Lab 2: Enzymes

Objectives

1. Explain how enzymes catalyze chemical reactions.
2. Explain how and why pH affects the enzymatic activity of lactase.
3. Use an absorption spectrum to determine which wavelength of light to use in spectrophotometry assay.
4. Use the equation from a standard curve to determine concentration.
5. Design your own experiment to test a hypothesis.

Enzymes are catalysts that speed up chemical reactions but are not themselves consumed or changed by the reactions. Without enzymes, most biochemical reactions would take place at a rate far too slow to keep pace with the metabolic needs and other life functions of organisms.

Most enzymes are proteins. A protein is a sequence of amino acids (primary structure) that is folded in a three-dimensional shape. The 3D shape is determined by many weak chemical bonds. Hydrogen bonds along the polypeptide backbone (secondary structure) create coils and folds in the protein that contribute to its overall shape. Hydrogen bonds, ionic bonds, and van der Waal's interactions between the side chains of the amino acids also contribute to the overall shape of the protein (tertiary structure). Although the chemical bonds are weak, a protein has many of these bonds, which is what keeps the protein folded in its unique 3D shape.

Enzymes speed up chemical reactions by lowering the activation energy required to make reactions proceed within the narrow range of temperatures in which living organisms function. There are many different types of enzymes. Each catalyzes only one or a few specific reactions. Enzymes have a complex three-dimensional structure consisting of one or more polypeptide chains folded to form an **active site** - a special area into which the **substrate** (material to be acted on by the enzyme) will fit (Figure 2.1). Enzyme specificity is due to the structure of the enzyme's active site which must match the shape of the substrate molecule it acts upon. The interaction of the active site with the substrate lowers activation energy.

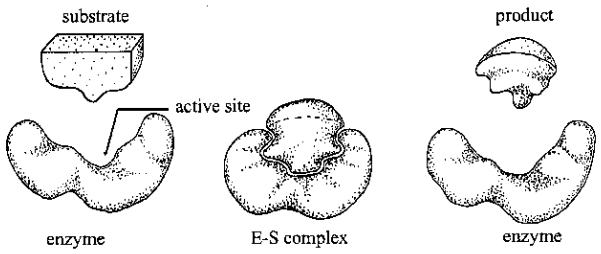


Figure 2.1. Induced fit model of enzyme function.

Enzymes are complex **proteins**, therefore, changes in temperature, alterations in pH, the addition of certain ions or molecules, and the presence of inhibitors all may affect the structure of an enzyme's active site and thus the activity of the enzyme and the rate of the reaction in which it participates. The rate of an enzymatic reaction can also be affected by the relative concentrations of enzyme and substrate in the reaction mixture.

Like most chemical reactions, the rate of an enzyme - catalyzed reaction increases as temperature increases, up to a point at which the rate is maximum. This temperature is usually close to the optimal temperature for an organism. The rate then declines with a further increase in temperature (Figure 2.2). Once the temperature climbs above 50°C, most proteins in living tissue become **denatured** - their secondary or tertiary protein structure breaks down due to the increased kinetic energy.

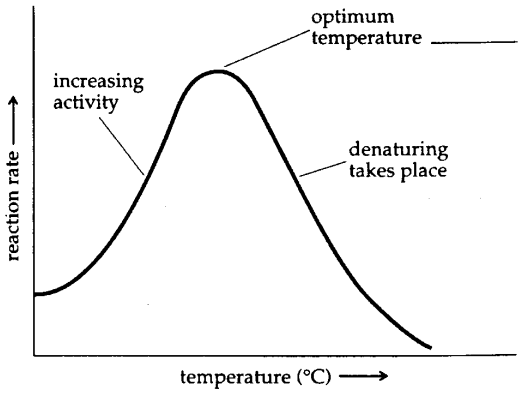


Figure 2.2. Effect of temperature on reaction rate.

The Effect of pH on Enzyme Action

The presence of various ions can interfere with the pattern of positive and negative charges within a protein molecule, thus changing the way the protein folds. In enzymes, the shape of the active site (Figure 2.1) may be changed. We should expect, then, that changes in pH (reflecting the concentrations of hydrogen and hydroxide ions) would affect the action of enzymes.

The most favorable pH value (the point at which the enzyme is most active) is known as the **optimum pH** (Figure 2.3). Extremely high or low pH values usually result in a complete loss of enzyme activity due to **denaturation**, the breakdown of the secondary and tertiary structure of a protein.

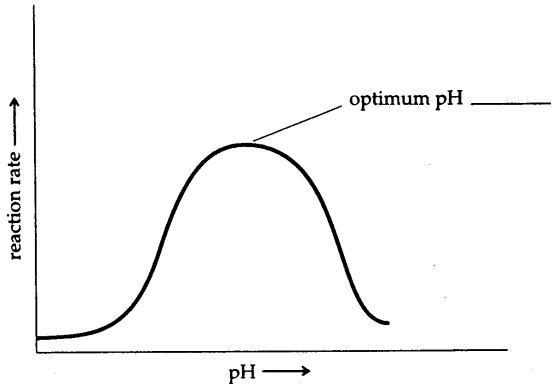


Figure 2.3. Effect of pH on reaction rate.

Lactase

In today's lab, you will investigate how changes in pH affect the activity of **lactase**, an enzyme that catalyzes the hydrolysis of lactose. Lactose is a disaccharide, made of glucose and galactose, that is found in dairy products. When a person consumes dairy products, the lactose is converted into galactose and glucose by the **lactase enzyme** (Figure 2.4).

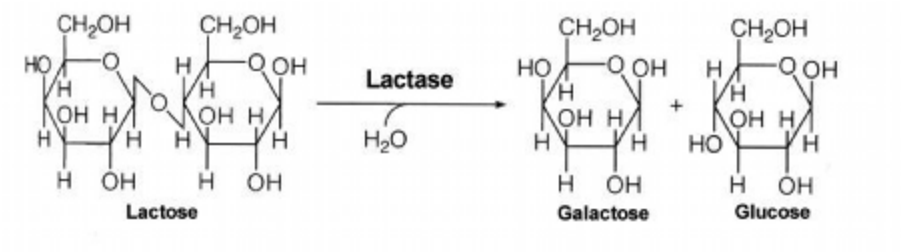


Figure 2.4. Enzymatic conversion of lactose into galactose and glucose.

Chances are you, or someone you know, is lactose intolerant, which means unable to digest lactose. People with lactose intolerance do not have enough lactase enzyme to digest lactose. This results in lactose accumulating in the large intestine, where it is fermented by bacteria. Fermentation of lactose by bacteria creates a lot of gas, which is the cause of abdominal discomfort for people who are lactose intolerant. Some people can consume small amounts of dairy products, whereas other people are intolerant to even small amounts; it all depends on how much lactase enzyme each person makes, which is determined by genetics.

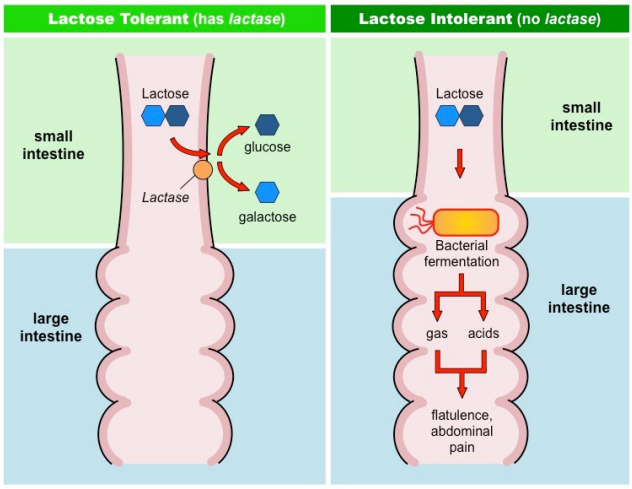


Figure 2.5. Insufficient lactase activity in the small intestine results in abdominal discomfort due to lactose fermentation by bacteria in the large intestine.

There are several over the counter products that contain lactase, such as Lactaid®, that can be used to treat lactose intolerance. These enzyme supplements come in a tablet form, typically taken with the first bite of food. The enzyme supplement must first pass through the stomach, where the pH can range from pH 1 (empty stomach) to pH 5 (full stomach), to get to the small intestine (pH 8) where the lactase will hydrolyze lactose into galactose and glucose. See Figure 2.6.

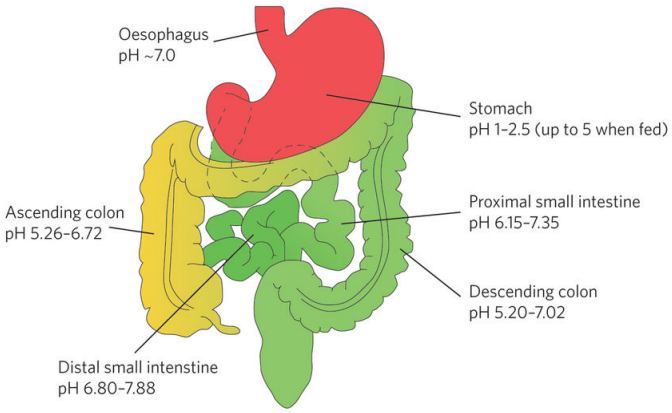
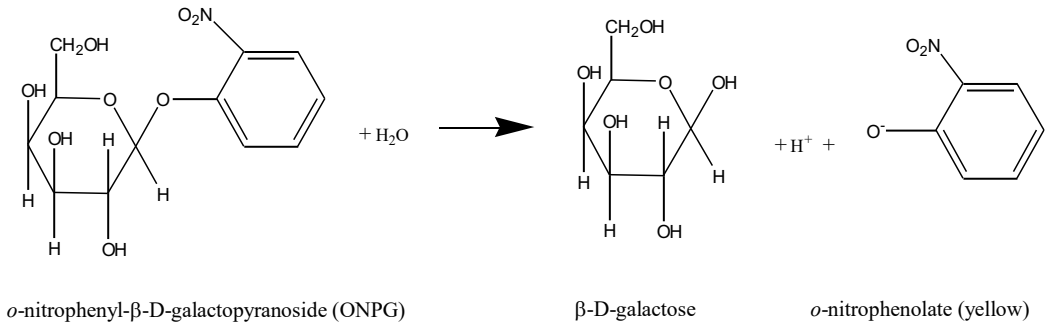


Figure 2.6. Morphology of the human gastrointestinal tract and pH values in different parts of the tract. Transit times for the esophagus and the small intestine are 10–14 seconds and 3.2±1.6 hours, respectively. For the colon, transit times are highly variable, dependent on bowel evacuations. The stomach half-empties typically in about 80.5 minutes. (Khutoryanskiy, V. 2015. Supramolecular materials: Longer and safer gastric residence. Nature Materials 14: 963–964)

Enzyme assay

To measure lactase activity, we could measure the products of the reaction (glucose and galactose). However, it is not easy to measure glucose or galactose in solution. Therefore, we will use a substrate that is similar to lactose, ONPG, which can be used in place of lactose. ONPG consists of galactose and o-Nitrophenol that are joined by a bond that is cleaved by

lactase (Figure 2.7). The products are galactose and o-nitrophenolate; o-nitrophenolate is yellow in color and can be measured by spectrophotometry.



o-nitrophenyl-β-D-galactopyranoside (ONPG) β-D-galactose *o*-nitrophenolate (yellow)

Figure 2.7. Lactase can convert ONPG (colourless) into o-nitrophenolate, a yellow compound which can be measured by spectrophotometry.

The absorption spectrum and standard curve for the yellow compound (o-nitrophenolate) have been done for you, see Figure 2.8 and 2.9. You will need these to determine which wavelength to use for the spectrophotometry assay, and to determine the concentration of o-nitrophenolate.

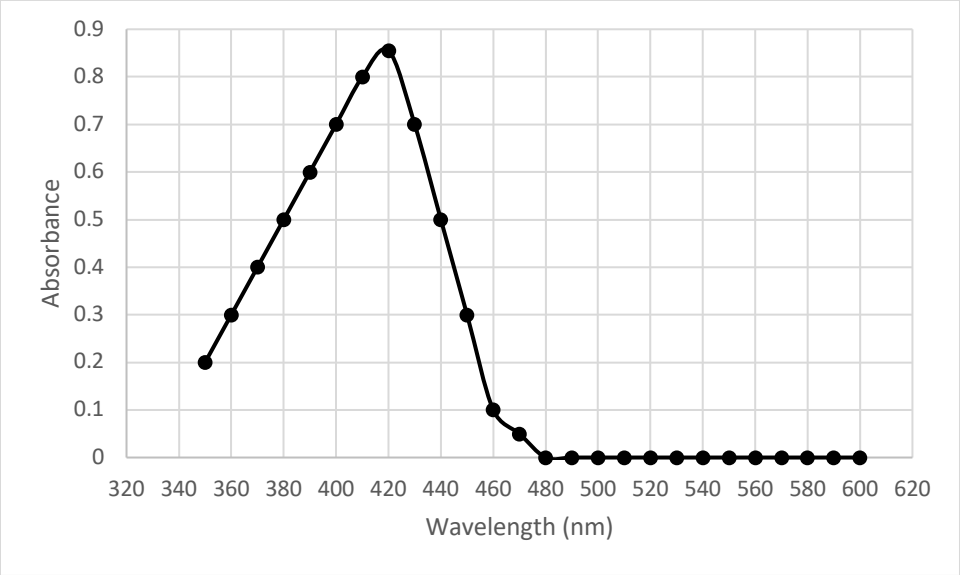


Figure 2.8. The absorption spectrum of o-nitrophenolate can be used to determine which wavelength you should use to measure o-nitrophenolate.

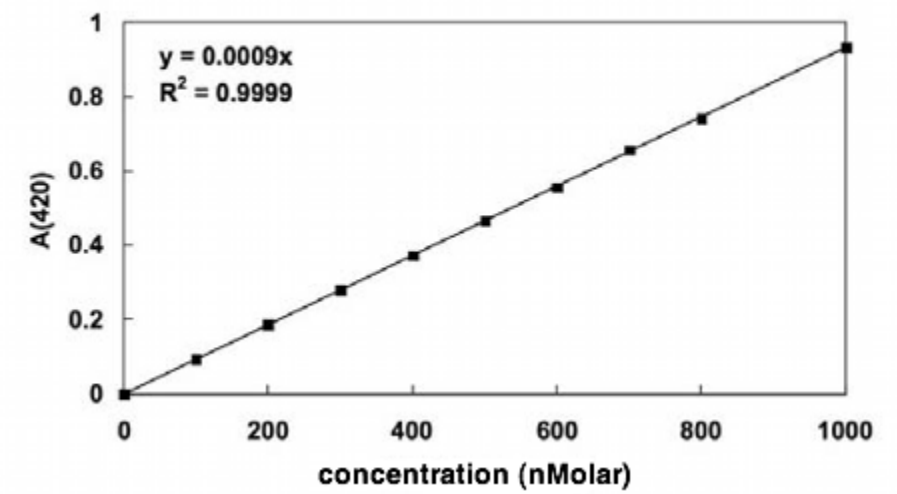


Figure 2.9 The standard curve for o-nitrophenolate can be used to determine the concentration at each absorbance.

Part A: The effect of pH on the lactase enzyme

Before you begin, use the absorption spectrum in Figure 2.8 to determine which wavelength you should use to measure 0-nitrophenolate, and record here: _____. Verify with your instructor that you are using the correct wavelength before you begin the experiment.

Experimental design:

Purpose:

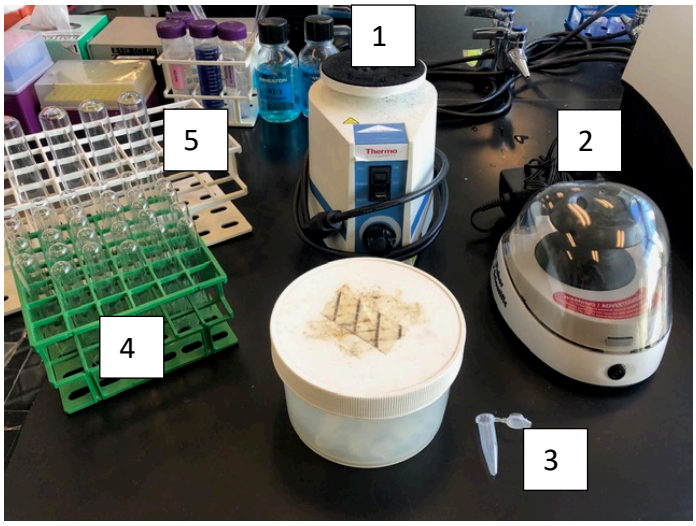
Hypothesis:

Independent variable:

Dependent variable:

Control:

Equipment list:



1. Vortex: Used to mix a solution. If you press down on the surface of the vortex it will shake to mix the solution.

2. Table top centrifuge: Centrifuges are the opposite of vortexes. They spin the samples around very fast which results in the separation of liquids of different density or the separation of liquids from solids.

3. Microfuge tubes: These small plastic tubes hold 1.5 ml and fit inside the centrifuge.

4. 5 ml test tubes: Small glass test tubes, these are the test tubes that fit in the spectrophotometer.

5. 10 ml test tubes: Larger glass test tubes.

Micropipettes: You used these in Lab 1 to measure precise volumes between 10- 1000 ul. Make sure you use the correct technique learned in Lab 1.

Spectrophotometers: You learned how to use the spec in Lab 1.

Procedure

I. Enzyme preparation

1. Add 1 ml phosphate buffer (pH 7) to the crushed lactaid (has been crushed for you) and wait for 5 minutes with periodic vortexing.
2. Spin your tube in the mini centrifuge for 5 minutes.
    a. Centrifuges must be balanced when you spin them, which means putting a tube of equal volume (= same weight) directly across from your tube. You can spin with the tube of the person beside you, or you can put 1 ml of water in a microfuge tube to use as a balance.
    b. Spinning the tubes will pellet the pill debris; the lactase enzyme will be in the supernatant.
3. Pour or pipette the 1 ml of supernatant (enzyme) into 9 ml of phosphate buffer (pH 7) in a large test tube. Avoid transferring any of the pellet/white debris into the large test

tube. Label this test tube: Lactase enzyme. This will be the working concentration of the lactase enzyme for your experiments. Be careful not to contaminate your bench or instruments with the enzyme.

II. Enzyme assay

1. Set up the **blanks** for the spectrophotometer (since you are measuring the enzyme activity at 4 different pH values, you will need 4 different blanks to set the specs to zero)

: = Lactase enzyme

3.6 ml phosphate buffer (pH1)
250 µl lactase

3.6 ml phosphate buffer (pH3)
250 µl lactase

3.6 ml phosphate buffer (pH7)
250 µl lactase

3.6 ml phosphate buffer (pH8)
250 µl lactase

Make sure you label these tubes with Blank pH 1, Blank pH 3, etc. The purpose of these tubes is to **blank the spec.** You are not using these tubes to measure the enzyme, so do NOT add ONPG to these tubes. Blanking the spec with phosphate buffer + enzyme will ensure that any light absorbed by the buffer or enzyme is removed so that in your reaction tubes, you will only be measuring ONP.

2. Set up the **reaction tubes** to test enzyme activity at each of the different pHs. For each pH: Add 3.5 ml of buffer and 250 µl lactase enzyme. Make sure you label each tube.

3.5 ml phosphate buffer (pH1)
250 µl lactase

3.5 ml phosphate buffer (pH3)
250 µl lactase

3.5 ml phosphate buffer (pH7)
250 µl lactase

3.5 ml phosphate buffer (pH8)
250 µl lactase

3. Blank the spec with the pH 7 blank (remember, the blank contains only buffer and enzyme, do not add ONPG to your blanks.)

4. Add 100 µl of ONPG to the pH 7 reaction tube, vortex, and **immediately** measure the absorbance every minute for five minutes. As soon as you add the ONPG, the reaction will proceed quickly, be organized! Vortex the tube briefly before each reading. Record the absorbance values in Table 1.

 = ONPG

5. Repeat this for pH1, 3 and 8. Be sure to blank the spec with the appropriate blank.

Table 1. Effect of pH on lactase activity. Values in the table below are reported as Absorbance.

pH	0min	1min	2min	3min	4min	5min
1						
3						
7						
8						

Using the equation for the standard curve (Figure 2.9), determine the concentration of o-nitrophenolate.

Table 2. Effect of pH on lactase activity. Values in the table below are reported as concentration of o-nitrophenolate.

pH	0min	1min	2min	3min	4min	5min
1						
3						
7						
8						

Analysis:

Determine the rate of reaction by graphing the results with **concentration on the Y axis** and **time (min) on the x-axis**. Graph paper is supplied in the lab so you can make this graph by hand before you leave the lab. Find the slope of the straightest portion of each curve as shown by the equation below. Ideally, the rate should include the time= 0 reading.

$$\text{Slope} = \frac{\text{concentration}_2 - \text{concentration}_1}{\text{time}_2 - \text{time}_1}$$

This determines the rate of the enzyme reaction rate in nmol/min.

Verify with your lab instructor that you have calculated rate correctly. You will enter the rates (nmol/min) for Part A into your lab's google sheet so that you have replicates to use to make your graph (with mean and standard deviation) for the Unit 2 assignment.

Part B: With your group, design an experiment to answer a question related to one of the following scenarios:

Scenario 1: The directions on the Lactaid® bottle indicate that you should take the enzyme supplement immediately before you eat any food containing lactose. You are meeting your friend for ice cream at 3:00 and haven't eaten anything since before your 8 AM class. Unfortunately, you didn't read the directions on the bottle, and took your Lactaid® 15 minutes before meeting your friend for ice cream (i.e.- you took the supplement on an empty stomach, which has a pH of ~2). Will the enzyme work as well after sitting in a pH of 2 (empty stomach) for 15 minutes compared to if you had taken it with your food (where pH of stomach will be ~5?) For this scenario, you will need to let the enzyme sit in pH 2 and in pH 5 for 15 minutes

before testing how will it functions in the small intestine (pH 8). (Note that this is different than Part A. In Part A, you tested enzyme function at the different pHs. In this experiment, you will test how well it works at pH 8 after going through pH 2 and pH 5.)

Scenario 2: According to the company that makes Lactaid®:
"Once Lactaid® tablets are ingested with the first bite of food, the lactase enzyme will be active for approximately 30-45 minutes. The pH of the stomach increases to 4.5 to 5.5 after a full meal. At this pH level, the lactase enzyme in Lactaid Tablets remains active for approximately 30 minutes."
You take a Lactaid® tablet with your meal, but then decide you want ice cream for dessert. You don't have any more Lactaid®, should you still eat the ice cream? (ie- how effective will the lactase be if more than 30 minutes has passed since you consumed it? You can use the enzyme prepped in Part A (more than 30 minutes will have passed since this enzyme was prepped and compare it to freshly made enzyme.)

Scenario 3: You usually buy the same brand of lactase supplements, but are curious if other brands work as effectively? Or is it worth it to buy the more expensive Lactaid® Fast Act caplets? Do the different kinds of Lactaid® have different rates of enzyme activity? For example, the regular strength tablets have 3000 lactase units, whereas the extra strength tablets have 4500 lactase units, and the fast act tablets have 9000 lactase units! In order to reduce waste, your instructor will organize all the groups who are testing this scenario. One pill will provide more than enough enzyme for many groups.

Scenario 4: You always leave your Lactaid® in the glovebox of your car. During the winter months, the Lactaid® will freeze, on an average summer day (21ºC) the internal temperature of your car will rise to 50ºC, on a hot day (32ºC) the internal temperature of your car could rise to 65ºC or higher! How well will the supplements work after being kept at these temperatures? If you are interested in testing this scenario, you will find Lactaid® tablets in the freezer, and in the water baths set to 50ºC and 65ºC. In order to reduce waste, your instructor will organize all the groups who are testing this scenario. One pill will provide more than enough enzyme for many groups. Remember, you are not testing how well the enzyme functions at these temperatures, as this would not be physiologically relevant! You are testing if storage at these temperatures reduces the effectiveness.

Extra Strength LACTAID® Tablets

Product Detail

If you get gas, bloating or diarrhea from milk or other dairy foods, it could be due to the milk sugar, lactose. Extra Strength LACTAID® Tablets contain lactase enzyme that breaks down the lactose into easily digested sugars to prevent uncomfortable symptoms.

Each Extra Strength LACTAID® Tablet contains 4500 Food Chemical Codex Lactase Units (FCCLU).

Available in packages of 40 and 80 tablets.

Directions for Use

Swallow or chew tablets **just before** eating any food containing lactose. The amount of Extra Strength LACTAID® you need depends on the amount of lactose in the food you eat. Start with 2 tablets and adjust up or down to find your own personal requirement. Four (4) tablets at any one time should prevent the symptoms caused by lactose intolerance. If symptoms persist, consult a doctor.

Caution

- Do not use if you have had a reaction to any LACTAID® product.
- Keep away from heat.
- Do not refrigerate Extra Strength LACTAID® Tablets.

Product Information

LACTAID® Fast Act Caplets

Product Detail

LACTAID® Fast Act lets you enjoy any dairy food. If you get gas, bloating or diarrhea from milk, or other dairy foods, it could be due to the milk sugar, lactose. LACTAID® Fast Act Caplets contain lactase enzyme that breaks down lactose into easily digested sugars to prevent uncomfortable symptoms.

Each LACTAID® Fast Act Caplet contains 9000 Food Chemical Codex Lactase Units (FCCLU).

Available in packages of 40 caplets.

The caplets are individually wrapped – ideal for on-the-go!

Directions for Use

Swallow one (1) LACTAID® Fast Act caplet **just before** eating any food containing lactose. The amount of LACTAID® Fast Act you need depends on the amount of lactose in the food you eat. Start with one (1) caplet. If needed, adjust dosage to 2 caplets. Two (2) LACTAID® Fast Act caplets at any one time should prevent symptoms caused by lactose intolerance. If symptoms persist, talk to a doctor.

Caution

- Do not use if you have had a reaction to any LACTAID® product.
- Keep away from heat and moisture.
- Do not refrigerate LACTAID® Fast Act Caplets.

Product Information

Regular Strength LACTAID® Tablets

Product Detail

If you get gas, bloating or diarrhea from milk or other dairy foods, it could be due to the milk sugar, lactose. Regular Strength LACTAID® Tablets contain lactase enzyme that breaks down the lactose into easily digested sugars to prevent uncomfortable symptoms.

Each Regular Strength LACTAID® Tablet contains 3000 Food Chemical Codex Lactase Units (FCCLU).

Available in packages of 100 tablets.

Directions for Use

Swallow or chew tablets **just before** eating any food containing lactose. The amount of Regular Strength LACTAID® you need depends on the amount of lactose in the food you eat. Start with three (3) tablets and adjust up or down to find your own personal need. Six (6) tablets at any one time should prevent the symptoms caused by lactose intolerance. If symptoms persist, consult a doctor.

Caution

- Do not use if you have had a reaction to any LACTAID® product.
- Keep away from heat.
- Do not refrigerate Regular Strength LACTAID® Tablets.

Clean-Up

- ☐ Empty the contents of your test tubes into the waste bottle in the **fume hood.** ONPG **cannot** go down the drain.
- ☐ Remove any tape from your test tubes, **rinse them out well** and place them upside down in the test tube racks on the bench.
- ☐ Turn off the spec.
- ☐ Spray your bench area with disinfectant and wipe down with paper towel. Wash your hands with soap and water.