**Objectives:**

* 1. To demonstrate activity of the enzyme polyphenol oxidase in crude extract prepared from potato.
	2. To demonstrate the chemical nature of the enzyme.
	3. To investigate the substrate specificity of the enzyme.
	4. To investigate the effects of various temperatures on the activity of the enzyme.

When we consider enzyme catalyzed reactions in the living cell, the reacting substances, upon which an enzyme acts, are termed the substrates. The substances produced as a result of the reaction are the products. Enzyme catalyzed reactions are mostly reversible and involve the formation of an intermediate enzyme-substrate complex.

The formation of an enzyme-substrate complex increases the possibility for chemical reaction by:

 1- Lowering the energy of activation.

2- Reducing the element of chance in the collisions of molecules or ions.

The rate of reaction is accelerated through the catalytic action of the enzyme. A single enzyme molecule, even though it can react over and over, is only capable of combining with a given total number of substrate molecules per minute. This number known as the turnover number varies from enzyme to enzyme. Many enzymes have a high turnover number. For example, catalase has a turnover number of 5 million per minute. Thus enzymes are generally effective in relatively minute concentrations in the living cell.

The formation of enzyme-substrate complex is confined to relatively small areas of the enzyme molecule, known as active sites. The structure of a particular substrate may induce the enzyme to "mold" itself over the substrate. This may be illustrated schematically in the following way:

The "induced fit" hypothesis suggests that differences in the surface configuration (three- dimensional shape) of the active site are essential to specificity. In other words, only certain types of substrate molecule would be able to establish a close fit with a given type of enzyme molecule. Because hundreds of reactions are simultaneously carried out in the living cell, it becomes difficult to study a single reaction in an intact living cell. However, it is possible to extract enzymes from cells and thus study enzyme catalyzed reactions in a test tube. In this experiment, a crude extract of the enzyme polyphenol oxidase will be prepared from the potato.

Polyphenol oxidase is a copper-containing enzyme with an optimum pH of 6.7. It catalyses the oxidation of di- and tri- hydroxyl phenol to the corresponding quinine.

This oxidation-reduction reaction is accompanied by a color change (quinines absorb light in the visible region of the spectrum). This reaction commonly occurs in nature and accounts for the "browning" of peeled potatoes and bruised fruits. You will familiarize yourself with the reaction catalyzed by the enzyme polyphenol oxidase, as it occurs removed from the intact living cell, i.e. in a test tube. This experiment is in four parts, corresponding to the four objectives listed on the first page.

Method: To detect and follow the progress of the reaction in this experiment a simple, qualitative method will be used. More sophisticated, quantitative methods of following enzyme catalyzed reactions will be introduced later in the course. In this experiment, record your observations according to the following scheme:

* No color change (colorless) −
* Faint color (just detectable) +
* Definite color ++
* Dark (deep) color +++

**Test tube enzymatic activity:**

* In this reaction we are looking for enzyme substrate reaction in general
* In this experiment you will notice the change qualitatively (change in the color).
* The intensity of this color (brown) will be proportional to the enzyme’s activity in the tube under observation.
1. **Label three clean test tubes A, B and C.**
2. **Prepare each tube as follows:**

|  |  |
| --- | --- |
| **Tube** | **Addition** |
|  **A** | * 15 drops of enzyme extract.
* 15 drops of 0.01M catechol solution
 |
|  **B** | * 15 drops of enzyme extract.
* 15 drops of distilled water.
 |
|  **C** | * 15 drops of 0.0M catechol solution.
* 15 drops of distilled water
 |

|  |  |
| --- | --- |
| **Incubation time (minutes)** | **Degree of color intensity (Symbol: −, +, ++ or +++)**  |
| **TUBE A** | **TUBE B** | **TUBE C** |
| **0** |  |  |  |
| **5** |  |  |  |
| **10** |  |  |  |
| **15** |  |  |  |
| **20** |  |  |  |

**c. Place all three tubes in a water bath at 37 ºC.**

**e. Every 5 minutes, after shaking, hold the tubes up to the light and examine.**

* **Record the color in each tube in the following table:**

*Chemical nature of PPO:*

 **Principle:**

* **As we all know majority of enzymes** are proteins. Some are made of RNA
* **They are high molecular** weight compounds made up principally of chains of amino acids linked together by peptide bonds.
* **It will be examined** the nature of polyphenol oxidase wither is it protein or not
* **TCA** is an analogue of acetic acid widely used in biochemistry **for precipitation of macromolecules, such as proteins, DNA, and RNA** .
* It will affect the PPO activity by altering the pH of solution leading to denatured and inactivated enzyme.

TCA treatments proves that the chemical nature of PPO **is protein**.

* **Phenylthiourea** has a very strong chemical affinity for the element copper (Cu+2). It is able to bind with Cu+2, even when the copper is attached to other chemical substances, as in the active site of PPO.

This shows that copper **is a cofactor** for PPO.

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube** | **(1)** | **(2)** | **(3)** |
| **A****(Control)** | Add 10 drops of* Enzyme extract.
* 0.01M catechol
 | Shake tube and place in water bath at 37 ºC for 10 minutes.* **As a Control!**
 |
|  **B** | Add 10 drops of* Enzyme extract.
* 5% trichloroacetic acid (TCA)
 | Shake tube very well and wait 5 minutes. | * Add 10 drops of 0.01M catechol solution.
* Place in water bath at 37 ºC for 10 minutes
 |
| **C** | * Add 15 drops of enzyme extract.
* Add a few crystals of phenylthiourea
 |

|  |  |  |
| --- | --- | --- |
| **Tube**  | **Treatment** | **Degree of color intensity (Symbol: −, +, ++ or +++)** |
| **A** | **Control** |  |
| **B** | **TCA** |  |
| **C** | **Phenylthiourea** |  |

* **Examine and compare with tube A.**

***3-Specificity of Enzymes:***

There are four distinct types of specificity:

* Absolute specificity - the enzyme will catalyze only one reaction.
* Group specificity - the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
* Linkage specificity - the enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
* Stereochemical specificity - the enzyme will act on a particular steric or optical isomer.

The three compounds used as substrates in this part of the experiment are (mono- and di-hydroxyl phenol)

Hydroquinone is a di-hydroxyl phenol that can slightly change the active site configuration depending on “induced fit model” and gives a fait color reacting with PPO.



1. ***Label three clean test tubes A, B and C.***

|  |  |  |
| --- | --- | --- |
| **Tube** | **(1)** | **(2)** |
| **A** | 15 drops of enzyme extract. | * Add 15 drops of 0.01M catechol solution
 |
| **B** | * Add 15 drops of 0.01M phenol solution
 |
| **C** | * 15 drops of 0.01M hydroquinone
 |

1. ***Shake the tubes gently and place them in a water bath at 37 ºC.***
2. ***Examine the tubes after 5 minutes and after 10 minutes.***
* ***Record the color in each tube, in the following table***

|  |  |
| --- | --- |
| Substrate | Degree of color intensity (Symbol: −, +, ++ or +++) |
| 5 minutes | 10 minutes |
| Catechol  |  |  |
| Phenol  |  |  |
| Hydroquinone  |  |  |

***4-Temperature And Enzymatic Activity:***

1. **Label three clean test tubes A, B and C.**

|  |  |  |  |
| --- | --- | --- | --- |
| **Tube** | **(1)** | **(2) Incubate for 10 min at:** | **(3)** |
| **A** | **Add 15 drops of enzyme extract to each tube.** | **0 ºC**  | **Add 15 drops of 0.01M catechol solution to each tube.** |
| **B** | **37 ºC.**  |
| **C** | **70 ºC.**  |

1. **Shake each tube gently and quickly return it to its proper temperature condition.**
2. **Wait for 15 minutes. After this time, examine each tube, without removing it from its temperature condition**
* **Record the color in each tube in the following table.**

|  |  |
| --- | --- |
| **Temperature (ºC)** | **Degree of color intensity (Symbol: −, +, ++ or +++)** |
| **0** |  |
| **37** |  |
| **70** |  |

 Questions:

In part 1 of the experiment, did you detect any reaction in tube B (enzyme extract and distilled water)?imagine that it will give a color, What could be the problem?

2- Can the enzyme be restored to an active state after TCA treatment (part 2)? Explain your answer.

3- What can you deduce about the specificity of polyphenol oxidase from part 3 of the experiment?

4- Explain the effects of temperature on the activity of polyphenol oxidase (part 4).

 5- Suppose that two solutions of equal concentration were prepared, one of purified polyphenol oxidase and one of purified trypsin. Which solution, would you expect, would lose its enzymic activity first and why?