Edvo-Kit # 193

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Forensics Enzymology

Experiment Objective:

The objective of this experiment is to study the ability of enzyme amylase, found in human saliva, to hydrolyze starch molecules. Students will perform two simple tests, the Iodine test for the disappearance of starch and the 3,5-Dinitrosalicylic acid test for the appearance of maltose produced from starch by amylase.

See page 3 for storage instructions.

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Table of Contents

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Experiment Components

	Component	Storage	Check (✓)
A	Simulated Negative Control saliva sample	Refrigerator	❑
B	Simulated Positive Control saliva sample	Refrigerator	❑
C	Simulated Driver #1's saliva sample	Refrigerator	❑
D	Simulated Driver #2's saliva sample	Refrigerator	❑
E	Starch solution	Refrigerator	❑
F	Iodine Solution	Refrigerator	❑
G	Acid Color Reagent G	Refrigerator	❑
H	Acid Color Reagent H	Refrigerator	❑

This experiment is designed for 10 lab groups.

Store all components below at room temperature.

Reagents and Supplies

- Microtiter plates ❑
- Microtest tubes with attached caps ❑
- Transfer pipets ❑

Requirements *(Not included with this kit)*

- Test tubes
- Test tube racks
- Lab permanent markers
- Beakers
- Distilled water
- Disposable lab gloves
- Safety goggles
- Automatic micropipettes, 0-50 µL and tips (recommended)
- 2 mL pipets
- Test tubes (12 x 75 mm)

All experiment components are intended for educational research only. They are not to be used for diagnostic or drug purposes, nor administered to or consumed by humans or animals.

Background Information

FORENSIC BASICS

Securing and Handling the Evidence

Forensic Scientists collect and analyze evidence from a crime scene in order to identify the nature of the evidence and its source. While this collection process takes place, the scientist cannot make any definitive statements about the nature of the evidence. Before making any conclusions, he or she must wait until extensive testing has revealed the nature of the evidence as well as information that can be gleaned from it. One cannot assume that a red stain on the floor or stain found by another detection method is actually blood. Only when you have determined the exact nature of the evidence, can further testing be done to produce additional information. Determining the nature of the evidence is a complex multi-step process. Forensic scientists use various assays to quickly and accurately determine the identity of a substance that must also satisfy the following criteria: the test must be quick, inexpensive, and most importantly, it must minimally affect the evidence. It is important that the initial testing be performed quickly and inexpensively in order to determine the direction of the investigation. A lengthy, expensive test would waste time and money if the sample being tested turns out to be something other than what it's thought to be. For example, attempting to generate a DNA profile from a potential bloodstain will give you conclusive results as to the nature of the stain but you will have spent several hours and hundreds of dollars doing so.

Maintaining the Integrity of the Evidence

In addition to collecting and testing evidence, the Forensic Scientist is also one of many people responsible for maintaining the integrity of the evidence itself. Steps must be taken to ensure that nothing is done to the evidence that would minimize or diminish it's value, thus making it less useful in an important situation, as in a courtroom/trial setting.

When evidence is collected, it is placed into a collection bag that is then sealed and taped shut, with the initials of the collector and collection date written across the tape. This is the first line of defense against any potential evidence tampering. In order to access the evidence, the seal on the bag/container must be broken. The evidence is then brought to a secure evidence room where signed records of its arrival are documented. Access to the room is restricted to only a few people who keep track of all the items. Any scientist who wishes to perform a test on the evidence must sign for the items they remove, noting the date and time as well. Eventually, these records provide a detailed picture of when the evidence was collected and every time it was moved or accessed for testing.

Testing procedures are affected in a variety of ways. After retrieving the evidence from the storage room a forensic scientist will note the condition of the container. Is the container properly sealed? Does the evidence tape sealing the openings look undisturbed? Are there any new openings in the container? All these questions must be answered and the condition of the container must be noted in the scientist's notebook before testing can begin. Next, the scientist must open the container without disturbing the prior sealing done by others. If at all possible, new openings should be made. This allows others who have made openings and sealed them to be able to say that their seal was undisturbed afterwards. This is important to show that the evidence wasn't tampered with and to document everyone who has tested the evidence. Often, the evidence bag will have numerous openings that have been resealed and signed.

Scientists need to take absolute care to ensure that evidence is never compromised to a point where contamination can occur. Contamination is the transfer of minute amounts of material from one piece of evidence to another that can drastically alter the value of the evidence. After testing is complete, all items are then signed back into the evidence room by the scientist who removed them. This procedure is used to document all of those who had access to the evidence.

PRINCIPLES OF ENZYME CATALYSIS

Enzymes as Biological Catalysts

A biological catalyst is used in trace amounts to accelerate the rate of a biochemical reaction without being consumed or transformed during the reaction. The equilibrium constant of reactions are not altered by catalysts. Only the rate of approach to equilibrium is changed.

Reactions in cells are catalyzed by biological catalysts known as enzymes which can accelerate reactions by as much as 10^{14} to 10^{20} times. Enzymes function best under physiological conditions at neutral pH, and temperatures of 37° C. Enzymes are generally very specific for the reactions they catalyze. Certain enzymes are regulated by intracellular concentrations of key metabolites that are not directly involved with the reaction they catalyze. This regulation (increase or decrease for an enzyme activity) is often regulated by a cell's physiological requirements at a given time. Enzymes that are regulated in this way are termed allosteric.

Measuring Enzyme Activity

The reactant molecule in an enzyme catalyzed reaction is called the substrate. The substrate (S) is transformed to product (P). Before the enzyme can transform the substrate it must first bind to it. Only a relatively small portion of the enzyme molecule is involved with substrate binding and catalysis. This region is called the active site. The active site contains the critical amino acid residues and, if applicable, the prosthetic groups required for activity.

Initial binding is non-covalent and can be in rapid equilibrium. After productive binding has been achieved, the enzyme-substrate complex begins to generate product (P) which is subsequently released. The free enzyme (E) can react with additional substrate (S) and this reaction is repeated rapidly and effectively. The reaction is summarized using a single substrate, single product in a non-reversible reaction:

E + S —> ES —> EP —> E + P

In this experiment, Amylase is an enzyme which catalyzes the hydrolysis of the polysaccharide starch to the disaccharide maltose. Salivary amylase is produced by the salivary glands. If amylase is added to a solution of starch, the starch will be digested to form maltose.

Substrate (S)	Enzyme (E)	Products (P)
Starch	————> Amylase	Maltose

The rate of the reaction is increased if the enzyme and substrate mixture is brought to body temperature (37° C) compared to room temperature.

The appearance of product (P) or the disappearance of substrate (S) can be measured as a function of time during a reaction. One can measure the amount of product formed or the decrease in substrate at regular intervals. This quantity can be plotted as a graph.

In this head-on car collision, each of the drivers claimed that the other fell asleep at the wheel in the early morning accident. The two passengers (one in each car) were critically injured but the drivers were only mildly hurt. When the police officer arrived at the scene of the accident, he recorded the required information and arranged for all to be taken by ambulance to the local hospital. The attending emergency room (ER) physician did a thorough examination of the two drivers, took blood and urine samples for analysis, took their temperature using a disposable plastic tongue depressor to examine for damage to their mouths and teeth. He placed the first used tongue depressor in the original plastic packaging and discarded it in the dedicated waste container. He did the same for the second tongue depressor (used on the second driver) but forgot to discard it and he left it on the examination table. Four hours after arrival to the hospital the two critically injured passengers in the hospital intensive care unit (ICU) were pronounced dead which made the case a potential homicide.

The attending emergency room (ER) physician remembered from his medical school days that the level of saliva amylase

increases due to sleep deprivation. He then recovered the two tongue depressors, labeled them by the names of the drivers, John Smith and Sam White, contacted the authorities and provided the forensics laboratory with the tongue depressors to test for levels of saliva amylase for both drivers.

Humans produce 1 to 1.5 liters of saliva per day. Saliva has a slightly alkaline pH and is composed of water, mucus, proteins, salts and enzymes. Saliva is used to lubricate food, to assist in swallowing and initiate food digestion by the enzyme amylase, which initiates digestion of starch. Amylases are ubiquitous and are found both in plants and animals. The enzyme is abundant in human saliva in easily detectable quantities. Amylases are subdivided into alpha and beta sub-types. The alpha is found in animals and the beta in plants. The catalytic function of alpha amylase present in human saliva is digestion of starch which is obtained from a variety of foods.

Amylase acts within the starch chains to produce small disaccharides known as maltose. This disaccharide is made up of two glucose units that can be further hydrolyzed to produce glucose that can serve as a source of energy. Alpha-amylase is present in many human tissues and secretions and can be a useful tool for forensics. It is estimated to be present in saliva at concentrations that are 50 fold higher than other human secretions. In humans there are two DNA loci that code for amylase. AMY1 and AMY2 that are found on chromosome 1. AMY 1 codes for amylase present in saliva, breast milk and perspiration and AMY 2 codes for amylase found in the pancreas and other human secretions.

In this simulation experiment students will determine the level of saliva amylase for the two drivers recovered from the tongue depressors to determine who was responsible for the accident due to falling asleep at the wheel. The reaction can be visualized by testing (1) the disappearance of the substrate (starch) or (2) the appearance of product (maltose). The Iodine test will be used for starch and Dinitrosalicylic acid test for maltose.

In the first module of this experiment, when a drop of iodine is added to a starch solution it will give a characteristic dark brown or blue color, depending on the starch concentration. If amylase is present, it then digests starch, the complex structure of starch is destroyed, and the intensity of the dark brown (or blue) color fades away. Lowering the shades of the blue color the starch iodine test can be correlated to the quantity of amylase that is present in the saliva sample. For this experiment a safe substitute {providine-iodine) will be used to provided the blue color for the starch test. Complete hydrolysis of starch is indicated by a yellowish color when combined with iodine.

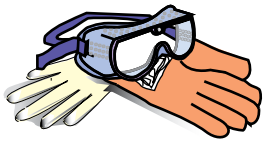
In the second module of this experiment, 3,5-Dinitrosalicylic acid (yellow color) is used as the color reagent in reactions of amylase, which is an enzyme that can break down starch. In this module, the presence of maltose, a reducing sugar, is detected by performing an oxidation – reduction reaction. Due to the presence of a carbonyl group (C=O), maltose participates in an oxidation – reduction with Dinitrosalicylic acid. This reaction causes a change in color from yellow to orange or red, depending on the concentration of maltose produced.

Experiment Overview

EXPERIMENT OBJECTIVE

The objective of this experiment is to study the ability of enzyme amylase, found in human saliva, to hydrolyze starch molecules. Students will perform two simple tests, the Iodine test for the absence of starch and the 3,5-Dinitrosalicylic acid test for the presence of maltose produced from starch by amylase.

LABORATORY SAFETY



No human materials are used in this experiment. Gloves and safety goggles should be worn as good laboratory practice.

LABORATORY NOTEBOOK RECORDINGS:

Address and record the following in your laboratory notebook or on a separate worksheet.

Before starting the Experiment:

- Carefully read the introduction and the protocol. Use this information to form a hypothesis for this experiment.
- Predict the results of your experiment.

During the Experiment:

- Record (draw) your observations, or photograph the results.

After the Experiment:

- Formulate an explanation from the results.
- Determine what could be changed in the experiment if the experiment were repeated.
- Write a hypothesis that would reflect this change.

Module I: Studying the Disappearance of the Substrate (Starch)

STUDYING THE DISAPPEARANCE OF THE SUBSTRATE (STARCH) USING MICROTITER PLATE METHOD

1. **LABEL** 4 plastic transfer pipets as follows:
   (-) (negative)
   (+) (positive)
   D1 – Simulated Driver #1's saliva sample
   D2 – Simulated Driver #2's saliva sample

NOTE: Use appropriately labeled plastic transfer pipet for addition of each 4 saliva samples as outlined in Modules I & II.

2. **PLACE** a microtiter plate piece as shown below. Across the top of the plate, **LABEL** the 4 wells as shown below using a laboratory marking pen. Also **MARK** the plate with your initials or lab group number:

   (-) (negative)
   (+) (positive)
   D1 – Simulated Driver #1's saliva sample
   D2 – Simulated Driver #2's saliva sample

IMPORTANT: Avoid cross-contamination by using a new pipet tip when using an automatic micropipette for each saliva sample.

PUT ON YOUR GLOVES NOW.

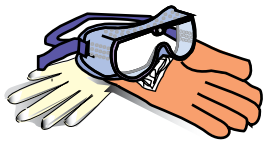
3. Using a different disposable pipet or pipet tip for each sample, **ADD** 3 drops or 50 µL of each saliva sample into the appropriately labeled well. For example, Simulated Negative Control saliva sample goes into the well labeled (-).

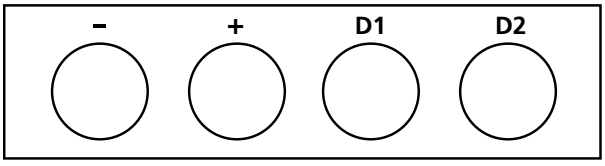
   REPEAT the same procedure for Simulated Positive Control saliva sample, Simulated Driver #1's saliva sample, and Simulated Driver #2's saliva sample. Each well requires 3 drops or 50 µL saliva sample.

4. Use a new pipet to **ADD** 50 µL of Starch Solution (Component E) into each of the wells.

5. Use a new pipet to **ADD** 10 µL of Iodine Solution (Component F) into each of the wells.

6. **RECORD** the color changes in each well in the diagram, below.





Module II: Studying the Appearance of Product (Maltose)

STUDYING THE APPEARANCE OF PRODUCT (MALTOSE) USING TEST TUBE METHOD

1. With a water resistant pen, **LABEL** 4 empty test tubes (at the top) as follows:
   - (-) (negative)
   - (+) (positive)
   - D1 – Simulated Driver #1's saliva sample
   - D2 – Simulated Driver #2's saliva sample

IMPORTANT: Avoid cross-contamination by using a new pipet tip when using an automatic micropipette for each saliva sample.

PUT ON YOUR GLOVES NOW.

2. Using a different transfer pipet or pipet tip for each sample, **ADD** 6 drops or 100 µL of each saliva sample into the appropriately labeled well. For example, Simulated Negative Control saliva sample goes into the well labeled (-).

   REPEAT the same procedure for Simulated Positive Control saliva sample, Simulated Driver #1's saliva sample, and Simulated Driver #2's saliva sample.

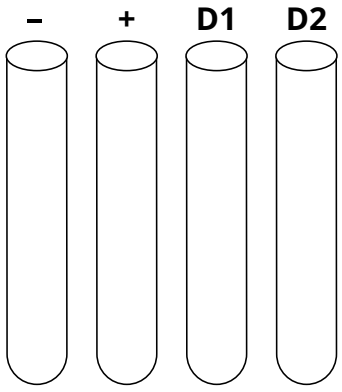
3. Use a new transfer pipet or pipet tip to **ADD** 6 drops or 100 µL of Starch solution to each of the test tube. **MIX** well by tapping or pipetting up and down. **INCUBATE** the tubes at room temperature for 5 minutes.

4. **ADD** 200 µL of Acid Color Reagent to each tube. **MIX** well by tapping or gently shaking. **COVER** each tube with a small piece of foil. **INCUBATE** the tubes in boiling (use caution) water bath for 1 minute.

5. **REMOVE** the tubes from the water bath and allow them to cool to room temp.

6. **ADD** 2 mL of distilled water to each tube. **MIX** well by tapping or pipetting up and down.

7. **RECORD** the color changes in each tube, below.



Study Questions

Answer the following study questions in your laboratory notebook or on a separate worksheet.

1. What is maltose and how is it obtained from starch?

2. Why did the doctor send the tongue depressor to the forensics laboratory?

3. What are the functions of saliva in human digestion?

4. How is glucose obtained from starch as a source of energy?

Instructor's Guide

NOTES TO THE INSTRUCTOR

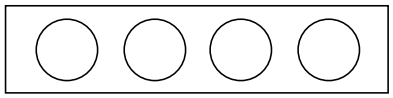
Students should be made aware of the safety concerns when working with human products even though all of the materials in this Edvotek kit are chemicals used to simulate saliva.

If you do not find the answers to your questions in this section, a variety of resources are continuously being added to the EDVOTEK® web site. In addition, Technical Service is available from 8:00 am to 5:30 pm, Eastern time zone. Call for help from our knowledgeable technical staff at 1-800- EDVOTEK (1-800-338-6835).

Safety Data Sheets can be found on our website: www.edvotek.com/safety-data-sheets

Pre-Lab Preparations

1. Label 10 microtest tubes "A".
2. Label 10 microtest tubes "B".
3. Label 10 microtest tubes "C".
4. Label 10 microtest tubes "D".
5. Label 10 microtest tubes "E".
6. Label 10 microtest tubes "F".
7. Label 10 microtest tubes "Acid".
8. Aliquot 160 µl of Simulated Negative Control saliva sample (component A) into each tube "A".
9. Aliquot 160 µl of Simulated Positive Control saliva sample (component B) into each tube "B".
10. Aliquot 160 µl of Simulated Driver #1's saliva sample (component C) into each tube "C".
11. Aliquot 160 µl of Simulated Driver #2's saliva sample (component D) into each tube "D".
12. Aliquot 700 µl of the Starch Solution (component E) into each tube "E".
13. Aliquot 50 µl of the Iodine Solution (component F) into each tube "F".
14. Component G will likely precipitate when stored properly in the refrigerator. Place the bottle in a warm waterbath for several minutes until the material goes into solution.
15. Add all of the Acid Color Reagent (comp. G, now back in solution) to the tube containing the Acid Color Reagent H (component H). Mix well. Label this solution as "Acid". Aliquot 850 µl of the "Acid" solution into each tube "Acid".
16. Each group will require the following materials:
    a. One tube of each A, B, C, D, E, F and Acid.
    b. One microtiter plate piece (1 row of 4 wells).



    c. Four glass test tubes.
    d. Eight plastic transfer pipets.

NOTE:
Due to the effect of temperature on reaction rates, all reactions should take place at room temperature. Materials stored in the refrigerator should be brought to room temp before performing the experiments.

NOTE:
- Tubes A, B, C, D, E, are used in both Modules I and II.
- Tube F is used in Module I only.
- Tubes "Acid" is used in Module II only.

Expected Results

MODULE I: STUDYING THE DISAPPEARANCE OF THE SUBSTRATE (STARCH) USING MICROTITER PLATE METHOD

The level of saliva amylase increases in human due to sleep deprivation. Normally a starch solution turns a dark brown (or blue) color when iodine is added. A dark brown color indicates the presence of starch at the end of the reaction (Case of Driver #1). On the other hand, if amylase works on starch, the complex structure of starch is destroyed, and the intensity of the dark brown (or blue) fades away. Complete hydrolysis of starch is indicated by a yellowish color when combined with iodine (Case of Driver #2).

MODULE II: STUDYING THE APPEARANCE OF PRODUCT (MALTOSE) USING TEST TUBE METHOD

In Module II, 3,5-Dinitrosalicylic acid (yellow color) is used as the color reagent in reactions of amylase, which is an enzyme that can break down starch. The presence of maltose, a reducing sugar, is detected by performing an oxidation reaction. This reaction causes a change in color from yellow to orange or red, depending on the concentration of maltose produced.

Forensic Data Analysis:

Laboratory results showed that saliva sample from Driver #2 produces more Maltose than Driver #1 does, which indicates that there is more amylase present in the saliva of Driver #2 than there is in Driver #1's sample. This again suggests that Driver #2 is sleep-deprived, and therefore, may be responsible for the accident.

Please refer to the kit insert for the Answers to Study Questions