12. Investigation of the effect of temperature on enzyme activities

Starch is a mixture of two polysaccharides, amylose and amylopectin. The relative amounts of the two polysaccharides vary widely in different types of starch. Amylose molecules consist of long straight chains of glucose units. Amylopectin has a complicated branched structure. The two polysaccharides combine with iodine to give the characteristic blue-black colour of the standard iodine test.

Through the process of hydrolysis, amylase degrades starch by splitting the long glucose chains into smaller intermediates, finally producing a complex mixture containing predominantly maltose, but with some glucose and some small branched oligosaccharides. This mixture does not react with iodine to give blue-black colour.

Amylases are found in almost all plants, animals and microorganisms. Large amounts of amylase occur in germinating cereals, and in the pancreas and saliva of higher animals.

The optimum pH of animal amylases is between 6.9 and 7.1, pH 7.0 is most suitable for their activities. They are activated by chloride ion. However, most amylases extracted from plants and microorganisms have an optimum pH of about 4.5 to 5.0. These amylases are not activated by chloride ion, but can be inhibited by heavy metal ions, iodoacetamide and urea.

In this investigation, amylase extracted from germinating mung beans is used to act on a given quantity of starch at
different temperatures. When nearly all the starch is hydrolysed, the reaction mixture will give a negative result to iodine test. The reciprocal of the time required for the disappearance of starch will give an indication of the relative rate of enzymatic reaction.

Procedure

A. **Extraction of amylase from mung beans**
   1. Amylase can be obtained from 20 mung beans that have been germinated for 72 hours between moist paper towels.
   2. Remove the testa. Grind the softened beans in a mortar with 2 cm$^3$ of stabilising peptone solution into a fine suspension.
   3. Dilute with another 8 cm$^3$ of peptone solution. After standing in the mortar for about 5 minutes with gentle stirring, the mixture is centrifuged for 5 minutes. The supernatant contains the amylase.
      (Another method: Filter the mixture through four layers of muslin and use the filtrate as the source of amylase.)

B. **Initial measurement of enzyme activity**
   1. Put 2 cm$^3$ of 1% starch solution and 2 cm$^3$ of pH 4.7 acetate buffer into a test tube.
   2. Use a dropper to add one drop of iodine solution to each cavity of a spot plate.
   3. Use a graduated pipette to add 1 cm$^3$ of the enzyme extract to the test tube, and start the stopwatch at the same time. Shake the tube vigorously, quickly transfer one drop of the reaction mixture onto one of the iodine samples and stir with a glass rod.
4. At 2-minute intervals, shake the tube, transfer a further drop of mixture onto a fresh sample of iodine solution. Continue until no positive results are obtained.

5. If the above reaction time is too short, dilute the enzyme extract with pH 4.7 acetate buffer so that 1 cm$^3$ of the diluted enzyme extract can hydrolyse most of the starch in about 10 minutes.

C. The effect of temperature on amylase activity

1. Use a dropper to add one drop of iodine solution to each cavity of seven spot plates.

2. Label 7 test tubes from 1A to 7A.

3. Use graduated pipettes to add 2 cm$^3$ of pH 4.7 acetate buffer and 1 cm$^3$ of diluted enzyme extract to each tube.

4. Label another 7 test tubes from 1B to 7B. Use a graduated pipette to add 2 cm$^3$ of 1% starch solution to each tube.

5. Place the above tubes in water baths maintained at different temperatures for 5 minutes as shown below.

<table>
<thead>
<tr>
<th>Water bath</th>
<th>Temperature of water bath (°C)</th>
<th>Tubes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1A &amp; 1B</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>2A &amp; 2B</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>3A &amp; 3B</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>4A &amp; 4B</td>
</tr>
<tr>
<td>5</td>
<td>40</td>
<td>5A &amp; 5B</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>6A &amp; 6B</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>7A &amp; 7B</td>
</tr>
</tbody>
</table>

6. Add the starch solution from each “B” tube to the corresponding “A” tube. Start the stopwatch immediately. Mix well and place the “A” tubes in their respective water baths described in step (5).
7. At 2-minute intervals, shake each tube and transfer one drop of reaction mixture onto an iodine sample. Note for the disappearance of blue-black colour and record the time.

(The test may be done at 1-minute intervals if the students can manage to do the test for all seven tubes within this time interval.)

**Note**

1. In carrying out the iodine test for starch in the assay of amylase activity, it is advisable to use a very dilute iodine solution.

2. As the brown colour will fade gradually, it is advisable not to leave the iodine solution in the cavities for too long.

3. A spot colour made up by mixing 0.01% of starch solution and iodine solution may be used as an arbitrary standard for the absence of starch.

**Preparation of Reagent**

1. Peptone solution

   A peptone solution is made by dissolving 20 g peptone in 1000 cm³ of hot distilled water. It must be freshly prepared and should not be kept for more than 24 hours.

2. pH 4.7 acetate buffer

   The buffer solution is prepared by using 8 g anhydrous sodium acetate and 6 cm³ glacial acetic acid made up to 1000 cm³ with distilled water. It should be freshly prepared and should not be kept for more than 24 hours.

3. Iodine solution

   A stock iodine solution is prepared by dissolving 1 g iodine and 2 g potassium iodide in 1000 cm³ of distilled water. 0.5 cm³ of the stock solution is added to 10 cm³ of distilled water prior to use.