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Expression, Purification and Characterization of RecJ Nuclease -An Integrated Biochemistry Laboratory Course for Undergraduates

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ABSTRACT

Here we describe a half-semester biochemistry laboratory course based on the expression, isolation, purification, and biochemical characterization of RecJ nuclease. The students transformed the expression plasmid of thermostable RecJ nuclease into the host bacteria *Escherichia coli* BL21(DE3), to induce the expression of the recombinant RecJ nuclease by IPTG. The expressed RecJ nuclease was purified from the cell lysates broken with sonication by Ni-affinity chromatography. SDS-denatured electrophoresis was utilized to identify the purity and size of the RecJ exonuclease. The activity of the enzyme was assessed by an *in vitro* exonuclease reaction using a fluorescent-labeled single-stranded DNA substrate. Through this lab course, students grasp the method of preparing and characterizing a recombinant protein and excise more than one biochemistry and molecular biology common techniques, which are great useful and significant to their future works.

Keywords: Biochemistry Laboratory Course; RecJ Nuclease; Protein Expression and Purification; Enzymatic Assay

Introduction

In traditional biochemical experiments, students passively accept knowledge. This type of experimental course has a fixed teaching model. Some teachers not only tell students the principles and procedures of experiments in the class, but also tell them the results of experiments. Students follow the experimental steps for mechanical operation without thinking or innovation. This procedural teaching method has seriously decreased students' interest in experimental classes. At the same time, it is difficult for students to apply the techniques learned in the experimental class to their own future research projects flexibly. Until now, many schools are always trying to optimize the teaching mode of the experimental course [1-6]. Teachers also tried to improve the curriculum mode in the integrated biochemistry experiment. The improved integrated laboratory course allows students to become explorers rather than learners. Students will complete the entire laboratory project independently through participating in each course stage, including pre-experiment preparation, experimental design, instrument use, experimental results analysis and laboratory report writing. The integrated biochemistry course provides students with an opportunity to perform a scientific research experience.

This is a half-semester (6 weeks) laboratory course. Schedule of this course is shown in Table 1. At the beginning of the semester, students are informed of the progress of the experiment, and each student is required to read the relevant literature and be familiar with the experimental content before each session. One or two students are randomly selected to explain the principles and operation steps of the experiment at the beginning of each class. The teacher only gives

supplementary explanations. The course takes a small class of 10 to 15 students for single-person operation, so that each person can complete his own experiments independently. At the end of the class, the students will prepare their own reagents for the next week's course under the guidance of the teacher. This comprehensive experiment has strong coherence. Students are involved in every aspect of the experiment and need to operate more instruments and grasp more techniques. Such an experimental course gives a better simulation of the experimental process of the student's independent research in the future, and the students' innovative ability is cultivated.

Table 1: Schedule of the biochemistry laboratory course.

Weeks	Schedule of Laboratory Course
Week 1	Overview of experimental courses and safety notes
Week 2	Preparation of host strains and transformation of recombinant plasmids
Week 3	Inducible expression of RecJ nuclease cryopreservation of bacteria
Week 4	Sonication of bacteria and purification of RecJ nuclease
Week 5	Characterization of RecJ protein size (SDS-PAGE)
Week 6	Activity identification of RecJ nuclease

The target protein selected in the course is thermostable RecJ nuclease. RecJ is an Mg^{2+}-dependent single-stranded DNA (ssDNA) exonuclease that degrades its substrates in the 5'-3' direction and participates in homologous recombination and mismatch repair [7]. The advantage of choosing this protein is that RecJ exonuclease is a highly conserved enzyme in bacteria and archaea, and it is easy to obtain its gene fragments [8-10]. In addition, the activity of the RecJ enzyme can be characterized quickly by the assaying of its ability to degrade single-stranded DNA molecules.

The host strain used in this experiment is BL21 (DE3), which is genetically modified to meet the needs of expressing a recombinant protein. The host strain contains a set of T7 expression system [11-13], and the target gene (RecJ) in the recombinant plasmid cannot be expressed under normal conditions unless induction by IPTG (Isopropyl Thiogalactoside) [14-16]. The recombinant plasmid used in the experiment is from pDEST17 vector with an in-frame inserted RecJ gene, which has antibiotic resistance to ampicillin. This recombinant plasmid was constructed by students themself in the first half of the semester which is another integrated experimental course. At the end of the first half of the semester, the students stored their own recombinant plasmids at -20°C. Therefore, what the students need to do in this experimental course is to transform their own recombinant plasmids into the host strain, then RecJ exonuclease is expressed by induction with IPTG, and finally students extract the expressed protein and characterize the size and activity of the enzyme.

Materials and Methods

Reagents, Materials and Equipment

The reagents and materials used for this laboratory course are commonly used in the biochemistry lab. Yeast extract, tryptone, agar powder, glycine, TEMED, acrylamide, etc. were purchase from Sinopharm Chemical Reagent Co (Shanghai, China). Sodium chloride, sodium hydroxide, etc. were purchase from Shanghai Lingfeng Chemical Reagent Co (Shanghai, China). IPTG was purchase from Beijing Dingguo Biotechnology Co. (Beijing, China). Coomassie brilliant blue fast staining solution was purchase from Solarbio Science & Technology Co (Beijing, China). The host strain used in this experiment was BL21 (DE3) and the plasmid vector was pDEST17. The recombinant plasmid has been sequenced to confirm successful insertion of the RecJ gene.

The following equipment is required for the experiment: Ultra-clean Worktable (AIRTECH Co., Suzhou, China, SW-CJ-2FD), Thermostat Water Bath (Shanghai Jing Hong Laboratory Instrument Co., Shanghai, China, DK-S24), Desktop Full Temperature Shaker (Shanghai Jing Hong Laboratory Instrument Co., Shanghai, China, THZ-421), Biochemical Incubator (Shanghai Heheng Instrument Equipment Co., Shanghai, China, LRH-250F), Single-beam UV-VIS Spectrophotometer (Shanghai Xinmao Instrument Co., Shanghai, China, UV-7504), Ultrasonic Crasher (Scientz Biotechnology Co., Ningbo, China, JY92-IDL), Vertical Electrophoresis Tank (Tanon Science & Technology Co., Shanghai, China, VE-180), FLA-9000 multifunctional laser imager (Fujifilm, Japan).

Laboratory Procedures

Inducible Expression of RecJ Nuclease-Figure 1 showed the flowchart for the laboratory practice. Students take their own recombinant plasmid (10 µl/tube) from the -20°C freezer and competent cells (100µl/tube) from -80°C freezer respectively. Mixed them gently and placed on ice for at least half an hour. The mixture need be heated shock at 42°C water bathing for 90s accurately, then put back on ice for 5 min. 1 ml of LB liquid medium added to the mixture. After culturing on shaker at 200 rpm at 37°C for 1 hour, students take 100µl culture for plating on LB plate containing ampicillin (50 µg/ml). The plate is kept at 37°C until monoclonal colonies appear. A colony is picked using pipette with a tip into 25 ml LB liquid medium containing ampicillin (50 µg/ml) and grow for overnight on shaker at 200 rpm at 37°C. The overnight bacterial cultures were diluted into 150ml fresh LB liquid medium containing ampicillin. When the OD_{600} of the cultures was 0.6 to 0.8, 0.5 mM of inducer IPTG is added and then the bacteria are further cultured for 3 hours [13-16].

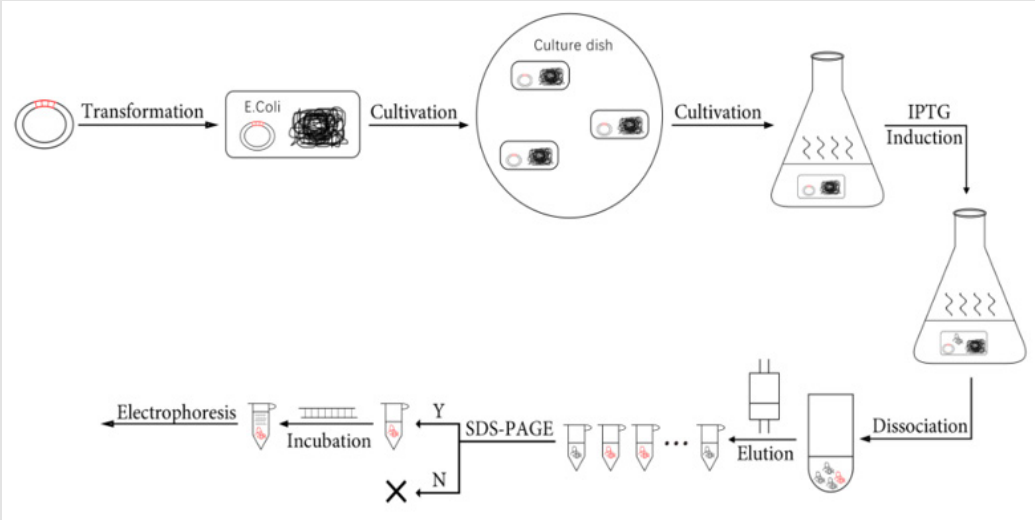

Figure 1: Flowchart for laboratory practice. The course takes 6 weeks and several of the most routinely used biotechniques include plasmid transformation, bacterial culture, inducible expression, sonication, Ni-affinity chromatography, SDS-PAGE etc.

Isolation and Purification of RecJ Nuclease-The bacterial cells were collected by centrifugation using a 50 ml sterile centrifuge tube after inducible expression. Each student added a 20 ml lysis buffer to resuspend the bacterial cells and then broke them ultrasonically (Crushing conditions: 300w power, 5s ultrasonic, 5s interval, repeat 100 times in ice water) [17]. After sonication, the cell lysates were centrifuged to remove the insoluble cell debris and the supernatant was taken for the next step purification. In this experiment, RecJ protein was purified by Ni-affinity chromatography. The column was pre-equilibrated with lysis buffer, and then the supernatant was slowly added into column to flow through the resin. After washing the impurity proteins using lysis buffer containing 20 mM imidazole, the adsorbed proteins were eluted by a high concentration of lysis buffer containing 200 mM imidazole [18,19]. Each student collected 5 tubes (each 1.0 ml) of purified protein solution.

Characterization of RecJ Nuclease-The purity and size of the purified protein was determined by SDS-PAGE. The 5 tubes of protein were loaded according to the order of elution for gel electrophoresis. After electrophoresis, the gel was stained with Bio-Safe Coomassie Blue G-250 and the bands were observed. The activity of enzyme was identified by exonuclease assay reaction [7,20]. The reaction system (15μl in total) contains reaction buffer (1.5 μl), substrate (5'-FAM-labelled-ssDNA, 4 pmole), enzyme (1μl, 2μl of purified protein, 1μl positive commercial exonuclease, negative control BSA) and deionized water. The reactions were incubated at 55°C for 20 minutes, then an equal volume (15 μl) of the reaction stopping solution was added to stop the reaction. 15μl reaction mixture was taken and loaded onto 8M urea gel for PAGE. After electrophoresis, the gel was scanned and taken a photo using the gel imaging system, and let the students analyze the bands on the gel.

Results and Discussion

After the recombinant plasmid is transformed into BL21 (DE3), the students will take a certain volume of bacterial cultures and apply it on the LB plate to obtain the single clone strain. To use a suitable inoculum for obtaining a single clone easily, the teacher would suggest that students set up 2-3 subgroups inoculum in the experiment, such as inoculation amount of 50μl and 150μl of bacteria cultures on two plates respectively. Due to the limited class hours, students can't carry out pre-experiment, so in the whole experiment process teachers will try to let students set more gradient to reduce the risk of experimental failure. At the same time, it can also cultivate students' consciousness of establishing gradient experiment at any time. The result shows that all the students could find a plate with moderate colony number in their own different gradient culture plates and easy to pick out the single clone strains. The student's picture is shown in Figure 2.

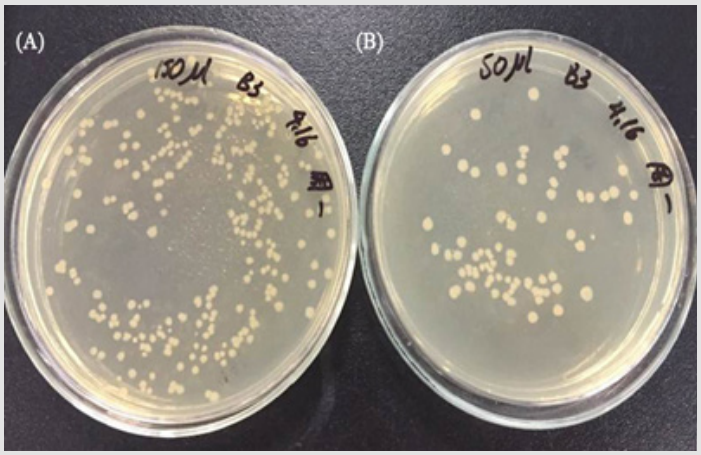

Figure 2: Plates with single clone strain. The inoculation amount of the two plates was (A) 50 µl and (B) 150 µl respectively.

At the stage of inducible expression, the growth rate of the bacterial culture varies from student to student because the amount of inoculation varies when each student picks up the single clone strain. The initial OD_{600} value of the bacterial culture was about 0.35-0.5 after inoculating into 150 ml of fresh LB medium. All students can obtain the bacterial culture with an OD_{600} value of 0.6-0.8 within 1 hour and accurately add the correct amount of IPTG. Currently, the bacteria are in the logarithmic growth phase, and the capability of induced expression is the best. In this step, students can take out 1 ml of bacterial culture without IPTG as their own negative control. There are many ways to lyse bacterial cells such as manual homogenization, sonication, freeze-thawing, alkaline treatment, high-pressure treatment [21,22]. To make students exposed to more laboratory equipment, this experiment adopts the method of sonication to break bacterial cells. Four ultrasonic crushers were available in the laboratory. The TA will demonstrate the use of the instrument and inform students the attentions. Students will operate the ultrasonic crusher independently for cell disruption. During the operation, students need to be reminded to prevent the ultrasonic horn from touching the wall of the centrifugal tube, to avoid damaging the probe. At the same time, students need to pay close attention to the fluid in the tube during the sonication. If a large amount of foam or liquid overflow occurs, the ultrasonic power should be reduced appropriately.

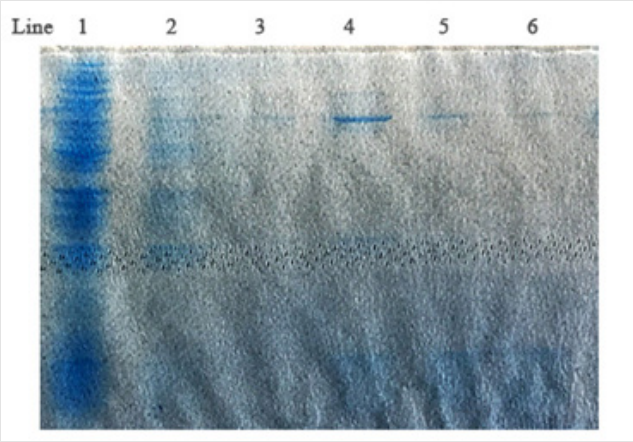

Figure 3: SDS-PAGE of purified RecJ nuclease. The 15% polyacrylamide gel was run at 100 V and stained with Bio-Safe Coomassie Blue G-250 stain. Line 1 was loaded with unpurified total protein solution. Line 2-6 were loaded with orderly eluted RecJ protein by Ni-affinity chromatography.

For many students it is the first time for them to visually see the presence of their own purified protein after SDS-PAGE. After loading onto gel wells according to the eluted order of the purified protein, the band of the target protein tends to become shallower during electrophoresis. However, minor students' first and second tubes of elutes almost do not contain any target protein or very little one. The size of the purified protein is approximately 55 kDa. After observing the bands on gel, the tube of target protein elutes with the bluest band is selected and frozen for activity identification of the enzyme. See Figure 3 for representative student data. The activity of purified proteins was measured by the ability of RecJ exonuclease to degrade single-stranded DNA. As shown in Figure 4, the lanes with the addition of the commercial RecJ nuclease and the purified one, respectively, appear a band of the digested DNA fragment besides the band of the substrate DNA. In the lane of the negative control, only the band of the substrate DNA is present. Moreover, the quantity of degraded fragments shows a gradient. As more purified RecJ enzymes are added, the shorter fragments are generated, and the darker the band color. Students can easily determine the activity of extracted RecJ enzyme based on the results of the 8M-urea denature electrophoresis. There are also failed experiments where some student did not see the band of degraded fragments. Once the experiment fails, the teacher will ask the students to analyze the possible reasons for the failure in the experiment report, where this student contributed the failure to that the band of purified protein is very light in gel of the SDS-PAGE experiment, indicating that the protein concentration he collected is extremely low. Therefore, the student concludes that it is most likely that there is a problem in sonication, for example, the ultrasonic horn is not set into the bacterial turbid liquid. In summary, more than 90% of the students in the class can get a clear identification result.

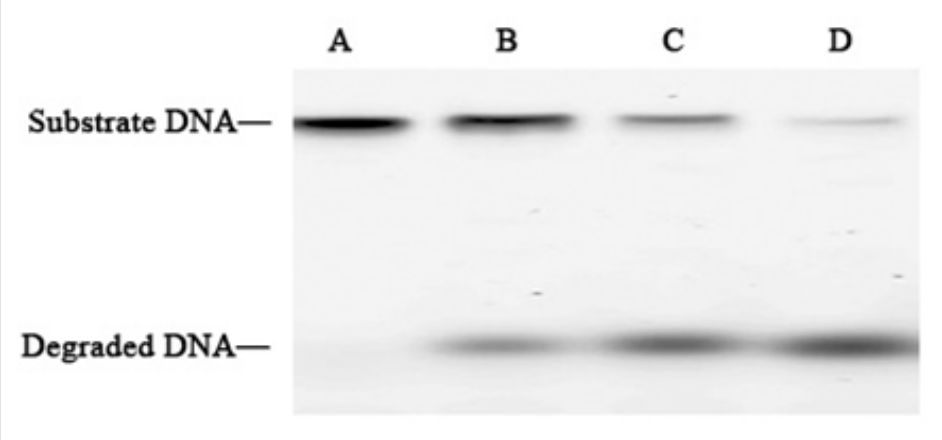

Figure 4: Verification of the 5′exonuclease activity of RecJ protein on the 5′-FAM-labelled-ssDNA. The 15% denature PAGE gel was run at 500 V and imaged by a laser scanner. Four lanes are:

A. negative control BSA,

B. 1 μl of purified RecJ nuclease,

C. 2 μl of purified RecJ nuclease,

D. Positive commercial exonuclease.

During the whole experiment, the students participated in almost all the steps, including explaining the experimental principle, operating the experiment independently and analyzing the results. The high-engagement experimental course fully ensures that students can apply the experimental techniques they have learned to their own future projects. To better communicate with students, the course has a teaching website (https://www.cnmooc.org/session/managerCourse.mooc). Every student needs to enter the website to register an account. At the end of each experimental session, students can raise questions and discuss on the website. At the same time, the teacher will post the experimental contents and notes for next week on the website every week and attach 1-2 relevant papers for students to read. At the end of the integrated laboratory course, each student is asked to submit an experimental report and upload it to the website. The final grade is not only judged by the success or failure of the experiment, but also by the usual pre-class report, class performance and the final report submitted. In the experimental report submitted by the students, it is obvious that the students have a thorough understanding of the whole experiment, and the analysis of the results is also reasonable. The students whose experimental results were not ideal also analyzed the reasons for the problems as much as possible. Most students say this course is a fun, enjoyable and valuable learning experience. Many students have made some suggestions and assessments for the course.

A. Some of the Remarks were as Follows:

"This lab is different from others. It is no longer a one-day thing. We participate personally in the entire process of expressing a protein from bacteria until it shows its function. This is amazing."

"This lab allows me to accumulate a lot of experience and meet many new instruments. I am confident that I can perform the expression, separation, and purification of a protein independently."

"I really enjoy this course. I not only learned a lot of experimental techniques, but also strengthened my ability to read literature and report."

"This course is too useful for me because it is relevant to my future goals."

"The lab is so interesting and makes sense to me."

Conclusion

In conclusion, students successfully induced and purified RecJ protein from the recombinant plasmid and identified it. And they also process and analyze the experimental pictures in the submitted laboratory report independently. Students said they have learned a lot on the course, not only in experimental skills but also in thinking methods. This integrated laboratory course model accomplished its goal of teaching students a series of biochemical techniques related to protein induction, purification, and characterization. Students control the whole experiment process independently just like a real scientific project and accumulate experience from the integrated experiment. This experience will help students in their future research and career.

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