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Incorporating Polyacrylamide Electrophoresis of Model Enzymes into Scenario Based Labs Investigating Protein Structure-function Relationships

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Scenario based exercises provide a relevant context for labs and demonstrate the application of methods for testing authentic hypotheses. Investigative labs also offer opportunities to familiarize students with a variety of technologies that researchers use in practice. In our introductory molecular and cell biology labs we have developed a three-week lab unit that engages students in hypothesis testing, protocol optimization, and testing functional consequences of changes in enzyme structure using model enzymes. We have adapted isozyme staining protocols to modified SDS-PAGE analysis to allow students to visualize differences in tertiary structure of active enzymes in tissue extracts simulating different genotypes for a hypothetical mutation in alkaline phosphatase. This workshop will demonstrate these techniques.

Keywords: enzyme, PAGE, protein

Introduction

The use of realistic scenarios to frame authentic experiments increases student understanding of the "why" behind lab exercises that otherwise seem mysterious or esoteric. This helps to promote student engagement and interest in doing experiments correctly. In molecular biology labs, scenarios frequently lead students to develop hypotheses about the differences between two or more molecules of interest. In authentic molecular biology research, separating molecules based size is often a first step in being able to analyze differences.

Electrophoresis is a key technology for separating biological macromolecules based on charge, and by far the dominant method for separation of proteins is SDS-PAGE (polyacrylamide gel electrophoresis). The addition SDS to PAGE buffer allows proteins to effectively be separated by size because the anionic detergent binds to peptides and "coats" them with uniform negative charge proportional to their mass. The interaction with SDS promotes denaturation of proteins and, in combination with other denaturating agents, allows the separation of individual peptides. Peptides can then be visualized through general staining, using reagents such as Coomassie Brilliant Blue or silver, or more specifically using antibodies (Western blots).

There are a number of drawbacks to standard SDS-PAGE that limit its utility in certain situations, such as arise in the teaching lab. The first is that standard staining techniques tend to take far more time than available in a single lab period, and they cannot differentiate between specific proteins. This becomes especially problematic in complex samples like tissue homogenates that may have hundreds of bands. Western blots have excellent specificity but are expensive, time consuming, and only work with proteins for which a good antibody is readily available. Visualization of enzymes as model proteins using colorimetric reactions, on the other hand, is fast, cheap, and simple, but the denaturing conditions of SDS-PAGE interfere with protein function. Denaturation can be avoided using non-denaturing or "native" PAGE methods, which do not use SDS, however without the anionic coating provided by SDS, proteins will migrate according to their net charge and may run in different directions or not at all. Also, the resolution of protein bands is far inferior to SDS-PAGE, and there is an increased likelihood of smearing at high protein concentrations.

In our introductory labs we have taken the approach of using in-gel enzyme reactions to visualize proteins, following "renaturation" of enzymes run under mild denaturation conditions with minimal SDS. Under these conditions, partially denatured proteins will refold within the polyacrylamide matrix if the SDS is removed following electrophoresis, resulting in "renaturation" of active enzymes (Lacks and Springhorn, 1980). Although SDS can be removed through simple diffusion by repeated washing in buffer this process takes a long time. The removal of SDS from proteins can by accelerated by the addition of a competing detergent such

as Triton X-100, which is non-ionic and generally does not interfere with enzyme function (Clarke, 1981).

Implementation

We use enzymes as model proteins in our introductory molecular and cell biology labs, including demonstrating the use of SDS-PAGE to separate proteins by size and charge, or what we define as "apparent molecular weight." This is part of a multi-week enzyme lab, where students test hypotheses about the function of normal and "mutant" alkaline phosphatases. We use wheat germ acid phosphatase as our hypothetical "mutant" enzyme, so there is a difference in activity as a function of pH between the two theoretical isoforms. As a part of this scenario based exercise students must determine the suitability of electrophoresis for differentiating between the normal and "mutant" enzymes in tissue extracts form homozygous and heterozygous individuals.

Scenario

Week 1

Metabolic acidosis is a physiological condition that arises from a failure to regulate the acid-base balance in the blood. There are a variety of conditions that can cause metabolic acidosis, including dietary acid loading, diabetes, kidney failure, and respiratory distress, among others. Long-term metabolic acidosis can cause an imbalance in mineral homeostasis leading to depletion of hypoxyapatite and loss of bone density.

As a student laboratory assistant working under Dr. Imma Phicsion, you have been measuring serum alkaline phosphatase levels in diabetic teenagers with moderate kidney dysfunction leading to metabolic acidosis. Dr. Phicsion believes that inhibition of the **alkaline phosphatase enzyme in osteoblasts by high acidity may be a primary caus**e of reduced bone density in this population.

While analyzing the measurements from 1500 samples you notice that 17 of the patients don't exhibit any change in alkaline phosphatase despite having significantly increased blood acidity. You bring these cases to the attention of Dr. Phicsion. After much head scratching and inspection of the data, Dr. Phicsion decides to examine these unusual cases more closely. She quickly discovers that those individuals are heterozygous for a mutation in the alkaline phosphatase protein that might cause the enzyme to misfold.

Week 2

After reporting the progress of your team in establishing a spectrophotometric assay for the AP enzyme you are given the green light to proceed with your experiment using valuable patient protein samples. However, before she will give you the samples, Dr. Phicsion insists that you state the hypothesis to be tested clearly, incorporating the information gained from your previous tests.

Dr. Phicsion credits your keen observational and analytical skills for the discovery this mutant enzyme and decides to give you the opportunity to investigate its function under acidic conditions. You are assigned the task of putting together a team to develop a standardized assay for alkaline phosphatase function and to determine if the misfolded enzyme behaves differently from the normal enzyme.

Week 3

Dr. Phicsion is thrilled by the success of your assay in demonstrating that there is a key functional difference between the normal and mutant variants of alkaline phosphatase from heterozygous individuals. Now she wants to see if you can develop an assay to detect these variants in the field using inexpensive and portable equipment. You suggest using polyacrylamide gel electrophoresis (PAGE) in combination with a test for catalytic activity similar to the *p*-nitrophenol assay.

Notes to the Instructor

Phosphatases as model enzymes

Alkaline phosphatase (AP) is an excellent model enzyme for studying protein structure and function in the teaching lab. Standard protocols are well established for measuring AP activity in solution using spectrophotometry, as well as colorimetric and fluorescent visualization on blots and in gels. AP enzymes are readily available in economical tissue lysates with reliable activity levels, and AP enzymes are very stable even under less than ideal conditions and in the hands of novice biology students. Another advantage is that other phosphatases with different biochemical and structural properties can used to simulate "mutant" isoforms of the model AP enzyme. In our labs we employ wheat germ acid phosphatase as a "mutant" isoform that functions at a different pH from normal AP enzyme.

Alkaline and acid phosphatase are also good model enzymes for electrophoresis because they differ in activity depending on tertiary structures. Alkaline phosphatase has almost no activity except as a tetramer, while the acid phosphatase has highest activity as a dimer, although tetrameric forms will also appear on gels.

Electrophoresis protocols

There were two factors that needed to be addressed to make SDS-PAGE suitable for in-gel enzyme staining protocols designed to work in a single lab period. The first obstacle was minimizing protein denaturation - this was accomplished by using non-denaturing loading buffer in combination with minimization of SDS concentrations in the running buffer. An SDS concentration of 0.05% in the running buffer proved sufficient to "coat" the proteins enough that they would have a net negative charge. Another factor we encountered was that standard tris-glycine running buffer made gels resistant to changes in pH post-electrophoresis. We used a sodium borate running buffer instead.

Staining protocols

The major hurdle to in-gel staining was getting rapid renaturation of enzymes, even using minimal SDS. A key step is the use of a gel wash buffer containing an excess of Triton X-100, a non-ionic detergent that displaces SDS from the proteins in the gels. Subsequent washes with AP buffer were used to remove the excess detergents from solution and to shift the pH of the gel.

Most enzyme staining methods are indirect, using separate substrate and dye molecules. For phosphatases we use 1(alpha)-naphthyl phosphate as a substrate in a coupled reaction with fast blue RR salt to give a brown-to-black precipitate. We use fast blue RR salt because it is relatively inexpensive compared to the more typical fast blue BB or fast red salts. Similarly, 1-naphthyl phosphate is a lot less expensive than other more phosphatase substrates more commonly encountered in molecular biology applications.

Coomassie G-250 staining protocol

For demonstration of general protein staining as a way of showing students why enzyme staining is advantageous, we have adopted a rapid Coomassie staining protocol that avoids exposure of students to hazardous materials. Standard Coomassie Brilliant Blue R-250 takes hours of staining and destaining to complete and involves solutions in methanol and acetic acid. "Fast" versions of R-250 staining involve microwaving methanol/acetic acid solutions and are not suitable for student use. This Coomassie Brilliant Blue G-250 protocol is quick, easy, and only requires the addition of HCl to the staining solution. Following electrophoresis, gels are washed with distilled water and then covered with staining solution. The gel and staining solution are put in the microwave for 10 seconds and them allowed to develop at room temperature. Bands become visible with 10 minutes and there is very little background so no destaining is required.

The alkaline phosphatase and acid phosphatase enzymes are active components of tissue lyophylates that are complex mixtures of many other molecules. When an SDS-PAGE gel is examined using Coomassie staining, students see the lanes are stained blue throughout, with a number of darker bands visible. If is difficult to visualize individual proteins, much less identify the one they are interested in.

Recipes

2X Sample (Loading) Buffer, Non-denaturing

Mix: 10 ml 1 M Tris, pH 8.0
30 ml glycerol
0.03 g Bromophenol blue (a pinch)
60 ml diH_2O
Store at 4°C, indefinitely

10X Tris/Glycine/SDS Running Buffer

Mix: 30.0 g Tris base
144 g glycine
430 ml diH_2O
10 ml 10% SDS (add after Tris/glycine are dissolved)

- Store at room temperature
- If SDS precipitates, warm to 37°C and stir to dissolve

10X Sodium Borate/SDS Running Buffer

1. Dissolve 24.7 g boric acid in 800 ml diH_2O.
2. Add 4.0 g NaOH and dissolve.
3. Add 10 ml 10% SDS.
4. Bring final volume to 1000 ml with diH_2O.

Store at room temperature. If SDS precipitates, warm to 37°C and stir to dissolve

Gel Wash Buffer, pH 6.0

Mix: 15.8 g Tris-HCl
2.9 g NaCl
990 ml diH_2O
10 ml Triton X-100 (add after Tris/NaCl are dissolved and pH adjusted)
Adjust to pH 6.0 with 10 N NaOH or conc. HCl

10X AP Buffer, pH 6.0

Mix: 158 g Tris-HCl
29 g NaCl
1000 ml diH_2O
Adjust to pH 6.0 with 10 N NaOH or conc. HCl

2X Citrate Buffer, pH 6 (Gomori, 1955)

Mix: 2.21 g citric acid
26.0 g trisodium citrate, dehydrate
1000 ml diH_2O
Mix fresh; use with 2 weeks

2X Carbonate Buffer, pH 10 (Delory and King, 1945)

Mix: 6.35 g sodium carbonate
3.36 g sodium bicarbonate
1000 ml diH_2O
Mix fresh; use within 1 week.

10X PBS (Phosphate Buffered Saline)

Mix: 10.9 g Na_2HPO_4, anhydrous
3.2 g NaH_2PO_4, anhydrous
90 g NaCl
1000 ml diH_2O
Store at room temperature. Discard if phosphates begin to precipitate

50X AP Dye Solution

1. Dissolve 2.5 g Fast Blue RR salt in 100 ml diH_2O.
2. Place at 4°C overnight; protect from light.
3. Vacuum filter (this should give a clear, deep yellow solution).

Store at 4°C, protected from light
Make fresh; use within 2 weeks (re-filter if brown precipitate forms)

50X AP Substrate Solution

Mix: 2.5 g 1-Naphthal phosphate
100 ml diH_2O
Store at 4°C, protected from light
Make fresh; use within 4 weeks

Coomassie G-250 Protein Stain (Lawrence and Besir, 2009)

1. Disolve 60-80 mg Coomassie Brilliant Blue G-250 in 1000 ml diH_2O.
2. Add 3 ml HCl (conc.)

Store in dark at room temperature

Use:

1. Wash gel with diH_2O.
2. Cover gel with stain solution
3. Shake gently until color develops.
4. Wash with diH_2O.

May be reused

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Literature Cited

Clarke, S. 1981. Direct renaturation of the dodecyl sulfate complexes of proteins with Triton X-100. *Biochimica et Biophysica Acta*, 670: 195-202.

Delory, G. E., and E. J. King. 1945. A sodium carbonate-bicarbonate buffer for alkaline phosphatases. *Biochemical Journal*, 39: 245.

Gomori, G. 1955. Preparation of Buffers for Use in Enzyme Studies. *Methods in Enzymology*, 1: 138-146.

Lacks, S.A., and S. S. Springhorn. 1980 Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. *The Journal of Biological Chemistry*, 255: 7467-7473.

Lawrence, A., and H. Besir. 2009. Staining of proteins in gels with Coomassie G-250 without organic solvent and acetic acid. *Journal of Visualized Experiments (JoVE)*, 30: e1350.

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Michael Keller earned a B.S. from the State University of New York at New Paltz, an M.S. from Villanova University, and a Ph.D. from the University of Missouri, Columbia. He was a post-doctoral fellow at the National Institute of Child Health and Human Development before accepting his current position as Lecturer and Lab Coordinator at the University of Maryland.

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