

Megazyme

ASSAY OF
endo-**PROTEASE**
USING
PROTAZYME OL
TABLETS

T-PROL 11/08



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 enzymes which are active on collagen. These tablets 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 (e.g. Subtilisin A), papain, trypsin and bromelain. Protazyme OL tablets are particularly useful for assaying *endo*-proteases with low pH optima (e.g. *Aspergillus niger* *endo*-proteases at pH 4.5). Unlike Protazyme AK (containing AZCL-Casein), the Protazyme OL tablets hydrate rapidly at pH 4.5 and do not need the extensive pre-hydration conditions required for Protazyme AK.

ASSAY OF PROTEASES AT pH 7.0

A. BUFFERS FOR EXTRACTION/DILUTION AND ASSAY:

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

Dissolve 17.8 g of di-sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) in 900 mL of distilled water and adjust the pH to 7.0 with 1 M HCl. Adjust the volume 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)

Dissolve 8.9 g of di-sodium hydrogen phosphate dihydrate ($\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$) in 450 mL of distilled water and then add 2.65 g of L-cysteine hydrochloride monohydrate (Sigma C-7880) and 5.5 g of ethylenediaminetetra acetic acid (EDTA; Sigma ED2SS) and dissolve. Adjust the pH to 7.0 with 1 M sodium hydroxide (40 g/L), and adjust the volume to 500 mL. Store at 4°C. Use on day of preparation.

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

Prepare this buffer immediately before use. Add 1.0 g of sodium lauryl sulphate (SDS; Sigma L-4509) to 100mL of Buffer A and dissolve by stirring and heating. Adjust the pH 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.

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 in all cases except where stated otherwise. For trypsin, chymotrypsin, bovine pancreatin and pepsin, Buffer A is employed. Where used, the SDS aids the swelling and dispersion of the substrate and minimizes “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 determined by using the various buffers to hydrate the tablet (Step 1 in assay procedure). Where buffers containing SDS are used, the reaction absorbance obtained should be at least as high as that obtained with buffers not containing SDS.

B. ENZYME EXTRACTION/DILUTION:

Suspend powdered enzyme preparation (1.0 g) in 50 mL of Buffer A or B and stir on a magnetic stirrer for about 15 min at room temperature (until the powder is completely dissolved or dispersed). Add liquid enzyme preparations (1.0 mL), using a positive displacement dispenser (e.g. Eppendorf Multipette), to 49.0 mL of Buffer A or B and mix thoroughly. Filter or centrifuge (1,500 g, 10 min) the preparations if necessary. Further dilute the extract in Buffer A or B until a concentration suitable for assay is obtained. For Alcalase (Novozymes), a dilution of 500-fold in phosphate buffer (pH 7.0) is required.

C. ASSAY PROCEDURE:

1. Add a Protazyme OL tablet and a magnetic stirrer bar (5 x 15 mm) to 1.0 mL of Buffer A or C in a glass test-tube (18 x 150 mm) and allow the tablet to hydrate and equilibrate (with gentle stirring) over 5 min at 40°C.
[Ideally use an arrangement which allows uniform stirring in a number of tubes with accurate temperature control (e.g. a Megazyme Multistir Incubation Bath with setting 350 on the IKA MINI MRI basic magnetic stirrer)].
2. Add an aliquot of enzyme (1.0 mL, at room temperature) in Buffer A or B to the stirred tube and allow the reaction 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. After 10 min, terminate the reaction by adding 10 mL of tri-sodium phosphate (2 % w/v, pH 11.0) with vigorous stirring on a vortex mixer.
4. Allow the tube to stand for 5 min at room temperature and filter the contents through a Whatman No. 1 filter circle.
5. Measure the absorbance of the filtrate at 590 nm against a substrate blank.

To prepare the substrate blank, add a Protazyme OL tablet to 2.0 mL of extraction buffer, incubate at 40°C for 10 min, add 10.0 mL of tri-sodium phosphate solution (2 %, adjusted to pH 11.0), mix and allow to stand at room temperature for 5 min. Filter the solution.

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 substrate blank.

STANDARDISATION:

A standard curve relating the activity of Subtilisin A on Protazyme OL (Lot 61002) (absorbance 590 nm) to *endo*-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 TCA soluble tyrosine per minute from soluble casein at pH 7.0. The casein/TCA method is available on request.

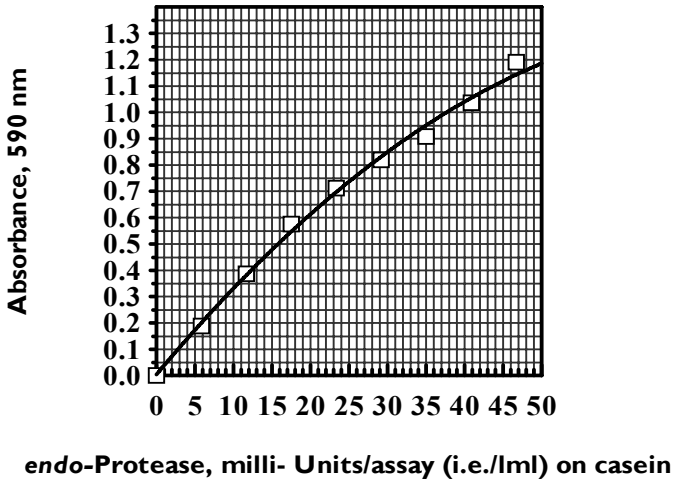


Figure 1. Standard curve relating activity of Subtilisin A on Protazyme OL Tablets (61002) at pH 7.0 (in 100 mM sodium phosphate buffer) and 40°C, to activity on casein at pH 7.0 and 40°C (Tyrosine Units).

ASSAY OF PROTEASES AT pH 4.5

A. BUFFER FOR EXTRACTION/DILUTION AND ASSAY:

Buffer D: (Sodium acetate, 100 mM, pH 4.5)

Add glacial acetic acid (6.0 g, 1.05 g/mL) to 800 mL of distilled water. Adjust the pH to 4.5 by the addition of 1M (20g/100 mL) sodium hydroxide solution. Adjust the volume to 1 litre. Store at 4°C.

B. ENZYME EXTRACTION/DILUTION:

Suspend powdered enzyme preparation (1.0 g) in 50 mL of Buffer D and stir on a magnetic stirrer for about 15 min at room temperature (until the powder is completely dissolved or dispersed). Add liquid enzyme preparations (1.0 mL), using a positive displacement dispenser (e.g. Eppendorf Multipette), to 49.0 mL of Buffer D and mix thoroughly. Filter or centrifuge (1,500 g, 10 min) the preparations if necessary. Further dilute the extract in Buffer D until a concentration suitable for assay is obtained. For Bioprotease A preparation (*A. niger*; Quest International) a dilution of 20-fold in sodium acetate buffer, pH 4.5 is required.

C. ASSAY PROCEDURE:

1. Add a Protazyme OL tablet and a magnetic stirrer bar (5 x 15 mm) to 1.0 mL of Buffer D in a glass test-tube (18 x 150 mm) and allow the tablet to hydrate (with gentle stirring) over 5 min.
[Ideally use an arrangement which allows uniform stirring in a number of tubes with accurate temperature control (e.g. a Megazyme Multistir Incubation Bath with setting 350 on the IKA MINI MRI basic magnetic stirrer)].
2. Add an aliquot of enzyme (1.0 mL, at room temperature) in Buffer D to the stirred tube and allow the reaction 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. Terminate the reaction by adding 10 mL of tri-sodium phosphate (2 % w/v, pH 11.0) with vigorous stirring on a vortex mixer.
4. Allow the tube to stand for 5 min at room temperature and filter the contents through a Whatman No. 1 filter circle.
5. Measure the absorbance of the filtrate at 590 nm against a substrate blank.

To prepare the substrate blank, add a Protazyme OL tablet to 2.0 mL of extraction buffer, incubate at 40°C for 10 min, add 10.0 mL of tri-sodium phosphate solution (2 %, adjusted to pH 11.0), mix and allow to stand at room temperature for 5 min. Filter the solution

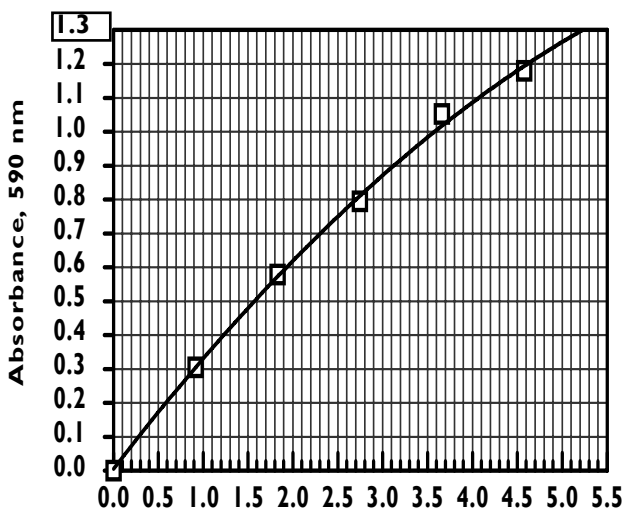
NOTE:

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 Bioprotease A *endo* protease (*Aspergillus niger* on Protazyme OL (Lot 61002) (absorbance 590 nm) to *endo*-protease activity is shown in Figure 2.

One Protease Unit (HUT) is defined as the amount of enzyme which will produce the equivalent of one micromole of TCA soluble tyrosine per minute from haemoglobin at pH 4.5 at 40°C. The haemoglobin/TCA reference method is available on request.



endo-Protease, milli-Units/assay (i.e./l.0ml) on Haemoglobin

Figure 2. Standard curve relating activity of *Aspergillus niger* *endo*-protease on Protazyme OL Tablets (61002) at pH 4.5 (100 mM sodium acetate buffer) and 40°C, to activity on haemoglobin at pH 4.5 and 40°C (HUT Units).

CALCULATIONS:

ASSAYS at pH 7.0

U/mL or gram of original preparation (e.g. Subtilisin A, pH 7.0):

= milliUnits per assay (i.e. per 1.0 mL) \times 50 \times 1/1000 \times Dilution.

where:

milliUnits per assay (i.e. /1.0 mL) is obtained by reference to the standard curve.

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

1/1000 = conversion from milliUnits to Units (if relevant).

Dilution = further dilution of the original extract.

ASSAYS at pH 4.5

U/mL or gram of original preparation (e.g. *A. niger*, pH 4.5):

= Units per assay (i.e. per 1.0 mL) \times 50 \times Dilution.



Megazyme

**Megazyme International Ireland,
Bray Business Park, Bray,
Co. Wicklow,
IRELAND**

Telephone: (353.1) 286 1220

Facsimile: (353.1) 286 1264

Internet: www.megazyme.com

E-Mail: info@megazyme.com

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