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Laboratory Exercises

A Complete Approach for Recombinant Protein Expression Training

FROM GENE CLONING TO ASSESSMENT OF PROTEIN FUNCTIONALITY*

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A practical course was given to undergraduate biology students enrolled in the elective course "Introduction to Genetic Engineering" at the Federal University of São Carlos (UFSCar), São Paulo, Brazil. The goal of the course was to teach current molecular biology tools applied to a real research situation that could be reported by the students themselves. The purpose was to produce a plant recombinant protein and demonstrate a heretofore unreported biological activity. Cystatins, natural inhibitors of cysteine proteases, were proposed for these studies. Initially, the students searched for plant cystatin cDNA sequences in the NCBI databases and selected the Oryzacystatin I gene (*ocI*) from rice, *Oriza sativa*, as the target gene for this study. Total RNA was extracted from rice-germinating seeds and primers containing restriction sites for NdeI and EcoRI were designed based on the *ocI* cDNA sequence and then used to amplify the open reading frame (ORF). RT-PCR amplification provided a band of the expected size for *ocI* ORF (309 bp). The PCR product was cut with NdeI and EcoRI restriction enzymes and cloned directly in the pET28a expression vector digested with the same enzymes. A pET28-*ocI* recombinant clone was selected, checked by sequencing, and used to transform *Escherichia coli* BL21 (DE3) expression strain. After induction of the bacteria with isopropylthiogalactoside and cellular disruption, the His-tagged OCI protein, present mainly in the soluble fraction, was purified by affinity chromatography in a nickel column. The purified protein was successfully used to inhibit fungal growth (*Trichoderma reesei*). The results were discussed extensively and the students contributed to the writing of this article, of which they are co-authors.

Keywords: Cystatin, oryzacystatin, protein expression, antifungal activity, laboratory classes.

Firsthand practice in the fields of biochemistry and molecular biology is essential in the learning process and can be used as a motivating tool for undergraduate students. Awareness of this fact motivated us to create a new subject in the undergraduate discipline "Introduction to Genetic Engineering" in which we propose essentially experimental models for molecular biology courses.

In line with this proposal, and based on theoretical classes and discussions about the chosen subject, the students participated in all the practical procedures, *i.e.* analyses of DNA/protein sequences in a data bank, selection of a gene to be studied, mRNA extraction, cDNA synthesis and cloning, heterologous expression in bacteria, protein purification, antifungal activity tests, including bibliographical research, and the production of this article.

The protein oryzacystatin I (OCI), which is an inhibitor of cysteine proteases, was chosen as the subject of the experimental procedure. This protein is produced in rice-germinating seeds and belongs to the superfamily of cystatins, which is classified into three different families based on the cystatin molecular structures [1]. Their synthesis is induced by defense mechanisms in plants in response to external agents such as plant pathogens. Fungi are important phytopathogens, and the potential antifungal activity of some cystatins has been described [2, 3].

In order to teach the use of current molecular biology tools applied to a real research situation, with intensive student participation throughout the process, we decided to produce a recombinant rice cystatin and investigate its inhibitory activity against the filamentous fungus, *Trichoderma reesei*. The students then reported the results and participated in the writing of this article.

PROCEDURES

"Introduction to Genetic Engineering" is a 90-h elective course given to undergraduate biology students who have already attended classes on Biochemistry, Microbiology,

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TABLE I
Technical steps and specific experimental topics

Step	Experimental topics	Timeline
Searching for a candidate gene for cloning	Students' interaction to select a gene Utilization of databases Bibliographic research Germination of selected seeds	2 weeks (2 classes)
mRNA extraction	RNA extraction by Trizol reagent Analysis by electrophoresis	1 week
RT-PCR	Analysis *in silico* of the cDNA sequence Design of primers with appropriate restriction sites cDNA synthesis and PCR amplification PCR product analysis by electrophoresis PCR product purification	2 weeks
cDNA cloning	Enzymatic digestion of PCR product Choice of an expression system Ligation in a plasmid vector *E. coli* transformation Selection of recombinant clones by PCR Plasmid mini-preparation	2 weeks
Sequence checking	Automatic sequencing	2 weeks
Protein expression	IPTG induction Cellular lysis and protein solubility tests SDS-PAGE analysis	2 weeks
Protein purification	Metal affinity chromatography Dialysis	1 week
Biological activity of the recombinant protein	Fungal culture and manipulation Fungal growth in the presence and absence of the recombinant protein	1 week
Elaboration of the manuscript	Establishment of an internet group to discuss and exchange information	3 weeks (including the one used for biological activity tests)

and Molecular Genetics. The team comprised the professor of the discipline (Coordinator), an assistant professor, two graduate assistants, one of them involved in a specific program in teaching (PESCD/CAPES),[1] and six undergraduate students. The latter were divided into two groups in the experimental part of the course.

The course began with the coordinator giving the students a theoretical class on cystatins and their cysteine proteases inhibitory activity. The students were also required to read specific literature about the subject matter of this class [2, 4]. Additional theoretical classes were given during the course to further elucidate the experiments and to discuss the principles involved in molecular biology techniques. The students carried out essentially practical experiments, starting from the search for the target gene in NCBI databases (www.ncbi.nlm.nih.gov) up to its cloning, protein expression in *Escherichia coli*, and protein purification. The requirements of the gene to be chosen should be to codify for a gene expressed in high levels in an accessible plant, have a known full-length cDNA sequence, and have an open reading frame (ORF)[2] containing no more than 700 bp to facilitate the cDNA synthesis. Finally, an "*in vivo*" test was carried out to investigate an as yet unreported biological activity of the chosen cystatin.

Basic protocols were provided to the students, who were required to adapt them during the experiments. The students drew up reports of each experimental step. Table I shows the steps of the course and the experimental techniques involved in each phase, including the timeline.

MATERIALS AND METHODS

Equipment

The laboratory work called for the following equipment: a PTC-100™ Thermocycler (MJ Research, Inc., Waltham, MA); an ABI Prism 377 automatic sequencer (Applied Biosystems, Foster City, CA); water bath (42 °C and 100 °C); a shaker for bacterial growth (37 °C); a sonicator; a laminar flow hood for sterile work; an incubator (37 °C); a microcentrifuge; a centrifuge (Sorvall), vertical and horizontal gel electrophoresis systems; electrophoresis power supply; an UV transluminator; micropipettes and sundry other tools.

Materials

The rice total RNA was extracted from germinated *Oryza sativa* seeds kept on wet cotton for at least 5 days. The expression vector used was pET28a (Novagen, Madison, WI), which provides a His-tag fusion to facilitate further protein purification. Strains of *E. coli* DH5-α and BL21 (DE3) were used, respectively, for plasmid propagation and protein expression. Enzymes were purchased from Amersham Biosciences (Piscataway, NJ). The recombinant protein was purified using a Ni-NTA minicolumn (Qiagen, Valencia, CA). Spores of *Trichoderma reesei* grown in potato dextrose agar were utilized to investigate the antifungal activity of the recombinant OCI.

Methods

Background—Because a variety of commercial kits and reagents were used in this course, the students were given explanations about the role of each component before beginning the experiments, considering the use of alternative kits including the ones prepared "in-house." We also discussed possible problems they might encounter and the proper procedures to overcome them (Table II).

[1] The Supervised Teacher Training Internship Program (PESCD) is a program supported by a Brazilian Research Funding Institution (Coordinating Bureau for the Improvement of Undergraduate and Post-graduate Students [CAPES]) to provide graduate students with teacher training.

[2] The abbreviations used are: ORF, open reading frame; IPTG, isopropylthiogalactoside.

TABLE II
Troubleshooting guide

Step	Possible problems	Comments and suggestions
mRNA extraction	Low yield of RNA	Incomplete homogenization or lysis of samples
		Final RNA pellet incompletely redissolved
	RNA degradation	Tissues were not immediately processed or frozen (–70 °C) after removal from the source
		Aqueous solutions or tubes were not RNase-free
		RNase contamination during manipulation or electrophoresis
RT-PCR and purification of the amplified product	No product amplified	Is the chosen gene highly expressed in the tissue used for RNA extraction?
		Low quality RNA (check in a gel the presence of ribosomal RNA)
		Check the sequence and quality of primers
		Increase template RNA
		Increase number of cycles in PCR
		Check sequence and Tm of the primers
		Check all reagents
	Little product on high background smear	Reduce number of cycles
		Reduce template
		Reduce the amount of enzyme
		Test different Mg^{2+} concentrations
	Low yield	Increase the number of cycles
		Increase the amount of template, enzyme, or Mg^{2+}
	Unspecific products	Try higher annealing temperatures
		Reduce the annealing time
		Reduce time of the elongation step
		Decrease the amount of enzyme
		Reduce the amount of template
		Reduce the number of cycles
		Add 1–2% DMSO
		Design new/longer primers
cDNA cloning	Few or no transformants	Poor quality of the competent cells (perform a test transformation using a known amount of supercoiled plasmid)
		Unsuccessful ligation: Check the quality of DNA ligase by performing a control ligation reaction using a linearized plasmid
	High background of nonrecombinants	Possibly the PCR product and/or vector were not efficiently cut by the restriction enzymes—check the quality of the enzymes and try new cleavages using longer times
		Unsuccessful purification of the insert (repeat the procedure)
		Low antibiotic concentration (supply medium with fresh antibiotic)
		Ratio of vector to insert is too high—adjust ratio to optimal (1:3)
Sequence checking	Sequence with bad quality or absent	Check the quality of template
		Check the primer sequence, quality and concentration
		Ensure that the chemicals are working (perform a control sequence)
		Check possible losses in the precipitation step
Protein expression	No or low expression	Check if the coding sequence is ligated into the correct reading frame
		Check the culture conditions (including IPTG concentration and quality)
		Protein is rapidly degraded—perform a time course to check the kinetics of growth and induction; consider adding protease inhibitor during cell lysis process; keep all samples and solutions at 4 °C
	Protein is insoluble	Expression level is too high and protein cannot fold correctly—modify growth and induction conditions (time, IPTG concentration, temperature)
		Check the levels of soluble protein remaining in the supernatant
		Try other fusions and bacteria strains
	Protein does not have the expected size	Check if the coding sequence is ligated into the correct reading frame
		Check the presence of unexpected stop codons in the cloned sequence
Protein purification	Protein does not bind to the nickel resin	His_6 tag is not present: check the sequence and verify if the frame is correct; check for putative internal translation starts
		His_6 tag is inaccessible: purify under denaturing conditions; fuse His tag to the opposite end of the protein
		Binding conditions incorrect: check buffers and solutions (especially pH) immediately before use
	Protein elutes in the wash buffer	Check pH of wash buffer
		Increase the pH
	Protein precipitation during purification	Protein forms aggregates: add 0.1% Triton X-100; NaCl (up to 2 M) or β-mercaptoethanol (up to 20 mM)
	Protein does not elute	Perform an imidazole gradient to determine the optimal concentration for elution
		Elute under denaturing conditions
	Protein with contaminants	Increase stringency by adding up to 20 mM imidazole to the binding and wash buffers
		Reduce the amount of nickel resin
		Add β-mercaptoethanol up to 20 mM to reduce probable disulfide bonds

Table II—*continued*

Step	Possible problems	Comments and suggestions
Biological activity test	Inhibition absent	Increase salt to disrupt nonspecific interactions Work at 4 °C and include protease inhibitors to prevent protein degradation Is the protein correctly folded? Is the target for the recombinant protein present or accessible in the cell? Increase protein concentration Try other fungi or microorganisms

RNA Extraction—Total RNA was extracted from about 200 mg of nongerminated and germinated rice seeds (about 5 days in wet cotton), previously macerated in liquid nitrogen. The RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA), following the manufacturer's instructions.

First-strand cDNA Synthesis—The reaction for the synthesis of the first-strand cDNA was carried out using 10 μl of total RNA (1 μg), 1 μl of primer *ocl*-R (10 pmol/μl) (5′-CCGAATTCTTAGGCATTTGCACTGGCATC-3′, EcoRI site underlined), and water to a final volume of 15 μl. After 5 min at 70 °C, 5 μl of 5× reverse transcriptase buffer M-MLV5 (Promega, Madison, WI), dNTP to 0.5 mM, 3 μl of "RNA Guard" (Amersham Biosciences) RNase inhibitor, and 1 μl of M-MLV5 RT enzyme were added. The reaction temperature was kept at 42 °C for 1 h.

ORF Amplification—A volume of 2.5 μl of the cDNA solution was used in a reaction containing 0.2 mM dNTP, 5 μl of 10× amplification buffer, 1.5 μl of $MgCl_2$, (stock 50 mM), 1 μl of primer *ocl*-R (10 pmol/μl) (5′-CCGAATTCTTAGGCATTTGCACTGGCATC-3′), 1 μl of primer *ocl*-F (10 pmol/μl) (5′-GGCATATGTCGAGCGACGGAGGGCCG-3′, NdeI site underlined), 2.5 U of Taq DNA polymerase (Amersham Biosciences) and water to a final volume of 50 μl. The amplification conditions were: 1× [94 °C for 1 min], 35× [94 °C for 1 min, 47 °C for 1 min, and 72 °C for 1.5 min], and 1× [72 °C for 5 min]. The PCR amplification was checked in a 1% agarose gel. The PCR product was then purified from the gel, using the "QIAquick PCR purification kit" (Qiagen), and further quantified in agarose gel.

Construction and Characterization of the Expression Vector—The amplified *ocl* fragment was cut with the restriction enzymes EcoRI and NdeI, following the protocol recommended by the manufacturer. The *ocl* ORF (309 bp) was then gel-purified and inserted into the pET28a vector cleaved with the same enzymes. The reaction contained 3 μl of *ocl* ORF (100 ng), 2 μl of pET28a (100 ng), 2 μl of buffer 5×, 1 μl of T4 DNA ligase, and 2 μl of water. Plasmid DNA of the recombinant clones obtained was sequenced in an ABI Prism 377 DNA sequencer by the dideoxy chain termination method, using the DYEnamic ET terminator Cycle Sequencing kit (Amersham Biosciences).

Recombinant Expression of Oryzacystatin I—The expression plasmid containing the insert (dubbed pET28-*ocl*) was used to transform calcium chloride-competent *E. coli* BL21 (DE3). A recombinant clone, selected by PCR, was cultivated at 37 °C in 500 ml of a selective medium containing kanamycin (25 μg/ml) under agitation until an optical density of 0.6 was reached at 600 nm. At that point, the recombinant OCI expression was induced by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 0.4 mM. Aliquots of 500 μl were taken up to 3 h (at 1-h intervals) of induction, and the cells were centrifuged, boiled for 5 min, and analyzed in SDS-PAGE 15% [5]. After 3 h of induction, the culture was harvested at 7,000 rpm and 4 °C for 5 min, and the pellet was suspended in 25-ml lysis buffer (100 mM NaCl; 10 mM Tris-HCl, pH 8; 50 mM NaH_2PO_4). Cells were then sonicated on ice (eight 1-min pulses), and the debris was centrifuged at 14,000 rpm and 4 °C for 10 min. The supernatant and precipitate were analyzed by SDS-PAGE 15% [5].

Purification of the Recombinant Oryzacystatin I—The soluble fraction was filtered through 0.22-μm membranes (Millipore, Bedford, MA), and the recombinant OCI was purified from the supernatant using an affinity Ni-NTA superflow column (Qiagen) equilibrated with lysis buffer. After washing the column with 10 ml of lysis buffer (two column volumes), the protein was eluted twice with each increasing concentration (25, 50, 75, 100, and 250 mM) of imidazole mixed in 5 ml of the same buffer. An aliquot of the purified product (15 μl) was analyzed by SDS-PAGE 15% [5]. The fractions containing the purified protein were dialyzed against lysis buffer. After sterile filtration with 0.22-μm membranes (Millipore), the protein concentration was determined by Bradford's method [6].

Growth Inhibition Assay of the Filamentous Fungus T. reesei—A fungal growth inhibition assay was performed, with a few adaptations [7]. Fifty microliters of a spore solution of *T. reesei* was placed in a hole punctured in a solid potato dextrose agar medium containing 0, 50, and 100 μg per ml of recombinant OCI. The fungal growth was monitored daily and documented photographically after 5 days.

Writing of the Manuscript—An internet group was established to discuss and exchange information about the results. The students were given guidelines about how to write the article based on the book *How to Write and Publish a Scientific Paper* by Robert Day [8]. Table III shows the most important points considered. The course coordinators put the finishing touches on the final version of the article.

RESULTS

RNA Extraction—About 20 μg of total RNA was obtained from each preparation. The RNA quality was assured by the presence of bands corresponding to 18S and 28S ribosomal RNA (Fig. 1*A*).

Isolation and Amplification of ocl ORF—The *ocl* ORF was obtained by RT-PCR, resulting in a product with about 300 bp, corresponding to the *ocl* ORF (only from RNA of germinated seeds). The PCR product is shown in Fig. 1*B*.

Cloning in pET28a—After the transformation of *E. coli* BL21 (DE3), 60 clones were chosen for screening by PCR. Some of them were found to contain the insert with a size of around 300 bp (Fig. 1*C*). The plasmid DNA of three of these clones was then extracted and sequenced. One clone had the correct sequence, but the other two failed to display good quality sequences.

Induction of Expression, Solubility Test, and Protein Purification—The protein expression was induced with 0.4 mM IPTG over a 3-h period. As shown in the 15% SDS-PAGE (Fig. 2*A*), the protein expression was induced after 1 h and did not apparently increase over the subsequent 2 h.

The recombinant OCI was obtained in both soluble and insoluble fractions (Fig. 2*A*). There was enough protein in the soluble fraction to purify in the native form. The purification was done with a nickel resin, and the protein elution began with 50 mM of imidazole. However, the best elution was achieved in the washing with 100 and 250 mM imidazole (Fig. 2*B*). The samples thus collected were pooled, and a total of 10 mg of protein per liter of culture was obtained. Dialysis was performed to remove excess imidazole.

Antifungal Activity Assay—The antifungal activity of the recombinant protein was tested against the filamentous

TABLE III
How to write and submit an article: some considerations[a]

Item	Comments
Title	A good title contains the fewest possible words that adequately describe the contents of the paper Remember the importance of syntax (not only in the title!)
Definition of authorship	It should include those who actively contributed to the overall design and execution of the experiments (in order of contribution) The sequence of authors should be decided unanimously
Preparing the abstract	Provide a clear, simple, and brief summary of each of the paper's main sections (introduction, materials and methods, results, and discussion) Do not exceed the size stipulated by the journal With rare exceptions, references should not be cited
Introduction	Supply sufficient background information to allow the reader to understand and evaluate the results Present the nature and scope of the problem investigated Review the pertinent literature State the method of the investigation
Materials and methods	For materials, include the technical specifications, quantities, and source Describe the experimental design and provide details so that the experiments can be repeated This section should be written in the past tense
Results	Start by describing some of the experiments, but do not repeat experimental details previously described in the "Material and Methods" Present the data in the past tense Present representative data, avoiding repetitive data This section should be short. Avoid using too many words or unnecessarily difficult words Avoid redundancy
Discussion	Discuss clearly, do not recapitulate the results Compare your results with previously published works Discuss theoretical implications and possible practical applications State your conclusions as clearly as possible
Acknowledgments	Acknowledge significant technical help; source of special equipment; cultures; financial assistance; critical reading of the manuscript and some contribution to discussion
References	List only significant published references Check carefully the original publication and if all references cited in the text are listed Follow the journal's guidelines for authors
Submitting the paper	Identify the journals that publish in your subject area Consider the option of publishing in an indexed journal Read the "Instructions for authors" carefully (this also applies to writing the article) The corresponding author should send a cover letter with the manuscript, including the authors' names, title, addresses, the journal to which the manuscript is to be submitted, agreement of all the authors and brief considerations about the paper

[a] Based on Ref. 8.

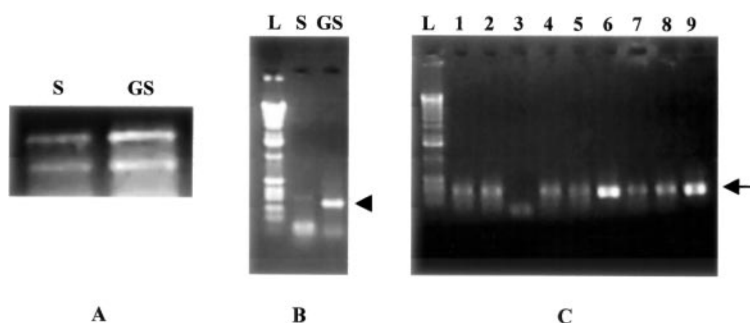

FIG. 1. **Cloning of oryzacistatin I ORF.** *A*, agarose gel electrophoresis of total RNA extracted from rice seeds (*S*) or germinating seeds (*GS*); *B*, respective RT-PCR products, showing *ocI* ORF amplification (309 bp, *arrowhead*) only from germinating seeds (*GS*); *C*, recombinant clones of pET28a-*ocI* in *E. coli* BL21 (DE3) identified by PCR screening of the colonies. One of these (clone 6), presenting a clear amplification band of about 300 bp (*arrow*), was selected for oryzacystatin I induction. *Lane L*, 1-kb DNA ladder (Invitrogen).

fungus *T. reesei*. Fungal growth was successfully inhibited in both concentrations tested (50 and 100 μg/ml) (Fig. 3).

Writing of the Manuscript—The results were compiled and discussed, after which the students wrote this article under the supervision of the course coordinators.

DISCUSSION

Courses involving protein expression and purification are important to prepare undergraduate students for assigning biological functions to the large number of novel genes available from genome projects. The first requirement for gene cloning and expression is to access and interpret information about genes and genomes available on specific websites. At the beginning of this course, searches on databases to choose a gene to be cloned provided opportunities for this kind of training.

The theoretical and practical aspects of the steps involving the choice of an expression vector and a specific host,

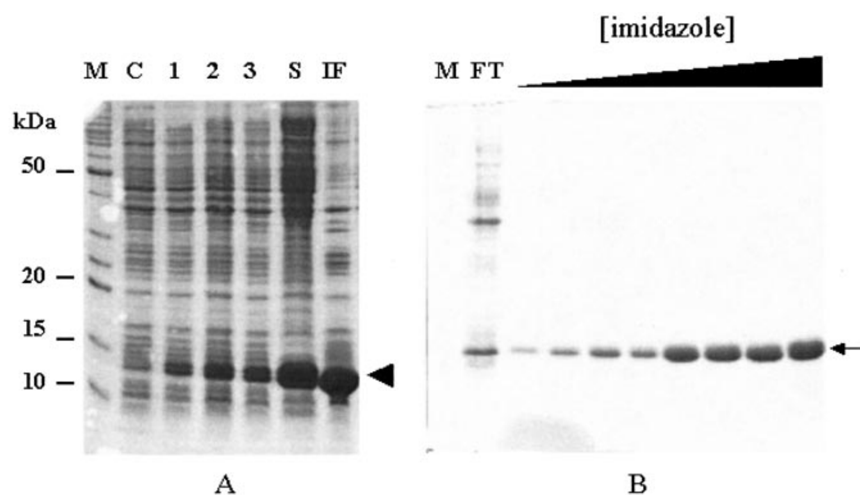

FIG. 2. *A*, time course for recombinant oryzacystatin I expression in *E. coli* BL21 (DE3) analyzed by SDS-PAGE. *Lane M*, protein standards; *lane C*, uninduced culture; *lanes 1–3*, induced culture with 0.4 mM IPTG for 1, 2, and 3 h, respectively; *lanes S* and *IF*, soluble fraction (*S*) and insoluble fraction (*IF*) of *E. coli* cells induced for 3 h with IPTG. The gel was stained with Coomassie Blue. The *arrowhead* indicates the recombinant OCI protein (~13.5 kDa). *B*, purification of recombinant OCI by affinity chromatography using a nickel column. *Lane M*, protein standards; *lane FT*, flow-through; *subsequent lanes*, lysis buffer with increasing concentrations of imidazole (from 50 to 250 mM). The gel was stained with Coomassie Blue. The *arrow* indicates the recombinant OCI protein (~13.5 kDa).

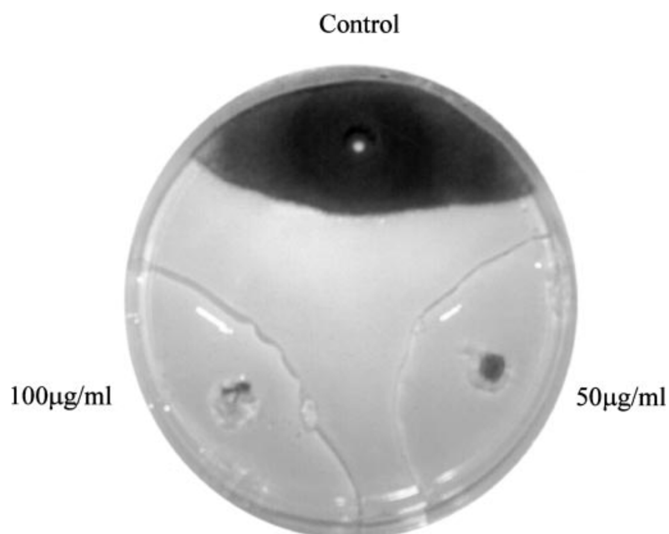

FIG. 3. **Growth inhibition assay of the filamentous fungus *T. reesei.*** Spores of *T. reesei* were placed on PDA medium containing 0 (control), 50, or 100 μg/ml of the recombinant OCI. The fungal growth was documented photographically after 5 days.

primer design, RT-PCR, gene cloning, and nucleotide sequencing were discussed during the experiments. A summary of some of the topics discussed is given below.

The discussion began with the question "why express recombinant proteins?," for which several main reasons were put forward, *i.e.* difficulties of protein extraction and purification from original tissues, possibility of inserting mutations in recombinant proteins, control of the expression process, production of large amounts of a specific gene product. Therapeutic antibodies, vaccines, hormones (insulin and growth hormone), and coagulation factors were used as examples of medical applications for recombinant proteins.

The requirements for an expression vector were pointed out: to ensure high-level expression of a heterologous gene product, efficient transcription is achieved from a strong promoter upstream of the gene of interest cloned in the multiple cloning sites of the plasmid vector. Transcription from these promoters is generally regulated in a negative manner, requiring the addition of a chemical inducer (*e.g.* IPTG) to activate the transcription of the gene of interest by reducing the affinity of the repressor for the operator. This regulation has important functions in *E. coli* systems: assurance of plasmid stability and production of the gene product that should be lethal to the host organism if the expression is constitutive [9].

Characteristics of vectors for improving expression and facilitating subsequent purification of expressed protein were also mentioned. Commercially available expression vectors such as pET28, pET32, pET42, pET37, and pET44 (Novagen) provide fusion of the gene of interest to a gene that expresses at high levels in *E. coli* or provides better protein solubilization and purification. Depending on the expression vector, the fusion may be a single stretch of histidines, thioredoxin, glutathione-*S*-transferase, cellulose binding domain, or others.

Other specific aspects of protein expression and purification using pET28 vector (Novagen) were discussed, as previously reported [10]. The advantages and disadvantages of using hosts other than *E. coli* were also discussed, including *Caulobacter crescentus*, *Bacillus subtilis, Saccharomyces cerevisiae*, *Pichia pastoris*, filamentous fungi, and insect and mammalian cells. The current production of recombinant proteins in transgenic animals was illustrated by the coagulation factor VIII.

Considering the antifungal activity of oryzacystatin I observed in this course and reported herein, the possibility of using this information to develop transgenic strains that express the protein in question, and the use of oryzacystatin I as a natural fungicide, were also considered. The possibility of using the present approach to investigate the activities of other cystatins and to bioprospect for new ones was also discussed.

The experiments and discussions about recombinant protein expression held during this course served as the basis of this manuscript, which was written with the enthusiastic participation of the students.

GENERAL CONCLUSIONS

This experimental course provided the students with hands-on contact with many current molecular biology techniques within the context of an actual investigation. The attribution of an as yet unreported biological function for a gene available in databases can be an interesting and promising approach for other experimental Molecular Biology courses. Finally, this course provided the students with an opportunity to write a scientific article and have it published.

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