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DIASTASE ACTIVITY (α-AMYLASE) IN HONEY

ASSAY PROCEDURE

T-AMZHY 05/14

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INTRODUCTION:

The traditional method for the measurement of diastase activity in honey is the Schade procedure[1]. One unit of diastase activity (or more specifically, α-amylase), the Schade or Gothe unit, is defined as that amount of enzyme which will convert 0.01 gram of starch to the prescribed end-point in one hour at 40°C under the conditions of the test. Results are expressed in Schade units per gram of honey and termed Diastase Number (DN). In this assay, a standard solution of starch, capable of developing, with iodine, a colour in a defined range of intensity, is acted upon by the enzyme in the sample under standard conditions. The diminution in the blue colour is measured at intervals. A plot of absorbance against time, or a regression equation, is used to determine the time t_x required to reach the specified absorbance, 0.235. The Diastase Number is calculated as 300 divided by t_x, provided the method is followed precisely.

An alternative procedure employing Phadebas® test tablets was introduced[2], and subsequently updated[3]. This method is based on the use of an insoluble, dyed starch substrate. As this substrate is hydrolysed by α-amylase, soluble dyed starch fragments are released into solution. The reaction is terminated and insoluble substrate removed by centrifugation. The absorbance of the supernatant solution (at 620 nm) is directly proportional to the diastase activity of the sample. This procedure has been widely adopted within the honey industry due to the convenience of a commercially available substrate and the simpler assay format. In 1992, Megazyme developed Amylazyme tablets for the measurement of α-amylase (diastase activity) in cereal and food products. These tablets are now widely used in the cereals and food industries and an assay format based on their use is a recommended procedure of the American Association of Cereal Chemists (AACC Method 22-05)[4,5] for the measurement of α-amylase in cereal grains and flours. These tablets can readily be adapted to the measurement of α-amylase (diastase activity) in a range of food products including honey.

PRINCIPLE:

Amylazyme tablets, in principle, are very similar to Phadebas® tablets. Amylazyme tablets contain dyed and crosslinked amylose (the linear fraction of starch). In contrast to the substrate in Phadebas tablets, when Amylazyme tablets are added to buffer solution, they rapidly hydrate and the particles of dyed, crosslinked amylose absorb the buffer solution. In the presence of α-amylase, the substrate is hydrolysed and soluble dyed products are released. The reaction is terminated and, following filtration, the absorbance of the filtrate is

measured at 590 nm. The absorbance of the filtrate is directly proportional to the diastase activity of the sample. **A major advantage of Amylazyme over Phadebas®** is that the degree of crosslinking in the Amylazyme tablets has been optimised to ensure rapid hydration and swelling of the substrate. In practice, this means that the tablet can be added to the enzyme containing solution, and the reaction performed with no need for stirring or shaking of the incubation mixture. The assay format has been adjusted to take advantage of this property.

Since the standard units employed by the honey industry are Schade units, for this application the Amylazyme has been standardised in Schade units. A curve showing the relationship between absorbance at 590 nm (Amylazyme tablets) and Schade units based on the dilution of a single standardised honey solution is shown in Figure 1. A correlation between Schade units and increase in absorbance at 590 nm (Amylazyme tablets) for a number of honey samples is shown in Figure 2. Use of the latter curve is recommended for the calculation of enzyme activity.

SPECIFICITY AND LINEARITY:

The Amylazyme test is absolutely specific for α-amylase. For α-amylase in a diluted honey sample, the curve is relatively linear over the absorbance range of 0.1 to 1.2 absorbance units, but the use of a polynomial regression equation is recommended (see Figure 1). In a comparison of the diastase activity in a number of samples analysed by the Schade method and the Amylazyme procedure, the line of best fit is linear and intersects the origin. The regression equations are very similar, however some samples deviate significantly from the curve of best fit. The reason for this is not clear.

INTERFERENCE:

The assay can be applied to all honey samples. At the sample dilution used, the concentration of sugars in the samples interferes with neither enzyme activity nor the filtration process.

SAFETY:

The reagents used in the determination of diastase activity in honey are not hazardous materials in the sense of the Hazardous Substances Regulations. The general safety measures that apply to all chemical substances should be adhered to.

REAGENTS AND SOLUTIONS:

All reagents must be of analytical grade.

1. **Amylazyme tablets.**
   Available from Megazyme in packs of 200 tablets. Ensure that the lot number relates to the standard curve in the booklet. Stable at room temperature for > 5 years.

2. **Sodium maleate buffer (100 mM, pH 5.6) plus calcium chloride (5 mM).**
   Add 11.6 g of maleic acid (Sigma cat. no. M-0375) and 0.735 g of calcium chloride dihydrate ($CaCl_2.2H_2O$) (Sigma cat. no. C-5080) to 800 mL of distilled water and adjust the pH to 5.6 with 2 M (8 g/100 mL) sodium hydroxide solution. Adjust the volume to 1 litre. Store the buffer in a 1 L Duran® bottle and overlay the solution with 2 drops of toluene to prevent microbial contamination. Stable at 4°C for approx. 2 months.

3. **Trizma base solution.**
   Add 20 g of Trizma base (Sigma cat. no. T-1503) to 900 mL of distilled water and dissolve. Check the pH and adjust to approx. 8.5 if necessary. Adjust the volume to 1 L with distilled water. Stable at room temperature for 12 months.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 120 mm, ~ approx. 17 mL capacity).
2. Micro-pipettor, e.g. Gilson Pipetman® (1 mL) for dispensing enzyme containing solutions.
3. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 25 mL Combitip® (to dispense buffer and diluted enzyme preparations).
4. Adjustable-volume dispenser (set at 10 mL) for dispensing Trizma base solution).
5. Analytical and top-pan balance.
6. Spectrophotometer set at 590 nm.
7. Thermostated water bath set at 40°C (e.g. Julabo PC®).
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Stop clock.
10. Whatman No.1 (9 cm) filter papers and filter funnels.

CONTROLS AND PRECAUTIONS:

1. The time of incubation of the enzyme extract with the Amylazyme tablet must be carefully controlled (i.e. 10.0 min).

2. Incubation temperatures must be accurately controlled (i.e. 40 ± 0.1°C).

3. When the Trizma base solution is added to the reaction tube, the tubes must be stirred vigorously to ensure thorough mixing. Leave the tubes at **room temperature** for 5 min and stir the contents again before filtration.

4. Following the addition of the Trizma base solution to the reaction tubes, the tubes must be stored at room temperature. At room temperature, the substrate suspension is stable for at least 1 hr. If this alkaline mixture is left in the water bath at 40°C, the dyed substrate will slowly degrade leading to elevated absorbance values on filtration.

5. With each set of analyses a single **reaction blank** should be run. This is performed by adding an Amylazyme tablet to the sodium maleate buffer and proceeding as for the enzyme assays.

6. Test tablets should be stored dry in well sealed containers at room temperature.

7. The Amylazyme assay is affected by the concentration of buffer salts. The optimal buffer salt concentration is 100 mM.

PREPARATION OF HONEY SAMPLES FOR ANALYSIS:

1. Since the α-amylase activity of honey can change on storage, the samples must be stored at approx. 5°C.

2. Weigh 2.00 g of honey into a 100 mL beaker and dissolve in 40 mL of 100 mM sodium maleate buffer (pH 5.6). Quantitatively transfer the solution to 50 mL volumetric flask and adjust to volume.

ASSAY PROCEDURE:

1. Dispense 1.0 mL of diluted honey sample to the bottom of a 16 x 120 mm tube and pre-incubate at 40°C for 5 min.

2. Using forceps, add an Amylazyme tablet to the tube without removing the tube from the water bath. Do not stir the tube, the tablet will hydrate rapidly and absorb most of the free liquid.

3. Incubate the tube at 40°C for exactly 10 min.

4. Add 10 mL of Trizma base solution (2% w/v, Sigma cat. no. T-1503 to terminate the reaction and stir the tube vigorously on a vortex mixer. Allow the tubes to sit at room temperature.

5. After approx. 5 min, stir the tubes again and filter the contents through Whatman No. 1 (9 cm) filter paper.
6. Measure the absorbance of the sample solutions at 590 nm against a reaction blank.

> **NOTE:**
>
> A single blank is required for each set of determinations and this is used to zero the spectrophotometer. The absorbance of the reaction solutions is then measured against the reaction blank (see Controls and Precautions 5).

STANDARDISATION:

Standard curves relating the activity of diastase activity in honey to absorbance at 590 nm on Amylazyme tablets are shown in Figures 1 and 2. In Figure 1, a single honey sample was employed at a range of dilutions. In contrast, the curve in Figure 2 relates the diastase activity of a number of samples in Schade units (determined using the Phadebas® method) to absorbance increase at 590 nm on incubation with Amylazyme tablets. The regression equation in Figure 1 is slightly curved, however, the curve in Figure 2 is essentially linear. **The regression equation from Figure 2 should be used to calculate the diastatic activity in unknown honey samples.**

CALCULATION OF ACTIVITY:

The α-amylase activity of a sample (as Schade or Gothe units per gram of honey) is determined by reference to the standard curve in Figure 2 or by use of the associated regression equation.

Diastase activity (DN) (Schade units/gram of honey)

= 20.0 x ΔAbs (Amylazyme Lot 40101)

This equation only applies if the sample has been diluted as described in the assay format, and the assay is performed exactly as described (e.g. 10 min, 40°C, pH 5.6 and sample volume of 1.0 mL).

If absorbance values for a sample are below 0.30, the sample should be re-assayed and the incubation time increased to 20 min (doubled). Diastatic activity calculated from the regression equation should then be divided by 2 to give the correct values in Schade units/gram.

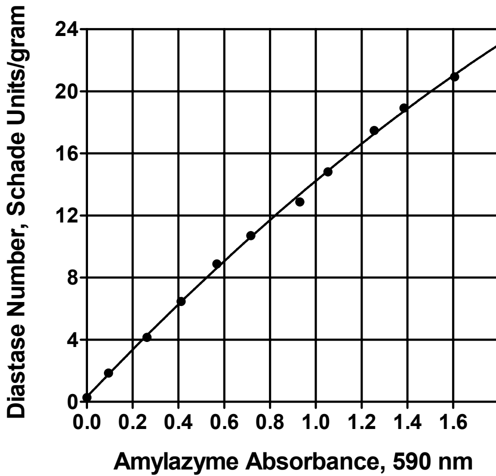

Figure 1. **Standard curve relating absorbance increase at 590 nm with the Amylazyme test (Amylazyme Lot 40101) to Diastase Number for a single honey sample at various dilutions.**

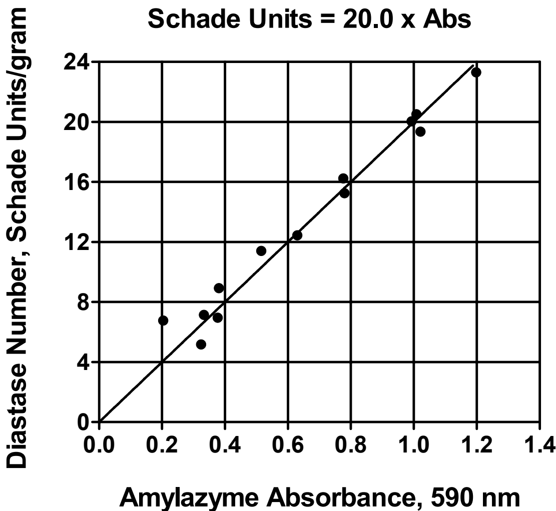

Figure 2. **Curve relating Diastase Number for a number of honey samples to absorbance increase at 590 nm with the Amylazyme test (Amylazyme Lot 40101) performed under standard conditions.**

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