Megazyme

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ASSAY OF

endo-PROTEASE

using

PROTAZYME AK TABLETS

T-PRAK 05/16

SUBSTRATE:

The substrate employed is Azurine-crosslinked casein (AZCL-casein). This substrate is prepared by dyeing and crosslinking casein 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 590 nm) can be related directly to enzyme activity. The substrate is a generally applicable, specific substrate for *endo*-proteolytic activity. It is supplied commercially, in a ready-to-use tablet form, as Protazyme AK Tablets.

APPLICATIONS:

Protazyme AK tablets can be used to assay all *endo*-acting, protease enzymes which are active on casein. Such enzymes include bacterial alkaline proteases (e.g. Subtilisin A, as in Alcalase from Novo Nordisk), papain, trypsin, fungal proteases and bromelain. In the current data booklet, standard curves are provided for Subtilisin A, papain, bromelain, ficin, Proteinase K, trypsin, α-chymotrypsin, bovine pancreatic protease and *A. oryzae* protease. Assays using this substrate have a sensitivity approx. 20 times that of assays based on casein, and about 5-fold greater sensitivity than that obtained with assays using a new, high-sensitivity, soluble Azo-Casein substrate offered by Megazyme (cat. no. **S-AZCAS**).

BUFFERS FOR EXTRACTION/DILUTION AND ASSAY:

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

Add 17.8 g of disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) to 900 mL of distilled water and dissolve by stirring. Adjust the pH to 7.0 with 1 M HCl and adjust the volume to 1 L with distilled water. Store at 4°C. Sodium azide (0.2 g; Sigma S2002) may be added as a preservative.

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

Add 8.9 g of disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) to 450 mL of distilled water and dissolve by stirring. Add 2.65 g of L-cysteine hydrochloride monohydrate (Megazyme cat. no. **G-LCYST200**) and 5.6 g of ethylenediaminetetra acetic acid (EDTA; Sigma cat. no. ED2SS) and dissolve by stirring. Adjust the pH to 7.0 with 1 M sodium hydroxide (40 g/L) and then adjust the volume to 500 mL. Store at 4°C. Use within 3 days.

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

Prepare this buffer just before use. Add 1.0 g of sodium lauryl sulphate (SDS; Sigma cat. no. L4509) to 100 mL of Buffer A and dissolve by stirring and gentle heating. Adjust the pH to 7.0 with 1 M sodium hydroxide (40 g/L). Store at room temperature. If SDS crystallises from solution, it can be redissolved with gentle stirring at 40°C.

BUFFER D: (Tris.HCl, 100 mM, pH 8)

Dissolve 12.1 g of Tris buffer salt (Megazyme cat. no. **B-TRIS500**) in 900 mL of distilled water and adjust the pH to 8.0 with 1 M HCl. Adjust the volume to 1 L with distilled water. Store at 4°C.

BUFFER E: (Tris.HCl, 100 mM, pH 8, plus SDS, 1% w/v)

Prepare this buffer just before use. Add 1.0 g of sodium lauryl sulphate (SDS; Sigma cat. no. L4509) to 100 mL of Buffer D and dissolve by stirring and gentle heating. Adjust the pH to 8.0 with 1 M sodium hydroxide (40 g/L). Store at room temperature. If SDS crystallises from solution, it can be redissolved with gentle stirring at 40°C.

BUFFER F: (Sodium acetate, 100 mM, pH 4.5)

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

BUFFER G: (Sodium acetate, 100 mM, pH 4.5, plus SDS, 1% w/v)

Prepare this buffer just before use. Add 1.0 g of sodium lauryl sulphate (SDS; Sigma cat. no. L4509) to 100 mL of Buffer F and dissolve by stirring and gentle heating. Adjust the pH to 4.5 with 1 M sodium hydroxide (40 g/L). Store at room temperature. If SDS crystallises from solution, it can be redissolved with gentle stirring at 40°C.

NOTE:

1. For the thiol-proteases (e.g. papain, bromelain and ficin) Buffer B (pH 7.0) is used for extraction and dilution. For other proteases, buffers A, D or E are employed.
2. Buffers containing SDS (buffers B, E and G) are used to hydrate the Protazyme AK tablet for all proteases reported here, **except for trypsin, chymotrypsin and bovine pancreatin.** For these proteases, Buffer A is employed because SDS inhibits these enzymes. SDS aids the swelling and dispersion of the substrate and minimises "sticking" of the substrate to the assay tube. However, before using SDS in these assay buffers, the stability of the particular protease in SDS needs to be established. This is easily determined by performing the assay in buffers with and without SDS added (step 1 in assay procedure). If a lower reaction absorbance value is obtained with buffers containing SDS compared to those without SDS, then don't include SDS.

ENZYME EXTRACTION/DILUTION:

Suspend powdered enzyme preparation (1.0 g) in 50 mL of appropriate buffer (see below) 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) to 49.0 mL of appropriate buffer using a positive displacement dispenser (e.g. Eppendorf Multipette) and thoroughly mix. Filter (Whatman No.1 filter paper) or centrifuge (1,000 *g*, 10 min) if necessary. These solutions are referred to here as the "**Original Extract**". Dilute these original extracts in appropriate buffer until a concentration suitable for assay is obtained [for Alcalase (Novozymes) a dilution of the "Original Extract" of 2500-fold is required].

Recommended buffers for tablet dispersion and extraction:

Enzyme	Extraction/Dilution	Added with Tablet
Papain	Buffer B	Buffer C
Bromelain	Buffer B	Buffer C
Ficin	Buffer B	Buffer C
Subtilisin A	Buffer D	Buffer E
Other Bacterial	Buffer B	Buffer C
Proteinase K	Buffer A	Buffer C
Trypsin	Buffer A	Buffer A
α-Chymotrypsin	Buffer A	Buffer A
A. oryzae (acid pH)	Buffer F	Buffer G

NOTE:

The active ingredient in Protazyme AK tablets, namely AZCL-casein, hydrates and swells rapidly (about 2 min) in buffers of pH 6 or higher. However, when using sodium acetate buffer, pH 4.5 (+ SDS), hydration takes approximately 1 h. Thus, at pH values below 5, the Protazyme AK tablet should be gently stirred in the appropriate buffer for approx. 1 h, before adding the enzyme preparation.

ASSAY PROCEDURE:

1. Add a Protazyme AK tablet and a magnetic stirrer bar (15 x 5 mm) to 1.0 mL of appropriate buffer (see "Added with Tablet" above) in a glass test-tube (18 x 150 mm) and allow the tablet to hydrate (with gentle stirring) for 5 min at 40°C (see note above for cases where pH is below 5.0). The ideal arrangement is one which allows uniform stirring in a number of tubes with accurate temperature control (e.g. using a Megazyme Multistir Incubation Bath, Megazyme cat. no. **D-IBMKIII**, with setting 350 on an IKA KMO 2 basic, magnetic stirrer).

2. Add 1.0 mL of enzyme (at room temperature) in appropriate buffer 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 carefully stirred by gentle hand agitation every few min).
3. Terminate the reaction by adding 10 mL of trisodium phosphate solution (2% w/v, pH ~ 12.0) with vigorous stirring on a vortex mixer.
4. Allow the tube to stand for approx. 5 min at room temperature and filter the contents through a Whatman No. 1 (9 cm) filter circle.
5. Measure the absorbance of the filtrate at 590 nm against a reaction blank.

Prepare a **reaction blank** by adding a Protazyme AK tablet to 2.0 mL of extraction buffer, with incubation at 40°C for 10 min. Remove the tube from the bath, add 10.0 mL of trisodium phosphate solution (2%, pH 12.0) and filter after 5 min at room temperature.

NOTE:
A single **reaction 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. The absorbance of the **reaction blank** should be less than 0.1.

If the absorbance of the reaction solution is above 1.5, an aliquot of the enzyme extract should be diluted with extraction buffer and **the assay repeated.**

CALCULATIONS:

U/mL or gram of original preparation:

= milliUnits per assay (i.e. per 1.0 mL) x 50 x 1/1000 x 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. 1 g/50 mL or 1 mL of enzyme diluted to 50 mL with buffer) to give the "Original Extract".

1/1000 = conversion from milliUnits to Units.

Dilution = further dilution of the "Original Extract".

STANDARDISATION:

The standard curve for Subtilisin A on Protazyme AK (Lot 140401) at 40°C and pH 8.0 to activity on casein at 40°C and pH 8.0, is shown in Figure 1. Standard curves relating the activity of various other proteases on Protazyme AK (Lot 140401) (Absorbance 590 nm) to activity on casein at pH 7.0 are shown in Figures 2-8.

In Figure 9, the standard curve relating *Aspergillus oryzae* protease activity on Protazyme AK tablets at pH 4.5 (the optimal pH for the action of this enzyme on this substrate) to activity on haemoglobin (2% w/v) at pH 3.0 is shown. For haemoglobin, the optimal pH for activity of this enzyme is 3.0.

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 40°C and the appropriate pH for the assay, i.e. pH 7 for all proteases reported here except subtilisin A (pH 8) and *A. oryzae* protease (pH 4.5). This assay method is available on request.

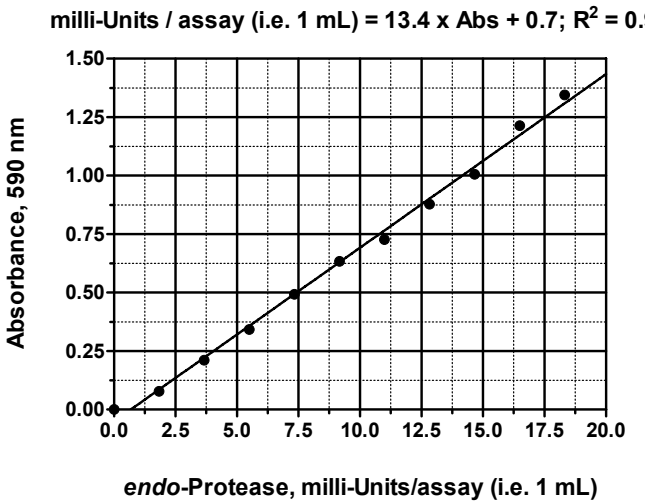

Figure 1. Standard curve relating activity of **Subtilisin A** on Protazyme AK Tablets (Lot 140401) at 40°C and pH 8.0 to activity on casein at 40°C and pH 8.0.

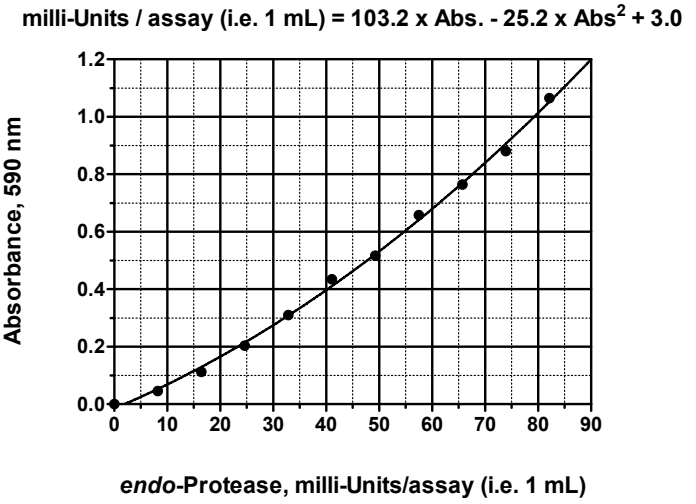

Figure 2. Standard curve relating activity of **papain** (from *Papaya latex*) on Protazyme AK Tablets (Lot 140401) at 40°C and pH 7.0 to activity on casein at 40°C and pH 7.0.

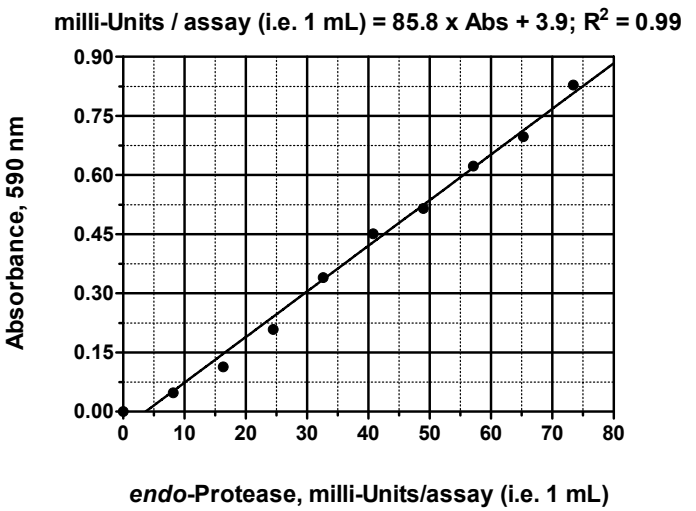

Figure 3. Standard curve relating activity of **bromelain** (from pineapple stem) on Protazyme AK Tablets (Lot 140401) at 40°C and pH 7.0 to activity on casein at 40°C and pH 7.0.

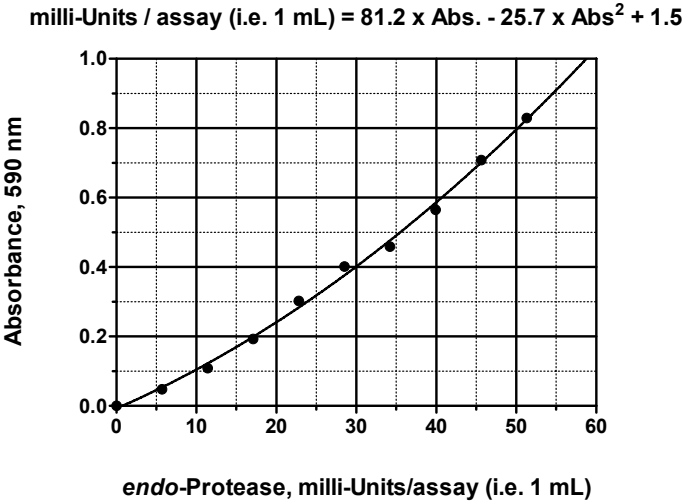

Figure 4. Standard curve relating activity of **ficin** (from figs) on Protazyme AK Tablets (Lot 140401) at 40°C and pH 7.0 to activity on casein at 40°C and pH 7.0.

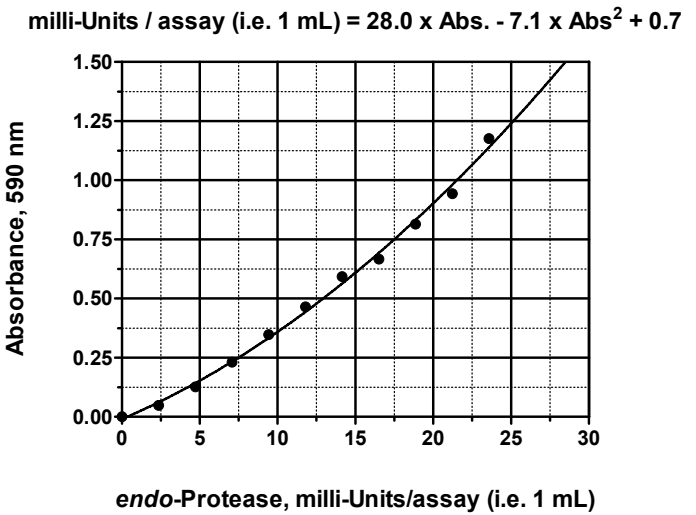

Figure 5. Standard curve relating activity of **Proteinase K** on Protazyme AK Tablets (Lot 140401) at 40°C and pH 7.0 to activity on casein at 40°C and pH 7.0.

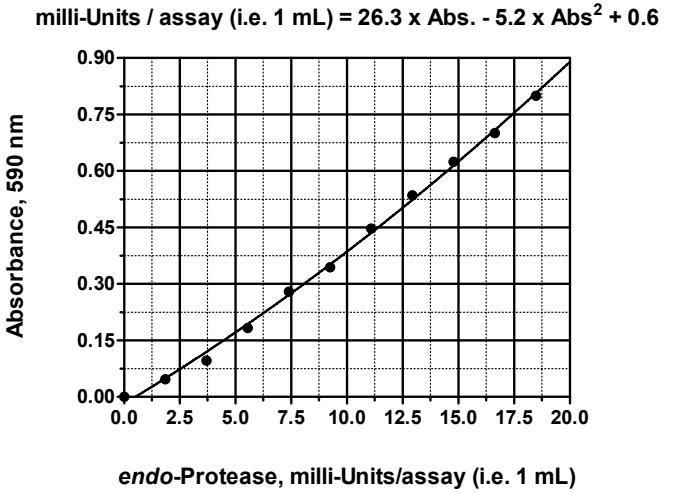

Figure 6. Standard curve relating activity of **trypsin** (from Porcine pancreas) on Protazyme AK Tablets (Lot 140401) at 40°C and pH 7.0 to activity on casein at 40°C and pH 7.0.

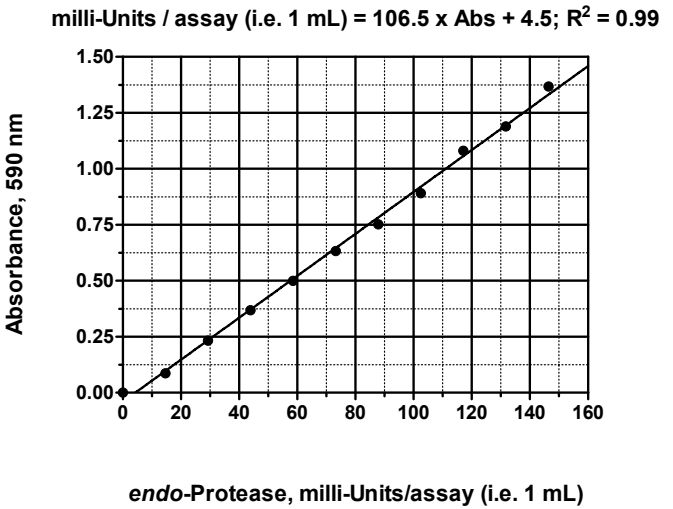

Figure 7. Standard curve relating activity of **α-chymotrypsin** on Protazyme AK Tablets (Lot 140401) at 40°C and pH 7.0 to activity on casein at 40°C and pH 7.0.

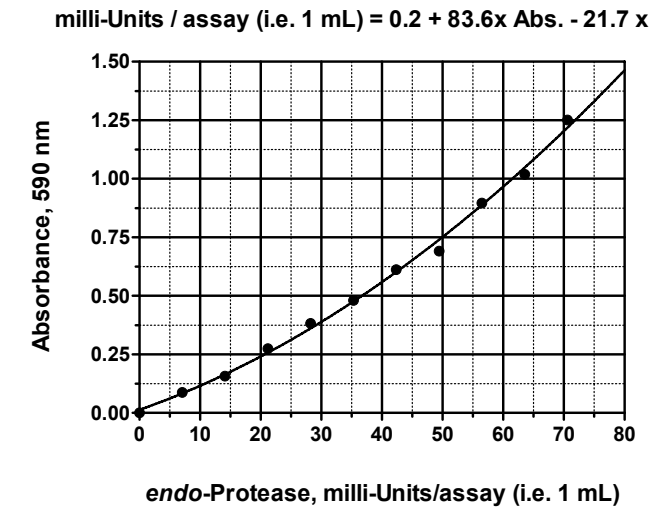

Figure 8. Standard curve relating activity of bovine pancreatic protease (mainly chymotrypsin) on Protazyme AK Tablets (Lot 140401) at 40°C and pH 7.0 to activity on casein at 40°C and pH 7.0.

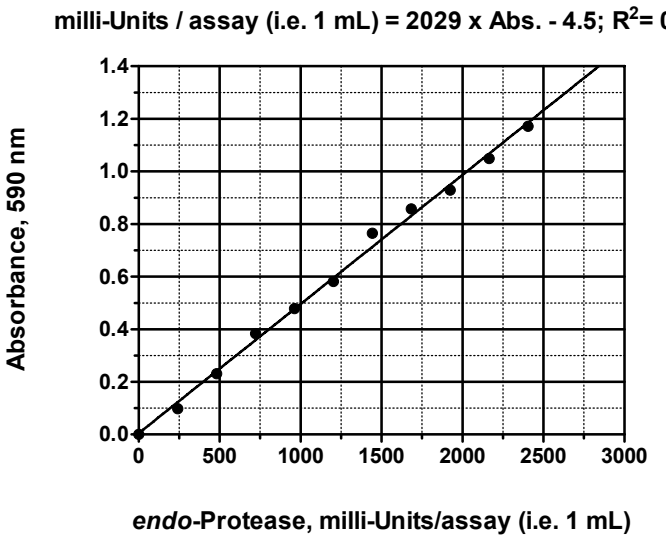

Figure 9. Standard curve relating activity of ***Aspergillus oryzae*** protease on Protazyme AK Tablets (140401) at 40°C and pH 4.5 to activity on haemoglobin at 40°C and pH 3.0 (standard HUT assay). Protazyme AK tablets were allowed to hydrate in acetate buffer for 1 h before adding enzyme.

NOTES:

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