

# Megazyme

---

## AMYLZYME ALPHA-AMYLASE ASSAY PROCEDURE

AMZ 7/98

FOR THE MEASUREMENT OF  
CEREAL AND MICROBIAL  
ALPHA-AMYLASES



## **INTRODUCTION:**

The level of  $\alpha$ -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  $\alpha$ -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  $\alpha$ -amylase in malt. Many of these are based on the measurement of the changes in colour produced on the interaction of iodine with the  $\beta$ -limit dextrin of starch. Due to the high levels of  $\alpha$ -amylase in malt, a range of other methods are also readily applicable, such as dye-labelled starch methods and the Ceralpha method<sup>1</sup>. The measurement of  $\alpha$ -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<sup>®</sup> (dye-labelled starch) method by Barnes and Blakeney<sup>2</sup> 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<sup>3</sup>, Europe<sup>4</sup> and Australia<sup>2</sup>. At present in Australia, the most widely used procedure for the measurement of  $\alpha$ -amylase in pre-harvest sprouted wheat and barley is the Amylazyme method<sup>5</sup>.

## **SUBSTRATE:**

The substrate employed is Azurine-crosslinked amylose (AZCL- Amylose). The substrate is prepared by dyeing and cross-linking amylose (Sigma cat. no. A-7043) to produce a material which hydrates in water but is water insoluble. Hydrolysis by  $\alpha$ -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  $\alpha$ -amylase in the presence of large excesses of  $\beta$ -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 on request. The Ceralpha assay is one of the few which employs a well defined substrate and allows the specific measurement of  $\alpha$ -amylase in the presence of other starch degrading enzymes and reducing sugars.

## **AVAILABILITY:**

Amylzyme 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  $\alpha$ -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 Amylzyme 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 II), which when used with an IKA MINI MRI 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 $\alpha$ -amylase)

[Sodium maleate (100 mM, pH 6.0) plus calcium chloride (5 mM) and Na azide (0.02%)].

Dissolve maleic acid (11.6 g, Sigma, cat. no. M-0375) in 900 mL of distilled water and adjust the pH to 6.0 with sodium hydroxide solution (2 M) (approximately 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 litre. **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* $\alpha$ -amylase)

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

Glacial acetic acid (5.9 mL, 1.05 g/mL) is added to 900 mL of distilled water. This solution is adjusted to pH 4.4 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approximately 30 mL is required). Calcium chloride dihydrate (0.74 g) and sodium azide (0.2 g) are added and the pH is readjusted to 4.4. The volume is adjusted to 1 litre. **Store at room temperature.**

#### C. Buffer C (for *Bacillus* sp. $\alpha$ -amylase)

[MOPS (100 mM, pH 7.0) plus calcium chloride (5 mM) and Na azide (0.02%).]

MOPS buffer salt (20.9 g acid form; Sigma, cat. no. M-1254) is added to 900 mL of distilled water. This is adjusted to pH 7.0 by the addition of 2 M sodium hydroxide (approximately 17 mL is required). Calcium chloride dihydrate (0.74 g) and sodium azide (0.2 g) are added and the pH is adjusted to 7.0. The volume is adjusted to 1 litre. **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 (eg. Gilson PipetmanR) (for the dispensing of enzyme preparation).
3. Adjustable-volume dispenser (for Trizma base solution).
4. Bench centrifuge (required speed 3,000 rpm), or Whatman GF/A glass fibre filter paper.
5. Analytical and top-pan balances.
6. Spectrophotometer set at 590 nm.
7. Vortex mixer (e.g. Thermolyne Maxi Mix II).
8. Thermostated water bath set at 40°C (eg. Julabo PC).
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 Amylzyme tablet must be carefully controlled (i.e. **10.0 min**).
2. Incubation **temperatures** must be accurately controlled (i.e. **40°C**).
3. After addition of Trizma base 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 Trizma 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 Amylzyme 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 Amylzyme 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:

Malt samples are milled to pass a 0.5 mm mesh. Malt flour (1.0 g) is suspended in Buffer A (20.0 mL), mixed by inversion and allowed to extract over 15 min. The slurry is then centrifuged (1,000 g, 10 min) or filtered through a Whatman GF/A glass fibre filter paper. An aliquot (0.5 mL) of the filtrate is then diluted 100-fold by addition to 49.5 mL of buffer A. Perform assays within 2 hours.

### 2. Wheat and Barley Flour:

Wheat or barley samples are milled to pass a 0.5 mm mesh. Samples are assayed as per Method 2 in this booklet.

### 3. *A. oryzae* Preparations:

Powder enzyme preparations (1.0 g) are added to 100 mL of Buffer B and allowed to extract over 10 min (with stirring).

An aliquot of the slurry is centrifuged or filtered as for the malt samples. These filtrates are then diluted 10-fold by adding an aliquot of the preparation (0.5 mL) to Buffer B (4.5 mL). This process of dilution is repeated until a suitable dilution for assay is achieved. For the preparation "Finizym" (Novo Industrias, Denmark), a dilution of 5,000-fold (of the original solution) is required.

Liquid enzyme preparations (1.0 mL) are diluted to 100 mL in Buffer B, and are then further diluted as for extracts of the powder samples.

### 4. *Bacillus* sp. Preparations:

Enzyme preparation (1.0 mL) is diluted to 100 mL in Buffer C. An aliquot (0.5 mL) of this solution is then diluted 10-fold by addition to 4.5 mL of Buffer C. This process of dilution is repeated until a suitable dilution for assay is achieved. For example, for the *Bacillus licheniformis*  $\alpha$ -amylase preparation from Megazyme (cat. no. E-BLAAM), a dilution of 200,000-fold is required.

## ASSAY PROCEDURE:

1. Aliquots (1.0 mL) of suitably diluted enzyme preparation or flour extract (in the appropriate buffer) are **pre-equilibrated** at 40°C for 5 min.
2. An Amylazyme tablet is added to each tube (**without stirring**). The tablet hydrates rapidly and absorbs most of the free liquid. The suspension **should not be stirred**. Incubate the tubes at 40°C for exactly 10 min.
3. Trizma base solution (10 mL, 2% w/v, Sigma cat. no. T-1503) is added after exactly 10 min (from time of addition of the tablet) to terminate the reaction. The tubes are stirred vigorously on a vortex mixer and left at **room temperature**.
4. After about 5 min the tubes are stirred again and the contents are **filtered** through Whatman No. 1 (9 cm) filter paper.
5. The absorbance at 590 nm of the filtrate is measured against the **reaction blank**.
6. A **substrate/enzyme blank** is prepared by adding Trizma Base to the enzyme solution before addition of the Amylazyme tablet.

### 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).
2. Since bacterial  $\alpha$ -amylases have optimal activity at pH 7.0, the Trizma 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, tri-sodium phosphate solution (1% w/v) adjusted to pH 11, can be used to terminate the reaction when bacterial enzymes are being assayed.

## STANDARDISATION:

Standard curves relating the activity of pure barley malt, *Aspergillus niger* and *Bacillus licheniformis*  $\alpha$ -amylases on Amylazyme (Lot 50504) 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  $\alpha$ -amylase assay procedure are given in Megazyme Data Booklet CER 6/93, 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 effects the assay. The optimal buffer salt concentration for each of the enzymes studied was 100 mM.

## CALCULATION OF ACTIVITY:

$\alpha$ -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:

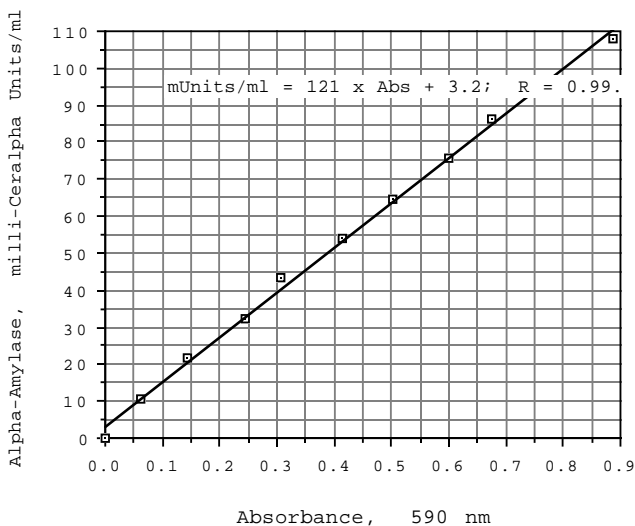
$$= \text{milliUnits/assay} \times \frac{1}{1000} \times 100 \times \text{Dilution}$$

where:

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

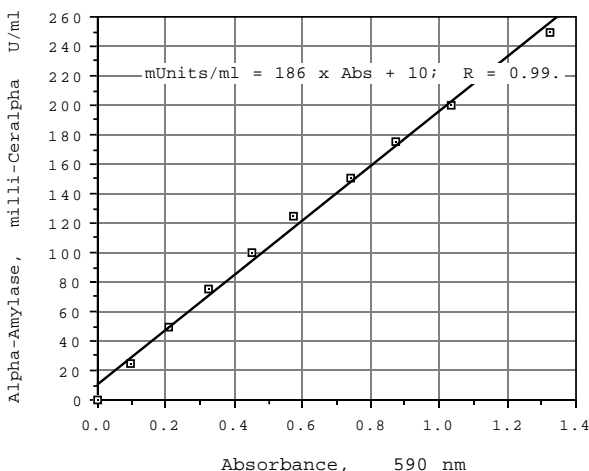
$$100 = \text{initial extraction volume (i.e. 100 mL per g of solid).}$$

Dilution = further dilution of the initial extraction solution.



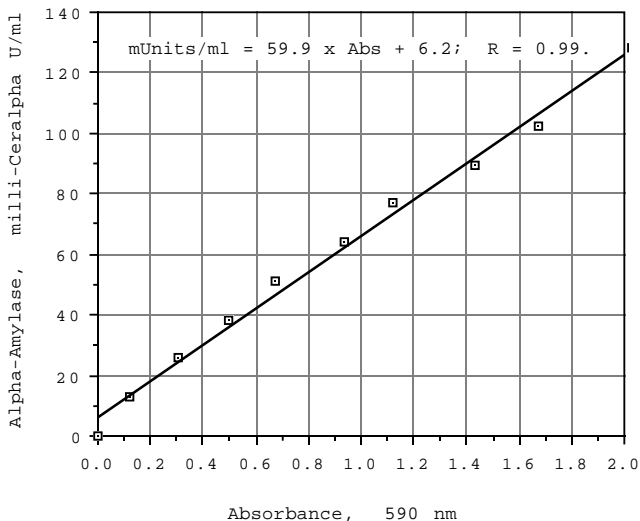
**Figure 1. Malted barley  $\alpha$ -amylase standard curve on Amylazyme (Lot 50504).**

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 CER 6/93 (Ceralpha), using purified malt  $\alpha$ -amylase.



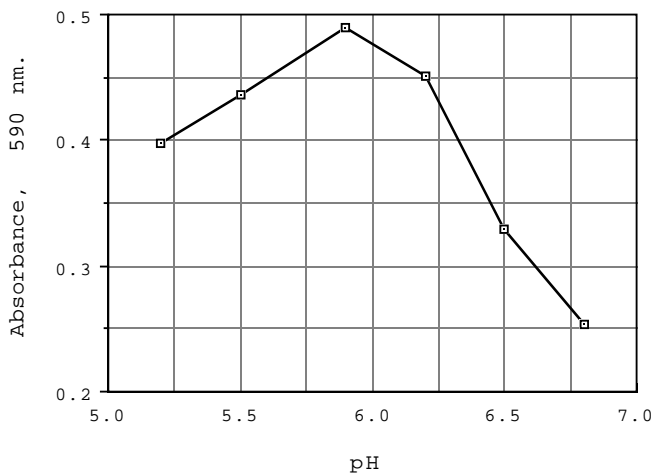
**Figure 2. *Aspergillus oryzae*  $\alpha$ -amylase standard curve on Amylazyme (Lot 50504).**

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 CER 6/93 (Ceralpha), using pure *A. oryzae*  $\alpha$ -amylase (Megazyme cat. no. E-ANAAM).



**Figure 3. *Bacillus licheniformis*  $\alpha$ -amylase standard curve on Amylazyme (Lot 50504).**

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 CER 6/93 (Ceralpha). The enzyme employed was purified *Bacillus licheniformis*  $\alpha$ -amylase (Megazyme cat. no. E-BLAAM).



**Figure 4. pH Activity curve for malt  $\alpha$ -amylase.**

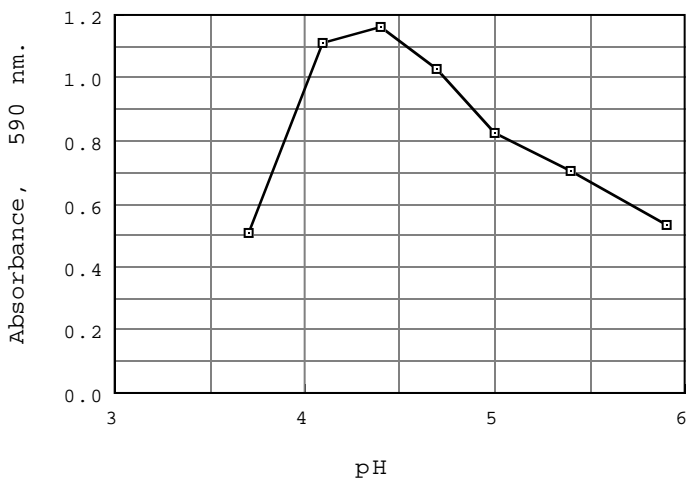


Figure 5. pH Activity curve for *A. niger*  $\alpha$ -amylase.

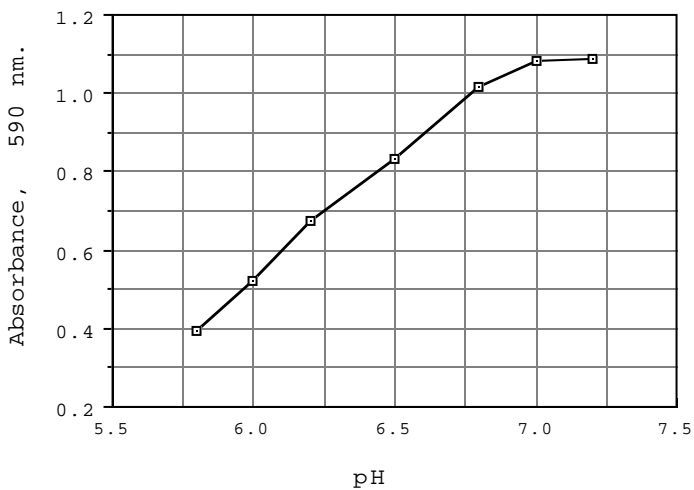
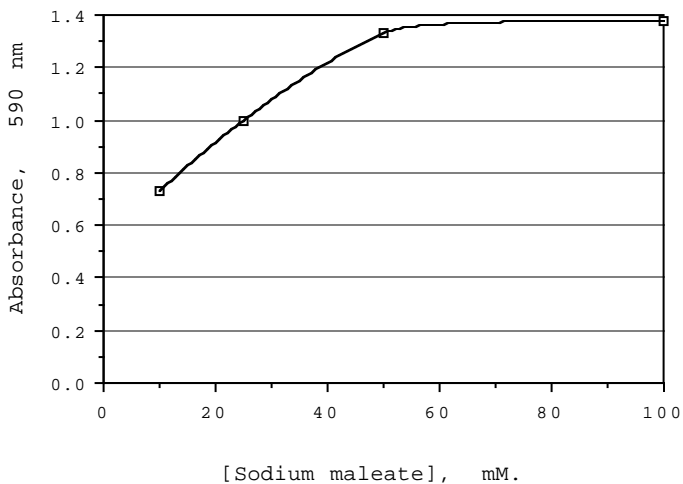


Figure 6. pH Activity curve for *Bacillus* sp.  $\alpha$ -amylase.



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

<b>METHOD 2:</b>	<b>FLOURS FROM PRE-HARVEST SPROUTED GRAINS AND FOOD PRODUCTS CONTAINING TRACE LEVELS OF <math>\alpha</math>-AMYLASE</b>
------------------	---

### **INTRODUCTION:**

In this assay format, enzyme extraction and assay is performed in the same test tube. 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  $\alpha$ -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  $\beta$ -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  $\beta$ -glucanase. As the tablet begins to disintegrate, the  $\beta$ -glucanase is released and depolymerises the  $\beta$ -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 II**).

Since assays are performed directly on flour slurries, starch in the flour sample acts as an alternative substrate and thus lowers the apparent  $\alpha$ -amylase activity. For this reason, standard curves have been prepared for pure wheat  $\alpha$ -amylase both in the absence and in the presence of added flour (0.5 g; essentially devoid of  $\alpha$ -amylase; Figure 8). The effect of adding the flour (" + F") is apparent, and we recommend the use of this curve in converting absorbance values to Ceralpha units. For barley  $\alpha$ -amylase, curves are presented for Amylazyme (50504) and Amylazyme BG (60801) (in the presence of added flour) (Figure 9).

## EQUIPMENT (RECOMMENDED):

1. Megazyme Incubation Bath Mk II.
2. IKA Labortechnik MINI MRI 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 Trizma base solution).
4. Top-pan balance (accurate to 0.01 g)..
5. Spectrophotometer set at 590 nm.
6. Vortex mixer (e.g. Thermolyne Maxi Mix II) (Sigma Z12, 099-5; 120 V or Z12, 100-2; 240 V).
7. Whatman GF/A (9.0 cm) glass fibre filter papers with filter funnels.

## ASSAY PROCEDURE:

1. Wheat or barley flour sample ( $0.5 \pm 0.01$  g) are accurately weighed into glass test tubes (20 x 150 mm) and the tubes are tapped to ensure all flour falls to the bottom. The tubes are placed into the tube rack in the Megazyme Incubation Bath Mk II (at 60°C) and a stir bar is added. The stirrer is adjusted to a setting of 850 r/min.
2. Sodium maleate buffer (5 mL, 100 mM, pH 6.0) (Buffer A, page 3), pre-equilibrated to 60°C, is added to each tube, and the tubes are stirred for 5 min.
3. An Amylzyme tablet (or Amylzyme BG tablet for barley flour samples) is added to each tube and the reaction is allowed to continue (with stirring) for exactly 5 min.
4. Trizma base (6 mL, 2% w/v, pH ~9) is added to each tube with vigorous stirring on a vortex mixer, to terminate the reaction. The tubes are left at room temperature.
5. After about **5 min**, the tubes are stirred again and filtered through Whatman GF/A glass fibre filter paper (9 cm circles).
6. The **absorbance** of the filtrate is measured (590 nm) against the reaction blank, and the activity is calculated by reference to a standard curve (curve "+ F" in Fig. 8, or the relevant curve in Fig. 9).

## NOTES:

1. A single blank is required for each set of determinations. This is prepared by adding the Trizma base solution to the flour slurry before the Amylzyme 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 Trizma 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 grams of flour).
3. **Barley flour slurries** are more viscous than wheat flour slurries and the repeatability of the assay is reduced. Consequently, we have developed alternative Amylzyme tablets containing bacterial  $\beta$ -glucanase (i.e. Amylzyme BG). This  $\beta$ -glucanase depolymerises the  $\beta$ -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 Amylzyme tablets. Consequently, Amylzyme BG tablets are recommended for use with barley flour samples.
4. **Standard curves** relating values obtained with the Amylzyme 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 at higher absorbance values is shown in **Figure 13**. The results shown in Figure 13 were obtained by diluting the filtered reaction solution 4-fold before measuring the absorbance.

#### CALCULATION OF ACTIVITY:

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

where:

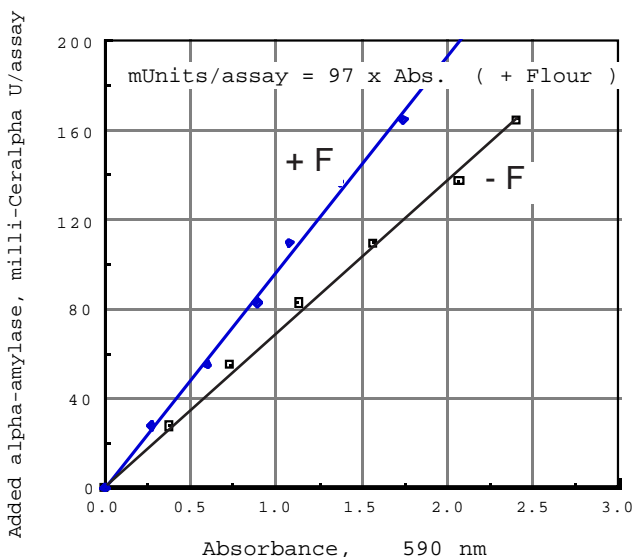
milliUnits (per assay) is obtained from **Fig. 8** (" + F" curve) or Fig. 9.

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

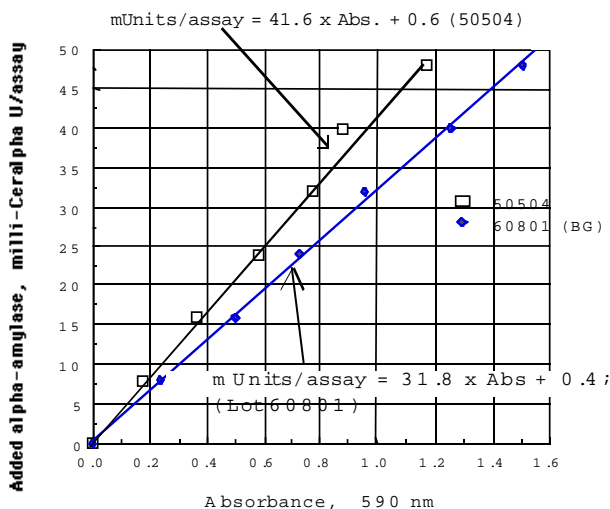
$\frac{1}{1000}$  = conversion from milliUnits to Units

#### REFERENCES:

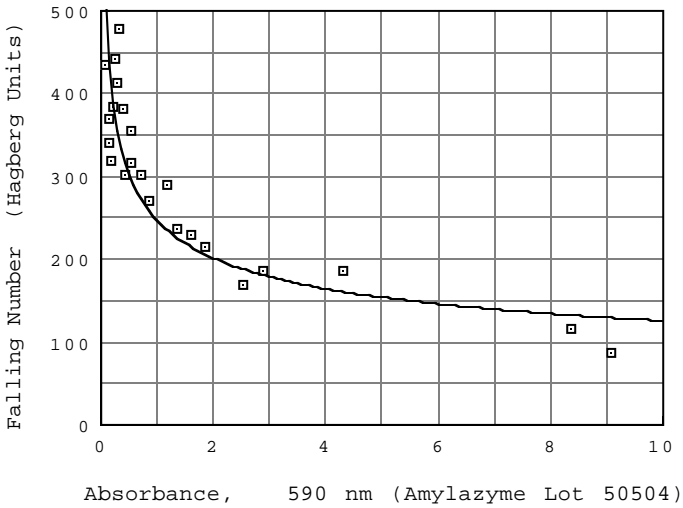
1. McCleary, B.V. and Sheehan, H. (1989). "Measurement of Cereal  $\alpha$ -amylase: A New Assay Procedure". **Journal of Cereal Science**, **6**, 237-251.
2. Barnes, W.C. and Blakeney, A.B. (1974). "Determination of Cereal Alpha Amylase" **Starch**, **26**, 193-197.
3. American Association of Cereal Chemists. Approved Methods (1985). **Method 22-06**. "Cereal  $\alpha$ -amylase" Approved 10-27-82.
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.



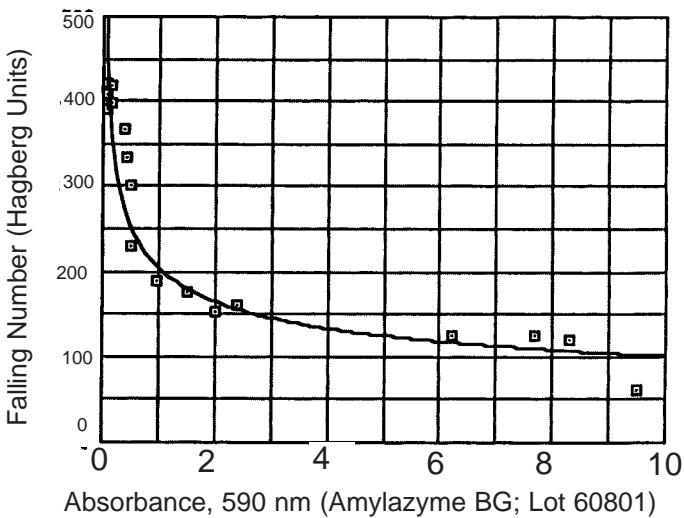
**Figure 8.** Standard curves relating the level of wheat flour  $\alpha$ -amylase (Ceralpha, milliUnits per assay) to absorbance at 590 nm using Amylazyme tablets (Lot 50504) under standard assay conditions (Method 2) with partially purified wheat  $\alpha$ -amylase in the absence (" - F"), or presence (" + F") of sound wheat flour (0.5 g).



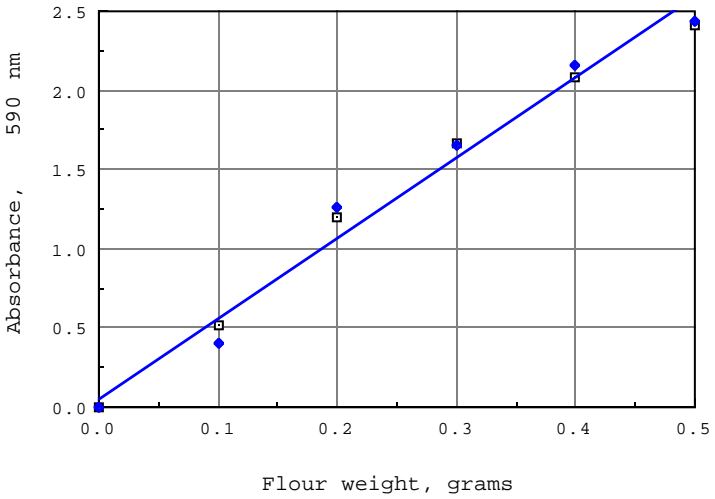
**Figure 9.** Standard curves relating the level of barley flour  $\alpha$ -amylase (Ceralpha, milliUnits per assay) to absorbance at 590 nm using Amylazyme tablets (Lot 50504) and Amylazyme BG (60801) under standard assay conditions (Method 2) with partially purified barley  $\alpha$ -amylase in the presence of sound barley flour (0.5 g).



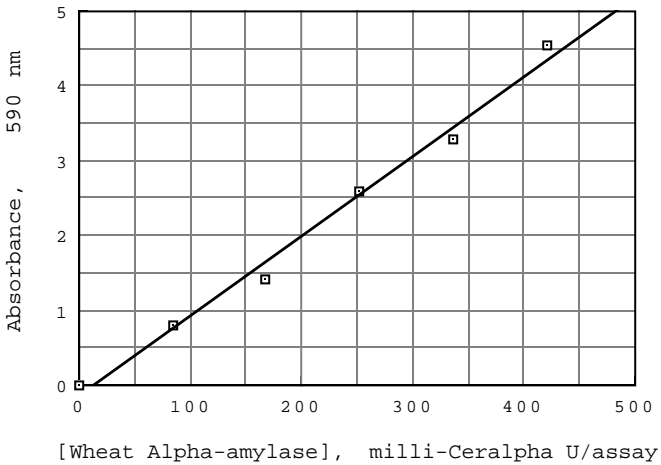
**Figure 10.** Comparison of values obtained for a range of weather damaged wheat flour samples using the Falling Number and Amylzyme methods (Method 2, tablet lot Amylzyme 50504).



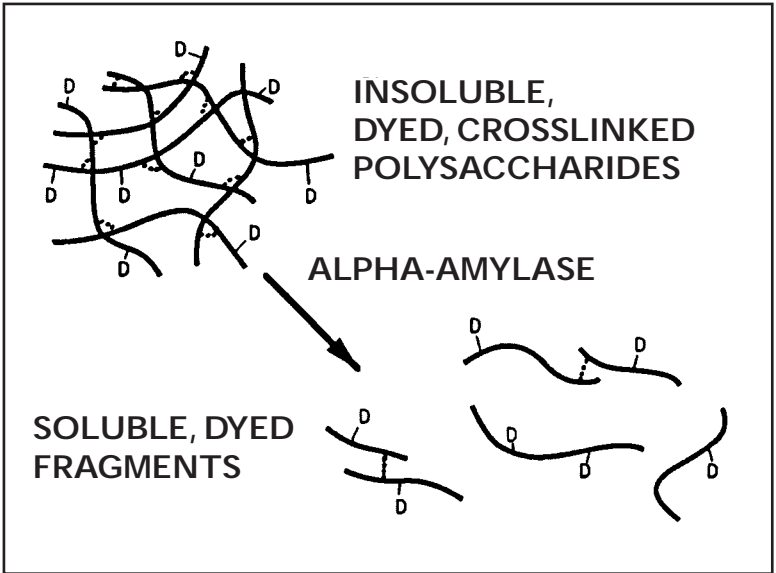
**Figure 11.** Comparison of values obtained for a range of weather damaged barley flour samples using the Falling Number and Amylzyme methods (Method 2, tablet lot Amylzyme BG 60801).



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



**Figure 13.** Linearity of the Amylzyme method at higher absorbance values (tablet lot 50504).



Scheme 1. Principle of the Amylazyme  $\alpha$ -amylase procedure.



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

---



**Megazyme International Ireland Ltd.,  
Bray Business Park, Bray,  
Co. Wicklow,  
IRELAND**

**Telephone: (353.1) 286 1220**

**Facsimile: (353.1) 286 1264**

**Internet: [www.megazyme.com](http://www.megazyme.com)**

**E-Mail: [info@megazyme.com](mailto:info@megazyme.com)**

---

**WITHOUT GUARANTEE**

The information contained in this booklet is, to the best of our knowledge, true and accurate, but since the conditions of use are beyond our control, no warranty is given or is implied in respect of any recommendation or suggestions which may be made or that any use will not infringe any patents.



**Megazyme International Ireland Ltd.,  
Bray Business Park, Bray,  
Co. Wicklow,  
IRELAND**

**Telephone: (353.1) 286 1220**

**Facsimile: (353.1) 286 1264**

**Internet: [www.megazyme.com](http://www.megazyme.com)**

**E-Mail: [info@megazyme.com](mailto:info@megazyme.com)**