Megazyme

www.megazyme.com

BETA-AMYLASE

ASSAY PROCEDURE

(BETAMYL-3® METHOD)

K-BETA3 12/19

(100/200 Assays per Kit)

Megazyme

© Megazyme 2019

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. Subsequently, Megazyme offered a product, **Betamyl**® (β-Amylase Assay Reagent) that comprised just PNPG5 and α-glucosidase,[2] which gave greater specificity. This reagent is now superceded by the Megazyme **Betamyl-3**®, β-Amylase assay reagent, which is more specific and considerably more stable than the **Betamyl**® reagent.

The Megazyme **Betamyl-3**®**,** β-amylase test reagent employs high purity β-glucosidase and *p*-nitrophenyl-β-D-maltotrioside (PNPβ-G3). The level of β-glucosidase used ensures maximum sensitivity of the assay. 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 β-glucosidase present in the substrate mixture (Scheme 1, page 11). 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 PNPβ-G3 plus β-glucosidase mixture contains stabilisers which significantly increase its stability. The blank absorbance value of the **Betamyl-3**® substrate solution, when stored at 4 or 20°C, increases much more slowly than similar increases for **Betamyl**® reagent

ACCURACY:

Standard errors of less than 5% are readily achieved.

SPECIFICITY:

The assay is highly selective for β-amylase. The substrate is hydrolysed by α-glucosidase and amyloglucosidase.

KITS:

Kits suitable for performing 100/200 assays are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: (x2) Each vial contains *p*-nitrophenyl-β-D-maltotrioside (PNPβ-G3) plus β-glucosidase (50 U) and stabilisers.
Stable for > 4 years below -10°C.

Bottle 2: Tris/HCl buffer (Extraction 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 3: MES buffer (Dilution Buffer) (48 mL, 1 M, pH 6.2) plus disodium EDTA (20 mM), BSA 10 mg/mL and sodium azide (0.09% w/v).
Stable for approx. 4 years at 4°C.

Bottle 4: Cysteine hydrochloride (16 g).
Stable for > 4 years at room temperature.

Bottle 5: Malt flour of standardised β-amylase activity (as specified on bottle label).
Stable for > 4 years; store sealed at room temperature.

LP 莆柧

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dissolve the contents of bottle 1 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. Do not dissolve the contents of the second bottle until required.
Stable for > 2 years below -10°C.

2. Dilute 2.5 mL of the contents of bottle 2 to 50 mL with distilled water. Before use, add 0.88 g of cysteine HCl (bottle 4; 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. This is Buffer A.

3. Dilute the entire contents of bottle 3 to 500 mL with distilled water. Stable for approx. 1 year at 4°C. This is Buffer B.

4. Use as supplied. Stable for > 4 years at room temperature.

5. Use the contents of bottle 5 as supplied.
Stable for > 4 years; store sealed at room temperature.

PREPARATION OF ADDITIONAL BUFFER A:

0.05 M Tris-HCl plus 1 mM EDTA

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. Stable for approx. 3 months at 4°C.

Immediately before use, add 1.75 g of cysteine HCl (Megazyme cat. no. **G-LCYST200**) to 100 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.

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)[3] and Santos and Riis (1996).[4] Enzyme extracted without added cysteine is termed "Soluble" β-amylase; that extracted with cysteine is "Total" β-amylase.

PREPARATION OF ADDITIONAL BUFFER B:

0.1 M MES buffer plus 1 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-MES500**) and 0.37 g of disodium EDTA (Sigma cat. no. E4884-500G) 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 on the day of preparation.

STOPPING REAGENT:

1% (w/v) Tris buffer (approx. 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. Disposable 13 mL polypropylene tubes e.g. Sarstedt cat. no. 60.541.685 PP (www.sarstedt.com).
2. Disposable 1.5 mL polypropylene screw cap microfuge tubes e.g. Sarstedt cat. no. 72.692 (www.sarstedt.com).
3. Disposable plastic micro-cuvettes (1 cm light path, 1.5 mL) e.g. Plastibrand®, semi-micro, PMMA; Brand cat. no. 7591 15 (www.brand.de).
4. Micro-pipettors, e.g. Gilson Pipetman® (100 µL and 200 µL).
5. 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).
6. Analytical balance.
7. Spectrophotometer set at 400 nm.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Stop clock.
10. Bench centrifuge or Whatman GF/A glass fibre filter paper circles.
11. Eppendorf centrifuge 54XX (13,000 rpm; 15,000 *g*).
12. Laboratory mill, e.g. Buhler Miag disc mill DLFU: Setting 0.2 mm (fine); Frisch Pulverisette 14® with 0.5 mm screen or Tecator Cyclotec® Mill.

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® reagent solution** and then add 0.2 mL of diluted malt extract. A single reagent blank determination is sufficient for each batch of assays.
3. If **reagent blank** absorbance value exceeds **0.3**, then the **Betamyl-3® substrate** should be discarded.
4. If **reaction values** exceed **1.8**, then the enzyme extract should be diluted in **Buffer B** (Bottle 3) 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.

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 substrate is rapidly hydrolysed by α-glucosidase and amyloglucosidase.

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 malt or barley to pass a 0.5 mm screen with a suitable laboratory mill (e.g. Buhler Miag disc mill DLFU; Setting fine, or Frisch Pulverisette 14® with 0.5 mm screen).
2. To exactly 0.5 g of flour in a 13 mL polypropylene tube (Sarstedt cat. no. 60.541.685 PP; www.sarstedt.com), **add** 5.0 mL of **Buffer A** (Bottle 2).
3. Allow the enzyme to extract over a **1 h** period at room temperature, with frequent vigorous stirring on a vortex mixer (approx. 5 times over the 1 h period). Alternatively, place the tube into Stuart Blood Tube Rotator (http://design.hileytech.com/fisher/Stuartblood.html) which allows end-over-end mixing and run the machine for 1 h.
4. Filter an aliquot of the enzyme preparation through Whatman GF/A glass fibre filter paper, or centrifuge in a bench or micro-centrifuge at a minimum of 2,000 *g* for 10 min.
5. Add 0.2 mL of filtrate to 4.0 mL of **Buffer B** (bottle 3), mix and use this for the assay of β-amylase activity.

NOTE:

If the level of α-amylase is to be assayed in the same extract using the Ceralpha method, proceed as follows:

1. Add 0.2 mL of the diluted malt or barley extract (as used in the Betamyl-3® assay above) to 3 mL of Ceralpha Buffer **A** (prepared as shown on page 3 of the Ceralpha Booklet - **K-CERA**).
2. Proceed with assay of α-amylase (page 7 of this booklet).

Assay of β-Amylase:

1. Dispense 0.2 mL aliquots of the diluted malt extract directly to the bottom of 13 mL polypropylene 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 diluted malt extract add 0.2 mL of **Betamyl-3® Substrate Solution**, 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** and stir the tube contents.
5. Read the absorbance of the **reaction solutions** and the **reagent blank** at **400 nm** against distilled water.

CALCULATION OF ACTIVITY:

Units of β-Amylase/g of 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/g Flour:

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

$$= \frac{\Delta A_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{5}{0.5} \times 21$$

$$= A_{400} \times 19.72$$

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	=	18.1 (at 400 nm) in 1% Tris buffer
Extraction volume	=	5 mL per 0.5 g of malt
Dilution	=	0.2 to final volume of 4.2 mL (*i.e.* 21 fold)

Assay of α-Amylase in malt extracts:

(Note: for complete details of Ceralpha method and required reagents, refer to the K-CERA booklet).

1. Dispense 0.2 mL aliquots of suitably diluted malt extract (see Note on page 5) directly to the bottom of 13 mL polypropylene tubes and pre-incubate the contents at 40°C for approx. 5 min.
2. Pre-incubate **Amylase HR Reagent®** at 40°C for approx. 5 min.
3. To each tube containing diluted malt extract add 0.2 mL of **Amylase HR Reagent®**, 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** and stir the tube contents.
5. Read the absorbance of the **reaction solutions** and the **reagent 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:

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

$$= \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{5}{0.5} \times 336$$

$$= \Delta E_{400} \times 315.6$$

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	=	18.1 (at 400 nm) in 1% Tris buffer
Extraction volume	=	5 mL per 0.5 g of malt

Dilution = 0.2 to volume of 4.2 mL (*i.e.* 21 fold)
then 0.2 mL to 3.2 mL (a further 16-fold)
= 21 x 16 = 336-fold.

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

Cereal α-amylase hydrolyses **Betamyl-3**® substrate at less than 1% the rate of β-amylase.

RELATIONSHIP BETWEEN UNITS OF ACTIVITY ON Betamyl (based on PNPG5) and Betamyl-3® (PNPβ-G3) SUBSTRATES for Barley Malt β-Amylase:

Units on Betamyl® substrate/**Betamyl-3® substrate** = 58.6

CONVERSION OF ACTIVITY on PNPβ-G3 TO INTERNATIONAL UNITS ON STARCH SUBSTRATE:

The activity of purified β-amylases (devoid of α-amylase, α-glucosidase and glucoamylase) on **Betamyl-3® 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 starch compared to **Betamyl-3®** for β-amylases from barley malt, wheat, soybean, sweet potato and *Bacillus cereus* are given below:

Source of β-Amylase	Ratio of Activity: Starch/Betamyl-3
Malted barley	39.7
Barley	40.1
Wheat	20.3
Soybean	21.2
Sweet potato	193.9
Bacillus cereus	352.2

Table 1. The ratio of activity of β-Amylase enzymes from different sources on starch compared to Betamyl-3 reagent.

REFERENCES:

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

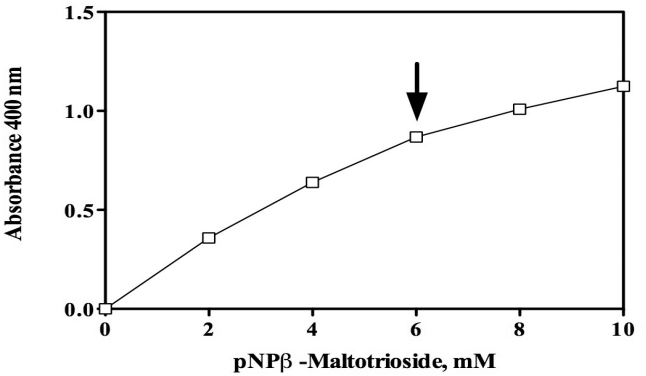


Figure 1. Effect of the substrate concentration (in the presence of excess β-glucosidase) on rate of hydrolysis of PNPβ-G3 by pure barley β-amylase. The arrow shows the final concentration in the Betamyl-3® reagent mixture.

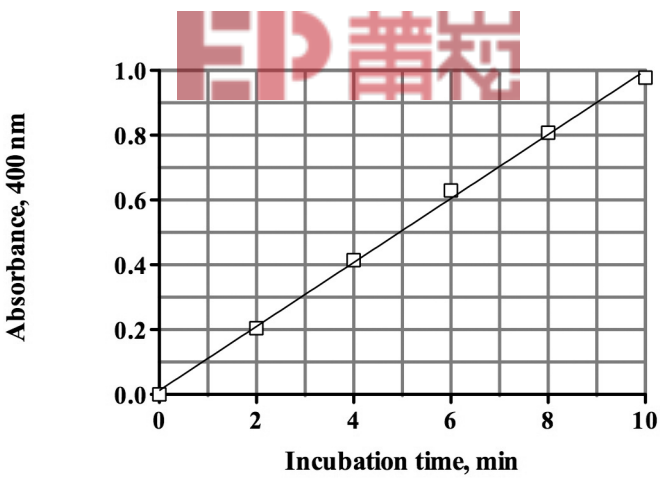


Figure 2. Time course of hydrolysis of PNPβ-G3 (in Betamyl-3® reagent) by pure barley β-amylase, as shown by the increase in absorbance at 400 nm.

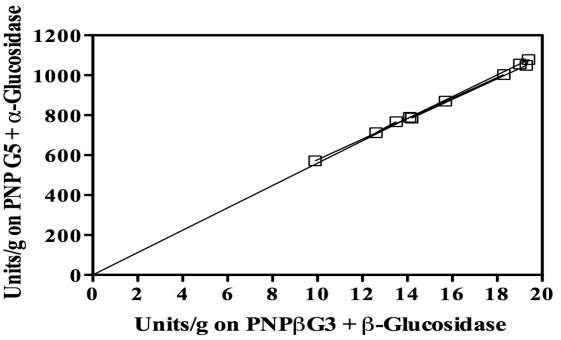
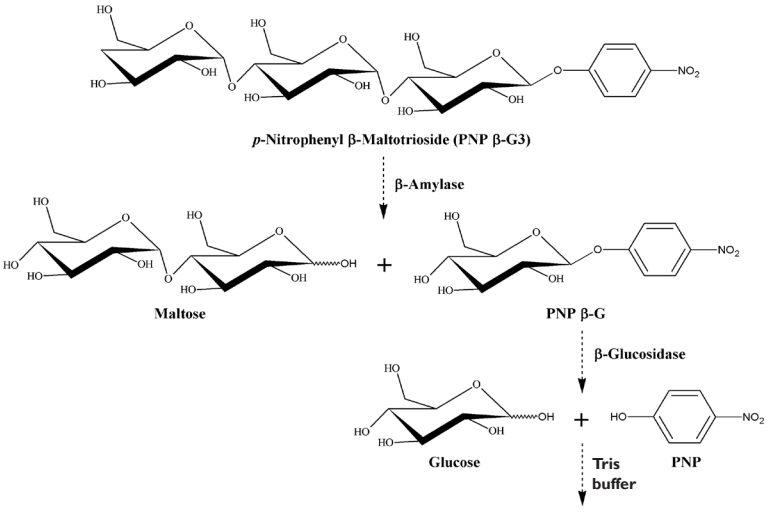


Figure 3. Comparison of activity of β-amylase in ten malt samples on Betamyl® substrate (PNPG5 plus α-glucosidase) and Betamyl-3® substrate (containing PNPβ-G3 plus β-glucosidase).
Units on Betamyl® Reagent = 58.6 x Units on Betamyl-3® Reagent.

Sample	Ceralpha (U/g)	Betamyl-3 (U/g)	Diastatic Power (EBC)WK (Units/100 g)	DP (°IoB) (Units/100 g)
A1	157.8	13.1	243	67.3
A2	153.7	13.8	239	66.2
A3	218.1	14.8	242	67.0
A4	258.8	16.4	212	59.2
A5	294.8	15.0	218	60.8
A6	324.1	16.2	251	69.4
A7	334.5	17.4	242	67.0
A8	268.6	10.5	226	62.9
A9	241.1	15.7	253	69.9
A10	187.8	15.1	229	63.6
B1	172.0	13.9	220	61.3
B2	186.8	17.7	228	63.4
B3	183.0	17.9	220	61.3
B4	174.2	17.4	233	64.7
B5	202.9	12.8	229	63.6
B6	192.5	16.8	253	69.9
B7	353.5	9.6	217	60.5
B8	167.3	14.4	230	63.9
B9	263.5	11.8	207	57.9
B10	278.7	13.1	233	64.7

Table 1. Comparison of values obtained for α-amylase (Ceralpha), β-amylase (Betamyl-3®) and Diastatic Power for a number of malt samples. Diastatic Power was determined using Analytica-EBC Method 4.12 (Diastatic Power of Malt), and DP (°IoB) calculated as DP (°IoB) = [DP (WK) + 16] / 3.85. As can be seen from the data, there is no correlation between DP and either β-amylase or α-amylase activity.



Scheme 1. **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.

Megazyme

Bray Business Park, Bray,
Co. Wicklow,
A98 YV29,
IRELAND.

Telephone: (353.1) 286 1220
Facsimile: (353.1) 286 1264
Internet: www.megazyme.com
E-Mail: info@megazyme.com

WITHOUT GUARANTEE

The information contained in this booklet is, to the best of our knowledge, true and accurate, but since the conditions of use are beyond our control, no warranty is given or is implied in respect of any recommendation or suggestions which may be made or that any use will not infringe any patents.