From cold cataracts to an undergraduate laboratory

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Introduction

I was an undergraduate student at the School of Sciences of the University of Buenos Aires in 1978 when I took a biochemistry class as part of my degree requirements. I had the fortune of taking the laboratory portion of that class with one assistant professor who was a member of the Campomar Institute, a research institute headed by Prof. Leloir who was a Nobel price in Medicine. I did not know exactly how our laboratory experiences (mostly isolation of DNA from rats whose reproductive organs were surgically removed) fitted into the large scheme of Prof. Leloir's research. However, I did had the feeling that I was doing something important --a "real life experience" in my undergraduate career that I will never forget.

Most of us try to incorporate in our classes pieces of our research projects but our success, sometimes, is limited. The engineering sciences have advanced so much that it is increasingly difficult to incorporate even small elements of our research projects into sophomore, junior or even senior classes. To make matters worse, we are often involved in research projects barely connected to what we like to call Engineering Fundamentals. The fact that the Chemical Engineering Curriculum of Missouri University of Science and Technology (formerly the University of Missouri-Rolla) includes a Biochemical Engineering Emphasis Program has facilitated the incorporation of research projects into my classes. Although this incorporation would have been quite more difficult in a traditional Chemical Engineering program, it is apparent to me that some (if not all) of the projects described in this paper can be incorporated into traditional unit operations laboratories.

Sometimes it is only possible to include small pieces of large research projects because of their complexity. I found that it is important to be somehow aggressive on this approach and above all, do not underestimate the students. The students' reactions may be mixed. Some of my students have used the expression "research laboratory" in a positive way while some others use it as a critique, mostly referring to the amount of hours that they are expected to spend in the lab.

Most of the experiments incorporated into our Bioseparation Laboratory were once either a main research project in our group or a source of raw material for more fundamental research projects. The two types of experiments have been received differently by different types of students. Laboratory experiences based on research projects aimed at the purification of a particular protein have been well-received by those students more inclined to work in the processing sector. Research-rooted projects have been, in general, better received by students inclined to pursue further studies (either graduate school or medical school). Still, it has been an excellent approach to attract students into our graduate program as many realize that to do research can be fun.

From the Research Group to the Trenches: A brief story.

Some of the projects that we have introduced into our undergraduate laboratory are: the isolation of transgenic antibodies from corn meal, the precipitation of antibodies by non-ionic polymers, the production and purification of alcohol dehydrogenase from yeast, the synthesis, purification and characterization of diblock peptides, and the fractionation of a family of proteins present in mammalian lenses. A few of these projects are described below.

Many years ago, our research group started investigating cold cataracts. In cold cataracts, mammalian lenses undergo a liquid-liquid phase transition as their temperature is lower below a critical temperature, which depends on the species and on the age of the specimen. As a result of that phase transition, a protein rich and a protein lean phases coexist. One of the proteins present in the lens (γ-crystallins) undergoes a phase transition similar to that of the whole lens. There is an interest in the study of solutions of crystallins because research in that direction is expected to suggest mechanisms for pathogenic or traumatic cataracts.

The α, β, and γ-crystallin subfamilies comprise most of the lens proteins in mammals. The α-crystallin subfamily consists of highly polydispersed proteins that constitute -40% of the protein content of the lens. The molecular weight of the α-subclass is approximately one million Da. β-crystallins are also polydispersed proteins and have a molecular weight ranging from 50,000 to 300,000 Da. γ-crystallins are a heterogeneous group of low molecular weight proteins, 30,000 Da, with subtle differences in their amino acid compositions. γ-crystallins can be divided into five subfamilies (γ-IV , γ-III, γ-II, γ-I, and γ-S). The fractionation of this subfamily into its components and their characterization by neutron and light scattering has been the focused of our work for quite some time.

The purification of crystallins was incorporated into a Bioseparations Laboratory, which is a mandatory class for those students in a Biochemical Engineering Emphasis Program that we started nearly 20 years ago. A number of different separation techniques are used to isolate γ-crystallins from whole calf lenses. A sequence of steps to purify the γ-fraction that is used routinely in our laboratory is the following (Petitt et al., 1997): 1. Cell Disruption; 2. Centrifugation; 3. Size exclusion chromatography ; 4. Dialysis; 5. Ion exchange chromatography; 6. Dialysis; and 7. Freeze Drying. The students characterize the preparation, explore new separation conditions (for example, new salt gradients in the ion-exchange step or different lengths of size exclusion columns) and study the proteins using static and dynamic light scattering. Good samples that are not used by the students are recycled into our research group. This laboratory requires some specialized equipment. A bench top or a floor centrifuge able to accommodate 50 mL tubes, a low pressure chromatography system, and a spectrophotometer are necessary. The freeze drier is, somehow, optional. The low pressure chromatography system can be replaced by some of its individual components like a flow cell spectrophotometer and a fraction collector. The pump of the chromatography system can be replaced by flow under gravity using a two-compartment gradient former. The most expensive materials are the chromatography gels (Sephadex G-75 and Sephadex C-50). We usually recycle gels from one year to the next without major problems. The low pressure columns are quite cheap in the sizes that we utilize (either 1.4 or 2.5 cm in diameter columns). Expendables are cheap. The lenses may be bought from some specialized vendor from whom

it is possible to buy lenses of various ages for the students to explore variations in the composition of the lenses as the animal ages or they can be gotten from the local slaughter house by free.

Figure 1. Solid state synthesizer

About ten years ago we began studying the behavior of plasma proteins at solid/liquid interfaces. Some time after, our interest switched to the study of short peptides at solid/liquid interfaces. These studies slowly evolved into an interest on the behavior of amyloid-forming peptides at solid/liquid interfaces. A few years ago, we acquired a solid-state synthesizer and immediately after we incorporated the synthesis of short peptides and their purification into the Bioseparations Laboratory. Every time that the synthesis is successful, the students are producing a tailored peptide that we can use to study, for example, its behavior at solid surfaces having diverse chemistries.

Solid state peptide synthesis is a powerful technique to prepare tailored peptides and even entire proteins (Merrifield, 1963). The fact that Prof. Merrifield won a Nobel Prize because of this work seems to have a positive effect on the students involved in this project.

The students learn how to synthesize a peptide using a solid state synthesizer (Figure 1), how to isolate the peptide by precipitation and how to identify (and purify further) the product by reverse phase chromatography. The basic pieces of equipment are a solid state synthesizer, a high speed centrifuge, a HPLC instrument and a freeze drier. The students are asked to explore different separation protocols in the reverse phase chromatography isolation of the target peptide. In addition, the students use a dynamic light scattering instrument to determine the thickness of the peptide adsorbed layer on poly(styrene) latex with different surface chemistries. This particular laboratory is relatively expensive because high pressure chromatography systems able to process hundreds of milligrams of peptides and the solid state peptide synthesizer are normally not found in a regular unit operations laboratory. It is possible to synthesize some simple peptides by wet chemistry with the consequent savings in equipment. However, the nature of the chemicals needed and the number of steps needed would make such a laboratory very difficult to pursue. The study of the behavior of these

peptides at solid/liquid interfaces requires a particle sizing instrument. We use in our laboratory a backscattering dynamic light scattering instrument from Brookhaven that has proven to be quite rugged, it is very easy to use and it is reasonable cheap.

A few years back we began exploring the isolation of human antibodies expressed in corn. The main objective of our work was to find suitable alternatives to the use of a protein A column, by far the most expensive element in the purification of antibodies. The transgenic production of antibodies in plants needs to be followed by a robust separation process. Otherwise, the cost advantages associated with the production of the antibody in plants are lost.

We have developed a new process by which human antibodies expressed in corn are isolated to high purity and yield using aqueous two-phase extraction (Lee and Forciniti, 2005). We have found that one or two extraction steps, where the target antibody concentrates in the bottom phase or top phase, followed by a second extraction step, where the target antibody precipitates at the interface, yields the best results. The optimum purification protocol consists of the following steps: 1) Extraction of the antibody (and contaminating proteins) from the corn meal using a NaCl solution. 2) Addition of PEG and a salt to a concentration high enough to induce the formation of two liquid phases at equilibrium. 3) Removal and disposal of the upper phase (PEG-rich). 4) Addition of PEG to the bottom phase to create a second ATPS. 5) Recovering of the antibody from the new liquid/liquid interface. 6) Removal of the excess salt by diafiltration 7) polishing of the product by protein A chromatography.

The students in this laboratory extract the antibody from corn meal and then study its partitioning behavior in a variety of aqueous two-phase systems. This particular project is well-suited for the statistical design of experiments. The equipment needed is quite standard. In addition to basic laboratory equipment, the students will need a rotary mixer, a spectrophotometer, a HPLC system and a centrifuge. The raw material in our case is corn meal containing a particular human antibody. Of course, most programs will not have access to that raw material. One option is to use commercial corn meal spiked with a commercial IgG. Another option is to spike the corn meal with hemoglobin rather than an antibody. Hemoglobin from pig is quite cheap and besides its presence may be detected by its absorbance at 450 nm. Thus, it is possible to measure total protein by a colorimetric assay and hemoglobin concentrations by UV spectroscopy. In addition, the students need PEG of various molecular weights and various salts. The cost of this laboratory varies depending on the approach. If the experiments are done with corn meal spiked with an antibody, the main cost is a protein A column and the corresponding chromatography hardware. If the experiments are done with corn meal spiked with hemoglobin the cost is very low. The aqueous two-phase systems by themselves are very cheap, particularly if dextran is replaced with a salt like phosphate or citrate. Most labs will have a centrifuge and a spectrophotometer. Even in the absence of a centrifuge, the systems can be allowed to separate under gravity. Details of this project are described in the following section as an example.

Example: Isolation of Antibodies from Corn Meal

When macromolecules, such as proteins, are dissolved in a two-phase system, they selectively distribute between the phases. A partition or distribution coefficient is defined by,

$$K = \frac{C_t}{C_b} \qquad (1)$$

where C_t and C_b are the concentrations of the protein in the top and bottom phase, respectively. The partition coefficient depends on the pH of the phases, temperature, type and concentration of salt added, and type and concentration of the polymers used as phase forming species. The fact that the partition coefficient is a function of so many variables makes this project particularly suited for optimization studies. For example, the students may choose three molecular weights of PEG, three different salts (varying in their chaotropic properties) and three pHs (near, above and below the isoelectric point of the protein) A three levels full factorial experimental design will consist then, of 27 experiments. Because duplication is a necessity in this type of experiments students need to prepare, sample and analyze 57 extraction experiments.

Materials

Polyethylene glycol of various molecular weights (for example, 3,500, 8,000 and 20,000), dextran 70,000, acetate and phosphate buffers spanning three pHs, sodium chloride, lithium chloride, potassium chloride, pig hemoglobin, and Bradford reagent.

Equipment

The following equipment is needed: A spectrophotometer or a plate reader, a centrifuge (optional), magnetic stirrers, hot plates, balances, a pH-meter, micropipettes, a rotary shaker (optional), and an ultrafiltration cell (optional).

Methods

"Extraction" of antibody from corn meal

1. Extract 1 g of corn meal with 10 ml of 150 mM NaCl for 8 to 12 hrs at 4 ^{0}C with stirring.
2. Remove the solid particles by centrifugation at 11,000 rpm for 1 hr at 4 ^{0}C.
3. Filter the supernatant through a filter paper
4. Filter again through a 0.45 μm membrane.
5. Add 1 mg/ml pig hemoglobin to this extract.
6. Determine total protein using the Bradford test.

Preparation of Stock Solutions

A) Dextran (30% *w/w)*

1. Weigh 30 g of dextran into a bottle
2. Dissolve the dextran in 30 g of deionized water
3. Mix the above, it will make a paste
4. Add the remaining 40 g of deionized water
5. Heat the solution up to 95° C to facilitate dissolution of the polymer

B) PEG (50% *w/w)*

1. Weigh 25 g of PEG into a bottle

2. Dissolve the PEG in 25 g of deionized water

Preparing the Phase Systems

1. Shake the stock solutions
2. Place a 15 ml centrifuge tube on a balance
3. Weigh out the stock solutions into the tube in order of their increasing densities
4. Add enough buffer (blanks) or buffer plus 1 g of corn meal extract to complete 10 g.
5. Mix the contents of the test tube thoroughly, first by hand, and then in a rotary shaker (20 minutes)
6. Centrifuge the tubes for 15 minutes at 1500 x g to allow the phases to separate
7. Sample the phases as described below
8. The pH in each phase is measured with a microelectrode directly on the undiluted phases. Because of the high viscosity of the phases, the pH measurements must be done over a relatively long period of time.

Sampling and analyzing the phases

1. Carefully pipet out 2 g of the top phase
2. Carefully pipet 2 g of the bottom phase
3. Leave the separated phases resting and stir again. Inspect the solution to detect density differences along the axial direction of the test tube.
4. Read absorbance of each phase sampled at 450 nm.
5. Perform the Bradford assay for each phase.
6. If readings are out of range then dilute the samples with buffer (can be done by volume)
7. Calculate the partition coefficient of PIG hemoglobin and the overall partition coefficient for each sample. Partition coefficient is reported as K = (Absorbance sample -Absorbance blank)$_{top}$ / (Absorbance sample - Absorbance blank)$_{bottom}$

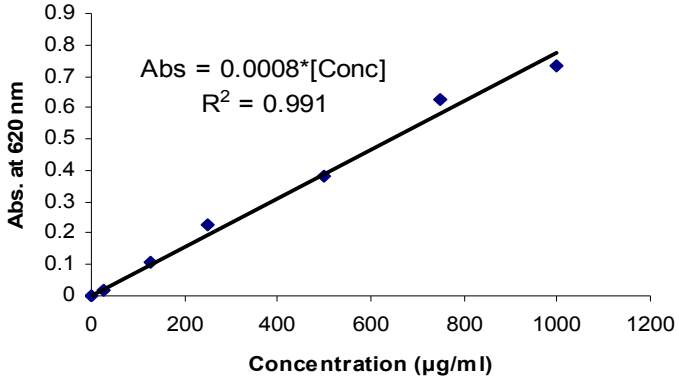

Figure 2. Bradford Standard Curve with BSA.

Bradford Assay Standard. The students create a standard curve for the Bradford Assay using standard solutions of BSA. A microplate procedure is ideal but, of course, the assay may be performed in a regular spectrophotometer. Figure 2 shows a typical standard curve. The assay is simple but careful (and polished) techniques are needed to obtain this type of results.

Results

The students process the raw adsorption data from the Bradford test and from absorbance at 450 nm. Proper blanks need to be discounted and dilution factors must be accounted for. The absorbance values are converted into concentrations using the appropriate calibration curves and those values plus the values of the volumes of each phase (using well-established correlations for densities of PEG and Dx solutions) are used to calculate recovery. Concentration values from Bradford (total protein) and absorbance at 450 nm (hemoglobin) are used to calculate purity and purification fold. Finally, the partition coefficients (total protein and target protein) are calculated and reported.

Figure 3 is a contour plot of the partition coefficient versus pH and PEG molecular weight. We found this presentation of the data is very illustrative.

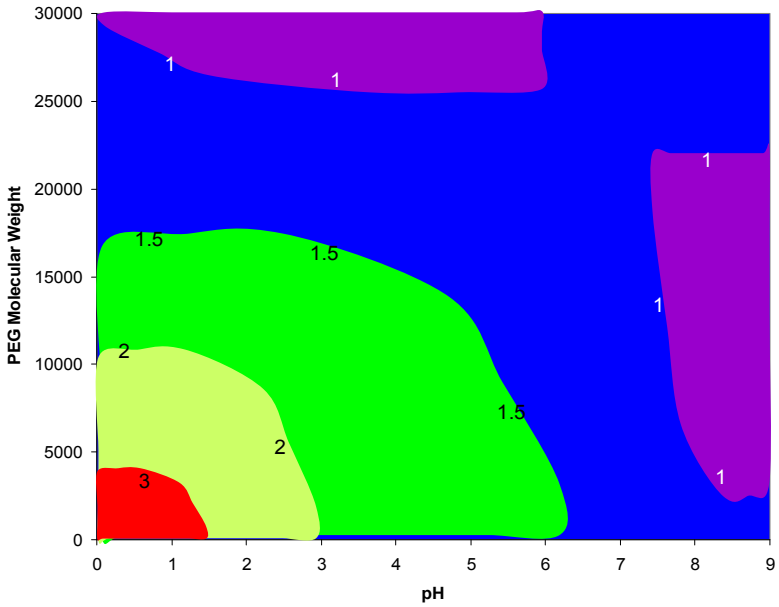

Figure 3. Contour plot of the partition coefficient (total protein) vs. PEG molecular

The partition coefficient data may be regressed to find a correlation between the partitioning coefficient with pH and PEG molecular weight for all salt types. One possible correlation is,

$$K = a + b[pH] + c[pH]^{-2} + d[MW] + e[MW]^{-1} + f\frac{[pH]}{[MW]} + g\frac{[pH]^2}{[MW]} + h\frac{[MW]}{[pH]} + i\frac{[MW]^2}{[pH]} + j\ln[pH] + k\ln[MW] \quad (2)$$

where MW is the molecular weight of PEG, pH is the pH, and “a” through “k” are adjustable parameters. Calculated and experimental partition coefficients are plotted in Figure 4.

Conclusions

Pieces of research projects have been successfully incorporated into the undergraduate curriculum. The students benefit because they are exposed to state of the art techniques, using equipment bought with research funds. In exchange, they contribute to a project by, for example, optimizing a particular separation or by producing materials that are fed into our research group.

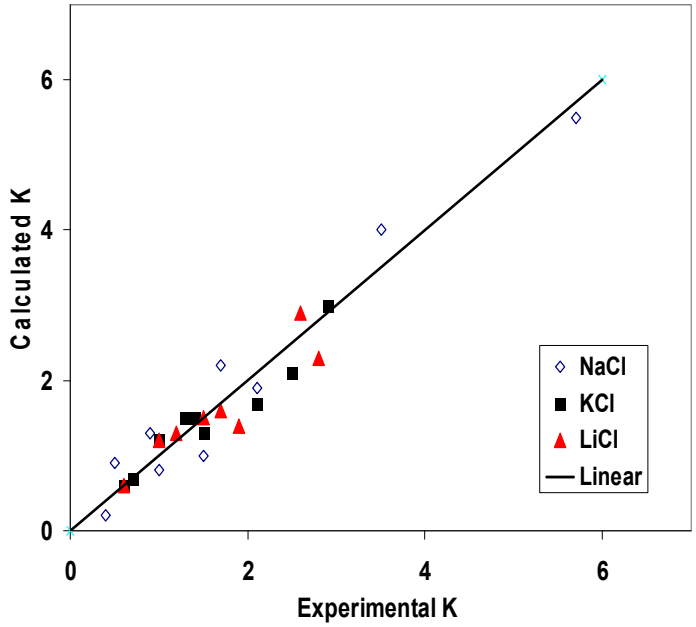

Figure 4. Calculated Partition Coefficient Versus Experimentally

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