Tested Studies for Laboratory Teaching
Proceedings of the Association for Biology Laboratory Education
Vol. 33, 174–186, 2012

Enzyme Explorations through Cheesemaking: A Qualitative Approach for Learning about Enzyme Function

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Typical general biology enzyme labs involve spectrophotometry, which, while quantitative, can be technologically challenging. Students can miss the essentials about the roles of temperature, pH, concentration, and substrate type in enzymology if they are overwhelmed by the equipment and analysis. Cheesemaking offers a practical and fun approach where students experiment with temperature, pH, enzyme concentration, and substrate type. While the experiment provides primarily qualitative data, the ability to see, feel, and taste the results aids their conceptual understanding and interest. the chymosin reaction in curd formation from milk.

Keywords: enzyme, chymosin, pH, temperature, concentration, substrate

Introduction

Enzyme properties are a commonly taught subject in college introductory biology courses. Typically we, as instructors, want students to gain a solid conceptual understanding in our introductory courses, knowing that if they advance in the discipline, this foundational knowledge will be supplemented with greater detail and depth in future coursework. Using spectrophotometry to teach about enzyme function can sometimes cloud a student's understanding because they have to learn the technology of spectrophotometry, the analytical skills of using a standard curve, as well as the concepts of enzymology.

This lab is designed for introductory biology students and focuses on the understanding of enzyme function concepts using familiar materials and procedures. Through guided inquiry, students learn the foundations of enzymology using milk and the enzyme chymosin to separate milk proteins into water soluble (whey) and water insoluble (curds) and then make mozzarella cheese with the curds. The specific objectives of the lab are for students to:

1. Understand that enzymes have optimum pH ranges and how pH affects enzymes;
2. Understand that enzyme reactions have optimum temperature ranges and how temperature affects enzymes;
3. Understand that reaction rates do not continue to increase with increasing enzyme concentration;
4. Understand that enzymes function only with specific substrates; and
5. Apply their knowledge from one specific enzyme reaction to predict the effects of temperature, pH, and substrate change in other enzyme reactions.

Although as written, this lab works best for advanced high school students or first year college students, with the addition of more detailed information about specific protein structure, micelle properties, the role of calcium in the enzyme reaction, and the optimization of pH, it could be adapted for a biochemistry course.

Preparation for this lab is modest, requiring approximately 0.5-hours to set up. The lab can be delivered in one 2-hour lab session, although 2.5-hours is more ideal. Alternatively, the lab can be broken up into two 1-hour sessions easily. Information about the different types of milk proteins and micelle structure can be given ahead of time, making it much easier to complete the lab in one 2-hour period. I precede this lab with other labs involving milk proteins (i.e., Bradford assay on protein concentration and SDS-PAGE electrophoresis of milk proteins) to accomplish this.

Student Outline

Background

Cheese is nearly a universal food and ranges widely in flavor and texture. While a quick survey of cheese types on Wikipedia will produce a list of over 3,000, more precise classifications result in lists of 400 to 1,000 varieties (Fox, 1993). Estimates of current world cheese production are about 20 billion kg/year, or about 45 billion pounds with Europeans being the largest producers and consumers (FAO, 2010).

Most of us have heard the legend of the first cheese that was produced by accident. If you haven't heard the story, imagine a nomadic traveler about 6000 years ago setting out on a journey across the desert. In preparation for his journey, he puts some milk (sheep or camel) in a container for nourishment along the way. The container he chooses is the stomach of a sheep. (Using internal organs for holding liquids was common practice given their abundance and water-tight characteristics.) Our nomadic friend travels for a while and then stops for a rest. But, when he attempts to drink from his sheep-stomach flask he finds that his milk has separated into a sweet yellowish liquid (whey) and some white chunky bits (curds). The conditions of the high temperature from the desert sun, the acidic stomach container, and the presence of a milk coagulating enzyme (discussed below) in the stomach all came together fortuitously. Being thirsty and hungry he consumes these parts and finds they are quite tasty and upon his return, spreads the word of his discovery, leading to the beginning of cheese production.

Whether this is the real story or not, it is likely that the first cheese was discovered accidentally by milk being fermented by bacteria or being accidentally introduced to enzymes that cause coagulation. It is widely believed that cheese originated in the Fertile Crescent after the domestication of sheep (about 8,000 years ago). Once the first cheese was produced, its value as a food source was likely quickly noticed. Like milk, cheese has high nutritional value. It is high in protein and fats, providing a rich source of energy. However, unlike milk, cheese is much easier to store and transport and in many cases can be kept edible for much longer periods of time (reviewed by Fox, 1993).

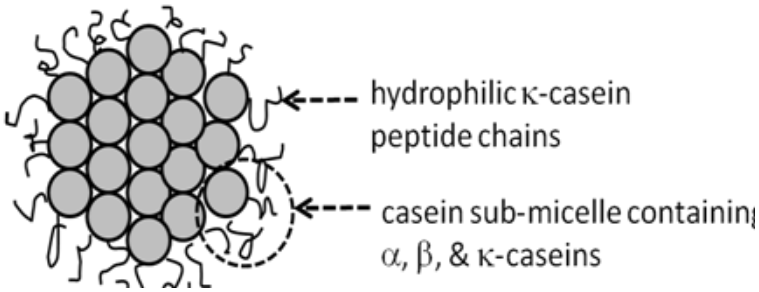

Figure 1. Casein micelle diagram.

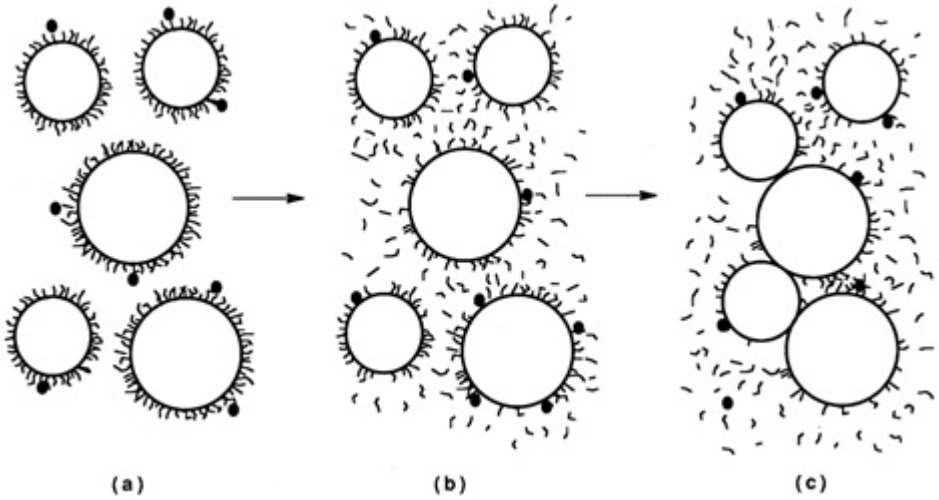

Figure 2. When κ-casein is intact, micelles remain whole and separate (a) but with the removal of the hydrophilic κ-casein chains (b) the now exposed hydrophobic casein micelles clump together (c) forming the coagulate that becomes the curd. (Figure from Dalgleish, 1993)

Milk is about 3-3.5% protein and the proteins in milk are classified into two broad categories: casein and serum (whey) proteins. Casein proteins, which make up about 82% of the protein in cow's milk, contain phosphorous and are the proteins that coagulate to form the curd. Because they contain phosphorous, casein proteins are able to bind calcium, in calcium phosphate salts, giving milk its high calcium content (Walstra, 1990). Serum proteins, the remaining 18%, do not contain phosphorous and remain in solution in milk, forming the whey.

There are a number of different types of casein proteins, including α-, β-, and κ-caseins. The α- and β-caseins are water insoluble but cluster together with κ-casein, which has a hydrophilic peptide chain. Think back to how phospholipids, which have a hydrophilic and hydrophobic component like κ-casein, arrange themselves in water. The caseins arrange themselves similarly, with the hydrophobic components being "hidden" from the water and the hydrophilic κ-casein chain on the outside (Holt and Horne, 1996). This arrangement causes the formation of casein micelles (Fig. 1) that are a couple hundred nanometers in diameter (McMahon and McManus, 1998), the size of some of the smallest bacterial cells. If the hydrophilic chains are disrupted or removed, the micelle has hydrophobic areas exposed, causing the micelle to clump together with adjacent micelles, forming casein coagulates, or curds (Fig. 2).

One way to disrupt the hydrophilic chain of κ-casein is through enzymatic means. Chymosin is a protease that cleaves the hydrophilic tail from κ-casein. Specifically, chymosin cleaves the peptide bond between the amino acids phenylalanine and methionine, in the 105 and 106 position (Fig. 3) leaving two units, para-κ-casein and a glycomacropeptide. Which do you think is the hydrophilic part? (Remember what "glyco" means.)

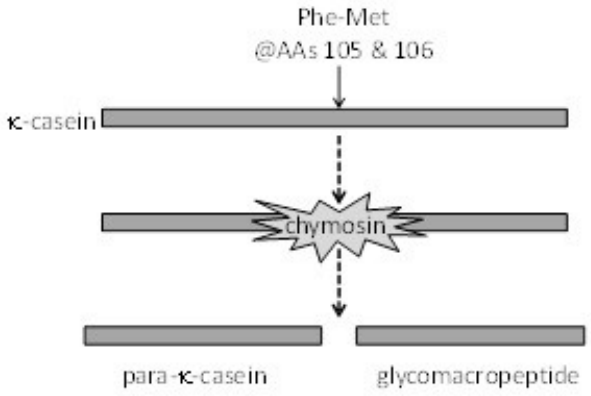

Figure 3. Chymosin separates the hydrophilic tail of κ-casein.

So where does chymosin come from? If you recall the legend of the first cheese, you should be able to guess. Chymosin is produced in the stomach of ruminants (actually, the abomasums, or the fourth chamber). It is also produced by gastric cells in infants. So why would an organism want to curdle milk in its stomach? Turning the milk into a coagulate means that it stays in the bowels longer, increasing the absorption time for nutrients.

Chymosin is also known as rennin. However, there are a number of enzymes that are collectively known as rennets and all have the ability to cause curd formation. Pepsin and gastricsin are two other mammalian enzymes that can be used as rennet. While chymosin is a neonatal enzyme, produced by those still feeding from mother's milk, and therefore harvested from calf stomachs for use, pepsin and gastricsin are produced by adult organisms as well, although at lower concentrations than chymosin in the unweaned. Because extracting proteases from animal stomachs is time consuming and relatively expensive, alternative rennets are also used. There are "vegetable" rennets which come from fungi such as *Mucor miehei*, *M. pusillus,* and *Endothia parasitica*. Additionally, recombinant DNA technology has been used to insert the calf chymosin gene into the bacterium *E. coli*, and fungi *Saccharomyces cerevisiae* (yeast), *Kluyveromyces lactis*, *Aspergillus nidulans*, *A. niger*, and *Tricoderma reesei* so that larger quantities can be produced without the reliance on using calf stomachs as a source (reviewed by Foltmann, 1993).

While coagulating the casein proteins (a.k.a., separating the curds from the whey) is only the first step in making cheese, it is an essential step in all cheesemaking. The variety of cheese come from what you do after this step. Additions of bacterial or fungal cultures, aging in different temperatures and humidity all create different flavors and consistencies. We will only be making a very simple cheese, mozzarella, that does not require aging or culture addition.

General Procedure

1. Produce the cheese curd.
    1) Heat milk to 13°C (~55°F) while stirring.
    2) Add citric acid. (1 teaspoon dissolved in ¼-cup water before adding)
    3) Heat to 31°C (~88°F) while stirring.
    4) Add enzyme. (1/8 teaspoon dissolved in ¼-cup water before adding)
    5) Heat to 40°C (~105°F) while stirring.
2. Separate the curd from the whey by straining. Squeeze as much of the whey from the curd as you can.
3. Put the curd into a glass bowl and heat in a microwave for about 20 seconds. Take the curds out of the microwave and knead, squeezing out more whey. Add about 3/4 teaspoon of salt (for flavor) and knead in.
4. Microwave again for about 20 seconds, remove curds and knead, draining whey. You may need to repeat this one or two more times. You are trying to get the temperature of the ball of cheese to about 54°C (~130°F). Once it starts feeling stretchy and smooth, you should be done.

Questions (post-demonstration but pre-experimentation)

Thinking about how enzymes work, answer the following:

1. What is the enzyme in this reaction and what is it doing?
2. Why do you think the temperatures are specified? What would happen if you tried to complete this reaction with lower temperatures? With higher?
3. Why do you think we added citric acid? What would happen if we didn't?
4. What do you think would happen if we added less rennet or more rennet? Why?

Experimentation

We are now going to try altering the recipe in some way that you think will affect the product. Will you change the temperatures? The amount of citric acid? The amount of rennet? The type of milk? The type of rennet? Each group will pick one variable to alter and repeat the cheese making process. You should make observations on how long it takes the curds to form and what the curds look like during the process. We will also weigh the cheese produced after each is finished and then compare them through taste and texture. You should record in your notebook the protocol you plan to follow and your prediction about the result. You should also construct a table to record observations on all the groups' products (Table 1).

Table 1. Example of a data table to reconstruct in your lab notebook for collection of qualitative observations on cheese.

Variable Changed	Observations on Curd Formation	Mass of Cheese	Observations on Taste & Texture
None (Control)			
(Group 1 change)			
(Group 2 change)			
(Group 3 change)			
(Group 4 change)			

Questions (post-experimentation)

1. With your knowledge of chymosin, draw the optimum pH and temperature ranges of this enzyme. Imagine instead we were working with an enzyme found in the blood of a deep sea fish. Draw the optimum pH and temperature ranges you would expect for this enzyme.
2. What happened, or what would you expect to happen, when chymosin is used with soy milk instead of cow milk? How do you think soy cheese is made?
3. What makes proteins different? How can different enzymes have different optimal temperatures and work with different substrates?
4. We considered pH and temperature independently in this lab. However, as is often the case with biology, interactions between two factors can have different effects. The graph below (Fig. 4) shows how pH and temperature can interact, in the absence of chymosin, to interrupt the casein micelles. (Remember: enzymes, like chymosin, don't make reactions happen that wouldn't typically happen; instead, they allow those reactions to proceed more quickly with lower energy input.) Based on the information in this graph, if you wanted to make cheese curd and did not have any chymosin, how would you alter the protocol we used?

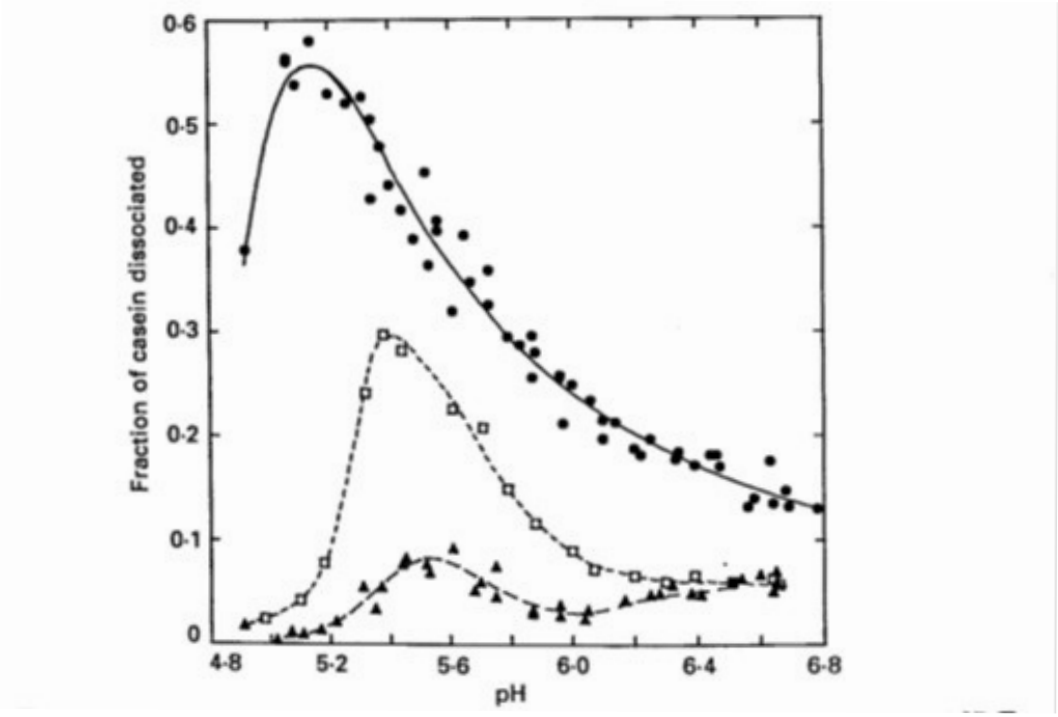

Figure 4. Total amounts of casein dissociated as functions of pH at temperatures of: 4°C □; 20°C ●; 30°C ▲. Points are expressed as fractions of the total casein (soluble and micellar). (Figure and caption from Dalgleish and Law, 1988.)

5. There is a cooking oil on the market called Enova™. The company claims that less of their oil is stored as fat when you eat it as compared to other oils. Most cooking oils are made primarily of triglycerides while EnovaTM is made primarily of 1,3-diglycerides. (Fig. 5; Remember, a triglyceride is a glycerol with three fatty acids.)

   When you consume fats, an enzyme breaks off the chains from the first and third carbons and all the components are transported across the cell membranes from inside your intestine into your body cells where enzymes reassemble the fat and then package it to be stored (Fig. 6).

   The company that makes Enova™ claims that the diglycerides in their oil are disassembled and transported in the same way as triglycerides, but cannot be reassembled. Therefore, instead of being packaged for fat storage, the unassembled pieces are sent to the liver to be used immediately for energy by breaking them down through cellular respiration.

   Based on what you've learned about how enzymes function, why do you think the disassembled diglycerides cannot be reassembled once they cross the membrane into the cell?

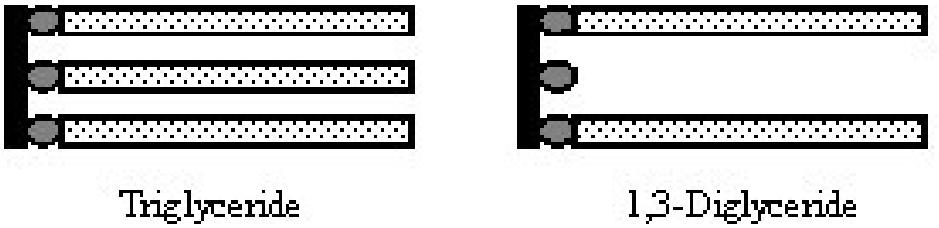

Figure 5. Schematic representation of the difference between triglycerides and diglycerides. Both contain a glycerol molecule but the diglyceride has only two fatty acid chains.

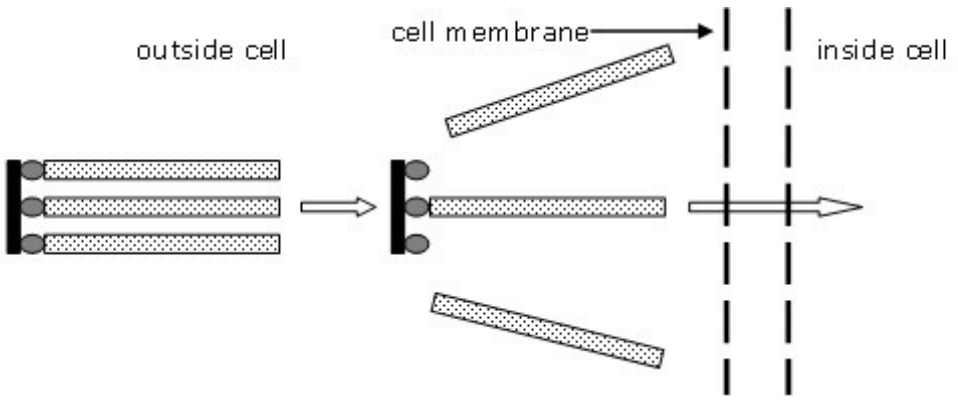

Figure 6. Schematic representation of how triglycerides are transported and reassembled across the cell membrane.

Materials

I generally do not put more than four to a group. Three students/group works quite well. So, if your lab has 25 students, I would suggest planning for 7-8 groups, plus supplies for a demonstration.

Supplies for each group of three students:

- Table cloth if using lab tables
- Hot plate
- ½ gallon milk (whole, store-bought milk works fine)
- Food-grade thermometer (I use the metal ones that clip on the side of the pan)
- 3-quart pan
- Wooden spoon
- Measuring cup
- Set of measuring spoons that includes a 1/8 teaspoon
- Colander
- Flour sack (or cheesecloth)
- Glass bowl
- Wax paper or freezer paper

Supplies for whole lab:

- Rennets (I use a variety, including an animal, fungal, and GMO rennet)
- Citric acid (enough for each group to have 1 teaspoon)
- Potable water (not chlorinated)
- Electronic balance
- Food-grade microwave (having at least two of these is helpful)
- Buckets for straining whey into
- Salt
- pH test strips (in the 8.0 to 4.0 range is best)
- Food-grade gloves
- Food-grade cleaning supplies for dishes

Notes for the Instructor

Safety Concerns

Obviously eating in lab is a reason for safety concerns. Ideally you would do this lab in a food prep area and if you have easy access to one, I would recommend using it. I have done this in the past, but found it to be too inconvenient given our facilities.

Currently I do this in a lab where labs with toxic chemicals are generally not taught. I clean all the tables beforehand and cover them completely with disposable tablecloths. All supplies (except hotplates) for this lab are stored separately from general lab supplies and kept for food-grade labs only. Additionally, I purchase separate supplies for cleaning all the dishware. I start the lab by explaining all this to students so they understand the precautions that were taken.

Expected Results

When students choose to use soy milk, they will get no curd formation. Generally they want to know why. You can use this opportunity to talk about enzyme-substrate specificity and how soy milk does not have caseins. At this point they will want to know how soy cheese is made. Tofu is really soy "cheese". Tofu is the curd from soy milk that is curdled using salts (calcium sulfate or magnesium chloride).

Generally anything that causes the curd formation to happen very quickly will result in bulkier curds that retain a lot more fluid and will ultimately result in a less pleasant cheese texture. Increasing the temperature drastically especially does this. As the micelles coagulate rapidly, they will trap more fluid.

If a group decides to not add citric acid, the enzyme will not function and there will be no curd formation. When a group decides to do this in my lab, we talk about it, understanding the importance of pH for enzyme function, and then I suggest they add the citric acid now (after the enzyme). When they do, curd formation will happen almost instantly. This is actually a vivid demonstration of the importance of pH.

Linking with Other Labs

While I don't do spectrophotometry with an enzyme lab, I do want students to learn the practice and value of it. I actually link the cheese making lab with two other milk protein labs. On the first week of the series we do the Bradford assay using spectrophotometry. This is an easy lab to use in learning spectrophotometry with as well as in practicing pipetting skills. (Bio-Rad actually makes an easy to use Bradford reagent kit.) Students do this assay with milk samples of a variety of types to determine protein concentration. The next week we do electrophoresis of the same milk samples (each group uses their own chosen samples) and they learn to identify different proteins. At this point I introduce them to caseins a bit and they see that caseins don't exist in vegetable-based milks.

One aspect of this lab that I like is the ability to refer back to it throughout the semester as we cover other topics in the course. There are links to DNA and protein synthesis (I use chymosin as an example and talk about what would happen if there was a mutation), to gene regulation (why and how chymosin production is stopped, why it is only produced in some cells), animal physiology (why only some animals produce it), evolution (similarities among chymosin and other proteases of different species), and genetically modified organisms (GMO chymosin, but see below).

Purchasing Supplies

Once the supplies and equipment are purchased initially, the cost of this lab is quite low. The only supply that needs to be purchased individually for each lab is milk. Eventually you will run out of citric acid and need to buy more and the rennet will expire (although I have used the same rennet for over two years just fine), but both of these are quite inexpensive for the volume you need.

The general kitchen items can be purchased easily at local stores. If you have a good natural food store locally, you can probably purchase everything you need without making an order. However, there are three items (citric acid, rennets, and thermometers) that I order specifically. The supplier I have used is New England Cheesemaking Supply Company (**http://www.cheesemaking.com**), however, there are other suppliers of cheesemaking ingredients and equipment, including The Cheesemaker (**http://www.thecheesemaker.com**) and Cheese Supply (**http://www.cheesesupply.com/**).

While most of the supplies for this lab are generic, there are a few items for which I recommend specifics:

1. Flour sacks – While cheesecloth is traditional, I much prefer using flour sacks. First, they are reusable (I wash them at home on the "sanitize" setting). Second, it is much easier to get the curd off of the cloth and without little bits of fabric stuck to it.
2. Thermometers – I really like the dial thermometers that clip on the side of the pan. New England Cheesemaking Supply has some that are shorter and fit perfectly in 3-quart pans.
3. Rennets – I prefer the liquid rennet. I also keep a variety on hand, including animal (veal), "vegetable" (usually fungal), and GMO (Chymostar or Chymax). However, recently there have been some changes to the labeling requirements for the GMO rennets and many cheesemakers have switched back to the animal rennet, making the GMO rennets difficult to find.
4. Water – My college has unchlorinated well water, so I use it straight from the tap. If your institution has chlorinated water, you should purchase some spring water to use. Chlorine interferes with the reaction.

Products

I have students lay out their cheeses on a table for tasting and comparing. The cheese with good flavor and texture generally ends up being divided up and taken with them. There is also a lot of whey produced in this lab. I encourage students to try a little of the whey, although few do. The whey, any uncurdled soy milk, and the cheese that is of poor flavor or texture I collect and give to local farmers for pig feed. I encourage you to find a local use of these high protein byproducts.

Acknowledgements

I wish to thank my students at Unity College who have helped me refine the procedures, especially Amanda Nelson. In addition, suggestions from the participants at the ABLE workshop in 2011 were invaluable.

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About the Author

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APPENDIX: Sample Results, Student Answers, and Student Evaluations

Sample Results

This is a qualitative lab and so results are descriptions of observations and the application of knowledge to answering questions.

Qualitative Data Collection

Table 2. Sample qualitative results comparing modifications to the cheese-making protocol

Variable Changed	Observations on Curd Formation	Mass of Cheese	Observations on Taste & Texture
None (Control)	Curds formation finished in about 25 seconds; curds fairly consistent in size, about 0.5-1.0cm	174g	Tastes like mozzarella, smooth, stringy
Substrate (Soy)	No curd formation	N/A	N/A
Temperature (raised all 10°C)	Curds formed very quickly, in less than 10 seconds; curds very large and bulky forming clumps over 5cm in some cases	211g	Very squishy, lots of moisture still in it
pH (no citric acid)	Curds did not form after addition of enzyme	N/A	N/A
Enzyme Type (GMO)	Curds formed in about 25 seconds and similar in size to control	181g	Tastes and feels same as control
Enzyme Conc (3X)	Curds formed in about 15-20 seconds and a little larger than control, more around 1cm	187g	A little more moisture than control, has an aftertaste

Sample Student Answers to Questions

A

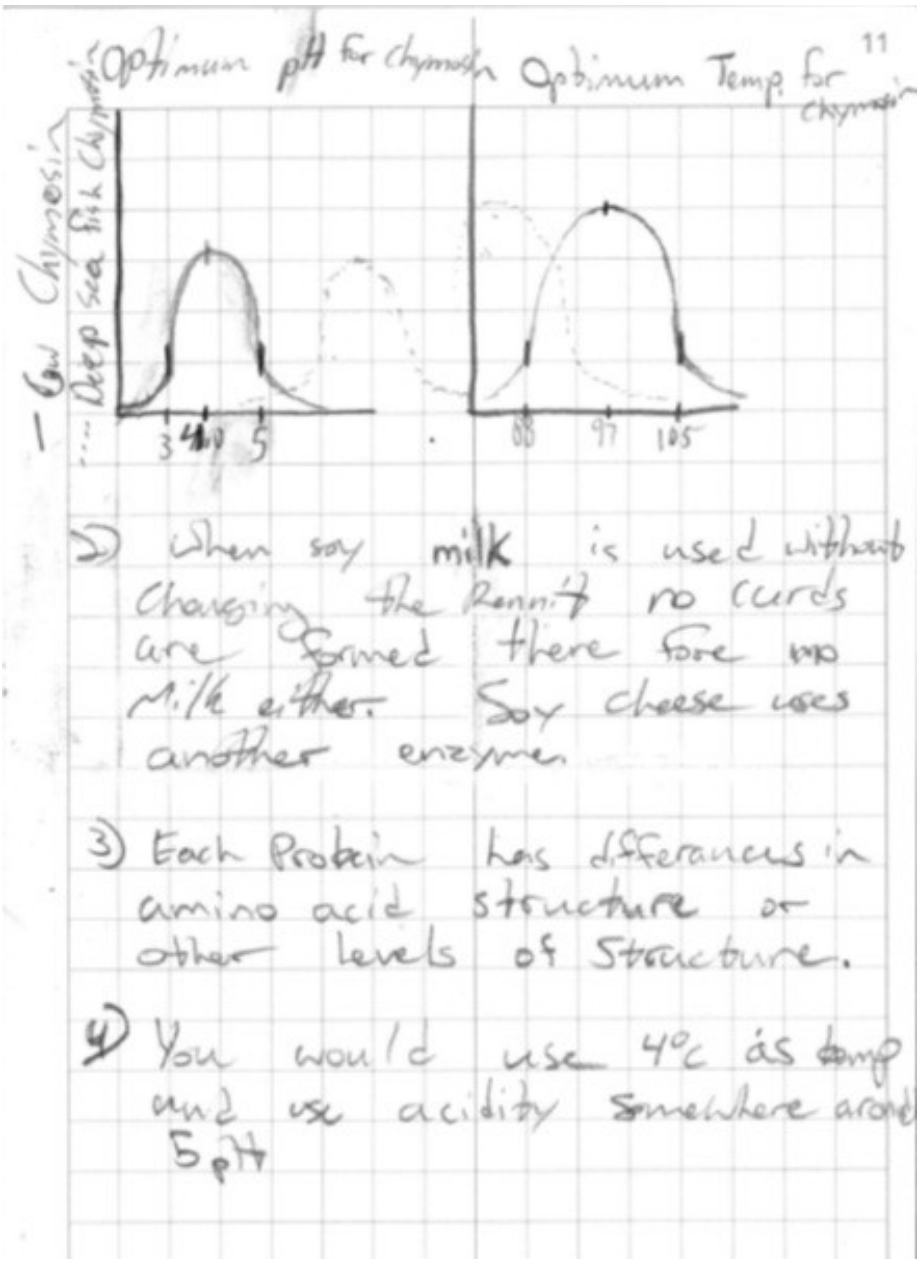
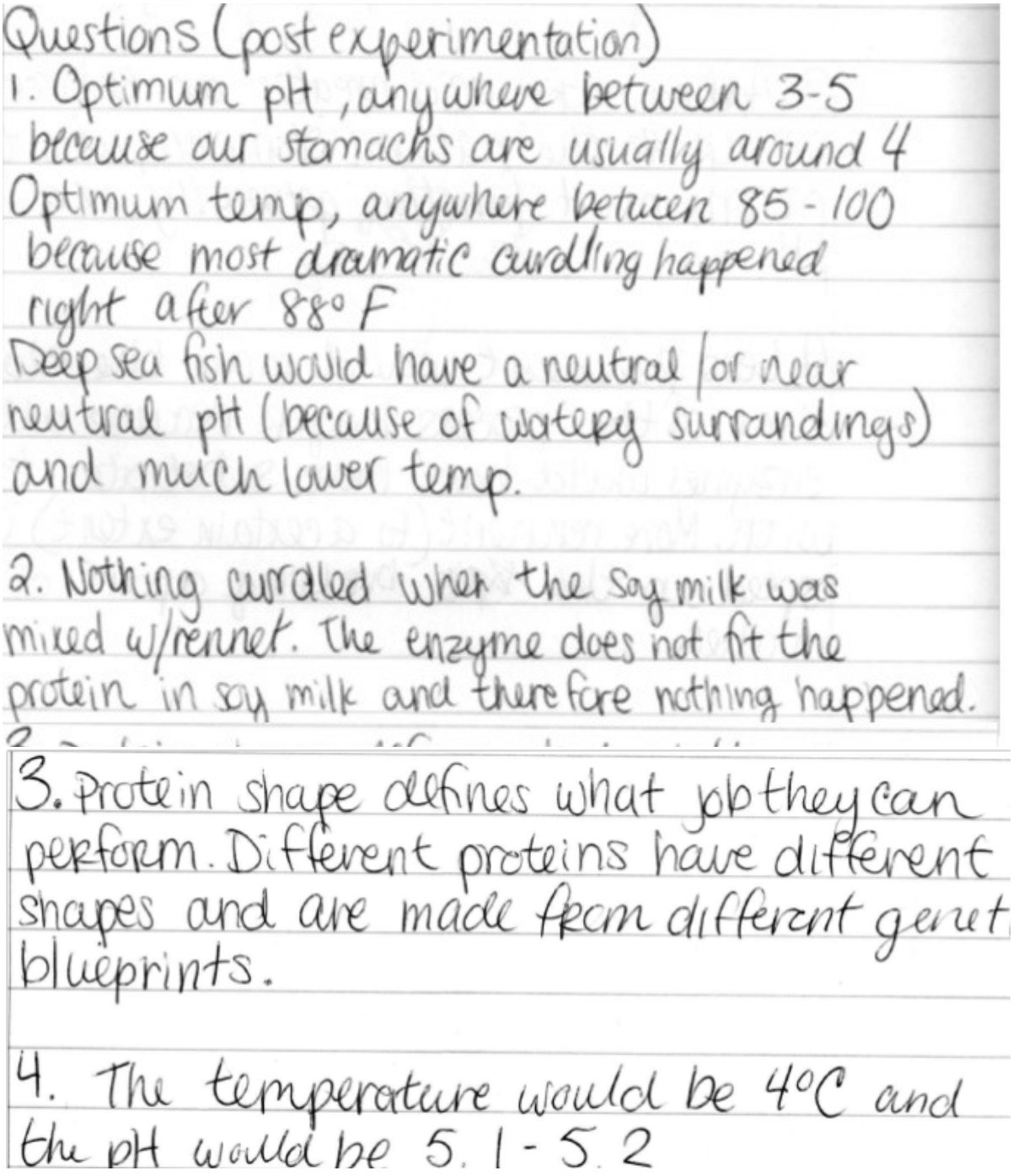


Figure 7a-b. Copies of student responses to post-experimentation questions from lab notebooks. I added question 5 of post-experimentation questions after I had scanned in sample responses from students. The answer for this question is that the enzyme that reassembles the triglycerides inside the cell has an active site that fits a monoglyceride and does not fit only a glycerol molecule that remains from the disassembling of the diglyceride. Because of enzyme-substrate specificity, the enzyme cannot reassemble the diglyceride.

Sample Student Answers to Questions

B



Questions (post experimentation)
1. Optimum pH, anywhere between 3-5 because our stomachs are usually around 4
Optimum temp, anywhere between 85 - 100 because most dramatic curdling happened right after 88° F
Deep sea fish would have a neutral /or near neutral pH (because of watery surroundings) and much lower temp.

2. Nothing curdled when the soy milk was mixed w/rennet. The enzyme does not fit the protein in soy milk and therefore nothing happened.

3. Protein shape defines what job they can perform. Different proteins have different shapes and are made from different genet blueprints.

4. The temperature would be 4°C and the pH would be 5.1 - 5.2

Student Evaluations of Exercise

Students were asked to evaluate this lab immediately after completion of the entire activity, including answering the post-experimental questions. Students were asked to rate their level of agreement (on a scale of 1-5, with 1 being "strongly disagree" and 5 being "strongly agree") for the following statements:

1. I enjoyed doing this lab.
2. I have a better understanding about the relationship between temperature and enzyme function after this lab.
3. I have a better understanding about the relationship between pH and enzyme function after this lab.
4. I have a better understanding about how substrates interact with enzymes after this lab.
5. I have a better understanding about the role of enzyme concentration in reaction rates after this lab.

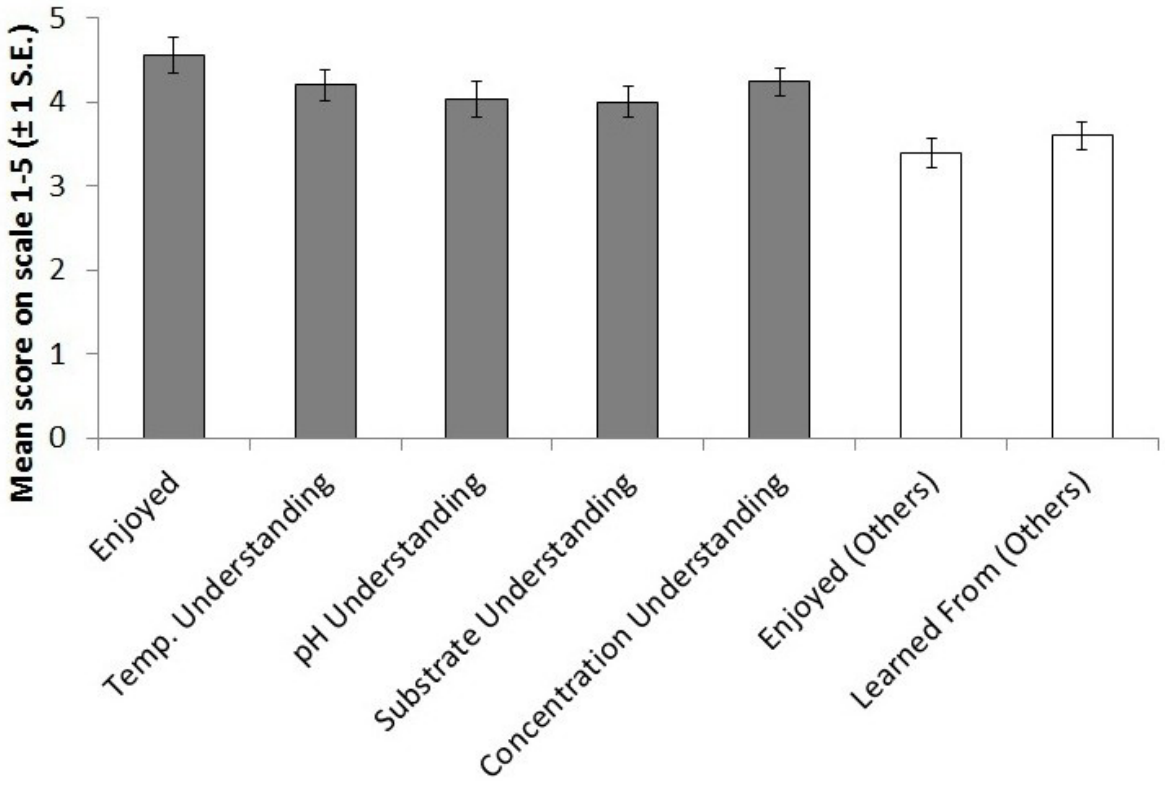


Figure 8. Student responses to the five post-lab evaluation questions. For comparison, the student ratings from other biology lab exercises for how much they enjoyed doing the labs and how much they perceive they learned from the labs are included.

Mission, Review Process & Disclaimer

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Citing This Article

Phillippi, A. 2012. Enzyme Explorations through Cheesemaking: A Qualitative Approach for Learning about Enzyme Function. *Tested Studies for Laboratory Teaching,* Volume 33 (K. McMahon, Editor). Proceedings of the 33rd Conference of the Association for Biology Laboratory Education (ABLE), 390 pages.
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