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EXPERIMENTAL ARTICLES

Induced Expression of *Eco*RI Endonuclease as an Active Maltose-Binding Fusion Protein in *Escherichia coli*[1]

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Abstract—Among the numerous bacterial Type II restriction enzymes, *Eco*RI endonuclease is the most extensively studied and is widely used in recombinant DNA technology. Its heterologous overexpression as recombinant protein has already been studied. However, very limited information concerning its fused product is available thus far. In the present study, the *Eco*RI restriction endonuclease gene was cloned and expressed as a part of maltose-binding fusion protein under the control of strong inducible *tac* promoter in TB1 strain of *Escherichia coli* cells. Transformed cells containing pMALc2X-*Eco*RI recombinant plasmid were unable to grow under experimental conditions. However, fused *Eco*RI protein was purified (with the yield of 0.01 mg/l of bacterial culture) by affinity chromatography from *E. coli* cells induced at the late exponential phase of growth. Restriction quality test revealed that the purified product could restrict a control plasmid DNA in vitro.

Keywords: *Eco*RI, endonuclease, fusion, maltose-binding protein, overexpression, restriction.

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Most bacteria posses a restriction–modification (R–M) system to serve as defense mechanism against intruding DNA molecules such as viral or plasmid DNAs. This system is composed of two separate enzymatic units including a restriction endonuclease that cleaves DNA at a specific recognition sequence and a DNA methyltransferase which is able to methylate the same sequence and to protect it against cleavage by the corresponding endonuclease. The restriction unit of the R–M system is generally classified into three classes Type I, Type II and Type III [1].

Type II DNA restriction enzymes are among the simplest sequence-specific DNA enzymes characterized so far. They only require Mg^{2+} as a cofactor and their DNA cleavage activity occurs at very specific sites that are within or close to the recognition sequence [1]. The vast majority of known restriction enzymes are of Type II, and the most characterized one is *Eco*RI which is staggered and its highly specific symmetrical recognition sequence is 5'-GAATTC-3' on duplex DNA molecule [2, 3]. It is composed of a single polypeptide (29 kDa) containing 277 amino acids that has been originally purified from a clinical strain of *Escherichia coli* RY13 a natural overproducer of *Eco*RI [4–6]. The physical and catalytic properties of *Eco*RI endonuclease and the regulation of its gene expression have been extensively studied by several groups [7–11].

A number of Type II restriction enzymes have been cloned, sequenced, over-expressed and purified because of their application potential in recombinant DNA technology and commercial benefits [12–16]. Since the isolation of these enzymes from their native host is often costly and results in very low yields upon purifications, overexpression of widely used restriction endonucleases in recombinant *E. coli* cells is advantageous for the high-yield recovery of these enzymes.

The recombinant expression of the *Eco*RI restriction–modification system has been investigated in both prokaryotic and eukaryotic systems. In bacteria, various genetically modified *Eco*RI overproducing strains of *E. coli* cells have been reported [17–19]. Based on the insertional inactivation and the lethality of *Eco*RI endonuclease, a series of bacterial positive selection vectors have been constructed and used in recombinant DNA technology [17, 20]. In addition to the use of strong regulable promoters, the induction conditions such as time and period of induction, inducer concentrations and temperature shifting have been found to be important in the overproduction of recombinant *Eco*RI endonuclease in recombinant bacteria [19]. In eukaryotes, expression of *Eco*RI restriction endonuclease has been studied in yeast cells under regulated conditions. It has been reported that expressed *Eco*RI protein interacts with the native

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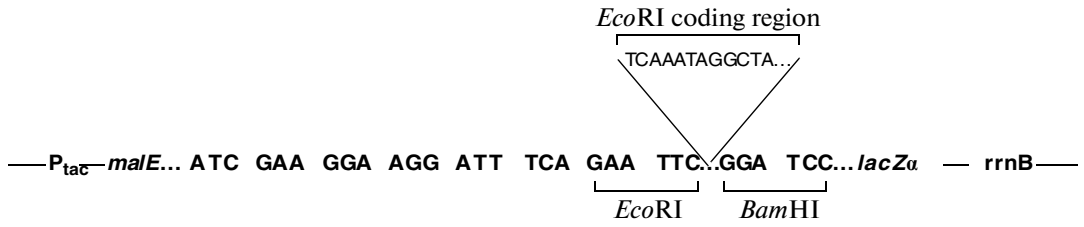


Fig. 1. The cloned map of the *EcoRI* gene on pMALc2X expression vector. *Eco*RI- and *Bam*HI-digested *EcoRI* gene was inserted, with its correct reading frame, downstream from the *malE* gene of *E. coli* resulting in an MBP (maltose-binding protein) fusion gene.

chromatin inside the yeast nucleus and cleaves the definition sites on the DNA molecules [21].

Despite these investigations, very limited information is available on fused products of *Eco*RI enzyme. Thus far, overexpression of *Eco*RI as a fusion protein consisting of protein. A from *Staphylococcus aureus* and *Eco*RI protein has been demonstrated [22, 23]. The bioactivity of this fused product has been found to be the same as of the native endonuclease. The controlled expression of this fused protein has also been recently studied by a three-plasmid harboring system [24].

Fusion tags are generally developed in order to facilitate protein purification and immobilization. They offer a powerful tool to enhance the folding and stability of the target protein moiety as fusion proteins can rapidly attain a native conformation. MBP fusion tags have often been found to minimally affect the bioactivity of the different fused products [25].

Here, we studied an overproducing MBP fusion construct in which the gene encoding *Eco*RI fused with a maltose-binding protein gene.

MATERIALS AND METHODS

Materials. *E. coli* strain RY13 was provided by Dr. M. Khosroshaheli (genetic laboratory, Department of Biology, Faculty of Science, University of Tabriz, Iran). *E. coli* strain TB1 and pMALc2X vector used for the recombinant construction and protein expression studies were supplied in a protein fusion and purification system Kit (Cat. no. E8000S; Bio New England Biolabs, UK). Plasmid vector pGEM-T for restriction test was from our laboratory stock. *Taq* DNA polymerase, buffer, dNTPs, and $MgCl_2$ used for PCR amplification were provided by CinnaGen, Iran. DNA Extraction Kit (Cat. no. K0513; Fermentas, Lithuania) was used for the purification of the PCR product from the agarose gel. Restriction enzymes *Eco*RI and *Bam*HI were purchased from CinnaGen Company. All the other chemicals used in this research work were of molecular biology grade.

Cloning of *EcoRI* restriction endonuclease gene. Cloning of *EcoRI* gene from *E. coli* strain RY13 was carried out by PCR using the sequence-specific primers (forward primer: 5'ACACGAATTCTCAAATAGGCTAACTGAAC3'; reverse primer: 5'ACCAGGATCCAAACAAGTCACGCCCCAAC3') were designed based on 5' and 3' ends of *EcoRI* gene sequence reported previously [26]. To do the directional cloning of the PCR-amplified fragment in *E. coli* expression vector, *Eco*RI and *Bam*HI restriction sites were included at the 5' ends of each primer. PCR reaction was carried out in a reaction volume of 20 μl containing 5× PCR buffer, 1 ng template DNA, 1 pmol of each primer, 10 mM dNTP mix, and 2 units *Taq* DNA polymerase. Amplification was performed in a Techneh (Germany) type thermal cycler, with 25 cycles of 1 min denaturation at 95°C, 2 min annealing at 55°C, and 2 min of extension at 72°C, ending with 10 min of incubation at 72°C. The amplified product was analyzed in 1% agarose gel using the TBE running buffer and finally extracted from the gel to proceed the next experiments.

Expression of the *EcoRI* gene as a fused product. The PCR amplification product after the agarose gel purification step was digested with *Eco*RI and *Bam*HI restriction enzymes and ligated into the pMALc2X expression vector which was linearized at the *Eco*RI and *Bam*HI sites within the multiple cloning region (Fig. 1). The ligation mixture was transferred into competent *E. coli* TB1 cells as follows: for the preparation of competent cells, the cells were grown in LB media up to $OD_{600} = 0.4$, incubated on ice for 15 min, centrifuged at 3500 rpm for 10 min at 4°C and washed with 10 ml of 100 mM $CaCl_2$. They were then centrifuged at 5000 rpm for 10 min, resuspended in 2 ml of chilled 50 mM $CaCl_2$ and stored on ice for 12 h. For transformation of bacterial cells, the ligation reaction mixture was supplemented with 25 μl competent cells, incubated on ice for 5 min, heated to 42°C for 2 min and after addition of 0.1 ml LB medium, it was incubated at 37°C for 20 min. The transformed cells were plated on LB medium (supplemented with Amp and X-gal) at 37°C and a recombinant clone was selected for gene expression studies.

Extraction and purification of expressed fusion protein. To extract the fusion protein, transformed cells were grown in 500 ml of rich broth/glucose/Amp up to $OD_{600} = 1.0$. For the induction of fused protein expression, the inducer IPTG was added to a final concentration of 0.3 mM; the mixture was then incubated for 2 h. The cells were harvested by centrifuga-

tion at 4000 *g* for 10 min and the pellet was dissolved in 25 ml of extraction buffer containing 20 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA, 1 mM sodium azide and 10 mM BME. The cells were frozen in the same buffer at –20°C overnight and were then sonicated in short pulses of 15 s. The sample was then centrifuged at 10000 *g* at 4°C for 20 min and the supernatant was used as a crude extract.

The fusion protein was purified from the crude extract by affinity column chromatography. It was done in a column packed with the amylase resin specific for the maltose-binding protein (MBP), which was a part of fused protein. The fusion protein was eluted out from the column with the column buffer (extraction buffer plus 10 mM maltose). The eluted product was analyzed for its homogeneity by SDS-PAGE electrophoresis using 10% polyacrylamide gel [27].

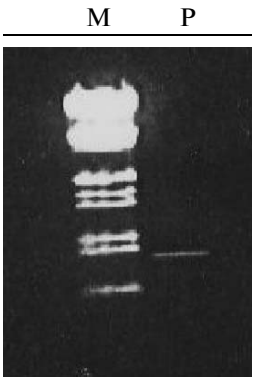
Protein content of the samples was determined by the Bradford method using BSA (bovine serum albumin) as a protein standard [28].

In vitro restriction activity test. To determine the endonuclease activity of the purified *Eco*RI fusion protein, pGEM-T easy plasmid containing two *Eco*RI recognition sites DNA was digested with different dilutions of the fused product [29]. The reactions were carried out at 37°C for 1 h. Digestion products were analyzed by electrophoresis in 0.8% agarose gel and compared with two control samples, including uncut plasmid DNA and the plasmid cut with commercialized *Eco*RI enzyme. The assessment was carried out by the unit definition which was derived from the highest dilution sample that still displayed complete digestion. One unit of *Eco*RI restriction activity was defined as the amount of enzyme required digesting 1 μg of plasmid DNA to completion in 1 h at 37°C.

RESULTS AND DISCUSSION

Cloning of *EcoRI* gene as fused DNA. In the present study, the *Eco*RI restriction endonuclease gene of *E. coli* strain RY13 was amplified using a pair of specific primers containing the *Eco*RI and *Bam*HI RI recognition sequences at their 5' ends. The amplification product was analyzed in 0.8% agarose gel and photographed (Fig. 2). The result showed a detectable amplified band of approx. 800 bp in size. The molecular mass of the detected product was consistent with the calculated size. However, further to confirm the correspondence of amplified product to *Eco*RI gene, its 5' end was partially sequenced by Microsynth DNA sequencing center in Switzerland. The sequence data is shown in Fig. 2. The sequence result was consistent with the *EcoRI* gene sequence reported previously [26].

For directional cloning of the *Eco*RI fragment, it was restricted with *Eco*RI and *Bam*RI enzymes and was then cloned into the *Eco*RI and *Bam*HI sites within the multiple cloning region of the pMALc2X expression vector with correct reading frame. Transformed TB1 cells harboring the recombinant pMALc2X-*Eco*RI vector were allowed to grow under the induced and non-induced conditions. The results showed that these cells could grow on LB plates containing ampicillin and X-gal in the absence of inducer molecules. However, they were not able to grow on the medium containing IPTG. This result suggests that *EcoRI* gene was expressed as an active fused product in the recombinant cells under induced conditions and there was no leaky expression of the fusion product at the lethal level under non-induced conditions.



5' ACACGAATTCTCAAATAGGCTA
ACTGAACAACATAAGTTATCTCAA
GGTGTAATTGGGATTTTGGGGAT
TATGCAAAAGCTCATGATCTCGCT
GTTGGTGAGGTTTCAAAATTAGTA
AAGAAAGCTCTTAGCAACGAATAC
CCTCAATTATCATTTCGATATAGA
GATAGTATAAAGAAAACAGAAATA
AATGAAGCTTTAAAAAAAATTGAC
CCTGATCTTGGCGGTACTTTATTT
GTTTCAAATTCCAGCATCAAACCT
GRTGGTGGAATTGTAGAGGTCAAA
GATGATT...3'

Fig. 2. Analysis of PCR-amplified *EcoRI* gene. The *EcoRI* gene was amplified from *E. coli* strain RY13 using a pair of specific primers designed based on the 5' and 3' ends of the previously reported gene sequence. Left: analysis of the PCR product in 0.8% agarose gel. M: *Eco*RI and *Hin*dIII double digested Lambda DNA marker; P: PCR product. Right: partial nucleotide sequence of the 5' end of amplified gene.

Late induction of fused *Eco*RI gene expression for purification of its product. Since induction with 0.3 mM IPTG at the early growth stage inhibited bacterial growth, bacterial cells were induced at the late exponential growth phase when the OD_{600} of the culture reached to 1.0. The induction period was extended for 2 h and the induced cells were used for the crude extract protein extraction and purification process by maltose-binding affinity chromatography as described in the Materials and Methods section. The purification yield of the expressed product was about 0.01 mg per liter of bacterial culture. SDS-PAGE analysis of the crude protein preparations obtained from non-induced and induced bacterial cultures on 10% polyacrylamide gel revealed that *Eco*RI protein is expressed as a part of the maltose-binding protein fused product with a molecular mass of about 72 kDa (Fig. 3). This was the expected size, considering the presence of MBP (42.482), the product of 270 amino acid residues of the *Eco*RI gene, and six amino acid residues from the expression vector. The purified fused product was also analyzed by SDS-PAGE and compared with crude samples as shown in Fig. 3.

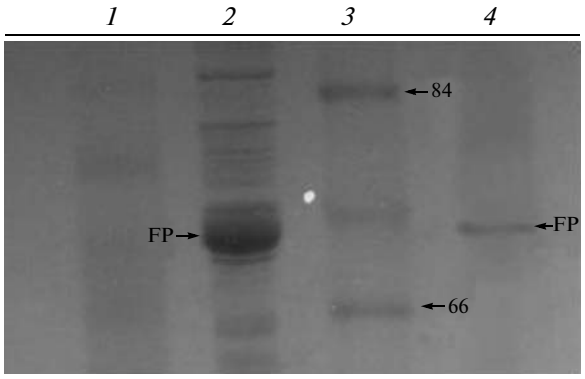


Fig. 3. SDS-PAGE analysis of the fused product expression. The crude protein extractions obtained from non-induced and induced bacterial cultures were electrophoresed in 10% polyacrylamide gel along with the purified fused protein prepared by affinity chromatography. The gel stained with coomassie brilliant blue dye and photographed. As it was shown, the expressed fused product (FP) was detected as a sharp band having a molecular size of about 72 kDa. Lane *1*: non-induced sample; lane *2*: induced sample; lane *3*: protein marker and lane *4*: purified sample.

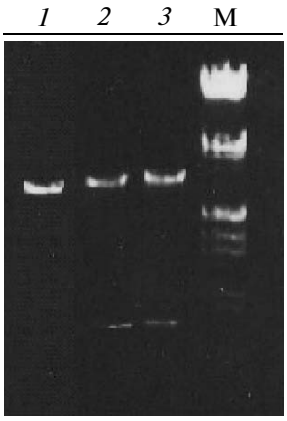


Fig. 4. Restriction quality test for the purified fused *Eco*RI. A standard plasmid DNA molecule (recombinant pGEM-T easy) containing two *Eco*RI recognition sites was restricted separately with one unit of each purified fused enzyme and commercial one. The restricted products were analyzed on 0.8% agarose gel and photographed. As it was shown, the fused enzyme was able to restrict plasmid DNA and its activity was comparable to the commercial enzyme activity. Lane *1*: uncut plasmid DNA; lane *2*: cut plasmid with purified fused MBP-*Eco*RI; lane *3*: cut plasmid with commercial enzyme; M: *Eco*RI and *Hin*dIII double digested Lambda DNA marker.

Restriction activity test on fused *Eco*RI enzyme. Induction of recombinant bacteria harboring the fused MBP-*Eco*RI protein at the beginning of their growth resulted in cell death. This phenomenon suggests that *Eco*RI enzyme might be active when it is expressed in recombinant cells as a part of maltose-binding fusion protein. To test this prediction, the purified fused protein prepared from late-induced cells was tested for in vitro specific restriction activity. The assessment was carried out using a circular plasmid DNA containing two *Eco*RI restriction sites as described in the Materials and Methods section. The specific activity of the fused enzyme was calculated to be about 2.9×10^3 units per mg of the purified fused product obtained by affinity chromatography. The product exhibiting one unit of endonuclease activity of the fused *Eco*RI was separated on 0.8% agarose gel and compared with two control samples including uncut plasmid DNA and digested plasmid using the commercial *Eco*RI enzyme (Fig. 4). The photograph demonstrates that the fused *Eco*RI enzyme could restrict plasmid DNA. One unit of *Eco*RI restriction activity was defined as the amount of enzyme required for digesting 1 μg of plasmid DNA to completion in 1 h at 37°C.

Since the discovery of the natural over-producer of *Eco*RI, *E. coli* RY13, several recombinant strains had been constructed and used for high-level production of this enzyme. Most of these studies were related to *Eco*RI gene expression under the control of λp promoters and inducible *lac* promoters with different induction parameters such as the inducer concentration, the period of induction and temperature shifting [2, 17–19]. Despite the recombinant studies on the *Eco*RI restriction system, very limited information concerning its fused products and its catalytic activities is available in the literatures. Overexpression of *Eco*RI endonuclease as a fusion protein consisting of protein A from *S. aureus* and *Eco*RI protein had been reported earlier [22, 23]. Under induced condition using the λp_R promoter, it has been found that the biological activity of the fusion protein was the same as that of the native endonuclease and there was no need for cleavage of the fusion product. The expression of the fusion protein SpA::*Eco*RI has been also studied in *E. coli* cells employing the combinations of three different types of multicopy plasmids. The fused enzyme activity has been reported to be higher in a specific plasmid combination and temperature shifting [23]. A new molecular model for the regulation of SpA::*Eco*RI fusion protein expression has been recently proposed and used to maximize the production of this protein [24].

In this present investigation, we used a different form of fusion construct in which the gene encoding *EcoRI* was fused downstream to the gene of maltose-binding protein on the pMALc2X expression vector. In MBP overproducing constructs, the strong '*tac*' promoter and the *malE* translation initiation signals are used to give high-level expression of the cloned sequences [30, 31]. On the other hand, a one-step purification method is used to purify the fusion protein using MBPs affinity for maltose [32].

Comparison of recombinant bacterial growth under the induced and non-induced conditions showed that the fused product is expressed as an active form which is lethal to recombinant bacteria. Also it

was deduced that there was no leaky expression of the fusion product at toxicity level under non-induced conditions. The lower purification yield of the expressed product (about 0.01 mg per liter of bacterial suspension under induced conditions) further confirmed that the fused *Eco*RI protein was toxic to recombinant bacteria.

The specific restriction activity measured for fused MBP-*Eco*RI was calculated to be about 2.9×10^3 units per mg of purified protein on plasmid DNA. This activity may be comparable to those of previously studied recombinant forms of the *Eco*RI enzyme [2, 6, 7, 33]. The recent results for the recombinant *Eco*RI specific activity were reported to be about 1×10^6 and 1×10^5 units per mg of recombinant enzyme purified from overproducing *E. coli* strains M5248 and 294, respectively [3]. Considering the highest molecular weight of fused MBP-*Eco*RI protein compared to the other recombinant forms of *Eco*RI enzymes, it seems that this fused product is an active protein. Therefore, it may not require cleavage of the fused product or further processing, as it has been already reported in the case of the SpA::*Eco*RI fusion protein [22, 23, 34].

Despite these experiments, several attempts may be needed concerning continuous/induced expression of this product in the presence of cognate methylase to improve the efficiency of MBP-*Eco*RI fusion protein production.

CONCLUSIONS

Using a different overproducing fusion construct in which the gene encoding *Eco*RI was fused with maltose-binding protein gene under the control of an inducible *tac* promoter, it was shown that the fused *Eco*RI enzyme may be active as like as other recombinant forms of the *Eco*RI enzyme.

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