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Eco1524I, A Type II Restriction Endonuclease

Isolation, Partial Purification, and Characterization

Hadeer Lazim,[1] Jytte Josephsen,[2] Assia Ben Hassen,[3] Omrane Belhadj,[1] and Férid Limam*,[4]

[1]Laboratoire de Biochimie, Faculté des Sciences de Tunis, Campus Universitaire 1060 Tunis, Tunisia; [2]Department of Food Sciences, Royal Veterinary and Agricultural University, D-1958 Frederiksberg C, Denmark; [3]Laboratoire de Microbiologie, Centre National de Greffe de Moelle osseuse, Bab Saadoun Tunis, Tunisia; [4]Laboratoire Interactions Légumineuses-Microorganismes, Institut National de Recherche Scientifique et Technique, 95-2050 Hammam-Lif, Tunisia, E-mail: ferid.limam@inrst.rnrt.tn

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Abstract

Various strains of *Escherichia coli*, isolated from different patients, were screened for type II restriction endonuclease activity. In 1 out of 23 patients, a type II restriction endonuclease activity was found. The restriction endonuclease designated Eco1524I was purified to near homogeneity, based on hydroxyapatite and heparin sepharose chromatography. Eco1524I exhibited endonuclease restriction activity in the pH range from 6.0 to 10.0 (maximum level at pH 8.0) and required Mg^{2+} as divalent cation. The enzyme was stable till temperature 55°C and pH range from 6.0 to 10.0. Eco1524I recognized the sequence 6-bp palindromic 5'AGG ↓ CCT 3', producing blunt end and is found to be an isoschizomer of Stu I.

Index Entries: *E. coli*; restriction/modification; type II endonuclease; restriction site; Stu I isoschizomer.

Introduction

More than 3000 restriction endonucleases (RE) have been isolated from bacteria sources *(1)* and most of them can be grouped into four classes based on subunit composition, cofactor requirement, methylation state, as well as type of DNA cleavage *(2)*. More than 98% of the known RE belong

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

to type II *(3)*. Restriction enzymes generally recognize palindromic sequence in DNA and cleave within or near their recognition sequences, producing DNA fragments of defined sizes *(4)*. These useful properties have led to the screening of diverse bacterial species for new restriction enzymes. Some of these enzymes have been studied extensively with respect to their structure, DNA binding, and reaction characteristics *(3)*. In spite of their importance to host bacteria, restriction enzymes have not been detected in all bacterial species investigated. However, some strains have more than one enzyme. For example *Neisseria* strains appear to be particularly rich in restriction endonucleases and their corresponding methyltransferase *(5)*. Type II restriction enzymes are abundant in the genus *Bacillus (6,7)*. Several restriction/modification (R/M) systems *(8)* have been isolated from *Lactococcus* spp. and *Escherichia coli*. Most of those which have been characterized belong to type II endonucleases *(9,10)*. A great number of bacterial species have been subjected to careful screening methods to isolate new restriction enzymes. In the present study we have surveyed 23 *E. coli* strains. Only *E. coli* strain 1524 exhibited type II restriction endonuclease activity which has been purified and characterized.

Materials and Methods

Strains and Growth Condition

Most *E. coli* strains were obtained from the "Laboratoire de Bactériologie, Centre National de Greffe de Moelle Osseuse," Tunis, Tunisia. *E. coli* XL1-Blue MRF was from (Stratagene La Jolla, CA). The strains were grown with shaking at 37°C in brain–heart infusion medium. For selection, ampicillin was added to a final concentration of 50 µg/mL. Blue/white screening was performed with 40 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 40 µg/mL isopropyl-β-D-thiogalactopyranoside (IPTG). pBluescript SKII+ (Stratagene La Jolla, CA) was used for cloning.

Preparation of Cell Extract for Screening

0.6 g of concentrate polymer: dextran T-70/polyethylene glycol (PEG) 6000, prepared as previously described *(11)* with minor modification (dextran-70 instead of dextran-500), was added to 4 tubes containing 1 mL of sonicated cell extract. In each tube, varying amounts of NaCl were added reaching concentrations of 50, 200, 400, and 800 m*M*. Mixture was then centrifuged at 4000*g* during 10 min at 4°C. The upper phase was collected from each tube and tested for endonuclease activity.

Protein Quantification

Total protein content was determined according to Bradford method *(12)*, using bovine serum albumin (BSA) as standard.

Restriction Endonuclease Assay

The standard restriction endonuclease assay was performed by incubating 5 µL cell extract with 1 µg λ DNA substrate in reaction buffer 10 m*M* Tris-HCl, pH 8.0 containing 7 m*M* 2-mercaptoethanol and 10 m*M* $MgCl_2$ in 20 µL final volume. After incubation at 37°C for 1 h, the reaction was stopped by addition of gel loading dye, and applied onto a 0.8% agarose gel for electrophoresis at 80 V during 2 h. The unit activity (U) of the enzyme was estimated by incubating various amounts of the enzyme with 1 µg λ DNA under standard assay conditions described above.

Purification of Enzyme From E. coli *15241*

All steps were carried out at 4°C. 40 g of cells obtained from 2 L of culture were harvested in stationary phase, washed with buffer A (10 m*M* Tris-HCl, pH 8.0 containing 7 m*M* 2-mercaptoethanol), and centrifuged. The cell paste was resuspended 1:3 (w/v) in buffer A, sonicated at 100 k cycles/s (intermittent sonication for 30 s followed by 30 s on ice to allow cooling). After centrifugation, supernatant was treated with PEG and NaCl mixture and applied onto hydroxyapatite column (1.6 × 30 cm), previously equilibrated in 10 m*M* phosphate buffer pH 7.0 containing 100 m*M* NaCl, 7 m*M* 2-mercaptoethanol, and 1 m*M* EDTA. The column was extensively washed with the same buffer and proteins were eluted with a linear gradient from 10 to 250 m*M* phosphate buffer pH 7.0 containing 100 m*M* NaCl and 7 m*M* 2-mercaptoethanol at a flow rate of 7 mL/h. Active fractions were pooled, dialyzed against distilled water, and lyophilized. The last step of purification was achieved by affinity chromatography. The lyophilized powder was resuspended in 3 mL of 10 m*M* Tris-HCl buffer pH 7.5 containing 7 m*M* 2-mercaptoethanol and 40 m*M* NaCl, and applied onto 1 mL HiTrap Heparin HP column at a flow rate of 7 mL/h. The column was pre-equilibrated in the same buffer and proteins were eluted with a linear NaCl gradient from 40 to 500 m*M*. Active fractions were pooled, dialyzed against distilled water and concentrated by lyophilization.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli *(13)*, using 12 % polyacrylamide gel. Silver staining was performed as previously described *(14)*.

Optimal Conditions for Eco1524I Restriction Endonuclease Activity and Stability

Optimum temperature for Eco1524I activity was determined by incubating 1 unit of enzyme at different temperatures (25, 35, 45, 55, 65°C) under the same conditions as the standard endonuclease assay (1 µg λ DNA in 10 m*M* Tris-HCl buffer pH 8.0 containing 7 m*M* 2-mercaptoethanol and 10 m*M* $MgCl_2$, 20 µL final volume, incubation time 1 h) and agarose electrophoresis was carried out thereafter.

Thermal stability was determined after pre-incubation of 10 units of enzyme at different temperatures (25, 35, 45, 55, 65°C) for 30 min in the same buffer. Residual activity was measured by using 1 unit of enzyme under the same conditions as the standard endonuclease assay.

Optimum pH for Eco1524I activity was determined by incubating 1 unit of enzyme in the following reaction buffers: 10 m*M* sodium phosphate pH 6.5, 10 m*M* Tris-HCl pH 7.0–8.5, and 10 m*M* glycine-NaOH pH 9.0–10.0 under the same conditions as the standard endonuclease assay. The pH stability was performed by pre-incubating 10 units of enzyme in buffers at different pH values varying from 6.5 to 10.0 for 30 min at 37°C. For measurement of residual activity, 1 unit of enzyme was incubated under the same conditions as the standard endonuclease assay.

Mg^{2+} and NaCl Effects

Mg^{2+} effect was determined by incubating 1 unit of enzyme with 1 µg λ DNA in the presence of increasing Mg^{2+} concentrations (0, 1, 5, 10, 15, 20, 25, 50 m*M*) under the same conditions as the standard endonuclease assay. NaCl effect was studied using the same protocol in the presence of different NaCl concentrations (0, 10, 25, 50, 75, 100, 150 m*M*).

Star Activity

Excess amount of enzyme (5 units) was incubated during 16 h with 1 µg λ DNA in a final volume of 50 µL at 37°C.

Substrate Specificity of Eco1524I

One unit of enzyme was incubated with 1 µg of DNA from phages λ and ϕX174, plasmids pUC18 and pBR322, type 2 adenovirus (Ad2), and SV40 virus under the same conditions as the standard endonuclease assay previously described.

Molecular DNA Techniques

Restriction enzymes, Klenow DNA polymerase, mung bean nuclease (New England Biolabs, Inc., Beverly, MA), T4 DNA ligase and alkaline phosphatase (USB Cleveland), and DNA molecular weight markers (GeneRuler 1 kb DNA Ladder from New England Biolabs, Inc, Beverly, MA) were used as recommended by supplier. Plasmid DNA and fragments were purified by QIAGEN kit (Qiagen, Inc., Chatsworth), GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences, Piscataway NJ). DNA sequencing was performed with the CEQ Dye Terminator Cycle Sequencing kit and CEQ 2000 DNA Analysis System (Beckman Coulter, Inc., Fullerton, CA). Sequence analysis was performed using genetic computer group sequence analysis software (Wisconsin, USA) and the BLAST local alignment tool at the National Center for Biotechnology Information (NCBI).

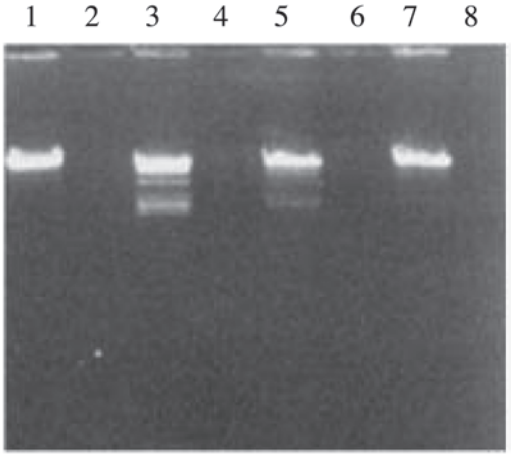

Fig. 1. Electrophoresis analysis of Eco1524I extracted from aqueous two phase system on 0.8% agarose gel. Increasing concentrations of NaCl (50, 200, 400, 800 m*M*) were used in the PEG phase partition as described in material and methods. The endonuclease activity was further tested on λ DNA substrate. (1) λ DNA + cell extract from 50 m*M* NaCl fraction; (2) Cell extract from 50 m*M* NaCl fraction; (3) λ DNA + cell extract from 200 m*M* NaCl fraction; (4) Cell extract from 200 m*M* NaCl fraction; (5) λ DNA + cell extract from 400 m*M* NaCl fraction; (6) Cell extract from 400 m*M* NaCl fraction; (7) λ DNA + cell extract from 800 m*M* NaCl fraction; (8) Cell extract from 800 m*M* NaCl fraction.

Determination of Recognition Sequence

About 1.5 kb Eco1524I fragment from λ DNA was subcloned into EcoRV site precut and dephosphorylated in pBluscript SKII+ after treatment with Klenow fragment of DNA polymerase I or mung bean nuclease. The ligation mixture was used to transform *E. coli* XL1-blue competent cells as previously described *(15)*. The junctions sites were identified after sequencing with primers T7 (3'-CGGGATATCACTCAGCATAATG-5') and T3 (5'-AATTAACCCTCACTAAAGGG-3') and comparing the sequence with the published sequence of λ.

Results

Screening of Restriction Endonuclease

Twenty-three clinical bacterial strains of *E. coli* were screened after PEG treatment for the presence of type II restriction endonuclease. Only one strain showed endonuclease activity on λ DNA substrate and named Eco1524I. The adjustment of salt concentration during the phase partition step leads to the separation of endonuclease activity from contaminants. The maximum endonuclease activity was obtained in the upper phase with 200 m*M* NaCl concentration (Fig. 1).

Table 1
Purification of Eco1524I Enzyme

Steps	Volume (mL)	Total protein (mg)	Total activity (unit)	Specific activity (U/mg)	Fold purification	Yield (%)
Cell extract	120	2800	36600	13	1	100
Partition phase	100	93.33	36000	386	29.50	98.50
Hydroxyapatite	36	10.60	15570	1468	112.30	42.50
Heparin HP	4	0.18	4076	22644	1732.50	11

One unit of activity (U) was defined as the amount of enzyme needed to cleave 1 μg of λ DNA in 1 h at 37°C.

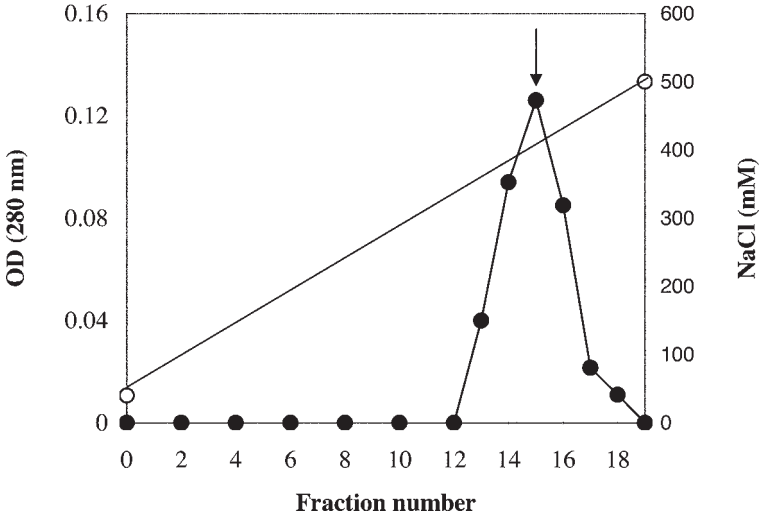

Fig. 2. Elution profile of Eco1524I enzyme from HiTrap Heparin HP column. Active fractions from Hydroxyapatite were pooled and subjected to Heparin column as described under "material and methods." Elution was carried out by 40–500 m*M* NaCl gradient (open circle) at a flow rate of 7 mL/h. OD was measured at 280 nm (full circle); Eco1524I activity was measured with λ DNA as substrate in the standard endonuclease assay. Maximal activity was indicated by the arrow.

Enzyme Purification

The strategy used for purifying Eco1524I from the crude extract is summarized in Table 1. The highest endonuclease activity was eluted from the Hydroxyapatite column at 150 m*M* phosphate buffer pH 7.0. The active fractions were pooled and subjected to affinity chromatography on HiTrap Heparin HP column. The Eco1524I enzyme was eluted as a single peak at 370 m*M* NaCl in 10 m*M* Tris-HCl buffer pH 7.5 containing 7 m*M* 2-mercapto-ethanol (Fig. 2). Purity of Eco1524I was analyzed by 12% SDS-PAGE which showed one major polypeptide with molecular mass of 44.55 kDa and a minor one with 55.52 kDa (data not shown).

Characterization of Eco1524I Enzyme

Mg^{2+} Effect

The Eco1524I activity was determined without NaCl in the presence of different Mg^{2+} concentrations. The same level of activity was found with concentrations varying from 1 to 50 m*M*, although no activity was detected without Mg^{2+} ions (data not shown). Consequently, 10 m*M* $MgCl_2$ were used in Eco1524I activity assay.

NaCl Effect

The effect of ionic strength on enzyme activity was determined by varying the NaCl concentrations from 0 to 150 m*M*. The enzyme was active up to 100 m*M* NaCl and even without NaCl. The highest activity was found from 0 to 50 m*M* NaCl. The activity decreased at a concentration higher than 100 m*M* NaCl (partial λ DNA digestion). Moreover, no λ DNA digestion occurred at 150 m*M* NaCl. Consequently NaCl was omitted in all Eco1524I activity assays.

Optimal Conditions for Enzyme Activity

Optimum temperature for Eco1524I activity was determined by carrying out the assays at temperatures varying from 25 to 65°C (Fig. 3A). Enzyme activity was unchanged from 35 to 55°C. Only 40% of this activity was lost at 65°C. Stability of Eco1524I was investigated for temperatures varying from 25 to 65°C. No loss of activity occurred at temperatures from 25 to 55°C. However, 80% of the initial activity was lost at 65°C.

The optimum pH for the enzyme activity was determined at a wide range of pH (6.0, 7.0, 8.0, 9.0, 10.0) (Fig. 3B). Results showed that Eco1524I exhibited a broad pH optimum culminating at pH 8.0. Stability determinations showed that Eco1524I was stable at pH range from 6.0 to 10.0.

Star Activity

Star activity assay was performed during 16 h in the presence of an excess of Eco1524I enzyme. Analysis of Eco1524I digestions gave a sharp banding pattern indicating no star activity.

Substrate Specificity of Eco1524I

The cleavage pattern of the enzyme was determined by using different DNA substrates such as λ, ϕX174, Ad2, SV40, pBR322 and pUC18. In our experimental conditions, cleavage profiles showed five sites with λ DNA and with SV40, eight sites with Ad2, one site with ϕX174, and no site with pBR322 and with pUC18 (Fig. 4).

Determination of the Recognition Sequence

Digestions of nonmethylated phage λ DNA with partially purified Eco1524I gave DNA fragments smaller than 2 kb. These fragments were

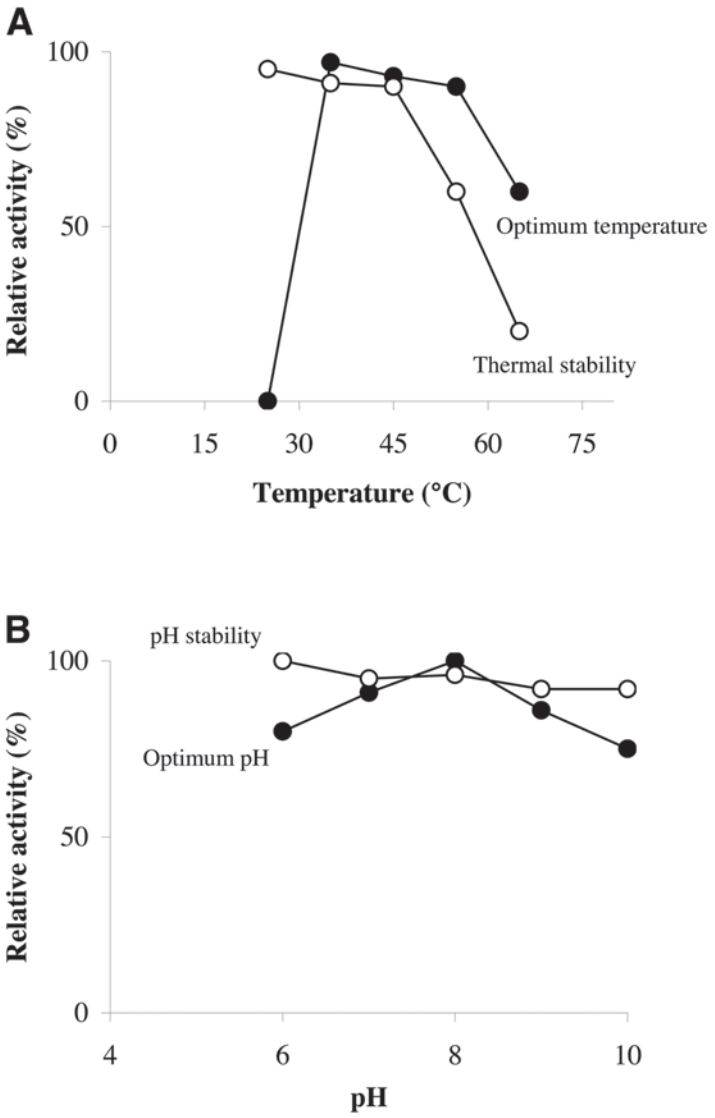

Fig. 3. Optimization of Eco1524I activity: effects of pH and temperature. **(A)** Optimum temperature and thermal stability; **(B)** Optimum pH and pH stability.

blunt ended with Klenow DNA polymerase or mung bean nuclease and subsequently ligated into pBluescript SKII+ digested with EcoRV. Plasmid DNA from white colonies was sequenced by using universal and reverse primers. Determinations of the junction sites revealed that Eco1524 recognized the sequence 5'AGG ↓ CCT 3' and cleaved as shown with an arrow (↓). This sequence is repeated six times in phage λ DNA. Restriction patterns of phage λ DNA, Ad2, and SV40 digested with Eco1524I and with the commercial isoschizomer StuI were identical (Fig. 5).

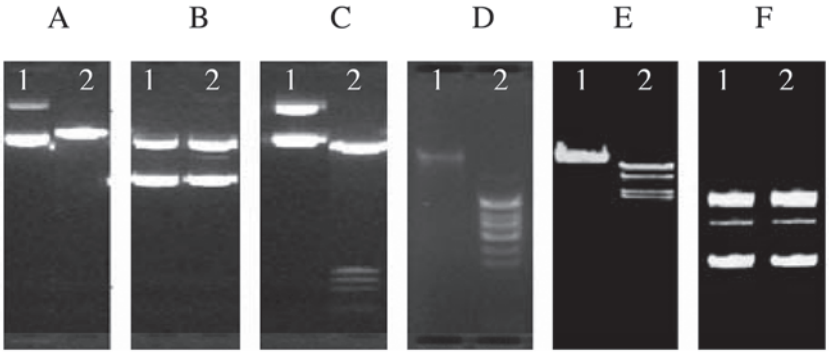

Fig. 4. Electrophoresis profiles of cleavage products of DNA. **(A)** ϕX174, **(B)** pUC18, **(C)** SV40, **(D)** Ad2, **(E)** λ, **(F)** pBR322. For each gel: (1) undigested DNA control, (2) DNA + Eco1524I. Electrophoresis was carried out on 0.8% (w/v) agarose gel in TBE buffer at 80 V during 2 h. Staining was performed with ethidium bromide.

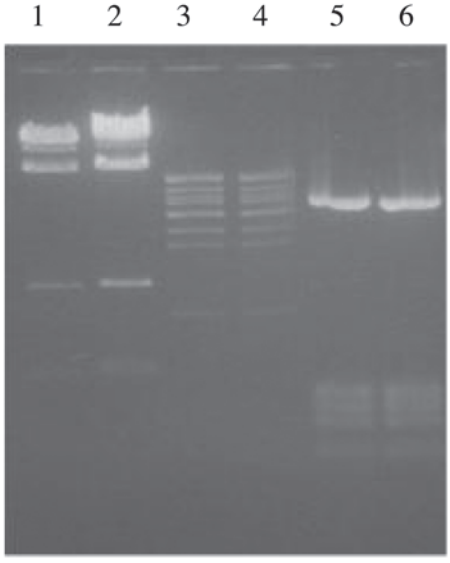

Fig. 5. Comparative cleavage patterns of Eco1524I and StuI restriction enzymes using different DNA substrates. (1) λ DNA digested with Eco1524I; (2) λ DNA digested with StuI; (3) Ad2 DNA digested with Eco1524I; (4) Ad2 DNA digested with StuI; (5) SV40 DNA digested with Eco1524I; (6) SV40 DNA digested with StuI. After electrophoresis on 0.8% agarose gel at 80 V during 2 h, staining was performed with ethidium bromide.

Discussion

In this study a type II restriction endonuclease called Eco1524I was isolated from *E. coli* 1524 strain. The enzyme was purified and characterized. The highest activity was obtained at pH 8.0 and 37°C in the presence of 10 m*M* $MgCl_2$. It recognized and cleaved the same sequence as StuI (5'AGG ↓ CCT 3').

Several methods have been used to detect R/M systems. Classical bacteriophage efficiency of plating (EOP) assay is a simple method to detect the presence of restriction enzymes. However, phages from many bacterial strains have not yet been isolated. Antirestriction systems, present in many phages, can also obscure the detection of R/M systems *(16)*. An alternate method to screen for bacterial type II endonucleases involves incubation of cell extracts with known DNA substrates and look for generation of distinct DNA fragments following agarose electrophoresis.

We have shown that aqueous two-phase system with PEG *(11)* is a rapid and inexpensive method which appeared very useful to screen bacteria for the presence of type II restriction endonucleases *(17)*. This method is not suitable for the detection of type I or type III endonucleases because of either nonspecific DNA cleavage or of the presence of nonspecific nucleases in cell extract. This method allowed us to detect a type II restriction endonuclease in a clinical isolate of *E. coli* named Eco1524I. Enzyme purification was highly simplified when starting from aqueous two-phase system. Eco1524I enzyme was separated with 200 m*M* NaCl, followed by hydroxyapatite and HiTrap Heparin chromatography. The enzyme was purified 1733-fold with an overall yield of 11%, exhibiting a high specific activity (22,644 units/mg) with λ DNA as substrate. Total enzyme activity produced per gram of cells was 36,600 units. After hydroxyapatite chromatography, the specific activity of the enzyme increased by 30-fold. The use of HiTrap Heparin HP column as a final purification step highly increased the specific activity of the enzyme by more than 100-fold. Purity and molecular weight of the enzyme were determined by 12% SDS-PAGE. Two bands were detected, a major one at 44.50 kDa and a minor one at 55 kDa.

Eco1524I enzyme exhibited the same level of activity at temperatures varying from 35 to 55°C. It showed high thermal stability till 55°C but lost 80% of its initial activity at 65°C. The enzyme maintained 75 to 100% of its activity at a wide pH range from 6.0 to 10.0 with an optimum near pH 8.0. It showed high stability, conserving 92 to 100% of its initial activity at a pH range from 6.0 to 10.0.

Ionic requirements for optimal Eco1524I activity were also investigated. Mg^{2+} ions are well known to be a necessary cofactor for DNA hydrolysis by type II restriction enzymes *(18)*. In our case, Eco1524I enzyme effectively required Mg^{2+} ions at a minimal concentration of 10 m*M*. NaCl was not required since it was slightly inhibitory; a characteristic shared by some but not all type II endonucleases. Indeed BstLVI showed low activity without NaCl but full activity in the presence of 50 m*M* NaCl *(19)*.

Star activity was often observed with restriction endonucleases. In our experiments, we never detected any star activity for Eco1524I enzyme even after 16 h of incubation with λ DNA. This property made the enzyme very suitable for many purposes in recombinant DNA technology.

The cleavage patterns generated by the enzyme on different DNA substrates were compared with those of other type II restriction enzymes.

The Eco1524I cleavage pattern looked like that generated by StuI produced by *Streptomyces tubercidicus*, as well as by some other commercially available enzymes (AaTI, Eco147I, PceI, and SseBI). StuI cleaved λ DNA in six fragments with sizes (19044, 12436, 7886, 6995, 1515, and 626 bp). The exact cleavage site of the enzyme was determined by nucleotide sequencing of the junction site of cloned digestion fragments. The fact that our enzyme recognized and cleaved the following sequence 5'AGG↓CCT3', shows that Eco1524I is an isoschizomer of StuI. However Eco1524I and StuI showed some different properties such as heat inactivation, star activity and high production.

Eco1524I enzyme could be a good alternative to StuI for several reasons: (1) generation time of *E. coli* is much shorter than *Streptomyces tubercidicus*, which allows a faster and easier production of high amount of enzyme, (2) Eco1524I is exonucleases free, (3) it has a high stability, and (4) no star activity.

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