**ENZYME ASSAY- AMYLASE & INVERTASE**

**INTRODUCTION:**

Amylase is enzyme which degrades starch and related polysaccharides, like glycogen. These are of two types. α-amylase hydrolyses starch into maltose and glucose while βamylase acts on starch to produce maltose. Both the enzymes cleave only I ,4 glycosidic linkages. Hence their action is stopped at 1 ,6 glycosidic linkages present in amylopectin. As a result of this, only about 55%of amylopectin. (branched component of starch) is hydrolysed, although amylose (linear component of starch) is completely hydrolysed. In case of amylopectin, the other products are limit dextrin. During germination, amylase is synthelized *de novo.*

**PRINCIPLE:**

Like any other enzyme, activity of amylase can be determined by measuring the amount of substrate that is utilized or the amount of product that is formed per unit time. In this case, the products are glucose and maltose which react with alkaline dinitrosalicylic acid to give an Orange colored product. The intensity of this color is proportional to the amount of glucose and maltose. Therefore, amylase activity is assayed by estimating the products of the reaction (glucose or maltose).

**AIM**:

To determine the enzyme activity of α Amylase

**MATERIAL REQUIRED:**

i. Seeds (e.g. barley or wheat).

ii. Sodium acetate buffer (0.1 M, pH 4.7)- Dissolve 8.203g of anhydrous CH3COONa in distilled water. Adjust pH to 4. 7 with acetic acid (CH3COOH) and make up volume to 1L with distilled water.

iii Calcium chloride (10 mM) -Dissolve 1.470g of CaCl2.2H20 in distilled water and make up volume to 1L.

iv Sodium hydroxide (1%) -Dissolve 10g NaOH in 1L distilled water.

v Dinitro salicylic (DNS) reagent (1%)- Dissolve 1g of dinitro salicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite (Na2SO3) in 100ml of 1% NaOH.

*vi* Sodium-potassium tartrate (40%) - Dissolve 20 g of Na-K tartrate in 50 ml distilled water.

vii Standard maltose solution (1 mg/ml) - 100 mg maltose dissolved in 100ml distilled water.

viii Working standard maltose(100µg/ml) - Dilute 10 ml of the standard solution to 100ml

with distilled water.

ix Mercuric chloride (0.02%)- Dissolve 100 mg HgCl2 in 500 ml dH20.

x Starch solution (1%) - 1 g starch dissolved in the sodium acetate buffer. Light warming

may be required.

xi Water bath (270C), spectrophotometer, centrifuge.

xii Test tubes, pipettes, mortar pestle, centrifuge tubes etc.

**Seed germination and enzyme extraction**

1. Surface sterilize 20 barley or wheat seeds by soaking in HgCl2 solution for 5 min.
2. Remove mercuric chloride by washing seeds for 3-4 times with dH2 0.
3. Allow them to germinate on sterile water, soaked filter paper sheets placed in petri plates at 25°C.
4. When embryos begin to germinate (3-4 days), weigh the seeds and grind them with 5 ml of ice cold 10mM calcium chloride solution.
5. Keep the extract at 25°C for atleast 3h. centrifuge the extract at 10,000 rpm for 10 min at 4ºC.
6. Take out the supernatant and make up its volume to 10 ml with the calcium chloride solution (enzyme extract). Dilute 1 ml of this extract with acetate buffer to 10 ml (diluted enzyme extract).

**Enzyme assay**

1. In each of the two test tubes, take 1 ml of the starch solution and 1 ml of the diluted enzvme extract (set up in ice).
2. Add 2 ml DNS reagent in one of the tubes, marked as control. Heat the test tube in boiling water bath for 5 min and then add 1 ml of sodium potassium tartrate solution. Meanwhile, incubate the other test tube at 27°C for 15 min.
3. Stop the reaction by adding 2 ml DNS reagent and heat the test tube in boiling water bath for 5 min.
4. Add 1 ml of sodium potassium tartrate while the tubes are warm.
5. Cool both the tubes under running tap water and make up the volume to 10 ml with distilled water.
6. Read absorbance at 540 nm against the control.
7. Calculate mg of maltose produced per 5 min (enzyme unit) using the standard curve of maltose.
8. Express the result as unit of amylase per g of germinating seeds.

**Preparation of standard curve of maltose**

1. Pipette out 0.2, 0.4, 0.6, 0.8 and 1.0 ml of working standard solution of maltose in different test tubes.
2. Make up the volume in each tube to 2 ml with distilled water. In control, take 2 ml distilled water only.
3. Add 2 ml DNS reagent to each tube, mix and heat in boiling water bath for 5 min.
4. Take out the tubes from water bath and add 1 ml of 40%Na-K tartrate solution
5. Cool the tubes under running tap water and make up the volume to I 0 ml with dH20.
6. Read absorbance at 540 nm against the control

**OBSERVATION** :

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| S.No | Volume of Working standard(ml) | Concentration of maltose(µg) | Volume of water (ml) | Volume of DNS (ml) | Incubation in hot water bath 5 min | Volume of 40% sodium potassium tartrate (ml) | OD at 540nm |
| 1 | 0 | 0 | 2.0 | 2.0 | 1.0 |  |
| 2 | 0.2 | 20 | 1.8 | 2.0 | 1.0 |  |
| 3 | 0.4 | 40 | 1.6 | 2.0 | 1.0 |  |
| 4 | 0.6 | 60 | 1.4 | 2.0 | 1.0 |  |
| 5 | 0.8 | 80 | 1.2 | 2.0 | 1.0 |  |
| 6 | 1.0 | 100 | 1.0 | 2.0 | 1.0 |  |

Plot a graph between quantity of maltose (µg) and OD at 540 nm. It should be a straight line passing through the origin.

**CALCULATION:**

**Enzyme assay**

i Weight of germinating seeds = 'm' g

ii Final volume of the enzyme extract= 10 ml

iii Aliquote taken for enzyme assay = 1 ml

iv Incubation period for enzyme assay = 15 min.

v O.D. of the contents in the sample tube= 'd'

Suppose 'd' absorbance corresponds to 'a' µg of maltose in the standard curve, that means:

1ml of the diluted enzyme extract in 15min produces ='a' µg maltose

10 ml enzyme extract in 5 min produces = a x 5 x 100 µg

(obtained from 'm' g seeds) 15

(Note : the enzyme extract was diluted 10 times)

= a mg

30

Hence, enzyme unit of amylase from 1 g seeds = a units

30xm

**Result**

Activity of amylase in the given seeds of barley (or wheat) -------units.

**B. INVERTASE**

**INTRODUCTION:**

Sucrose, commonly known as table sugar, is a disaccharide composed of an alpha-D-glucose molecule and a beta-D-fructose molecule linked by an alpha-1,4-glycosidic bond. When this bond is cleaved in a hydrolysis reaction, an equimolar mixture of glucose and fructose is generated. This mixture of monosaccharides is called *invert sugar*.Sucrose can be hydrolyzed in the presence of an enzyme called *invertase* or *sucrase*.

Sucrose + H2O ---> glucose + fructose

The official name for invertase is beta-fructofuranosidase, this enzyme involves the hydrolysis of the terminal non-reducing beta-fructofuranoside residues in beta-fructofuranosides. Invertase is mainly used in the food (confectionery) industry. A wide range of microorganisms produce invertase and can, thus, utilize sucrose as a nutrient. Commercially, invertase is biosynthesized chiefly by yeast strains of *Saccharomyces cerevisiae* or *Saccharomyces carlsbergensis*. Even within the same yeast culture, invertase exists in more than one form. For example, the intracellular invertase has a molecular weight of 135,000 Daltons, whereas the extracellular variety has a molecular weight of 270,000 Daltons.

**Aim:** To determine the enzyme activity of invertase

**Principle:**

Invertase assay uses a yellow reagent called DNS (43.9 mM 3,5-dinitrosalicylic acid and 250mM NaOH) that changes to an amber color in the presence of reducing sugars, such as glucose and fructose. Reducing sugars can act as mild reducing agents due to an aldehyde group. Because sucrose is not a reducing sugar, the activity of the invertase enzyme can be determined by the intensity of the amber color in the reaction tubes after time is allowed for sucrose hydrolysis and subsequent addition of DNS reagent. Invertase catalyzes the conversion of sucrose into glucose and fructose, which results in the DNS reagent turning amber. The intensity of the amber color can be measured in a spectrophotometer as absorbance, which is proportional to the amount of glucose and fructose produced during hydrolysis

**Materials and reagents:**

1. Enzyme

* + Invertase from Baker's yeast, 32 units/mg. (See Note 1.)
  + Stock solution: 1g/l.

2. 0.1 M Sodium acetate buffer: pH 4.7: Dissolve 3.4g of sodium acetate in 200ml of distilled water and adjust the pH to 4.7 by adding glacial acetic acid and make the volume up to 250ml.

4. Dinitrosalicylic acid:

Solution A: Dissolve 1 g of DNS in 20ml of 2N NaOH solution.

Solution B: Dissolve 30g of sodium Potassium tartarate in 60ml of distilled water. Mix solutions A and B and heat up to 80-90oC.The resulting solution is clear DNS

4. Standard sucrose solution: Stock solution :1g/100ml, working solution 0.4,0.8,1.2,1.6,2.0 mg/ml.

6. Enzyme dilution: Dilute crude enzyme in the ratio 1:25

**Procedure:**

1. Take 9 test tubes.

2. Pipette standard sucrose solutions to test tubes and make the volume 2 ml by adding distilled water and name the test tubes from 1-6.

3. Add 1 ml of sucrose solutions to 7, 8 and 9 test tubes respectively.

4. Add 1, 0.5 and 0 ml Sodium acetate buffer to 7,8 and 9 test tubes respectively.

5. Add 0, 0.5 and 1ml of diluted enzyme to 7,8 and 9 test tubes respectively.

6. Vertex the test tubes and incubate at 37o C for 15 min.

7. Arrest the enzyme substrate reaction by adding 1ml of DNS solutions to all test tubes.

8. Boil the test tubes in water bath for 5 min.

9. Add 5 ml of distilled water to all test tubes and vertex

10. Check the OD of solutions for all test tubes.

**Observation**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| S.No | Volume of sucrose (ml) | Concentration (µg) | Volume of water (ml) | Volume of DNS (ml) | Incubation in hot water bath 5 min | Volume of distilled water (ml) | OD at 540nm |
| 1 | 0 | 0 | 2.0 | 1.0 | 5.0 |  |
| 2 | 0.4 | 400 | 1.6 | 1.0 | 5.0 |  |
| 3 | 0.8 | 800 | 1.4 | 1.0 | 5.0 |  |
| 4 | 1.2 | 1200 | 0.8 | 1.0 | 5.0 |  |
| 5 | 1.6 | 1600 | 0.4 | 1.0 | 5.0 |  |
| 6 | 2.0 | 2000 | 0 | 1.0 | 5.0 |  |

**Enzyme activity**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Test tubes | Vol of substrate (ml) | Vol of buffer (ml) | Vol of enzyme (ml) | Incubate for 15 min at room temperature | Volume of DNS (ml) | Incubation in hot water bath 5 min | Vol of distilled water (ml) | OD at 540nm |
| 7 | 1.0 | 1.0 | 0 | 1.0 | 5.0 |  |
| 8 | 1.0 | 0.5 | 0.5 | 1.0 | 5.0 |  |
| 9 | 1.0 | 0 | 1.0 | 1.0 | 5.0 |  |

**Calculation:**

Enzyme activity =……………..µg/15min/ml of diluted enzyme

=……………..µg/min/ml of diluted enzyme

Enzyme activity for undiluted enzyme=………………….X25

=µg/min/ml of undiluted enzyme

Total volume of enzyme extract =……………………………..

Total enzyme activity for ………x……….ml of enzyme taken from …y…….g of source

= …………\*x/y Total α Amylase Enzyme activity in y g of source= ………./1000

= ……………….mg/min/ml

**RESULT**: Total α Amylase Enzyme activity = ……………….mg/min/ml