Megazyme

BETA-AMYLASE

ASSAY PROCEDURE

(BETAMYL METHOD)

K-BETA 12/04

(150 Assays per Kit)

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INTRODUCTION:

β-Amylase plays a central role in the complete degradation of starch to metabolisable or fermentable sugars during the germination or malting of cereal grains. It also finds considerable application, together with starch debranching enzymes, in the production of high maltose syrups. 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.

A major advance in the assay of β-amylase was introduced by Mathewson and Seabourn[1] who found that the Calbiochem Pantrak® serum α-amylase reagent could be used to measure β-amylase in the presence of cereal α-amylase. The reagent (Pantrak) consists of a mixture of *p*-nitrophenyl-α-D-maltopentaoside (PNPG5) and *p*-nitrophenyl-α-D-maltohexaoside (PNPG6). These substrates are rapidly hydrolysed by β-amylase, but are only slowly cleaved by cereal α-amylase, which requires a longer stretch of α-1,4-linked D-glucosyl residues to satisfy the substrate sub-site binding requirements. In fact, PNPG6 is cleaved by cereal α-amylase at about five times the rate for PNPG5, whereas β-amylase cleaves both substrates at very similar rates.

The Megazyme **Betamyl** β-amylase test reagent[2] employs high purity α-glucosidase and PNPG5, and the level of α-glucosidase used ensures maximum sensitivity of the assay. On hydrolysis of *p*-nitrophenyl-α-D-maltopentaoside to maltose and *p*-nitrophenyl-α-D-maltotrioside by β-amylase, the *p*-nitrophenyl-α-D-maltotrioside is immediately cleaved to glucose and free *p*-nitrophenol by the α-glucosidase present in the substrate mixture (Scheme 1). 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 Trizma base solution.

The PNPG5 plus α-glucosidase mixture contains stabilisers which significantly increase its stability i.e. reduce the rate of cleavage of PNPG5 by α-glucosidase while being stored. The blank absorbance value of the **Betamyl** substrate, when stored in liquid form at 4 or 20°C, increases at only 5-10 % the rate of that for the reagent described by Mathewson and Seabourn[1] (i.e. the substrate is 10-20 times more stable).

ACCURACY:

Standard errors of less than 7 % are readily achieved.

SPECIFICITY:

The assay is highly selective for β-amylase.

KITS:

Kits suitable for performing 150 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: (x3) Each vial contains *p*-nitrophenyl -α-D-maltopentaoside (PNPG5; 47.5 mg) plus α-glucosidase (1000 U) and stabilisers.

Bottle 2: Malt flour of standardised β-amylase activity (as specified on bottle label).

PREPARATION OF REAGENT SOLUTIONS:

1. Dissolve the contents of bottle 1 in 10 mL of distilled water. This is **Betamyl Substrate Solution**. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and on ice during use. Do not dissolve the contents of the other bottles until required. Once dissolved, the reagent is stable for > 2 years at -20°C.
2. Use the contents of bottle 2 as supplied. Stable for > 4 years at room temperature.

EXTRACTION BUFFER (Buffer A):

0.05 M Tris-HCl plus 1 mM EDTA

Dissolve 6.06 g of trizma base (Sigma cat. no. T-1503) and 0.37 g of disodium EDTA (Sigma cat. no. ED2SS) in 700 mL of distilled water. Adjust the pH to 8.0 with 1 M HCl and the volume to one litre. Stable for approx. 3 months at 4°C.

Immediately before use, add 1.75 g of cysteine HCl (Sigma cat. no. C-7880 to 100 mL of the buffer (final concentration of cysteine HCl approx. 100 mM). Adjust pH to 8.0. 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.

DILUTION/ASSAY BUFFER: (Buffer B)

0.1 M Maleate buffer plus 1 mM EDTA, 1.0 mg/mL of BSA and 0.02 % sodium azide.

Dissolve 11.6 g of maleic acid (Sigma cat. no. M-0375), 0.37 g of

disodium EDTA (Sigma cat. no. ED2SS) and 1.0 g of BSA (Sigma cat. no. A-2153) 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 litre. Add 0.2 g of sodium azide as a preservative. Stable for > 2 years at 4°C.

CAUTION

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.

STOPPING REAGENT:

1 % (w/v) Trizma base (approx. pH 8.5)

Dissolve 10 g of Trizma base (Sigma cat. no. T-1503) in 900 mL of distilled water. Adjust the volume to 1 litre. Stable for approx. 1 year at room temperature.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Micro-pipettors, e.g. Gilson Pipetman® (100 µL and 200 µL).
3. 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).
4. Analytical balance.
5. Spectrophotometer set at 400 nm.
6. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
7. Stop clock.
8. Bench centrifuge or Whatman GF/A glass fibre filter paper circles.

CONTROLS AND PRECAUTIONS:

1. β-Amylase is extremely unstable when highly diluted in buffers not containing other proteins. It is thus **essential** that the extraction buffers are 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** reagent solution and then add 0.2 mL of diluted β-amylase solution. A single reagent blank determination is sufficient for each batch of assays.
3. If **blank absorbance** values exceed **0.3**, then the Betamyl substrate should be discarded.

4. If **reaction values** exceed **0.8**, then the enzyme extract should be diluted in Buffer B and re-assayed. Appropriate corrections to the calculations should then be made.
5. It is essential that an incubation time of 10 min is strictly adhered to. Due to an initial lag phase in the cleavage of *p*-nitrophenyl-α-D-maltotriose by α-glucosidase, the assay is not linear with time. However, it is linear with enzyme concentration up to a reaction absorbance value of 0.8.
6. PNPG5 is resistant to cleavage by cereal α-amylases, but some α-amylases, particularly those of fungal origin, cleave it rapidly. Thus, this assay cannot be used to specifically measure β-amylase in materials which also contain substantial levels of fungal α-amylase activity e.g. wheat flours to which fungal α-amylase has been added. The ratio of activity of purified *A. niger* α-amylase (devoid of amyloglucosidase and α-glucosidase) on PNPG5 compared to starch (Nelson/Somogyi assay) is 0.69.

USEFUL HINTS:

1. On dissolution of Betamyl reagent, the PNPG5 is slowly cleaved by the high levels of α-glucosidase present. To minimise this and to ensure maximum stability of the substrate, it is suggested that the contents of vials (on dissolution) be divided into 2-3 mL aliquots and stored frozen in polypropylene containers. 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 at -20°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 substrate concentration or the incubation time.

ASSAY PROCEDURE:

Enzyme Extraction:

1. Mill malt or barley to pass a 0.5 mm screen with a suitable laboratory mill (e.g. Tecator Cyclotec® Mill).
2. To exactly 0.5 g of flour, **add** 5.0 mL of Extraction Buffer (Buffer A) (with or without added cysteine).
3. Allow the enzyme to extract over a **1 hour** period at room temperature, with frequent vigorous stirring on a vortex mixer (approx. 5 times over the 1 hour period).
4. Filter an aliquot of the enzyme preparation through Whatman GF/A glass fibre filter paper, or centrifuge at 1,000 *g* for 10 min.
5. Dilute 0.2 mL of filtrate to 10.0 mL in Buffer B, mix, and then further dilute 0.2 mL of this solution to 5.0 mL in Buffer B.

Assay of β-Amylase:

1. Dispense 0.2 mL aliquots of **Betamyl** Substrate Solution into the bottom of glass test tubes and pre-incubate the contents at 40°C for approx. 5 min.
2. Pre-incubate the enzyme preparation at 40°C for approx. 5 min.
3. To each tube containing **Betamyl** Substrate Solution, add 0.2 mL of pre-equilibrated (and suitably diluted) enzyme preparation directly to the bottom of the tube, mix, and then incubate 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 and stir the tube contents.
5. Read the absorbance (at 400 nm) of the **reaction solutions** and the **reagent blank** against distilled water.

CALCULATION OF ACTIVITY:

Units/g of flour:

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

where:

ΔA_{400}	=	Absorbance (sample) - Absorbance (blank)
Incubation time	=	10 min
Total volume in cell	=	3.4 mL (or 1.7 mL)
Aliquot assayed	=	0.2 mL (or 0.1 mL)
ε_{mM} *p*-nitrophenol in 1 % Trizma base	=	18.1 (at 400 nm)
Extraction volume	=	10 mL per g (wheat, barley or malt)
Dilution	=	1250 fold

Thus:
Units/g of flour:

$$= \frac{\Delta A_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{10}{1} \times 1250$$

$$= A_{400} \times 1174$$

One **Unit** of activity is defined as the amount of enzyme required, in the presence of excess α-glucosidase, to release one micromole of *p*-nitrophenol from PNPG5 in one minute under the defined assay conditions, and is termed a **Betamyl Unit**.

DETERMINATION OF, AND ALLOWANCE FOR, THE DEGREE OF α-AMYLASE INTERFERENCE IN THE ASSAY:

PNPG5 is cleaved by both α-amylase and β-amylase, but the rates of cleavage, relative to the rates of hydrolysis of starch, are quite different. β-Amylase cleaves PNPG5 at about the same rate as for starch, but the rate of cleavage of PNPG5 by cereal α-amylase is only approx. 4 % the rate of hydrolysis of starch. Cereal α-amylase can be conveniently assayed using the Megazyme Cereal α-Amylase Test Kit (Ceralpha) which employs end-blocked *p*-nitrophenyl maltoheptaose (BPNPG7) as substrate. The ratio of cleavage of BPNPG7 (at pH 5.2) and PNPG5 (at pH 6.2) by purified wheat or barley α-amylases is approximately 40:1. Thus, having assayed the activity on BPNPG7, the activity of α-amylase on PNPG5 will be one fortieth this value. This calculated value can then be subtracted from the activity value (Units/g) on PNPG5, to give a "true" measure of β-amylase activity.

$$\text{i.e. } \beta\text{-Amylase} = \text{Units on PNPG5} - \frac{\text{Units on BPNPG7}}{40}$$

In practice, however, even for malt flour extracts, the contribution to the hydrolysis of PNPG5 by the α-amylase present is less than 0.5 % that of β-amylase i.e. it is within the limits of experimental error of the method, and thus can be ignored.

CONVERSION OF ACTIVITY TO INTERNATIONAL UNITS ON STARCH SUBSTRATE:

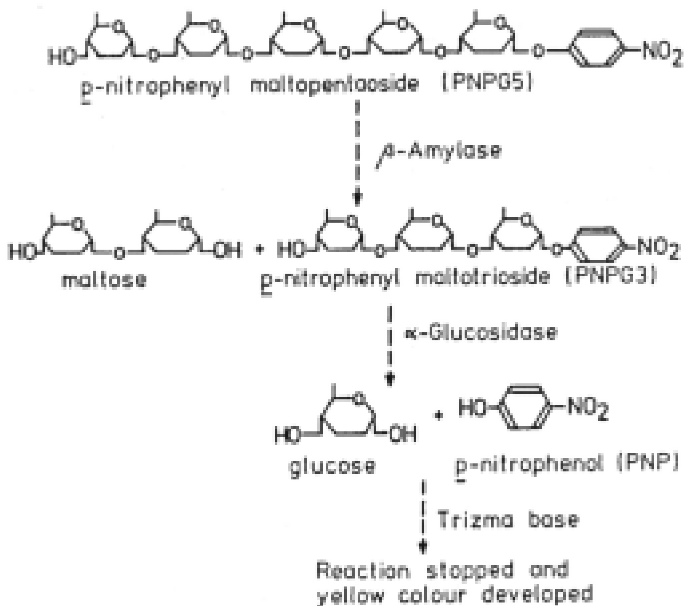
The activity of purified β-amylases (devoid of α-amylase, α-glucosidase and glucoamylase) on PNPG5 substrate and on soluble starch (assayed using the Nelson/Somogyi reducing sugar procedure) have been compared and conversion factors obtained. The ratios of activities on PNPG5 compared to starch for β-amylases from wheat, soybean, sweet potato and *Bacillus cereus* are 1.25, 1.03, 1.01 and 0.77, respectively.

CORRELATION BETWEEN TOTAL β-AMYLASE IN BARLEY AND DIASTATIC POWER IN MALT:

Santos and Riis (1996) have shown that there is a high correlation between total β-amylase in barley and DP in final malt, provided identical maltings are performed.

REFERENCES:

1. Mathewson, P. R. and Seabourn, B. W. (1983). *Journal of Agriculture and Food Chemistry*. **31**, 1322-1326.
2. McCleary, B. V. and Codd, R. (1989). *Journal of Cereal Science*. **9**, 17-33.
3. Erdal, K., Jensen, M. O., Kristensen, M., Krogh, J. J., Riis, P. and Vaag, P., E. B. C. *Proceedings of the 24th European Brewery Convention Congress*, Oslo, 1993, 147.
4. Santos, M. M. and Riis, P. (1996). *Journal of the Institute of Brewing*. **102**, 271-275.



Scheme 1. Theoretical basis of the Betamyl β-amylase assay procedure. When PNPG5 is cleaved to PNPG3, the latter is rapidly cleaved to *p*-nitrophenol and glucose by the excess quantities of α-glucosidase which are an integral part of the substrate mixture.

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