

Megazyme

ASSAY OF
endo-PROTEASE
USING
PROTAZYME OL
TABLETS

POL 11/98



SUBSTRATE:

The substrate employed is Azurine-crosslinked collagen (AZCL-collagen). It is prepared by dyeing and crosslinking collagen, to produce a material which hydrates in water but is water insoluble. Hydrolysis by proteases produces water soluble dyed fragments, and the rate of release of these (increase in absorbance at 590nm) can be related directly to enzyme activity. The substrate is a specific substrate for *endo*-proteolytic activity and is hydrolysed by a wide range of *endo*-proteases. It is supplied in a ready-to-use tablet form as Protazyme OL.

APPLICATIONS:

Protazyme OL tablets can be used to assay any *endo*-acting protease enzyme which is active on collagen. It can directly replace Hide Powder (collagen) Azure in assays where this substrate is used. The current substrate has the major advantage of being supplied in tablet form, which avoids the need to weigh the substrate into each assay tube. Protazyme OL can be used to assay such enzymes as bacterial alkaline proteases (eg: Subtilisin A, as in Alcalase from Novo Nordisk), papain, trypsin, fungal proteases and bromelain. Assays employing Protazyme OL tablets, have a similar sensitivity to that obtained with assays employing a new, high-sensitivity, soluble, Azo-Casein substrate supplied by Megazyme.

BUFFERS FOR EXTRACTION/DILUTION AND ASSAY:

BUFFER A: (Sodium phosphate, 100 mM, pH 7)

17.8 g of di-sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) is dissolved in 900 mL of distilled water and the pH is adjusted to 7.0 with 1 M sodium hydroxide (40 g/L). The volume is adjusted to 1 litre. Store at 4°C. Sodium azide (0.2 g; Sigma S-2002) may be added as a preservative.

BUFFER B: (Sodium phosphate, 100 mM, pH 7),
with cysteine (30 mM) and EDTA (30 mM)

8.9 g of di-sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) is dissolved in 450 mL of distilled water and L-cysteine hydrochloride monohydrate (2.65 g; Sigma C-7880) and ethylenediaminetetra acetic acid (5.6 g, EDTA; Sigma ED2SS) are added and dissolved. The pH is adjusted to 7.0 with 1 M sodium hydroxide (40 g/L), and the volume is adjusted to 500 mL. Store at 4°C. Use within 3 days.

BUFFER C: (Sodium phosphate, 100 mM, pH 7), with SDS (1% w/v)

This buffer should be prepared fresh before use. Add 1.0 g of sodium lauryl sulphate (SDS; Sigma L-4509) to 100ml of Buffer A and dissolved by stirring and heating. The pH is adjusted to 7.0 with 1 M sodium hydroxide (40 g/L). Store at room temperature. Sodium azide (0.02 g) may be added as a preservative. If SDS crystallises from solution, a new lot of buffer should be prepared.

ENZYME EXTRACTION/DILUTION:

NOTE:

1. For the thiol-proteases (e.g. papain, bromelain and ficin) Buffer B is used for extraction and dilution. For other proteases, Buffer A is employed.
2. Buffer C is used to hydrate the Protazyme OL tablet for all of the proteases reported here except for trypsin, chymotrypsin, bovine pancreatin and pepsin, in which case, Buffer A is employed. Where used, the SDS aids the swelling and dispersion of the substrate and minimises "sticking" of the substrate to the assay tube. Before using SDS in these assay buffers, the stability of the particular protease in SDS needs to be established. This is easily achieved by using both Buffer A and C to hydrate the tablet (Step 1 in assay procedure). When Buffer C is employed, the reaction absorbance obtained should be at least as high as that obtained with Buffer A.

Powdered enzyme preparation (1.0 g) is suspended in 50 mL of Buffer A or B and stirred on a magnetic stirrer for about 15 min. at room temperature (until the powder is completely dissolved or dispersed). Liquid enzyme preparations (1.0 mL) are added, using a positive displacement dispenser (e.g. Eppendorf Multipette) to 49.0 mL of Buffer A or B and thoroughly mixed. The preparations are then filtered or centrifuged (1,000 g) if necessary. They are then further diluted (1 mL to 9 mL of Buffer A or B) until a concentration suitable for assay is obtained (the dilution for Alcalase is 400).

ASSAY PROCEDURE:

1. A Protazyme OL tablet and a magnetic stirrer bar (15 x 6 mm) are added to 1.0 mL of Buffer C in a glass test-tube (18 x 150 mm) and allowed to hydrate and equilibrate (with gentle stirring) over 5 min. (Ideally in an arrangement which allows uniform stirring in a number of tubes with accurate temperature control (e.g. with a Megazyme Multistir Incubation Bath with setting 350 on the IKA MINI MR1 basic magnetic stirrer)].

2. An aliquot of enzyme (1.0 mL, at room temperature) in Buffer A or B is added to the stirred tube and the reaction is allowed to continue for exactly 10 min. (If a continuous stirring arrangement is not available, the tube contents should be stirred carefully by gentle hand agitation (every few minutes).
3. The reaction is terminated by the addition of tri-sodium phosphate (10 mL, 2% w/v, pH-12.3) with vigorous stirring on a vortex mixer.
4. The tube is allowed to stand for approx. 2 min at room temperature and the contents are filtered through a Whatman No. 1 filter circle.
5. The absorbance of the filtrate is measured at 590 nm against a substrate blank.

The substrate blank is prepared by adding a Protazyme OL tablet to 2.0 mL of extraction buffer, incubating at 40°C for 10 min, adding 10.0 mL of tri-sodium phosphate solution (2%, adjusted to pH 12.3) and filtering after 2 min at room temperature.

NOTE:

A single blank is required for each set of determinations and this is used to zero the spectrophotometer. The absorbance of the reaction solutions are measured at 590 nm against the blank.

STANDARDISATION:

A standard curve relating the activity of Subtilisin A on Protazyme OL (Lot 61002) (Absorbance 590 nm) to protease activity is shown in Figure 1.

One Protease Unit is defined as the amount of enzyme which will produce the equivalent of one micromole of tyrosine per minute from soluble casein at pH 7.0 and at 40°C. This casein/TCA reference method is available on request.

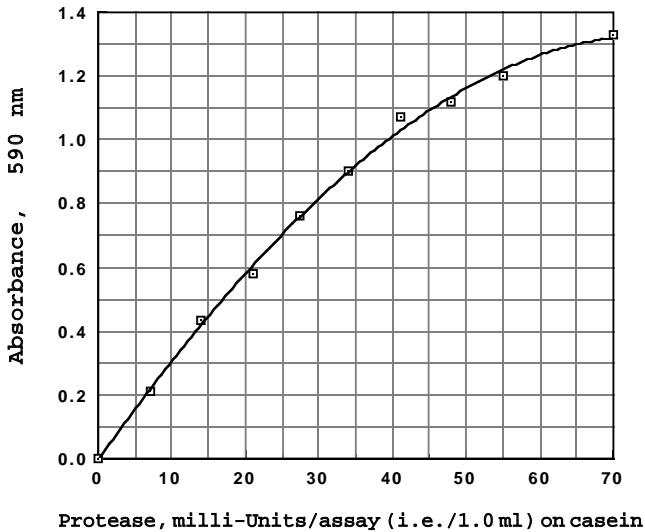


Figure 1. Standard curve relating activity on Protazyme OL Tablets (61002) at 40°C and pH 7.0 to activity on casein at 40°C and pH 7.0.

CALCULATIONS:

U/ml or gram of original preparation:

$$= \text{milliUnits per assay (i.e. per 1.0 ml)} \times 50 \times 1/1000 \times \text{Dilution.}$$

where:

milliUnits per assay (i.e./1.0 ml) is obtained by reference to the standard curve or to the relevant Regression Equation.

50 = the volume of buffer used to extract the original preparation (i.e. 1g/50ml or 1ml of enzyme added to 49ml of buffer).

1/1000 = conversion from milliUnits to Units.

Dilution = further dilution of the original extract (about 400-fold for Alcalase from Novo).



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