

# Megazyme

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ASSAY OF  
ALPHA-AMYLASE  
using  
RED-STARCH

RSTAR 11/99



## PRINCIPLE:

The assay procedure employing Red Starch is relatively specific for  $\alpha$ -amylase, however, the substrate is also hydrolysed at a slow rate by pullulanase (limit-dextrinase). On incubation of Red-Starch with  $\alpha$ -amylase the substrate is depolymerised by an *endo*-mechanism to produce low-molecular weight dyed fragments which remain in solution on addition of ethanol to the reaction mixture. High molecular weight material is removed by centrifugation, and the colour of the supernatant is measured at 510 nm.  $\alpha$ -Amylase in the assay solution is determined by reference to a Standard Curve, through which, activity is converted to Ceralpha Units (CU). The Ceralpha method is the only procedure which employs a well defined substrate (ie. end-blocked *p*-nitrophenyl maltoheptaoside). Factors to convert Ceralpha Units to International units, Dextrinising Units, SKB Units or Farrand Units can be obtained by reference to the Ceralpha Booklet (CER 1/99).

## SUBSTRATE:

The substrate is partially depolymerised starch which is dyed with Procion Red MX-5B to an extent of about one dye molecule per 30 sugar residues.

Powdered substrate (1.0g) is added to 50ml of 0.5 M potassium chloride and stirred vigorously at 60°C until it completely dissolves (about 10 min). This substrate should be stored at 4°C between use; it is stable for several months in the liquid form if stored in a well sealed glass bottle, overlain with a few drops of toluene to prevent microbial infection.

## BUFFERS:

### A. Buffer A (for cereal and fungal $\alpha$ -amylase)

Malic acid (Sigma M-0875; 1M)	134.1 grams/litre
Sodium hydroxide	70 grams/litre
Calcium chloride.2H <sub>2</sub> O (40 mM)	5.9 grams/litre
Sodium azide (Sigma S2002; 0.1%)	1.0 grams/litre

Add malic acid and sodium hydroxide to 800 ml of distilled water and dissolve by stirring. Allow the solution to cool to room temperature and add the calcium chloride. Adjust the pH to 5.4 by dropwise addition of sodium hydroxide (4 M) or HCl (4 M).

**Then** add the sodium azide. Adjust volume to 1 litre. Store at room temperature.

**For use, dilute 100ml of this concentrated buffer solution to 1 litre with distilled water.**

## CAUTION

Dissolve the reagents and adjust the pH to 6.0 before adding the sodium azide. Adding sodium azide to an acidic solution results in the release of a poisonous gas.

Powdered malic and maleic acids are irritants, and thus should be handled with due care.

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

Maleic acid (Sigma M-0375; 0.1M)	23.2 grams/2 litres
Calcium chloride. 2H <sub>2</sub> O (2 mM)	0.6 grams/2 litres
Sodium azide (Sigma S2002; 0.01%)	0.2 grams/2 litres

Add maleic acid to 1600 ml of distilled water and adjust the pH to 6.5 with 4M sodium hydroxide (160g/litre).

Add the sodium azide and adjust the volume to 2 litres. Store at room temperature between use.

**Use this buffer directly without further dilution.**

## ENZYME PREPARATION:

### A. Malt Flours:

1. Mill malt (20 g sample) to pass a 0.5 mm screen.
2. Accurately weigh 1.0 g malt flour into a 50 ml volumetric flask.
3. To the volumetric flask add Buffer A and adjust to volume.
4. Allow the enzyme to extract for 15-20 min at room temperature, with occasional stirring.
5. Filter an aliquot of the solution through a Whatman GF/A glass fibre filter paper, or centrifuge at 1,000g for ten minutes.
6. Dilute 1.0 ml of the filtrate with 9.0 ml of Extraction Buffer Solution (ie. 10-fold). Assay activity within two hours.

## B. Microbial Preparations:

### Liquid preparations:

1. Add 1 ml of liquid enzyme preparation (using a positive displacement dispenser) to **buffer A or B** (49 ml, pH 5.4 or 6.5) and mix thoroughly. This is termed the **Original Extract**.
2. Dilute an aliquot of **original extract** (1.0 ml) 10-fold by addition to 9.0 ml of appropriate **buffer (A or B)** and mix thoroughly. Repeat this step until a dilution suitable for assay is obtained. For example, for the industrial enzyme preparation, Bacterial Alpha-Amylase (from Novo Nordisk) a dilution of the **original extract** of approximately 500-fold is required.

### Powder preparations:

1. Add 1 g of enzyme powder preparation to 50 ml of **buffer A or B** (pH 5.4 or 6.5) and gently stir the slurry over a period of about 15 min or until the sample is completely dispersed or dissolved.
2. Clarify this solution (the **Original Extract**) by centrifugation (1,000g, 10 min) or filtration through Whatman No. 1 (9 cm) filter circles.
3. Dilute an aliquot of this solution (1.0 ml) 10-fold by addition to 9.0 ml of appropriate **buffer (A or B)** and mix thoroughly. Repeat this step until a dilution suitable for assay is obtained [ie. 2,000-fold for Fungamyl powder(1767 FAU; Novo Nordisk)] .

## ASSAY PROCEDURE:

### Malt and microbial preparations:

1. Dispense 0.5ml aliquots of Red Starch solution (2% w/v, in 0.5 M KCl) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate buffered malt or microbial preparation extract at 40°C for 5 min.
3. To each tube containing **Red Starch** Solution (0.5 ml), add an aliquot (1.0 ml) of pre-equilibrated (and suitably diluted) microbial enzyme or malt extract directly to the bottom of the tube. Incubate at 40°C for exactly 10 min (from time of addition). Terminate the reaction after 10 min by adding 2.5ml of ethanol (95% v/v) with vigorous stirring for 10 seconds on a vortex mixer. The reaction tubes are allowed to equilibrate to room temperature for 10 minutes and are then centrifuged at 3,000 rpm (1,000g) for 10 minutes.

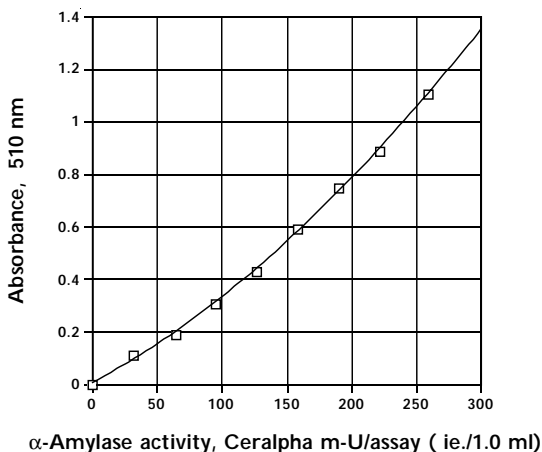
- The supernatant solution is poured directly from the centrifuge tube into a spectrophotometer cuvette and the absorbance of blank and reaction solutions are measured at 510nm against distilled water. Activity is determined by reference to a Standard Curve.

The blank is prepared by adding ethanol to the Red-Starch substrate before addition of the enzyme. Usually, a single blank is required with each set of determinations.

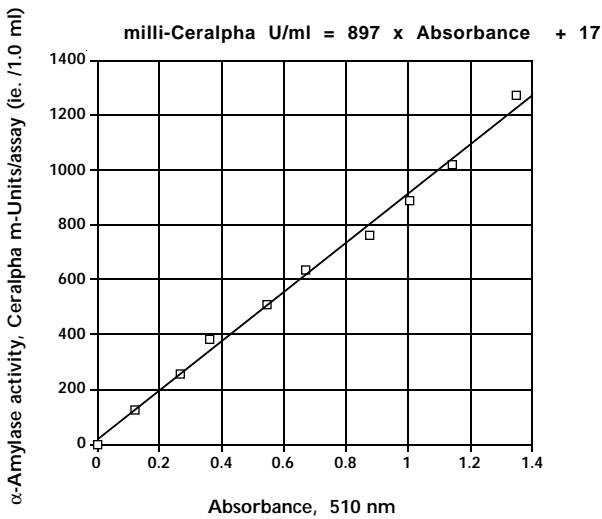
### STANDARD CURVE:

Standard curves for malt, fungal and bacterial  $\alpha$ -amylases are shown in Figures 1-3. Enzyme activity is standardised against the Ceralpha method (employing end-blocked *p*-nitrophenyl maltoheptaoside in the presence of excess quantities of thermostable  $\alpha$ -glucosidase).

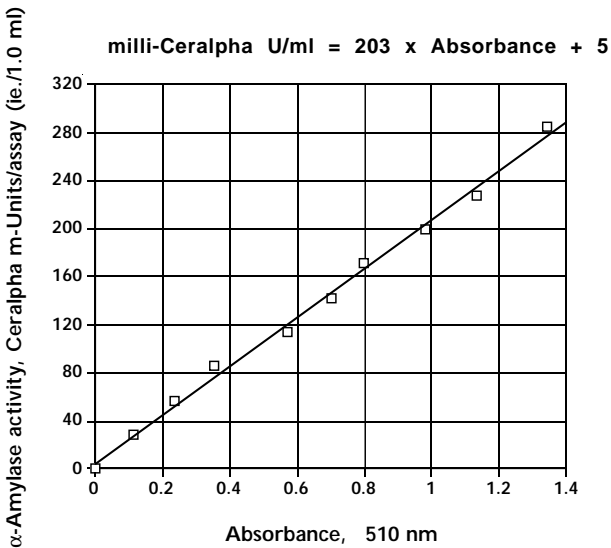
**One Unit of activity is defined as the amount of enzyme required to release one micromole of *p*-nitrophenol from blocked *p*-nitrophenyl maltoheptaoside per minute under the defined assay conditions of temperature and pH.**



**Figure 1.** Standard curve relating activity of malted barley  $\alpha$ -amylase on Red Starch (lot 90101)(Abs. 510 nm) to Ceralpha Units (milli-Units/assay).



**Figure 2.** Standard curve relating activity of *A. niger*  $\alpha$ -amylase on Red Starch (lot 90101)(Abs. 510 nm) to Ceralpha Units (milli-Units/assay).



**Figure 3.** Standard curve relating activity of *B. subtilis*  $\alpha$ -amylase on Red Starch (lot 90101)(Abs. 510 nm) to Ceralpha Units (milli-Units/assay).

## CALCULATIONS:

Units/ml of original solution

$$= \text{milliUnits per assay (ie. per 1.0 ml)} \times 50 \times \frac{1}{1000} \times \text{Dilution}$$

### where:

milliUnits per assay is determined by reference to the Standard Curve

50 = Initial extraction or dilution volume (e.g. 1g per 50ml)

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

Dilution = further dilution of the original enzyme extract  
( = 10-fold for malt extracts).



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