Megazyme

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MALT-AMYLASE

ASSAY PROCEDURE

BETAMYL-3® and CERALPHA® METHODS

K-MALTA 08/18

(50 assays of each per Kit)

INTRODUCTION:

α-Amylase and β-amylase play central roles in the complete degradation of starch to metabolisable or fermentable sugars during the germination or malting of cereal grains. Numerous methods have been developed for the measurement of α-amylase but the only procedure that employs a well defined substrate is the Ceralpha® method as described here. β-Amylase is usually measured using non-specific reducing sugar assays with starch as substrate. In some methods, the α-amylase is first inactivated by treatment at low pH.

The Ceralpha® procedure 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 (Amylase HR® reagent). The thermostable α-glucosidase 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 9) and the linearity of the assay is shown in Figure 1 (page 10).

The Megazyme **Betamyl-3®,** β-amylase test reagent employs high purity, thermostable β-glucosidase and *p*-nitrophenyl-β-D-maltotrioside (PNPβ-G3). The thermostable β-glucosidase has no action on the native substrate due to its inability to cleave α-linked D-glucosyl residues. On hydrolysis of PNPβ-G3 to maltose and *p*-nitrophenyl-β-D-glucose by β-amylase, the *p*-nitrophenyl-β-D-glucose is immediately cleaved to D-glucose and free *p*-nitrophenol by the excess quantities of β-glucosidase present in the substrate mixture (Scheme 2, page 10). Thus, the rate of release of *p*-nitrophenol relates directly to the rate of release of maltose by β-amylase. The reaction is stopped, and the phenolate colour is developed, on addition of a high pH Tris buffer solution. The time course of hydrolysis of PNPβ-G3 by pure barley β-amylase is shown in Figure 2 (page 11).

ACCURACY:

Standard errors of less than 5% are readily achieved.

SPECIFICITY:

The Ceralpha® assay is highly specific for α-amylase and the Betamyl-3® assay is highly selective for β-amylase. The Betamyl-3® substrate is hydrolysed by α-glucosidase and amyloglucosidase, whereas Amylase HR® reagent (Ceralpha® substrate) is not hydrolysed by these enzymes.

KITS:

Kits suitable for performing 50 assays of both α-amylase and β-amylase are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: **Ceralpha® substrate.** Blocked *p*-nitrophenyl-α-D-maltoheptaoside (BPNPG7) plus thermostable α-glucosidase (125 U) and stabilisers.
Stable for > 4 years below -10°C.

Bottle 2: **Betamyl-3® substrate.** *p*-Nitrophenyl-β-D-maltotrioside (PNPβ-G3) plus thermostable β-glucosidase (50 U) and stabilisers.
Stable for > 4 years below -10°C.

Bottle 3: **Ceralpha® Buffer A.** Sodium malate/sodium chloride (1 M) and $CaCl_2$ (40 mM) (50 mL, pH 5.4) plus sodium azide (0.02% w/v).
Stable for approx. 4 years at 4°C.

Bottle 4: **Betamyl-3® Buffer A.** Tris/HCl buffer (25 mL, 1 M, pH 8.0) plus disodium EDTA (20 mM) and sodium azide (0.02% w/v).
Stable for approx. 4 years at 4°C.

Bottle 5: **Betamyl-3® Buffer B.** MES buffer (48 mL, 1 M, pH 6.2) plus disodium EDTA (20 mM), BSA 10 mg/mL and sodium azide (0.10% w/v).
Stable for approx. 4 years at 4°C.

Bottle 6: Malt flour of standardised α-amylase and β-amylase activity (as specified on bottle label).
Stable for > 4 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS:

1. Dissolve the contents of bottle 1 in 10 mL of boiled and cooled distilled water. This is **Ceralpha® Substrate Solution**. Divide into appropriately sized aliquots and store in polypropylene tubes below -10°C between use. Store unused reagent on ice awaiting use.

2. Dissolve the contents of bottle 2 in 10 mL of boiled and cooled distilled water. This is **Betamyl-3® Substrate Solution**. Divide into appropriately sized aliquots and store in polypropylene tubes below -10°C between use. Store unused reagent on ice awaiting use.

Be careful not to mix Ceralpha® and Betamyl-3® substrates.

3. Diluted **Ceralpha® Buffer A**. Dilute the entire contents (50 mL) (plus a crystalline precipitate which may be present) to 1000 mL with distilled water before use. The pH should be 5.4; adjust if necessary.
Stable for > 12 months at 4°C.

4. Diluted **Betamyl-3® Buffer A**. Dilute 2.5 mL of the contents of bottle 4 to 50 mL with distilled water. Before use, add 0.88 g of cysteine HCl (Megazyme cat. no. **G-LCYST200**) (final concentration of cysteine HCl approx. 100 mM) and adjust the pH to 8.0 with 4 M NaOH.
Stable for 8 h at 4°C.

Cysteine is added to Buffer A (followed by pH adjustment) immediately prior to use of the buffer. Cysteine is required to extract the "insoluble" β-amylase present in ungerminated grain. This buffer has been changed from our original recommendation, based on research by Erdal (1993) and Santos and Riis (1996). Enzyme extracted without added cysteine is termed "Soluble" β-amylase; that extracted with cysteine is "Total" β-amylase.

5. Diluted **Betamyl-3® Buffer B**. Dilute the entire contents of bottle 5 to 500 mL with distilled water.
Stable for approx. 1 year at 4°C.

6. Use the contents of bottle 6 as supplied.
Stable for > 4 years stored dry at room temperature.

PREPARATION OF ADDITIONAL EXTRACTION BUFFERS:

A. Ceralpha® Buffer A (for α-amylase)

Stock Buffer:

Malic acid (Sigma M0875; 1 M)	134.1 grams/litre
Sodium hydroxide	70.0 grams/litre
Sodium chloride	58.4 grams/litre
Calcium chloride.$2H_2O$ (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 (working buffer):

Dilute 50 mL of this concentrated buffer solution to 1 L with distilled water. Adjust pH to 5.4 if necessary.

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

B. Betamyl-3® Buffer A - ready to use (for β-amylase)

[50 mM Tris/HCl buffer (pH 8.0) plus 1 mM disodium EDTA and 0.02% w/v sodium azide].

Dissolve 6.06 g of Tris buffer (Megazyme cat. no. **B-TRIS500**) and 0.37 g of disodium EDTA (Sigma cat. no. E4884-500G) in 700 mL of distilled water. Adjust the pH to 8.0 with 1 M HCl and the volume to 1 L. Add 0.2 g of sodium azide.
Stable for > 4 years at 4°C.

Immediately before use, add 0.88 g of cysteine HCl (Megazyme cat. no. **G-LCYST200**) to 50 mL of the buffer (final concentration of cysteine HCl approx. 100 mM). Adjust pH to 8.0 with 4 M NaOH.
Stable for 8 h at 4°C.

C. Betamyl-3® Buffer B - ready to use (for β-amylase)

[0.1 M MES buffer (pH 6.2) plus 2 mM EDTA, 1.0 mg/mL of BSA and 0.02% w/v sodium azide].

Dissolve 21.3 g of MES monohydrate (Megazyme cat. no. **B-MES250**) and 0.74 g of disodium EDTA (Sigma cat. no. ED2SS) in 700 mL of distilled water. Adjust the pH to 6.2 with 4 M (16 g/100 mL) sodium hydroxide and the volume to 1 L. Add 1.0 g of BSA (Sigma cat. no. A2153) and 0.2 g of sodium azide as a preservative.
Stable for > 2 years at 4°C.

NOTE:

1. Do not add the sodium azide to the buffer until it has been adjusted to pH 6.2. Adding sodium azide to an acidic solution results in the release of a poisonous gas.
2. If buffer is prepared without adding sodium azide as a preservative, then it should be used within a week and must be stored at 4°C.

STOPPING REAGENT:

[1% (w/v) Tris buffer solution (pH 8.5)]

Dissolve 10 g of Tris buffer (Megazyme cat. no. **B-TRIS500**) in 900 mL of distilled water. Adjust the pH to 8.5 (if necessary) and the volume to 1 L. Stable for approx. 1 year at room temperature.

EQUIPMENT (RECOMMENDED):

1. DLFU disc mill (Buhler Miag) as specified in EBC method 1.1. Gap between grinding discs is 0.2 mm. Alternatively, a Frisch Pulverisette 14® with 0.5 mm screen or Tecator Cyclotec® Mill can be used.
2. Disposable 13 mL polypropylene tubes, e.g. Sarstedt cat. no. 60.541.685 PP (www.sarstedt.com).
3. Disposable 1.5 mL polypropylene screw cap microfuge tubes, e.g. Sarstedt cat. no. 72.692 (www.sarstedt.com).
4. Glass 12 mL test tubes.
5. Disposable plastic cuvettes (1 cm light path, 3.0 mL), e.g. Plastibrand®, PMMA disposable cuvettes; Cat. No. 759105 (www.brand.de).
6. Micro-pipettors, e.g. Gilson Pipetman® (100 µL and 200 µL).
7. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of substrate solution).
   - with 25 mL Combitip® (to dispense extraction buffer and 3.0 mL of Stopping Reagent).
8. Analytical balance, accurate to 0.01 g.
9. Spectrophotometer set at 400 nm.
10. Vortex mixer (e.g. IKA® MS3 basic Compact Orbital Shaker).
11. Stop watch.
12. Bench centrifuge or alternatively Whatman GF/A glass fibre filter paper circles.
13. Eppendorf centrifuge 54XX (13,000 rpm; 15,000 *g*).
14. Waterbath (40 ± 1°C).

CONTROLS AND PRECAUTIONS:

1. β-Amylase is extremely unstable when highly diluted in buffers not containing other proteins. It is thus **essential** that additional dilution/assay buffer is prepared **exactly** as described and particularly that bovine serum albumin (BSA) is included.
2. For each set of assays, a **reagent blank** value should be determined. To obtain this value, add 3.0 mL of **Stopping Reagent** to 0.2 mL of pre-equilibrated **Betamyl-3® substrate solution** or **Ceralpha® substrate solution** and then add 0.2 mL of diluted malt extract. A single reagent blank determination for each type of assay (Ceralpha® or Betamyl-3®) is sufficient for each set of assays.
3. If **reagent blank** absorbance value exceeds **0.3**, then the substrate should be discarded.
4. If **reaction values** exceed **1.8**, then the enzyme extract should be further diluted in the assay buffer and re-assayed. Appropriate corrections to the calculations should then be made.
5. PNPβ-G3 is very resistant to cleavage by cereal α-amylases, but some α-amylases, particularly those of fungal origin do cleave it. Therefore, this assay cannot be used to specifically measure β-amylase in materials which also contain substantial levels of fungal α-amylase activity, e.g. flours to which fungal α-amylase has been added. The substrate is rapidly hydrolysed by α-glucosidase and amyloglucosidase. Malt does not contain amyloglucosidase and the level of α-glucosidase is not significant in terms of the assay.

USEFUL HINTS:

1. The substrate should be stored frozen between use and on ice after thawing. In the lyophilised powder form (as supplied), the substrate mixture is stable for > 4 years below -10°C.

2. The number of assays which can be performed per kit can be doubled by halving the volumes of all the reagents used and by employing semi-micro spectrophotometer tubes. Do not alter the concentration of substrate in the final reaction mixture.

ASSAY PROCEDURE:

Enzyme Extraction:

1. Mill 50 g malt with Buhler Miag disc mill. Gap between the grinding discs is 0.2 mm.
2. To 0.5 +/- 0.01 g of flour (note exact weight) in a 13 mL polypropylene tube **add** 5.0 mL of **Betamyl-3® buffer A** (reagent 4, page 3). Vortex briefly.

3. Allow the enzyme to extract over a **1 h** period at room temperature after placing the tubes into a Stuart Blood Tube Rotator (http://design.hileytech.com/fisher/Stuartblood.html), or manually vortex the tubes for 5 sec at approx. 10 min intervals.
4. Centrifuge the tubes in a bench centrifuge or microfuge at a minimum of 2,000 *g* for 10 min. Alternatively filter an aliquot of the extract solution through Whatman GF/A glass fibre filter paper.
5. Add 0.2 mL of filtrate to 4.0 mL of diluted **Betamyl-3® buffer B** (reagent 5, page 3), mix (this is **Extract A**) and use this solution for the assay of both α-amylase and β-amylase activities as described below:

Assay of β-Amylase:

1. Dispense 0.2 mL aliquots of diluted malt extract (**Extract A)** directly to the bottom of 12 mL glass test tubes and pre-incubate the tubes at 40°C for approx. 5 min.
2. Pre-incubate **Betamyl-3® substrate solution** at 40°C for approx. 5 min.
3. To each tube containing **Extract A** add 0.2 mL of **Betamyl-3® substrate solution**, briefly stir on a vortex mixer and incubate tubes at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add 3.0 mL of **Stopping Reagent** with multipipettor and stir the tube contents.
5. Read the absorbance of the **reaction solutions** and the **reagent blank** (see Controls and Precautions, 2, page 6) at **400 nm** against distilled water.

CALCULATION OF ACTIVITY:

Units of β-Amylase / g of malt flour:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable β-glucosidase, required to release one micromole of *p*-nitrophenol from PNPβ-G3 in one minute under the defined assay conditions and is termed a **Betamyl-3® Unit.**

Units of β-Amylase / g of flour = ΔE_{400} x 19.7

Assay of α-Amylase:

1. Make a further dilution of the malt extract by adding 0.2 mL of **Extract A** to 3.0 mL of **diluted Ceralpha® Buffer A** (reagent 3, page 3). This is **Extract B**.
2. Dispense 0.2 mL aliquots of **Extract B** directly to the bottom of 12 mL glass test tubes and pre-incubate the contents at 40°C for approx. 5 min.
3. Pre-incubate **Ceralpha® substrate solution** at 40°C for approx. 5 min.
4. To each tube containing diluted malt extract add 0.2 mL of **Ceralpha® substrate solution**, briefly stir on a vortex mixer and incubate tubes at 40°C for exactly 10 min (from time of addition).
5. At the end of the 10 min incubation period, add 3.0 mL of **Stopping Reagent** and stir the tube contents.
6. Read the absorbance of the **reaction solutions** and the **reagent blank** (see Controls and Precautions, 2, page 6) 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 of α-Amylase / g of flour = ΔE_{400} x 315.6

Details of the Calculation of α- and β-Amylase Activities:

Units of Amylase (α- or β-) / g of flour =

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

where:

ΔE_{400}	=	Absorbance (sample) - Absorbance (blank)
Incubation time	=	10 min
Total volume in cell	=	3.4 mL
Aliquot assayed	=	0.2 mL
ε_{mM} *p*-nitrophenol	=	18.1 (at 400 nm) in 1% Tris buffer solution
Extraction volume	=	5 mL per 0.5 g of malt
Sample weight	=	grams
Dilution	=	0.2 mL to volume of 4.2 mL (i.e. 21-fold) for β-amylase; then a further 0.2 mL to 3.2 mL (16-fold) for α-amylase (i.e. total 336-fold).

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7. Santos, M. M. & Riis, P. (1996). Optimized McCleary method for measurement of total β-amylase in barley and its applicability. *Journal of the Institute of Brewing*, **102**, 271-275.

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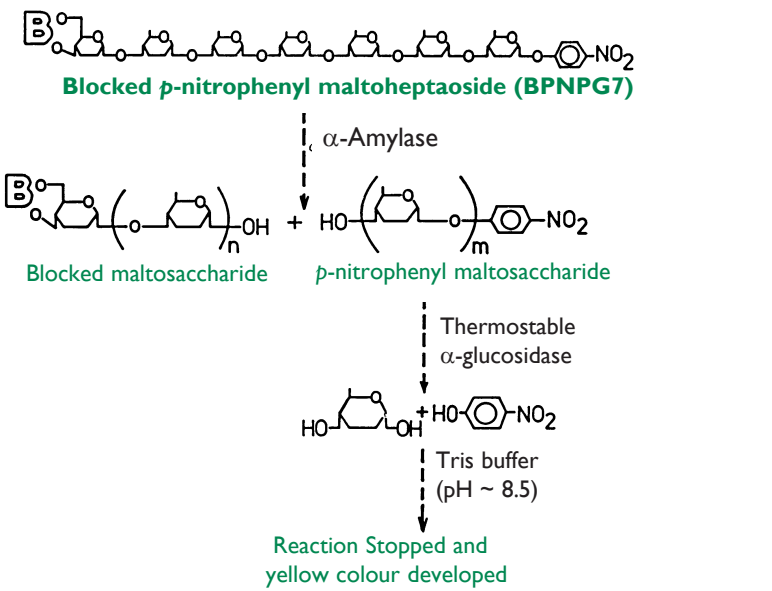
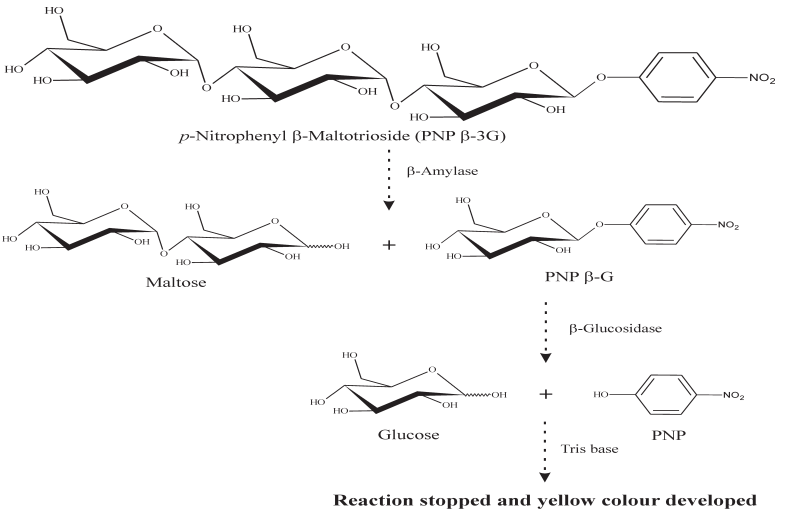


Scheme 1. **Theoretical basis of the Ceralpha® α-amylase assay procedure**. Immediately α-amylase cleaves a bond within the blocked *p*-nitrophenyl maltosaccharide substrate, the non-blocked reaction product containing the *p*-nitrophenyl substituent is instantly cleaved to glucose and free *p*-nitrophenol 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 Tris buffer (pH ~ 8.5).



Scheme 2. Theoretical basis of the Betamyl-3® β-amylase assay procedure. When PNPβ-G3 is cleaved to maltose and PNPβ-G, the latter is rapidly cleaved to *p*-nitrophenol and glucose by the excess quantities of β-glucosidase which is an integral part of the reagent mixture.

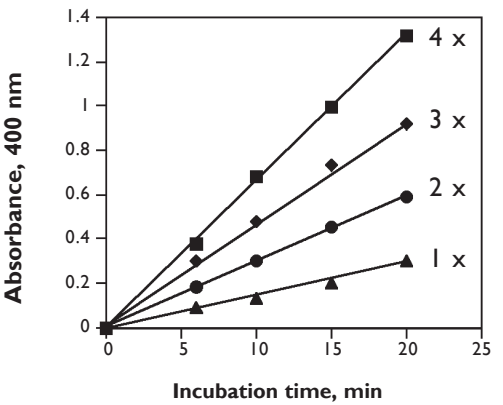


Figure 1. **Linearity of the the Ceralpha® assay with malt α-amylase in sodium malate buffer (pH 5.4).** The assay was performed with four concentrations of enzyme (1x, 2x, 3x and 4x). Reaction was terminated at various times by adding Tris buffer (3.0 mL, 1% w/v, pH 8.5).

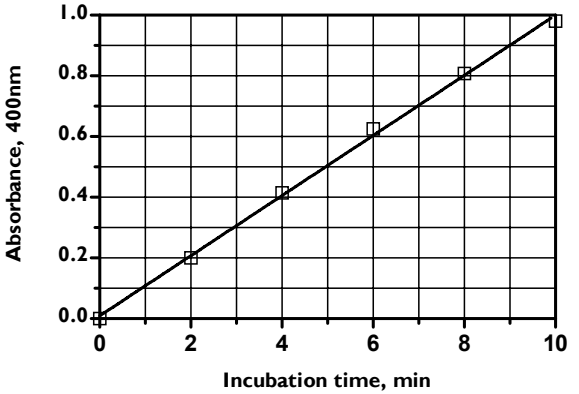


Figure 2. Time course of hydrolysis of PNPβ-G3 (in Betamyl-3® reagent) by pure barley β-amylase as shown by increase in absorbance at 400 nm.

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