A Project-Oriented Biochemistry Laboratory Course

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A biochemistry major was recently established at the Rochester Institute of Technology. To complete the undergraduate experience of the biochemistry majors, we needed an effective laboratory course in which students are exposed to modern techniques and analytical thinking in the design of experiments. For the past four years we have offered a course, "Biochemistry: Experimental Techniques Laboratory", which has been taken by students from the biology, biotechnology, biochemistry, chemistry, and clinical chemistry programs at RIT. This course involves mainly protein chemistry, because excellent courses in molecular biology and genetic engineering are available in the Department of Biology.

The course as first offered used experiments from a variety of laboratory manuals and the chemical education literature. This was inadequate for two reasons: (i) the experiments were designed separately and did not relate directly to each other, and (ii) only about half of the experiments worked. Therefore, before the course was offered a second time, the curriculum was modified to revolve around a single project: the purification, characterization, and molecular biology of threonine dehydrogenase (TDH) from Escherichia coli (1). This enzyme initiates one of three known pathways for threonine degradation and is found in many prokaryotic and eukaryotic organisms. Administration of threonine has been shown to relieve some of the muscular rigidity in persons suffering from multiple sclerosis, suggesting that products of the pathway initiated by TDH have a role in neurochemical pathways in the body (2–5). Recently, tdh, the gene encoding TDH, has been identified and sequenced in the plant pathogen Xanthomonas campestris (6, 7).

The integrated laboratory approach shares many features with other biochemistry laboratory programs that have been described in the literature:

All the experiments are focused on the purification and characterization of a single enzyme or protein (8–10).

The experiments are integrated so that each builds on the previous one (11–14).

The course incorporates the use of computers for data analysis and database searching (11, 13).

Several groups have advocated a project-oriented or research-based approach to their biochemistry laboratory course. In the Laboratory

The focus of the course is threonine dehydrogenase, an enzyme from E. coli that I studied in graduate school (1). The pathway initiated by threonine dehydrogenase is shown in Figure 1. The course schedule, specific techniques, and instrumentation for each part of the course are listed in Table 2.

The course is divided into four parts: enzyme purification, enzyme characterization, molecular biology, and an original project. Several factors make TDH an excellent choice for the course:

- Growth of the cells that express the enzyme is simple and rapid.
- Purification of the enzyme is straightforward and reliable.
- The TDH gene has been cloned into a plasmid based on the vector pBR322 (19), facilitating studies on the gene sequence and expression.
Table 1. Project-Oriented Biochemistry Laboratory Proposals Funded by NSF

<table>
<thead>
<tr>
<th>No.</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>8951782</td>
<td>Modular Approach to Undergraduate Biochemistry Laboratory Instruction</td>
</tr>
<tr>
<td>9151155</td>
<td>Development of Undergraduate Biochemical Engineering Laboratory at Tri-State University</td>
</tr>
<tr>
<td>9152722</td>
<td>Integrated Undergraduate Laboratories for Biochemistry and Molecular Biology</td>
</tr>
<tr>
<td>9250473</td>
<td>A Revitalized Undergraduate Biochemistry Laboratory Experience</td>
</tr>
<tr>
<td>9351350</td>
<td>Development of a Project Based Laboratory Sequence in Molecular Biology and Chemistry</td>
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<tr>
<td>9451580</td>
<td>Establishment of a Cellular and Molecular Biochemistry Laboratory for Undergraduate Teaching and Research</td>
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<tr>
<td>9452118</td>
<td>Equipment to Implement a New Undergraduate Biochemistry Laboratory Curriculum</td>
</tr>
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<td>9550975</td>
<td>Crossing Boundaries—A Bioorganic Approach to Chemistry at Pomona College</td>
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<tr>
<td>9602356</td>
<td>Modular Approach to Biotechnology Laboratory Instruction Based on a Novel Green-Fluorescent Protein</td>
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<td>96500095</td>
<td>Development of a Biochemistry Laboratory Using Physiologically Relevant Samples</td>
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<td>9651351</td>
<td>Purification and Characterization of Luciferase in the Undergraduate Laboratory</td>
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<td>A Project-Oriented Undergraduate Laboratory Course in Cell Biology</td>
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<td>9751332</td>
<td>A Research-Oriented, Evolution-Based Biochemistry Laboratory</td>
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<td>9751664</td>
<td>Cytochrome c: A Biochemistry Laboratory</td>
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<tr>
<td>9752329</td>
<td>Development of an Undergraduate Biochemistry Laboratory Project</td>
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Course Schedule

**Week 1: Activity and Protein Assays (20)**

Before beginning the purification of the enzyme, the students first learn how to find it in a cell-free extract. Protein concentrations are determined using the Bradford protein assay (21) and two different activity assays: (i) rate of NADH formation followed by a UV–vis spectrophotometer (22) and (ii) a colorimetric microplate assay for rapid detection of TDH activity eluting from a chromatography column. To facilitate the development of a quantitative approach to biochemistry, students are required to analyze the Bradford assay data in a spreadsheet format, using a template from an analytical chemistry textbook (23).

**Weeks 2 & 3. Enzyme Purification (24)**

The enzyme source for this project is a strain of *E. coli* that has a plasmid, pDR121 (19), which contains the gene for TDH. The presence of the plasmid makes this strain an overproducer of the enzyme. During the first week students prepare media, grow the cells, and prepare a cell-free extract by sonication. The cell-free extract is recovered by centrifugation in a superspeed centrifuge. This is followed by anion exchange chromatography, in which students use a Waters 650E protein chromatography system equipped with POROS DEAE columns. The use of three protein chromatography workstations enables students to (a) optimize stationary phase and mobile phase conditions in the ion exchange step during one lab period, then (b) scale up and complete the anion chromatography step during the subsequent lab period. Before performing the ion exchange chromatography step, students are encouraged to explore ion exchange chromatography using IonEx, a simulation of ion exchange chromatography developed at RIT for the Windows environment (25). The simulation allows them to examine the effects of salt concentration, pH, and protein load on the separation process.

The dye–ligand chromatography step is still done by gravity feed chromatography, but it is very straightforward and can be completed in one or two lab periods. Similar purification procedures for dehydrogenases have been described previously for undergraduate biochemistry lab courses (26, 27). Students then perform protein and activity assays and construct a protein purification table. Of the experiments in the course, the enzyme purification step is the only one that

![Figure 1. L-Threonine catabolic pathway initiated by threonine dehydrogenase. The pathway begins with the NAD-dependent oxidation of threonine by TDH, which is coupled to a ligation reaction to form acetyl CoA plus glycine in the cell. This figure includes the electron transfer reagents used to develop the colorimetric assay described below.](image)

Table 2. Instrumentation and Techniques in the Biochemistry Lab Course

<table>
<thead>
<tr>
<th>Week</th>
<th>Experiment</th>
<th>Instrumentation</th>
<th>Techniques</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Activity Assays</td>
<td>UV–vis spectrophotometer</td>
<td>Enzyme activity assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Micropipette reader</td>
<td>Bradford protein assay</td>
</tr>
<tr>
<td>2, 3</td>
<td>Purification</td>
<td>Superspeed centrifuge</td>
<td>Ion exchange chromatography</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Protein chromatography system</td>
<td>Affinity chromatography</td>
</tr>
<tr>
<td>4a</td>
<td>Electrophoresis</td>
<td>Protein electrophoresis apparatus</td>
<td>SDS-PAGE</td>
</tr>
<tr>
<td>4b, 5</td>
<td>Enzyme Kinetics</td>
<td>UV–vis spectrophotometer</td>
<td>Enzyme activity assays using two substrates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Micropipette reader</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Expression &amp; Sequencing</td>
<td>Thermal cycler Active DNA Sequencer</td>
<td>Sterile technique</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Microcage</td>
<td>Plasmid isolation</td>
</tr>
<tr>
<td>7-9</td>
<td>Independent Project</td>
<td>Combination of all the above</td>
<td>Depends on student design</td>
</tr>
<tr>
<td>10</td>
<td>Sequence Homology</td>
<td>Computer network with Internet access</td>
<td>Database searching</td>
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In the Laboratory
In the Laboratory

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regularly exceeds scheduled class time. This generally does not pose a problem because the students work in teams and there is less time pressure from exams early in the quarter.

Week 4a. Electrophoresis (28)

Aliquots from the various stages of purification are analyzed by SD-S–polyacrylamide gel electrophoresis (SDS–PAGE) for purity and to estimate the subunit molecular weight. In preparation for this experiment, students unfamiliar with electrophoresis are encouraged to visit a Web-based simulation of electrophoresis of protein and DNA (29) (http://www.rit.edu/~pac612/electroE_Sim.html). In addition to showing the effect of voltage and protein molecular weight on migration patterns, it also demonstrates the use of SDS–PAGE to estimate molecular weight. Please note that this simulation is a JAVA applet that works best with Netscape 3.0.

Weeks 4b, 5. Enzyme Kinetics (30)

Using their purified enzymes, teams of two or three students prepare a series of assay solutions and determine \( K_m \) and \( V_{\text{max}} \) values for TDH. This is perhaps the most difficult experiment, since we are analyzing a two-substrate, two-product reaction. Even with the \( K_m \) values for both substrates to assist them in selecting substrate concentrations, students have a difficult time obtaining reliable kinetic data. Despite the difficulties, I like this experiment because it forces the students to improve their pipetting technique in the lab and their quantitative analysis skills during report preparation.

Week 6. Gene Expression and Sequencing (31)

Students isolate the pDR121 plasmid from a small liquid culture of E. coli cells, using a commercially available kit that employs alkaline lysis followed by a chromatography step (5 Prime 3 Prime, Boulder, CO). Plasmid isolation is done with a commercial kit rather than a traditional mini-prep to insure the success of the DNA sequencing portion of this experiment, based on the recommendation of the manufacturer of the DNA sequencing system (32). The isolated plasmid is then used to transform E. coli cells that lack a functional \( \text{tdh} \) gene. Successful transformants are identified by their ability to grow on L-threonine as sole carbon source and to grow in the presence of ampicillin. The plasmid is also used for cycle sequencing of \( \text{tdh} \) using the ABI PRISM dye terminator sequencing protocol (Perkin Elmer Applied Biosystems, Foster City, CA). Students learn about the polymerase chain reaction (PCR) by performing the sequencing reactions; they set up the sequencing reactions (primer, dNTP’s, ddNTP’s, Taq polymerase, buffers, Mg\(^{2+}\)), which are done with our thermal cycler. The product mixtures are submitted to the nucleic acid core laboratory of a local research university, where the PCR extension products are analyzed. Students then evaluate the resulting DNA sequences using the BLAST server (33).

Weeks 7–9. Original Research Project

Each year a simple, well-defined research project that relates directly to threonine dehydrogenase is included in the class. The projects are designed so students have to apply techniques and principles that they learned in the first six weeks of the course. This really is a great adventure for the class, since the outcome is unknown. The original project portion of the lab is described in more detail below.

Week 10. Sequence Homology (34)

This exercise focuses on the use of information available in the primary literature and the Internet to design an experiment in site-directed mutagenesis of the \( \text{tdh} \) gene from E. coli. Students begin by submitting the known sequence of the gene to the BLAST (35) server at the National Center for Biotechnology Information (33). Each student then prepares a list of ten proteins from the BLAST search to study in greater detail. They use additional resources on the Internet, including SwissProt (36) and the Protein Data Bank (37), focusing on proteins with a known three-dimensional structure or well-characterized metal-ion binding sites. With these data in hand, they search MEDLINE for references on any of the proteins they have found. They are required to find links to the primary literature throughout the exercise. The goals of this exercise is for students to learn how to collect and sort information in designing an experiment.

Safety

The experiments in this course do not require any unusual safety precautions. Throughout the course students are required to wear goggles. Gloves are required during procedures using hazardous chemicals such as ethidium bromide or acids and bases, or when working with bacterial cultures. Prepared polyacrylamide gels are used for protein electrophoresis to avoid exposing students to the neurotoxic effects of the acrylamide monomer. All growth media are sterilized before disposal. To minimize waste production, ethidium bromide (for DNA gels) and Coomassie blue (for protein gels) are used repeatedly.

Original Research Projects

I select one original project for the course each year based on techniques the students have used during the course. The project is described in very general terms and the student teams then develop a strategy for attacking the problem. For example, the original project this year is to purify the threonine dehydrogenase from Klebsiella pneumoniae. The students will select the type and sequence of purification steps to use, as well as the characterization procedures. They have access to all equipment and reagents in the lab and to anything they can borrow from other labs in the building.

Three years’ experience with this approach have revealed several factors that are key to success in executing these projects:

1. The project must be very narrowly defined. If students are asked to think creatively on an original project, it should be built on things they have already done and there should be one or two obvious paths to take to solve the problem. Otherwise the students can become confused, sidetracked or frustrated.

2. The project should be in an area where the instructor is highly qualified.

3. Six 3-hour lab sessions are set aside for the project. The project should have built-in assessment points whereby the students can easily evaluate their progress at the end of each lab period and, if necessary, adjust their focus or their goals.

4. The project should be integrated into the overall direction of the course. Students must be able to understand how this project relates to their earlier experiments and how it will contribute to future developments in the course.
Three different projects have been attempted in the past three years: isolation of the tdh gene from Salmonella typhimurium, site-directed mutagenesis of the E. coli enzyme, and development of a colorimetric assay for the enzyme. The first two experiments were narrowly defined and fit nicely into the overall context of the course (criteria 1 and 4). However, they were unsuccessful because they did not meet the criteria 2 and 3. The third project involved developing a rapid colorimetric assay for TDH activity. This was in my area of expertise—analytical biochemistry—and it was a very successful experience for the students.

**Rapid Colorimetric Assay for TDH**

Threonine dehydrogenase from E. coli catalyzes the NAD-dependent oxidation of threonine to form 2-amino-3-ketobutyrate and NADH (Fig. 1). To date the activity of this enzyme has been followed spectrophotometrically (based on the 340 nm absorbance of NADH) or colorimetrically (based on the formation of aminoacetone) (38). Both assays are quantitative and specific for TDH, but they are also quite time consuming, requiring more than 5 minutes per assay. This is particularly cumbersome when analyzing the 80 fractions collected during chromatographic purification of the enzyme.

A colorimetric stain based on nitro blue tetrazolium has been used to detect TDH activity bands in electrophoresis gels (22). Similarly, a stain based on another tetrazolium dye, p-iodonitrotetrazolium violet, has been used to identify bacterial colonies expressing TDH activity on agar plates (19). On the basis of these references, the students developed a colorimetric assay for TDH activity. In the assay electron transfer is from NADH (which is transparent in the visible range) to p-iodonitrotetrazolium violet (ITV) using phenazine methosulfate (PM S) as an electron-transfer catalyst. The resulting formazan dye absorbs at 497 nm with an extinction coefficient of 19,000 M\(^{-1}\)cm\(^{-1}\) (39).

To begin the project, the students demonstrated that the rate of color development in the assay was linear, proportional to enzyme concentration and specific for TDH, using a UV-vis spectrophotometer (Fig. 2). The assay was performed with a crude extract of E. coli containing TDH to see if other enzymes present in the crude extract contributed to color development. The rate of color development in the absence of L-threonine was equivalent to the spontaneous rate of color development in the absence of enzyme, indicating that this assay is specific for TDH. The students subsequently obtained similar results using a microplate reader (Fig. 3). The rapid assay was then used to evaluate separations under a variety of pH conditions on a chromatographic column. One separation is illustrated in Figure 4.

The ITV colorimetric assay for THD is specific and the rate of spontaneous color development is low enough that enzyme activity can be detected in dilute solutions. This rapid colorimetric assay has subsequently been used extensively by students in the laboratory course to locate TDH as it elutes from a variety of chromatography columns.

**Future Plans**

This course is designed to develop over time to include new technologies that become available in our department. Rather than developing the new experiments by myself, it is my plan to do some preliminary investigation, then have the students actually develop new experiments as the original research portion of the course. Additional course modules will be designed to take full advantage of several instruments the department has obtained through NSF-ILI grant support (DUE-9650559, DUE-9551406): protein chromatography stations, capillary electrophoresis, and NMR.

**Protein Chromatography Stations**

With the analytical methods in place to identify and isolate TDH from E. coli, the original project for this year is to isolate TDH from another species, K. pneumoniae, using gel filtration, ion exchange and dye-ligand chromatography. We have already completed preliminary studies in our research group to demonstrate that TDH is expressed at detectable levels in this organism.

**Peptide Mapping of E. coli TDH Using Capillary Electrophoresis**

The purified enzyme from E. coli will be reduced and alkylated, then digested with trypsin and analyzed by capillary electrophoresis (40). The photo diode array detector in this system will enable the students to identify tryptic peptides containing aromatic residues.

**SDS-PAGE Using Capillary Electrophoresis**

To supplement their understanding of electrophoresis and to expose them to more modern technology, I will have the students analyze their purified enzyme samples using a Hewlett-Packard 3D Capillary Electrophoresis System to obtain a second estimate of molecular weight and a quantitative estimate of the purity of their protein (41).

**NMR of Unstable Reaction Intermediates**

The initial product of the reaction catalyzed by TDH is an unstable intermediate, 2-amino-3-ketobutyrate (AKB; Fig. 1). Using our 300 MHz NMR, students will follow the appearance of AKB and aminoacetone.
Summary

In this course, students learn techniques expected after completion of a biochemistry lab course. Teamwork and creative problem-solving skills are developed throughout the course and especially during work on the original projects. This approach can be applied successfully in undergraduate courses and especially during work on the original projects. Students develop creative problem-solving skills throughout the completion of a biochemistry lab course. Teamwork and creativity are essential components of this approach.

During the course.

Selection of the system to study is critical. It is most helpful if the protein is easily purified and stable for several months at 4°C and if the gene for the protein is available on a plasmid. While it is recommended that instructors design a similar course around a single system with which they are quite familiar, the pDR121 plasmid is available upon request. The course handouts can be found at this URL: http://www.rit.edu/~pac8612/505/.

At RIT, the course meets twice per week for 10 weeks (20 sessions). A few significant adjustments would be required to adapt this approach for a one-semester laboratory course meeting four hours per week for 14 weeks (14 sessions). It would be necessary to eliminate two of the exercises. For example, for students who are required to take a separate molecular biology laboratory course as biochemistry majors, removal of the gene expression–sequencing lab and the sequence homology labs would reduce the course by four lab sessions. The enzyme purification session (which requires four lab periods in our approach) could be reduced to two periods by limiting the purification to a single chromatography step and having the students work with the partially purified enzyme, or by providing the students with a partially purified enzyme at the beginning of the course so they can purify it with one further chromatography step.

Acknowledgments

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Literature Cited

34. Research Collaboratory for Structural Bioinformatics. Protein Data Bank; http://www.rcsb.org; (formerly Brookhaven National Laboratory. Protein Data Bank; http://www.pdb.bnl.gov/).