APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 9, 161–172 (1984)

Immobilization of the Restriction Endonuclease PstI

PRATAP SINGH†

Southern Biotech, Inc., 128 S. Moon Avenue, Brandon, FL 33511

Received August 15, 1983; Accepted November 24, 1983

ABSTRACT

PstI has been immobilized in agarose. A solution of low melting agarose containing 1,6-hexamethylenediamine and PstI formed a gel that was effective in the linearization of pBR322 DNA. The gel containing PstI could be treated with 1,5-bis(*N*-acetylamino-*N*-succinimidoxy carbonyl)pentane, a crosslinking agent, without affecting the enzyme activity. Polymerization of acrylamide in presence of PstI led to conisderably reduced enzyme activity, although EcoRI under identical conditions showed high activity.

It was found that acetylation of amino groups in PstI, by reaction with hydroxysuccinimide acetate, led to total inactivation of the enzyme activity. This reaction showed the presence of reactive amino groups that were essential for the enzyme activity of PstI. Involvement of these amino groups in binding to activated Sepharose 4B, during covalent immobilization, was responsible for inactive enzyme preparations.

Index Entries: Restriction endonuclease immobilization; endonuclease PstI, immobilization of; PstI, immobilization of the endonuclease; immobilization, of restriction endonuclease PstI; agarose, enzyme immobilization; structure–function relationship, of endonuclease; DNA cleavage, by immobilized enzyme; polyacrylamide, enzyme immobilization on; endonuclease, presence of active amino groups on; immobilized enzyme, treatment with crosslinking agent.

†Present address: Clinical Chemistry Division, American Dade, PO Box 520276, Miami, Florida 33152.

0273–2289/84/0400–0161$02.40

INTRODUCTION

The unique ability of type II restriction endonucleases to recognize and cleave DNAs at a limited number of specific sites (*1*) has found very broad applications in molecular biology. Most of these enzymes have been utilized in partially purified preparations and, except for EcoRI (*2, 3*), very little information is available about their physical or catalytic properties.

Besides their applications in analytical and commercial processes, the immobilization of enzymes has been utilized for structure–function studies (*4,5*) and also in mimicking the behavior of these enzymes when membrane-bound in their natural environment. It has been found recently that subunits of EcoRI participate (*3*) in the binding and cleavage of substrate DNAs and also that the rate of cleaveage of DNA by PstI (*6*) and other endonucleases (*7*) is dependent on the specific nucleotide sequence present near the site of cleavage. These phenomena can be better understood by immobilization of the enzymes (*8*), in addition to the operational advantages offered by such an immobilization. Although a few endonucleases have been immobilized by reaction with CNBr-activated Sepharose (*9*) or adsorption on Tritylagarose (*10*), there is no information available on the immobilization of PstI. This report describes the results of efforts to immobilize PstI on different solid supports.

Covalent immobilization of PstI, involving the reaction of its amino groups with the activated Sepharose, led to inactive enzyme preparations. Presence of reactive amino groups at or near the active site of enzyme was found to be responsible for such inactive preparations. Immobilization of PstI in low melting agarose gave rise to a gel that showed the same pattern of cleavage for pBR322 DNA as that expected from reaction of the enzyme in homogenous solution.

MATERIALS AND METHODS

All organic chemicals were obtained from Aldrich Chemical Company. Agarose (type I), low melting agarose (type VII; low gelling temperature with "first melt" at < 30°C), bovine serum albumin (BSA) and bromophenol blue were obtained from Sigma. Acrylamide, *N,N'*-methylenebisacrylamide (BIS), *N,N,N',N'*-tetramethylenediamine (TEMED) and ammonium persulfate were obtained from Biorad. Glycerol was obtained from BRL. TLC was carried out on silica gel plates with fluorescent indicator (Brinkman Instruments).

Enzymes

EcoRI and PstI were obtained from BRL and Biotech. One unit of enzyme activity hydrolyzed 1 μg of DNA solution after 2 h at 25°C.

Buffers

50 m*M* Tris (pH 7.5), 5 m*M* $MgCl_2$, 2 m*M* mercaptoethanol, 50 m*M* NaCl, 100 μg/mL BSA (Buffer A); Buffer A with 10% glycerol (Buffer A-glycerol); 20 m*M* Tris (pH 7.5), 10 m*M* $MgCl_2$, 1 m*M* DTT, 50 m*M* NaCl, 100 μg/mL BSA (Buffer B); Buffer B containing 10% glycerol (Buffer B-glycerol).

DNA

pBR322 DNA isolated from *E. coli* strain HB101 was kindly provided by Dr. V. B. Reddy. An aqueous solution containing 100 μg/mL was used for digestion studies. Gel electrophoresis of this solution gave at least four bands representing different forms of DNA, namely form I, form II, and polymers derived from these two forms. Complete digestion of this DNA with PstI or EcoRI gave rise to a single band in gel electrophoresis representing the linear form III. This linear form moved faster than either of the four bands present prior to digestion with the endonuclease.

Gel Electrophoresis

Analysis of DNA was carried out by horizontal gel, 20 × 20 × 0.3 cm, containing 0.7% agarose (type I). Gel preparation and electrophoresis was performed in the standard (E) buffer containing 0.04*M* Tris-acetate, 0.02*M* sodium acetate and 2 m*M* EDTA (pH 7.9). DNA solutions, for analysis, were combined with 10 μL of 0.02% bromophenol blue in 50% glycerol and 40 μL of this solution was placed in the gel slot. After electrophoresis for 4 h, the gel was stained with ethidium bromide and photographed as described earlier (*11*).

Synthesis of 1,5,-Bis(N-acetylamino-N-succinimidoxycarbonyl) pentane (BASP)

A mixture containing 1 g of 1,5-diaminopimelic acid, dioxan (5 mL), and acetic anhydride (5 mL) was stirred for 16 h at 25°C. This solution, obtained after stirring for an additional 2 h with 3 mL of 1.0*N* NaOH, was adjusted to pH 4.0. It was evaporated to dryness under reduced pressure, the semisolid residue so obtained gave a negative TNBS test for free amino groups. The semisolid was dissolved in 20 mL dioxan containing 1.15 g *N*-hydroxysuccinimide (NHS, 0.5*M*) and 2.06 g *N,N'*-dicyclohexylcarbodiimide (DCC, 0.5*M*) and mixture stirred at 25°C for 16 h. The clear dioxan solution obtained on filtration was evaporated to dryness and residue crystallized three times from ethyl acetate–hexane and twice from isopropanol to give 1.0 g of white needles (yield 41%). TLC showed R_f of 0.5 and 0.9 for BASP using ethyl acetate–hexane and ethyl acetate–acetic acid (9 : 1), respectively, whereas NHS showed R_f values of 0.1 and 0.5, respectively, in the same solvent systems. Purity of the

sample was confirmed from the fact that two moles of NHS were generated from each mole of BASP (in dioxan solution) when added to 0.1*M* phosphate–0.1*M* ethylenediamine, pH 7.4. The amount of NHS liberated was estimated from the absorbance of the conjugate base of NHS at 260 nm (ϵ 10,000 $m^{-1}cm^{-1}$). BASP obtained was found to be at least 98% pure from these measurements.

Synthesis of N-Hydroxysuccinimide Acetate (NHA)

NHA prepared from NHS and acetic anhydride (*12*) was crystallized three times from ethyl acetate–hexane and twice from isopropanol. Purity of the sample was checked as in the case of BASP; for each mole of NHA 1 mol of NHS was liberated as measured by absorption at 260 nm.

Reaction of PstI with NHA

A solution of PstI (10 U/μL) was diluted with equal volumes of 0.1*M* phosphate, pH 7.4, and 10 m*M* phosphate, 20 μ*M* EDTA, 0.15% Triton X-100, 50% glycerol (pH 7.4). Total reaction mixture of 20 μL contained 5 μL (16.5 U) of diluted PstI, 2–10 μL of a solution of 75 m*M* NHA in 50% glycerol (so as to give final concentrations of 7.5, 18.75, and 37.5 m*M*) and the remaining volumes compensated with 50% glycerol. Reaction mixture was incubated for 2 h at 25°C. Assay of enzyme activity was carried out by further incubation of this reaction mixture, for 1 h at 25°C, with 5 μL of DNA solution and 20 m*M* Tris, 10 m*M* $MgCl_2$, 50 m*M* ammonium sulfate, 100 μg/mL BSA (pH 7.4) to a total volume of 50 μL. A solution of 0.02% bromophenol blue in 50% glycerol (10 μL) was added to the assay mixture and this solution subjected to gel electrophoresis.

In control experiments, the reaction mixture of 20 μL contained 5 μL of diluted PstI solution, 5 μL of 100 m*M* NHS in 50% glycerol (to give a final concentration of 25 m*M*), and 10 μL of 50% glycerol.

Immobilization of EcoRI in Polyacrylamide

A solution containing EcoRI (20 U in 20 μL), 50% acrylamide (24 μL), 2.5% BIS (120 or 240 μL), 1% ammonium persulfate (13 μL), 10 m*M* Tris, 50 m*M* NaCl, 0.2 m*M* EDTA, 0.15% Triton X-100, 50% glycerol, pH 7. 5 (110 or 0 μL) was flushed with nitrogen for 2 min by bubbling a slow stream of gas through the solution. This solution was cooled in an ice bath, 1% TEMED (13 μL) added and mixture was allowed to warm to 25°C. After 30 min, the gel so formed was passed through a 23 gage needle and the gel particles were washed six times with 1 mL of Buffer A by shaking the gel suspension for 5 min each time on a vortex mixer and removing the supernatant solution after centrifugation. For reaction with DNA, one-fourth portion of the gel suspension in 70 μL of Buffer A containing 7 μL of DNA was shaken at 37°C for 3 h and the supernatant solution analyzed by gel electrophoresis.

Immobilization of EcoRI in Agarose

A solution of low melting agarose (1%) was prepared by heating a suspension of agarose (10 mg) in 1 mL of Buffer A-glycerol. This solution (0.2 mL) containing 47 m*M* 1,6-hexamethylenediamine (HMD) was incubated at 37°C for 15 min. A solution of EcoRI (10 U/μl) was added and the mixture placed in an ice bath immediately. After 15 min, the gel formed was passed through a 23 gage needle and then washed six times with 1 mL of Buffer A–glycerol as described above. For reaction with DNA, the gel particles were mixed with 70 μL of the washing buffer containing 7 μL of DNA and then shaken gently on a vortex mixer for 2 h at 25°C. The supernatant solution was analyzed by gel electrophoresis.

Immobilization of PstI in Agarose

A mixture containing 1% agarose solution (0.2 mL in Buffer B-glycerol), HMD (25–120 mM) and PstI (5 U/10 μL) was cooled in an ice bath and gel particles were washed six times with 1 mL of Buffer B-glycerol. The gel was reacted with DNA by shaking gently on a vortex mixer, at 25°C for 2 h, with 100 μL of the washing buffer containing 10 μL of DNA and the supernatant solution analyzed by gel electrophoresis.

For reaction with BASP, the gel particles were suspended in 0.2 mL of Buffer B-glycerol and 5–10 μL of a dioxan solution (2.5–5.0 m*M*) or 10 μL of 50% glycerol solution (2.0 m*M*) of BASP was added and the suspension shaken on a vortex mixer for 5 min. The gel was washed and reacted with 20 μL of the DNA solution as described above.

Treatment with Glutaraldehyde

A solution (5 μL) of 10% glutaraldehyde was added to the gel suspension in 0.2 mL of Buffer B and this mixture was shaken for 5 min at 25°C. This gel was washed as described above and then utilized for cleavage of the DNA solution.

RESULTS

Reaction of PstI with Sepharose 4B preactivated with a variety of methods (*13*), all involving reaction of the active groups on solid support with amino groups of the protein, did not show any detectable enzyme activity on Sepharose as judged by ability of the immobilized enzyme for its cleavage of DNA (data not shown). These results indicated the presence of reactive amino groups that appeared to be essential for the enzyme activity of PstI. In order to confirm this finding a homogenous mixture of PstI and NHA was incubated for 2 h at 25°C. A stock solution of NHA in 50% glycerol, rather than in dioxan, was utilized for this reaction as it was found that presence of 5–10% dioxan in the reaction mixture

was detrimental for the enzyme activity of PstI. Figure 1 shows that treatment of PstI solution with either an 18.75 or 37.5 m*M* solution of NHA caused total loss of enzyme activity for the cleavage of DNA, whereas there was only partial loss of the activity at the lower concentration (7.5 m*M*) of NHA. Control experiments, in which PstI was incubated either in the presence of 50% glycerol or a solution of NHS in 50% glycerol (25 m*M*), showed no effect on the enzyme activity. It was found that during the incubation conditions only 28% of the added NHA had decomposed by hydrolysis; this was calculated from an increase in absorption at 260 nm due to formation of NHS (see Methods).

Very little information is available (*14*) on the structure of PstI. Furthermore, the presence of reactive amino groups at or near the active site of PstI prompted me to look at alternative methods of immobilization, especially the ones that did not involve chemical reaction of the enzyme with the activated solid support. Initial screening of various methods (*15*) was carried out by using EcoRI. Most promising results were obtained when a solution of acrylamide containing the enzyme was polymerized and when a solution of low melting agarose containing the enzyme and HMD was cooled to form a gel; these two methods were evaluated further for the immobilization of PstI.

A mixture containing 4% acrylamide, 1 or 2% BIS, and 6.5% of a solution of EcoRI was polymerized to form a gel. This gel, when treated with DNA solution at 37°C for 3 h, showed the same cleavage pattern for DNA as that expected from reaction of the enzyme in solution (Fig. 2). These gels could be used at least twice without loss of enzyme activity. To immobilize PstI, several combinations of acrylamide and BIS were utilized, including the ones found successful for EcoRI. The acrylamide gel containing PstI showed barely detectable enzyme activity, as evi-

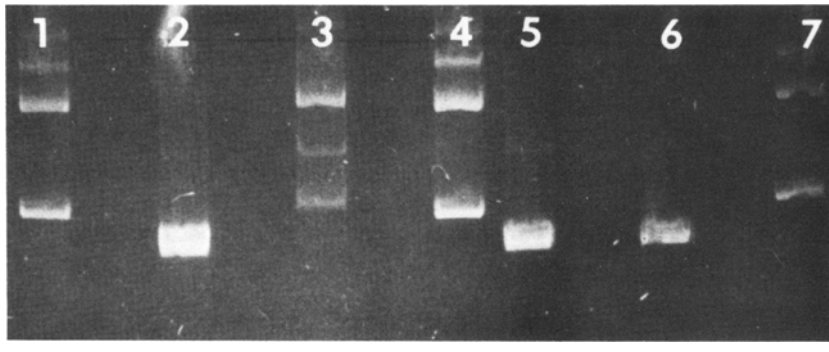

Fig. 1. Effects of different concentrations of NHA on the enzyme activity of PstI. Agarose gel electrophoresis of pBR322 DNA solution was carried out after its reaction with PstI, that had been preincubated for 2 h at 25°C, with a solution of 7.5 m*M* NHA (lane 3), 18.75 m*M* (lane 1), 37.5 m*M* NHA (lane 4), 25 m*M* NHS (lane 6), and 50% glycerol (lanes 2 and 5). Untreated DNA was in lane 7.

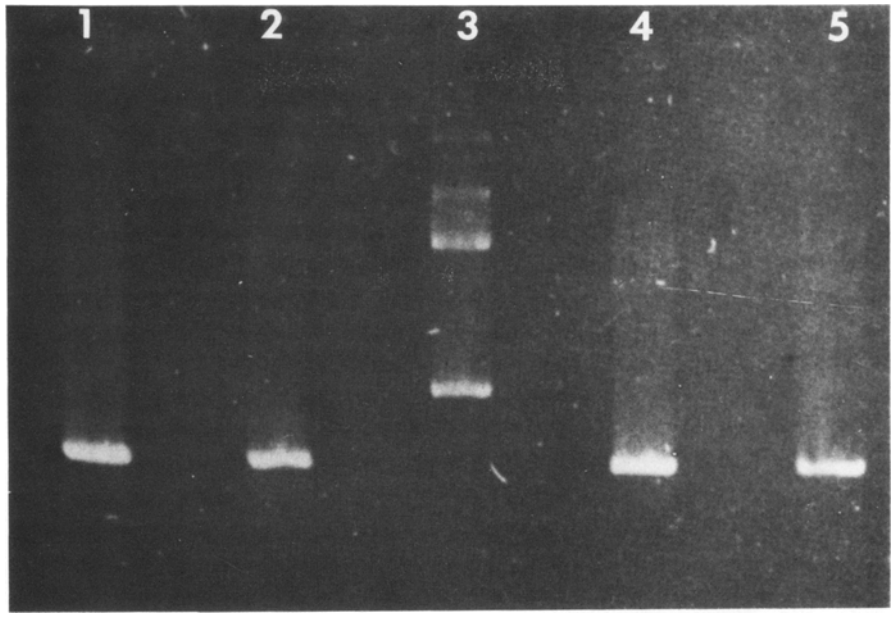

Fig. 2. Cleavage pattern of DNA with EcoRI immobilized in polyacrylamide gel. Immobilization was carried out by polymerizatin of a 4% solution of acrylamide containing 1% BIS (lane 1) and 2% Bis (lane 2); the supernatant solutions were analyzed by gel electrophoresis. These gels were washed and treatment with DNA solutions repeated (lanes 4 and 5, respectively). Lane 3 contained untreated DNA solution.

denced by incomplete DNA cleavage, even after treatment of the DNA solution for 48 h at 37°C.

Low melting agarose solution (1%) containting EcoRI and HMD formed a gel when cooled to 4°C. This gel was washed six times to remove any enzyme loosely bound on the gel surface and showed high activity for the linearization of DNA when reactions were carried out at 25–30°C. About 90% of the enzyme activity used for the preparation of this gel appeared to be maintained in the gel. Presence of HMD was found to be essential for high enzyme activity (see Discussion).

Crosslinking agents such as glutaraldehyde have been utilized to increase gel strength and also to prevent leaching of the immobilized enzyme because of formation of a large network around the enzyme. In experiments where agarose or polyacrylamide gels containing the enzyme was treated with low concentrations of glutaraldehyde, it appeared that most of the DNA from solution was bound to the gel as the supernatant solution, when analyzed by electrophoresis showed highly reduced intensity of the bands for DNA after staining with ethidium bromide.

PstI was also immobilized in 1% agarose solution containing HMD. The gel containing PstI, after washing six times with the reaction buffer, retained about 85% of the enzyme activity toward DNA cleavage (2 h,

25°C) giving rise to a single band on electrophoresis as was obtained from reaction of PstI and DNA in the homogenous solution. Prior to gel formation, varying the concentration of HMD between 25 and 120 m*M* in agarose solution had very little effect on the cleavage of DNA by the immobilized enzyme. The gel containing PstI could be treated with low concentrations of BASP, a newly synthesized crosslinking agent, without affecting enzyme activity towards its cleavage of DNA (Fig. 3). These preparations could be used at least twice, with about 20% loss of activity when used second time. Control experiments, in which the immobilized enzyme was incubated with buffer for 2 h at 25°C, showed undetectable enzyme activity in the supernatant buffer as no effect was evident on DNA when it was incubated with the supernatant buffer.

BASP was synthesized by acetylation of 1,5-diaminopimelic acid and then reacting with NHS in presence of DCC. Purity of the sample was checked on TLC and from the amount of NHS liberated when treated with a nucleophile.

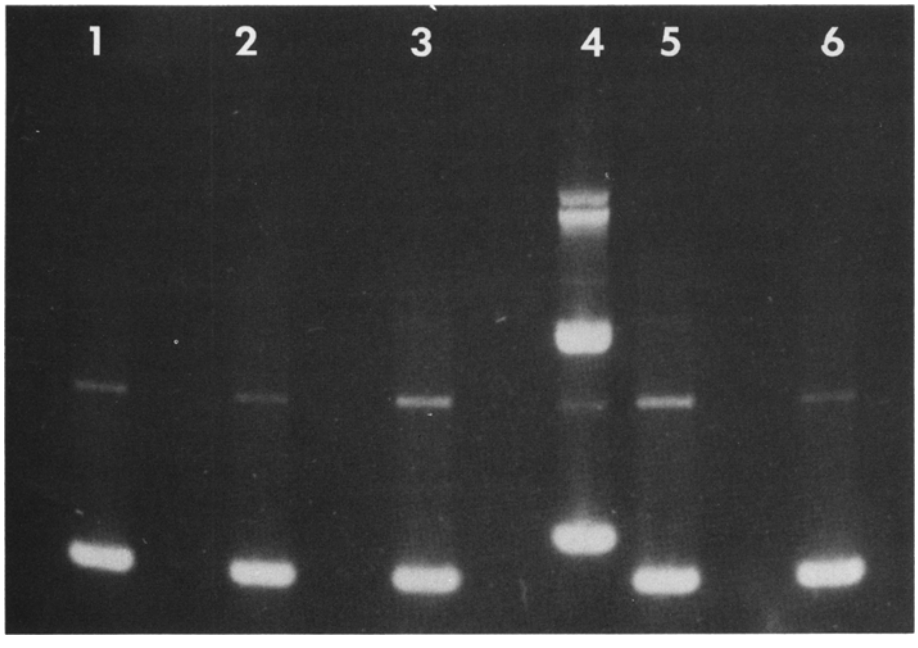

Fig. 3. Effect of treatment with BASP on the enzyme activity of PstI immobilized in low melting agarose, containing 35 m*M* HMD, was studied by the reaction of DNA solution with the gel that had not been treated with BASP (lane 1) and the gels that were treated with 2.5 m*M* solution of BASP in dioxan for 5 min (lane 2), with 5.0 m*M* BASP in dioxan (lane 5), and 2.0 m*M* BASP in 50% glycerol (lane 6). Lane 3 contained DNA solution that had been treated with PstI in solution. Untreated DNA was placed in lane 4.

DISCUSSION

A wide variety of methods is available for the covalent immobilization of a protein on solid supports. Most commonly employed such methods involve reaction of amino groups of the protein with the activated solid support. In such reactions, nucleophilic attack of the free amino groups of lysine residues in the protein plays a very important role (*16*). Covalent immobilization of PstI, involving reaction of its amino groups with active groups on Sepharose (13), led to inactive preparations.

Selective acetylation of amino groups in insulin (*17*) and phenylalanyl-tRNA (*18*) has been carried out by reaction with NHA. Modification of amino groups in these cases had no effect on the biological activity because of the small size of the protective group. On the other hand, acetylation of the amino groups in PstI with NHA caused total loss of the enzyme activity towards its linearization of pBR322 DNA (Fig. 1), and thereby showed the presence of reactive amino groups at or near the active site of this enzyme. Reaction of an activated solid support with the enzyme would involve interaction with the reactive and easily accessible amino groups on the protein. On the other hand, a low molecular weight substrate, such as NHA, would indiscriminately react with amino groups that are readily accessible on the enzyme molecule as well as those groups that are embedded deep into the three-dimensional structure of the protein. Loss of activity in both cases suggested the presence of reactive amino groups that appeared to be essential for the enzyme activity of PstI. Although this reaction with NHA did not preclude the possibility of presence of other highly reactive groups, such as thiol, it has been shown (*14*) that disulfide linkages rather than free thiol groups are involved in the catalytic site of PstI and thus indicated that the presence of free amino groups was essential for the enzyme activity of PstI. Not surprisingly, involvement of amino groups in the immobilization reaction would give rise to inactive preparations as was the case when efforts were made to immobilize PstI on activated Sepharose.

Polymerization of a solution containing acrylamide, BIS and PstI gave rise to a gel that showed poor activity towards its cleavage of DNA. This loss of activity on immobilization could not be caused by failure of the substrate DNA to interact with the enzyme entrapped in polyacrylamide network, since EcoRI when immobilized under identical conditions could cleave DNA in the same way as expected from reaction of the enzyme in solution. Presence of free radicals, inadvertent rise in temperature, or reaction of the enzyme with monomers present during the polymerization reaction may have contributed to the partial inactivation of PstI. Heat lability of this enzyme has been documented (*19*).

Both EcoRI and PstI were immobilized successfully in low melting agarose containing HMD. Prior to gel formation, HMD was added to agarose for protection of the enzyme activity both during immobilization and a subsequent reaction of the immobilized enzyme with BASP. HMD was expected to neutralize anionic groups such as sulfate and also polyanionic enzyme inhibitors, agaropectins, present in commercial agarose and thereby helping in protection of the enzyme activity during immobilization. Presence of such inhibitors in DNA, isolated from agarose gels, has been shown by Bouche (*20*) who demonstrated that preincubation of the isolated DNA with a polyamine, like spermidine, was necessary for uninhibited digestion of DNA with an endonuclease. Reaction of a crosslinking agent, BASP, and the gel containing PstI did not affect enzyme activity of the gel (Fig. 3). Enzyme activity was protected during this reaction since, because of the presence of a relatively large proportion of HMD, its amino groups reacted with BASP in preference to the reaction of amino groups of PstI, which were found to be essential for the enzyme activity.

Crosslinking with bis- or multifunctional reagents has been a standard practice for increasing gel strength and preventing leaching of the enzyme when it is entrapped in large molecular weight matrices. There was no indication that enzyme activity of PstI, immobilized in agarose and treated with BASP, was a result of the leaching of the enzyme since no tendency towards linearization of DNA could be detected in the control experiments (see Results). Retention of total enzyme activity after treatment with BASP showed that the enzyme was not adsorbed on the gel surface as the enzyme should have been inactivated by such a treatment as was shown by reaction of the enzyme with NHA in solution (Fig. 1). Furthermore, the fact that the immobilized enzyme could be treated with BASP in dioxan solution (Fig. 3), whereas treatment of the enzyme solution with dioxan or NHA in dioxan caused total loss of the enzyme activity (see Results), also indicated that the enzyme was entrapped in the matrix and not adsorbed on the surface.

Immobilized heparin has been utilized for the purification of many nucleic acid binding proteins including PstI and EcoRI (*21*). It has been suggested (*22*) that strong interaction of these proteins with heparin, a polysaccharide, results from the recognition of heparin by these enzymes as a nucleic acid analog, rather than being caused by ion exchange. It is reasonable to expect similar strong interaction between agarose, a polysaccharide that was utilized in the present study for immobilizations, and the endonucleases PstI and EcoRI. This strong interaction with a polysaccharide backbone and treatment with the crosslinking agent, BASP, would prevent leaching of the enzyme during its reaction with the substrate DNA under normal experimental conditions utilized for such reactions. Treatment with the crosslinking agent, obviously, did not affect reaction of different forms of DNA in solution (present in the mixture used

for the digestion studies) and the immobilized enzyme. Molecular weight of the substrate DNA had no effect on the cleavage reaction as shown by reaction of both the monomeric (containing 4362 nucleotides) and polymeric forms of pBR322 DNA. In addition, DNA that had been pretreated with EcoRI also underwent cleavage with immobilized PstI generating the same fragment as expected from reaction with PstI in solution (result not shown).

Almost 85% of the enzyme activity was retained on immobilization and a loss of about 20% of this activity was observed when immobilized PstI was used for second time. This loss might have been caused by the slow denaturation of the enzyme during use, although some loss on washings can not be completely ruled out. Control experiments, however, suggest that any activity that may have been washed out in buffer represented only a very small fraction of the total activity present in the immobilized form. In principle, a loss on washings can be avoided if crosslinking was carried out with reagents that react rather slowly with amino groups (in cases where an enzyme like PstI is to be immobilized) such as a bis-maleiimide in the presence of added dithiol.

Immobilized restriction endonuclease offers a distinct operational advantage in simplifying the separation of cleaved DNA from the contaminating enzyme by eliminating the phenol extraction step when cleavage is carried out in solution. It has been recognized recently (*6,7*) that identical recognition sites in DNA, for a specific endonuclease, are cleaved at markedly different rates because of the presence of specific neighboring nucleotide sequences. By immobilization of these endonucleases, it should be possible to carry out only partial cleavage because of the very easy separation of the product from the immobilized enzyme. Additionally, immobilization of endonucleases over different solid supports so as to mimic the effects of a specific nucleotide sequence, some sites may be cleaved in preference to others. Such studies should be helpful in a better understanding of the mechanism of action of the endonuclease and also of the structure of DNA. The approach described here may also be useful in the immobilization of other labile enzymes and thereby leading to better understanding of these enzymes and their substrates.

ACKNOWLEDGMENT

The author is grateful to Dr. Charles Hsu for his interest.

REFERENCES

1. Roberts, R. J. (1983), *Nucl. Acids Res.* **11,** r135.
2. Jack, W. E., Terry, B. J., and Modrich, P. (1982), *Proc. Natl. Acad. Sci. USA* **79,** 4010.

3. Alves, J., Pingoud, A., Langowski, J., Urbanke, C., and Maass, G. (1982), *Eur. J. Biochem.* **124,** 139.
4. Klibanov, A. M. (1983), *Science* **219,** 722.
5. Werner, M., Garrett, C., Chiu, A., and Klempner, L. (1982), *Clin. Chem.* **28,** 2351.
6. Armstrong, K., and Baur, W. R. (1982), *Nucl. Acids Res.* **10,** 993.
7. Malcolm, A. D. B., and Moffatt, J. R. (1981), *Biochim. Biophys. Acta* **655,** 128.
8. Bickerstaff, G. F. (1980), *Int. J. Biochem.* **11,** 201.
9. Lee, Y. H., Blakesley, R., Smith, L. A., and Chirikjian, J. G. (1980), *Meth. Enzymol.* **65,** 173.
10. Cashion, P., Javed, A., Harrison, D., Seeley, J., Lentini, V., and Sathe, G. (1982), *Biotech. Bioeng.* **24,** 403.
11. Sugden, B., DeTroy, B., Roberts, R. J., and Sambrook, J. (1975), *Anal. Biochem.* **68,** 36.
12. Ames, D. E., and Grey, T. F. (1955), *J. Chem. Soc.* 631.
13. Singh, P., Goldman, J., and Jackson, C. E. (1982), *Artificial Organs* **6,** 145.
14. Nath, K. (1981), *Arch. Biochem. Biophys.* **212,** 611.
15. Chibata, I., and Tosa, T. (1981), *Ann. Rev. Biophys. Bioeng.* **10,** 197.
16. Srere, P. A., and Uyeda, K. (1976), *Meth. Enzymol.* **44,** 11.
17. Lindsay, D. G., and Shall, S. (1971), *Biochem. J.* **121,** 737.
18. Lapidot, Y., DeGroot, N., and Fry-Shafrir, I. (1967), *Biochim. Biophys. Acta* **145,** 292.
19. Smith, D. I., Blattner, F. R., and Davies, J. (1976), *Nucl. Acids. Res.* **3,** 343.
20. Bouche, J. P. (1981), *Anal. Biochem.* **115,** 42.
21. Wu, X., Kong, Y., Zhu, S., and Dang, J. (1982), *Chem. Abstrs.* **96,** 195600h.
22. Pirrotta. A., and Bickle, T. A. (1980), *Meth. Enzymol.* **65,** 89.