Megazyme

Measurement of

endo-Protease

and

α-Amylase

in

Biological Washing Powders & Liquids

using

AZO-CASEIN

and

AMYLAZYME TABLETS

ACS 10/13

Megazyme

INTRODUCTION:

α-Amylase and protease are routinely added to biological washing powders and liquids to assist in the solubilisation and removal of biological stains of starch or protein origin. In the current document, a simple procedures for the quantitative measurement of both α-amylase and protease in the presence of the washing powder components is described.

The most commonly employed α-amylase in washing powders is thermostable α-amylase from *Bacillus licheniformis* , and a commonly used protease is also from *Bacillus licheniformis* (namely, Subtilisin A). The specific procedures described here are for these two enzymes and other enzymes with similar properties.

α-Amylase is measured using Amylazyme Tablets (containing dyed and crosslinked amylose) and is standardised against the Megazyme Ceralpha method (employing Amylase HR reagent; which contains blocked *p*-nitrophenyl maltoheptaoside in the presence of thermostable α-glucosidase) at pH 7.0 and 40°C.

Protease is measured using Azo-Casein (dyed casein), and this is standardised against a Neutral Protease assay procedure. One Neutral Protease Unit is defined as the amount of enzyme that will produce the equivalent of one micromole of tyrosine per minute from soluble casein at pH 8.0 and 40°C.

EXTRACTION AND DILUTION BUFFER

A. Sodium chloride (1 % w/v) plus calcium chloride (5 mM)
Add 10 g of sodium chloride and 0.74 g of calcium chloride dihydrate to 950 mL of distilled water. Dissolve by stirring and adjust the volume to 1 litre.

B. MOPS (100 mM, pH 7.0) plus calcium chloride (5 mM) and Na azide (0.02%).
Add 20.9 g of MOPS buffer salt (acid form; Megazyme cat. no. B-MOPS250) to 900 mL of distilled water. Adjust to pH 7.0 by the addition of 2 M sodium hydroxide (approximately 17 mL is required). Add 0.74 g of calcium chloride dihydrate and 0.2 g of sodium azide and adjust the pH to 7.0. Adjust the volume to 1 litre.
Store at room temperature.

ENZYME EXTRACTION AND DILUTION:

1. Add 10 g (or 10 mL) of detergent sample to 100 mL of extraction solution A in a 100 mL polypropylene sealed container and stir for 15 min at room temperature.

2. Centrifuge an aliquot (~ 10 mL) at 3,000 rpm (1,000 *g*) for 10 min. Alternatively, filter some of the solution through a Whatman No. 1 (9 cm) filter paper.

3. Dilute an aliquot (1.0 mL) of the supernatant to 10 mL (1:10) in buffer B (100 mM MOPS buffer, pH 7.0).

MEASUREMENT OF PROTEASE ACTIVITY

SUBSTRATE:

Azo-Casein is prepared by dyeing casein with sulphanilic acid. The dyeing level is carefully controlled to produce a substrate which has about 5-times the sensitivity of similar products from other commercial suppliers (e.g Sigma Chemical Co., Azo-Casein Lot. 74H7165).

DISSOLUTION:

To powdered substrate (2 g) in a 120 mL beaker, add 4 mL of ethanol or industrial methylated spirits (IMS). Stir this is on a magnetic stirrer to remove all "lumps", and then add 96 mL of MOPS buffer (100 mM, pH 7.0). Stir the suspension vigorously on a magnetic stirrer until the substrate is completely dissolved (about 10 min). Dislodge any Azo-Casein which sticks to the edge of the beaker with a small spatula. Store the solution in a well sealed glass Duran bottle, and overlay with 2 drops of toluene to prevent microbial contamination. This solution is stable for several weeks at 4°C.

ASSAY PROCEDURE:

1. Add 1.0 ml of pre-equilibrated enzyme solution (in buffer B) to 1.0 ml of pre-equilibrated substrate solution (2 % w/v Azo-Casein in buffer B).

2. Mix the solution on a vortex stirrer and incubate at 40°C for exactly 10 minutes.

3. Add 6.0 ml of 5 % trichloroacetic acid (TCA) to terminate the reaction and precipitate non-hydrolysed Azo-Casein. Stir the tube vigorously for 5 seconds on a vortex mixer.

4. Allow the reaction tubes to equilibrate to room temperature for 5 minutes and then filter the contents through Whatman No.1 (9 cm) filter circles. Alternatively, centrifuge the suspension at 3,000 rpm (1,000*g*) for 10 min.

5. Read the absorbance of all filtrates (or supernatant solutions) against the reaction blank at 440 nm.

Prepare Reaction Blanks by adding the TCA to the substrate solution immediately before the enzyme preparation is added.

STANDARDISATION:

A standard curves relating the activity of Subtilisin A on Azo-Casein (Lot 81001; absorbance 440 nm) at pH 7.0 and 40°C, to *endo*-protease activity on casein (pH 7.0 and 40°C) is shown in Figure 1.

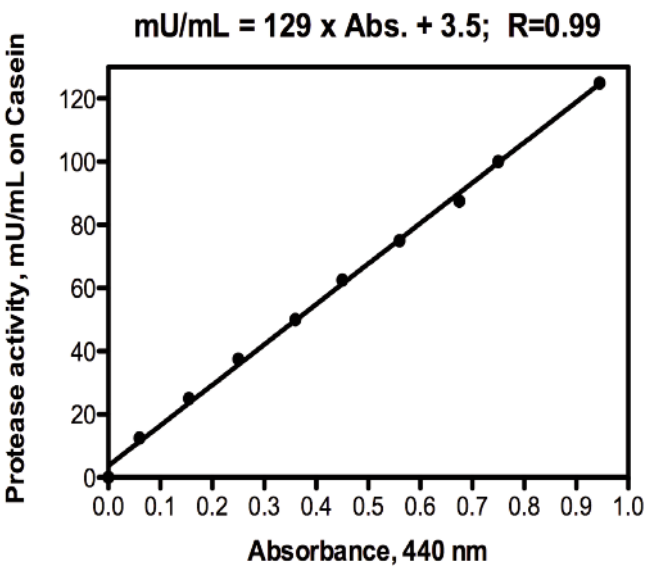

Figure 1. Subtilisin A Standard Curve on Azo-Casein (Lot 81001). Assay conditions; 10 min; pH 7.0; 40°C.

CALCULATION OF ACTIVITY:

Protease activity is determined by reference to the standard curve (Figure 1) or to the regression equation to convert absorbance units to milli-Units of protease activity per assay (i.e. per 1.0 mL) and then calculated as follows:

Units/g or ml of Original Preparation:

$$= \text{milli-Units/per assay} \times 10 \times \frac{1}{1000} \times \text{Dilution}$$

where:

milli-Units per assay (i.e./1.0 mL) is obtained by reference to the standard curve or to the regression equation.

10 = the volume of buffer used to extract the original preparation (i.e. 100 mL per 10 g or 10 mL of detergent preparation).

1/1000 = conversion from milli-Units to Units.

Dilution = dilution of the original enzyme preparation.

MEASUREMENT OF α-AMYLASE:

ASSAY PROCEDURE:

1. Add 1.0 mL of the diluted extract (in 100 mM MOPS buffer, pH 7.0) to a glass test-tube (16 x 120 mm) and pre-equilibrate at 40°C for 5 min.
2. Add an Amylazyme tablet to the tube (without stirring) and incubate at 40°C for 10 min.
3. Terminate the reaction after exactly 10 min by the addition of 10 mL of tri-sodium phosphate (2 % w/v, pH 12), and immediately stir the tube on a vortex mixer. Leave the tube at room temperature.
4. Stir the tube again after 5 min and filter the contents through a Whatman No. 1 (9 cm) filter paper.
5. Read the absorbance of the filtrate at 590 nm against the reaction blank.
6. Prepare the reaction blank by adding an Amylazyme tablet to MOPS buffer (100 mM, pH 7.0), incubating for 10 min at 40°C, and adding 10 ml of tri-sodium phosphate, followed by mixing and filtration (as for the enzyme assay).

7. Calculate the α-amylase activity using the *Bacillus licheniformis* α-amylase standard curve for Amylazyme (see Figure 2).

STANDARDISATION:

A standard curves relating the activity of *Bacillus licheniformis* α-amylase on Amylazyme Tablets (Lot 40101; absorbance 590 nm) at pH 7.0 and 40°C, to α-amylase activity on Ceralpha reagent (pH 7.0 and 40°C) is shown in Figure 2.

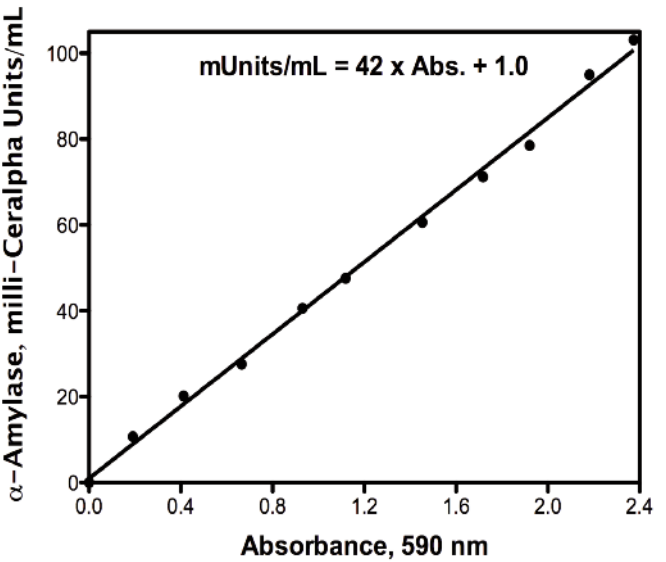

Figure 2. *Bacillus licheniformis* α-amylase standard curve on Amylazyme (Lot 40101).
The Amylazyme assay was performed at pH 7.0 and Ceralpha assay at pH 6.5 under standard conditions as described in this booklet and in Megazyme Booklet K-CERA 01/12 (Ceralpha; using Amylase HR Reagent). The enzyme employed was purified *Bacillus licheniformis* α-amylase (Megazyme cat. no. E-BLAAM).

CALCULATIONS:

Units/g or mL of Original Preparation:

$$= \text{milli-Units/assay (from standard curve)} \times \frac{1}{1000} \times 10 \times \text{Dilution}$$

$$= \text{milli-Units/assay} \times 0.01 \times \text{Dilution}$$

where:

milli-Units/assay is determined by reference to the standard curve for *Bacillus licheniformis* α-amylase on Amylazyme (eg. Lot 40101). Activity is expressed in Ceralpha Units (details available from the Megazyme web site; www.megazyme.com).

1/1000 = conversion from milli-Units to Units.

10 = initial extraction volume (i.e. 100 mL per 10 g or 10 mL of detergent preparation).

Dilution = further dilution of the initial extraction solution (i.e. 10-fold).

EFFECT OF DETERGENT ON ENZYME ACTIVITY

From the results shown in Figures 3 and 4, it is evident that at the concentration of detergent in the diluted washing powder extract, there is no effect of the detergent on the level of determined enzyme activity

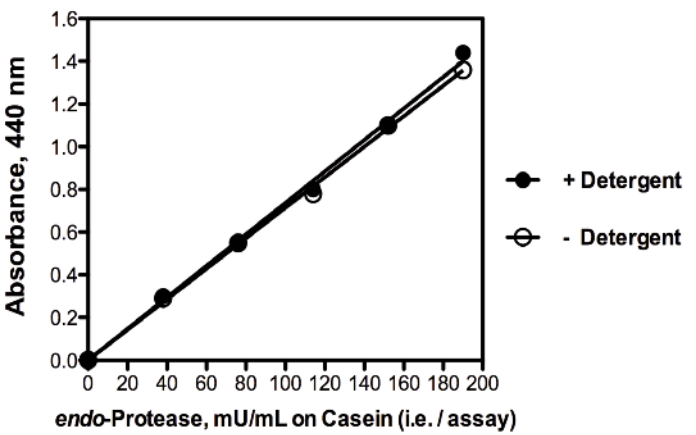

Figure 3. Effect of detergent on measurement of Subtilisin A protease using Azo-Casein. The level of detergent is that present in the diluted detergent extract used in the assay.

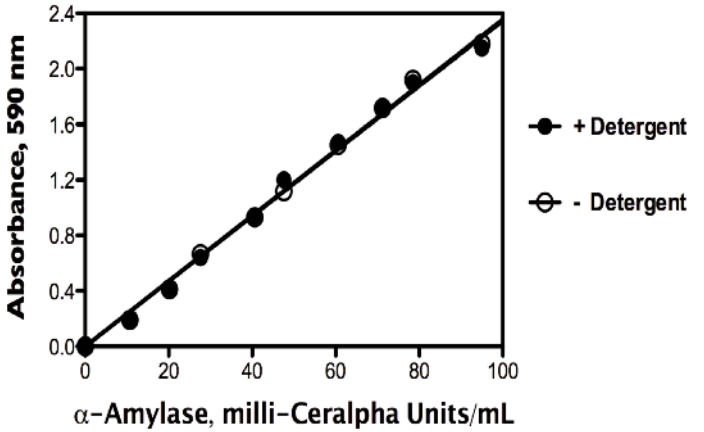

Figure 4. Effect of detergent on the measurement of α-amylase using the Amylazyme assay. The level of detergent is that present in the diluted detergent extract used in the assay.

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