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Sequence-specific DNA affinity chromatography: application of a group-specific adsorbent for the isolation of restriction endonucleases

C. Pozidis and G. Vlatakis

Institute of Molecular Biology and Biotechnology, Enzyme Technology Division, P.O. Box 1515, Heraklion 711 10, Crete (Greece)

V. Bouriotis

Institute of Molecular Biology and Biotechnology, Enzyme Technology Division, P.O. Box, 1515, and Department of Biology, Division of Applied Biology and Biotechnology, University of Crete, P.O. Box 1470. Heraklion 711 10, Crete (Greece)

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ABSTRACT

The use of sequence-specific DNA affinity adsorbents for the isolation of restriction endonucleases *Eco*RI and *Sph*I to near homogeneity has been reported. However, the high cost of these adsorbents is a limiting factor for their wider application. This paper reports the application of sequence-specific DNA affinity ligands containing recognition sequences for 34 restriction endonucleases as group-specific ligands in the isolation of restriction endonucleases. Crude samples of six restriction endonucleases, namely *Bsh*FI, *Bam*HI, *Sma*I, *Sac*II, *Pvu*II and *Sal*I, were shown to bind to these adsorbents and could be eluted at different KC1 concentrations. High purification factors and recoveries were obtained. Restriction endonuclease *Bsh*FI, an isoschizomer of *Hae*III, from the microorganism ***Bacillus sphaericus*** was purified to near homogeneity employing a two-step procedure which involves DNA-cellulose chromatography and oligonucleotide–ligand affinity chromatography. The enzyme exists as a monomer with an apparent relative molecular mass of 34 000 as determined by both sodium dodecyl sulphate–polyacrylamide gel electrophoresis and size-exclusion chromatography.

INTRODUCTION

Sequence-specific DNA affinity chromatography has been successfully applied to the isolation of proteins that interact with specific DNA sequences [1–6]. The use of this technique for the isolation of restriction endonucleases to homogeneity has also been reported [7,8]. Although several procedures have been published regarding the purification of restriction endonucleases, most of them involve lengthy and laborious protocols [9,10]. However, rapid two-step chromatographic procedures involving triazine dye adsorbents, HPLC and affinity partitioning have been reported, resulting in partially purified restriction endonucleases free from contaminating nuclease activities [11-13]. Although sequence-specific DNA affinity chromatography compares favourably with other adsorbents used for the isolation of restriction endonucleases to homogeneity, the high cost of this technique, resulting from the different synthesis of the ligand for each restriction endonuclease, is a limiting factor for its wider application.

Affinity chromatography using specific ligands requires a different and elaborate synthesis for each purification problem. Further, as the choice of the ideal ligand is still largely empirical, extensive ex-

Correspondence to: V. Bouriotis, Institute of Molecular Biology and Biotechnology, Enzyme Technology Division, P.O. Box 1515, Heraklion 711 10, Crete, Greece.

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perimentation is needed to achieve satisfactory separations [14,15].

Group-specific adsorbents exhibit affinity for whole groups of enzymes and therefore display great versatility in application. The development of oligonucleotide affinity adsorbents containing sites for a number of restriction enzymes which can therefore be used as group-specific adsorbents could potentially solve this problem.

This paper describes the synthesis of two group-specific DNA affinity adsorbents and their potential application in the isolation of a number of restriction endonucleases recognizing sequences within this oligonucleotide ligand. The purification of restriction endonuclease *Bsh*FI, an isoschizomer of *Hae*III, [16] to homogeneity employing a two-step procedure is also presented.

EXPERIMENTAL

Materials

Restriction endonucleases (*Bsh*FI, *Bam*HI, *Pvu*II, *Sma*I, *Sac*II, *Sal*I), λDNA, λDNA–HindIII digest and T_4 DNA ligase were kindly supplied by Minotech (Heraklion, Greece), T_4 polynucleotide kinase was obtained from New England Biolabs (Beverly, MA, USA), DNA cellulose from Sigma (St. Louis, MO, USA), cyanogen bromide-activated Sepharose 4B from Pharmacia (Uppsala, Sweden) and a Sep-Pak C_{18} column from Waters (Milford, MA, USA).

Yeast extract, tryptone and other chemicals were purchased from Merck (Darmstadt, Germany) and agarose for gel electrophoresis from Bethesda Research Labs. (Bethesda, MD, USA).

Growth of cells

Bacillus sphaericus (*Bsh*FI) was grown at 30°C until late logarithmic phase on a medium containing yeast extract 5 g/l, tryptone 10 μg/l and NaCl 5 g/l with a yield of 4 g of cell paste per litre of growth medium. Cells were harvested at 4°C by centrifugation and stored frozen at −70°c.

Enzyme assays

Routine assays for locating *Bsh*FI, *Bam*HI, *Pvu*II, *Sma*I, Sac11 and *Sal*I endonuclease activity during the chromatographic runs were performed as described previously [12]. One unit of enzyme activity is defined as the amount of enzyme required to produce a complete digest of 1 .O μg of λDNA at 37°C (25°C for *Sma*I) for l h in a total volume of 50μl.

Preparation of oligodeoxynucleotide affinity adsorbents

Two complementary oligodeoxynucleotides of the sequence 5′-GATCGCATGCCGCGGATCC-CGGGCCCAGGTGGCCAGCTGTCGAC-3’ and 3′-CGTACGGCGCCTAGGGCCCGGGTC-CACCGGTCGACAGCTGCTAG-5’ were synthesized (1-μmol scale; Applied Biosystems) and kindly provided by the Microchemistry Department of IMBB, already deprotected in ammonia solution.

The crude oligodeoxynucleotides were evaporated to remove ammonia, dissolved in water, centrifuged to remove benzamides formed during protection, evaporated and resuspended in 50 m*M* triethylammonium acetate (TEA-Ac) (pH 7.0). An initial purification step was then performed with a size-exclusion G-25 Sephadex gel column, followed by a desalting step with a Sep-Pak C_{18} column [7]. Annealing of strands was performed in 100 μl of buffer containing 50 m*M* NaCl, 1 m*M* EDTA and 10 m*M* Tris–HCl (pH 8.0) by heating at 90°C for 2 min, then at 65°C for 90 min and 55 °C for 30 min and cooling slowly to room temperature.

Preparation of oligodeoxynucleotide affinity adsorbent I

After annealing had been performed, 1.8 mg of the DNA was phosphorylated in a reaction mixture containing 1800 U/ml T4 polynucleotide kinase, 5 m*M* ATP (containing 5 μCi of [γ-^{32}P]ATP), 70 m*M* Tris–HCl (pH 7.6), 10 m*M* $MgCl_2$ and 5 m*M* dithiothreitol (DTT) for 2 h at 37°C. The DNA was precipitated with ethanol and dried *in vacuo*. The oligonucleotide was dissolved in ligation buffer containing 66 m*M* Tris–HCl (pH 7.5), 10 m*M* $MgCl_2$, 15 m*M* DTT, 1 m*M* spermidine–HCl and 1 m*M* ATP and was polymerized with T4 DNA ligase (50 Weiss units) for 18 h at 15°C. DNA was extracted with phenol-chloroform, precipitated with ethanol and resuspended in water, to be used for coupling to cyanogen bromide-activated Sepharose gel (3.5 g) according to the method of Kadonaga and Tjian [4]. By measuring the radioactivity that remained in the washings after the coupling, the concentration

of bound DNA was calculated to be cu. 60 μg DNA/ml gel (10 ml total gel).

Preparation of oligodeoxynucleotide affinity adsorbent II

A 1.8mg amount of the annealed DNA was phosphorylated as above and then coupled to 3.5 g of cyanogen bromide-activated Sepharose gel [4]. After coupling the concentration of bound DNA was ca. 70 μg DNA/ml gel (10 ml total gel).

Protein assay und electrophoresis techniques

Protein determination was performed according to Bradford using bovine serum albumin as protein standard [17]. Polyacrylamide gel electrophoresis (PAGE) of proteins in the presence of sodium dodecyl sulphate (SDS) was performed according to the method of Laemmli [18]. Agarose gel electrophoresis of DNA fragments was performed as described previously [12].

Comparison of properties of oligonucleotide affinity adsorbents I and II

The same sample of a partially purified preparation of BshFI (20 ml; 4 mg; 900 000 U/mg; 3 600 000 U) was applied to the same size of column of both affinity adsorbents 1 and II (2 × 1.6 cm I.D.; 4 ml). The columns were washed with 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) (pH 7.8), containing 7 m*M* β-mercaptoethanol, 1 m*M* EDTA and 10% (v/v) glycerol until no absorption at 280 nm was evident in the effluents, and then developed with a 0.3 *M* step gradient of KCl.

Fractions with BshFI activity eluted with the 0.3 A4 KC1 step gradient from adsorbent I (0.32 mg; 6 400 000 U/mg; 2 050 000 U) and adsorbent II (0.168 mg; 11 309 520 U/mg; 1 900 000 U) were pooled and stored at 4°C.

Enzyme purification procedures

For purification of BshFI, frozen cell paste (8 g) was thawed with 80 ml of 20 m*M* potassium phosphate buffer (pH 7.0) containing 7 m*M* β-mercaptoethanol, 1 m*M* EDTA and 5% (v/v) glycerol (buffer A), sonicated in an ice-bath for a total of 7 min (14 × 30 s) and centrifuged at 4°C for 1 h at 100.000 g. The supernatant (80 ml; 2 mg/ml; 160 mg) was loaded onto a dsDNA cellulose column (4 × 4.4 cm I.D.; 60 ml) previously equilibrated in buffer A. The column was washed with buffer until no adsorption at 280 nm was evident in the effluents and then developed with a linear gradient of NaCl (600 ml total volume, O-O.8 *M*) in buffer A at a flow-rate of 40 ml/h. Fractions (5 ml) with BshFI activity corresponding to ca. 0.15 *M* NaCl in the gradient were pooled (80 ml), concentrated with PEG (M_r 30 000) treatment and dialysed against 25 m*M* HEPES (pH 7.8) containing 7 m*M* β-mercaptoethanol, 1 m*M* EDTA, 10% (v/v) glycerol (buffer B) and 0.1 A4 KCl.

A sample (20 ml; 1 mg; 1 920 000 U) from the latter preparation was applied to an adsorbent bearing immobilized ligand II (adsorbent II, 5 × 1.6 cm I.D.; 10 ml) which had previously been equilibrated in buffer B containing 0.1 *M* KCl. The column was washed with buffer B containing 0.1 *M* KC1 (56 ml) and subsequently with buffer B containing 0.2 *M* KC1 (78 ml) and then developed with a linear gradient of KC1 (114 ml; 0.2-0.4 *M*) in buffer B at a flow-rate of 9 ml/h. Fractions (2.8 ml) with BshFI activity corresponding to cu. 0.25 A4 KC1 in the gradient were pooled (36.4 ml; 1.35 μg; 40 U/μl; 1 456 000 units) and stored at 4°C.

Purification of restriction endonucleases BshFI, *Bam*HI, *Sma*I, *Sac*II, PvuII *and Sal*I

Partially purified preparations of restriction endonucleases BshFI (1.26 mg; $3.96 \cdot 10^6$ U), *Bam*HI (0.2 mg; 20 000 U), *Sma*I (0.46 mg; 40 000 U), *Sac*II (3.22 mg; 42 500 U), PvuII (0.5 mg; 196 000 U) and Safl(0.35 mg; 80 000 U) were separately applied to Affinity adsorbent II (5 × 1.6 cm I.D.; 10 ml) which had previously been equilibrated in buffer B. The column was washed with buffer B until no absorption at 280 nm was evident in the effluents and then three successive step gradients consisting of 0.1, 0.2 and 0.3 *M* KC1 in buffer B were applied at a flow-rate of 9 ml/h. BshFI (22.5 μg; $3.4 \cdot 10^6$ U) and *Bam*HI (0.5 μg; 15 000 U) were eluted with 0.3 *M* KCl, *Sma*I (9.2 μg; 35 000 U) and *Sac*II (25 μg; 40 000 U) with 0.2 *M* KC1 and PvuII (4 μg; 168 000 U) and *Sal*I (35 μg; 64 000 U) with 0.1 A4 KCl. Fractions with BshFI, *Bam*HI, *Sma*I, *Sac*II, PvuII and *Sal*I activities were pooled and stored at 4°C.

RESULTS AND DISCUSSION

Several purification procedures have been described for the isolation of restriction endonucleases free from contaminating nuclease activities. Among these, recent methods based on dye-ligand chromatography [I 1], HPLC [12] and affinity partitioning [13], although effective, required the use of two or three columns, resulting in partially purified restriction enzyme preparations.

Sequence-specific DNA affinity chromatography has been successfully applied for the isolation of several proteins to homogeneity [1–6]. This technique has also been employed in order to prepare restriction endonucleases of high purity [7,8]. A limiting factor in the application of this technology is the high cost involved, resulting from the expensive synthesis of the oligonucleotide ligands. The construction of two oligonucleotide affinity ligands containing recognition sequences for 34 restriction endonucleases is reported in this paper. The restriction enzymes, their recognition sequences and recognition sites (number of first base) within the oligonucleotide affinity ligand are illustrated in Table I. One of the two affinity ligands was constructed after annealing of two oligonucleotides and subsequent polymerization and the other after annealing of two oligonucleotides without any further polymerization (Fig. 1). Both affinity ligands were subsequently coupled to cyanogen bromide-activated Sepharose. The oligonucleotides were designed in such a way that the resulting DNA affinity ligands contained S-single-strand overlapping termini in order that primary amines of the free bases could be coupled to activated agarose.

The resulting affinity adsorbents having roughly the same ligand concentration were compared in terms of capacity and specificity using partially purified preparations of the restriction endonuclease *Bsh*FI. Although both adsorbents exhibited similar capacities (ca. 500 000 U/ml gel) the adsorbent that resulted from the unligated ligand (adsorbent II) exhibited higher specificity (42 μg of protein/ml of gel were bound) than that resulting from polymerization of the oligonucleotide (80 μg of protein/ml of gel were bound, adsorbent I). We therefore employed adsorbent II for all subsequent experiments.

In order to hydrolyse DNA, restriction enzymes require the presence of divalent metal ions, usually Mg^{2+}. For this reason, the addition of EDTA in all buffers for chelating the metal ion is necessary. The same method is used for DNA–cellulose or DNA–agarose in order to avoid hydrolysis from non-specific nucleases [19].

In order to examine the potential application of affinity adsorbent II as a group-specific adsorbent for the isolation of restriction endonucleases, we used partially purified enzyme preparations of

TABLE I

RESTRICTION ENZYME RECOGNITION SITES CONTAINED IN SEQUENCE-SPECIFIC DNA AFFINITY LIGANDS I AND II

Enzyme	Recognition sequence[a]	Recognition site (number of first base)
ACCI	GTMKAC	39
*Alu*I	AGCT	35
*Alw*I	GGATC	14
*Apa*L	GGGCCC	21
*Ava*L	CYCGRG	18
Ball	TGGCCA	30
*Bam*HI	GGATCC	14
*Ban*II	GRGCYC	21
*Bgl*I	GCCNNNNNGGC	23
*Bsp*12861	GDGCHC	21
*Bst*NI	CCWGG	25
*Bst*UI	CGCG	II
*Bst*YI	RGATCY	14
*Eae*I	YGGCCR	30
*Fnu*4HI	GCNGC	9
*Hae*III	GGCC	22, 31
*Hinc*II	GTYRAC	39
*Hpa*II	CCGG	19
*Mbo*I	GATC	15
*Nci*I	CCSGG	18, 19
*Nla*III	CATG	6
*Nla*IV	GGNNCC	14.21
*Nsp*I	RCATGY	5
*Nsp*BII	CMGCKG	10, 34
*Pvu*II	CAGCTG	34
*Sac*II	CCGCGG	10
*Sal*I	GTCGAC	39
*Scr*FI	CCNGG	18. 19, 25
*Sec*I	CCNNGG	10, 18, 24
*Sfi*I	GGCCNNNNNGGCC	22
*Sma*I	CCCGGG	18
*Sph*I	GCATGC	
*Taq*I	TCGA	40
*Xma*I	CCCGGG	18

[a] D = GorAorT;H = AorCorT;K = GorT;M = A or C; N = AorCorGorT:R = A or G; S = GorC;W = A or T; Y = T or C.

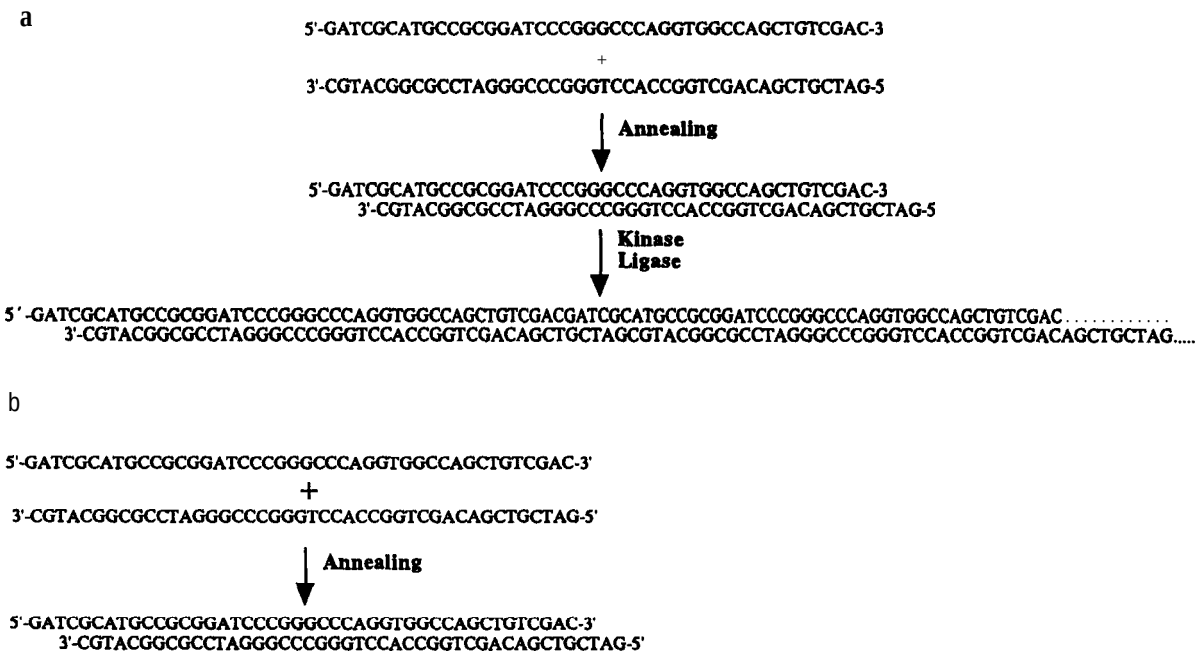

Fig. 1. Preparation of two sequence-specific DNA affinity ligands containing recognition sequences for 34 restriction endonucleases. (a) The two oligonucleotides were annealed, phosphorylated at the 5' protruding ends with T_4 polynucleotide kinase and ATP and complementary oligonucleotides were polymerized with T_4 DNA ligase and ATP (affinity ligand I). (b) The two oligonucleotides were annealed without subsequent polymerization (affinity ligand II).

*Bsh*FI, *Bam*HI, *Sma*I, *Sac*II, *Pvu*II and *Sal*I, which have recognition sequences within the oligonucleotide ligand. All enzyme preparations tested were bound to this adsorbent and were subsequently eluted at different salt concentrations by step gradients of KCl. The ability of adsorbent II, which was designed in order to interact specifically with restriction enzymes, was evaluated on the basis of the KC1 concentration that was necessary for the desorption of the enzymes. *Bsh*FI and *Bam*HI were eluted with 0.3 *M*, *Sma*I and *Sac*II with 0.2 *M* and *Pvu*II and *Sal*I with 0.1 *M* KCl, which is an indication of the different affinities of the enzymes for the ligand in the absence of Mg^{2+} (Table II). The purification factors obtained varied from 8- to greater than 300-fold and the recoveries varied from 75 to 94% (Table II). The estimated association constants of the interaction between the above restriction endonucleases and their recognition sites (2.4 μM ligand concentration) varies from $1.2 \cdot 10^6$ to $6.5 \cdot 10^6$ 1 mol^{-1}, under the buffer conditions described [20]. After each application the affinity column was regenerated with 2.5 *M* KC1 in order to wash out traces of bound material. Despite the lability of the DNA ligand, the column showed very good stability and could be used more than ten times for the purification of the above enzymes.

TABLE II

ELUTION OF RESTRICTION ENDONUCLEASES FROM OLIGONUCLEOTIDE AFFINITY ADSORBENT II AT VARIOUS KC1 CONCENTRATIONS

Enzyme	Elution [KCl (*M*)]	Purification (-fold)	Recovery (%)
*Bsh*FI	0.3	48	86
*Bam*HI	***0.3***	***> 300***	75
*Sma*I	***0.2***	***43***	***88***
*Sac*II	***0.2***	***122***	***94***
*Pvu*II	0.1	108	86
*Sal*I	0.1	8	80

Employing a two-step procedure including DNA-cellulose and affinity adsorbent II, we puri-

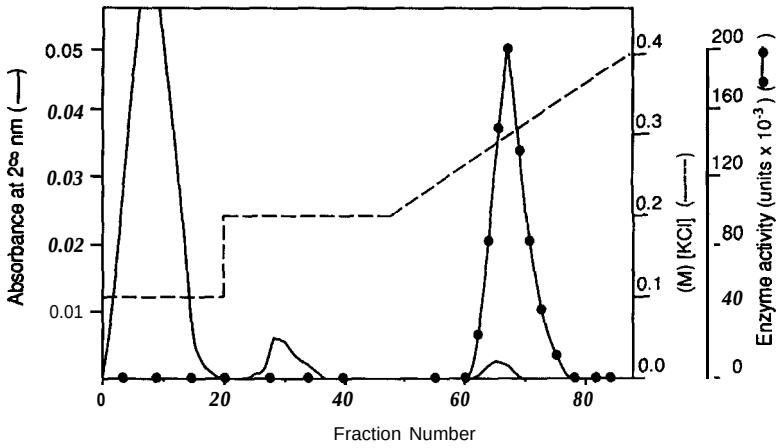

Fig. 2. Purification of *Bsh*FI by chromatography on oligonucleotide affinity adsorbent II. A sample (20 ml, 1 mg; $1.92 \cdot 10^6$ units) of partially purified *Bsh*FI on a dsDNA–cellulose column was applied to an oligonucleotide affinity adsorbent (5 × 1.6 cm; 10 ml) previously equilibrated in buffer B containing 0.1 M KCl. The affinity column was washed with buffer B containing 0.1 *M* KC1 (56 ml) and subsequently with buffer B containing 0.2 M KC1 (78 ml) and then developed with a linear gradient of KC1 (114 ml total volume; 0.2–0.4 *M*) in buffer B at a flow-rate of 9 ml/h. Fractions (2.8 ml) with *Bsh*FI activity correspondend to ca. 0.25 M KCl. The protein content was followed with a UV monitor at 280 nm. 0.05 a.u.f.s. (solid line). Dashed line, KCl concentration. ● = Enzyme activity.

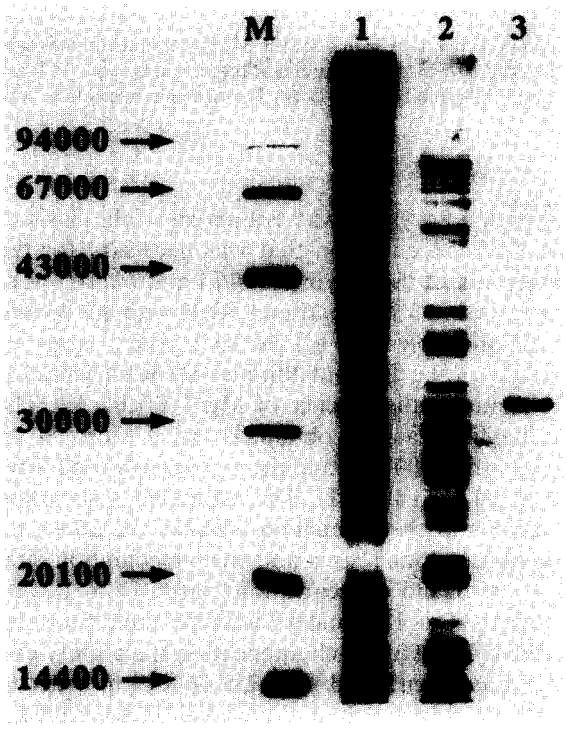

Fig. 3. Denaturing SDS-PAGE of the eluate containing *Bsh*FI activity and obtained from the oligodeoxynucleotide affinity adsorbent II. Lanes: 1 = crude protein mixture obtained from *Bacillus sphaericus* strain after cell lysis; 2 = fractions with *Bsh*FI activity eluted from the DNA-cellulose adsorbent; 3 = fractions with *Bsh*FI activity eluted from the oligonucleotide affinity adsorbent II; M = relative molecular mass markers, with the values shown on the left.

fied restriction endonuclease *Bsh*FI to homogeneity (Figs. 2 and 3, Table III). The successful isolation of the enzyme is mainly due to the effectiveness of the affinity chromatographic column. The enzyme preparation was enriched 562-fold and a 76% yield was obtained (Table II). The affinity column could be used repeatedly more than 20 times to isolate restriction endonuclease *Bsh*FI without any decrease in its performance. The enzyme exists in its native form as a monomer with an apparent relative molecular mass of 34 000 as determined by both SDS-PAGE and size-exclusion chromatography.

DNA-cellulose proved to be a very effective step in order to remove proteins that exhibited non-specific interaction with DNA from the crude extract, as reported previously [8].

A possible explanation for the weak interaction of *Pvu*II and *Sal*I with affinity adsorbent II is that the specific interaction of the enzymes with their recognition sequence might require the presence of Mg^{2+} ions. The only enzymes that have been studied and for which it has been demonstrated that the specific interaction with DNA is independent of the presence of Mg^{2+} ions are the enzymes EcoRI and *Bgl*II [19,21]. Further, it has been reported that in the absence of Mg^{2+} the binding affinities of EcoRI for specific and non-specific DNA differ by a factor of $1 \cdot 10^5$ [21–23]. Similar results were obtained with

TABLE III

SUMMARY OF THE PURIFICATION PROTOCOL FOR *Bsh*FI

Step	Protein (mg)	Enzyme activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	160	–	–	–	–
DNA-cellulose	1	$1.92 \cdot 10^6$	$1.92 \cdot 10^6$	1	100
Adsorbent II	$1.35 \cdot 10^{-3}$	$1.46 \cdot 10^6$	$1.08 \cdot 10^9$	562	76

*Rsr*I, an isoschizomer of *Eco*RI [24], whereas with *Eco*RV the ratio of binding affinities is 1 [25].

This study has demonstrated the effectiveness of a group-specific oligonucleotide affinity adsorbent for the isolation of restriction endonucleases. It is expected that restriction endonucleases that exhibit higher affinity for specific rather than non-specific DNA sequences will be successful candidates for purification by sequence-specific DNA affinity chromatography.

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