A Comparative Study on the Recovery of EcoRI Endonuclease from Two Different Genetically Modified Strains of Escherichia coli

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A laboratory scale procedure developed for the purification of EcoRI restriction endonuclease was applied to two different Escherichia coli strains, E. coli 294 and E. coli M5248, which are genetically modified to overproduce the enzyme. The purification method consisted of three successive chromatographic steps including phosphocellulose and hydroxyapatite columns and further fractionation in a second phosphocellulose column. It was shown that the second phosphocellulose separation can be omitted in the case of E. coli 294. Quality control tests indicated enzyme preparations free of contaminants and endo- or exo-nucleases. The yields obtained at the final stage of the purification were 1.3x10^5 U/g cells for E. coli M5248 and 3.3x10^6 U/g cells for E. coli 294.

Key Words: EcoRI endonuclease, recombinant E. coli, purification, enzyme recovery

Introduction

Restriction enzymes have extensive applications in recombinant DNA technology. They are used in the preparation of recombinant molecules, and they provide an attractive system for the analysis of sequence specific DNA-protein interactions. Escherichia coli RI (EcoRI) endonuclease is a well-known restriction enzyme that recognizes the symmetrical hexanucleotide sequence GAATTC on duplex DNA and cleaves each strand between G and A residues.

Physical and catalytic properties of EcoRI restriction endonuclease have been extensively studied by several groups and different purification protocols have been described. In addition to the natural overproducer of EcoRI, E. coli RY13, genetically modified overproducing strains were also used to produce the enzyme. The gene encoding EcoRI endonuclease was placed under the control of the ApL promoter in these genetically modified, overproducing strains. The application of different purification protocols made it difficult to compare the yield and the quality of these strains in the production of EcoRI.

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In the present study, we compare the production of EcoRI endonuclease from two different genetically modified strains of *E. coli*, namely *E. coli* 294 (pPG430) and *E. coli* M5248 (pSCC2), using the same small scale purification protocols. *E. coli* 294 carries a plasmid, pPG430, in which the genes encoding EcoRI endonuclease and methylase are placed under the control of the lac promoter. The second strain, *E. coli* M5248, contains these genes under the control of the *pL* promoter on plasmid pSCC2.

**Materials and Methods**

**Bacterial Strains and Plasmids:** *E. coli* 294 cells containing the plasmid pPG430, which is a derivative of pBR322, were kindly provided by Dr. Herbert Boyer (University of California, San Francisco). *E. coli* M5248 (λ bio275 cI857 HI), *E. coli* N99 (λ+ str+ su-) and plasmid pSCC2 were kindly provided by Dr. Paul Modrich (Duke University, Medical Center, Durham, North Carolina).

**Enzymes:** T4 ligase, BamHI, PstI and PvuII used in the experimental work were purchased from New England Biolabs (USA).

**Chemicals:** Phosphocellulose (P11) from Whatman (UK), hydroxyapatite (HA) from Bio-Rad (USA) and acrylamide and agarose from Sigma (USA) were used in all the experiments. All other chemicals were analytical grade and supplied by either Merck AG (Germany) or Sigma (USA).

**Growth of Cells:** The culture medium used was LB medium containing 1% (w/v) bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco) and 1% (w/v) NaCl. *E. coli* M5248 was cultured in LB medium supplemented with 0.01M K-phosphate (pH 7.0), 0.01% (w/v) thymine, 0.005% (w/v) thiamine and 5% (w/v) glucose. Both media were supplemented with ampicillin to a final concentration of 80 μg/ml to prevent the overgrowth of plasmid-free cells that do not have the ability to synthesise the product. Ten ml of pre-culture was used to inoculate sterile 1L LB medium and then placed in the orbital shaker at the specified temperatures for each *E. coli* strain.

The *E. coli* M5248 strain was first transformed by the plasmid pSCC2, which was obtained from the *E. coli* N99 strain, and the transformed cells were grown at 30-32°C to an absorbance value of 1.0 at 590 nm. The culture temperature was then raised to 42°C to induce product synthesis, and incubation was continued over a period of 4-5 hours as described by Cheng et al.10. The cells were harvested by centrifugation at 2603 g (4000 rpm in a GSA rotor) for 15 min at 4°C and then stored at -20°C until further purification.

The second strain used in the study was *E. coli* 294, carrying the plasmid pPG430 containing EcoRI endonuclease and methylase under the control of the lac promoter. The *E. coli* 294 strain was grown at 37°C to an absorbance value of 1.2 at 595 nm. A parametric study was conducted to optimize induction conditions, cells were induced by the addition of 0.1mM isopropyl-β-D-thiogalactoside (IPTG), and incubation was continued over a period of 6 hours. The cells were collected by centrifugation at 2603 g for 15 minutes at 4°C. Plasmid stability of the strains was determined via the replica plating technique10.

**Purification of EcoRI Endonuclease:**

**a) Preparation of Crude Extract:** *E. coli* 294 (4.375 g, wet weight) and *E. coli* M5248 (4.923 g, wet weight) were thawed, suspended in Buffer A (20mM K-phosphate which was prepared by adding 20mM KH2PO4 to K2HPO4 until the pH of the solution was neutral, 1 mM 2-mercaptoethanol, 1mM ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, pH 7.0) and supplemented with 0.8M NaCl and 0.1M phenyl methyl sulphonyl fluoride (PMSF) at final concentration. The cell suspensions were then sonicated while kept on ice to prevent heating. The crude extract was dialysed for 16 hours against
Buffer A containing 0.4M NaCl, after which cell debris were removed by centrifugation at 10786 g (9500 rpm in a SS34 rotor) for 15 min at 4°C using a Sorvall RC-28S centrifuge. All steps of the purification were performed at 0-4°C.

b) First Phosphocellulose Column Chromatography: The dialysed fraction was applied to a phosphocellulose column (50cm x 2cm diameter) equilibrated with Buffer A containing 0.4M NaCl. The subsequent elution was carried out stepwise by Buffer A containing increasing concentrations of NaCl (from 0.4 to 1 M).

c) Batchwise Hydroxyapatite Chromatography: Active fractions eluted with 0.6M NaCl were pooled and applied to batchwise hydroxyapatite chromatography which was equilibrated with Buffer A containing 0.6M NaCl. The elution was carried out stepwise by increasing concentrations of K-phosphate ranging from 0.1M to 0.6M in Buffer A containing 0.6M NaCl.

d) Second Phosphocellulose Column Chromatography: The active enzyme fractions were pooled and diluted four times with Buffer A and applied to a second phosphocellulose column (10 cm x 1 cm diameter). The elution was carried out as in the first phosphocellulose column. Finally, active fractions were supplemented with 50 µg/ml BSA and dialysed against storage buffer containing 50% (v/v) glycerol, 10 µg/ml BSA, 10 mM K-phosphate, 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 0.1% TritonX-100, pH 7.0.

Electrophoresis: Homogeneity of the purified enzyme was tested by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using a slightly modified procedure described by Laemmlli under denatured conditions\(^\text{11}\).

Enzyme Assays: One unit of enzyme activity was defined as the amount of enzyme required to produce a complete digestion of 1.0 µg λDNA at 37°C in 1 hour in a total reaction volume of 10µl. The enzymatic activity was determined using serial dilutions of enzyme preparations. λDNA having 5 recognition sites for EcoRI was used as substrate in this study, and the completion of the digestion was checked on 0.8% agarose gels. The activity in units was derived from the dilution factor by determining the highest dilution that still displays complete digestion.

Protein Measurement: Protein contents of the samples were determined by the Bradford method using bovine serum albumin (BSA) as protein standard\(^\text{12}\).

Quality Control Tests: Two different quality control tests were carried out in order to check the existence of any potential endo and exonuclease and the ligation inhibitor.

1) Overdigestion Test: Each EcoRI preparation was tested for contamination by other endodeoxyribonucleases capable of digesting DNA at either random or specific sites. One microgram of substrate DNA is digested with 20 units of the enzyme for 5 hours at an appropriate temperature. This represents 100-fold excess digestion as compared to 1 unit for 1 hour as described by the manufacturers\(^\text{9}\).

2) Cut-Ligate-Recut Test: EcoRI restriction endonuclease was also tested for the presence of contaminants that would inhibit ligation or degrade termini. The restored sites were cleaved by the same enzyme. The initial cleavage of λDNA was performed with the EcoRI isolated in this study, and the DNA fragments were extracted by phenol and chloroform, followed by precipitation with ethanol. T4 ligase was used to ligate the fragments obtained from the initial cleavage. Ligation was performed at 16°C for 4 hours under conditions described by the manufacturer. T4 ligase was inactivated by heating the reaction mixture for 15 min at 65°C. Ligated fragments were recut by using the same enzyme preparation used in the initial cleavage of DNA\(^\text{9}\).
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Results

In the present work, EcoRI endonuclease was purified from both E. coli 294 (pPG430) and E. coli M5248 (pSCC2) overproducing strains using similar protocols, and the enzyme yields were compared. In plasmid pPG430, genes encoding EcoRI endonuclease and methylase are oriented under the control of the lac promoter\textsuperscript{13}, whereas in plasmid pSCC2, they are placed under the control of $P^L_3$.

a) Enzyme Purification: Table 1 summarises the purification of EcoRI endonuclease from E. coli 294 (pPG430) and E. coli M5248 (pSCC2). This is a modified purification method developed by Luke and Halford\textsuperscript{6} on a genetically modified strain, E. coli 1100, to overproduce enzyme EcoRI. Luke and Halford have modified the purification used by Botterman and Zabeau\textsuperscript{3} on the same strain, and produced a tenfold increase in the specific activity of the enzyme. Botterman and Zabeau\textsuperscript{3} have applied the supernatant of the sonicated cell suspension to phosphocellulose and then to hydroxyapatite chromatography. The modification that Luke and Halford applied was to include a dialysis step after sonicating the cell suspension to decrease the aggregation of the protein and therefore to increase its specific activity\textsuperscript{6}. In this work, a dialysis step was included for a similar purpose, to minimise the formation of insoluble intracellular aggregates before hydroxyapatite chromatography and also a second phosphocellulose chromatography was also applied after hydroxyapatite chromatography on two different genetically modified E. coli strains.

Table 1. Purification of EcoRI endonuclease

<table>
<thead>
<tr>
<th>E. coli 294 strain (4.375 g wet cells)</th>
<th>Fractions</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant of disintegrated cell suspension</td>
<td>100</td>
<td>356.875</td>
<td>1.5x10\textsuperscript{7}</td>
<td>42 031</td>
<td>100</td>
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<tr>
<td>First phosphocellulose chromatography</td>
<td>84</td>
<td>43.75</td>
<td>1.47x10\textsuperscript{7}</td>
<td>336 000</td>
<td>98</td>
<td>8.0</td>
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<tr>
<td>Batchwise hydroxyapatite chromatography</td>
<td>48</td>
<td>13.775</td>
<td>1.44x10\textsuperscript{7}</td>
<td>1 045 372</td>
<td>96</td>
<td>24.9</td>
<td></td>
</tr>
<tr>
<td>Second phosphocellulose chromatography</td>
<td>55</td>
<td>9.023</td>
<td>9.6x10\textsuperscript{6}</td>
<td>1 066 718</td>
<td>64</td>
<td>25.4</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>E. coli M5248 strain (4.923 g wet cells)</th>
<th>Fractions</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant of disintegrated cell suspension</td>
<td>150</td>
<td>932</td>
<td>1.05x10\textsuperscript{6}</td>
<td>1 127</td>
<td>100</td>
<td>1.0</td>
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<tr>
<td>First phosphocellulose chromatography</td>
<td>80</td>
<td>64</td>
<td>8.9x10\textsuperscript{5}</td>
<td>13 906</td>
<td>85</td>
<td>12.3</td>
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<tr>
<td>Batchwise hydroxyapatite chromatography</td>
<td>48</td>
<td>24</td>
<td>7.5x10\textsuperscript{5}</td>
<td>30 992</td>
<td>71</td>
<td>27.5</td>
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<tr>
<td>Second phosphocellulose chromatography</td>
<td>64</td>
<td>6.1</td>
<td>6.4x10\textsuperscript{5}</td>
<td>104 918</td>
<td>60</td>
<td>93.1</td>
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</table>
Cells that were induced for the synthesis of EcoRI either by the addition of 0.1 mM IPTG in the case of *E. coli* 294 (pPG430) or by a temperature shift at 42°C for a period of 5 hours in the case of *E. coli* M5248 (pSCC2), were disrupted by sonication. The crude extracts were dialysed, and cell debris was removed together with precipitated proteins by centrifugation. The supernatant was applied to a phosphocellulose column as described in the Materials and Methods section. The elution profiles for *E. coli* 294 and *E. coli* M5248 are given in Figures 1 and 2, respectively. The active fractions were pooled and applied to batchwise HA chromatography, which resulted in 71 and 96% recovery of EcoRI endonuclease with *E. coli* M5248 and *E. coli* 294 respectively (Figure 3). The application of a second phosphocellulose column only increased the specific activity of EcoRI threefold in the case of *E. coli* M5248 by eliminating contaminating proteins (Figure 4). Since the degree of purification was not improved any further by a second phosphocellulose column in the case of *E. coli* 294, it was concluded that the application of the second phosphocellulose column could be omitted. This observation allowed the development of a simple two-step procedure consisting of only two chromatographic separations for the preparation of pure EcoRI for commercial use from the genetically engineered overproducing *E. coli* 294.

The activity measured in the clarified cell extract of the EcoRI enzyme isolated from *E. coli* 294 (pPG430) was found to be 3.43x10^6 U/(g-wet cells) whereas it was 2.13x10^5 U/(g-wet cells) in the case of *E. coli* M5248 (pSCC2). The existence of a 16-fold difference in the clarified cell extracts has clearly indicated that *E. coli* 294 with pPG430 is a better source for the efficient production of EcoRI endonuclease. The application of a two-step protocol for *E. coli* 294 (pPG430) and a three-step protocol for *E. coli* M5248 (pSCC2) resulted in enzyme yields of 3.3x10^6 units and 1.3x10^5 units of EcoRI endonuclease per gram wet cells with 96% and 61% recovery respectively. 3.1 and 1.2 mg of final product were obtained per gram of wet cells with specific activities of 1x10^6 U/mg and 1x10^5 U/mg from *E. coli* 294 (pPG430) and *E. coli* M5248 (pSCC2), respectively. SDS-PAGE analyses of the enzyme preparations obtained from the two different overproducing strains showed patterns identical to that of commercial EcoRI (Figure 5).
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Figure 3. The change in total protein concentration with respect to K-phosphate concentration in hydroxapatite chromatography.

Figure 4. Elution profile of the second phosphocellulose column chromatography for the *E. coli* M5248 strain. Arrows indicate the points where the buffer has been changed.

b) Quality Test Results: An overdigestion quality test indicated the absence of endo- and exo-nucleases in the final enzyme preparations (Figure 6). The same EcoRI preparations were also tested for their ability to ligate, and recut restriction fragments of λDNA and were found to be free of contaminants that would inhibit ligation or degrade termini (Figure 7). These results have clearly shown that the enzyme preparations were suitable for use in molecular biology.

Figure 5. SDS-PAGE analysis of the purified EcoRI endonuclease.
Lane 1 and 4: Commercial EcoRI endonuclease
Lane 2: EcoRI endonuclease purified from the *E. coli* M5248 (pSCC2) strain
Lane 3: EcoRI endonuclease purified from the *E. coli* 294 (pPG430) strain

Figure 6. Overdigestion of λDNA by EcoRI endonuclease purified from the *E. coli* 294 (pPG430) (lanes 1 and 2) and the *E. coli* M5248 (pSCC2) strain (lanes 3 and 4)
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Figure 7. Ligation-Recut analysis of EcoRI endonuclease
Lane 1: Initial cleavage of DNA by EcoRI endonuclease purified from the E. coli M5248 (pSCC2) strain
Lane 2: Initial cleavage of DNA by EcoRI endonuclease purified from the E. coli 294 (pPG430) strain
Lane 3: Ligation of DNA fragments produced by EcoRI endonuclease purified from the E. coli M5248 (pSCC2) strain
Lane 4: Ligation of DNA fragments produced by EcoRI endonuclease purified from the E. coli 294 (pPG430) strain
Lane 5: Recut of ligated fragments by EcoRI endonuclease purified from the E. coli M5248 (pSCC2) strain
Lane 6: Recut of ligated fragments by EcoRI endonuclease purified from the E. coli 294 (pPG430) strain

Discussion

The restriction endonuclease EcoRI has been purified in many laboratories using different purification procedures. Greene et al.\textsuperscript{5} have developed a method for the purification of EcoRI endonuclease from E. coli RY 13 strain. Their yield was 13 U/gcell. Modrich et al.\textsuperscript{1} have modified this method to increase the yield of the enzyme to 190 U/gcell from the same strain. Vlaktais and Bouritis\textsuperscript{14} have purified EcoRI endonuclease from the same strain by applying sequence specific DNA affinity chromatography and ended up with a yield of $1.8 \times 10^5$ U/g cell. Mehra et al.\textsuperscript{9}, on the other hand, have applied the dye-ligand chromatography to purify EcoRI endonuclease from E. coli RY 13. Their yield was $3 \times 10^4$ U/g cell. Cheng et al.\textsuperscript{2} have constructed an overproducing strain, E. coli M5248 (pSCC2), to purify EcoRI restriction and modification enzymes. Their purification method involves streptomycin and ammonium sulphate fractionations followed by phosphocellulose and hydroxyapatite chromatographies respectively. They have obtained 500 mg of enzyme per kg cell paste with a recovery of 47%. The specific activity of the EcoRI endonuclease was reported to be $4.5 \times 10^4$ U/mg protein. It can be calculated that Cheng et al.\textsuperscript{2} have obtained approximately $2.25 \times 10^4$ units of enzyme per gram cell. In the present study, an almost tenfold increase was obtained in the yield of the EcoRI endonuclease from E. coli M5248 (pSCC2) by the application of a new purification scheme.

On the other hand, the yield and the specific activity of the enzyme produced by E. coli M5248 (pSCC2) in this work was at a lower level when compared with the results reported by Luke and Halford\textsuperscript{6}. These investigators have used a different overproducing construct in which the gene encoding EcoRI was placed under the control of the same pL promoter, but the genes carrying the EcoRI methylase and cl-coded temperature sensitive repressor were on separate compatible plasmids. There are substantial differences in
both enzyme yield and enzyme specific activity between the two strains used by Cheng et al.\textsuperscript{2} and Luke and Halford\textsuperscript{6}, and these may be due to the lower expression of the M5248 (pSCC2) strain, resulting either from the distance between the pL promoter and the gene for EcoRI endonuclease or from the simultaneous placement of EcoRI and methylase genes on the same plasmid. The nature of the host cells and the plasmid copy number may be other important factors that lead to lower enzyme recovery in the case of the \textit{E. coli} M5248 (pSCC2) strain.

It has to be noted that several improvements may be possible at the fermentation level to improve the productivities of EcoRI endonuclease. A careful investigation of growth characteristics, recombinant gene expression and plasmid stability in these recombinant strains may allow the development of good model systems for predicting the yield of recombinant protein production in induced cultures.

### Acknowledgement

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### References

