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Optimization of Starting Time and Period of Induction and Inducer Concentration in the Production of the Restriction Enzyme *Eco*RI from Recombinant *Escherichia coli 294*

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Induction parameters including inducer concentration, period of induction and the cell concentration at which inducer is to be added to the fermentation broth were optimized in order to increase the yield of the *Eco*RI restriction endonuclease isolated from recombinant *E. coli*. Bacterial cells harboring the plasmid pPG430 containing *Eco*RI endonuclease and the methylase genes under the control of *lac* promoter were used in the experiments where induction was accomplished by using lactose isopropyl-β-D-thiogalactoside (IPTG). An IPTG concentration of 0.1mM, the late exponential phase of growth (at an optical density of 1.2 at 595 nm) and an induction period of 6 hours were determined to be the optimum conditions for induction.

Keywords: *Eco*RI, restriction enzyme, induction, enzyme purification.

Introduction

Restriction enzymes are very important tools of recombinant DNA technology. A considerable number of highly efficient expression vectors have been engineered in the past few years to obtain high yields of those enzymes encoded by cloned genes of procaryotic and eucaryotic origins[1]. Strong controllable promoters are preferred in the construction of the expression vectors to enhance the synthesis of the product. The induction conditions are considered to be important parameters in the production of the recombinant proteins and they need to be carefully optimized to increase the yield of the product.

*Eco*RI restriction endonuclease has proven extremely useful as a reagent in the analysis and manipulation of DNA molecules, and therefore it is one of the most widely used restriction enzymes. It recognizes a specific sequence on a double-stranded DNA molecule and it cleaves the sequence GAATTC between G and A on both strands[2]. In addition to the natural overproducer of *Eco*RI, *E. coli* RY13, genetically modified overproducing strains were also used to produce the enzyme. In many of these studies, the gene encoding *Eco*RI endonuclease was placed under the control of λp_L promoter and the production of the enzyme upon induction by a temperature shift was investigated[3].

In the present study, the plasmid pPG430 containing *Eco*RI endonuclease and methylase gene under the control of a *lac* promoter was used for the high level production of *Eco*RI endonuclease. The chemical induction of the *lac* promoter was accomplished by the addition of the non-hydrolizable analog of lactose isopropyl-β-D-thiogalactoside (IPTG). In order to increase the yield of the *Eco*RI endonuclease from this strain, the effects of the induction parameters on the production of the enzyme, i.e., the inducer concentration, the period of induction and the cell concentration when IPTG should be added to the fermentation broth were investigated and optimized.

Materials and Methods

Chemicals: Tryptone and yeast extract came from Difco (USA), phosphocellulose from Whatman (UK), hydroxyapatite from Bio-Rad (USA), IPTG, PMSF, BSA, Triton X-100, and acrylamide and agarose from Sigma (USA). All other chemicals were analytical grade and were supplied by Merck (Germany).

Bacterial Strain and Plasmids: *E. colu* 294 containing the plasmid pPG430, which is a derivative of pBR322, was kindly provided by Dr. Herbert Boyer (Department of Biochemistry, University of California, San Francisco). Plasmid pPG430 carries the genes encoding *Eco*RI endonuclease and methylase.

Medium and Culture Conditions: The culture used for all experiments was a classical L-broth containing 1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, and 1% NaCl (w/v)(pH 7.0). The culture medium was also supplemented with antibiotics to a final ampicillin concentration of 80 μg/ml for inoculum preparations to prevent the overgrowth of plasmid-free cells. Cells were grown at 37° C in shake flasks located in an orbital shaker at 180 rpm to specified absorbance values at 595 nm and then induced by a specified IPTG concentration over the specified induction period. The cells were harvested by centrifugation at 4000 rpm for 15 min with an Sorvall GSA rotor at 4° C and stored at $-20°$ C until further purification.

Purification of *Eco*RI Endonuclease: The methods described in the literature[2−9] were modified to develop a new protocol[10] that was used in the purification of *Eco*RI from recombinant *E. coli* 294. All the steps in this protocol were performed at $0-4°$ C. After thawing the cells, the cell paste was suspended in a lysis buffer composed of Buffer A (20 mM K-PO_4-pH 7.0, 2-mercaptoethanol, 1 mM EDTA, 0.2% Triton X-100) supplemented with a final concentration of 0.8 M NaCl and 0.1 M PMSF (phenylmethylsulfonyl fluoride). The cell suspension was sonicated during 5×30 sec time intervals while the suspension was kept on ice to prevent heating-up. The sonicated cell extract was dialysed against Buffer A containing 0.4 M NaCl. The lysate was clarified by centrifugation at 9500 rpm for 15 min by sorvall SS34 rotor at 4° C. The fraction at this step was named Fraction I and applied to the phosphocellulose column (2×50 cm) equilibrated with Buffer A containg 0.4 M NaCl. The subsequent elution was carried out stepwise with Buffer A containing increasing concentrations of NaCl (from 0.4 to 1.0 M). Active fractions which eluted at about 0.6 M NaCl were pooled and named Fraction II. Hydroxyapatite chromatography was applied to Fraction II, which was equilibrated with Buffer A containing 0.6 M NaCl. The elution was carried out by increasing concentrations of K-phosphate ranging from 0.1 M to 0.6 M in Buffer A with 0.6 M NaCl. The fractions eluted with 0.4 M K-phosphate containing the active enzyme were pooled and named Fraction III. Fraction III was diluted with Buffer A and applied to a second phosphocellulose column (1×10 cm). The elution was carried out as in the first phosphocellulose column. Finally the active fractions were pooled and named Fraction IV; this final fraction was supplemented with 50 μg/mL BSA (bovine serum albumin) and dialyzed against a storage buffer containing 50% glycerol, 10 μg/mL BSA, 10 mM K-phosphate (pH 7.0), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, and 0.1 % Triton X-100.

Enzyme Assays: The enzyme was assayed by the standard unit definition. One unit of enzyme

activity was defined as the amount of the enzyme required to produce a complete digestion of 1.0 μg λDNA at 37° C in 1 hour in a total reaction volume of 10 μL. The activity in units was then derived from the dilution factor by determining the highest dilution that still displays complete digestion, as described by Yıldır[10]. Specific activity was defined as the enzyme activity per mg of total protein. Complete digestion of λDNA results in the production of 6 fragments (21226, 7421, 5804, 5643, 4878 and 3530 bp, respectively), which are observed as five distinct bands on 0.8% agarose gels (Figure 1). The completion of the digestion was estimated visually under a transilluminator. Protein determination was conducted according to the Bradford method using BSA as the protein standard[11].

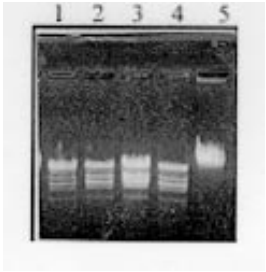

Figure 1. Electrophoretic analysis of λDNA digested with increasing dilutions of *Eco*RI restriction enzyme on 0.8 % agarose gels. Lanes 1-5: 2.5, 2.0, 1.5, 1.0 and 0.5 units of *Eco*RI restriction endonuclease, respectively, used for digesting one microgram of λDNA.

Results and Discussion

A parametric study was conducted to optimize the induction conditions in the recovery of the *Eco*RI enzyme from the overproducing strain of *E. coli* 294 (pPG430). In the plasmid pPG430, *Eco*RI endonuclease and methylase genes were under the control of the *lac* promoter. IPTG was used to induce the *lac* promoter for the synthesis of *Eco*RI endonuclease.

The Growth of Recombinant *E. coli* Cells

The growth behaviour of recombinant *E. coli* 294 cells containing the *Eco*RI overproducing plasmid pPG430 was investigated at 37° C. A lag phase of around three hours was observed in the growth of the cells. The exponential phase of cell growth was completed within 16 hours of inoculation, after which the cells entered their stationary phase. The growth curve is shown in Figure 2. The arrows on the growth curve indicate the OD_{595} values of the different phases of cell growth. Induction of cells were conducted at these stages of growth.

Time Course of Induction

In order to determine the time course of induction, two shake flask fermentations were performed in parallel. The first culture was grown at 37° C in LB medium until the OD_{595} reached 1.2, and then 0.1 mM IPTG was added to the medium. The same amount of IPTG was added to the second culture when its absorbance at 595 nm reached 0.5. After the addition of IPTG to the fermentation broths, incubation of flasks at 37° C was continued and samples were taken every hour from both cultures to determine the specific activity of the *Eco*RI endonuclease, as indicated above in the Enzyme Assays section. The enzyme activities were determined in cell extracts obtained after sonication without further purification in order to minimize other effects that may arise during purification. The maximum specific activity of the enzyme was found to be attained six hours after the addition of IPTG to the medium, after which a decrease in the

specific activity was observed in both of the shake flask fermentations (Figure 3). The addition of IPTG in the early or the late exponential phase did not have any meaningful effect on the time course of induction of enzyme synthesis. The highest level of enzyme synthesis was obtained by six hours of induction of the *E. coli* cells.

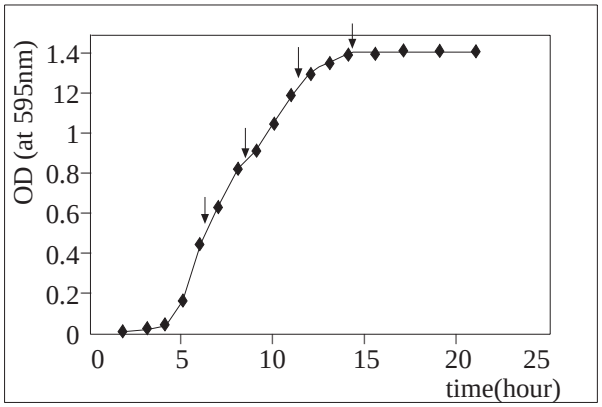

Figure 2. Growth curve of *E. coli* 294 (pPG430) (Arrows indicate the induction times where IPTG was added).

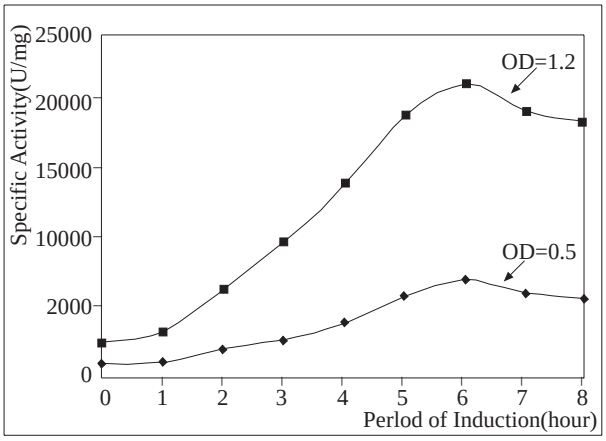

Figure 3. The effect of the period of induction on the *Eco*RI specific activity.

Optimum Starting Time of Induction

In order to determine the optimum starting time of induction, four shake-flask fermentations were performed in parallel, and each culture was induced at different phases of growth. The absorbance values 0.45, 0.9, 1.2 and 1.4 at 595 nm represent the early exponential, exponential, late exponential and stationary growth phases, respectively. Following the addition of 0.1 mM IPTG to each of the culture media, the incubation was continued over a 6-hour period, and enzymatic activity was determined in cell extracts after sonication. The results are presented in Table 1. The specific activity (U/mg) and the yield of the enzyme, i.e., the enzyme produced per gram cell (U/gcells), increased as the cell concentration increased until the stationary phase. A decrease in the *Eco*RI enzyme activity was observed if the induction was made at the stationary phase. This observation is in agreement with the results reported by Botterman et.al.[6], who showed that in the production of the *Eco*RI enzyme from *E. coli* 1100, an induction of gene expression by a temperature-shift during the exponential phase resulted in an approximately fivefold increase in productivity as compared to that of late induction during the stationary phase. The highest values of specific activity and enzyme yield were obtained when 0.1 mM IPTG was added to the medium at the late exponential phase

of growth (OD_{595} = 1.2). Therefore, the optimum starting time of induction for *E. coli* 294 (pPG430) was chosen to be the late exponential phase of growth.

Table 1. The effect of the starting time of induction on the production of *Eco*RI endonuclease from *E. coli* 294 (pPG430)

OD_{595}	C_{IPTG} (mM)	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Enzyme Yield (U/g cells)
0.45	0.1	100	246	1.5×10^6	6098	3.5×10^5
0.92	0.1	100	328	4.0×10^6	12195	9.0×10^5
1.2	0.1	100	416	9.8×10^6	23558	2.1×10^6
1.4	0.1	100	398	7.5×10^6	18844	1.7×10^6

The Effect of Inducer Concentration on the Efficiency of Induction

After determining the optimum starting time of induction, the effect of the inducer (IPTG) concentration on the efficiency of the induction was examined by using 0.1, 0.5 and 1 mM IPTG concentrations. Three parallel shake-flask fermentations were performed, and all cultures were grown until the OD_{595} reached 1.2 in the late exponential phase. Subsequently, 0.1, 0.5 and 1.0 mM IPTG was added to each culture flask, respectively, and the flasks were incubated for 6 hours. Cell extracts obtained after sonication were separately assayed for *Eco*RI endonuclease activity. A control experiment was also performed in which the addition of IPTG was omitted. In the control experiment, the cells also grew until the late exponential phase, and then their cell extracts were analyzed for *Eco*RI activity. Table 2 shows that IPTG induction resulted in approximately a 10-fold increase in the total activity and the specific activity of the enzyme. However, an increase in the IPTG concentration from 0.1 mM to 1 mM did not result in any significant improvement in enzyme production.

Table 2. The effect of IPTG concentration on the production of *Eco*RI endonuclease from *E. coli* 294 (pPG430) at OD_{595} = 1.2

C_{IPTG} (mM)	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Increase in Specific Activity	Enzyme Yield (U/g cells)
0	100	202	6.0×10^5	2970	1	1.5×10^5
0.1	100	403	1.05×10^7	26054	8.8	2.4×10^6
0.5	100	438	1.2×10^7	27397	9.2	2.9×10^6
1.0	100	452	1.3×10^7	28761	9.7	3×10^6

Purification of *Eco*RI Endonuclease

*Eco*RI endonuclease was purified both from cell cultures induced by 0.1 mM IPTG at an optical density of 1.2 at 595 nm and from uninduced cell cultures. Enzyme recoveries from these two cultures are compared in Table 3 and Table 4, with the results indicated at each purification step. The product yields obtained were 8.82×10^4 U/g cells and 2.3×10^6 U/g cells from uninduced cultures and cultures induced by 0.1 mM IPTG, respectively. A 25-fold increase in the enzyme yield was achieved after inducing the cell cultures by 0.1 mM IPTG, which was chosen as the optimum concentration for further experiments, since even a tenfold increase in the IPTG concentration does not lead to any significant improvement in enzyme production.

Table 3. Purification of *Eco*RI Endonuclease from cultures induced with 0.1 mM IPTG at OD_{595} = 1.2 (4.21 g cells)

Fractions	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Recovery (%)
Cell Extract	100	585.275	1.50×10^7	25 629	100
Fraction I	100	356.875	1.50×10^7	42 031	100
Fraction II	84	43.75	1.47×10^7	336 000	98
Fraction III	48	13.775	1.44×10^7	1 045 372	96
Fraction IV	55	9.023	9.63×10^6	1 066 718	64

Table 4. Purification of *Eco*RI endonuclease from uninduced cultures (5.1 g cells)

Fractions	Volume (mL)	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Recovery (%)
Cell Extract	100	389.421	9.0×10^5	2 311	100
Fraction I	100	264.182	7.5×10^5	2 838	83
Fraction II	84	41.250	6.75×10^5	16 363	75
Fraction III	48	18.29	5.25×10^5	28 704	58
Fraction IV	55	10.781	4.5×10^5	41 740	50

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