JOURNAL OF CHEMICAL EDUCATION

Laboratory Experiment

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Effect of pH on the Heat-Induced Denaturation and Renaturation of Green Fluorescent Protein: A Laboratory Experiment

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Supporting Information

ABSTRACT: A fluorescence spectroscopy experiment is described where students integrated biochemistry and instrumental analysis, while characterizing the green fluorescent protein excitation and emission spectra in terms of its phenolic and phenolate chromophores. Students studied the combined effect of pH and temperature on the protein's fluorescence, which led them to conclude that its fluorescence, denaturation, and renaturation was pH dependent. The importance of temperature control in fluorescence spectroscopy, and of control samples in experiments, was also stressed. This experiment is suitable for an upper-level biochemistry laboratory, as an extension of experiences involving bacterial transformation and hydrophobic interaction chromatography to purify the protein. It is also appropriate for an instrumental analysis laboratory using a provided protein sample.

KEYWORDS: *Upper-Division Undergraduate, Analytical Chemistry, Biochemistry, Interdisciplinary/Multidisciplinary, Laboratory Instruction, Hands-On Learning/Manipulatives, Fluorescence Spectroscopy, pH, Proteins/Peptides*

As evidence of the impact of the green fluorescent protein (GFP) in science, Shimomura, Chalfie, and Tsien were awarded the 2008 Nobel Prize in Chemistry for the discovery and development of GFP.[1] Hicks introduced the idea of using GFP in undergraduate laboratory curricula.[2] GFP is a 27 kDa globular protein consisting of an 11-stranded β-barrel with short helices that run through the cylinder. The GFP chromophore is in the center of the β-barrel, formed by an intramolecular cyclization reaction involving serine-65, tyrosine-66, and glycine-67.[3] Formation of the chromophore is autocatalytic, needing only O_2 and occurs after protein folding.[4,5] The phenolic form of the GFP chromophore is responsible for the 395 nm fluorescence excitation peak. It can be converted to the phenolate chromophore by deprotonation of the tyrosine hydroxyl group, leading to a highly conjugated resonance structure responsible for the 475 nm fluorescence excitation peak.[3] The fluorescence emission spectrum exhibits a peak near 508 nm using 395 nm excitation, and a peak near 503 nm using 475 nm excitation.[3] Most of the GFP amino acid sequence (94−97%) is needed for chromophore formation or fluorescence.[6,7] GFP has been widely used as a tag and an indicator.[3] Other interesting GFP uses are found in Marc Zimmer's Web site.[8]

Proteins are susceptible to denaturation by altering the balance of noncovalent forces that maintain their native conformation. Some denatured proteins undergo renaturation if they can refold, regaining their three-dimensional structure and conformationally sensitive properties. The GFP chromophore inside the β-barrel is protected from quenching by oxygen[9] and is fairly resistant to heat, certain pH changes, proteases, detergents, and chaotropic agents.[10,11] Fluorescence is completely lost when GFP is denatured[12] but is regained when the β-barrel reforms.[13] Thus, onset of fluorescence after GFP denaturation can be an indicator of protein renaturation.

Part I of this experiment studied the effect of pH on GFP fluorescence excitation and emission spectra. Part II, based on the Alkaabi et al. work,[10] studied the effect of pH on the heat-induced protein denaturation and renaturation using fluorescence excitation spectra. A classroom activity of fluorescence has been reported in this *Journal*,[14] as well as several protein denaturation experiments.[15−18] This laboratory experience taught students how to (i) differentiate between a fluorescence excitation and emission spectra; (ii) characterize GFP excitation spectra in terms of its phenolic/phenolate chromophores; (iii) examine whether GFP fluorescence, denaturation, and renaturation are pH dependent; (iv) study the effect of temperature on GFP fluorescence; and (v) confirm the importance of using control samples in experiments.

EXPERIMENTAL DETAILS

Students worked in pairs and needed about 75 min to perform the fluorescence spectroscopy. Each student group was assigned two pH conditions (8 and either 3, 6, 10, or 12) for Part I of the experiment, and one pH condition (6, 8, 10, or 12) for Part II. Because of limited instrument availability, some student groups performed the spectroscopy in out-of-class time periods. On other occasions, students performed different experiments while select student groups performed the fluorescence spectroscopy. Procedure details are found in the Supporting Information.

Published: December 11, 2012

dx.doi.org/10.1021/ed2006949 | J. Chem. Educ. 2013, 90, 248−251

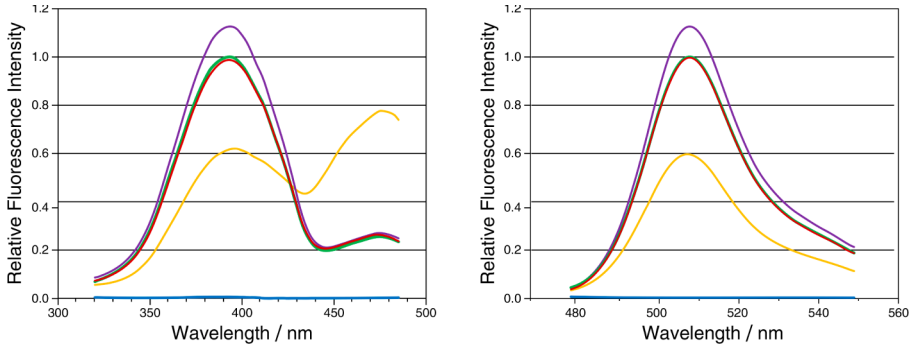

Figure 1. Effect of pH on GFP normalized fluorescence excitation (left, λ_{em} = 505 nm) and emission (right, λ_{ex}= 395 nm) student spectra in 50 mM phosphate solutions at 25 °C and various pH conditions: 3 (blue), 6 (red), 8 (green), 10 (purple), and 12 (yellow).

HAZARDS

The use of ultraviolet-rated safety glasses and latex gloves is recommended. Phosphate solutions should be discarded in hazardous waste containers.

RESULTS

Analysis of the GFP sample by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed the presence of bacterial proteins in addition to GFP. In spite of using a crude preparation of GFP in this experiment, the fluorescence excitation and emission spectra were close to those reported in the literature.[2,3] A major excitation peak by the phenolic chromophore was detected near 395 nm, and a minor excitation peak by the phenolate chromophore was evident at 475 nm (Figure 1, left). An emission peak near 508 nm was observed using 395 nm excitation (Figure 1, right). The GFP fluorescence excitation and emission spectra were completely eliminated at pH 3. However, GFP fluorescence was surprisingly resistant to pH 12. Because the intensities recorded in Part I of the experiment depended greatly on sample aliquoting variations, students were instructed to use the ratio of the intensities at 475 and 395 nm from each spectrum, to study the effect of pH on GFP fluorescence. The fluorescence intensity ratio 475/395 at pH 12 was higher than at any other pH (1.237 ± 0.030, Table 1), indicating that a larger fraction of phenolic chromophores deprotonated at pH 12, increasing the number of phenolate chromophores responsible for the 475 nm excitation peak, as reported in the literature.[3] The 475/395 ratio values at pH 6, 8, and 10 were closer to each other (0.267 ± 0.007, 0.254 ± 0.006, 0.243 ± 0.005, respectively). The highly variable ratio value at pH 3 (0.871 ± 1.135) was considered meaningless because GFP was assumed denatured due to very low fluorescence. From these results, students observed how greatly pH 3 and pH 12 affected GFP fluorescence.

Table 1. GFP Intensity Ratio Values at Various pH

Sample pH[a]	Fluorescence Intensity Ratio[b]
3	0.871 ± 1.135
6	0.267 ± 0.007
8	0.254 ± 0.006
10	0.243 ± 0.005
12	1.237 ± 0.030

[a]50 mM phosphate solutions at 25 °C. [b]The 475/395 ratio values are averages ± standard deviation from at least two student triplicate normalized excitation spectra for each pH, using 505 nm emission.

The fluorescence intensities of GFP samples incubated at 0 °C were consistently greater than those of the 25 °C samples, at all pH conditions (Figure 2). Students realized that this could be due to a decrease in collisional deactivation of the electronic excited states, generally observed in fluorescence at lower temperatures. This result stressed the importance of temperature control when performing fluorescence spectroscopy. Interestingly, the fluorescence intensity percentage of the 25 °C GFP sample compared to the 0 °C sample did not seem to depend that much on pH. In fact, the average intensity percentage at 395 and 475 nm for the 25 °C sample compared to the 0 °C sample at all pH conditions was 81.0 ± 2.6 and 86.1 ± 1.1, respectively (Table 2).

The lower fluorescence intensity percentage observed for the 80 °C-treated GFP sample at all pH conditions was probably due to denaturation, which involved protein unfolding and loss of the GFP β-barrel structure needed for chromophore fluorescence. Students observed that the 80 °C-temperature effect on the GFP excitation spectra was dependent on pH (Figure 2), and that GFP sample fluorescence was more resistant to 80 °C-denaturation at pH 6 or 8 than at pH 10 or 12 (Table 3). Thus, students concluded that GFP denaturation was pH dependent. This result stressed the importance of pH control when studying biomolecules.

Students also concluded that GFP renaturation was pH dependent. A second GFP control sample was needed for the renaturation treatment because fluorescence dropped somewhat when incubating the diluted GFP sample at 25 °C for 20 min. This finding stressed the importance of using control samples in experiments. Student results showed that GFP renaturation reached a higher extent at pH 8 than at pH 6 (Table 3), similar to what Alkaabi et al. reported,[10] where GFP denaturation was less effective and renaturation was more effective at pH 8.5 than at pH 6.5.

DISCUSSION

This experiment integrated important biochemistry and instrumental analysis lessons while exposing students to the green fluorescent protein. Students distinguished between recording the excitation and emission spectra. They characterized the GFP excitation and emission spectra in terms of its phenolic and phenolate chromophores. Students also studied the effect of pH and temperature on GFP fluorescence, concluding that denaturation and renaturation was pH dependent, and they realized the importance of using control

dx.doi.org/10.1021/ed2006949 | J. Chem. Educ. 2013, 90, 248–251

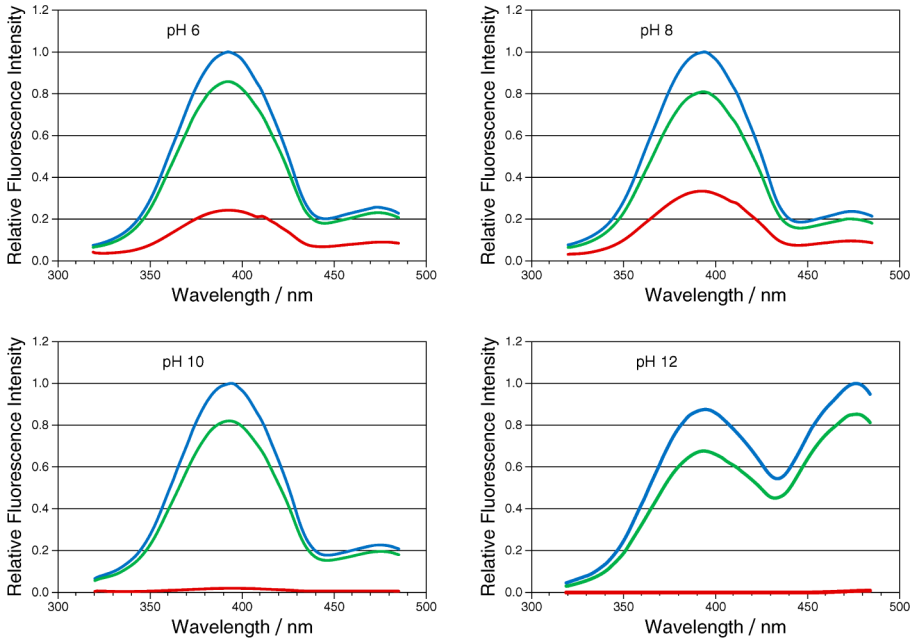

Figure 2. Effect of temperature on GFP normalized excitation student spectra in 50 mM phosphate solutions at various pH conditions. Samples were incubated at 0 °C (blue), 25 °C (green), and 80 °C (red) for 10 min.

Table 2. Comparison between Fluorescence Intensity of GFP Sample at 25 and 0 °C

	Fluorescence Intensity of 25 °C Sample Compared to 0 °C Sample[a] (%)	
Sample pH	395 nm	475 nm
6	83.3 ± 4.3	87.4 ± 4.9
8	80.9 ± 3.6	85.1 ± 6.3
10	82.3 ± 1.6	86.5 ± 1.5
12	77.3 ± 2.1	85.5 ± 2.4
Average	**81.0 ± 2.6**	**86.1 ± 1.1**

[a]Percentage fluorescence intensity values were averages ± standard deviation from at least two student triplicate excitation spectra for each pH, using 505 nm emission.

Table 3. Effect of pH on the Denaturation and Renaturation Treated GFP Samples

	Fluorescence Intensity of Treated Sample Compared to 25 °C Control Sample[a] (%)			
	395 nm		475 nm	
Sample pH	Denaturation	Renaturation	Denaturation	Renaturation
6	28.4 ± 0.4	47.4 ± 2.3	39.1 ± 0.7	48.5 ± 2.1
8	41.6 ± 12.0	70.5 ± 3.7	48.6 ± 12.5	68.9 ± 1.4
10	2.4 ± 1.6	16.1 ± 8.3	3.4 ± 1.6	17.6 ± 7.4
12	−5.5 ± 1.6	−5.2 ± 1.1	0.7 ± 0.1	2.0 ± 0.3

[a]Samples were heated at 80 °C for 10 min (denaturation) or heated at 80 °C for 10 min and incubated at 25 °C for 20 min (renaturation). Values were averages ± standard deviation from two student triplicate excitation spectra for each pH, using 505 nm emission.

samples in experiments. Thus, student goals for the experiment were achieved. The experiment was run three times in a teaching laboratory with consistent results, twice in an upper-level biochemistry laboratory with 19 students and once in an instrumental analysis laboratory with 13 students. Variations in results were more evident when studying the pH effect on GFP fluorescence using normalized spectra (Figure 1), probably due to student sample aliquoting variations when acquiring the 2 μL GFP stock solution that was used to prepare the sample. However, using the fluorescence intensity 475/395 ratio to study the pH effect on GFP fluorescence produced student results that agreed better, presumably because the effect of sample aliquoting variations was minimized. Proper water bath temperature control (80 °C) and incubation time control (Table A of Supporting Information) when performing GFP denaturation was important to ensure success in the laboratory experiment. Finally, the possible effect on GFP denaturation or renaturation, of the bacterial protein impurities present in the GFP stock solution, was assumed to be minimal because student results shown in Table 3 were consistent with those reported by Alkaabi et al.[10] using a more pure GFP sample. A survey of student perception regarding attainment of key laboratory objectives with this experiment indicated a 3.8 ± 0.1 average response, where 4.0 referred to "strongly agree" (see the Supporting Information).

■ ASSOCIATED CONTENT

Supporting Information

Student manual, instructor notes, student survey, spreadsheet file for analyzing spectral data. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

R.V.F. gratefully acknowledges support from U.S. Department of Education Minority Science and Engineering Improvement

Program, Pre-Minority Access for Research Careers Program, and participating laboratory students.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published on the Web on December 11, 2012, showing a slightly outdated protein structure in the Supporting Information. The corrected version was reposted on February 4, 2013.