 100 μg/mL BSA, 1 m*M* dithiothreitol, and 25 m*M* Tris, pH 7.8, in a volume of 16 μL. The mix for Tthlll I and *Hin*d III was similar except for the NaCl, which was added to a level of 50 m*M*. The *Eco*RI, BamHI, and *Hin*d III digests were incubated at 37°C for 1 h. Tthlll I was incubated at 65°C.

The digests were brought to 20 μL with the addition of 4 μL of 20% (w/v) ficoll and 0.25% bromophenol blue in 40 m*M* Tris-acetate, pH 7.8. Electrophoresis was conducted in 0.8% agarose gels at 40–50 V, as described (*12*).

After electrophoresis, the gels were stained with ethidium bromide at a concentration of 0.5 μg/mL for 20–30 min and destained in distilled–deionized water for 10–15 min. Gels were then photographed with Type 55 Polaroid film under short wave UV light with an F stop setting of 4.5 and exposure time of 4–6 min.

RESULTS AND DISCUSSION

Most protocols for the handling and utilization of DNA restriction endonucleases call for the use of Tris-based buffers. Since Tris contains a primary amino group that can react strongly with glutaraldehyde, it was important to show that restriction enzymes retain activity after exposure to other buffers. The four enzymes used in this study all retained complete activity after 15 min in 20 m*M* HEPES, MOPS, and phosphate (data not shown).

Exposure to glutaraldehyde affected each of the four restriction enzymes in a similar manner; i.e., above a critical level partial cutting was observed and at higher concentrations activity was completely lost. Maximal exposure levels are summarized in Table 1. Figures 1 and 2 show the effect of glutaraldehyde on *Eco*RI in the absence and presence of BSA and are representative of the gels obtained in the concentration-dependence studies.

TABLE 1
Maximal Glutaraldehyde Exposure Levels of Restriction Endonucleases[a]

Enzyme	Glutaraldehyde alone[b]	Glutaraldehyde and 0.1% BSA[b]
Hind III	0.075	0.20
BamHI	0.07	0.07
*Eco*RI	0.01	0.03
Tth111 I	0.0025	0.0075

[a]In % w/v.
[b]Values represent the highest concentration of glutaraldehyde in which complete cutting of pBR322 is obtained.

In the absence of BSA, bands representing partially cut and nicked pBR322 began to appear at 0.02% and above (Fig. 1). At 0.01% and below, complete cutting was obtained (gel not shown). The presence of 0.1% BSA (Fig. 2) exerted a protective effect, with complete cutting obtained at levels up to 0.03% glutaraldehyde.

The thermophilic restriction enzyme Tthlll I was the most sensitive of the activities tested (Table 1), with full activity expression at 0.0025 and

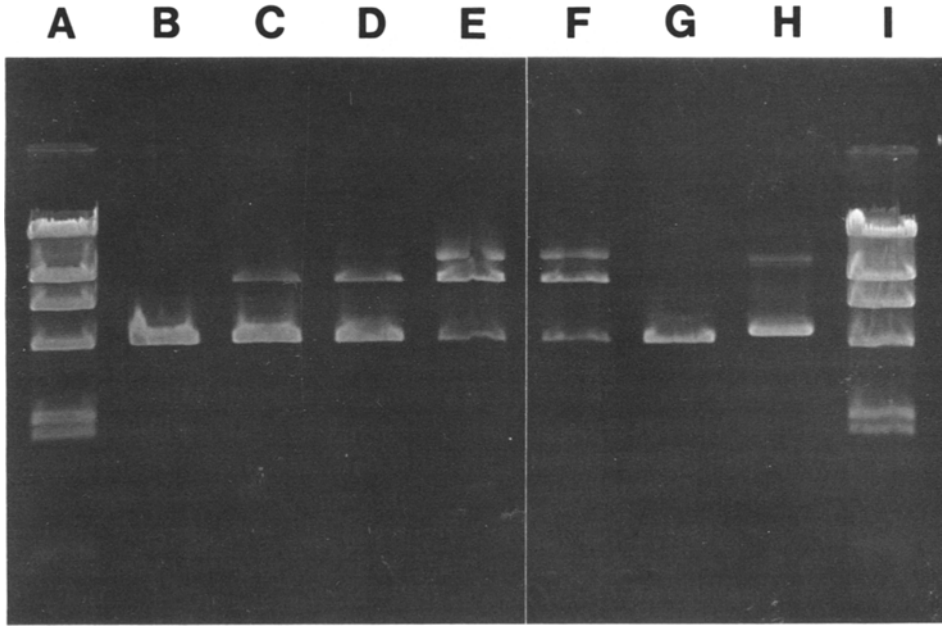

Fig. 1. Effect of glutaraldehyde on the activity of *Eco*RI. Lanes A and I, *Hind* III lambda digest molecular weight markers; B and G, control-*Eco*RI digests incubated in the absence of glutaraldehyde; C–F, *Eco*RI treated with glutaraldehyde at levels of: C. 0.02%, D. 0.03%, E. 0.04%, and F. 0.06%; H, undigested pBR322 in the form of supercoiled concatenated dimer.

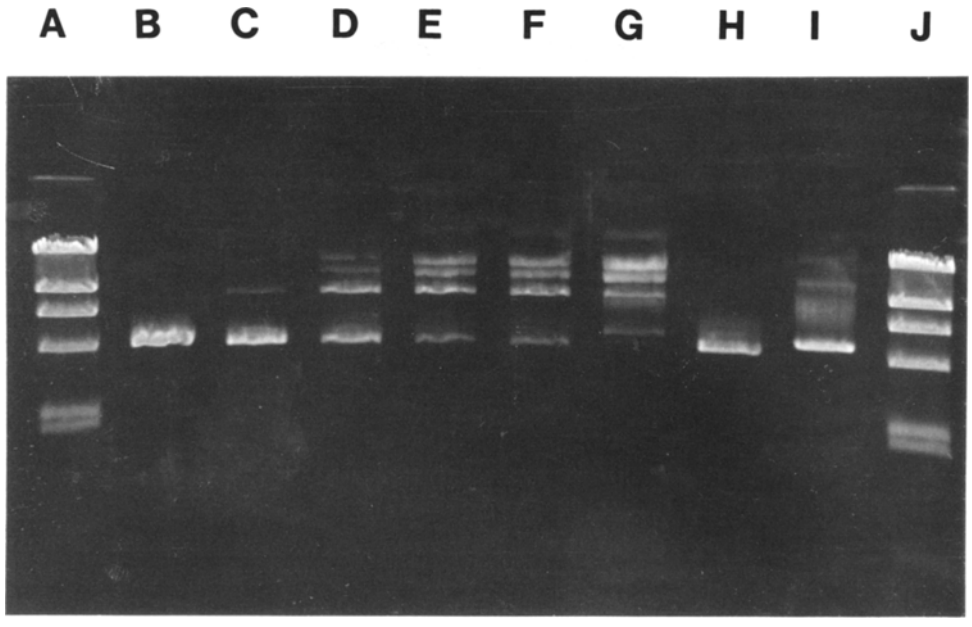

Fig. 2. Effect of glutaraldehyde on the activity of *Eco*RI in the presence of 0.1% BSA. Lanes A and J, *Hin*d III lambda digest molecular weight markers; B and H, control-*Eco*RI digests incubated in the absence of glutaraldehyde; C–G, *Eco*RI treated with glutaraldehyde at levels of: C. 0.03%, D. 0.04%, E. 0.05%, F. 0.06%, and G. 0.07%; I, undigested pBR322 in the form of supercoiled concatenated dimer.

0.0075% glutaraldehyde in the absence and presence of BSA, respectively.

The use of glutaraldehyde as a crosslinking agent for coupling enzymes to inorganic matrices has become quite widespread and has applicability toward a broad group of enzymes. Since glutaraldehyde and its polymerization products react primarily with lysine (*13–15*), enzymes with lysine residues at their active site may be susceptible to activity losses upon exposure to this reagent. Indeed, some enzymes, such as glucoamylase (*16,17*) and bovine catalase (*18,19*), are gradually inactivated upon exposure to glutaraldehyde, whereas others, such as glucose oxidase (*10,20*), are completely resistant. These enzymes are available in relatively large quantities. Thus, small losses in activity during immobilization may be acceptable. Highly purified DNA restriction enzymes, on the other hand, are handled and used in submicrogram quantities. Maximal activity expression is therefore essential. To design practical immobilization procedures, especially of the type in which adsorbed enzyme is crosslinked *in situ*, it is necessary to define maximum exposure limits to chemical modification reagents, such as glutaraldehyde.

This study examined the glutaraldehyde stability of *Eco*RI, BamHI, *Hin*d III, and Tthlll I and found that restriction enzymes inactivate at

glutaraldehyde levels one to two orders of magnitude lower than the concentrations of 0.5–2.0% commonly cited in the immobilization literature. Levels of 0.075–0.01% may appear infinitesimal, but on a molar basis (calculated using the molecular weight of the glutaraldehyde monomer) they correspond to a concentration range of 1.0–7.5 m*M*. These concentrations should be sufficient to obtain crosslinking, particularly if the restriction enzymes have been preadsorbed onto the immobilization matrix by noncovalent interactions. Haynes and Walsh (*8*) effectively used a glutaraldehyde concentration of 0.06% to crosslink proteases on colloidal silica beads.

Also demonstrated in this study was that BSA exerted a protective effect with three of the endonucleases.

Although this study did not probe which specific amino acid residues reacted with glutaraldehyde, one may speculate that these enzymes contain several subclasses of glutaraldehyde-reactive groups. When a folded molecule such as an enzyme encounters glutaraldehyde, outside amino groups are likely to react first. It is possible that at the low glutaraldehyde concentrations, the exterior noncatalytic residues are reacting to glutaraldehyde and that the active-site lysine residues are shielded. Reaction with external residues may deplete much of the free glutaraldehyde when present at low concentrations. Obtaining kinetic data to confirm this theory is currently difficult for this class of enzymes and would be greatly simplified by the availability of a rapid quantitative assay for analyzing restriction enzyme activity. A study focusing on the application of these results toward the development of a method for immobilizing restriction endonuclease on inorganic support surfaces is currently in progress.

ACKNOWEDGMENTS

This research was supported in part by Grant BRC 1 RO3 RR01830-01A1 from the National Institutes of Health, a Rutgers Biomedical Research Support Grant, and the New Jersey Agricultural Experiment Station with State and Hatch Act Funds. New Jersey Agricultural Experiment Station Publication No. D-10530-1-85.

REFERENCES

1. Szalay, A. A., Mackey, C. V., and Langridge, W. H. R. (1979), *Enz. Microb. Tech.* **1,** 154.
2. Smith, H. O. (1979), *Science* **205,** 455.
3. Fuchs, R., and Blakesley, R. (1983), *Methods Enzymol.* **100,** 3.
4. Cashion, P., Javed, A., Harrison, D., Seeley, J., Lentini, V., and Sathe, G. (1982), *Biotechnol. Bioeng.* **24,** 403.
5. Singh, P. (1984), *Applied Biochem. and Biotech.* **9,** 161.

6. Lee, Y. H., Blakesley, R. W., Smith, L. A., and Chirikjian, J. G. (1978), Nucleic Acids Res. **5,** 679.
7. Bulow, L., and Mosbach, K. (1982), Biochem. Biophys. Res. Comm. **107,** 458.
8. Haynes, R. and Walsh, K. A. (1969), *Biochem. Biophys. Res. Comm.* **36,** 235.
9. Robinson, P. J., Dunnill, P., and Lilly, M. D. (1971), *Biochim. Biophys. Acta* **242,** 659.
10. Wasserman, B. P., Hultin, H. O., and Jacobson, B. S. (1980), *Biotechnol. Bioeng.* **22,** 271.
11. Oguntimein, G. B., and Reilly, P. J. (1980), *Biotech. Bioeng.* **22,** 1127.
12. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982), *Molecular Cloning* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
13. Quiocho, F. A., and Richards, F. M. (1966), *Biochemistry* **5,** 4062.
14. Habeeb, A. F. S. A., and Hiromoto, R. (1968), *Arch. Biochem. Biophys.* **126,** 16.
15. Monsan, P., Puzo, G., and Mazurguil, H. (1975), *Biochimie* **57,** 1281.
16. Stanley, W. L., Watters, G. G., Kelly, S. H., and Olson, A. C. (1978), *Biotechnol. Bioeng.* **20,** 135.
17. Wasserman, B. P., Burke, D., and Jacobson, B. S. (1982), *Enzyme Microbial Technol.* **4,** 107.
18. Herzog, V., and Fahimi, H. D. (1977), *J. Cell Biol.* **60,** 303.
19. Wasserman, B. P., and Hultin, H. O. (1981), *Arch. Biochem. Biophys.* **212,** 385.
20. Karube, I., Hirano, K. I., and Suzuki, S. (1977), *Biotech. Bioeng.* **19,** 1233.