Purification of Three Spectral Isoforms of Recombinant Fluorescent Protein from Insect Bioreactors Infected with Recombinant Baculovirus

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Abstract: Recombinant peptides and proteins have a wide range of applications, especially in medicine and biomedical research. Methods to rapidly and inexpensively manufacture recombinant proteins are needed to realize the full potential of proteins in medicine and to broaden their applications. A one-semester-length laboratory exercise is reported here demonstrating the use of the insect bioreactor system to create recombinant proteins. These laboratory exercises are suitable for students majoring in biochemistry or biotechnology and can be used to teach students basic biochemistry laboratory techniques including bacterial transformation, plasmid DNA isolation, insect cell culture, production of recombinant virus, use of recombinant viruses to deliver transgenes to a living organism, protein isolation by precipitation and chromatography, and protein quantification and protein characterization by electrophoresis and western blotting. The techniques acquired can also be used as the basis for related independent research projects in the second semester.

Introduction

Natural products have been used in medications since antiquity. Protein- and peptide-based drugs isolated from natural sources have had a place in medicine since the early part of the twentieth century. Starting in the late 1970s with the cloning of human insulin in *Escherichia coli* [1], recombinant proteins emerged as a new means of treating human disease. Still, many human proteins with potential medical benefits cannot be synthesized in bacteria because they lack the post-transcriptional mechanisms to process the recombinant proteins as done by humans. Phosphorylation, glycosylation, and myristillation, for example, are not performed identically on proteins in humans and bacteria. The need for more efficient means to produce recombinant human proteins with the correct post-translational modifications can be seen in two currently used drugs. First, Avonex has recently shown promise in treating some patients with multiple sclerosis [2]. Avonex is recombinant human interferon β-1a (a glycoprotein) and is currently made in cultured Chinese Hamster Ovary (CHO) cells [3]. The cost of Avonex preparation in CHO cells results in a drug with annual costs exceeding $10,000 per patient [4]. Second, Cerezyme, also a glycoprotein produced in CHO cells [3], has an annual cost of approximately $200,000 [5].

One possible method for producing large, inexpensive quantities of recombinant protein is to examine other species for use as bioreactors. Lepidopteran larvae, larvae from butterflies or moths, have emerged as one possibility. In *Trichoplusia ni* (cabbage loopers), protein production levels of 1.58 mg/larva, or 26% of total larvae protein have been obtained [6]. Commercial producers are able to supply larvae expressing recombinant proteins from hundreds to thousands of larvae [7], meaning that gram quantities of purified recombinant protein can be made available, and this could drastically lower costs for patients. While post-translational modifications are probably not identical between insect larvae and human cells, both *Trichoplusia ni* and *Bombyx mori* (silk worms) have been shown to glycosylate bovine interferon γ [8]. The ability of various insect cells infected with different recombinant viruses to glycosylate human placental-secreted alkaline phosphatase has been shown to be dependent upon a complex set of factors [9]. The ability to make transgenic insects by viral infection [10] also means the transgenic larvae could be produced with the necessary human proteins to properly complete post-translational modifications.

Currently the most common method for delivering genes of recombinant proteins to lepidopteran larvae is via the *Autographa californica* nuclear polyhedrosis virus (AcNPV). Recombinant AcNPV can be produced in cultured insect cells. The recombinant virus can be isolated from cultured cells and used to infect large numbers of larvae. The larvae can be reared to maximize protein expression, which occurs nearly simultaneously with insect death as the virus spreads throughout the insect. The recombinant protein can then be purified from the insect bioreactor.

These techniques are demonstrated here using three spectral isoforms (blue, green, and yellow) of the green fluorescent protein (GFP) as a marker of expression. The GFPs are valuable biological markers for many research and teaching applications and have been thoroughly reviewed [11–13]. The structures have also been included in newer editions of at least two commonly used undergraduate texts [14, 15]. These 28 kDa proteins have been cloned from cnidarians (jellyfish) and anthozoans (coral), and various mutations have led to proteins emitting blue, cyan, green, yellow, and red light. Understanding the molecular basis for the fluorescence requires an understanding of the structures of the fluorophores in the various GFP spectral isoforms. The wild-type GFP is composed of 238 amino acids. A triad of adjacent amino acids: serine-65, tyrosine-66, and glycine-67 (65SYG67), form the fluorophore by condensation of the carbonyl group of the

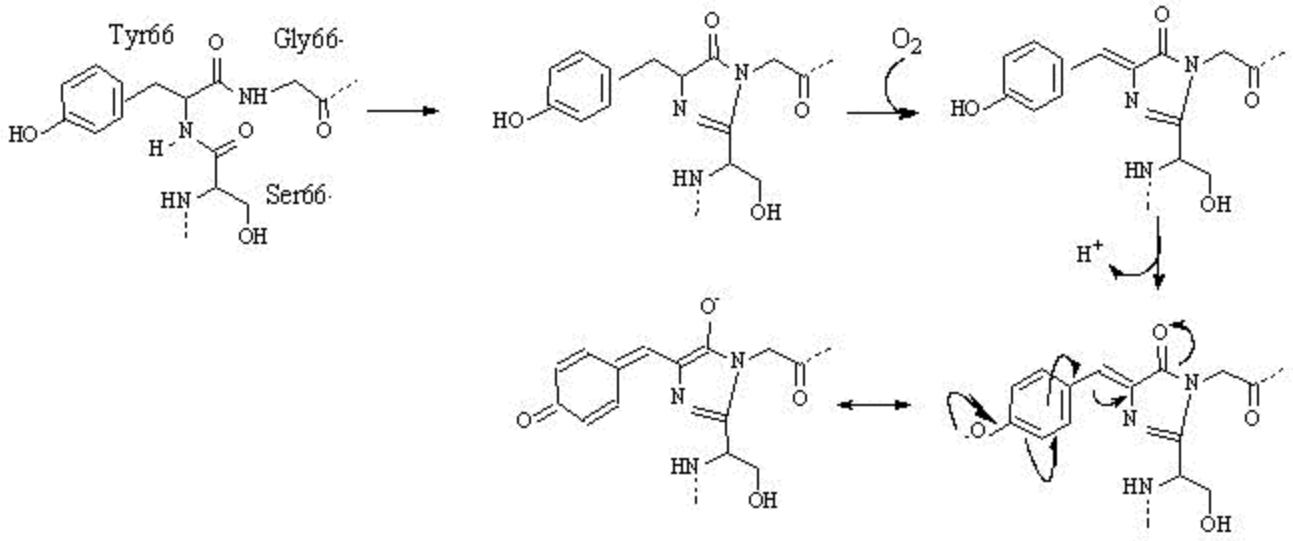

Figure 1. Mechanism of formation of the wild-type GFP chromophore.

serine residue with the amido group of the glycine residue in the protein interior. This is followed by oxidation from molecular oxygen to form a double bond to the imidazole-5-one ring as depicted in Figure 1. The phenolic form of tyrosine-66 is responsible for the UV excitation peak of GFP near 390 nm. Deprotonation of the tyrosine hydroxyl group leads to the planar, highly conjugated, quinonoid resonance structure with the red-shifted excitation peak near 490 nm [16, 17]. Mutations can be designed that favor the visible excitation, but it can also be promoted by elevating the pH (causing deprotonation of tyrosine-66) on wild-type GFP [18].

The GFPs are also a fantastic protein for laboratory exercises designed to introduce high school students [19] and undergraduates to protein biochemistry [20, 21]. Because the crystal structures for most of the spectral variants are available in the Protein Data Bank (PDB), this laboratory work can be accompanied with protein structural work on the computer using the PDB files. By performing these laboratory exercises, students not only gain insight and experience in traditional biochemistry techniques, they acquire understanding and expertise in large-scale production of recombinant proteins. We take advantage of this experience in the second semester and provide students with various independent GFP-related research projects. This experience, however, could certainly be applied in a post-graduate academic or industrial setting utilizing methodologies for making recombinant protein-based pharmaceuticals.

Experimental Section

Safety Precautions. Like all biologicals, especially viruses, there may be unforeseen hazards; thus all work utilizing the intact AcNPV should follow NIH guidelines for work with recombinant DNA. It is noteworthy that several recent articles on AcNPV point to effects in cultured mammalian cells [22–24], and, more importantly, that AcNPV is an effective vector for DNA delivery in cultured human cells [25] with potential applications in human gene therapy. The product literature from BD Pharmingen states, "The absence of a product warning is not to be construed as an indication that the product is safe. All possible hazards of many biological products may not be known at this time." Still, if appropriate safety practices are followed, including wearing gloves, washing hands before and after use, cleaning all surfaces with 70% ethanol, treating all viral solutions with 10% bleach prior to disposal, making sharps containers available, and autoclaving all solid waste, the project can be performed without impact to students or the environment.

Students like to view the fluorescent proteins with the hand held UV lamps. While the longer, 365-nm lamp is less dangerous than the 254-nm lamp, it is still necessary to remind students to avoid shining the lamps toward their skin or eyes and to minimize illumination times. Ideally, most illumination should be done in a hood away from other students, and UV-protective eyewear should be used.

Materials. All chemicals were from Sigma, Aldrich, or Fisher Scientific unless otherwise stated. *E. coli*, strain JM109 or DH5α, were obtained from Promega. LB media, agar, and disposable plastic Petri dishes were from Fisher Scientific. Precast agarose gels were from Sigma. Cyber Green DNA stain was from Molecular Probes. The BD Baculogold starter package from BD Pharmingen contains live *S. frugiperda* (Sf9) cells, TNM-FH insect cell medium (with antibiotics), BD Baculogold linearized AcNPV DNA (with lethal mutation), calcium phosphate transfection buffers, a wild-type viral stock solution, and some control transfer vectors. This kit provides enough reagents for at least 9 pairs of students to make recombinant virus. In addition, we ordered the pAcHLT-A-GFP transfer vector set from BD Pharmingen that includes transfer vectors expressing GFP, BFP, or YFP with a 6xHis tag, a thrombin cleavage site, and a multi-cloning site for introduction of other genes [26]. All manipulations of the Sf9 cells were performed in a Forma Scientific sterile cabinet. Sephadex G-75 was from Sigma. Ni-NTA agarose was from Qiagen. Precast SDS PAGE gels and minigel apparatus were from Bio-Rad. Cabbage looper eggs used for this work and the looper diet were the generous gift of Dr. Patrick Hughes at the Boyce–Thompson Institute, Cornell University. Other commercial vendors (Carolina Biological Supply and Benzon Scientific) of insect eggs/larvae, however, are currently being utilized. Protein was quantified with a Pierce Coomassie Plus kit. Fluorescence intensity of pooled protein fractions was quantified with a Varian spectrofluorimeter. All other reagents were obtained from Sigma/Aldrich or Fisher Scientific.

Figure 2. Container for growing the lepidopteran larvae.

Procedures: Cloning the Transfer Vector in E. coli and Isolation of Cloned Plasmid DNA. The bacteria were transformed with the fluorescent protein transfer vectors by standard methods [27]. Briefly, a frozen 200-μL aliquot of competent cells in a calcium chloride buffer was thawed on ice. To the cells, 3 μL of DMSO and 20 ng of transfer vector were added. The cells were then incubated on ice for 10 min, followed by 2 cycles of freezing in liquid nitrogen and thawing in a 37 ºC water bath. The transformed cells were diluted to 1 mL with LB medium and incubated at 37 ºC for 45 min. The transformed cells were plated in aliquots (either 10, 100, or 890 μL) onto 60 cm LB agar plates containing 100 μg/mL ampicillin. Plates were stored for 24 h in a 30 ºC incubator.

Plasmid DNA was, likewise, isolated by standard methods [28]. Briefly, transformed colonies were used to inoculate 2 mL of LB medium containing 100 μg/mL ampicillin and grown at 37 ºC overnight. The 2 mL was divided among six 1.5-mL microcentrifuge tubes. An equal volume of 0.2 M NaOH containing 1% (m/v) SDS was added to each tube to lyse the cells. To the lysates was added an equal volume of 3 M potassium acetate, pH 5.5. This mixture was centrifuged for 10 min at maximum speed in a centrifuge (>6,000g). The supernatant was decanted and mixed with an equal volume of isopropyl alcohol (or ethanol) to precipitate the plasmid DNA, and the DNA was isolated by centrifugation at maximum speed (>6000g). The alcoholic solution was carefully decanted and the resulting pellet resuspended in 50 μL of TE containing 1 mg/mL RNAse. A small portion of this was used for agarose gel analysis; the remainder was precipitated from the TE/RNAse solution for transforming the insect cells. The DNA was electrophoresed in a 1% agarose gel in an Owl Scientific submarine apparatus at 15 V/cm for 4 h, stained with Cyber Green from Molecular Probes, and photographed on a UV light box. DNA standards (1 μg of λ phage digest) were used as positive controls and for semiquantitation of plasmid DNA yields.

Insect Cell Culture and Production of Recombinant Baculovirus. BD Pharmingen supplies an extensive 100-page instruction manual with the baculovirus kit [29]. The Sf9 cells were cultured in a Lab-Line incubator at 28 °C under air (no special gas mixture like 5% carbon dioxide commonly used for mammalian cells is required for these insect cells). Cells were grown in 65-cm Nunc culture flasks in TNM-FH medium, passed into Costar 6-well plates, and transfected the next day. The cells are plated in the 6-well plates at six different densities, and the two wells in the 6-well plate in which the cells are dense, but not quite confluent are chosen. Cotransfection of the linear DNA and transfer vectors was done by the calcium phosphate precipitation method as described in the instruction manual, except that we used only about half the recommended amount of linear DNA and transfer vector (wells in the 6-well plates are smaller than the recommended 60-cm dishes in the manual). Incubate the cells for 3–5 hours in the DNA transfection solution. After 3–5 hours, remove the transfection buffer and add 2 mL of fresh TNM-FH medium. One day (12–24 h) after transfection, remove 1 mL of the medium and add 2 mL of fresh medium. Keep the cells at 28 °C for 4–6 days in this medium without changing or feeding them. Examine the cells occasionally under the fluorescent microscope for cells expressing the GFP. We used a Zeiss Axiovert 25CFL with a 10 × objective for cell observation and 40 × 0.5-NA objective for fluorescence microscopy. Digital images were captured with a Kodak MDS100 camera. The filter sets for BFP and GFP (also used for YFP) were obtained from Omega Optical. Because these insect cells can utilize the polyhedron promoter in the GFP transfer vector, some simple transient transfection will also occur. It is important to wait for visual fluorescence persisting for at least 6 days beyond the date of cotransfection. At this point, 10–100 μL of the culture supernatant is removed and used to inoculate a fresh 65-cm flask of Sf9 cells. The virus is expanded until the majority of the cells in the flask fluoresce. The recombinant virus is isolated by removing 8 mL of media from the flask, spinning at low speed in a 15-mL plastic tube to remove cell debris, and then pelleting from the low-speed supernatant in a Beckman ultracentrifuge in a Type 75Ti rotor at 50,000 rpm for 1 hour. The viral pellet is resuspended in 500 μL of cell medium.

Looper Rearing and Infection with Recombinant Baculovirus. The looper diet is prepared by boiling 16 g of dry mix (mostly wheat germ) with the 1.2 g of agar in 82 mL of water and adding 1 g of multivitamin mix to the cooled, but still warm solution. About 20 to 30 mL of the warm media is poured into plastic containers with a lid (sealed tight enough to keep the small larvae in, but not air tight so some evaporation can occur). Looper eggs are kept at room temperature until they hatch and then three young loopers (first instar) are transferred to each plastic container (Figure 2). Loopers are infected by one of three methods: (1) recombinant virons can be added directly to the diet surface in the containers, (2) virons can be injected into the looper, and (3) loopers can be coated with viron solution. While all three methods worked in our hands, the first method was slower and had the lowest efficiency, and the second method caused many loopers to die, so we routinely infect loopers by bathing them. This is most efficient if done in the third instar and repeated during the fourth instar by simply rubbing about 10 to 20 μL of viral solution over the body of the insect using a yellow tipped pipetman. After infection, loopers are monitored daily with a 365-nm UV light to examine spread of the viral infection. Within 1-2 days, those infected will show one or a few small fluorescent spots, but the spots will grow within 2 to 4 days until the entire insect fluoresces.

Protein Purification and Characterization. Fluorescent insects were stored at –80 ºC until ready to be used. The frozen insects were combined with 5 to 10 volumes of homogenization buffer (10 mM Tris, pH 8.0, containing 1 mM dithiothreitol) and the mixture homogenized in a glass–Teflon homogenizer. It is important to include the reducing agent or the solution will turn dark brown to black upon storage. An aliquote of the homogenate was kept for protein, fluorescence, and SDS PAGE analysis. The loopers contain something that emits blue light upon UV illumination at 365 nm, so, especially for the BFP, it is best to quantify fluorescence-emission intensity with a visible excitation (390 nm for BFP, and preferably closer to 490 nm for GFP and YFP).

Protein in the homogenate is first purified and concentrated by three phase portioning [30]. To the homogenate an equal volume of 4 M ammonium sulfate is added dropwise in a beaker on a magnetic stirrer. To this solution a volume of dry *t*-butanol (over molecular sieves) equivalent to the amount of ammonium sulfate is added, also with stirring. This solution was transferred to a 35-mL centrifuge tube

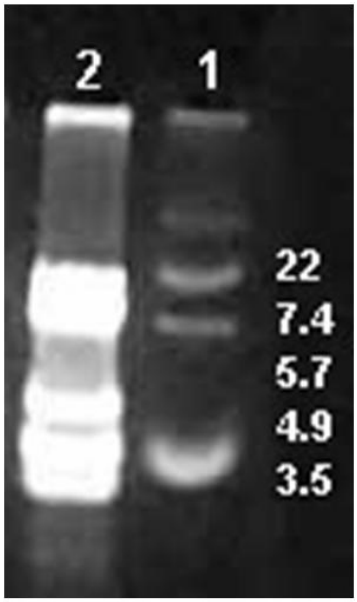

Figure 3. Plasmid DNA preparation analyzed by agarose gel electophoresis on a 1% agarose gel. Lane 1 plasmid prep from 1 pair of students, lane 2 DNA standards from λ phage. The sizes of the standards, in kb, are indicated on the right.

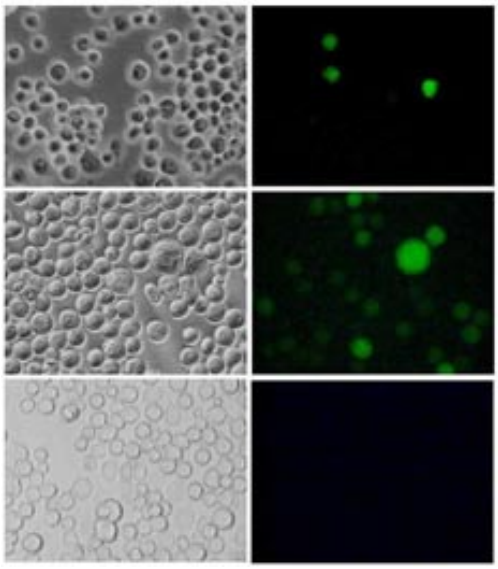

Figure 4. Transfected Sf9 cells producing virus. Right panels, phase micrographs, left panels fluorescence micrographs. Top day 5, middle day 7, bottom control (no DNA). It is not uncommon to see some virally infected cells to swell in culture as seen in the day 7 culture.

and spun in a Sorval SA-600 rotor in an RC3B centrifuge at 3000 rpm for 10 min. Centrifugation results in three layers separated by a protein "cake" pelleted between the dense aqueous ammonium sulfate and upper *t*-butanol layers. The GFP should be in the protein cake, but it is important to verify this with a UV lamp. If so, decant the *t*-butanol and carefully remove the protein cake with a spatula. If not, decant the wet *t*-butanol and replace it with fresh, dry *t*-butanol and spin again. The protein cake is resuspended in homogenization buffer and allowed to stand at 4 ºC overnight. The resuspended protein cake is centrifuged to remove proteins irreversibly denatured by the ammonium sulfate/*t*-butanol precipitation. An aliquot of this material is saved for SDS PAGE analysis, and the remainer quantified by fluorimetery.

The clarified supernatant is decanted and separated by size-exclusion chromatography using a 1-by-20-cm Sephadex G-75 column. Homogenization buffer was used as the mobile phase. Fractions of about 2 mL were collected and analyzed, first using a hand held UV lamp and later by spectrofluorimetry. The two peak fractions were pooled and a small aliquot saved for SDS PAGE analysis.

The pooled fractions from the size-exclusion chromatography column were run onto a 2-mL Ni-NTA affinity resin poured in a Pasteur pipet with a glass-wool plug. The column was washed once with homogenization buffer and once with homogenization buffer containing 0.2 M NaCl to remove nonspecifically bound proteins. The fluorescent protein was eluted with homogenization buffer containing either 500 mM of histidine or 500 mM of imidazole, adjusted to pH 8. The 1-mL fractions were inspected, first using a hand held UV lamp and later by spectrofluorimetry. The two peak fractions were pooled and a small aliquot saved for SDS PAGE analysis.

Protein analysis on all the aliquots was performed using the Pierce Coomassie Blue Plus protein assay. For each group, 20 μL of each fraction was removed, combined with 2× sample treatment buffer and boiled for 5 min. The denatured protein–glycerol solution was chilled on ice before loading onto a Bio-Rad precast minigel with a 10 to 20% linear gradient. Proteins were electrophoresed at 25 mV for 4 h and Sigma rainbow markers were used as a positive control and for molecular weight determination. The gel was stained with Coomassie Blue dye and scanned on a flatbed scanner for documentation.

The absorption and emission spectra of the various spectral isoforms of recombinant GFPs were recorded on a Varian spectrofluorimeter. Spectral differences between isoforms were correlated with differing amino acid substitutions in the proteins, and the rusulting changes in the chromophore structure or environment. Protein structures were obtained from the Protein Data Bank [31] and viewed using Rasmol software.

Results and Discussion

All of the data shown below are from student groups (students worked in pairs).

The plasmid DNA preparation showed the three familiar bands of the nicked, relaxed and supercoiled plasmid DNA (Figure 3). No effort was made to do a restriction analysis because there was only one plasmid present in the transformed bacteria. We did, however, do an experiment to demonstrate the hyperchromic effect by comparing the $A_{260\ nm}$ for isolated plasmid DNA before and after boiling.

The isolated plasmid DNA was used to cotransfect Sf9 cells with the linearized baculovirus DNA containing the lethal mutation (that is rescued by recombination with the transfer vector). Because AcNPV is an envelope virus, it spreads from cell to cell in culture (note the increase in number of fluorescent cells in Figure 3, top and middle right panels); however, the transfer-vector promoter can also be read by the Sf9 cells, so transient transfection is also possible. To ensure that fluorescence observed is due to viral infection and not to simple transient transfection, cells should be monitored for at least 7–10 days (Figure 4). Furthermore, transferring a small portion of the cell supernatant solution to a new flask of cells ensures that only viral transfection is occurring. In the expanded cultures it is normal to see an increase in the number of fluorescent cells with increasing time until nearly all cells are fluorescent, and this would not be expected for transient trnasfections. While individual viral plaques can be isolated, and this should be done for more in-depth work because of the mutation rate of the recombinant viral DNA, we have had

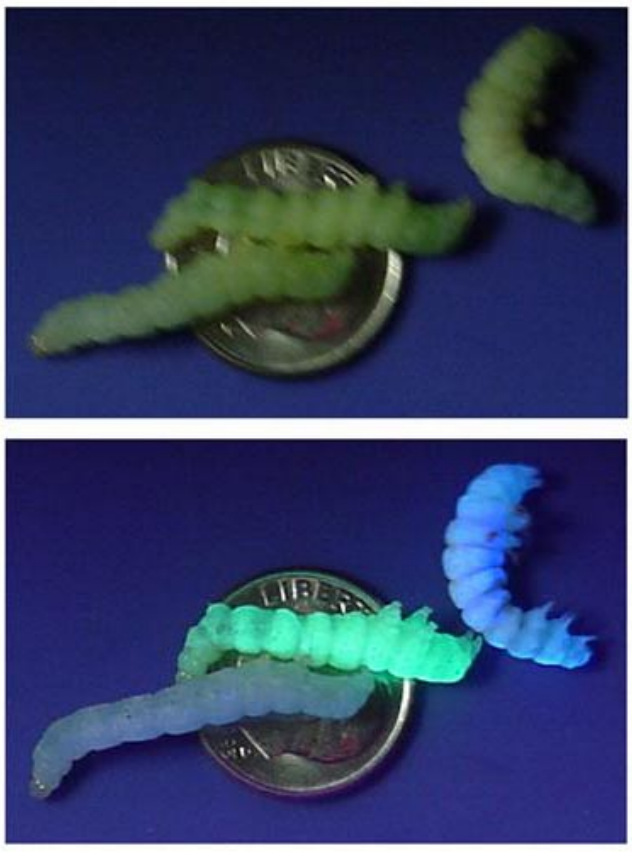

Figure 5. Digital images of live, virally-infected loopers expressing GFP or BFP, and one control (not infected). The top panel shows loopers under white light illumination, and the bottom panel shows both white and UV illumination. The dime is used for a size reference. These images were collected with a SONY Mavica camera and processed with Microsoft PhotoEditor software.

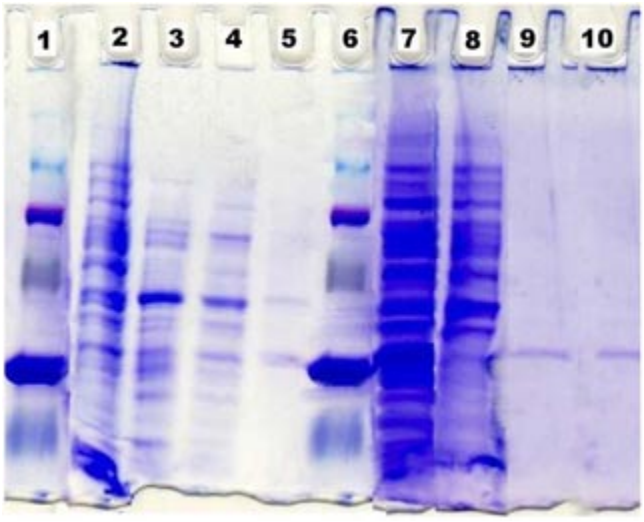

Figure 6. SDS PAGE analysis of the fluorescent protein purification from two different student groups. Lanes 1 and 6 are Sigma rainbow markers, lanes 2 and 7 are crude homogenates, lanes 3 and 8 are from the three phase partioning, lanes 4 and 9 from the size exclusion chromatography, and lanes 5 and 10 from the Ni-NTA affinity column. The molecular weight standards are (top to bottom) myosin (205 kDa), galactosidase (116 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20.1 kDa).

success infecting insect larvae without purifying virons from individual plaques.

The virus isolated from the expanded culture is used to infect the cabbage loopers in the third instar (about 5–7 days after hatching). The infected loopers will eventually exhibit strong fluorescence throughout their body and are easily visualized under a long wavelength UV lamp (Figure 5). The upper panel shows the appearance of the insects under white light while the lower panel shows them also with UV illumination. The bottom looper in this figure is a control, not infected with virus. As mentioned previously, these insects also possess a faint blue emission with UV illumination so BFP quantification cannot be done with 365 nm illumination. The loopers will live for several days while completely fluorescent. It is important to monitor the infected cabbage loopers to decide when to freeze them, as they will begin to turn dark brown/black as they die, and this is likely accompanied by proteolysis and other problems that can result in reduced yield of recombinant protein.

The fluorescent proteins were purified from the intact insect larvae to homogeneity in three steps, as assessed by SDS PAGE (Figure 6) using Coomassie Blue Staining. In addition, students prepared protein purification tables on the pooled fractions from each step. This was done in two ways, comparing the ratio of A_{390}/A_{280} and by quantitating fluorescence (relative to an arbitrary standard) intensity per milligram of protein for each step. Both of these measures of purity increased for most groups in almost every step, but purification factors for each step varied among groups. For both the size-exclusion column and the Ni/NTA affinity column students were required to make plots of fluorescence intensity versus fraction number. We did not do total protein analysis, or A_{280}, on individual fractions, but this could be included to show how each column step aided in purification of the recombinant GFPs. While the Ni-NTA affinity column provides the greatest purification and manufacturers often claim purity can be accomplished in a single step, adding the three-phase partitioning and the size-exclusion steps allow addition instruction on other protein purification techniques and ensure that the final product is truly clean, as it is not uncommon for proteins in crude homogenates to bind nonspecifically to Ni/NTA resins. The most intense band in the standards (lanes 1 and 5) is carbonic anhydrase with an apparent molecular mass of 29 kDa. The recombinant GFPs all have masses of about 30 kDa because these proteins are slightly larger than the native GFPs of 28 kDa. The increased mass is due to the addition of the 6xHis tag and a thrombin cleavage site in the carboxy terminus (The transfer vectors are designed for production of GFP fusion proteins that can be readily isolated and purified and then separated from the GFP fusion partner by selective proteolysis); thus, the recombinant GFPs migrate just above carbonic anhydrase in the gel. Students were required to estimate the molecular mass of the purified protein by constructing log molecular mass (standards) versus mobility (mm) plots. While the gel shows that the recombinant GFPs are abundant in the crude homogenate, it also clearly demonstrates selective enrichment of the fluorescent proteins by our three-step purification procedure.

The purified proteins were examined by fluorimetry. Both excitation and emission spectra were obtained by each group of students. The combined results for the BFP and GFP are

Table 1. Some GFP-Related Biochemistry Research Projects at the USAFA, 2000 – 2002

Project	"Collaborator"/Institution	Materials Provided
Green-Fluorescent Beer	Brendon Cormack/Stanford Univesity	DNA
GFP-Expressing Transgenic Insects	Al Handler/USDA	DNA
Red-Fluorescent Porphyrin	Charles Roessner/Texas A&M	Transformed E. coli
Transplantation of GFP Bone Marrow	Gen Kondoh/Osaka Univeristy, Japan	Transgenic mice
from Transgenic GFP-Expressing Mice	Jennifer Alfieri/UC Davis	
	Mike Wilcox/USAFA	Surgical supervision
Food-Based Vaccines for Trout	Dave Harris/CO Division of Wildlife	Rainbow Trout fry
GFP-Transgenic Zebrafish	Ann Davidson/University of Minnesota	Transgenic zebrafish
Making GFP-Transgenic Plants	C. Neal Stewart/UNC Greensboro	Transformed agrobacteria
GFP Silk	Hajime Mori/Kyoto Institute, Japan	Recombinant virus, DNA
Rat Myeloma with YFP Mitochondria	-	
Rat Monoclonal and Polyclonal	-	
Antibodies against GFP		
A Flavin-Containing YFP	Thomas Baldwin/Texas A&M	Transformed E. coli
A Point Mutation in YFP	Rebekka Wachter/University of Oregon	DNA

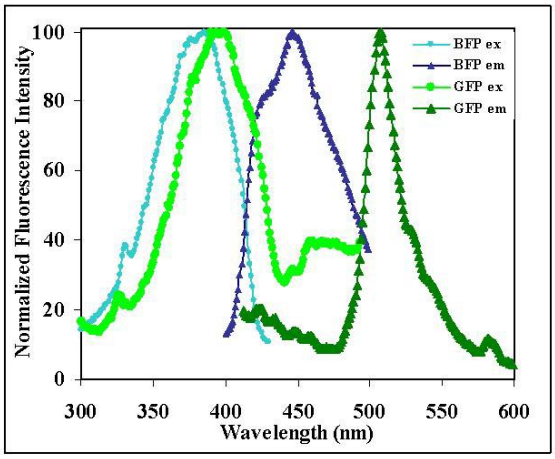

Figure 7. The excitation (light lines) and emission (dark lines) spectra for blue and green fluorescent proteins purified from the insect bioreactors. The YFP excitation and emission are quite similar to the GFP with a slight shift in the wavelength of maximum emission, and the presence of a red shift shoulder in the emission spectrum so they were omitted for clarity.

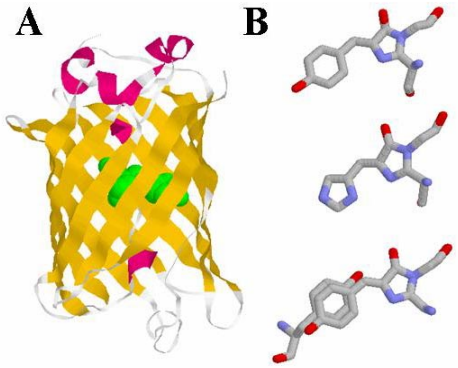

Figure 8. A. Ribbon structure of the GFP (PDB entry 1EMG) showing the β-barrel structure of all the fluorescent proteins with the centralized chromophore displayed in "space filling" mode using Rasmol software. **B**. Structures of the chromophore site in (top to bottom) green, blue and yellow fluorescent proteins displayed in "sticks" mode. The top shows the tyrosine side chain in GFP, the middle the imidazole side chain in BFP resulting from a tyr to his mutation, and the bottom the inclusion of another tyrosine ring (resulting from a thr to tyr mutation) adjacent to the chromophore in YFP.

shown in Figure 7. All three recombinant GFPs have excitation peaks near 390 nm, while the GFP and YFP have additional exciation peaks near 490 nm (depending upon the charge on the tyrosine in the GFP and YFP chromophores). In their final reports, students were required to explain the Stoke's shift using a Jablonski diagram.

The variations in spectra were correlated with changes in primary sequence of the recombinant proteins and with the differences in protein tertiary structure by examining the PDB structures. There are 23 entries for various isoforms of recombinant GFPs (including DsRed) in the protein data bank with high-resolution structures, and students must include PDB structures in their final reports. This exercise, perhaps more clearly than any other method, demonstrates the profound effects that single amino acid substitutions are capable of producing. Students can readily see with their own eyes the effect of the mutation, and then correlate it to an imperceptible alteration in tertiary structure. All three proteins retain the β-barrel configuration (Figure 8A), despite the chromophore mutations. The amino acids in or near the chromophore are unique in each case. In BFP, a histidine residue is substituted for the GFP tyrosine residue in the chromophore and in YFP an additional tyrosine residue is placed near the planar chromophore where the aromatic stacking alters the electronic environment and hence the color of emission (Figure 8B).

This semester-length project introduces students to a variety of techniques in biochemistry. In the second-semester laboratory we do independent research projects. The unifying theme of these projects is the GFP, although not all projects necessarily utilize one of the GFP variants. Project ideas are selected from reviews of the scientific literature on GFP using

Medline or SciFinder databases. After reading the abstracts, principle investigators at academic or industrial research institutions around the world are contacted to ask if they would be willing to assist us in our research projects. From those that are willing to participate, I compile a list of potential projects. Projects that we have attempted in the past two years, along with the assisting researchers and the materials they provided are shown in Table 1. For these projects students are given little more than the overall objective and a few background references; however, it is necessary to provide numerous checkpoints throughout the semester to ensure the students are staying on task, and to make sure they are not getting disappointed.

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Suporting Materials. Class handouts provided to USAFA cadets for background and methods prior to class are available upon request by contacting the author.

References and Notes

1. Goeddel, D. V.; Kleid, D. G.; Bolivar, F.; Heyneker, H. L.; Yansura, D. G.; Crea, R.; Hirose, T.; Kraszewski, A.; Itakura, K.; Riggs, A. D**.** *Proc. Natl. Acad. Sci. USA* **1979,** 76*,* 106–110.
2. Jacobs L.; Rudick R.; Simon, J.; *J Neuroimmunol* **2000,** 107*,* 167–173.
3. *The Physician's Desk Reference*, 53rd Edition; Medical Economics Company Inc.: Montvale, NJ, 1999; pp 734–738, 1078.
4. Rubin, R**.** In USA Today Health. http://www.usatoday.com/life/health/general/lhgen087.htm (accessed Oct 2001).
5. Alper, J. *Science* **2001,** *291,* 2343.
6. Cha, H. J.; Minh-Quan P.; Rao, G.; Bentley, W. E. *Biotechnol. Bioeng*. **1997,** *56,* 239–247.
7. For example, Agrivirion, Inc. http://www.agrivirion.com/ (accessed Oct 2001).
8. Murakami, K.; Uchiyama, A.; Kokuho, T.; Mori, Y.; Sentsui, H.; Yada, T.; Tanigawa, M.; Kuwano, A.; Nagaya, H.; Ishiyama, S.; Kaki, H.; Yokomizo, Y.; Inumaru, S. *Cytokine* **2001,** *13,* 18–24.
9. Joshi, L.; Davis, T.; Mattu, T.; Rudd, P.; Dwek, R.; Shuler, M.; Wood, H. *Biotechnol. Prog*. **2000,** *16,* 650–656.
10. Liu, Z.; Yang, F.; Qi, Y.; Zhu, Y.; Zhu, F. *Kunchong Xuebao* **2001,** *44,* 1–8.
11. Methods in Molecular Biology. Green Fluorescent Protein: Applications and Protocols; Hicks, B. W., Ed.; Humana Press: Totowa, NJ, 2001, in press.
12. Tsein, Y. *Annu. Rev. Biochem*. **1998,** *67,* 509–544.
13. Meth. in Enzym. Volume 302: Green Fluorescent Protein; Conn, M. P., Ed.; Academic Press: San Diego, CA, 1999.
14. Nelson, D. L.; Cox, M. M. *Lehninger's Principles of Biochemistry,* 3rd ed.; Worth Publishers: New York, NY; p 187.
15. Garrett, R. H.; Grisham, C. M. *Biochemistry,* 2nd ed.; Saunders College Publishing: Fort Worth, TX; pp 92, 416.
16. Heim, R.; Prasher, D. C.; Tsien, R.Y. *Proc. Natl. Acad. Sci USA* **1994**, *91,* 12501–12504.
17. Ormo, M.; Cubitt, A.; Kallio, K.; Gross, L.; Tsien, R. *Science* **1996,** *273,* 1392.
18. Ward, W. W.; Bokman, S. H. *Biochemistry* **1982,** *21,* 4535–40.
19. Bio-Rad's Biotechnology Explorer Education Products. http://www.bio-rad.com (accessed Oct 2001).
20. Hicks, B. W. *J. Chem. Educ.* **1999,** *76,* 409–415.
21. Cutler, M.; Davies, D.; Ward, W. In *Bioluminescence and Chemicluminescence. Fundamentals and Applied Apects;* Campbell, A.; Kricka, L.; Stanley, P., Eds.; Wiley: Chinchester, England, 1994; pp 383–384.
22. Van Loo, N.-D.; Fortunati, E.; Ehlert, E.; Rabelink, M.; Grosveld, F.; Scholte, B**.** *J. Virol*. **2001,** 75*,* 961–970.
23. Ma, L.; Tamarina, N.; Wang, Y.; Kuznetsov, A.; Patel, N.; Kending, C.; Hering, B.; Philipson, L. *Diabetes* **2000,** *49,* 1986–1991.
24. Gronowski, A.; Hilbert, D.; Sheehan, K. F.; Garotta, G.; Schreiber, R. *J. Virol*. **1999,** *73,* 9944–9951.
25. Ma, L.; Tamarina, N.; Wang, Y.; Kuznetsov, A.; Patel, N., Kending, C.; Hering, B. J.; Philipson, L. H. *Diabetes* **2000,** *49,* 1986–1991.
26. While the initial cost of obtaining these reagents may seem expensive (about $1000), if this or a similar laboratory is performed on a yearly basis, costs can be substantially reduced the second and each subsequent time the laboratory is performed (for example, there is no need to order transfer vectors, cells, or calcium phosphate transfection medium).
27. Doyle, K., Ed. *Promega Protocols and Applications Guide,* 3rd ed.; Promega Corporation: Madison, WI, 1996.
28. Cormack, R.; Somssich, I. Technical Tips Online 1: 25:T01210. http://research.bmn.com/tto (accessed Oct 2001).
29. *Instruction Mannual,* 6th ed.; Pharmingen, a BD Company: San Diego, CA.
30. Dennison C; Lovrien, R. *Protein Expression and Purification* **1997,** *11,* 149–61.
31. The Protein Data Bank. http://www.rcsb.org/pdb/index.html (accessed Oct 2001).