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AMYLAZYME

ALPHA-AMYLASE

ASSAY PROCEDURE

T-AMZ 05/18

For the Measurement of Cereal and Microbial α-Amylases

AACC Method 22-05.01

INTRODUCTION:

The level of α-amylase in wheat and barley grains and malted barley is a key quality parameter. Elevated levels of this enzyme in wheat and barley are used as an indication of pre-harvest sprouting. In malted barley, high levels of α-amylase are required to catalyse starch solubilisation and dextrinisation during the mashing process of beer manufacture.

Numerous procedures have been developed for the measurement of α-amylase in malt. Many of these are based on the measurement of the changes in colour produced on the interaction of iodine with the β-limit dextrin of starch. Due to the high levels of α-amylase in malt, a range of other methods are also readily applicable, such as dye-labelled starch methods and the Ceralpha method.[1] The measurement of α-amylase in the flours from weather damaged (pre-harvest sprouted) wheat and barley grains presents a special problem. The levels of enzyme to be measured are very low, and extracts of the flour contain high levels of starch which acts as an alternative substrate. However, due to the industrial importance of pre-harvest sprouting, numerous methods have been developed, and continue to be developed. The traditional "Falling Number Method" and "Amylograph Viscosity Method" are widely used by millers, bakers and plant breeders. Where large numbers of assays need to be performed, various improved iodine-based or dye-labelled substrate methods are employed. Detailed evaluation of the Phadebas® (dye-labelled starch) method by Barnes and Blakeney[2] clearly demonstrated a good correlation between this method and the Falling Number and Amylograph methods. Procedures based on the use of crosslinked, dye-labelled starch substrates have been adopted as standard methods in America[3], Europe[4] and Australia.[2] In Australia, the Amylazyme procedure[5] is widely used for the measurement of α-amylase in pre-harvest sprouted wheat and barley.

SUBSTRATE:

The substrate employed is azurine-crosslinked amylose (AZCL- Amylose). This is prepared by dyeing and cross-linking high amylose maize starch to produce a material which hydrates in water but is water insoluble. Hydrolysis by α-amylase produces water soluble dyed fragments, and the rate of release of these (increase in absorbance at 590 nm) can be related directly to enzyme activity. This substrate specifically measures α-amylase in the presence of large excesses of β-amylase and amyloglucosidase. The substrate is supplied commercially in a ready-to-use tablet form as Amylazyme tablets. All activities are converted to Ceralpha Units through standard curves. Details of the Ceralpha method are available at www.megazyme.com. The Ceralpha assay is one of the few assays for α-amylase which employs a well defined substrate and allows the specific measurement of α-amylase in the presence of other starch degrading enzymes and reducing sugars.

AVAILABILITY:

Amylazyme tablets are available directly from Megazyme in pack sizes of 200 or 1000 tablets. The tablets are unbuffered, allowing their use over a wide pH range. The substrate is very stable and can be used at temperatures up to 60°C. However, the substrate is not stable in alkaline solutions at high temperatures.

PRINCIPLE:

Formats have been developed for the assay of samples containing trace quantities of α-amylase (e.g. as in weather damaged wheat and barley grains) and those with high enzyme activity (e.g. malted barley and microbial enzyme preparations).

Activity in malted barley and microbial preparations is measured simply by adding an Amylazyme tablet to an aliquot (1 mL) of pre-equilibrated and suitably diluted (in the assay buffer) enzyme preparation. The tubes are incubated without stirring under controlled conditions of time and temperature. The reaction is terminated with a weak alkaline solution, the slurries are filtered and the absorbances of the filtrates are measured (refer to Method 1).

With flours from weather-damaged wheat and barley, the enzyme is simultaneously extracted and assayed. Assays are performed at 60°C to increase the sensitivity of the test (refer to Method 2). To facilitate these assays, Megazyme has developed and supplies an incubation bath (Megazyme Incubation Bath Mk III), which when used with an IKA Labortechnik KMO 2 basic magnetic stirrer and an immersion heater (e.g. Julabo PC), allows controlled stirring and incubation of 16 samples simultaneously.

ASSAY METHOD 1: MALT AND MICROBIAL ENZYME PREPARATIONS

EXTRACTION AND DILUTION BUFFERS:

A. Buffer A (for cereal α-amylase**)**

[Sodium maleate (100 mM, pH 6.0) plus calcium chloride (5 mM) and sodium azide (0.02% w/v)]

Dissolve maleic acid (11.6 g, Sigma cat. no. M0375) in 900 mL of distilled water and adjust the pH to 6.0 with sodium hydroxide solution (2 M) (approx. 80 mL is required). Add calcium chloride dihydrate (0.74 g) and sodium azide (0.2 g) and readjust the pH to 6.0. Adjust the volume to 1 L. Store at room temperature.

NOTE: Do not add the sodium azide until the pH is adjusted. Acidification of sodium azide releases a poisonous gas.

B. Buffer B (for *A. oryzae* α-amylase)

[Sodium acetate (100 mM, pH 4.4) plus calcium chloride (5 mM) and sodium azide (0.02%)]

Add 5.9 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 4.4 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 30 mL is required). Add 0.74 g of calcium chloride dihydrate and 0.2 g of sodium azide and re-adjust the pH to 4.4. Adjust the volume to 1 L. Store at room temperature.

C. Buffer C (for *Bacillus* sp. α-amylase)

[MOPS buffer salt (100 mM, pH 7.0) plus calcium chloride (5 mM) and sodium azide (0.02%)]

Add 20.9 g of MOPS buffer salt (acid form; **Megazyme B-MOPS250**) to 900 mL of distilled water. Adjust the pH to 7.0 by the addition of 2 M sodium hydroxide (approx. 17 mL is required). Add 0.74 g of calcium chloride dihydrate and 0.2 g of sodium azide and re-adjust the pH to 7.0. Adjust the volume to 1 L. Store at room temperature.

EQUIPMENT RECOMMENDED:

1. Glass test tubes (16 x 120 mm, ~ 17 mL capacity, round bottomed).

2. Micro-pipettors, 1.0 mL (e.g. Gilson Pipetman®) (for the dispensing of enzyme preparation).

3. Adjustable-volume dispenser (for Tris Base solution).

4. Bench centrifuge (required speed 3,000 rpm, ~ 1,800 g), or Whatman GF/A glass fibre filter paper (9 cm diameter).

5. Analytical and top-pan balances.

6. Spectrophotometer set at 590 nm.

7. Vortex mixer (e.g. Vortex Genie 2®).

8. Thermostated water bath set at 40 ± 0.1°C (e.g. Julabo ED).

9. Stop clock.

10. Whatman No. 1 (9 cm) filter circles 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. After addition of Tris Base solution (**Megazyme B-TRIS500**; 2% w/v, pH 9) to the reaction tube, the tubes must be stirred vigorously to ensure thorough mixing. Tubes are left at room temperature for 5 min before filtration.

4. On addition of the Tris Base solution to the reaction tubes, the tubes must be stored at room temperature. Under these conditions, the substrate is stable for several hours. Storage of the substrate suspension under alkaline conditions at elevated temperatures will lead to slow hydrolysis and release of dye molecules, resulting in elevated blank and/or reaction values.

5. With each set of analyses a single reaction blank should be run. This is performed by adding an Amylazyme tablet to the appropriate 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 significantly affected by the concentration of buffer salts. The optimal buffer salt concentration is 100 mM.

ENZYME EXTRACTION AND DILUTION:

1. Malt Flour:

Mill malt samples to pass a 0.5 mm mesh. Add 1.0 g of malt flour to 20.0 mL of Buffer A, mix by inversion and allow to extract over 15 min. Centrifuge the slurry at 1,000 g for 10 min or filter through a Whatman GF/A glass fibre filter paper. Dilute an aliquot (0.5 mL) of the filtrate 100-fold by addition to 49.5 mL of buffer A. Perform assays within 2 h.

2. Wheat and Barley Flour:

Mill wheat or barley samples to pass a 0.5 mm mesh. Assay samples as per Method 2 (page 12) in this booklet.

3. *A. oryzae* and *A. niger* Preparations:

Add 1.0 g of powdered enzyme preparation to 100 mL of Buffer B and allow to extract over 10 min (with stirring). Centrifuge an aliquot of the slurry or filter as for the malt samples. Dilute these filtrates 10-fold by adding 0.5 mL of the preparation to 4.5 mL of Buffer B. Repeat this process of dilution until a concentration suitable for assay is achieved. Typically, for concentrated industrial preparations, dilutions of up to 10,000-fold (of the original solution) may be required.

For liquid enzyme preparation, dilute 1.0 mL of the preparation to 100 mL in Buffer B, and then further dilute as for extracts of the powder samples.

4. *Bacillus* sp. Preparations:

Dilute 1.0 mL of liquid enzyme preparation to 100 mL in Buffer C. Dilute an aliquot (0.5 mL) of this solution 10-fold by addition to 4.5 mL of Buffer C. Repeat this process of dilution until a concentration suitable for assay is achieved. For example, for the *Bacillus licheniformis* α-amylase preparation from Megazyme (cat. no. **E-BLAAM**), a dilution of 100,000-fold is required.

ASSAY PROCEDURE:

1. Pre-equilibrate 1.0 mL of suitably diluted enzyme preparation or flour extract (in the appropriate buffer) at 40°C for 5 min.

2. Add an Amylazyme tablet to each tube (without stirring). The tablet hydrates rapidly and absorbs most of the free liquid. **Do not stir the suspension**. Incubate the tubes at 40°C for exactly 10 min.

3. After 10 min from the time of addition of the tablet, add 10.0 mL of Tris Base solution (pH 9, 2% w/v, **Megazyme B-TRIS500**) and vigorously stir the tubes on a vortex mixer. Leave the tubes at room temperature.

4. Stir the tubes again after approx. 5 min and filter the contents through Whatman No. 1 (9 cm) filter paper.

5. Measure the absorbance of the filtrate at 590 nm against the reaction blank.

6. Prepare a substrate/enzyme blank by adding Tris Base to the enzyme solution before addition of the Amylazyme table.

NOTE:

1. A single blank is required for each set of determinations and this is used to zero the spectrophotometer. The absorbance of the reaction solutions are then measured against the reaction blank (see Controls and Precautions 5, page 4).

2. Since bacterial α-amylases have optimal activity at pH 7.0, the Tris Base solution (pH ~ 9.0) does not completely terminate the enzyme reaction. It is thus essential that these reaction solutions are filtered within 5 min of "terminating" the reaction. Alternatively, the reaction can be terminated with tri-sodium phosphate solution (pH 11, 1% w/v).

STANDARDISATION:

Standard curves relating the activity of pure barley malt, *Aspergillus* niger and *Bacillus licheniformis* α-amylases on Amylazyme (Lot 40101) and Ceralpha reagent are shown in Figures 1-3. With the Ceralpha method, one Unit of enzyme activity is defined as the amount of enzyme required to release one micromole of *p*-nitrophenol from blocked *p*-nitrophenyl maltoheptaoside substrate mixture per minute at 40°C and the defined pH conditions. Full details of the Ceralpha α-amylase assay procedure are given in Megazyme Data Booklet K-CERA (www.megazyme.com), and the assay conditions are given in the figure captions (Figures 1-3).

pH Activity curves and the effect of buffer salt concentrations on these assays are shown in Figures 4-7. It is evident that the optimal pH for activity of each of these enzymes is quite different. The concentration of buffer salt affects the assay. The optimal buffer salt concentration for each of the enzymes studied was 100 mM.

CALCULATION OF ACTIVITY:

α-Amylase activity is determined by reference to the appropriate standard curve to convert absorbance (590 nm) to milli-Ceralpha Units of activity per assay (i.e. per 1.0 mL), and are then calculated as follows:

Units/gram or mL of original preparation:

$$= \text{milliUnits/assay} \times \frac{1}{1000} \times 100 \times \text{Dilution}$$

where:

$\frac{1}{1000}$ = conversion from milliUnits to Units

100 = initial extraction volume (i.e. 100 mL per g of solid)

Dilution = further dilution of the initial extraction solution

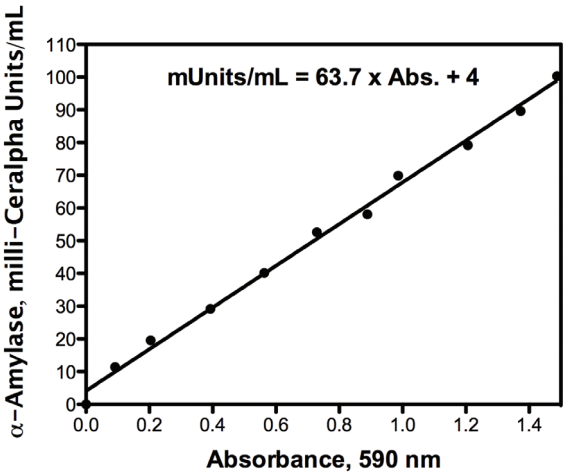

Figure 1. **Malted barley α-amylase standard curve on Amylazyme (Lot 40101).**
Amylazyme assay was performed at pH 6.0 and Ceralpha assay at pH 5.2 under standard conditions as described in this booklet and in Megazyme Booklet K-CERA (Ceralpha), using purified malt α-amylase.

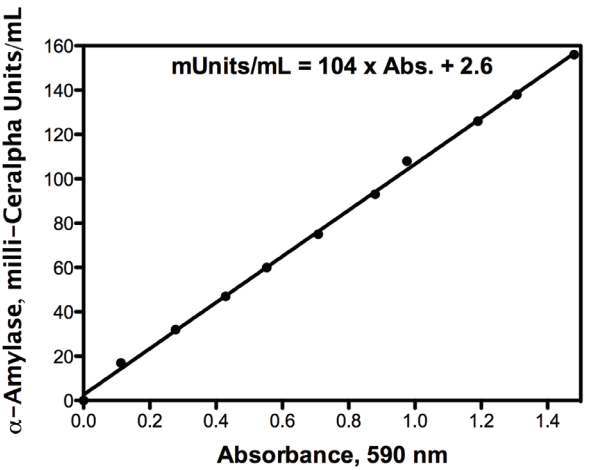

Figure 2. ***Aspergillus oryzae*** **α-amylase standard curve on Amylazyme (Lot 40101).**
The Amylazyme assay was performed at pH 4.4 and the Ceralpha assay at pH 5.0 under standard conditions as described in this booklet and in Megazyme Booklet K-CERA (Ceralpha), using pure A. oryzae α-amylase (Megazyme cat. no. E-ANAAM).

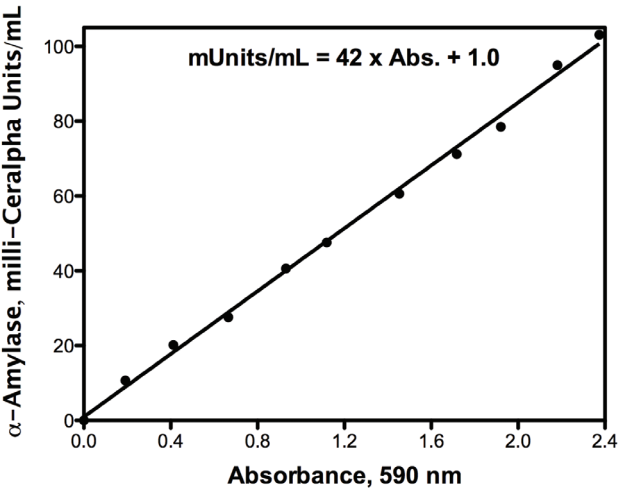

Figure 3. ***Bacillus licheniformis*** **α–amylase standard curve on Amylazyme (Lot 40101).**
The Amylazyme assay was performed at pH 7.0 and Ceralpha assay at pH 6.0 under standard conditions as described in this booklet and in Megazyme Booklet K-CERA (Ceralpha). The enzyme employed was purified Bacillus licheniformis α-amylase (Megazyme cat. no. E-BLAAM).

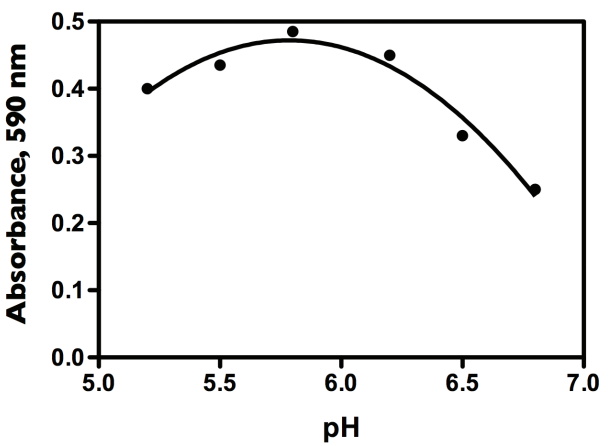

Figure 4. **pH Activity curve for malt α-amylase.**

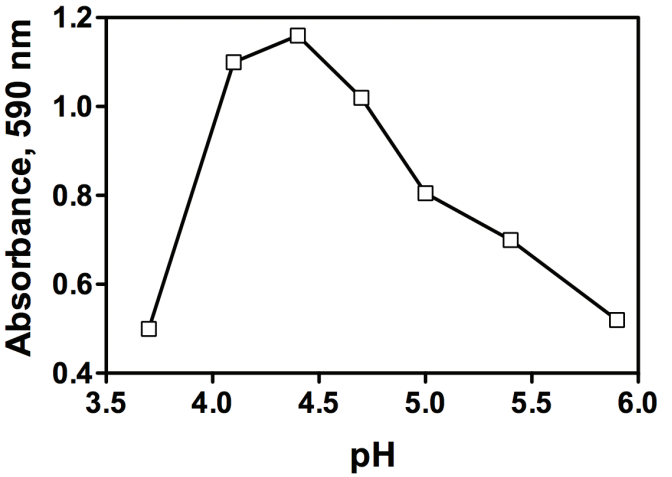

Figure 5. **pH Activity curve for *A. niger* α-amylase.**

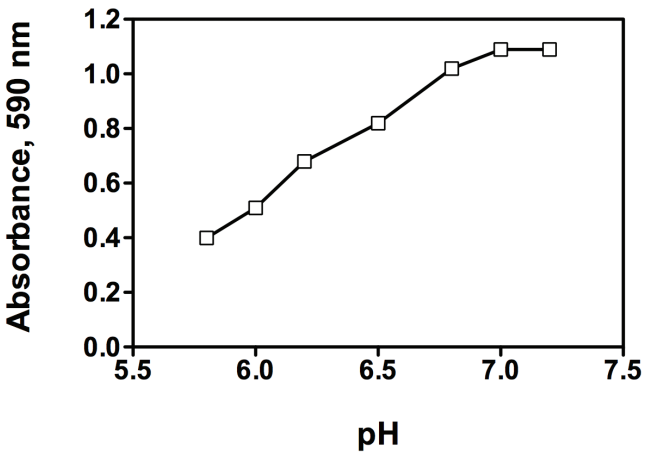

Figure 6. **pH Activity curve for *Bacillus* sp. α-amylase.**

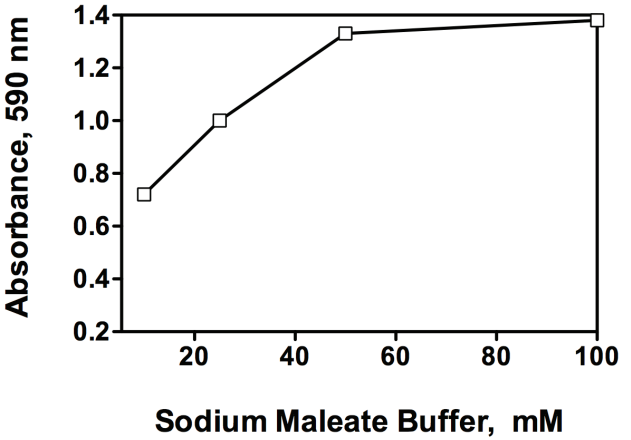

Figure 7. **Effect of buffer salt concentration on the activity of malt α-amylase on Amylazyme.**

ASSAY METHOD 2: FLOURS FROM PRE-HARVEST SPROUTED GRAINS AND FOOD PRODUCTS CONTAINING TRACE LEVELS OF α-AMYLASE

INTRODUCTION:

In this assay format, enzyme extraction and assay are performed concurrently. This allows the rapid analysis of large numbers of samples, which is essential in monitoring materials produced in plant breeding programs. This also allows the measurement of trace levels of α-amylase present in various food products such as custard powder mixtures.

PRINCIPLE:

Wheat flour samples are pre-equilibrated and extracted in maleate buffer (pH 6.0) with continuous stirring for 5 min at 60°C. An Amylazyme test tablet is added and stirring is continued for exactly 5 min at 60°C. The reaction is terminated and the slurry is filtered. The absorbance of the filtrate is measured at 590 nm and the activity is calculated.

With barley flour samples, the β-glucan in the sample results in the extract solution being highly viscous, and this adversely affects the stirring of the sample, and thus the disintegration of the substrate tablet. Uneven rates of disintegration of the substrate tablets can lead to poor reproducibility of the assay. To resolve this problem, tablets have been prepared (Amylazyme BG) which contain an active β-glucanase. As the tablet begins to disintegrate, the β-glucanase is released and depolymerises the β-glucan. This results in a rapid decrease in viscosity and rapid tablet disintegration.

To facilitate these assays, Megazyme has developed, and supplies, a purpose-built incubation bath (Megazyme Incubation Bath Mk III).

Since assays are performed directly on flour slurries, starch in the flour sample acts as an alternative substrate and thus alters the apparent α-amylase activity. For this reason, a standard curve has been prepared for partially purified wheat α-amylase in the presence of added flour (0.5 g; essentially devoid of α-amylase; Figure 8, page 15). This curve is used for the conversion of absorbance values to Ceralpha units. For barley α-amylase, the curve obtained with Amylazyme BG (Lot 60801) (in the presence of added barley flour) is shown in Figure 9, page 15.

EQUIPMENT (RECOMMENDED):

1. Megazyme Incubation Bath Mk III (or similar).
2. IKA Labortechnik KMO 2 basic, magnetic stirrer and PTFE coated stir bars (6 mm x 15 mm).
3. Julabo PC immersion heater (or similar) set at 60°C.
4. Glass test tubes (20 x 150 mm, ~ 30 mL capacity, round bottomed) (18 x 150 mm tubes can also be used).
5. Adjustable volume dispensers:
   - 5.0 mL (for maleate buffer)
   - 6.0 mL (for Tris Base solution).
6. Top-pan balance (accurate to 0.01 g).
7. Spectrophotometer set at 590 nm.
8. Vortex mixer (e.g. Vortex Genie 2®).
9. Whatman GF/A (9.0 cm) glass fibre filter papers with filter funnels.

ASSAY PROCEDURE:

1. Weigh 0.5 ± 0.01 g of wheat or barley flour sample into glass test tubes (20 x 150 mm) and tap the tubes to ensure all flour falls to the bottom. Place the tubes into the tube rack in the Megazyme Incubation Bath Mk III (at 60°C) and add a stir bar. Adjust the stirrer to a setting of 850 rpm.
2. Add 5 mL of sodium maleate buffer (100 mM, pH 6.0) (Buffer A, page 3), pre-equilibrated to 60°C, to each tube, and stir the tubes for 5 min.
3. Add an Amylazyme tablet (or Amylazyme BG tablet for barley flour samples) to each tube and allow the reaction to continue (with stirring) for **exactly 5 min**.
4. Add 6 mL of Tris Base (2% w/v, pH ~ 9.5) to each tube with vigorous stirring on a vortex mixer to terminate the reaction. **Leave the tubes at room temperature.**
5. After approx. **5 min**, stir the tubes again and filter the contents through Whatman GF/A glass fibre filter paper (9 cm circles).
6. Measure the **absorbance** of the filtrate at 590 nm against the reaction blank, and calculate the activity by reference to a standard curve (Figure 8, page 15).

NOTES:

1. A single blank is required for each set of determinations. This is prepared by adding the Tris Base solution to the flour slurry before the Amylazyme tablet. This slurry is stirred and **stored at room temperature** for 5 min before filtration.
2. In situations where the absorbance value is **greater than 2.0**,

simply dilute the blue filtrate with Tris Base (2% w/v) and re-measure the absorbance. The curve is essentially linear up to an undiluted absorbance value of 6.0. Alternatively, repeat the assay with a smaller sample size (e.g. 0.1 or 0.2 g of flour).

3. **Barley flour slurries** are more viscous than wheat flour slurries and the repeatability of the assay is reduced. Consequently, an alternative Amylazyme tablet containing bacterial β-glucanase (i.e. Amylazyme BG) has been developed. This β-glucanase depolymerises the β-glucan in barley flour samples giving a rapid viscosity drop. Results obtained for barley flour samples using these tablets are much more reproducible than those using standard Amylazyme tablets. Consequently, Amylazyme BG tablets are recommended for use with barley flour samples.
4. **Standard curves** relating values obtained with the Amylazyme method to those obtained with other methods are shown in **Figures 8-11**. The effect of the weight of flour analysed in the assay, is shown in **Figure 12,** and the linearity of the assay (in the presence of 0.5 g of flour) at higher absorbance values is shown in **Figure 13**. The results shown in Figures 12 and 13 were obtained by diluting the filtered reaction solution 4-fold before measuring the absorbance.

CALCULATION OF ACTIVITY:

$$\text{Units/g} = \text{milliUnits/0.5 g (i.e. per assay)} \times 2 \times \frac{1}{1000}$$

where:

milliUnits (per assay) is obtained from Figure 8 or Figure 9

2 = conversion from 0.5 g (as assayed) to 1 g of flour

$\frac{1}{1000}$ = conversion from milliUnits to Units

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1. McCleary, B. V. and Sheehan, H. (1989). "Measurement of Cereal α-amylase: A New Assay Procedure". **Journal of Cereal Science 6**, 237-251.
2. Barnes, W. C. and Blakeney, A. B. (1974). "Determination of Cereal Alpha Amylase". **Starch 26**, 193-197.
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4. European Brewing Convention, Analytica EBC (fourth edition; 1987). **Method 4.12.3**. "Alpha-Amylase (Colorimetric Method)".
5. McCleary, B. V. (1991). "Measurement of Polysaccharide Degrading Enzymes Using Chromogenic and Colorimetric Substrates". **Chemistry in Australia**, 398-401.

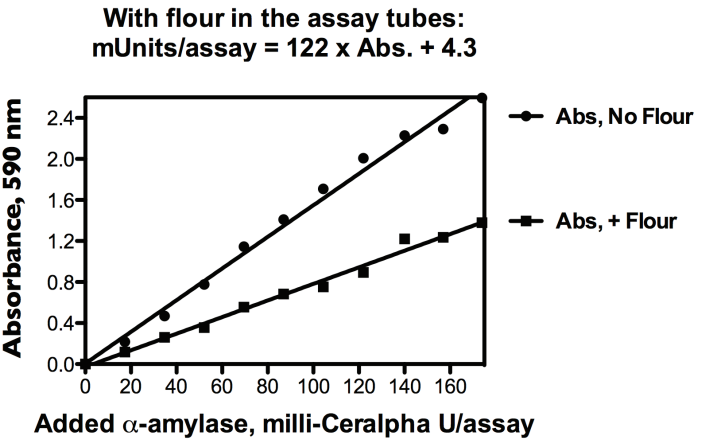


Figure 8. **Standard curve relating the level of wheat flour α-amylase (Ceralpha, milliUnits per assay) to absorbance at 590 nm using Amylazyme tablets (Lot 40101)** under standard assay conditions (Method 2) with partially purified wheat α-amylase in the presence of sound wheat flour (0.5 g). Ceralpha Units determined at 40°C.

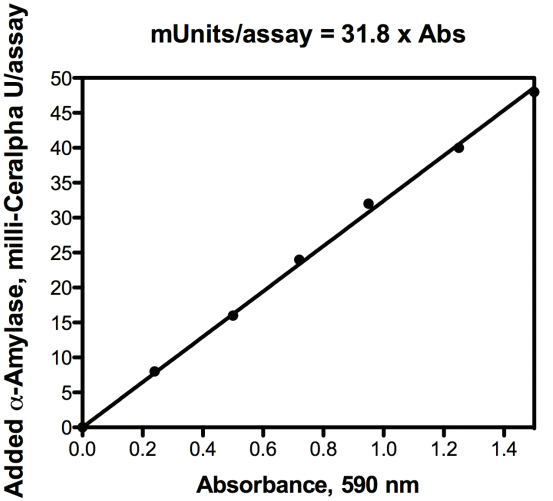


Figure 9. **Standard curves relating the level of barley flour α-amylase (Ceralpha, milliUnits per assay) to absorbance at 590 nm using Amylazyme BG (Lot 60801)** under standard assay conditions (Method 2) with partially purified barley α-amylase in the presence of sound barley flour (0.5 g).

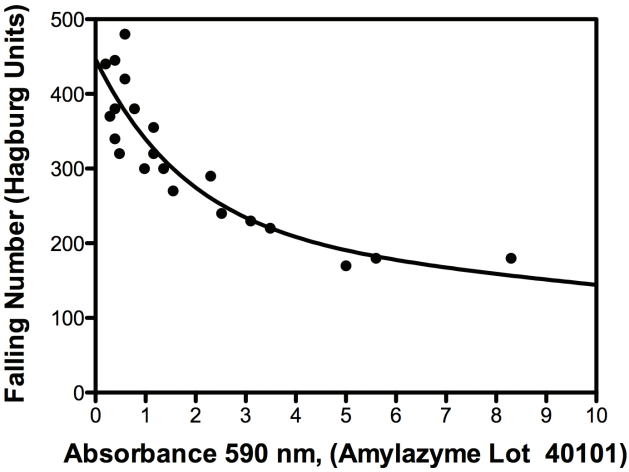


Figure 10. **Comparison of values obtained for a range of weather damaged wheat flour samples using the Falling Number and Amylazyme methods** (Method 2, Amylazyme Lot 30602).

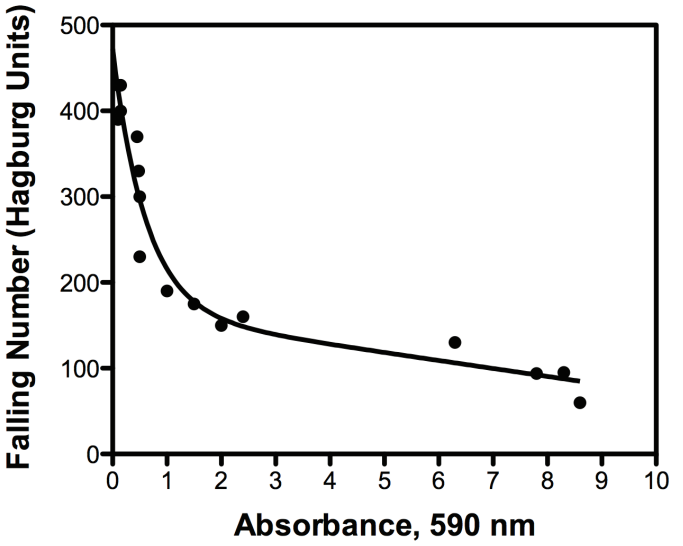


Figure 11. **Comparison of values obtained for a range of weather damaged barley flour samples using the Falling Number and Amylazyme methods** (Method 2, Amylazyme BG Lot 60801).

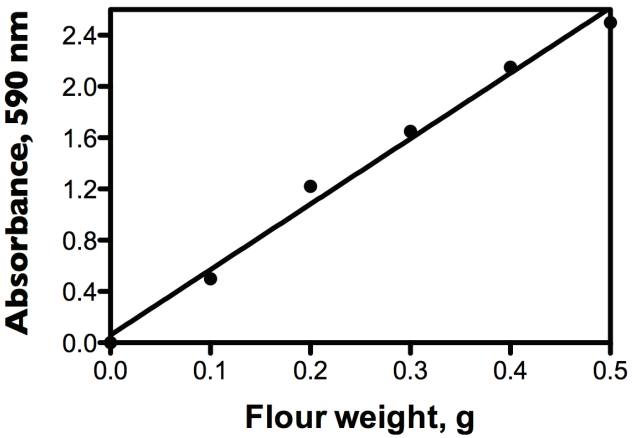


Figure 12. **The effect of the weight of the wheat flour sample used in the assay on Amylazyme absorbance value using standard assay conditions (Method 2).**

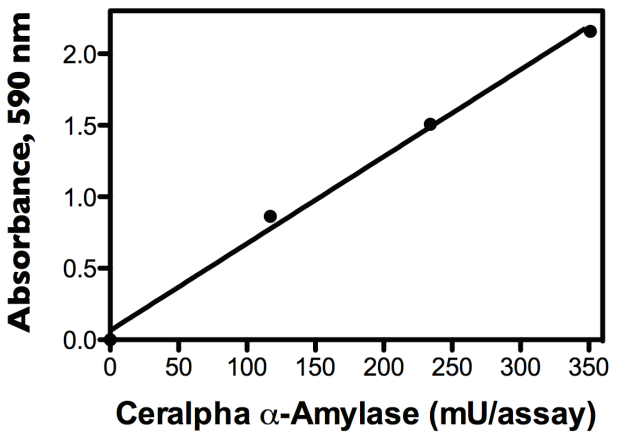
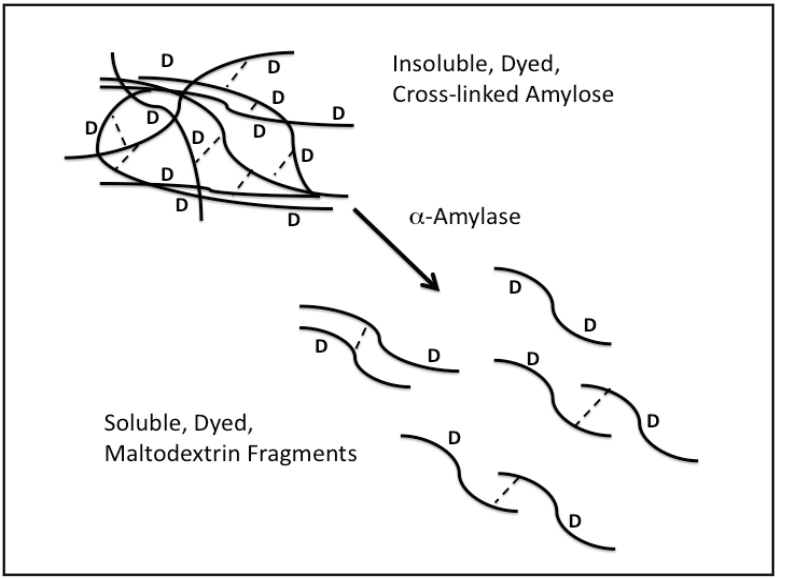
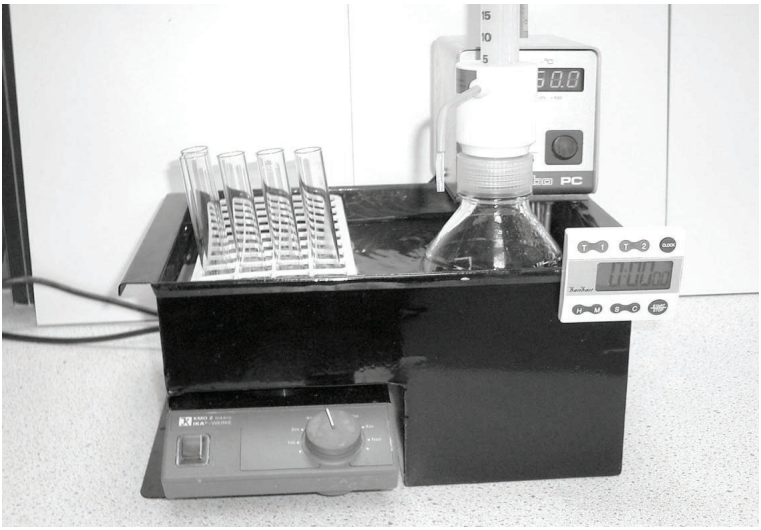


Figure 13. **Linearity of the Amylazyme method at higher absorbance values.**



Scheme 1. Principle of the Amylazyme α-amylase procedure.



The Megazyme Incubation Bath Mk III (plus tube rack) is designed for use in conjunction with an IKA Labortechnik KMO 2 basic magnetic stirrer and a precision immersion heater (e.g. Julabo PC) and Amylazyme or Amylazyme BG test tablets. With this system, sixteen tubes can be stirred and thermoregulated simultaneously.

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