Megazyme

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AMYLAZYME RED TABLETS

For the measurement of cereal and microbial α-amylases

T-AMZRD 08/16

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" methods and the newer "Stirring Number" 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, 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]. At present in Australia, the most widely used procedure for the measurement of α-amylase in pre-harvest sprouted wheat and barley is the Amylazyme method[5].

Amylazyme Red tablets are quite similar to Amylazyme tablets (blue), however the dyeing procedure has been altered and a new dye employed, with the aim of increasing sensitivity of the assay.

SUBSTRATE:

The substrate employed is Red-crosslinked amylose (RCL-Amylose), which is prepared by dyeing and cross-linking high-amylose starch to produce a material which hydrates in water but is water insoluble. Hydrolysis by α-amylase produces water soluble, red-dyed fragments, and the rate of solubilisation of these (increase in absorbance at 510 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 Red tablets.

All activities are converted to Ceralpha Units through standard curves. Details of the Ceralpha method are available on the Megazyme website (**K-CERA**).

The Ceralpha assay is one of the few methods 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 Red tablets are available 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 it 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 Red tablet to an aliquot (0.5 mL) of pre-equilibrated and suitably diluted enzyme preparation in the assay buffer. Reaction tubes are incubated without stirring under controlled conditions of time and temperature. The reaction is terminated by addition of a weak alkaline solution, slurries are filtered and the absorbances of the filtrates are measured (refer to Method 1, page 3).

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, page 11). To facilitate these assays, Megazyme has developed and supplies an incubation bath (Megazyme Incubation Bath), 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.

REFERENCES:

1. McCleary, B. V. & Sheehan, H. (1989). Measurement of Cereal α–amylase: A New Assay Procedure. *Journal of Cereal Science*, **6**, 237-251.
2. Barnes, W. C. & 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 α–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.

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 11.6 g maleic acid (Sigma cat. no. M0375) in 900 mL of distilled water and adjust the pH to 6.0 with 2 M NaOH (approx. 80 mL is required). Add 0.74 g of calcium chloride dihydrate and 0.2 g of sodium azide. Adjust the pH to 6.0 and 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% w/v).

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

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

MOPS (100 mM, pH 7.0) plus calcium chloride (5 mM) and sodium azide (0.02% w/v).

Dissolve 20.9 g of MOPS buffer salt (Megazyme cat. no. **B-MOPS250**) in 900 mL of distilled water. Adjust the pH to pH 7.0 with 2 M NaOH (approx. 17 mL is required). Add 0.74 g of calcium chloride dihydrate and 0.2 g of sodium azide. Adjust the pH to 7.0 and 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, 0.5 mL (e.g. Gilson Pipetman®) for the dispensing of enzyme preparation.

3. Adjustable-volume dispenser (for Tris base solution. Megazyme cat. no. **B-TRIS500**).
4. Bench centrifuge (required speed 1,500 *g*).
5. Analytical and top-pan balances.
6. Spectrophotometer set at 510 nm.
7. Vortex mixer (e.g. Thermolyne Maxi Mix II).
8. Thermostated water bath set at 40°C (e.g. Julabo PC).
9. Stop clock.
10. Whatman No. 1 and Whatman GF/A glass fibre (9 cm) filter circles and filter funnels.

CONTROLS AND PRECAUTIONS:

1. The time of incubation of the enzyme extract with the Amylazyme Red tablet must be carefully controlled (i.e. 10 min).
2. Incubation temperatures must be accurately controlled (i.e. 40°C).
3. After addition of Tris base to the reaction tube, the tubes must be stirred vigorously to ensure thorough mixing. Leave tubes 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 Red 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 Red assay is significantly affected by the concentration of buffer salts. The optimal buffer salt concentration is 100 mM.

ENZYME EXTRACTION AND DILUTION:

1. Malt Flours

Mill malt samples to pass a 0.5 mm screen. Accurately weigh 1.00 g of sample into a 100 mL flask and add 20.0 mL of buffer A. Mix the slurry thoroughly over 15 min by stirring or inversion. Centrifuge an aliquot of the slurry at 1,500 *g* for 10 min, or filter through Whatman GF/A glass fibre filter paper. Dilute 0.2 mL of the supernatant or filtrate to 40 mL (200-fold) in buffer A. Perform the assays within 2 h.

2. Wheat and Barley Flours

Mill wheat or barley samples to pass a 0.5 mm mesh. Extract and assay samples according to Method 2 in this booklet (page 11).

3. *A. oryzae* Preparations

Using a positive displacement dispenser, transfer 1.0 mL of enzyme preparation to a 100 mL volumetric flask and dilute to volume with Buffer B. Dilute 0.5 mL of this solution 10-fold by adding 0.5 mL to 4.5 mL of Buffer B. Repeat this process of step-wise 10-fold dilutions until an enzyme concentration suitable for assay is achieved (approx. 100-fold). For example, the *Aspergillus oryzae* α-amylase preparation from Megazyme (cat. no. **E-ANAAM**), a dilution of 10,000-fold is required.

4. *Bacillus* sp. Preparations

Using a positive displacement dispenser, transfer 1.00 mL of enzyme preparation to a 100 mL volumetric flask and dilute to volume with Buffer C. Dilute 0.5 mL of this solution 10-fold by adding 0.5 mL to 4.5 mL of Buffer C. Repeat this process of step-wise 10-fold dilutions until an enzyme concentration suitable for assay is achieved (approx. 10,000-fold). For example, the *Bacillus licheniformis* α-amylase preparation from Megazyme (cat. no. **E-BLAAM**), a dilution of 100,000-fold is required.

ASSAY PROCEDURE:

1. Equilibrate 0.5 mL aliquots of suitably diluted enzyme preparation or flour extract (in the appropriate buffer) for 5 min at 40°C.

2. Add an Amylazyme Red tablet to each tube. Do not stir the tube contents; the tablet hydrates rapidly and absorbs most of the free liquid. Incubate the tubes for exactly 10 min at 40°C.

3. Terminate the reaction by adding 10 mL of Tris base solution (2% w/v, pH ~ 10; Megazyme cat. no. **B-TRIS500**). Stir the tubes vigorously on a vortex mixer and let them stand for 5 min at room temperature.

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

5. Measure the absorbance of the filtrate against a **reaction blank** at 510 nm.

6. Prepare a **reaction blank** by adding Tris base to the enzyme solution before addition of the Amylazyme Red 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 α-amylases have optimal activity at pH 7.0, the Tris base solution (pH ~ 10) does not completely terminate the enzyme reaction. It is thus essential that tri-sodium phosphate solution (1% w/v adjusted to pH 11), is used to terminate the reaction when bacterial enzymes are being assayed.

STANDARDISATION:

Standard curves relating the activity of pure malted barley, *Aspergillus niger* and *Bacillus licheniformis* α-amylases on Amylazyme Red tablets (Lot 160711) to activity on Ceralpha reagent are shown in Figures 1-3 (pages 7 & 8). 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 at a defined pH. Full details of the Megazyme Ceralpha α-amylase assay procedure can be obtained from the web site and the assay conditions are given in the Figure captions (Figures 1-3).

The effect of buffer salt concentration on the assay and pH activity curves for these assays are shown in Figures 4-7 (pages 9 & 10). The optimal buffer salt concentration for all assays is 100 mM.

CALCULATION:

α-Amylase activity is determined by reference to the appropriate standard curve to convert absorbance readings at 510 nm to Ceralpha Units of activity per assay (i.e. milli-Units per 0.5 mL), and are then calculated as follows:

Units/g or mL of original preparation:

$$= \text{milliUnits/assay} \times 2 \times \frac{1}{1000} \times 100 \times \text{Dilution}$$

where:

2	=	factor to convert from 0.5 mL as assayed to 1.0 mL.
$\frac{1}{1000}$	=	conversion from milli-Units to Units.
100	=	initial extraction volume (i.e. 100 mL per mL or g of enzyme).
Dilution	=	further dilution of the initial extract.

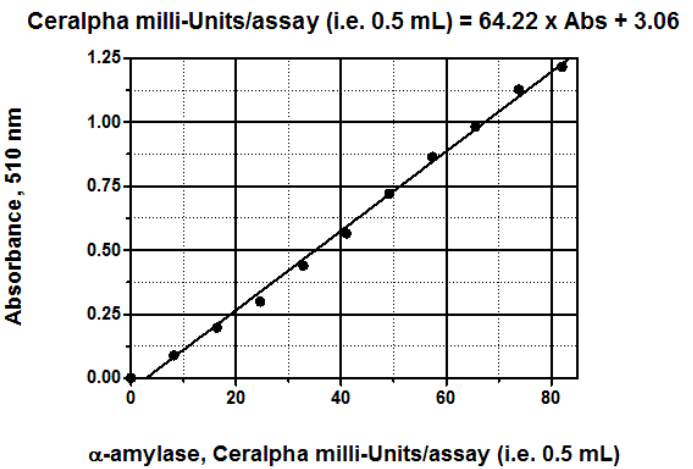

Figure 1. **Standard curve for barley malt α-amylase on Amylazyme Red tablets (Lot 160711).**
Amylazyme Red tablet assay performed at pH 6.0 (buffer A) under standard conditions. Ceralpha assay performed in sodium malate buffer, pH 5.4. (See Ceralpha kit booklet; **K-CERA**).

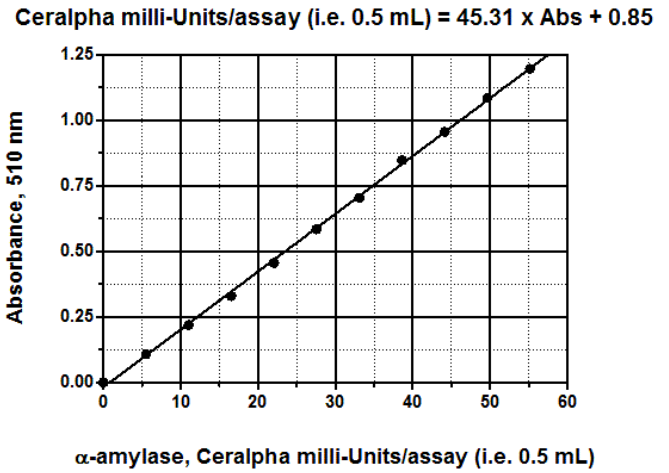

Figure 2. **Standard curve for *Aspergillus oryzae* α-amylase on Amylazyme Red tablets (Lot 160711).**

Amylazyme Red tablet assay was performed at pH 4.4 (buffer B) and the Ceralpha assay at pH 5.4 under standard conditions as described in this booklet using pure *A. oryzae* α-amylase (Megazyme cat. no. **E-ANAAM**).

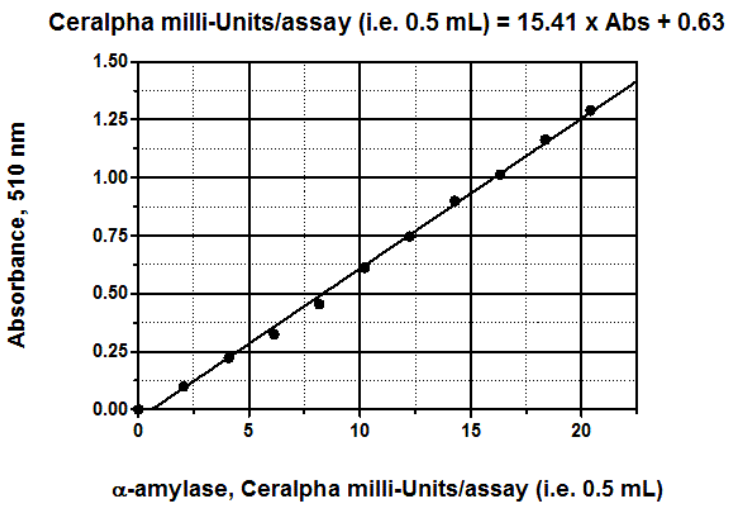

Figure 3. **Standard curve for *Bacillus licheniformis* α-amylase on Amylazyme Red tablets (Lot 160711).**

Amylazyme Red tablet assay was performed at pH 7.0 (buffer C) and Ceralpha assay at pH 6.0 under standard conditions as described in this booklet using purified *Bacillus licheniformis* α-amylase (Megazyme cat. no. **E-BLAAM**).

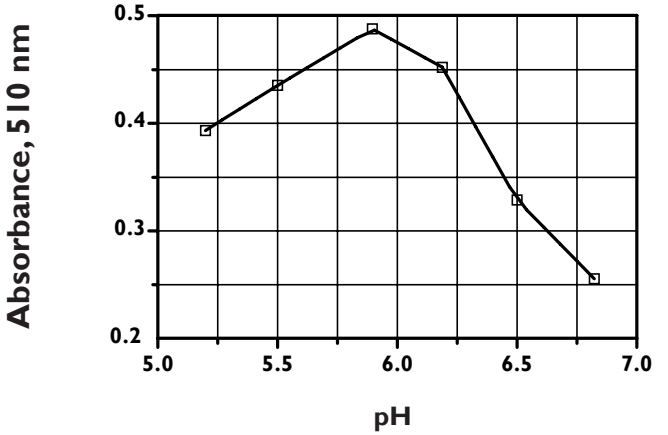

Figure 4. **pH Activity curve for malt α-amylase on Amylazyme Red tablets.**

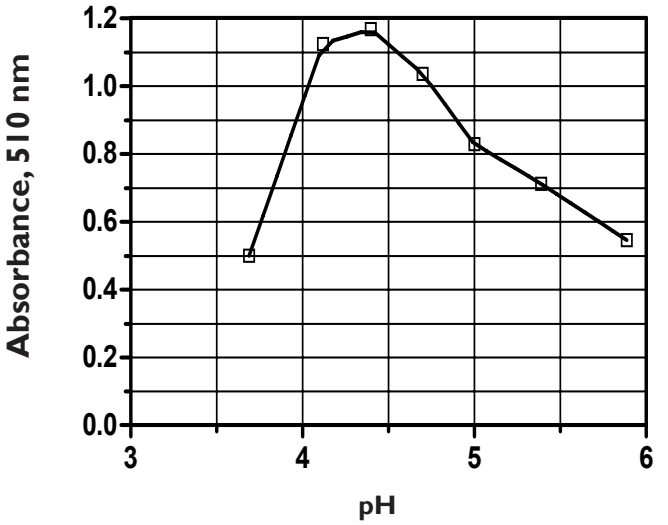

Figure 5. **pH Activity curve for *A. oryzae* α-amylase on Amylazyme Red tablets.**

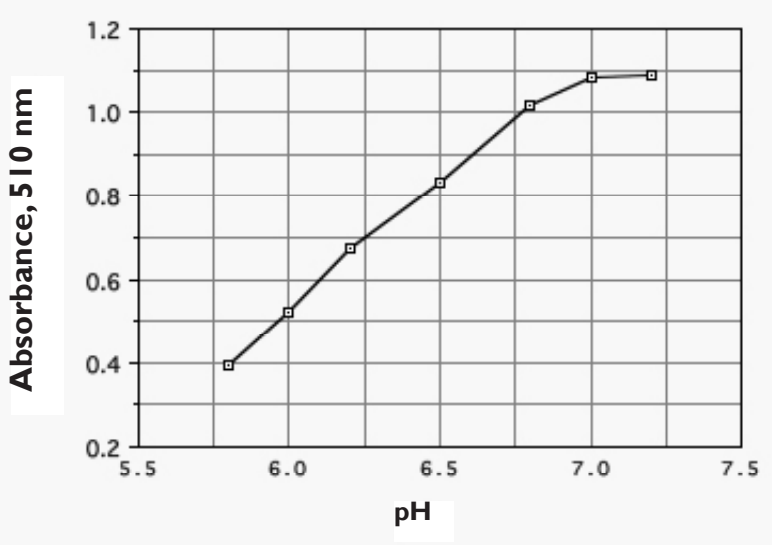

Figure 6. **pH Activity curve for *B. licheniformis* α-amylase on Amylazyme Red tablets.**

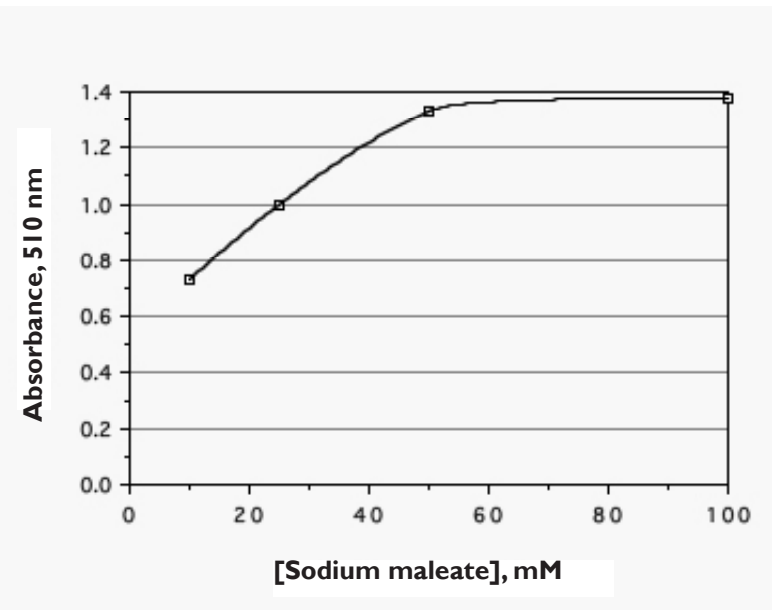

Figure 7. **Effect of buffer salt concentration on the activity of malt α-amylase on Amylazyme Red tablets.**

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 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 α-amylase present in various food products such as custard powder mixtures.

For the measurement of α-amylase in wheat and barley flours, standard Amylazyme tablets have the same sensitivity as Amylazyme Red tablets, therefore we recommend the use of that product (**T-AMZ**). Amylazyme tablets have been used for this application over the past decade, and the assay parameters and the significance of the results are well understood.

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