

Megazyme

ALPHA-AMYLASE ASSAY PROCEDURE (CERALPHA METHOD)

CER 7/98

FOR THE MEASUREMENT OF
CEREAL AND MICROBIAL
ALPHA-AMYLASE

ICC Standard No. 303



INTRODUCTION:

The level of α -amylase in cereal grains and products significantly affects the industrial exploitation of these commodities. In bread-making, the level of α -amylase must be sufficient to produce saccharides which can be absorbed and utilised by yeast, but not so high as to cause excessive starch dextrinisation which can lead to sticky crumb and problems in processing. In the brewing industry, the level of malt α -amylase is a key quality parameter. The need for a rapid, specific, convenient and reliable method for the assay of α -amylase by plant breeders, bakers, maltsters, brewers, and enzyme manufacturers and users is well documented.

PRINCIPLE:

In the Ceralpha procedure for the assay of α -amylase, an aliquot of a cereal flour extract or fermentation broth is incubated with substrate mixture under defined conditions and the reaction is terminated (and colour developed) by the addition of Trizma base solution. The absorbance at 410 nm is measured and this relates directly to the level of α -amylase in the sample analysed.

The substrate is a mixture of the defined oligosaccharide "non-reducing-end blocked *p*-nitrophenyl maltoheptaoside" (BPNPG7) and excess levels of amyloglucosidase and α -glucosidase (which have no action on the native substrate due to the presence of the "blocking group"). On hydrolysis of the oligosaccharide by endo-acting α -amylase, the excess quantities of amyloglucosidase and α -glucosidase present in the mixture, give instantaneous and quantitative hydrolysis of the *p*-nitrophenyl maltosaccharide fragment to glucose and free *p*-nitrophenol. The assay format is shown in Scheme 1 (page 11) and the linearity of the assay is shown in Figure 1 (page 10).

This substrate mixture can be used to quantitatively assay cereal, fungal and bacterial α -amylases. However, due to the pH and temperature sensitivities of the amyloglucosidase and α -glucosidase, the assay can be used only in the pH range of 5.0 to 6.0 and at, or below, 40°C.

Reagent mixtures employing blocked *p*-nitrophenyl maltoheptaoside as substrate do not distinguish between fungal, cereal and bacterial α -amylases. However, if the reagent mixture contains *p*-nitrophenyl maltopentaoside (BPNPG5) as substrate, it can be used in combination with the standard substrate to distinguish between the cereal and fungal enzymes. Thus, the relative rates of hydrolysis of BPNPG5/BPNPG7 by fungal α -amylase is 70.6:100, and by malt flour α -amylase is 2.9:100. This difference in relative rates can be used to measure the proportional amounts of cereal and fungal α -amylase in mixtures e.g. bread improver mixtures.

ACCURACY:

Standard errors of less than 5% are achieved routinely (refer to Table 1).

KITS:

Kits suitable for carrying out 100/200 assays are available from Megazyme and consist of:-

1. Full assay method;
2. Freeze dried BPNPG7 plus glucoamylase and α -glucosidase;
3. Concentrated Extraction Buffer;
4. Concentrated Stopping Reagent;
5. Control Malt Flour.

SPECIFICITY:

The assay is absolutely specific for α -amylase.

Table 1: Reproducibility of the Ceralpha assay for the measurement of wheat-flour α -amylase^d

Sample	Absorbance (410 nm)								Average
	Day 1		Day 2		Day 3		Day 4		
A	0.365	0.390	0.365	0.354	0.384	0.379	0.385	0.398	0.378
B	0.486	0.534	0.463	0.502	0.502	0.507	0.514	0.486	0.499
C	0.255	0.259	0.270	0.286	0.265	0.287	0.264	0.284	0.271
D	0.142	0.146	0.143	0.150	0.142	0.137	0.135	0.153	0.143
S.E.M. ^b	0.0134		0.0134		0.0134		0.0134		0.0067

^aDuplicate analyses of single extracts made on four separate days.

^bBased on a pooled estimate of the variance for each sample mean.

S.D. of single observation (for comparisons on same and different days) = 0.0189.

C.V. (%) = 4.05.

ENCLOSED SUBSTRATE:

Blocked *p*-nitrophenyl maltoheptaoside (BPNPG7, 54.5 mg)
glucoamylase (100U at pH 5.2)
 α -glucosidase (100U at pH 5.2), per vial.

Dissolve the entire contents of one vial in 10.0 mL of distilled water. Divide into 2-3 mL aliquots and store frozen between use. At 0-5°C the dissolved substrate is stable for seven days; in the frozen state it is stable for at least 12 months.

ENCLOSED MALT FLOUR:

Malt flour of standardised α -amylase activity (as specified on the bottle label).

It is recommended that the user standardises at least one batch of user's own wheat or malt flour to be employed as a secondary reference flour.

ENCLOSED SOLUTIONS:

(1) Concentrated Extraction Buffer:

1 M sodium malate
1 M sodium chloride
40 mM calcium chloride
0.1% sodium azide

Dilute the entire contents (50 mL) (plus a precipitate which may be present) to 1000 mL with distilled water before use. Stable at 0-5°C for 12 months. The pH should be 5.2; adjust if necessary.

(2) Concentrated Stopping Reagent: [20% (w/v) Trizma base]

Dilute the entire contents (25 mL) to 500 mL with distilled water. Stable at room temperature for twelve months.

References:

McCleary, B.V. and Sheehan, H. (1987). "Measurement of Cereal α -Amylase: A New Assay Procedure". *Journal of Cereal Science*, **6**, 237-251.

Sheehan, H. and McCleary, B.V. (1988). "A New Procedure for the Measurement of Fungal and Bacterial α -Amylase". *Biotechnology Techniques*, **2**, 289-292.

PREPARATION OF ADDITIONAL CONCENTRATED EXTRACTION BUFFER:

Malic acid (Sigma M0875; 1M)	134.1 grams/litre
Sodium hydroxide	70 grams/litre
Sodium chloride	58.4 grams/litre
Calcium chloride. 2H ₂ O (40 mM)	5.9 grams/litre
Sodium azide (Sigma S2002; 0.1%)	1.0 grams/litre

Add malic acid, sodium chloride and sodium hydroxide to 800 mL of distilled water, allow to cool to room temperature and add the calcium chloride. Adjust the pH to 5.2 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.

CAUTION

Dissolve the reagents and adjust the pH to 5.2 before adding the sodium azide. Adding sodium azide to an acidic solution results in the release of a poisonous gas.

Powdered malic acid is an irritant, and thus should be handled with due care.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (12 mL capacity).
2. Pipettors, 0.1 and/or 0.2 mL (e.g. Gilson Pipetman[®]) to dispense enzyme extract and substrate.
3. Adjustable-volume dispenser:
 - 0-10 mL (for Extraction Buffer)
 - 0-5 mL (for Stopping Reagent).
4. Top-pan balance
5. Spectrophotometer set at 410 nm.
6. Vortex mixer (optional).
7. Thermostatted water bath set at 40.0°C.
8. Stop Clock.
9. Bench centrifuge or Whatman GF/A glass fibre filter paper circles.

CONTROLS AND PRECAUTIONS:

1. α -Amylase is an enzyme present at high levels in all body fluids. It is thus recommended that disposable gloves are used when handling and dispensing the substrate mixture.
2. It is essential that the water used to dissolve the Ceralpha substrate mixture is high purity. If freshly distilled water is not available, heat the water to boiling and cool it to less than 30°C before using. Algal growth in water in wash bottles can produce sufficient α -amylase to significantly reduce the long-term stability of the reagent dissolved in such water.
3. The freeze-dried substrate is extremely stable at room temperature, however, when dissolved it should be stored at 0-5°C during use and at -20°C between use. If the number of assays performed at any one time is limited, it is recommended that the substrate be divided into 2-3 mL aliquots and stored in the frozen state.
4. On storage at 0-5°C, the blank absorbance values will increase from 0.03 to about 0.06 in 5 days, this does not affect the performance of the substrate, but obviously these values must be determined at the same time as the assay is performed. Blank absorbance values as high as 0.50 do not affect the reliability or accuracy of the assay.

A single Reaction Blank only is normally sufficient for each batch of samples being analysed. To obtain this blank value, 3.0 mL of **stopping reagent** should be added to 0.2 mL of **substrate** solution. Then add 0.2 mL of **enzyme** preparation.

5. The spectrophotometer employed should be standardised with a *p*-nitrophenol standard in 1% Trizma Base ($E_{mM} = 17.8$). *p*-Nitrophenol solution (10 μ moles per mL) can be obtained from Sigma Chemical Company (cat no. 104-1). An aliquot of this solution when diluted 200-fold in 1% Trizma base gives an absorbance of 0.890 at 410 nm.
6. The assay format should be standardised with the enclosed malt flour. The activity of this flour is shown on the enclosed vial. A wheat flour standard can be provided on request.
7. The time of extraction of **wheat flours** should be controlled carefully (5 ± 1 min). With longer extraction times, α -amylase binds to the starch granules resulting in lower activity in the extract.
With **malt flour** samples, the optimal extraction time is 15 min.

USEFUL HINTS:

1. If absorbance values for a particular assay are greater than 1.50, then the enzyme extract should be diluted appropriately with Extraction Buffer and re-assayed. Appropriate corrections to the calculations should then be made.
2. The number of assays which can be performed per kit can be doubled by halving the volumes of all reagents used and employing semi-micro spectrophotometer tubes.

ASSAY PROCEDURE:

Enzyme Extraction (Wheat and Barley Flours):

1. Mill wheat, barley or other grain (approximately 2-10 g sample) to pass a 0.5 mm screen (e.g. with Tecator Cyclotec[®] mill).
2. Weigh accurately 0.5 g of flour into centrifuge tubes of 12 mL capacity.
3. To each tube add a 5.0 mL aliquot of Extraction Buffer solution (pH 5.2) and stir the contents vigorously.
4. Allow the enzyme to extract over a 5±1 minute period at room temperature, with occasional mixing.
5. Filter the solution through a Whatman GF/A glass fibre filter paper, or centrifuge at 1,000g for ten minutes. **Assay activity** within two hours.

Enzyme Extraction (Malt Flour):

1. Mill malt (20 g sample) to pass a 0.5 mm screen.
2. Weigh accurately 0.5 g malt flour into a 100 mL volumetric flask.
3. To the volumetric flask add a solution of 1% sodium chloride plus 0.02% calcium chloride plus 0.02% sodium azide; adjust to volume.
4. Allow the enzyme to extract for approximately 15 minutes at room temperature, with occasional mixing.
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 0.5 mL of the filtrate with 9.5 mL of Extraction Buffer Solution. Assay activity within two hours.

Assay of α -Amylase:

1. Dispense 0.2 mL aliquots of **Ceralpha** Substrate Solution into test tubes and pre-incubate the tubes and contents at **40°C** for five minutes.
2. Pre-incubate wheat, barley or malt extract at 40°C for five minutes.
3. To each tube containing **Ceralpha** Substrate Solution (0.2 mL), add an aliquot (0.2 mL) of pre-equilibrated (and suitably diluted) wheat, barley or malt extract directly to the bottom of the tube. Incubate at 40°C for exactly ten minutes (from time of addition).
4. At the end of the ten minute incubation add exactly 3.0 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance (at 410 nm) of the solutions and the reaction blank against distilled water.

CALCULATION OF ACTIVITY:

Units/g Flour:

$$= \frac{\Delta E_{410}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{E_{mM}} \times \frac{\text{Extraction Volume}}{\text{Sample Weight}} \times \text{Dilution}$$

Where:

ΔE_{410} = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 mins

Total Volume in Cell = 3.4 mL (or 1.7 mL)

Aliquot Assayed = 0.2 mL (or 0.1 mL)

E_{mM} of *p*-nitrophenol in 1% Trizma base = 17.8

Extraction volume = 5 mL per 0.5 gram (wheat and barley) or
100 mL per 0.5 gram (malt)

Dilution = 20 fold (for malt extracts only).

Thus for Wheat and Barley:

Units/g flour:

$$\begin{aligned} &= \frac{\Delta E_{410}}{10} \times \frac{3.4}{0.2} \times \frac{1}{17.8} \times \frac{5}{0.5} \\ &= \Delta E_{410} \times 0.955 \end{aligned}$$

For Malt:

$$\begin{aligned} &= \frac{\Delta E_{410}}{10} \times \frac{3.4}{0.2} \times \frac{1}{17.8} \times \frac{100}{0.5} \times 20 \\ &= \Delta E_{410} \times 382 \end{aligned}$$

One Unit of activity is defined as the amount of enzyme, in the presence of excess α -glucosidase and glucoamylase, required to release one micromole of *p*-nitrophenol from BPNPG7 in one minute under the defined assay conditions, and is termed a **Ceralpha Unit**.

CONVERSION OF ACTIVITY TO INTERNATIONAL UNITS ON STARCH SUBSTRATE:

The activity of pure wheat α -amylase on BPNPG7 substrate and soluble starch (1% w/v) in extraction buffer solution (assayed using the Nelson-Somogyi reducing sugar procedure) have been compared and a conversion factor of 3.0 obtained; i.e. Units of activity on soluble starch (Nelson/Somogyi assay) equals 3.0 x Units of activity on BPNPG7.

A similar value (3.2) has been obtained for a temperature stable *Bacillus subtilis* α -amylase preparation.

COMPARISON OF CERALPHA AND FARRAND METHODS FOR WHEAT AND FUNGAL α -AMYLASES:

A slightly modified version of the Ceralpha method has been developed for this purpose. This document can be accessed from the Megazyme Internet Site under "Ceralpha Vs Farrand Methods".

ASSAY OF α -AMYLASE IN MICROBIAL FERMENTATION BROTHS:

The procedure described here can be directly adapted to the assay of α -amylase in bacterial and fungal fermentation broths. The assay should be performed exactly as described for cereal flour extracts with the modification that the assay pH should be adjusted to suit the particular α -amylase being measured. Fungal α -amylases are usually assayed at pH 5.0, while bacterial α -amylases are assayed at pH 6.0. Thus to adapt this kit for the assay of fungal α -amylase, the pH of the Extraction Buffer should be adjusted to 5.0 (with 1 M hydrochloric acid) and the fermentation broth serially diluted in this buffer to obtain an activity value within the measurable range. For bacterial α -amylases, the Extraction Buffer should be adjusted to pH 6.0 (with 2 M sodium hydroxide).

MEASUREMENT OF RATIOS OF FUNGAL AND CEREAL α -AMYLASE IN MIXTURES:

This can be achieved by assaying extracts both with Ceralpha reagent (Blocked *p*-nitrophenyl maltoheptaoside) and with a reagent containing Blocked *p*-nitrophenyl maltopentaoside. This latter substrate is readily hydrolysed by fungal α -amylase, but not by cereal α -amylase. Further information is available from Megazyme International Ireland Ltd.

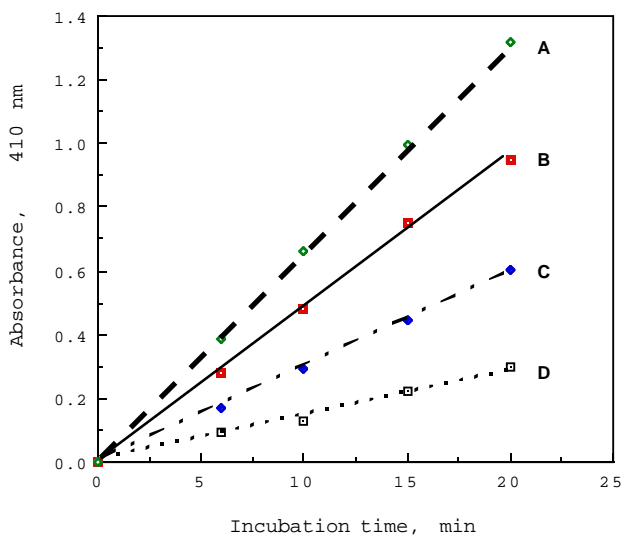
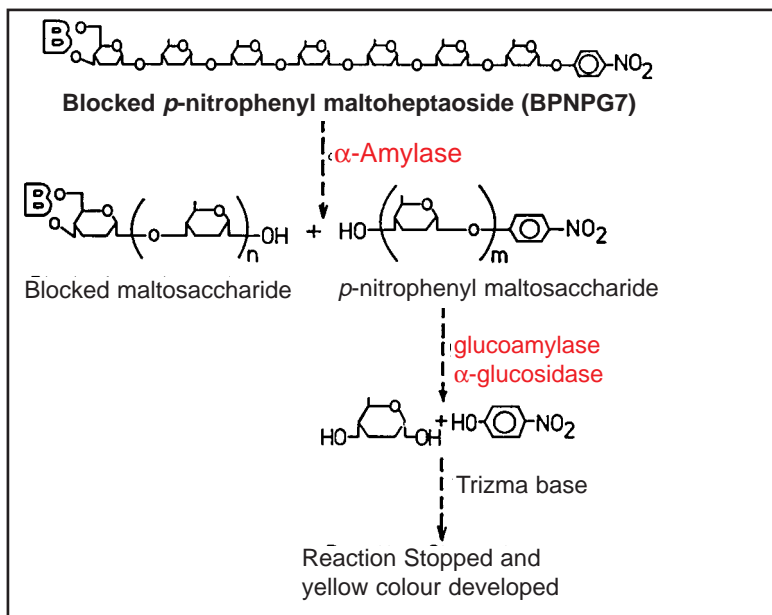


Figure 1. Linearity of the Ceralpha α -amylase assay with enzyme concentration and incubation time.
 D = x; C = 2 x; B = 3 x; A = 4 x.



Scheme 1. Theoretical basis of the Ceralpha α -amylase assay procedure. Immediately α -amylase cleaves a bond within the blocked *p*-nitrophenyl maltosaccharide substrate, the non-blocked reaction product containing the *p*-nitrophenyl substituent is instantly cleaved to glucose and free *p*-nitrophenol by the excess quantities of glucoamylase and α -glucosidase which are integral parts of the substrate mixture, and free *p*-nitrophenol is released. The reaction is terminated and the phenolate colour is developed on addition of Trizma base.



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