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ALPHA-AMYLASE

ASSAY PROCEDURE

(CERALPHA METHOD)

K-CERA 06/18

FOR THE MEASUREMENT OF PLANT AND MICROBIAL ALPHA-AMYLASES

(100/200 Assays per Kit)

AOAC Method 2002.01
AACC Method 22-02.01
ICC Standard No. 303

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INTRODUCTION:

Microbial α-amylases find widespread application in the modification of starch in cereal products and in cereal processing. The level of endogeneous 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. α-Amylase also finds application as a silage additive, to assist in the degradation of starch and thus to provide fermentable sugars for bacterial growth. Bacterial, fungal and cereal α-amylases can all be measured with Amylase HR reagent, however assay conditions (specifically pH) need to be modified to suit each particular enzyme. Amylase HR reagent is specific for α-amylase. The substrate is absolutely resistant to hydrolysis by *exo*-enzymes such as β-amylase, amyloglucosidase and α-glucosidase.

PRINCIPLE:

The Ceralpha procedure (employing Amylase HR reagent) for the assay of α-amylase employs, as substrate, the defined oligosaccharide "non-reducing-end blocked *p*-nitrophenyl maltoheptaoside" (BPNPG7) in the presence of excess levels of a thermostable α-glucosidase (which has 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 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 17) and the linearity of the assay is shown in Figure 1 (page 11).

Essentially, 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 a weak alkaline solution. The absorbance at 400 nm is measured (previously absorbance values were measured at 410 nm in line with literature values, however the true absorption peak is at 400 nm) (see Figure 4, page 12) and this relates directly to the level of α-amylase in the sample analysed.

Amylase HR reagent mixture can be used to quantitatively assay cereal, fungal and bacterial α-amylases. With the replacement of amyloglucosidase and yeast α-glucosidase (as present in the original Ceralpha Reagent mixture) by thermostable α-glucosidase, the assay

can now be used over a broader pH range (5.2 to 7.0) and at temperatures of up to 60°C. With this new reagent, the optimal pH for activity of cereal α-amylases is 5.2-5.4 (see Figure 7, Page 16). Furthermore, in this pH range, the activity values obtained for cereal α-amylases with Amylase HR reagent are essentially the same as those obtained with Ceralpha reagent (containing amyloglucosidase and α-glucosidase) at pH 5.2. Reagent mixtures employing blocked *p*-nitrophenyl maltoheptaoside as substrate do not distinguish between fungal, cereal and bacterial α-amylases.

ACCURACY:

Standard errors of less than 5% are achieved routinely.

KITS:

Kits suitable for performing 100/200 assays are available from Megazyme and consist of:

1. Full assay method
2. Freeze dried BPNPG7 plus thermostable 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[a]**

	Absorbance (400 nm)				
Sample	Day 1	Day 2	Day 3	Day 4	Average
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

[a]Duplicate analyses of single extracts made on four separate days.
[b]Based 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)
Thermostable α-glucosidase	(125 U at pH 6.0), 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 7 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 vial 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: (Buffer A)

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

Dilute the entire contents (50 mL) (plus a crystalline 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 5.4; adjust if necessary.

(2) Concentrated Stopping Reagent: (20% [w/v] tri-sodium phosphate solution, pH ~ 11)

Dilute the entire contents (25 mL) to 500 mL with distilled water. Stable at room temperature for 3 months.

PREPARATION OF ADDITIONAL EXTRACTION BUFFERS:

A. Buffer A (for cereal and fungal α-amylase):

Malic acid (Sigma M0875; 1 M)	134.1 grams/litre
Sodium hydroxide	70 grams/litre
Sodium chloride	58.4 grams/litre
Calcium chloride dihydrate (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 5.4 by dropwise addition of sodium hydroxide (4 M) or HCl (4 M). **Then** add the sodium azide. Adjust volume to 1 L. Store at room temperature. **For use, dilute 50 mL of this concentrated buffer solution to 1 L with dist. water.**

CAUTION

Dissolve the reagents and adjust the pH to 5.4 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. α-amylase):

Maleic acid (Sigma M0375; 0.1 M)	23.2 grams/2 litres
Sodium chloride	11.6 grams/2 litres
Calcium chloride dihydrate (2 mM)	0.6 grams/2 litres
Sodium azide (Sigma S2002; 0.01% w/v)	0.2 grams/2 litres

Add the maleic acid and sodium chloride to 1600 mL of distilled water and adjust the pH to 6.5 with 4 M (160 g/L) sodium hydroxide. Add the calcium chloride and sodium azide and adjust the volume to 2 L. Store at room temperature between use.
Use this buffer directly without further dilution.
Some bacterial α-amylases are unstable on dilution. This problem is usually resolved by inclusion of BSA (0.5 mg/mL) in the buffer.

PREPARATION OF ADDITIONAL STOPPING REAGENT:

Dissolve 10 g of tri-sodium phosphate (anhydrous) in 1 L of distilled water and adjust the pH to approx. 11.0. Stable at room temperature for at least 3 months.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (12 mL and 20 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. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.5 mL aliquots of concentrated enzyme solutions).
   - with 25 mL Combitip® (to dispense various aliquots of dilution buffers).

5. Top-pan balance.
6. Spectrophotometer set at 400 nm.
7. Vortex mixer (optional).
8. Thermostated water bath set at 40°C.
9. Stop Clock.
10. Bench centrifuge or Whatman GF/A glass fibre filter paper circles (9 cm diameter).

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 of 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 below -10°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 0.05 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.

NOTE:
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% tri-sodium phosphate (ε_{mM} = 18.1). *p*-Nitrophenol solution (10 mM) can be obtained from Sigma Chemical Company (cat. no. N7660). An aliquot of this solution when diluted 200-fold in 1% tri-sodium phosphate gives an absorbance of 0.905 at 400 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 carefully controlled (15-20 min). With **malt flour** samples, the optimal extraction time is also 15-20 min.

USEFUL HINTS:

1. If the absorbance values for a particular assay are greater than 1.20, the enzyme extract should be diluted with the appropriate buffer and re-assayed.
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 cuvettes.

ENZYME EXTRACTION:

A. Wheat and Barley Flours:

1. Mill wheat, barley or other grain (approx. 10-50 g sample) to pass a 0.5 mm screen (e.g. with a Fritsch centrifugal mill).
2. Accurately weigh 3.0 g of flour into a flask of 50 mL capacity.
3. To each flask add 20.0 mL of Extraction Buffer solution (pH 5.4) and stir the flask contents vigorously.
4. Allow the enzyme to extract over 15-20 min at 40°C with occasional mixing.
5. Filter an aliquot of the solution through a Whatman GF/A glass fibre filter paper, or centrifuge an aliquot at 1,000 *g* for 10 min. **Assay enzyme activity** within 2 h.

NOTE:
The control wheat flour from Megazyme is very homogeneous. Consequently, a smaller sample weight can be extracted (e.g. 1.0 g per 6.0 mL of extraction buffer.

B. Malt Flours:

1. Mill malt (20 g sample) to pass a 0.5 mm screen.
2. Accurately weigh 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 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 1,000 *g* for 10 min.
6. Dilute 0.5 mL of the filtrate with 9.5 mL of Extraction Buffer Solution. **Assay enzyme activity** (see page 6, section A, point 5) within 2 h.

C. 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 1.0 mL of **Original Extract** 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 Kerry Ingredients, Ireland) a dilution of the **Original Extract** of approx. 4,000-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 (1,000 *g*, 10 min) or filtration through Whatman No. 1 (9 cm) filter circles.
3. Dilute 1.0 mL of this solution 10-fold by addition to 9.0 mL of appropriate **Extraction/Dilution buffer** and mix thoroughly. Repeat this step until a dilution suitable for assay is obtained.

ASSAY PROCEDURE:

A. Wheat and barley flours:

1. Dispense 0.2 mL aliquots of **Amylase HR Reagent** solution (unbuffered) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate cereal extract at 40°C for 5 min.
3. To each tube containing **Amylase HR Reagent** solution (0.2 mL), add 0.2 mL of pre-equilibrated wheat or barley extract directly to the bottom of the tube. Incubate at 40°C for exactly **20 min** (from time of addition).
4. At the end of the **20 min** incubation period, add exactly 3.0 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at 400 nm against distilled water.

B. Malt and microbial preparations:

1. Dispense 0.2 mL aliquots of **Amylase HR Reagent** solution (unbuffered) 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 **Amylase HR Reagent** solution (0.2 mL), add 0.2 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).
4. At the end of the **10 min** incubation period, add exactly 3.0 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at 400 nm against distilled water.

CALCULATION OF ACTIVITY:

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

Units/g Flour:

$$= \frac{\Delta E_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\varepsilon_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution}$$

where:

ΔE_{400} = Absorbance (reaction) - Absorbance (blank)
Incubation Time = 10 min (malt and microbial preparation extracts)
= 20 min (wheat and barley extracts)
Total Volume in Cell = 3.4 mL
Aliquot Assayed = 0.2 mL
ε_{mM} of *p*-nitrophenol (at 400 nm) in 1% tri-sodium phosphate = 18.1
Extraction volume = 20 mL per 3 gram (wheat and barley)
100 mL per 0.5 gram (malt)
50 mL per 1 g or 1 mL of microbial preparation
Dilution = Dilution of the original extract
(= 20-fold for malt extracts)

Thus:

A. For Wheat and Barley:

Units (CU)/g flour:

$$= \frac{\Delta E_{400}}{20} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{20}{3.0}$$

$$= \Delta E_{400} \times 0.313$$

B. For Malt:

Units (CU)/gram of milled malt:

$$= \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{100}{0.5} \times 20$$

$$= \Delta E_{400} \times 376$$

C. For microbial preparations:

Units (CU)/mL or gram of original preparation:

$$= \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{50}{1.0} \times \text{Dilution}$$

$$= \Delta E_{400} \times 4.7 \times \text{Dilution}$$

where:

ΔE_{400} = Absorbance (reaction) - Absorbance (blank)
Incubation Time = 10 min
Total volume in cell = 3.4 mL
Aliquot Assayed = 0.2 mL
ε_{mM} of *p*-nitrophenol (at 400 nm) in 1% tri-sodium phosphate = 18.1
Extraction vol = 50 mL/1.0 g (or 49 mL plus 1 mL of enzyme concentrate)
Dilution = further dilution of the **Original Extract**

APPENDIX:

A. Linearity of Ceralpha assay with enzyme concentration and incubation time.

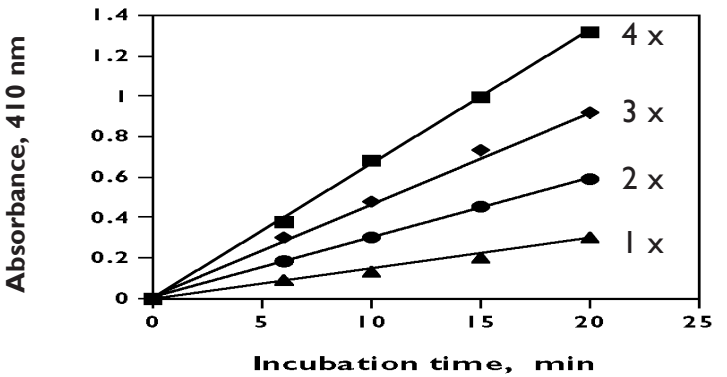


Figure 1. **Linearity of the Ceralpha assay with malt α-amylase in sodium malate buffer (pH 5.4).** The assay was performed with four concentrations of enzyme (1x, 2x, 3x and 4x). Reaction was terminated at various times by adding tri-sodium phosphate (3.0 mL, 1% w/v).

B. Effect of the concentration of thermostable α-glucosidase in the reagent solution on determined α-amylase values.

From the results shown in Figure 2, it is evident that the concentration of α-glucosidase required to saturate the reaction is 12 U/mL in the substrate solution.

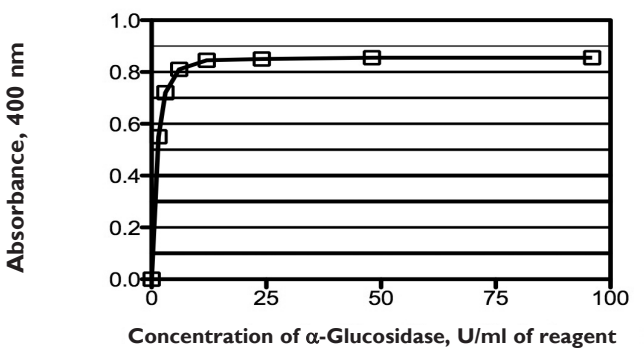


Figure 2. **The effect of the concentration of α-glucosidase in the substrate reagent solution on the determined absorbance values.**

C. Stability of reagent mixture at 60°C.

The stability of the reagent solution was determined by incubating aliquots of this solution at 60°C for 0-20 min. These solutions were then used to assay the activity of fungal α-amylase (at 40°C). From the data shown in Figure 3, it is evident that the reagent is very stable at 60°C. Over a 20 min incubation period, the blank absorbance values increased by less than 0.01 absorbance units, and the determined activity decreased by less than 3% (of the non pre-incubated reagent).

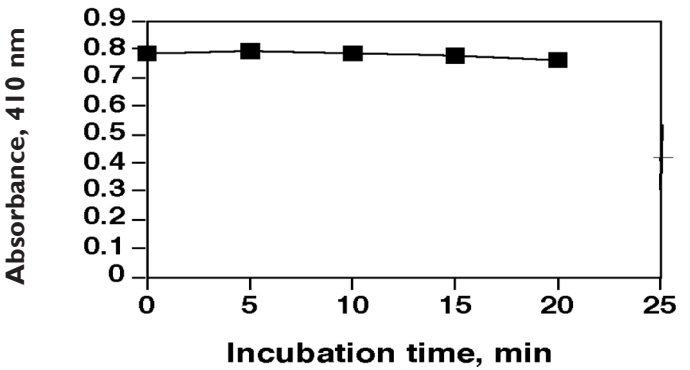


Figure 3. **Temperature stability of Amylase HR assay reagent.** Aliquots of the reagent were stored at 60°C for 0-20 min, cooled to room temperature, and used to assay fungal α-amylase at 40°C.

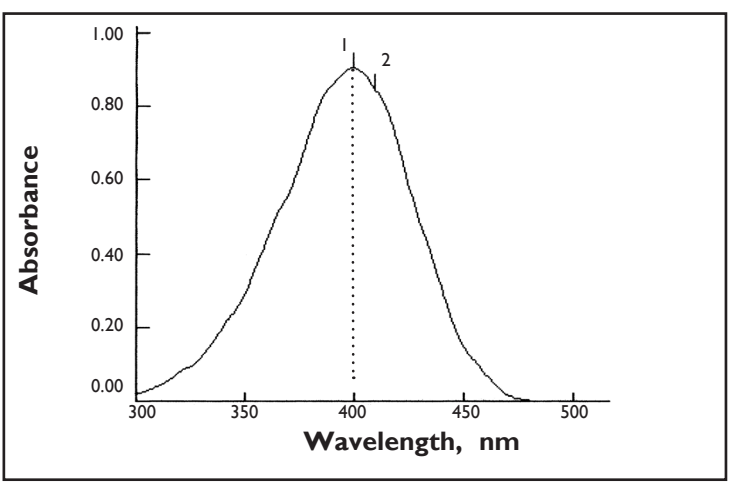


Figure 4. **Absorbance curve for *p*-nitrophenol in 1% tri-sodium phosphate at pH 11.0.**

D. Conversion of Ceralpha Units (CU) to International Units (IU) on Starch Substrate.

The activity of pure ***Bacillus subtilis***, ***Aspergillus niger*** and barley malt α-amylases on Amylase HR Reagent and on ACS soluble starch (1% w/v; assayed using the Nelson-Somogyi reducing sugar procedure; International Units) was determined and the conversion factors are:

A. niger (both assays performed at pH 5.4)
International Units on Starch = 0.94 x Ceralpha Units.

B. subtilis (both assays performed at pH 6.5)
International Units on Starch = 4.6 x Ceralpha Units.

Barley malt (both assays performed at pH 5.4)
International Units on Starch = 4.1 x Ceralpha Units.

One International Unit (IU) of activity is defined as the amount of enzyme required to release one micromole of glucose reducing-sugar equivalents per minute under defined conditions of temperature and pH.

E. Comparison of the Ceralpha and Farrand Methods for the Measurement of Wheat and Fungal α-Amylase.

The Farrand method employs a β-limit dextrin of starch as substrate, and measures the decrease in the colour of the starch/iodine complex on depolymerisation of the substrate. The assay is performed in the presence of excess quantities of β-amylase which, for cereal samples, originate from the flour extract. For fungal samples, a pure β-amylase has to be added. The Farrand method was commonly used in the United Kingdom and employed a β-limit dextrin preparation supplied by Rank Hovis. [A purified β-limit dextrin (maltose removed) is now available from Megazyme International].
In the standard Farrand method, the extract is unbuffered and the pH of the flour extracts is approx. 5.8. In an interlaboratory comparison of the Farrand and Ceralpha methods, coordinated by Campden-Chorleywood Food Research Association, the correlation between Farrand and Ceralpha Units for **wheat flour** α-amylase was found to be:

Farrand Units = Ceralpha Units x 57 - 1.9

A very similar correlation for wheat α-amylase was previously reported by McCleary and Sheehan (1987), namely:

Farrand Units = Ceralpha Units x 57.1

For fungal preparations, the regression equation obtained by McCleary ***et al***. was:

Farrand Units = Ceralpha Units x 69

The measurement of α-amylase in wheat flour supplemented with fungal α-amylase is complicated by the problems associated with the blending of two components, one of which has a low level of enzyme and the other of which has a level several thousand-fold higher than the first component. To try to minimise the errors associated with possible incomplete mixing of these components, duplicate samples should be assayed and larger samples should be extracted (~ 6 g/40 mL).

F. Comparison of the Ceralpha Method (CU), the ASBC Method (DU) and AACC Method 22-01 (SKB Units) for the Measurement of α-Amylase:

AACC method 22-01 (SKB Units) uses a β-limit dextrin prepared from a "special" lintner starch supplied by AACC/ASBC. The method measures the time to reach a particular colour with iodine, on incubation of α-amylase with the β-limit dextrin at 30°C.

The ASBC/EBC/International Method (Dextrinising Units, DU) uses the same substrate and the same concentration of enzyme as employed in AACC Method 22-01, and the Units of activity are calculated the same way. However, since the assay is performed at 20°C, DU values for a particular malt sample are approximately half the SKB value for the same malt.

The correlation between AACC Method 22-01 (SKB Units) and Ceralpha Units (CU) for malt flours is shown in Figure 5. The correlation between the ASBC (International Method) for α-amylase (DU) and the Ceralpha method (CU) is shown in Figure 6.

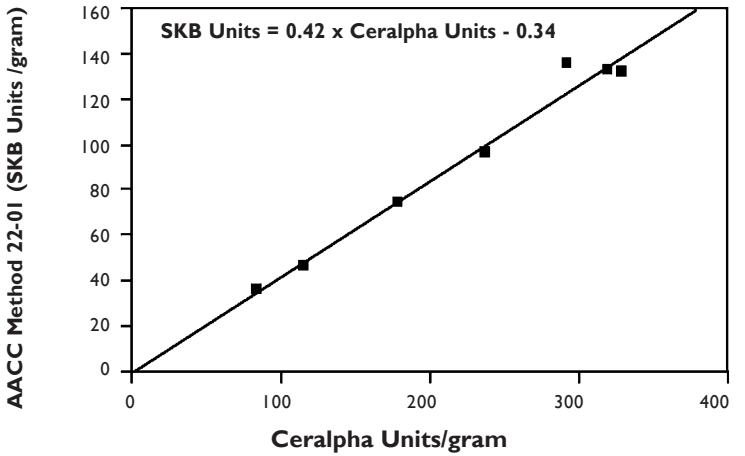


Figure 5. **Comparison of the Ceralpha method and AACC Method 22-01 (SKB) for the measurement of α-amylase in malt flours.**
Seven malt samples were analysed in duplicate by the two methods.

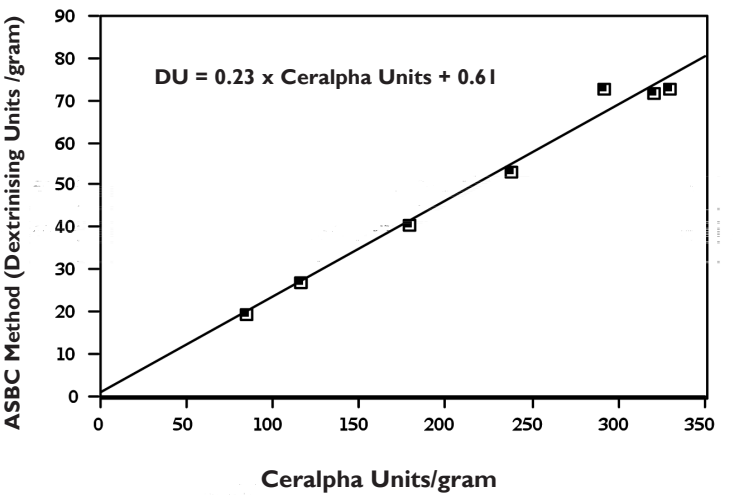


Figure 6. **Comparison of the Ceralpha and the ASBC (International) methods for the measurement of α-amylase in malt flours.**
Seven malt samples were analysed in duplicate by the two methods.

The conversion factors relating SKB Units to Ceralpha Units for malt, fungal and bacterial α-amylases are:

Malt α-amylase:

SKB Units = 0.42 x Ceralpha Units (CU) - 0.34.

(SKB performed at pH 4.7; Ceralpha performed at pH 5.4).

Fungal α-amylase:

SKB Units = 0.60 x Ceralpha Units (CU).

(SKB performed at pH 5.4; Ceralpha performed at pH 5.4).

Bacterial α-amylase:

SKB Units = 1.8 x Ceralpha Units (CU).

(SKB performed at pH 6.5; Ceralpha performed at pH 6.5).

G. pH Activity Curves for Cereal, Fungal and Bacterial α-Amylases.

pH Activity curves for malted wheat, malted barley, fungal (***A. niger***) and bacterial (***B. subtilis***) α-amylases were determined using the Ceralpha method with Amylase HR reagent. For cereal and fungal α-amylases, the pH curve was prepared using malate and maleate buffers (100 mM, pH 5.0-6.4); for bacterial α-amylase, maleate and Bis-Tris Propane buffers were employed (pH 5.6-9.0). All buffers contained 10 mM calcium chloride. The curves for purified malted wheat and fungal α-amylases are shown in Figure 7. Malted barley α-amylases gave the same curve as malted wheat. The pH activity curve for ***B. subtilis*** α-amylase is shown in Figure 8.

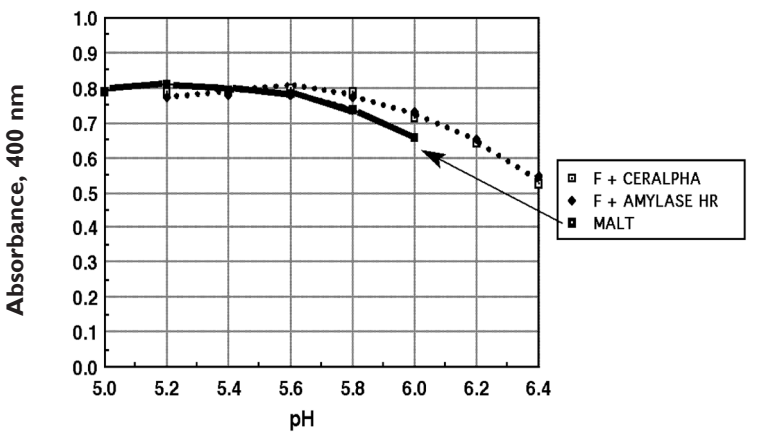


Figure 7. **pH activity curves for fungal (*A.niger*) and malted wheat α-amylases.** Fungal α-amylase was assayed with both Amylase HR reagent and Cereal α-Amylase assay reagent (containing amyloglucosidase and yeast α-glucosidase). The curves with the two reagents were the same.

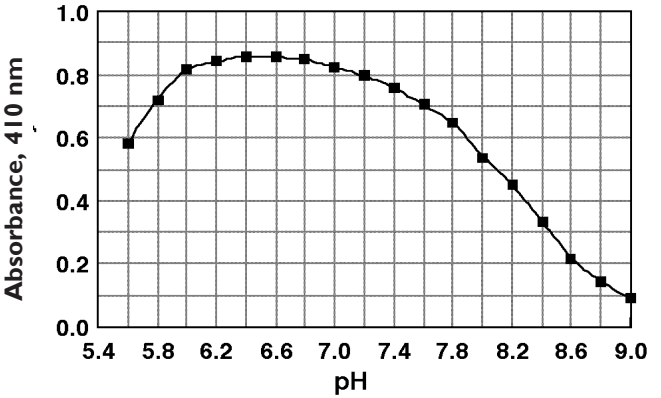
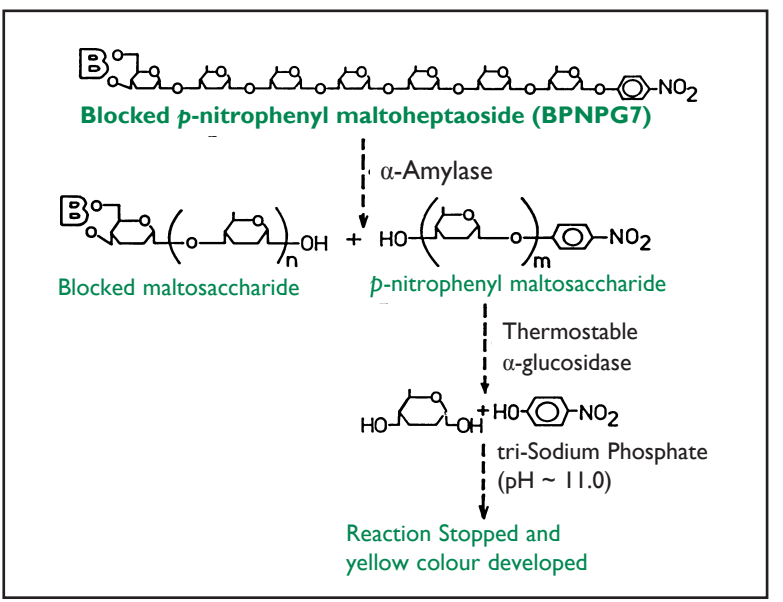


Figure 8. **pH activity curves for *B. subtilis* α-amylase.**



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 thermostable α-glucosidase which is an integral part of the substrate mixture, and free *p*-nitrophenol is released. The reaction is terminated and the phenolate colour is developed on addition of tri-sodium phosphate (pH ~ 11.0).

REFERENCES:

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