Purifacation of Wild Type EcoRⅠ Endonuclease and EcoRⅠ Endonuclease RS187 Crystal Growth of WT EcoRⅠ Endonuclease-DNA Complex and Endonuclease RS187-DNA Complex

by

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EcoRⅠ endonuclease is a very useful tool to study the structural mechanism of protein-DNA recognition. In this work, wild type EcoRⅠ Endonuclease and EcoRⅠ Endonuclease mutant RS187 were purified to high purity. Crystals of wild type EcoR Ⅰ Endonuclease-DNA 13mer complex have been obtained with good size and shape. Some small crystals of EcoRⅠ Endonuclease RS187-DNA 13mer complex were also grown.

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1.0 INTRODUCTION

DNA has stored the biological information for many significant biological processes. Important steps in the processing of the information stored in the DNA of every living organism are carried out by protein molecules. The recognition of base sequences in double helical DNA is central to this process. Explanation of the molecular basis of protein-DNA interactions could help us to understand regulation of gene expression, site specific recombination and design novel therapeutic agents and new generation of research tools in genetic engineering (Grigorescu et al., 2003).

DNA binding proteins can recognize a specific sequence from much of other sequences present in the DNA. Most of DNA binding proteins recognize their cognate sequence through a facilitated diffusion mechanism involving transient, non-specific interactions with the DNA molecules. Tight binding only occurs at the target position. This recognition mechanism makes the process of protein-DNA recognition more complex.

1.1 ENERGETIC BASIS FOR DNA RECOGNITION

To form the different complexes with various DNA sites, proteins are able to select the DNA sequences through modulated affinity and differential stability. Some proteins show very strong recognition, but others can discriminate against nonspecific sequences by modulated affinity.

The complex formed by DNA binding proteins such as repressors, transcriptional activators or restriction endonucleases, with the target DNA site, is considered as the specific or cognate complex. Type Ⅱ restriction endonucleases (EcoRⅠ, BamHⅠ, EcoRⅤ, MunⅠ, BglⅡ, etc), could recognize hexanucleotide palindromic sites that differ by two or more base-pairs from their target site and are considered as non-specific sites(Grigorescu et al., 2003). The DNA sites selected by Type Ⅱ restriction endonucleases that differ by only one base-pair from their target sequence are considered as miscognate sites. Different from restriction endonucleases, transcriptional factors or nuclear hormone receptors can recognize various related DNA sites by tuning binding affinity.

Binding specificity can elucidated energetically. For instance, with the binding specificity ratio that is referred as the ratio of the intrinsic binding constants for a specific and a non-specific binding site, respectively, the DNA binding specificity can be measured. According to Jen-Jacobson' research, repressors and restriction endonucleases have the highest specificity ratios (105-107 under physiological conditions). Because solution conditions such as pH, temperature, nature and concentrations of ions, osmotic strength, and even the DNA flanking sequences surrounding the target site, are able to influence specific and non-specific binding constants of some DNA binding proteins such as restriction endonucleases, the binding specificity ratio is sensitive to the solution conditions and sequence context.

Free energy differences ($\triangle\triangle G^0$bind) between different complexes, could be used to measure binding specificity. The binding free energy ($\triangle G^0$bind) could be calculated through the measured equilibrium binding constant (KA) as:

$$\triangle G^0\text{bind} = -RT\ln KA$$

If we measure the equilibrium binding constants under common solution conditions, the relative free energy differences in respect to a reference DNA fragment $\triangle\triangle G^0$bind can be calculated. Therefore $\triangle\triangle G^0$bind is a useful tool to measure the binding specificity (Grigorescu, 2003).

During non-specific binding, the mobile solvent molecules appear to be retained at the loose and dynamic interface formed by the protein and DNA molecules. In this binding mode, DNA binding proteins are able to bind a generic DNA molecule regardless of their sequence. However, in the process of specific binding, the stabilization of the interface of protein-DNA complex is involved in the formation of direct and water mediated hydrogen bond, electrostatic and van der waals contacts, and often accompanied by obvious conformational changes in the protein and DNA molecules (Grigorescu et al., 2003, D.F. Cao, 2002). The study also displays that much mobile water molecules are released from the interface upon specific binding (Grigorescu et al., 2003). Many studies on the binding free energy have been carried out for some specific DNA binding proteins such as cro (Takeda et al 1992), trp (Otwinowski, 1988), lac and λ repressors, TATA binding protein (Grigorescu et al., 2003), EcoRI and BamHI restriction endonucleases (Eftink, 1983, Duan, 1996, Jen-Jacoboson et al 2000b), transcriptional activator GCN4-bZIP (Grigorescu et al., 2003). All of the mentioned studies show that the formation of the specific protein-DNA complexes is not driven by any universal electrostatic process. By contrast, because of the specific function of the protein, the affinity of each DNA binding protein for its specific site is carefully regulated and optimized (Grigorescu, 2003).

It is only the first step for understanding the critical factors that control protein-DNA recognition to analyze the several binding models. To deeply understand the mechanism of DNA

sequence recognition, more structural studies on free DNA binding proteins and protein-DNA complex should be performed to characterize the structural basis for DNA recognition..

1.2 STRUCTURAL BASIS FOR DNA RECOGNITION.

In the process of "direct read-out", through hydrogen bonds and van der Waals contacts, a DNA binding protein could interact with the functional groups exposed at the edges of the bases in the DNA sequence. If we check the structures which are stored in the Protein Data Bank, we can easily find extensive direct or water mediated interactions between side chain or main chain atoms of the protein and the function of groups of the DNA in these structures. In the process of "Indirect read-out", by identifying precise sugar and phosphate geometries, and conformational properties of the DNA backbone, the DNA binding proteins are able to recognize their cognate sites. According to the structural studies, "Indirect read-out" has the similar effect on the DNA sequence recognition as the direct read-out. Besides of sequence recognition, DNA distortion also contributes to the ability of the DNA binding proteins to discriminate the related DNA sequences (Grigorescu et al., 2003). Many structural studies have shown that the type and degree of DNA distortion varies widely in different protein-DNA complexes. For example, in some complexes, proteins can dramatically bend, kink and unwind the DNA, but in other complexes, the proteins induce very little DNA distortion. It has been proposed that the degree of DNA distortion is related to the thermodynamic features of site-specific binding (Jen-Jacobson et al. 2000b).

Many DNA binding proteins utilize "clamp phosphate" contacts (phosphate neutralization at precise positions), insertion of protein side chains between the DNA bases to

induce and stabilize the DNA distortion which facilitates the formation of complementary interface between protein and DNA molecules. From an energetic perspective the energetic cost of DNA distortion can help the protein to distinguish between related DNA fragments (Jen-Jacobson 1997, Chen et al 2001).

To form the large interface with DNA molecules, the globular protein mainly uses three structural mechanisms to extend the area of the interface between protein and DNA. In the first mechanism, dependent on inducing the DNA distortion, some DNA binding proteins such as TATA box binding protein, PU. 1 EST domain and CAP activator (Grigorescu et al., 2003), can create a large size of interface. In second mechanism, some DNA binding proteins such as DNA enzymes (Jones et al. 1996, 1999) can make use of some specific flexible domains that can fold into specific conformations such as arm-like structure to envelope the DNA and extend the interface. In third mechanism, some DNA binding proteins such as Zif268 including leucine zipper bZIP fold (Grigorescu et al., 2003), contain reading domain or reading heads that fold upon DNA binding.

In order to increase the ability of the protein to discriminate between closely related sequences, Bacterial endonucleases and repressors can make the recognition of two symmetric DNA sites translate into interaction between identical protein subunits through functioning as homodimers and checking the symmetry of their target sits. Eukaryotic transcriptional and regulatory mechanism may use a similar principle to enhance the cooperation between several proteins bound to different DNA sites (Grigorescu et al., 2003).

Structural studies on protein-DNA complexes also show that most of structural elements such as α helices, 3_{10} helices, β strands, hairpins and loops are involved in DNA recognition.

Therefore the structural strategies utilized by protein to discriminate the DNA fragments are very different (Jen-Jacobson et al., 2000b).

In order to deeply understanding the significant factors control the DNA recognition, we should carefully research the proteins from different families and the classes. Type Ⅱ restriction endonucleases provide us the good model systems to study protein-DNA interactions.

1.3 TYPE Ⅱ RESTRICTION ENDONUCLEASES

Type Ⅱ restriction –modification systems consist of two separate enzymes, a homodimeric restriction endonuclease and a monomeric methyltransfrerase. Type Ⅱ restriction endonucleases are able to protect bacterial cells against bacteriophage infection, by specifically and accurately recognizing target sites without an energy consuming proof-reading mechanism. A particular palindromic and unmethylated sequence which is typically 4-8 base pairs in length, can be recognized and cut by all of these restriction endonucleases. Now it is clear that the cut sites are precisely positioned in the cognate sequence, and in a single DNA binding process, double DNA helices can be cleaved. A metal cofactor such as Mg^{2+} is also required to hydrolyze the phosphodiester bonds (Grigorescu et al., 2003).

Although most Type Ⅱ restriction endonucleases, are homodimers and are of the similar size, these proteins do not share similarity in protein sequence. On the other hand, Type Ⅱ restriction endonucleases contain a similar α / β conformation and a common core motif which consists of a mixed β sheet surrounded by two α helices. However, idiosyncratic and unique structural elements that consist of small domains or long loops, sometimes even the segments

interspersing the common-core motif, can be identified in all of these proteins (Grigorescu et al., 2003).

By analyzing the structure of several complexes formed by Type Ⅱ restriction endonucleases with their target DNA sequences (Kim et al 1990, Winkler et al, 1993, Athanasiadis et al 1994, Newman et al 1994, Diebert et al 1999), we can find that the structural strategies for dimerization and DNA binding vary widely. For example EcoRⅠ, BamHⅠ, MunⅠ bind in the major groove of the DNA molecule, but EcoRⅤ and PvuⅡ bind in the minor groove. In addition, these restriction endonucleases in their DNA binding complexes can induce a varied degree of DNA distortion. Though the obvious differences exist between these complexes, we also can find all of the restriction endonucleases recognize target DNA in an envelope mode, and interact with the sugar-phosphate backbone outside their binding site. The interfaces between endonuclease and DNA molecules have the similar area and complementarity (Grigorescu et al., 2003).

At energetic level, Jen-Jacobson and coworkers (Jen-Jacoboson et al 2000a, Engler, 1998, Lesser et al), have analyzed the interaction between EcoRⅠ, BamHⅠ and EcoRⅤ with DNA molecules. For instance, at thermodynamic level, the studies for specific and non-specific binding of EcoRⅠ, BamHⅠ and EcoRⅤ have been carried out. Especially, the energetic dissection of the sensitivity of EcoRⅠ and BamHⅠ to base analog substitution and flanking sequences in DNA molecule has been invesgated by Jen-Jacoboson (2000a). All of these studies have revealed that in the specific binding process heat capacity upon complex formation shows a large negative change.

Structural studies on the DNA binding have been performed. Winkler (1993) and Viadiu (2000) have respectively reported the crystal structures of the complex formed by EcoRⅤ with

two short DNA oligomers, and the complex formed by BamHⅠ with a non-target DNA duplex. At the loose and dynamic protein-DNA interfaces in these crystal structures, the structural complementarity can not be found, and the mobile water molecules are retained. Jen-Jacoboson and Cao also reported in the non-specific binding process of EcoRⅠ, BamHⅠ and EcoRⅤ, few water molecules are released from these loose interfaces. In contrast, in specific binding mode, the mobile water molecules are released from the interfaces.

Many studies have revealed that the DNA recognition is closely related to the cleavage function of endonucleases (Rosenberg 1991, Jen-Jacoboson 1997, Pingoud and Jeltsch 1997). In the non-specific complexes, restriction endonucleases maintain their overall conformation but the local segments of the enzyme are more disorded than in the specific complexes. In the cases of EcoRⅤ and BamHⅠ, the lack of a tight complementary interface which is formed by conformational changes in DNA or protein, could influence their recognition and catalytic function (Grigorescu et al., 2003).

The studies on different type Ⅱ restriction edonucleases can help us to deeply understand the critical factors that control the DNA recognition. For this purpose, the structure of free endonucleases, DNA molecules and protein-DNA complexes are required to analyze these factors. The work described in the chapter 2 and chapter 3 has focuses on the purification and protein-DNA crystal growth of wild type and mutated endonuclease EcoRⅠ.

1.4 ECORⅠ RESTRICTION ENDONUCLEASE

EcoRⅠ endonuclease is a member of the Type Ⅱ restriction endonuclease family, which can protect host bacterial cell through rapidly hydrolyzing foreign DNA injected into the host cell. EcoRⅠ endonuclease whose monomer is only 277 amino-acids long, can form stable,

catalytically active homodimers in solution. Without the help of the cofactor, Mg^{+2}, EcoR Ⅰ can recognize its target DNA site (G/AATTC), but not hydrolyze DNA molecule. Because of these advantages mentioned above, EcoR Ⅰ endonuclease becomes a good research model for a X-ray structure determination of a protein-DNA complex (reviewed by Jen-Jacoboson 1997, Heitman 1990a and 1990b, Rosenberg 1991). In 1986, Rosenberg firstly determined the crystal structure of EcoR Ⅰ-DNA complex. By X-ray crystallography and MIR phasing, the structures at 3.0Å and 2.7Å were determined (Berman 1986, McClarin et al 1986, Kim et al 1990, Grigorescu et al., 2003).

In these structures of EcoR Ⅰ-DNA complex, we can find the homodimeric enzyme whose dimensions is 55 X 65 X 50Å, binds to the DNA major groove. This complex consists of a DNA duplex and two identical protein subunits related by a central axis of two-fold totational symmetry. The molecular architecture of EcoR Ⅰ endonuclease in the complex shows that the core of the EcoR Ⅰ monomer is a parallel five-strended β sheet. In this β-sheet, the second strand (β2) is anti parallel to the other four strands. The significant residues: Asp^{91}, Glu^{111} and Lys^{113} in active site of EcoR Ⅰ are located in a β meander formed by β1 and β3. With the help of two crossover helices (α4 and α5), β3 and β5 can form a binding fold motif named as half Rossmann motif which is the core of both the DNA-endonuclease and endonuclease-endonuclease interfaces. Besides of the core motif, β bridge (regions 58-90), inner arm (regions 116-140), outer arm (region 171-200) and β hairpin (region 221-232) also play significant roles in dimerization and DNA recognition (Venclovas, 1995, Grigorescu et al., 2003). Through hydrogen bonds between the peptide amide atoms and the phosphate group of DNA, the DNA molecule can is bound in the cleft formed by β bridge. Inner arm and outer arm participate in the

sequence-specific contacts to the bases, and wrap the DNA and hold it against the recognition interface (Grigorescu et al., 2003).

The sequence-specific interactions in EcoR Ⅰ-DNA complex can be divided into three modes. The first mode is direct interaction that is formed by interaction between protein and the bases in target site. The second mode is indirect interaction that is formed by intra DNA interaction contributing to the deformability of DNA at target site. The third mode is "Buttressing" interaction that is formed by the interaction between amino acids contacting DNA target site. All of three interactions form an extensive recognition network to stabilize the protein-DNA interface. In this recognition network, there are several driving forces (hydrogen bonds, Van der Waals contacts and electrostatic interactions) which make the interaction between protein and the bases of the target sites or the sugar-phosphate backbone both inside and outside the target site (Grigorescu et al., 2003).

In the direct interactions of EcoR Ⅰ-DNA complex, there are seven hydrogen bonds that are formed by the interaction between amino acid side chains and purines. There are also two hydrogen bonds which are formed by the interaction between protein backbone and pyrimidines. At the complete EcoR Ⅰ-DNA interface, there are a total of 18 protein-base hydrogen bonds. Depending on the different number of hydrogen bonds formed by purines and pyrimidines, EcoR Ⅰ can discriminate the different bases. In the high resolution crystal structure of EcoR Ⅰ-DNA complex, a new hydrogen bond, CH-O hydrogen bond formed by Gly^{140} C α atom and the O4 of T2 has been identified. It is believed that this CH-O hydrogen bond is a important structural feature to contribute to the DNA recognition. The bound water molecules at interfaces such as W1 which bind with Arg^{200} and Arg^{203}, can facilitate the sequence-specific binding. Recently, 20 bound water molecules have been found to participate in the interaction between protein and

DNA. In these water molecules there are five water molecules which are only found in the high resolution crystal structure. Therefore, it is very important for structural study of EcoRⅠ-DNA complex to achieve high purity protein sample and the crystal with high resolution (Grigorescu et al., 2003).

It is interesting to note that the solution condition such as elevtated pH, Mn^{2+}, low ionic strength or organic solvent, can make EcoRⅠ lose the normal tight specificity (Grable 1984, 1990). So through carefully adjusting the conditions of crystal growth and purification, we can analyze the structural basis for different EcoRⅠ-DNA specific binding under different conditions to identify the significant factors that control the DNA recognition.

In the follwing work, wild type EcoRⅠ endonuclease is purified, and the crystals of WT EcoRⅠ endonuclease-DNA 13mer are grown (chapter2). DNA 13mer is synthesized by DNA synthesis facility of university of Pittsburgh, the sequence of which is TCGCGAATTCGCG.

2.0 PURIFICATION OF WILD TYPE ECOR Ⅰ ENDONUCLEASE AND CRYSTAL GROWTH OF WT ECOR Ⅰ ENDONUCLEASE-DNA 13MER (TCGCGAATTCGCG) COMPLEX

2.1 EXPERIMENTAL METHODS AND RESULTS

2.1.1 Transformation

Wild type EcoR Ⅰ endonuclease is expressed from Escherichia coli strain ER2566/pAXU22-8. This Escherichia coli strain provided by the Jen-Jacobson lab, contains the T7 RNA polymerase gene inserted into the lac z gene. Transcription of T7 RNA polymerase is under control of the lac promoter. The Escherichia coli strain has been transformed with plasmid pAXU22-8 which contains the EcoR Ⅰ methyltransferase expressed from a constitutive promoter. Therefore, if Escherichia coli strain is not transformed with plasmid pAXU22-8, it will be lethal for Escherichia coli cells to express EcoR Ⅰ restriction endonuclease. This plasmid confers resistance to the antibiotic chloramphenicol (Cam) on the cells that harbor the plasmid.

Besides plasmid pAXU22-8, Escherichia coli cells should also be transformed with plasmid pPS12a. Plasmid pPS12a derived from a commercially available pET vector, contains a gene encoding EcoR Ⅰ restriction endonuclease which is inserted into the NheI/Bam HI sites of the vector. Expression of EcoR Ⅰ restriction endonuclease is under control of T7 RNA

polymerase gene which in turn is under control of the lac promoter. So it is clear that only grown in the LB medium, EcoR Ⅰ restriction endonuclease can be expressed. This plasmid confers resistance to the antibiotic Kanamycin (Kan) on the cells that contain the plasmid. Unlike plasmid pAXU22-8, usually plasmid pPS12a is not transformed into E.coli cells. So the transformation experiments need be carried out by following steps.

1. Competent cells (E.coli cells with pAXU22-8) which are stored at -80°C, is thawed on the ice for 20-30 minutes. Like competent, plasmid pPS12a also is thawed on ice for about 15 minutes.
2. Two 1.5ml microtubes were added with 40μl competent cells respectively. Then 2μl plasmid was added into one microtube (named as tube A), but not into the other microtube (tube B) which is considered as a control. It must be noted that before transformation, the tubes carrying medium, microtubes should be sterilized and sealed carefully.
3. After mixing competent with plasmid for a while, the competent in tube A and tube B are transferred into two electroporation cuvettes respectively on ice.
4. Using the electroporation device in Jen-Jacoboson lab, two cuvettes are electropermeabilized for about a minute.
5. The competent in tube A is immediately transferred in to a new 5 ml tube containing 2ml sterile LB medium.
6. After incubation for about three hours at 37°C, the grown cells in tube A and B are plated on two LB-Kan-Cam plates, respectively. Then the two plates are incubated overnight at 37°C. In the process of plating, all the tools should be sterilized with flame and alcohol very carefully.

If E. coli cells are transformed with pPS12 successfully, the cells will grow on the medium supplemented with Cam and Kan. So in one of plates, clear cell colonies will be found.

If the colonies are not visible in two plates, the experiment should be repeated again to transform the competent with plasmid. Actually, after repeating the experiments for three times, we got a successful transformation.

2.1.2 Cell growth

After transformed with plasmid pPS12a, EcoR Ⅰ restriction endonuclease will be grow in cell culture. Prior to cell growth, the flasks containing LB medium and tips are sterilized with autoclave machine in public lab of University of Pittsburgh, and then sealed carefully. Using filter with ultrafiltration membrane, the 20% (w/v) stock glucose can be sterilized. In the process of preparing the first cell culture for incubation, three components will be added into a 250ml flask containing 100ml LB medium: 5ml 20% glucose (the final concentration is 1%), 50μl of Kan from a 50mg/ml stock (the final concentration is 25μg/ml) and 50μl of Cam from a 30mg/ml stock (the final concentration is 15μg/ml). After all of these components are added into the flask, one single and clear colony of ER2566/pAXU22-8+pPS12a can be transferred into the flask from the plate which has been incubated for overnight. It must be noted that all of experimental tools and table should be sterilized with flame or ethanol, and experimental operation should be completed very fast to avoid the sterile flask exposed to air for a long time. Finally the sealed flask with cell culture will be incubated at 37°C overnight in the rollerdam.

In second day, if the color of cell culture changes from yellow to beige, we can consider the concentration of the cells in the culture is enough for next cell growth. Prior to experiment, seven sterile flasks: four 1000ml flasks with 500ml LB and three 2000ml flasks with 1000ml LB have been prepared. According to table 1, the required culture components including the above

overnight cell culture are added into seven flasks. As mentioned above, the flasks, tools and table have been sterilized. After all operations have been finished, the flasks are placed in the incubator at 37°C and shaking at rpm=110.

Table 1. The components of sterile medium

Flask:	20% Glucose	Kan (50mg/ml)	Cam (30mg/ml)	Cell Culture
1000ml	25ml	250µl	250µl	5ml
2000ml	50ml	500µl	500µl	5ml

To making sure that the cells are ready for induction (maximizing the expression of EcoR Ⅰ), the concentration of cell in the culture will be checked. After incubation for about three hours, two flasks were randomly chosen from these flasks. 1ml cell culture taken from one flask was added into cuvette A. And 1ml cell culture taken from another flask was added into cuvette B. Using sterile LB without cell culture as reference solution (blank) to set zero on spectrophotometer in Rosenberg's lab, The OD values at 595nm of two cuvettes were read. The first average OD of two cuvettes is 0.481. This OD was close to 0.5, so the OD reading of cell culture was taken every 10 minutes (the steps were mentioned above). After 30 minutes, the average OD was equal to 0.568 (the OD greater than 0.55 means that Cell concentration is proper for induction). Therefore, IPTG was quickly added into each flask from the 1M stock IPTG (500µl IPTG added into 1000ml flask with 500ml LB; 1ml IPTG added into 2000ml flask with 1000ml LB). After induction, the cells are allowed to grow overnight at 18°C with rpm=110, and harvested in the morning. To keep the temperature of incubator low than 18°C in the whole night, about half of water in bath of incubator was drained out, and an equal amount of ice was added into the bath to keep temperature low than 10°C.

2.1.3 Harvesting cells

In the morning, the cell culture was transferred from the flasks into the centrifuge bottles. These tubes were balanced in pairs very carefully. Then they are spun in the GS 3 rotor for 15 minutes at 5K. After centrifuge stopped, the supernatant solution in tubes was discarded. The rest cell culture was poured into these bottles, and spun in the GS 3 rotor again. Because the total volume of cell culture is about 5 liters, the spin of cell culture was repeated for three times. Using about 100ml tris buffer (50mM, pH 7.0), the cells in the bottles were resuspended, and the cell solution in 6 bottles was transferred into one bottle. The cell solution was balanced with water, and then spun for 15 minutes at 5K. After the supernatant was discarded, the bottle including cells was weighed. The total weight of the bottle is 75.89g. Because the weight of empty bottle is 65.40g, the weight of grown cells is 10.49g. Finally, the cells was resuspended in 40ml sonication buffer (20mM BTP, 300mM KCl, 1mM EDTA, 7mM BME, 1mM NaN_3, pH 7.0), and poured into 50ml tube and placed in the -80°C refrigerator.

2.1.4 Cell lysis

Before cell lysis with Dr. Hempel's sonicator, the cells were thawed on ice. Then 80µl PMF was added into cell solution to protect endonuclease by inhibiting the protease. After the probe of sonicator was cleaned with ethanol and water, about 20ml cell solution was poured into a small beaker which was placed on ice. The probe was lowered into the beaker just under the surface of the cell solution, and then cell lysis start and went on for 40 seconds (pulse intensity set at 60). It

must be noted that in the process of cell lysis, the position of probe should be adjusted to avoid producing large bubbles. After one round of cell lysis was finished, the probe was taken out of the cell solution and cooled for about 40 seconds. The cell lysis was repeated for six rounds to ensure a complete lysis has occurred. At this time, the color of cell solution has become brown.

After all of cells were lysed, the solution was balanced in pairs and spun in SS 34 rotor for 20 minutes at 10K. At last the brown supernatant was poured into a beaker for loading on P11 phosphocellulose column (the total volume of cell lysate was about 100ml).

2.1.5 Purification through P11 phosphocellulose column

Firstly, cell lysate was poured into the semipermeable membrane (12-14K MWCO). The membrane should be carefully checked to see whether the membrane contains the micropores. Then the membrane containing cell sample was sealed with two clips, and taken into a 1000ml measuring cylinder which contained 500ml P11 column starting buffer (20mM Na_2HPO_4, 300mM NaCl, 10% glycerol (v/v), 1mM EDTA, 1mM NaN_3, 1mM DTT). 400µl PMSF was added into P11 starting buffer. Finally, the measuring cylinder was placed on the magnetic stirrer, and the dialysis began. After dialyzing for half an hour, the old starting buffer was poured from the measuring cylinder, and the equal volume of fresh P11 starting buffer was taken into the measuring cylinder. 400µl PMSF was also added into the buffer. So the cell sample was dialyzed into the fresh P11 starting buffer. After changing P11 starting buffer for four times (every hour these steps were repeated again), the cell lysate was totally dialyzed into P11 starting buffer. The cell sample was poured into a beaker from the membrane for loading onto the P11 column.

P11 phosphocellulose resin made by Whatman is bifunctional cation exchanger containing both strong and weak acid groups based on an ester-linked orthophosphate functional group. The used column has been packed by Ingrid in Rosenberg's lab. Based on the procedures in instruction manual, the P11 column can be regenerated. Firstly, the old P11 resin was poured into 1L of 0.5N NaOH and left for 5 minutes. The supernatant was discarded, and the medium was washed with water in a funnel until the filtrate pH was 11.0 or below (the actual pH was 10.58). Secondly, the P11 resin was poured into 1L of 0.5N HCl and left for 5 minutes. Then the supernatant was decanted off, and the P11 resin was washed with water in funnel until the filtrate pH was above 3.0 (the actual pH was 4.17). Finally, regenerated P11 was poured into the column slowly. After all of the medium was added into the column, the column outlet was opened, and gel was packed under flow. When the resin bed was stable, the P11 column was equilibrated with P11 starting buffer. After the P11 column was washed with 3L starting buffer (about 25 bed volumes), the pH of solution passed through the column was 6.98, which means that the column has been equilibrated for sample loading. Purification was performed in following steps.

1. Loading: Cell lysate was loaded on to the top of column, and infiltrated into the column. The solution passed through the column was collected in a flask. 100μl of this solution named as "passing through" was taken into a 1ml tube. This "passing through" solution could be analyzed with SDS-PAGE gel to check whether endonuclease could bind with the beads in the column well.

2. Washing: After all of crude cell lysate was loaded into the column, the column was washed with about 4 bed volumes of P11 starting buffer to remove contaminating proteins which could not bind to the groups in P11 resin.

3. Gradient elution: Because the column should be eluted with total 4 bed volumes of buffer (the bed volume is about 120ml), 250ml of P11 start buffer and 250ml of P11 final buffer (20mM Na_2HPO_4, 1M NaCl, 10% glycerol (v/v), 1mM EDTA, 1mM NaN_3, 7mM DTT), were added into two bottles in gradient maker (in Rosenberg's lab) respectively. It must be noted that the valve could not be opened, until gradient elution begins. After the column was connected to gradient maker and round auto-collector in Rosenberg's lab, respectively, the mixing valve in the maker was open, and stirrer was turned on. Then the different protein components binding to the beads in column was eluted and collected in the tubes of the collector (120 drops per tube), which were marked with numbers.

After gradient elution was finished, all of the tubes marked with odd numbers were taken out of the collector and placed onto the ice. Then these tubes were carried out of the cold room to read the OD at 280nm using spectrophotometer. Using the solution in tube 1 as reference to set zero, the OD_{280} values of solutions in all of 50 tubes were read.

Table 2: The OD at 280nm of 10 tubes in group1 (tube 41-tube 59)

Tubes	41	43	45	47	49	51	53	55	57	59
OD	0.042	0.086	0.138	0.199	0.298	0.402	0.368	0.280	0.199	0.183

Table 3: The OD at 280nm of 8 tubes in group2 (tube 61-tube 75)

Tubes	61	63	65	67	69	71	73	75
OD	0.219	0.317	0.629	1.125	0.976	0.487	0.181	0.127

Table 4: The OD at 280nm of 8 tubes in group3 (tube 77- tube 91)

Tubes	77	79	81	83	85	87	89	91
OD	0.132	0.136	0.142	0.191	0.271	0.227	0.090	0.016

Table 5: Samples loaded onto the lanes of Gel Ⅰ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	Marker	PT	45	49	51	55	63	65	67

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes.

Table 6: Samples loaded onto the lanes of Gel Ⅱ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	69	71	72	81	85	87	87	61	62

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

In all of 50 tubes, The OD_{280} values of protein samples in tubes of group1, 2 and 3 was much higher than that of reference and samples in other tubes, the OD_{280} values of most of which were lower than 0.05 (not shown here). For example, the average OD_{280} of group1 was about

0.21 (Table 2). The average OD_{280} of group2 was about 0.51 (Table 3). The average OD_{280} of group3 also reached 0.13 (Table 4). Therefore, most of protein components including EcoRⅠ endonuclease should be collected in the tubes of group1, 2 and 3. The average OD_{280} of group2 was higher than other two groups, especially, the OD_{280} of protein samples in tube 67 and tube 69 was highest among all of the tubes (Table 3), so it was most possible that EcoRⅠ endonuclease should be collected in tubes 61-71. To prove this possibility, the protein samples in tubes of group1, 2 and 3 were analyzed with two 12% SDS-PAGE gels.

According to instruction manual, two 12% SDS-PAGE gels, each of which contained ten lanes for sample loading, were made. In Gel Ⅰ, besides standard EcoRⅠ endonuclease (ST), molecular marker and "pass through" sample (PT) which was collected in loading section, the protein samples in tubes of group 1 and 2 were loaded onto the lanes 4-10 (Table 5). In Gel Ⅱ, standard EcoRⅠ endonuclease (ST) was loaded onto lane1, and the protein samples in tubes of group 2 and 3 were loaded onto lanes 2-8 (Table 6).

As shown in figure 2.1, In lane3 of Gel Ⅰ, the band standing for EcoRⅠ could not be found, so it was clear that no EcoRⅠ left in "pass through" solution, in another words, most of EcoRⅠ endonuclease has bound to the beads in the column. In lanes 4, 5, 6 and 7, the single sharp bands of contaminating protein, the weight of which is about 97Kda, could be identified easily. Reversely, in these lanes, the bands of EcoRⅠ were very faint. So we could say that the main protein component in tubes of group1 (table 2), was the contaminating proteins, and EcoRⅠ were almost not collected in these tubes.

In lanes 7-9 of Gel Ⅰ (figure 2.1), the sharpness of the single bands of EcoRⅠ was very high. However, the single bands for contaminating proteins were very clear. Especially, the weight of

one of contaminating proteins was very close to EcoR Ⅰ. These results suggested that the concentration of EcoR Ⅰ in tubes 63-67 should be high, but in the these tubes, the content of two contaminating proteins was high too. So in next steps of purification, the two contaminating proteins should be removed.

As shown in figure 2.2, in lanes 5, 6, 7, 8 of Gel Ⅱ, there were only some single bands of contaminating proteins, the weights of which were lower than EcoR Ⅰ. It could be considered that the tubes 81-91 did not contain EcoR Ⅰ. In the lanes 2, 3, 4, 9 and 10 of Gel Ⅱ, according to the sharpness of bands and the OD_{280} values of samples, it could be considered that in tubes 69-72, although the content of proteins was lower than that of tubes 63-67, the concentration of EcoR Ⅰ was still high. Contaminating protein has been removed from the solutions in these tubes, though the concentration of other contaminating proteins were relative high (figure 2.2). As for the solutions in tubes 61 and 62 (lane 9 and lane 10 of Gel Ⅱ), it was obvious that the content of EcoR Ⅰ in these tubes was not high, but the concentration of contaminating proteins was very low (figure 2.2). So the protein samples in these two tubes could be used for future purification. As a result, the protein samples in tubes 61-72 were pulled into a beaker. This collection could achieve most of EcoR Ⅰ, simultaneously, avoid collecting too much contaminating proteins. The volume of protein sample in the beaker is 66ml. Because in the process of future purification, the protein solution should be concentrated, in order to keep the concentration of EcoR Ⅰ in solution, the collected protein sample was concentrated in a contractor with a 12-14K semipermeable membrane (Rosenberg’s lab). After

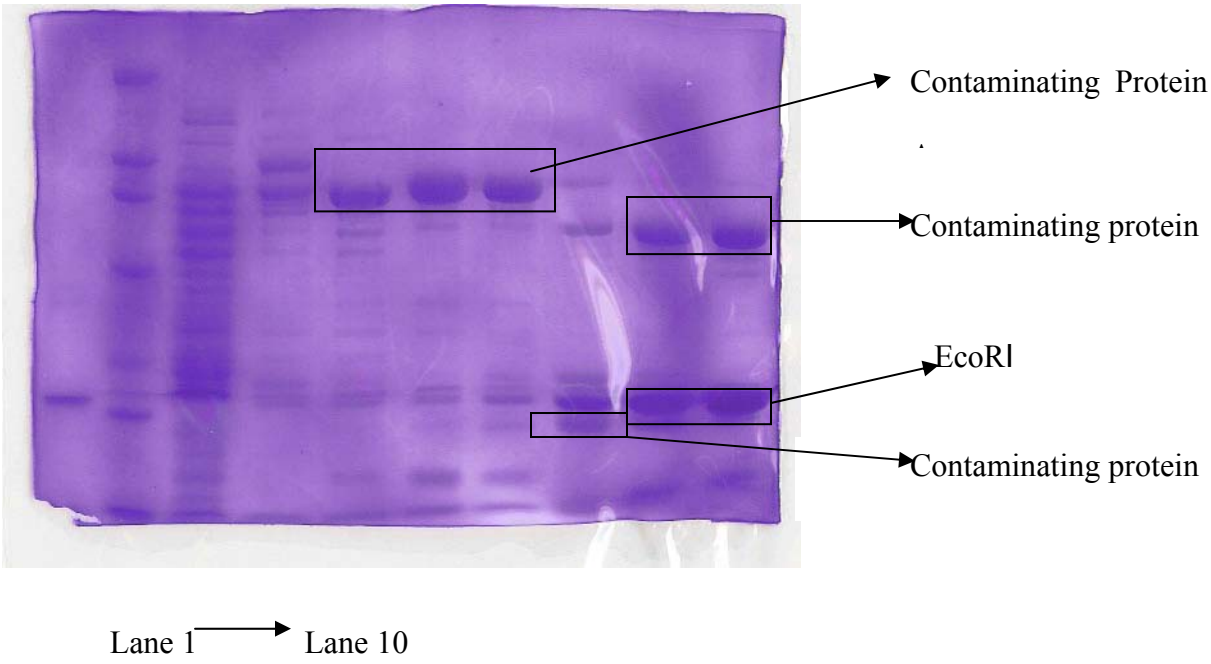

Figure 2.1. The SDS-PAGE gel Ⅰ of WT EcoRⅠ endonuclease through P11 column.

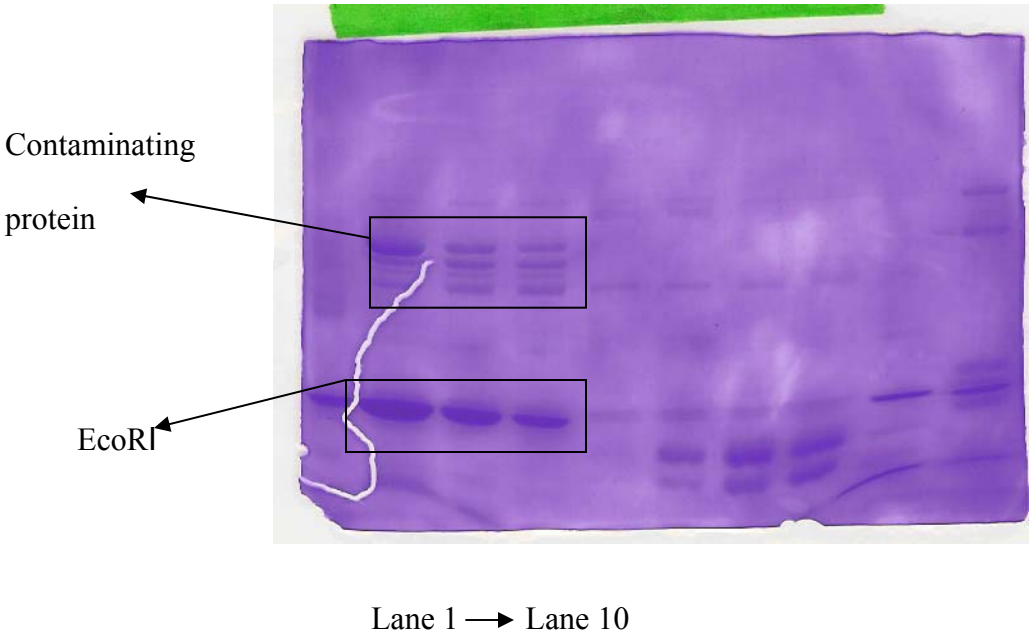

Figure 2.2. The SDS-PAGE gel Ⅱ of WT EcoRⅠ endonuclease through P11 column

concentration, the volume of the protein sample was concentrated from 66ml to 40ml. Then as mentioned above, the protein sample was transferred into a 12-14K dialysis bag, and dialyzed against five changes of 500ml of HAP hydroxyapatite start buffer (10mM K_2HPO_4, 10% glycerol (v/v), 0.1mM EDTA, 0.1M NaCl, 1mM DTT, 1mM NaN_3; pH 7.0) (no PMSF). Finally, the protein sample was dialyzed into HAP hydroxyapatite start buffer.

2.1.6 Purification through HAP hydroxyapatite column

The used HAP hydroxyapatite column was prepared by Ingrid in Rosenberg'lab (the bed volume was 110ml). According to the instruction manual, in order to regenerate HAP column, the used HAP hydroxyapatite column firstly was washed with one bed volume of 0.5M K_2HPO_4 to remove the contaminated sample in the column. The top layer of HAP column bed was removed too. Then the remainder HAP column was washed with one bed volume of 1M NaCl again, and equilibrated with at least six bed volumes of HAP start buffer until pH was 6.97 (pH should be very close to 7.0). At this time, purification could be carried out in following steps.

1. Loading: The protein sample dialyzed into HAP start buffer was collected into the beaker, and loaded onto HAP column. As mentioned in section 2.1.5, the "pass through" (PT) solution which has been passed through the column during loading, was collected in the flask for analysis with SDS PAGE gel.

2. Washing: After loading protein sample into the column, the HAP column was washed with four bed volumes of HAP start buffer to remove the protein components which could not bind to the beads in HAP column.

3. Gradient elution: Because the bed volume of HAP column was about 110ml, as mentioned in section 2.1.5, 240ml HAP start buffer and 240ml final buffer (0.5M K_2HPO_4, 10% glycerol (v/v), 0.1mM EDTA, 0.1M NaCl, 1mM DTT, 1mM NaN_3; pH 7.0), were added in the gradient maker. All of experimental operations have been mentioned in section 2.1.5. After gradient elution was finished, most of protein samples have been collected in the tubes of autocollector.

50 tubes marked with odd number were placed onto the ice to read OD at 280nm (the solution in tube 1 as reference to set zero). The OD_{280} values of tubes which could contain proteins have been listed in tables 7, 8 and 9.

Table 7: The OD at 280nm of 6 tubes of group 1(tube 25- tube 35)

Tubes	25	27	29	31	33	35
OD at 280nm	0.022	0.023	0.100	0.060	0.057	0.065

Table 8: The OD at 280nm of 5 tubes of group 2 (tube 39-tube 47)

Tubes	39	41	43	45	47
OD at 280nm	0.068	0.148	0.115	0.059	0.058

Table 9: The OD at 280nm of 5 tubes of group 3 (tube 61-tube 69)

Tubes	61	63	65	67	69
OD at 280nm	0.230	1.009	0.689	0.247	0.110

According to the results in table 7 and table 8, the average OD_{280} values of protein samples in tubes of group1 and group 2 were 0.06 and 0.09. Because the OD_{280} values of solution in other tubes which did not belong to groups 1, 2 and 3, were even lower than that of

reference fraction (not shown here), the results in group1 and 2 suggested that some protein samples were contained in the tubes of group1 and 2, but the contents of these protein should be low. In contrast, the average OD_{280} value of protein samples in tubes of group 3 is 0.46 (table 9). This value is much higher than the average values of protein samples in tubes of group 1 and 2, so it was most possible that EcoRⅠ endonuclease was collected in tubes 61-69.

Table 10: Samples loaded onto the lanes of Gel Ⅰ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	Marker	PT	29	33	35	39	41	43	45

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes.

Table 11: Samples loaded onto the lanes of Gel Ⅱ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	Marker	61	62	63	64	65	66	67	69

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

Firstly, in Gel Ⅰ (figure 2.3), there were not almost any bands in lane 3. It means that "pass through" solution did not contain any protein samples including EcoRⅠ, in another word, all of the protein samples have been loaded onto the HAP column. Secondly, in lane 4, lane 8 and lane 9 of Gel Ⅰ, three single sharp bands standing for contaminating proteins, the weights of which were very close to EcoRⅠ, could be identified easily (figure 2.3). In other lanes (5, 6, 7 and 10), the bands for contaminating proteins or EcoRⅠ could not almost be found. These results suggested that the contaminating proteins, which were shown in lane 8 and lane 9, have been separated from EcoRⅠ (figure 2.3).

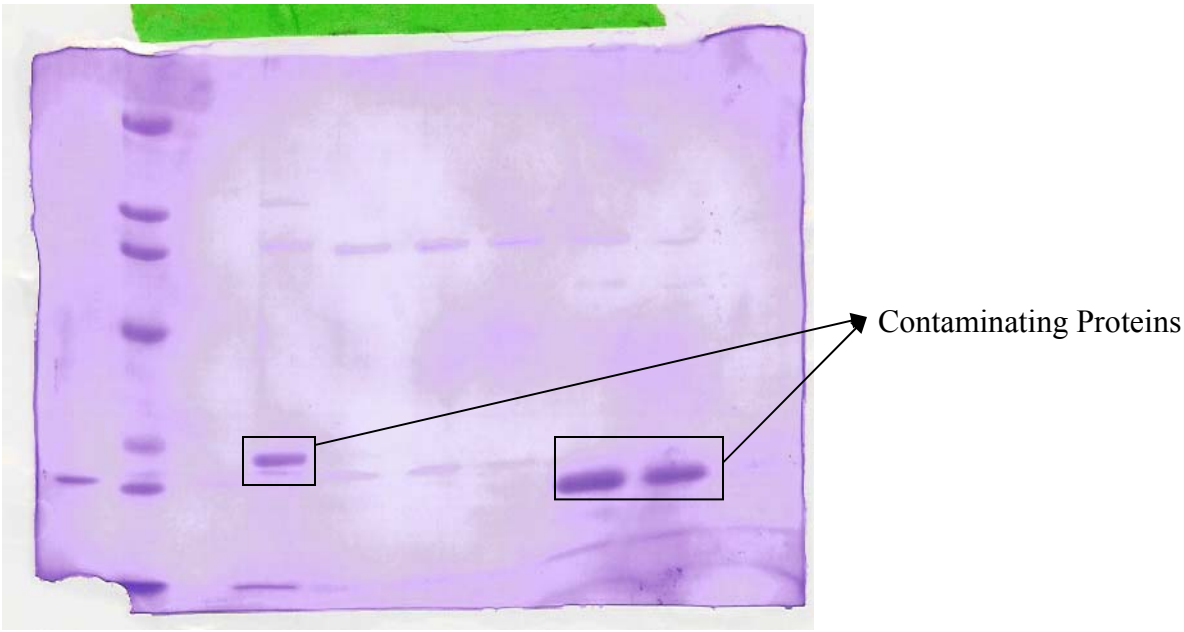

Figure 2.3. The SDS-PAGE gel of WT EcoR Ⅰ endonuclease through HAP column

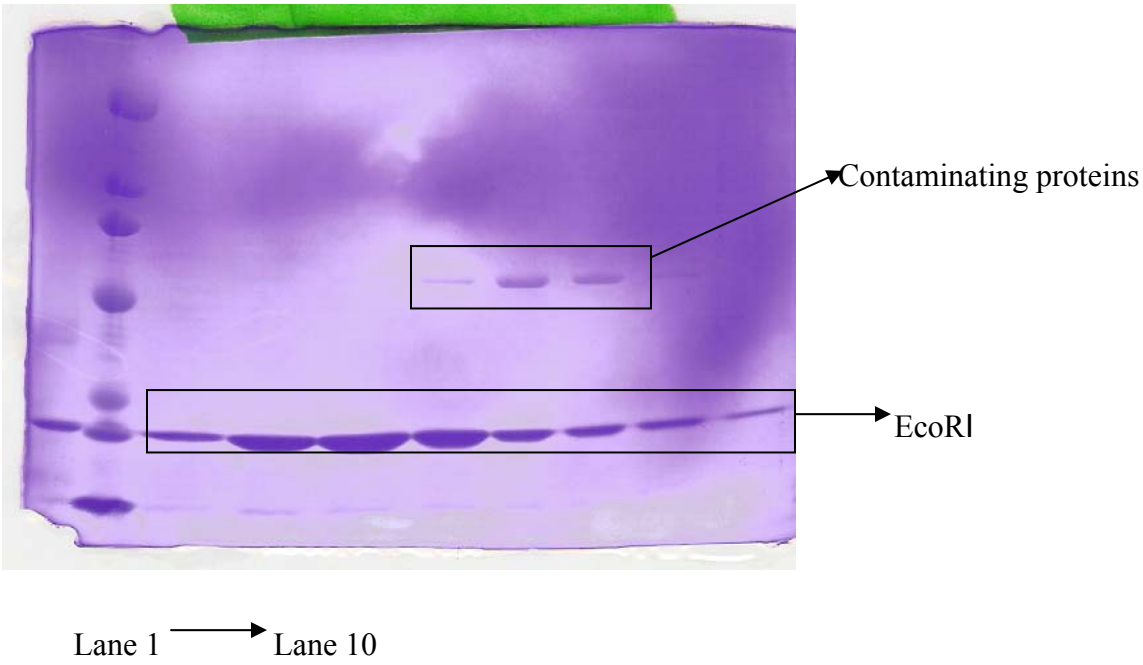

Figure 2.4. The SDS-PAGE gel Ⅱ of WT EcoR Ⅰ endonuclease through HAP column

As shown in figure 2.4, in lanes 3-7 of Gel Ⅱ, the single sharp bands for EcoR Ⅰ could be recognized clearly. So it could be suggested that most of EcoR Ⅰ is present in tubes 61-69. Although in lane 6, 7 and 8 of Gel Ⅱ, some thin and unclear bands standing for contaminating proteins could be found (figure 2.4), comparison with the bands for EcoR Ⅰ in the same lanes, it could be said that in solutions of tubes 64-66, the content of contaminating protein was much lower than that of EcoR Ⅰ. In conclusion, after purification by HAP column, EcoR Ⅰ has been concentrated in tubes 61-69, and most of two main contaminating proteins have been removed or separated from EcoR Ⅰ. In the last step of purification, the purity of EcoR Ⅰ should be further increased.

As mentioned in 2.1.5, firstly, the protein samples in tubes 61-69 were collected into concentrator with 12-14K semipermeable membrane. The volume of solution was concentrated from 46ml to about 30ml. Then the protein samples were transferred into 12-14K dialysis membrane, and dialyzed against five changes of 500ml of Bio-Rex 70 start buffer (10mM K_2HPO_4, 0.3M NaCl, 10% glycerol (v/v), 1mM EDTA, 7mM DTT, 1mM NaN_3; pH 7.0). Finally after the protein samples were dialyzed into Bio-Rex 70 start buffer, the solution was transferred into a beaker for sample loading.

2.1.7 Purification through Bio-Rex 70 column

The used Bio-Rex 70 column has been prepared by Ingrid in Rosenberg's lab. According to instruction manual, in order to regenerate the column, Bio-Rex column was washed with 4 bed volumes of 0.5N NaOH to change the resin to sodium form. This conversion is complete when pH of "pass through" solution became greater than 9. Then the column was rinsed with 4 bed

volumes of deionized water. Finally, Bio-Rex column was equilibrated with 6 L of Bio-Rex column start buffer, until pH became 7.0. As mentioned in 2.1.5 and 2.1.6, purification by Bio-Rex 70 column was also performed in following steps.

1. Loading: The protein samples were loaded onto the top of Bio-Rex 70 column, at the same time, "pass through" solution was collected for analysis with SDS PAGE gel.

2. Washing: After sample loading was finished, Bio-Rex 70 column was washed with 4 bed volumes of Bio-Rex start buffer to remove the protein components which could not bind to the beads in Bio-Rex 70 column.

3. Gradient elution: In order to elute the column with 4 bed volumes of elution buffer (the bed volume is about 120ml), 250ml of Bio-Rex start buffer and 250ml of Bio-Rex final buffer (10mM K_2HPO_4, 0.3M NaCl, 10% glycerol (v/v), 1mM EDTA, 7mM DTT, 1mM NaN_3; pH 7.0), were added into gradient maker. The elution fraction was collected in 60 drops per tube to decrease the loss of EcoRⅠ. The tubes in autocollector were marked with numbers too.

After gradient elution was completed, 60 tubes marked with odd numbers were taken to read OD at 280nm. Because of the effect of DTT, the water was used as reference to set zero (the OD_{280} value of solution in tube 1 was 0.344). All of the OD_{280} values of 60 tubes showed that the OD_{280} values of the solutions in tubes 63-89, average of which was 0.6, were higher than that of tube 1 (listed in table 12). The OD_{280} values of the solutions in other tubes were very close to, or even lower than the OD_{280} of the solution in tube 1 (not shown here). These results strongly suggested that EcoRⅠ endonuclease was present in tubes 63-89.

Table 12: The OD at 280nm of 14 tubes of group 1 (tubes 63-89)

Tubes	63	65	67	69	71	73	75	77	79	81	83	85	87	89
OD	0.39	0.40	0.42	0.45	0.46	0.48	0.49	0.59	1.13	1.21	0.83	0.63	0.49	0.39

In order to prove this conclusion, the protein samples were analyzed with two 12% SDS PAGE gels. Besides standard EcoRⅠ (ST), molecular marker, loading sample (SP) which has been dialyzed into Bio-Rex start buffer, but not loaded onto the column and "pass through" solution (PT), the protein samples of tube 29 (OD_{280} is 0.345) and tube 43 (OD_{280} is 0.35) were loaded onto lane 3 and lane 4 of Gel Ⅰ as the control fractions. The rest of lanes in Gel Ⅰ were loaded with protein samples of group 1 (table 13). In Gel Ⅱ, besides standard EcoRⅠ, two protein samples of tube 93 (OD_{280} is 0.345) and tube 105 (OD_{280} is 0.335) were also load onto lane 9 and lane 10 as the control. The protein samples of group 1 were loaded onto the rest of lanes of Gel Ⅱ (table 14).

As shown in figure 2.5, in Gel Ⅰ, there was only a single sharp band standing for EcoRⅠ in lane 3, but not any bands for protein samples could be identified in lane 4. These results suggested that purity and concentration of EcoRⅠ in the protein solution for sample loading, were very high. And almost all of protein samples in loading solution have been loaded into Bio-Rex 70 column.

Table 13: Samples loaded onto the lanes of Gel Ⅰ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	Marker	SP	PT	29	43	63	65	67	69

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

Table 14: Samples loaded onto the lanes of Gel Ⅱ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	73	77	79	81	85	87	89	93	105

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

The clear and sharp bands for EcoRⅠ were found in lanes 7-10 of Gel Ⅰ and lanes 2-8 of Gel Ⅱ (figure 2.5 and figure 2.6). In accordance with the OD_{280} values of tubes 63-89, These results further proved that there is a higher concentration of EcoRⅠ endonuclease in solutions of tubes 63-89. In lane 9 and 10 of Gel Ⅰ and lanes 2-4 of Gel Ⅱ (figure 2.5 and figure 2.6), five thin and faint bands for contaminating proteins, could be identified, which means the content of contaminating protein was much lower than EcoRⅠ. In conclusion, the final concentration and purity of EcoRⅠ should be high in tubes 63-89.

The protein samples in tubes 63-89 were pooled into the concentrator. Then the total volume of protein solution was concentrated from 54ml to about 21ml. EcoRⅠ sample was transferred into 12-14K dialysis membrane, and dialyzed against five changes 400ml of BTP buffer (40mM BisTris propane, 0.5M NH_4Cl, 1mM EDTA, 15% Dioxane (v/v); pH 8.5).

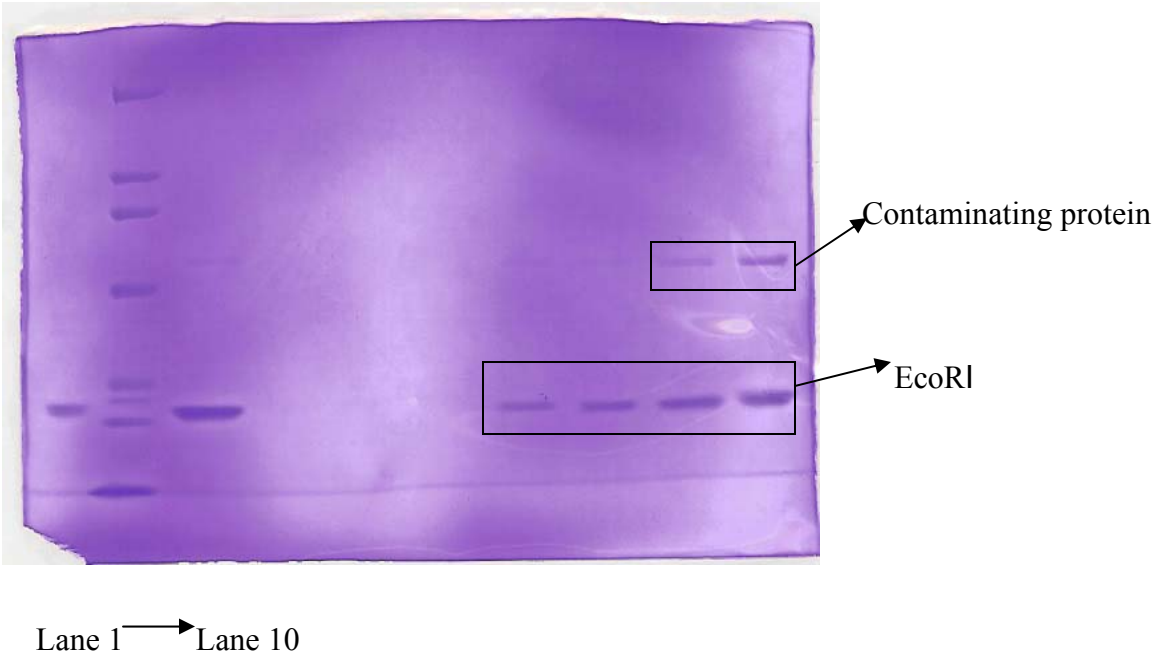

Figure 2.5. The SDS-PAGE gel Ⅰ of WT EcoRⅠ endonuclease through Bio-Rex 70 column

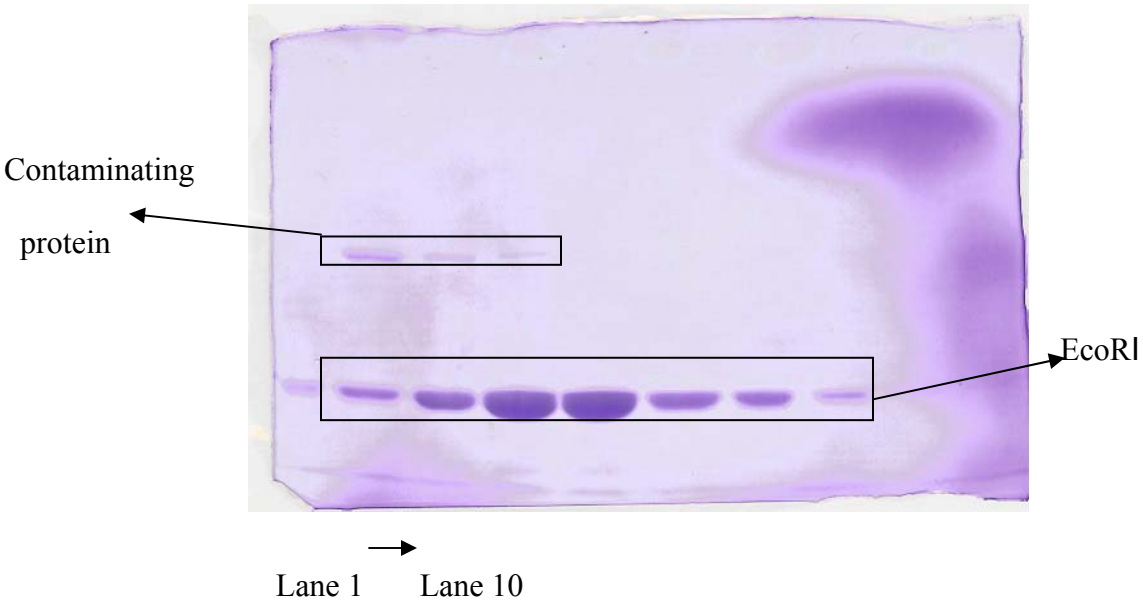

Figure 2.6. The SDS-PAGE gel Ⅱ of WT EcoRⅠ endonuclease through Bio-Rex 70 column

After EcoR Ⅰ was dialyzed into BTP buffer, the concentration of EcoR Ⅰ was set with Bradford assay. According to the instruction manual, bovine serum albumin (BSA) was used as standard reference. Firstly, a series of BSA standards were made at the following concentration: 0mg/ml, 0.1mg/ml, 0.2mg/ml, 0.3mg/ml, 0.4mg/ml, 0.5mg/ml and 0.6mg/ml. Secondly, 14 1.5ml Eppendorf tubes were prepared for assay. Then 500μl of Bio-Rad reagent made before was added to each tube. 20μl of each of BSA standards made above was also added to each tube. When Bio-Rad reagent turned BSA blue, each tube was shaken to mix protein and reagent well. Thirdly, each of the samples was taken an OD reading at 595nm on the spectrophotometer in Rosenberg's lab (the vial containing 0mg/ml BSA standard served as the blank). Finally, each concentration of BSA standard had two OD values. These OD readings were taken an average value. These numbers could be graphed and served as the solutions for the standard curve.

Similarly, a series of 20μl dilutions of EcoR Ⅰ were made at the following ratios: 1:20, 1:10 and 1:4. After 6 1.5ml Eppendorf tubes were prepared, each of which containing 500μl Bio-Rad reagent, 20μl of the solutions of EcoR Ⅰ was added to each tube. Then the OD values of the solutions of these tubes were read as mentioned above. Finally, two OD values of each concentration of EcoR Ⅰ were taken an average value. On the computer, a graph of absorbance versus concentration for BSA standards could be made. Three dilutions could get three protein concentrations. The average of the concentration is the concentration of EcoR Ⅰ.

Table 15: The concentrations of EcoR Ⅰ with different volumes

Volume	21ml	7.0ml	2.6ml
Concentration	0.94mg/ml	3.48mg/ml	7.48mg/ml

The EcoR Ⅰ dialyzed into BTP buffer was firstly set to 0.94mg/ml (table 15). This concentration was low for crystal growth (the concentration should be greater than 6mg/ml). Therefore, EcoR Ⅰ sample was transferred into 12-14K dialysis membrane, the size of which is 28mm. After dialysis membrane was sealed, the aquacide was placed on the surface of membrane to absorb the solvent in the solution to concentrate the protein solution. After absorbance overnight, the volume of the solution became one third of the original solution. The concentration of solution with Bio-Rad assay was set to 3.43mg/ml (table 15). So the concentration was continued until the concentration was 7.48mg/ml (the volume was about 2.6ml). This concentration was proper for crystal growth

2.1.8 Crystal growth of wild type EcoR Ⅰ endonulease -DNA (13mer) complex

The crystals of the complex of wild type EcoR Ⅰ endonulease with DNA 13mer were grown with sitting drop vapor diffusion. Based on the conditions used in Rosenberg's lab, reservoir buffer and precipitant (PPT) were made. Reservoir buffer includes two components: 16% PEG 400 (w/v) and 40mM BTP (PH 6.5). Precipitant (PPT) consists of two components: 40% PEG 400 and 40mM BTP (pH 7.0). DNA sample (13mer), the concentration of which was 10mg/ml, has been purified by Ingrid in Rosenberg's lab. The concentration of EcoR Ⅰ endonuclease sample which was in BTP buffer mentioned in 2.1.7, was 7.48mg/ml. The setting up of crystallization in the tray was listed in table 16.

Table 16: The setting up of crystallization in the tray

	1	2	3	4	5	6
A: DNA	7µl	7µl	7µl	6µl	6µl	6µl
Protein	6µl	6µl	6µl	5µl	5µl	5µl
PPT	3µl	3µl	3µl	3µl	3µl	3µl
B: DNA	7µl	7µl	7µl	6µl	6µl	6µl
Protein	5µl	7µl	8µl	5µl	6µl	7µl
PPT	3µl	3µl	3µl	2.5µl	2.5µl	2.5µl
C: DNA	5µl	4µl	3µl	5µl	4µl	3µl
Protein	4µl	4µl	4µl	3µl	3µl	3µl
PPT	2µl	2µl	2µl	2µl	2µl	2µl
D: DNA	7µl	7µl	7µl	6µl	6µl	5µl
Protein	5µl	4µl	7µl	5µl	6µl	5µl
PPT	2µl	2µl	2µl	3µl	3µl	3µl

After two weeks, the tray was checked. It was very clear that in most of drops, the crystals could be identified very clearly. In some pools such as C1 and C4 (figure 2.7 and figure 2.8), the crystals with good shape and size were very good for diffraction in the next step structural research .

2.2 DISCUSSION AND CONCLUSION

The protein sample with high purity and concentration is the critical basis of crystal growth. In the works mentioned above, plasmid transformation was the first critical and difficult step. Besides the quality of cells and plasmids, some operations, especially, electroporation could affect the success of transformation very much. So sometimes, the transformation experiment had to be repeated for several times to achieve well grown cell colonies. In this work, after two times of transformations, much transformed cell colonies were grown well in the plate.

In the process of cell growth, all of the flasks, mediums and tools were sterilized very well to avoid contaminating cell culture. Before inducing the expression of EcoR Ⅰ endonuclease, the OD_{595} of cell culture reached 0.568 which stood for a proper cell concentration for induction. Too low or too high concentration of cells in the culture would affect the expression level of EcoR Ⅰ endonuclease. After harvesting E. coli cells, the weight of crude cells was 10.49g. It means that the level of cell growth was very high.

After purification through P11, HAP and Bio-Rex columns, it could be found that firstly, the protein samples could be load onto all of the columns very well. During purification the loss of protein sample was very small. Two main contaminating proteins were successfully separated from EcoR Ⅰ endonuclease step by step. The content of contaminating protein was also reduced very much in protein sample. So the purity of EcoR Ⅰ endonuclease was very high. The OD_{280} values of protein samples and the sharpness of bands standing for EcoR Ⅰ endonuclease in SDS-PAGE gels revealed that the concentration of EcoR Ⅰ endonuclease should be high in protein solution. After three concentrations with concentrator and aquacide, the final concentration of

EcoRⅠ endonuclease was 7.48mg/ml. The volume of EcoRⅠ endonuclease was about 2.6ml, which was enough for crystal growth.

In most of the drops in the tray, the crystals of EcoRⅠ-DNA complex appeared. This result more proved that a little of contaminating protein in EcoRⅠ endonuclease sample did not affect the crystal growth. Because the conditions of crystallization focused on adjusting the ratio of the volumes between protein and DNA in precipitant, the results in crystal growth revealed that the changes of relative concentrations of EcoRⅠ endonuclease and DNA sample in precipitant did not affect crystal growth very much. The quality of protein sample, pH and ionic strength should be more critical factors which determine crystal growth. In a word, the good crystal of complex of EcoRⅠ with DNA fragment makes the good basis for diffraction and data collection.

Figure 2.7. The crystals of WT EcoRⅠ-DNA 13mer complex in C1**.**

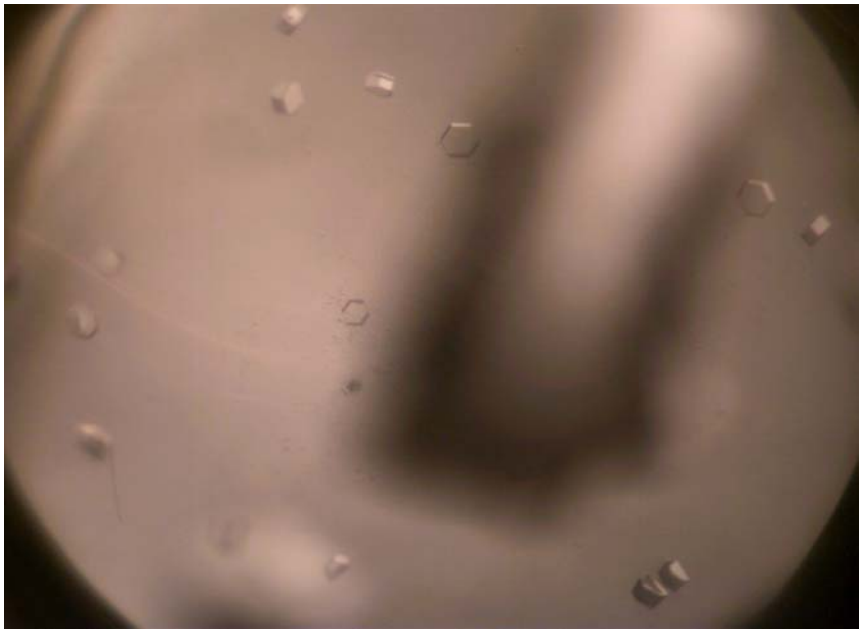

Figure 2.8. The crystals of WT EcoRⅠ-DNA 13mer complex in C4

3.0 PURIFICATION OF ECOR Ⅰ ENDONUCLEASE RS187 AND CRYSTAL GROWTH OF ECOR Ⅰ ENDONUCLEASE RS187-DNA 13MER (TCGCGAATTCGCG) COMPLEX

3.1 INTRODUCTION

In early researches of EcoR Ⅰ restriction endonuclease, mutagenesis has been used to study catalytic and DNA recognition mechanism of EcoR Ⅰ endonuclease(Jeltsch, 1992, McLaughlin, 1987). EcoR Ⅰ mutant EQ111 (Glu^{111}→Gln) could bind DNA more tightly than wild type EcoR Ⅰ in the absence of divalent cation, but this mutant did not cleave DNA even when divalent cation was present (King et al., 1989). Similarly, the EcoR Ⅰ mutant RK145 (Arg^{145}→Lys) could significantly lower the activity of EcoR Ⅰ endonuclease (Wolfes, 1986, Yanofsky, 1987, Heitman and Model, 1990b). In structural study, mutagenesis can help to more deeply understand the important factors which control the DNA recognition mechanism of EcoR Ⅰ endonuclease.

The two regions 116-140 and 170-196 of EcoR Ⅰ endonuclease form two "arms": inner arm and outer arm. These two "arms" in apo- EcoR Ⅰ endonuclease (the protein in the absence of DNA) are less ordered than in the DNA-containing complexes (Jen-Jacoboson, 1986, Grigorescu et al., 2003). A series of structural studies on apo-EcoR Ⅰ endonuclease by

Chandrasekhar, Wilkosz and Rosenberg, reveal that four main chain- main chain hydrogen bonds made between residues Asp^{185}, Gly^{186}, Arg^{187}, play important roles in crystallographic packing (Grigorescu et al., 2003). In another word, the intermolecular interactions made by subunits in the complex are the factors responsible for stabilizing the local structure of its arms. Similarly, in other structural studies (Thielking, 1991, Freitag et al., 1997; Perona and Martin, 1997a; Raghunathan et al., 1994; Vigil et al., 2001), the relationship between intermolecular interaction and stabilization of the conformation of large protein region has been reported.

Different from apo-EcoRⅠ endonuclease, in the protein-DNA complex, the arms wrap the DNA and make it against the recognition interface between EcoRⅠ and DNA fragment. In this wrapping process, the domains connecting the arms to the main region of EcoRⅠ form a series of highly specific interactions with the DNA bases of the target site (Grigorescu et al., 2003). Therefore, in protein-DNA complex, the interaction between protein and DNA stabilize the conformation of arms. Besides the structural study, the thermodynamic study also reveals an order-disorder transition upon DNA binding that Spolar and Record attributed to the arms. Furthermore, it is reported that apo-EcoRⅠ is very sensitive to proteolysis while EcoRⅠ-DNA complex resists proteolysis very much (Jen-Jacobson et al., 1986). It can be hypothesized that ordering and binding of the domain connecting the arms with the main domain, cause the ordering of the arms.

The mutations in primary domain reveal that the residues in the main region of EcoRⅠ play key roles in maintaining the correct conformation and dimerization of protein (Heitman and Model, 1990a; Muir et al., 1997). In contrast, the mutations in domain with low structural stability, such as Glu^{192} and Tyr^{193} (in the outer arm), Ala^{138} and His^{114} (at the interface between the arm and the primary domain), reduce the sequence specificity of EcoRⅠ endonuclease

(Heitman and Model, 1990b). These results suggest that the ability of sequence discrimination is related to the ordering of binding of the local domains including arms with low structural stability.

All of results above suggest that two arms play key roles in molecular packing of apo-EcoR Ⅰ endonuclease, protein-DNA binding and the conformation of protein-complex conformation. Arg^{187} plays an important role in maintaining the structure of outer arm. In the mutant RS187 (Arg^{187}→Ser), the disordered tendency of outer arm may be increased. Early study history of mutant RS187 in Rosenberg’s lab showed that order-disorder transitions make this mutant difficult to purify and crystallize. In order to achieving the crystal structure of mutant RS187-DNA complex to help us understand the function and role of Arg^{187} in DNA binding, the following work focuses on the purification of EcoR Ⅰ mutant RS187 and the crystal growth of mutant-DNA complex to make a good basis for future diffraction and data collection.

3.2 EXPERIMENTAL METHODS AND RESULTS

3.2.1 Transformation

The detailed experimental methods have been mentioned in section 2.1.1. Mutant RS187 was also expressed from Escherichia coli strain ER2566/pAXU22-8. Because the plasmid pPS12a containing a gene encoding EcoR Ⅰ mutant RS187 provided by Ingrid in Rosenberg' lab, has been transformed into Escherichia coli cells by Ingrid in Rosenberg's lab, the transformed competent cells stored at -80°C were plated on the LB-Kan-Cam plate. The plate was incubated overnight at 37°C. In the morning, much single cell colonies appeared in the plate. Therefore, transformed cells were ready for the next cell growth.

3.2.2 Cell growth

As the detailed procedure mentioned in section 2.1.2, firstly, one of cell colonies was transferred and grown in sterile 100ml of LB medium overnight at 37°C. Secondly, in second day, the first grown cells were added into 7 sterilized flasks, all of which held total 5 L of sterile medium (table 1). After incubation in incubator at at 37°C with rpm=110 for about four hours, the OD readings were taken at 595nm on spectrophotometer at Rosenberg's lab every 15 minutes. Thirdly, when the third OD value reached 0.585, IPTG was added into all of the cell culture to maximize the expression of EcoR Ⅰ endonuclease. Finally, the heating power of incubator was cut off. And the ice was added into the incubators to keep the temperature lower than 10° C. Then the cell culture was incubated overnight.

3.2.3 Harvesting cells

As the detailed process discussed in section 2.1.3, the cell culture was balanced pairs in the centrifuge bottles. Then the cell culture was spun in the GS 3 rotor for 15 minutes at 5K. When centrifuge was finished, the supernatant was discarded. All of the cells extracted from culture were resuspened in 50mM tris buffer, and were spun for 15 minutes at 5K again. After discarding the supernatant, we got 9.29g cells. Finally, the cells were resuspended in 40ml sonocation buffer, and stored at -80°C.

3.2.4 Cell lysis

As the detailed procedures mentioned in section 2.1.4, using Dr. Hempel's sonicator, the E.coli cells were broken, until the color of cell solution became brown. Then the cells were balanced pairs and spun in the SS 34 rotor for 20 minutes at 10K. Finally, the supernatant was poured into a beaker for sample loading on the P11 phosphocellulose column.

3.2.5 Purification through P11 phosphocellulose column

The experimental methods and used buffers have been discussed in detail in section 2.1.5. Similar to the purification of wild type EcoR Ⅰ endonuclease, firstly used P11 column was regenerated and equilibrated. Then EcoR Ⅰ mutant was purified in the three main steps: sample loading, washing and elution, and collected in the tubes in auto-collector. The OD_{280} values of protein samples in 50 tubes marked with odd numbers were read with spectrophotometer (the solution in tube 1 as the blank).

The OD_{280} values of solutions in tubes of two groups were much higher than that of the solutions in other tubes (table 17 and table 18). The average OD_{280} values of group 1 and group 2 were 0.63 and 0.47, respectively. So EcoRⅠ endonuclease might be collected in the tubes of group 1 and group 2, especially, in the tubes of group 1.

Table 17: The OD at 280nm of 8 tubes in group1 (tube 21-tube 35)

Tubes	21	23	25	27	29	31	33	35
OD	0.182	0.272	0.433	0.710	0.877	0.901	0.878	0.792

Table 18: The OD at 280nm of 7 tubes in group2 (47-59)

Tubes	47	49	51	53	55	57	59
OD	0.508	0.481	0.477	0.507	0.507	0.44	0.368

Table 19: Samples loaded onto the lanes of Gel Ⅰ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	Marker	SP	PT	19	20	21	23	25	27

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

Table 20: Samples loaded onto the lanes of Gel Ⅱ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	29	31	33	34	Marker	47	53	57	59

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

The protein samples distributed in tubes of group 1 and group 2 were loaded onto two 12% SDS-PAGE gels (table 19 and table 20). In lanes 8, 9 and 10 of Gel Ⅰ (figure 3.1), the bands standing for EcoRⅠ mutant were very thin and unclear. In contrast, the bands for

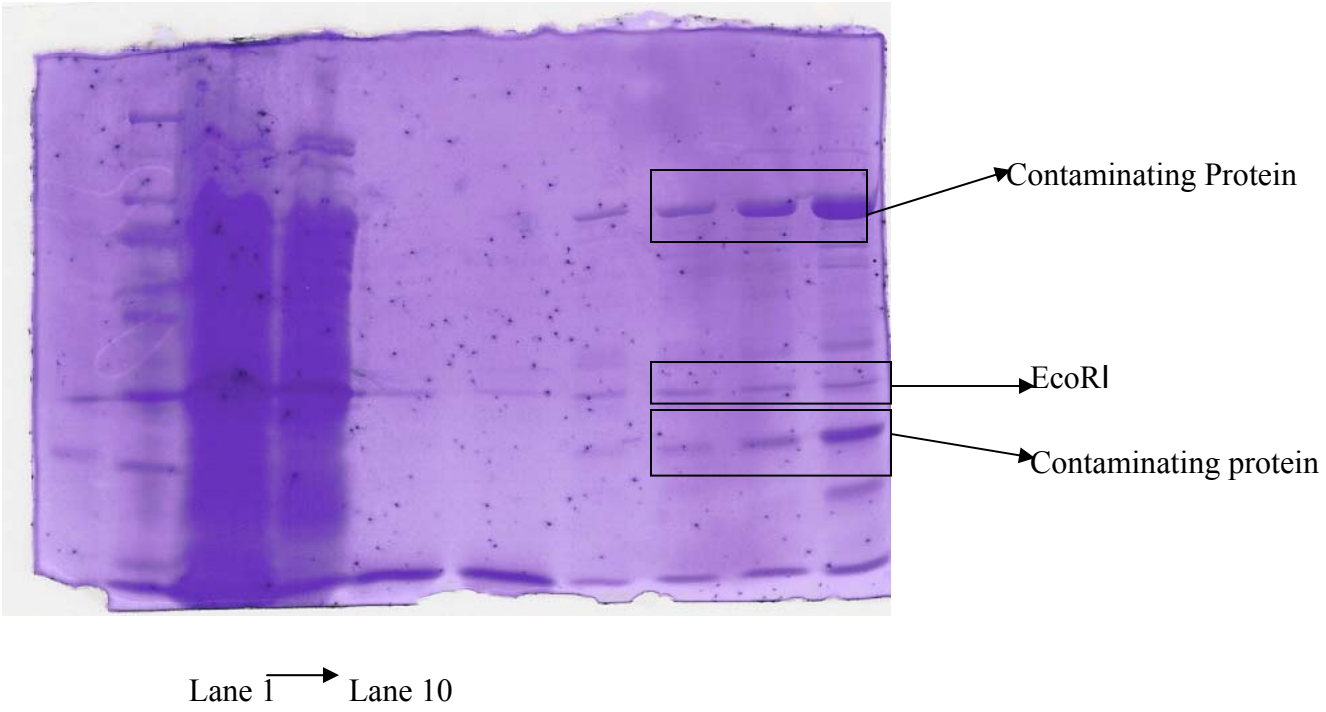

Figure 3.1 The SDS gel Ⅰ of EcoR Ⅰ RS187 through P11column in the first round purification.

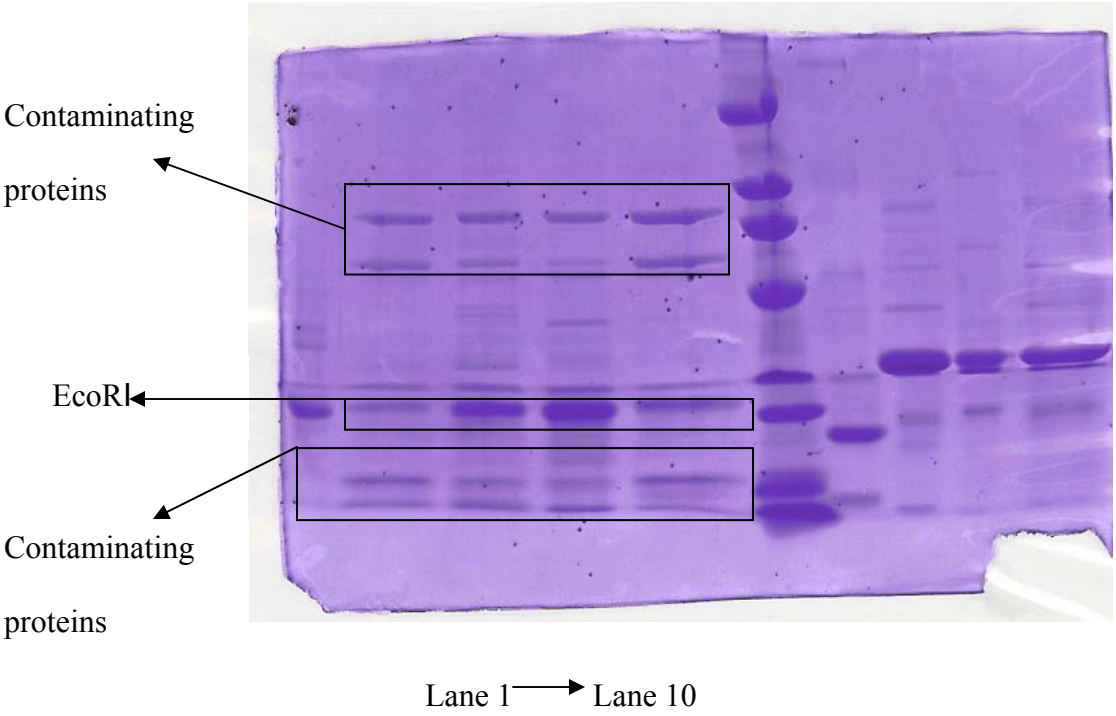

Figure 3.2. The SDS gel Ⅱ of EcoR Ⅰ RS187 through P11column in the first round purification.

contaminating proteins (figure 3.1), were very clear. These results revealed that the content of EcoRⅠ mutant in tubes 23, 25 and 27 was much lower than that of contaminating proteins. In lanes 2, 3, 4 and 5 of Gel Ⅱ (figure 3.2), the bands for EcoRⅠ mutant was much clearer. It means the content of EcoRⅠ mutant in tubes 29-34 was higher than that of mutant in tubes 23-27. However, the content of other contaminating proteins in these tubes was very high (lanes 2-5 in Gel Ⅱ). Not like wild type EcoRⅠ endonuclease, the relative concentration of EcoRⅠ mutant was low in the solutions of these tubes. In order to increase the concentration of mutant in these solutions and reduce the loss of mutant in following purification, after the protein samples of tubes 22-34 were pooled together (total volume is about 60ml), the solution was added into concentrator, the volume of which was concentrated to about 20ml. Then the concentrated solution was dialyzed into HAP start buffer.

3.2.6 Purification through HAP hydroxyapatite column

According to methods and procedures mentioned in section 2.1.6, old HAP column was regenerated and equilibrated. The following purifications were performed in three steps: sample loading, washing and elution. Finally, using the solution in tube 1 as the blank, the OD_{280} values of protein samples in tubes marked with odd numbers were read.

The tubes which might contain protein samples were divided into three groups (1, 2 and 3). The average OD_{280} values of group 1, group 2 and group 3 were 0.08, 0.13 and 0.12, respectively (table 21, 22 and 23). All of three average OD_{280} values were low, which suggested that the content of protein samples, especially EcoRⅠ mutant in these tubes should be very low. EcoRⅠ mutant should be collected in group2 or group 3.

Table 21: The OD at 280nm of 4 tubes in group1 (tube 23-tube 29)

Tubes	23	25	27	29
OD	0.044	0.069	0.124	0.066

Table 22: The OD at 280nm of 6 tubes in group2 (tube 45-tube 55)

Tubes	45	47	49	51	53	55
OD	0.094	0.150	0.165	0.133	0.133	0.121

Table 23: The OD at 280nm of 6 tubes in group2 (tube 57-tube 67)

Tubes	57	59	61	63	65	67
OD	0.117	0.120	0.115	0.119	0.119	0.125

Table 24: Samples loaded onto the lanes of Gel Ⅰ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	Marker	SP	PT	25	27	45	47	49	50

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

Table 25: Samples loaded onto the lanes of Gel Ⅱ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	51	53	54	55	57	59	61	65	67

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

The protein samples in tubes of group 1, 2 and 3 were loaded onto two 12% SDS-PAGE gels (table 24 and table 25). In lane 3 of Gel Ⅰ (figure 3.3), which was loaded with sample prior to loading onto HAP column, besides EcoR Ⅰ mutant, other three contaminating proteins could be identified clearly. The content of one contaminating protein was much higher than that of

other protein components including EcoRⅠ mutant. Not any protein samples were shown in lane 4 of Gel Ⅰ (figure 3.3), which means that all of protein samples have been loaded into HAP column. The only single sharp band standing for contaminating protein in lane 6 of Gel Ⅰ (figure 3.3) revealed that most of this contaminating protein has been separated from EcoRⅠ mutant.

In lanes 8, 9 and 10 of Gel Ⅰ (figure 3.3), only the bands standing for EcoRⅠ mutant could be identified, which means the relative purity of EcoRⅠ mutant in the solutions of tube 47-tube 50 was high. However, according to the sharpness of these bands, it could be determined that the content of EcoRⅠ mutant was low. In lanes 2, 3, 4 and 5 of Gel Ⅱ (figure 3.4), some faint bands standing for EcoRⅠ mutant and contaminating protein could be found, which means that the content of EcoRⅠ mutant and this contaminating protein in solutions of tube 51-tube 55 was very low. The single sharp bands for contaminating proteins in lanes 2, 3, 4 and 5 of Gel Ⅱ (figure 3.4), revealed that the contaminating proteins were the main component in solutions of tube 51-tube 55. Therefore, the protein samples in tubes 46-50 were pooled together. The total volume of the solution was 56ml.

Now the main difficulty was the content or concentration of EcoRⅠ mutant was very low. The further loss of EcoRⅠ mutant could be predicted during purification through Bio-Rex column. Therefore, after all of purifications were finished, the amount of EcoRⅠ mutant should have not been enough for crystal growth. In order to increase the amount of purified EcoRⅠ mutant for crystal growth, before purification through Bio-Rex column, the second round purification of EcoRⅠ mutant must be performed. Naturally, the collected protein solution was concentrated to 20ml, and stored in ice box.

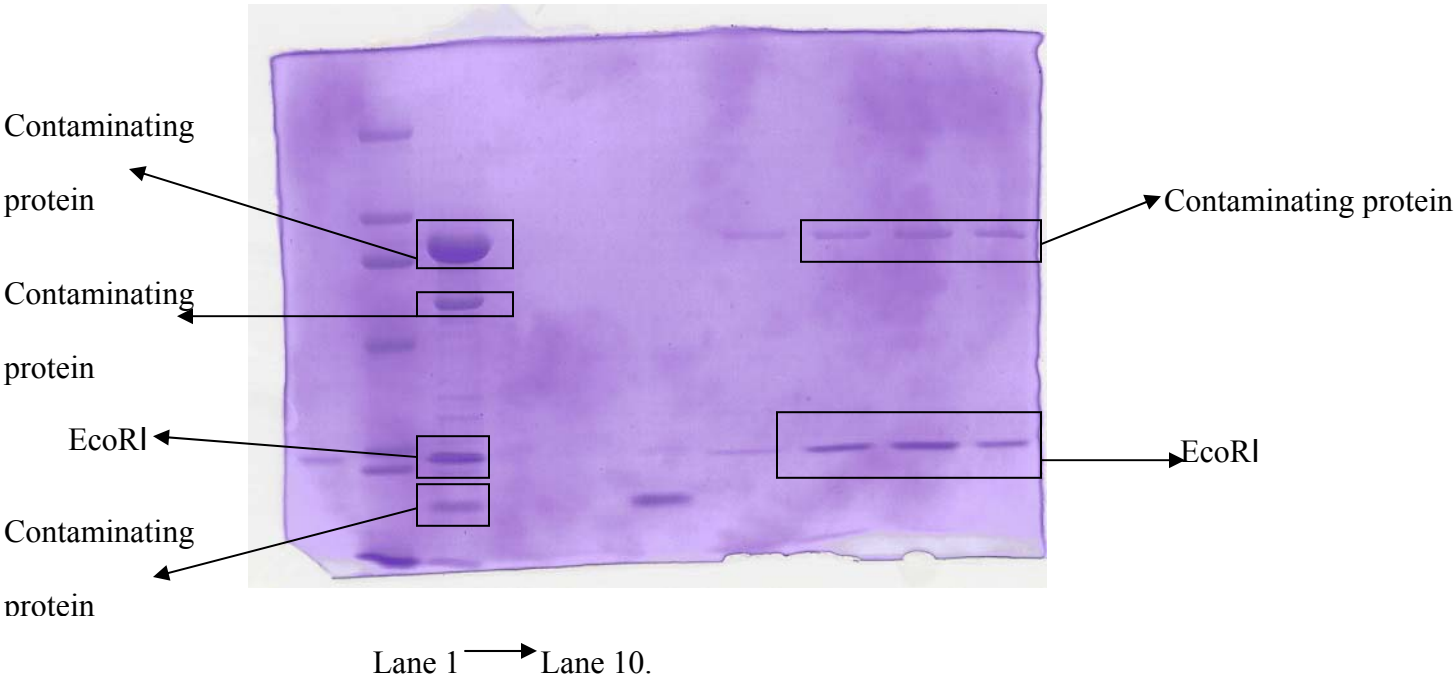

Figure 3.3. The SDS gel Ⅰ of EcoRⅠ RS187 through HAP column in the first round purification

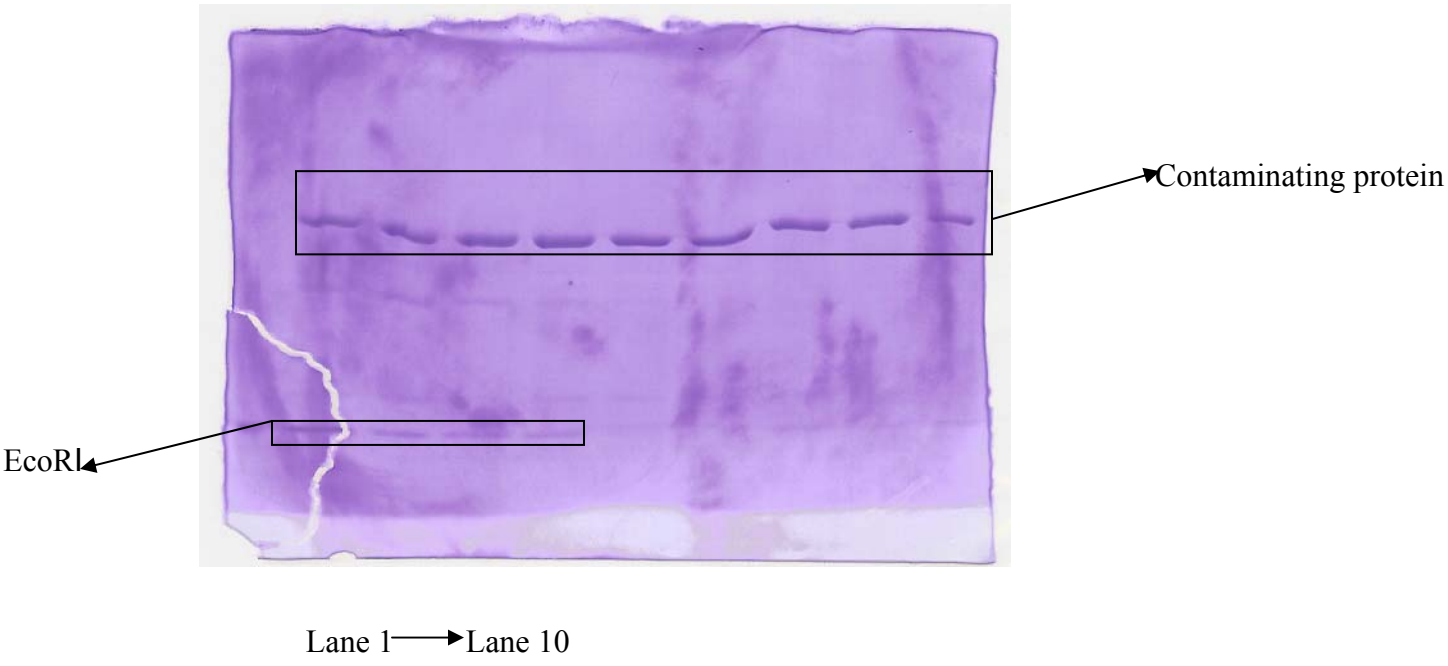

Figure 3.4. The SDS gel Ⅱ of EcoRⅠ RS187 through HAP column in the first round purification

3.2.7 The second round purification of EcoR Ⅰ endonuclease RS187

With the transformed cell colonies stored in refrigerator, the second round of cell growth could be carried out. As mentioned above, after cell growth, expression induction and harvesting cells, 8.48g E.coli cells expressing EcoR Ⅰ mutant were achieved. Then the cells were lysed with sonicator, and the crude cell lysate was dialyzed into P11 start buffer for sample loading.

Before sample loading, used P11 column was regenerated and equilibrated well, and then crude cell lysate was loaded onto P11 column. Through washing and gradient elution, the expressed protein components were collected in the tubes marked with numbers of auto-collector. Then the OD_{280} values of the solutions in tubes marked with odd numbers were read on spectrophotometer.

The tubes which might collect protein components were classed into three groups (listed in table 26, table 27 and table 28). The average of OD_{280} values of group1, group2 and group3, were 0.33, 0.24 and 0.27, respectively. It was most possible for EcoR Ⅰ mutant to be distributed in tubes of group1.

Some protein samples in tubes of group1, group2 and group3, were loaded onto two 12% SDS-PAGE gels (table 29 and table 30). Because flask holding “pass through” solution was contaminated during the experimental operation, the protein sample prior to dialysis into P11 start buffer (SP1) and the protein sample after dialysis into P11 start buffer (SP2) were loaded onto lane 3 and lane 4 respectively.

Table 26: The OD at 280nm of 10 tubes in group1 (tube 29-tube 47) for P11 purification

Tubes	29	31	33	35	37	39	41	43	45	47
OD	0.22	0.35	0.49	0.48	0.41	0.34	0.29	0.25	0.24	0.23

Table 27: The OD at 280nm of 6 tubes in group2 (tube 49-tube 59) for P11 purification

Tubes	49	51	53	55	57	59
OD	0.23	0.264	0.272	0.248	0.227	0.225

Table 28: The OD at 280nm of 5 tubes in group3 (tube 61-tube 69) for P11 purification

Tubes	61	63	65	67	69
OD	0.243	0.279	0.282	0.265	0.259

Table 29: Samples loaded onto the lanes of Gel Ⅰ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	Marker	SP1	SP2	27	29	30	31	33	35

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes.

Table 30: Samples loaded onto the lanes of Gel Ⅱ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	37	39	41	43	45	47	53	36	65

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes.

The bands standing for EcoRⅠ mutant (RS187) in lanes 5-10 of gel Ⅰ (figure 3.5) were thin and faint, which means that in tubes 27-35, the content of EcoRⅠ mutant was very low. Similar to the first round purification through P11 column, the bands standing for EcoRⅠ

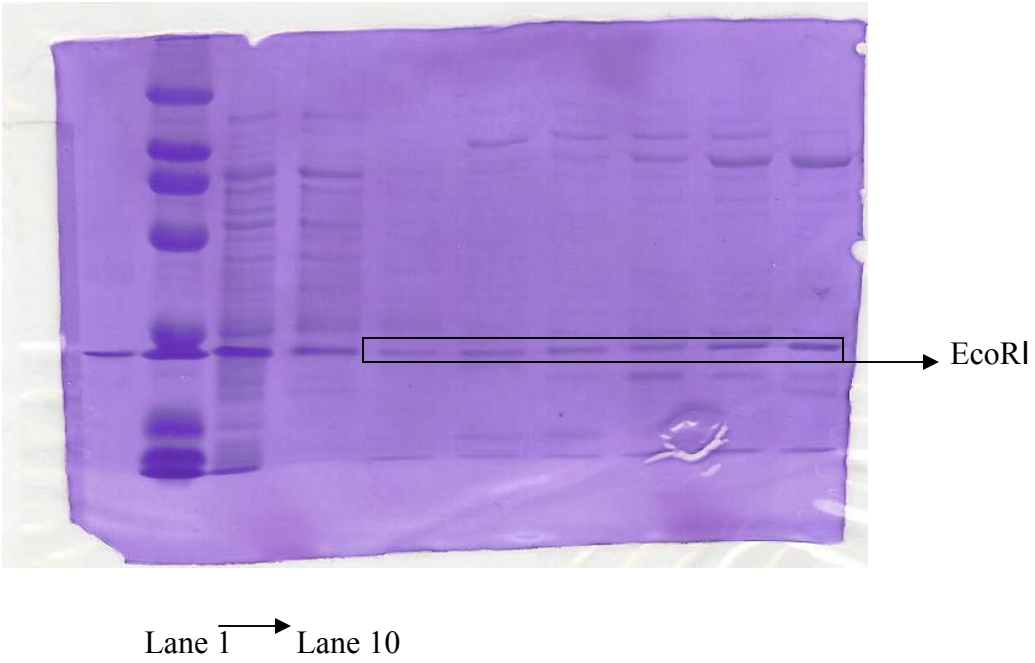

Figure 3.5. The SDS gel Ⅰ of EcoRⅠ RS187 through P11column in the second round purification

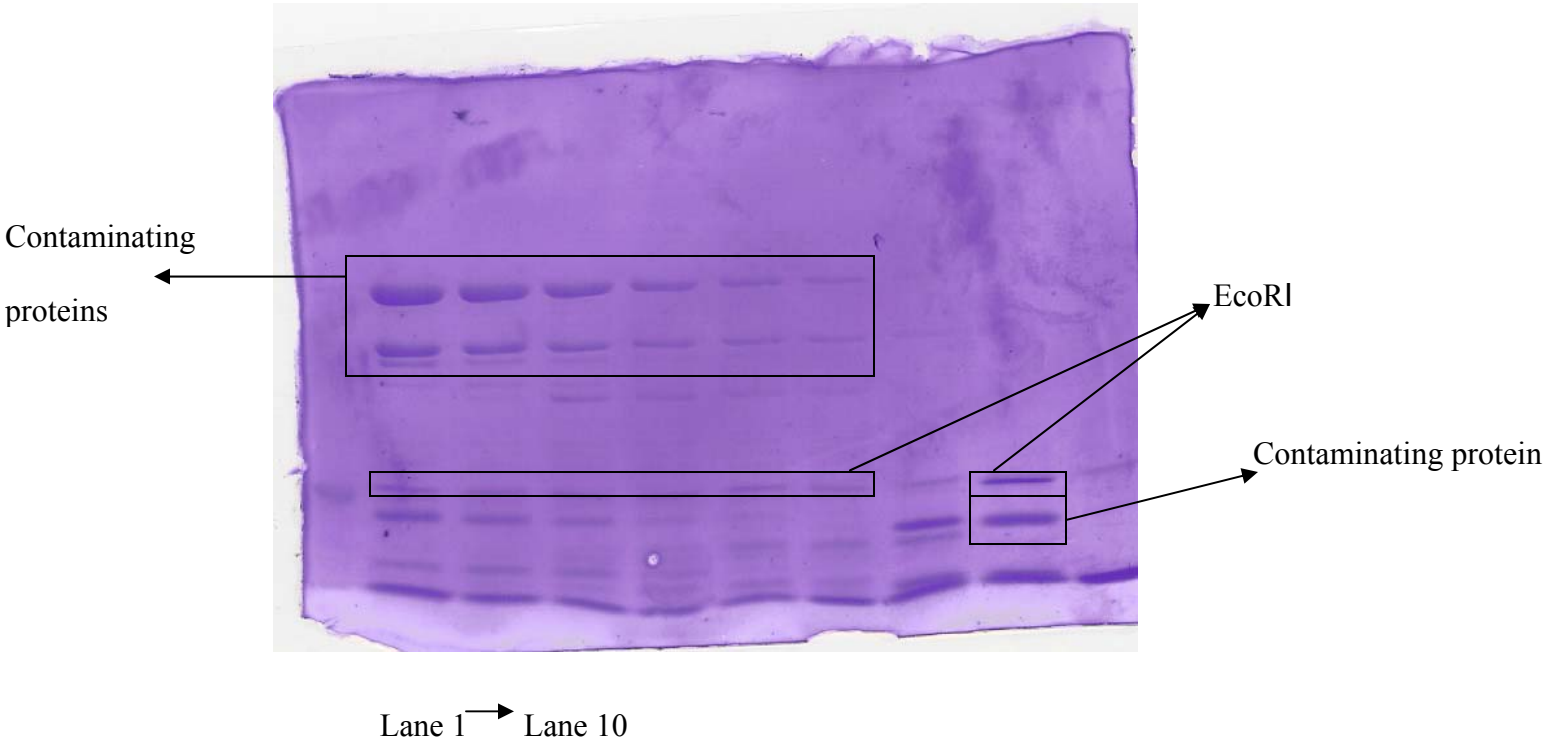

Figure 3.6. The SDS gel Ⅱ of EcoRⅠ RS187 through P11column in the second round purification.

mutant and contaminating proteins in lanes 2-7 and lane 9 of gel Ⅱ (figure 3.6) revealed that the content of contaminating proteins was much higher than that of EcoRⅠ mutant. In another word, the content and relative concentration of EcoRⅠ mutant in solutions of tubes 36-47 were very low. The bands standing for EcoRⅠ mutant almost could not be identified in lane 8 and lane 10 in gel Ⅱ (figure 3.6). So EcoRⅠ mutant could not be present in the tubes of group2 and group3.

Although the content and relative concentration of EcoRⅠ mutant in solutions of tubes 27-47 were very low, the protein samples in these tubes were pooled together and concentrated to 25ml.

Table 31: The OD at 280nm of 6 tubes in group1 (tube 31-tube 41) for HAP purification

Tubes	31	33	35	37	39	41
OD	0.07	0.091	0.104	0.086	0.078	0.078

Table 32: The OD at 280nm of 7 tubes in group2 (tube 45-tube 57) for HAP purification

Tubes	45	47	49	51	53	55	57
OD	0.082	0.093	0.095	0.098	0.113	0.127	0.122

Table 33: The OD at 280nm of 4 tubes in group3 (tube 59-tube 65) for HAP purification

Tubes	59	61	63	65
OD	0.155	0.182	0.150	0.144

Table 34: The OD at 280nm of 7 tubes in group4 (tube 71-tube 83) for HAP purification

Tubes	71	73	75	77	79	81	83
OD	0.147	0.149	0.154	0.157	0.162	0.168	0.170

The concentrated protein solution was dialyzed into HAP start buffer. Then the protein samples were loaded onto HAP column which had been regenerated and equilibrated well. After purification through washing and gradient elution, different protein components were collected in the tubes marked with numbers of round auto-collector. Then the OD_{280} values of the solutions in 50 tubes marked with odd numbers were read.

The protein samples were distributed in the tubes of four groups: group1, group2, group3, and group4. The average OD_{280} values of the solutions in tubes of groups 1, 2, 3 and 4, were 0.08, 0.1, 0.16 and 0.16 (tables 31, 32, 33 and 34). All of the OD_{280} values of solutions in tubes of four groups were very low. Therefore, the contents of all kinds of protein components including EcoRⅠ mutant were very low in the solutions of tubes of four groups. EcoRⅠ mutant was collected in the tubes of group2 or group3. The high concentration of salt in solution may cause the relative high OD_{280} values of solutions in the tubes of group4.

The protein components of four groups were loaded onto two 12% SDS-PAGE gels (table 35 and table 36). There was only one band standing for contaminating protein in lane 4 of Gel Ⅰ (figure 3.7). So this contaminating protein has been separated from other protein components. Only two unclear bands for EcoRⅠmutant could be identified in lane 9 and lane 10 of Gel Ⅰ (figure 3.7). It means that EcoRⅠ mutant only was present in the solutions of tubes 50-53, but the content of EcoRⅠ mutant in these tubes was very low. The bands for EcoRⅠ mutant became more clearer in lanes 2-5 of Gel Ⅱ (figure 3.8), which means the content of

EcoR Ⅰ mutant in tubes 54-57 was higher than in tubes 50-57, but compared with the bands for contaminating proteins in lanes 2-5 of Gel Ⅱ, it could be considered that the content of contaminating proteins was higher than that of EcoR Ⅰ mutant. In lanes 6-10, there were not any bands for EcoR Ⅰ mutant (figure 3.8). It means that EcoR Ⅰ mutant was not distributed in tubes of group3 and group4.

Table 35: Samples loaded onto the lanes of Gel Ⅰ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	Marker	PT	35	41	45	47	49	51	53

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

Table 36: Samples loaded onto the lanes of Gel Ⅱ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	54	55	56	57	58	61	75	81	83

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

At last the protein samples in tubes 50-57 were pooled together, though the content or relative concentration of EcoR Ⅰ mutant in these tubes were very low. This collected protein solution was mixed with the protein solution in the first round purification. The mixed solution was transfered into the concentrator, the volume of which was concentrated from about 65ml to 15ml. This concentrated solution was dialyzed into Bio-Rex start buffer for sample loading. The used Bio-Rex 70 column has been regenerated and equilibrated well.

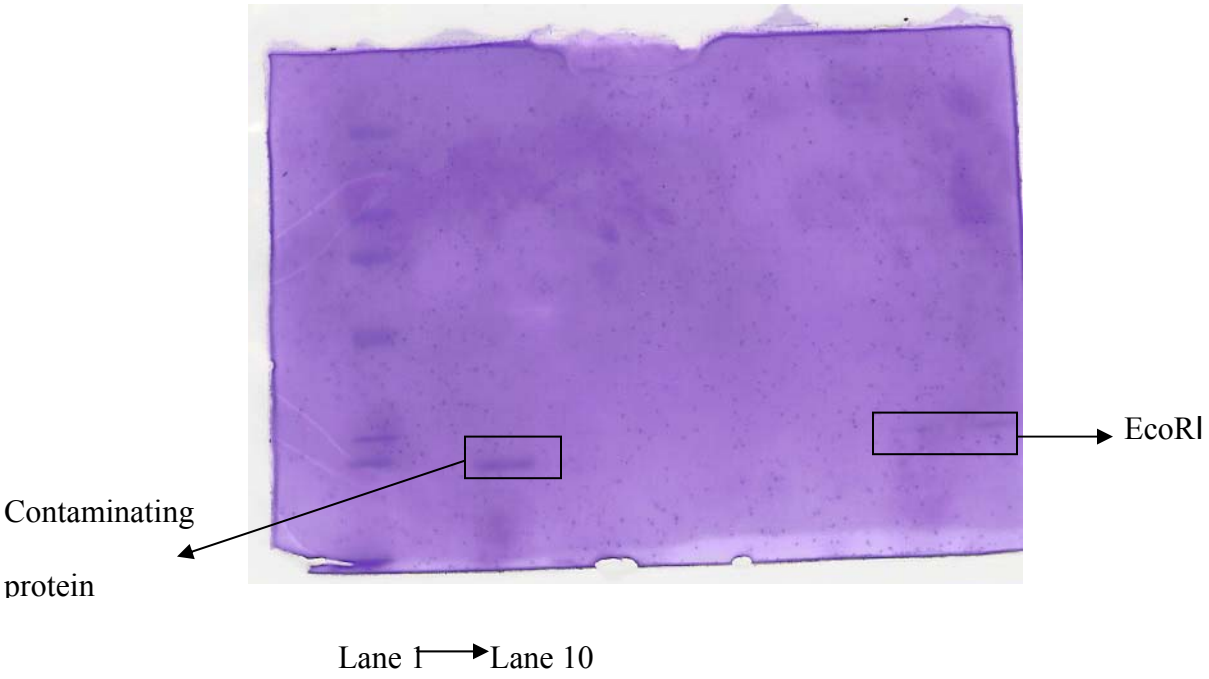

Figure 3.7. The SDS gel Ⅰ of EcoRⅠ RS187 through HAP column in the second round purification

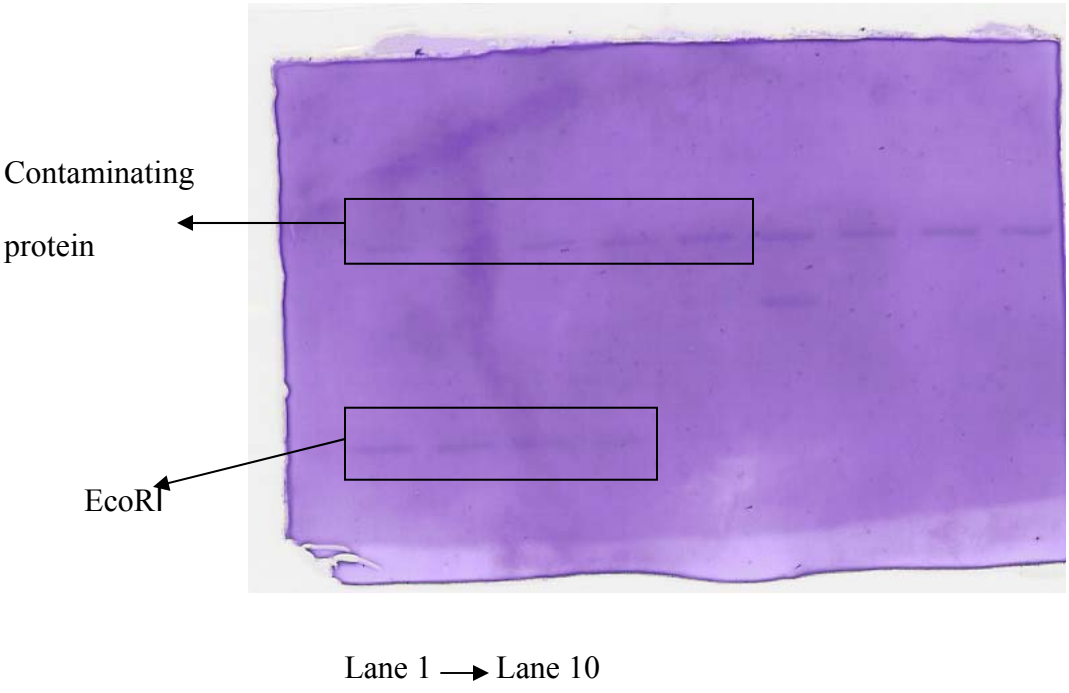

Figure 3.8. The SDS gel Ⅱ of EcoRⅠ RS187 through HAP column in the second round purification

3.2.8 Purification through Bio-Rex 70 column

The experiments were carried out as the steps mentioned in section 2.1.7. EcoR Ⅰ mutant which has been loaded into Bio-Rex 70 column, was purified through washing and gradient elution. Then the protein components collected in tubes marked with odd numbers were taken to read the OD_{280} values. Not like the OD_{280} reading in section 2.1.7, in this OD_{280} reading, the solution in tube 1 was used as the blank to set zero.

Table 37: The OD at 280nm of 9 tubes in group1 (tube 43-tube 59)

Tubes	43	45	47	49	51	53	55	57	59
OD	0.012	0.012	0.012	0.013	0.021	0.038	0.029	0.018	0.009

Table 38: Samples loaded onto the lanes of Gel Ⅰ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	Marker	SP	42	43	44	45	46	47	49

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

Table 39: Samples loaded onto the lanes of Gel Ⅱ

Lanes	1	2	3	4	5	6	7	8	9	10
Sample	ST	50	51	52	53	54	55	56	57	58

Numbers stand for the tubes from which protein samples were taken to be loaded onto the lanes

According to the OD_{280} values of the solutions in tubes, protein samples were mainly collected in the tubes of group 1 (table 37). However, the OD_{280} values of the solutions in tubes of group 1 were very low. The average OD_{280} value of the solutions in these tubes was 0.02. So the content of protein components in these solutions should be very low.

The samples loaded onto two 12% SDS-PAGE were listed in table 38 and table 39. According to the bands standing for EcoR Ⅰ mutant and contaminating protein in lanes 4-10 of Gel Ⅰ (figure 3.9), the content of the contaminating protein decreased gradually. In contrast, the content of EcoR Ⅰ mutant went up obviously. Especially, in lane 8, lane 9 and lane 10 of Gel Ⅰ (figure 3.9), the contents of EcoR Ⅰ mutant were higher than that of contaminating protein. So the concentration of EcoR Ⅰ mutant in tubes 46-49 was higher than that of contaminating protein.

Table 40: The concentrations (mg/ml) of EcoR Ⅰ mutant (RS187) with different volumes

Volume	8ml	800µl	400µl	120µl
Concentration	0.054mg/ml	0.61mg/ml	1.17mg/ml	3.82mg/ml

In lanes 2-10 of Gel Ⅱ (figure 3.10), only the single bands for EcoR Ⅰ mutant could be identified clearly. The bands for contaminating protein were very faint. These results revealed that the purity of EcoR Ⅰ mutant in tubes 50-58 was high. Finally, EcoR Ⅰ mutant in tubes 46-58 was pooled together, and dialyzed into BTP buffer (40mM BisTris propane, 0.5M NH_4Cl, 1mM EDTA, 15% Dioxane (v/v); pH 8.5). After dialysis was finished, the protein solution was concentrated using 12-14K concentrator. In the first concentration, the volume of protein solution changed from 34ml to 8ml. The concentration of this solution was determined with Bradford assay mentioned in section 2.1.7, and set to 0.054mg/ml (table 40). This concentration was too low for the requirement of crystal growth. So the protein solution was further concentrated.

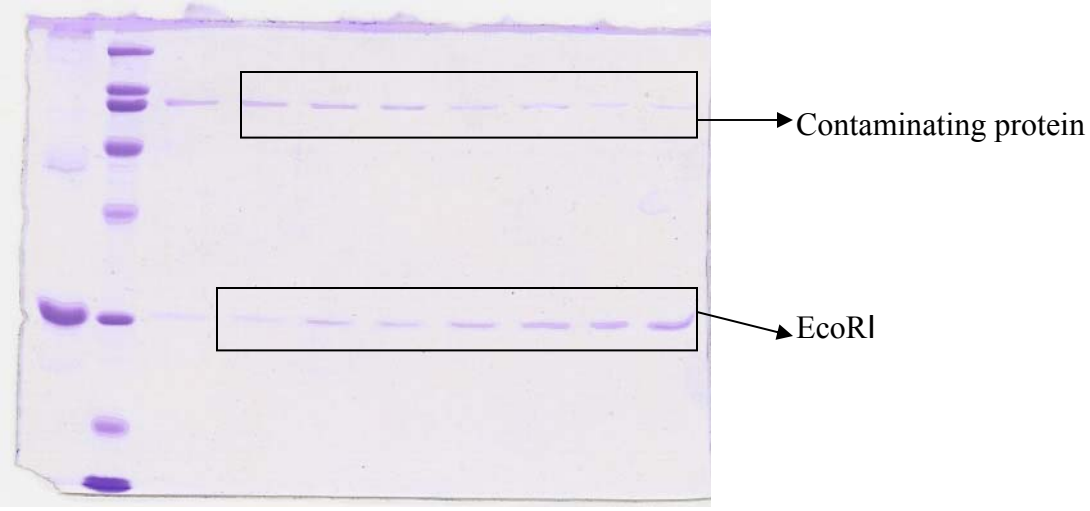

Figure 3.9. The SDS gel Ⅰ of EcoRⅠ RS187 through Bio-Rex 70 column

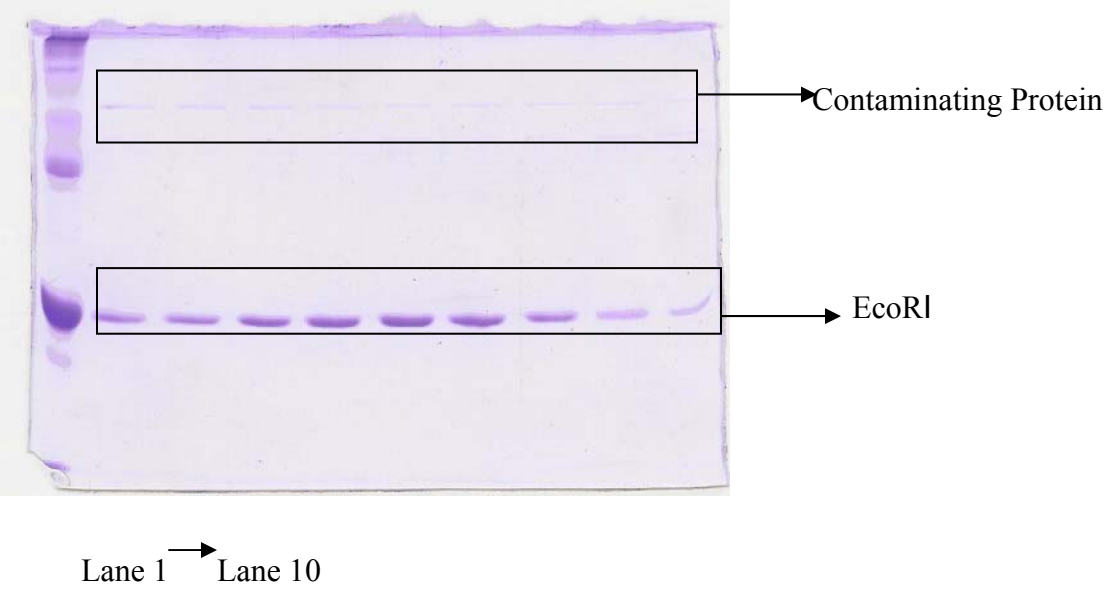

Figure 3.10. The SDS gel Ⅱ of EcoRⅠ RS187 through Bio-Rex 70 column

In second round concentration, the protein solution was transferred into 12-14K dialysis membrane with the size of 28mm. Aquacide was placed on the surface of dialysis membrane to absorb the solvent. When the volume decreased to about 800μl, the concentration was set to 0.61mg/ml (table 40). This concentration was also too low. In order to further concentrate protein solution, the solution was transferred into another 12-14K dialysis membrane with the size of 10mm. Using aquacide, the volume was concentrated to about 400μl. The third concentration was set to 1.17mg/ml. When the final volume was 120μl, the concentration was set to 3.82mg/ml. Although the proper concentration for crystal growth should be about 6-7mg/ml, further concentration could cause much loss of protein sample in dialysis membrane. Therefore, the concentration was left at 3.82mg/ml. Then the solution was transferred from dialysis membrane into a tube, and stored in ice box for crystal growth.

3.2.9 Crystal growth of EcoRⅠ mutant (RS187)-DNA 13mer complex

The crystals of EcoRⅠ mutant (RS187)-DNA 13mer complex were grown with sitting drop vapor diffusion. Reservoir buffer used in this crystal growth comprised 16% PEG 400 (w/v) and 40mM BTP (pH 6.5). Precipitant (PPT) consisted of 40% PEG 400 and 40mM BTP (pH 7.0). Based on the conditions of crystal growth for WT EcoRⅠ-13mer complex (section 2.1.8), the conditions of crystal growth for EcoRⅠ mutant-13mer complex could be determined. Because of the very limited volume of EcoRⅠ solution (120μl), only two rows in the tray could be set up for crystal growth. The setting up of crystallization in the tray was listed in table 41.

Table 41: The setting up of crystallization in the tray

	1	2	3	4	5	6
A: DNA	5μl	4μl	3μl	2μl	5μl	4μl
Protein	3μl	3μl	3μl	2μl	2μl	2μl
PPT	2μl	2μl	2μl	2μl	2μl	2μl
B: DNA	5μl	5μl	5μl	7μl	7μl	7μl
Protein	5μl	5μl	4μl	5μl	5μl	4μl
PPT	3μl	3μl	3μl	2μl	3μl	3μl

After about 18 days, some very small crystals were grown in drop B1, B2 and B3 (figure 3.11, figure 3.12 and figure 3.13). Although the size and shape of these crystals were not as well as the crystals of WT EcoRⅠ-13mer complex, this crystal growth has made a good basis for future crystal growth and diffraction.

3.3 DISCUSSION AND CONCLUSION

The methods and procedures of purification of EcoRⅠ mutant (RS187) are almost as same as those of wild type EcoRⅠ. Although the growth levels of E. coli cells expressing EcoRⅠ RS187 in two rounds of purification were close to cells expressing wild type EcoRⅠ, the expression levels of EcoRⅠ RS187 were much lower than that of wild type EcoRⅠ. In contrast, the expression levels of main contaminating proteins in mutation cells were close to the levels of WT cells. Therefore, the relative concentration of EcoRⅠ mutant was very low. In order to

increase or keep the concentration of EcoRⅠ mutant during purification through Bio-Rex 70 column, a total of 10 L of cell culture was incubated in two rounds. Furthermore, after purification was finished, the EcoRⅠ mutant solution was concentrated from 34ml to 120μl to maximize the concentration.

Although the content of EcoRⅠ mutant (RS187) in protein solution was very low, most of contaminating proteins were separated from the EcoRⅠ mutant. The content of contaminating protein which could not be totally separated from the EcoRⅠmutant, could be reduced very much in purification through Bio-Rex 70 column. So the homogenous purity of purified EcoRⅠ mutant was very high.

Some small crystals were observed in B1, B2 and B3 drops, in which the conditions of crystal growth were same. On one hand, it means that the quality and purity of purified EcoRⅠ RS187 were good for crystal growth. On the other hand, the relative low concentration of EcoR Ⅰ mutant probably prevented growth of these crystals.

To improve the quality of the crystal, firstly, the concentration of EcoRⅠ mutant should be increased. Therefore, in future study, at least 15 L of cell culture should be incubated to express enough amount of EcoRⅠ RS187. During purification, to minimize the loss of protein, loading sample should be concentrated to much smaller volume, and eluted protein fractions should be collected in the tubes with 40 drops per tube. If we could express and purify enough amount of EcoRⅠ RS187, more conditions can be scanned. However, the crystals obtained are probably adequate for structure determination.

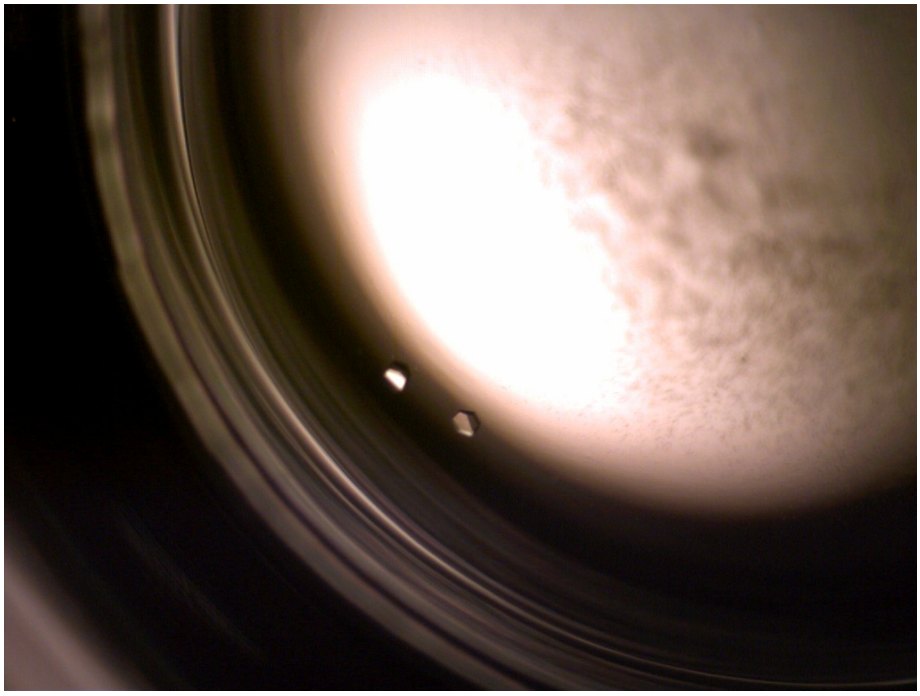

Figure 3.11 The crystals of EcoR Ⅰ RS187-DNA complex in B1

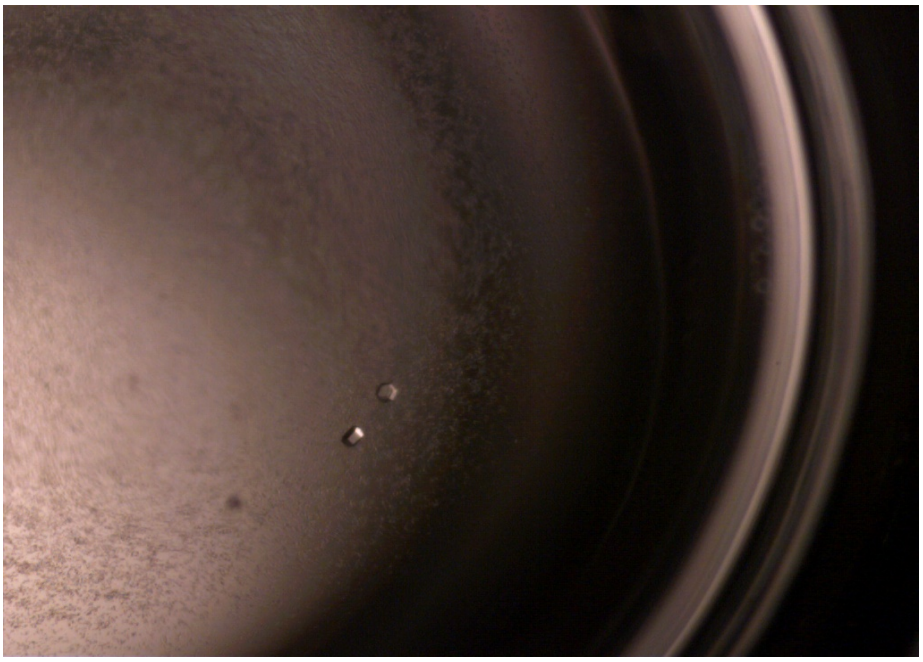

Figure 3.12 The crystals of EcoR Ⅰ RS187-DNA complex in B2

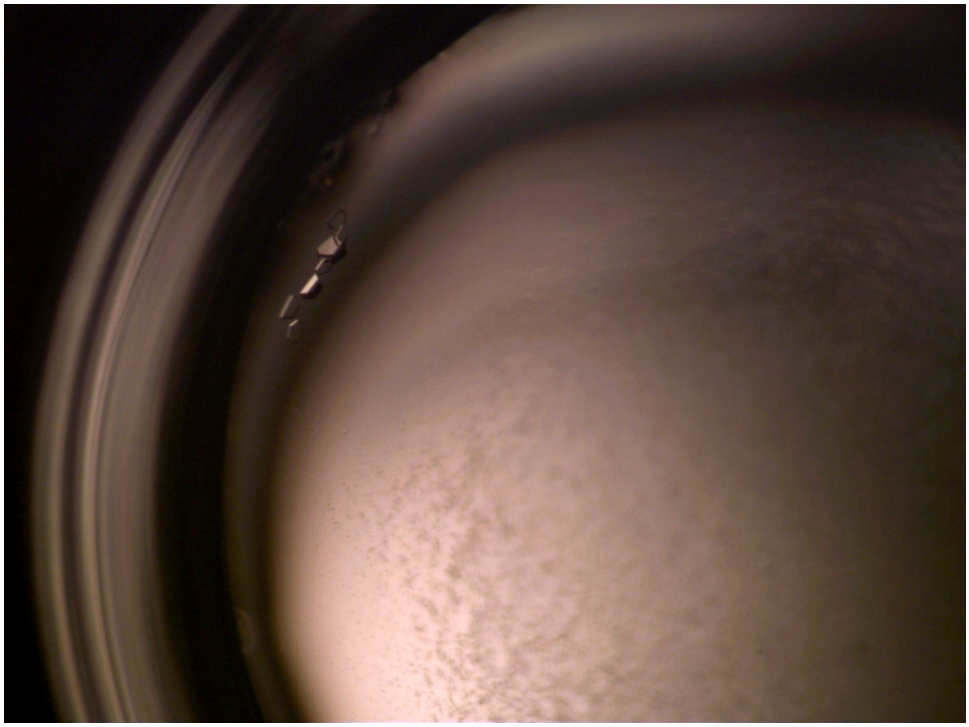

Figure 3.13 The crystals of EcoR Ⅰ RS187-DNA complex in B3

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