Megazyme

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ASSAY OF

endo-PROTEASE

using

Azocasein

S-AZCAS 06/20

CAS NUMBER: 102110-74-7

Substrate:

Azocasein (Azo-casein) is prepared by dyeing casein with sulphanilic acid. The dyeing level is carefully controlled to produce a substrate which has approximately 5-times the sensitivity of similar products from other commercial suppliers (e.g. Sigma, Azo-Casein cat. no. A2765) and for most proteases, a linear reaction curve up to an absorbance of 1.0 absorbance units at 440 nm.

Dissolution:

Weigh 2 g of Azocasein into a 120 mL beaker. Add 4 mL of ethanol or industrial methylated spirits (IMS) and stir on a magnetic stirrer for a few seconds to remove all "lumps". Immediately add 96 mL of sodium phosphate buffer (100 mM, pH 7.0; Buffer A) or Tris-HCl buffer (100 mM, pH 8; Buffer C) at approx. 40°C. Vigorously stir the suspension on the magnetic stirrer until the substrate is completely dissolved (appox. 10 min). Dislodge any Azocasein which sticks to the edge of the beaker with a small spatula. Store the solution in a well-sealed glass duran bottle at 4°C and add 2 drops of toluene to prevent microbial contamination.

This solution is stable for more than 4 weeks at 4°C. Alternatively, aliquots (approx. 50 mL) can be stored frozen in 120 mL polypropylene containers for over 2 years. On thawing these solutions, heat the container and contents at approx. 40°C for 10-20 min and obtain complete dissolution by shaking the container vigorously by hand for a few min.

Applications:

Azocasein can be used to assay the activity of all *endo*-proteases which are active on casein. Such enzymes include bromelain, papain, ficin, Proteinase K, fungal and bacterial proteases (e.g. Subtillisin A) trypsin and chymotrypsin. *endo*-Proteases can also be assayed using Protazyme AK tablets from Megazyme and assays employing these tablets are 2-5-fold more sensitive than assays employing Azocasein.

In this booklet, standard curves for activity of a range of proteases at pH 7.0 or pH 8.0 are shown. For further information on activity measurement under different conditions, please contact Megazyme.

Buffers for Extraction, Dilution and Assay:

Buffer A - Stock Buffer - *(Sodium phosphate, 100 mM, pH 7)*
Add 35.6 g of di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$; MW = 178 g/mol) to 1800 mL of distilled water and dissolve with stirring. Adjust the pH to 7.0 with 1 M HCl. Adjust the volume to 2 L. Stable at 4°C for 4 weeks. For longer term storage, add sodium azide (0.4 g) as a preservative.

Buffer B - Extraction and Dilution (*Sodium phosphate, 100 mM, pH 7) with cysteine (30 mM) and EDTA (30 mM).*
To 450 mL of Buffer A, add 2.65 g of L-cysteine hydrochloride monohydrate (MW 175.6; Sigma cat. no. C7880 and 5.6 g of ethylenediaminetetraacetic acid (EDTA (MW = 372.2 g/mol); Sigma cat. no. ED2SS) and dissolve. Adjust the pH to 7.0 with 1 M sodium hydroxide (40 g/L), and adjust the volume to 500 mL with Buffer A. Stable at 4°C for approx. 2 days.

Buffer C - Extraction and Dilution (*Tris-HCl, 100 mM, pH 8)*
Dissolve 24.2 g of Tris buffer salt (Megazyme cat. no. **B-TRIS500**) in 1800 mL of distilled water and adjust the pH to 8.0 with 1 M HCl. Adjust the volume to 2 L with distilled water. Stable at 4°C for 4 weeks. For longer term storage, add sodium azide (0.4 g) as a preservative.

Trichloroacetic Acid (TCA) - *(5% w/v)*

Dissolve 50 g of TCA (Sigma cat. no. T6399) in 950 mL of distilled water and adjust to volume. Store the solution in a well sealed glass duran bottle.
Stable at room temperature for > 5 years.

Enzyme Extraction and Dilution:

NOTE: *Papain, bromelain and ficin (thiol proteases) are extracted and diluted in Buffer B. Trypsin, Chymotrypsin and Proteinase K are extracted and diluted in Buffer A and Subtilisin A is extracted and diluted in Buffer C.*

Add 1.0 g of powdered enzyme preparation to 50 mL of Buffer A, B or C and stir on a magnetic stirrer for approx. 15 min at room temperature (until the powder is completely dissolved or dispersed). Filter the preparation through a Whatman No. 1 filter sheet or centrifuge an aliquot in a microfuge for 3 min at 13,000 rpm or in a bench centrifuge at ~ 3,000 rpm for 10 min, if necessary. Dilute this **original extract** 10-fold (1 mL to 9 mL of Buffer A, B or C) and further, until a concentration suitable for assay is obtained.

For liquid preparations, use a positive displacement dispenser to transfer 1.0 mL of liquid enzyme preparation to 49.0 mL of Buffer A, B or C and thoroughly mix the solution. If necessary, filter or centrifuge as above. Dilute this **original extract** 10-fold (1 mL to 9 mL of Buffer A, B or C) and further, until a concentration suitable for assay is obtained. For Alcalase preparation, a further dilution of 200-fold is appropriate.

Assay procedure:

1. Add 1.0 mL of Azocasein solution (2% w/v) to a 16 x 160 mm) glass test tubes and equilibrate at 40°C for 5 min.
2. Pre-equilibrate enzyme solution (~ 5 mL) at 40°C for 5 min
3. Add 1.0 mL of pre-equilibrated enzyme solutions to tubes containing Azocasein solution, stir on a vortex mixer for a few seconds and incubate at 40°C for 10 min.
4. Add 6.0 mL of TCA solution (5% w/v) and stir tube contents vigorously on a vortex mixer for 5 sec to terminate the reaction and to precipitate non-hydrolysed Azocasein.
5. Store the reaction tubes at room temperature for ~ 5 min and then filter the contents through Whatman No. 1 (9 cm) filter circles. Alternatively, centrifuge the suspensions at ~ 3,000 rpm for 10 min (in this case, some floating "bits" may be observed; carefully decant the solution to avoid these bits).
6. Read the absorbance of all filtrates (or supernatant solutions) against the reaction blank at 440 nm.
7. Prepare the **reaction blank** by adding the TCA solution to the enzyme preparation with mixing, before adding the Azocasein substrate solution.

Standardisation:

Standard curves relating the activity of bromelain, papain, Subtilisin A, trypsin and chymotrypsin on Azocasein (Lot 110602) at pH 7.0 or 8.0 and 40°C, to protease activity (tyrosine Units) on casein, sodium salt (Sigma cat. no. C8654-500G) at pH 7.0 or 8.0 and 40°C are shown in Figures 1-5. The standard curves for bromelain, papain, ficin, proteinase K and Subtilisin A are linear, whereas those for chymotrypsin and trypsin are not. The Regression equations for a number of proteases are shown below. The equations for Subtilisin A, Ficin and Proteinase K are very similar. Differences in the relative activity of various

proteases on casein and Azocasein most likely relates to the chemical modification of the Azocasein.

REGRESSION EQUATIONS:

Papain (from *Papaya* latex) (pH 7.0):

Protease (milli-Units/mL) = 235 x Abs. (440 nm) + 5.4; R = 0.99
Linear absorbance range = 0.1 to 1.0

Bromelain (from pineapple stems) (pH 7.0):

Protease (milli-Units/mL) = 221 x Abs. (440 nm) + 8; R = 0.99
Linear absorbance range = 0.1 to 1.1

Ficin (from figs) (pH 7.0):

Protease (milli-Units/mL) = 142 x Abs. (440 nm) + 6; R = 0.99
Linear absorbance range = 0.1 to 1.1

Subtilisin A (from *Bacillus licheniformis*) (pH 8.0):

Protease (milli-Units/mL) = 135 x Abs. (440 nm) - 0.5; R = 0.99
Linear absorbance range = 0.1 to 1.0

Proteinase K (from *Tritirachium album*) (pH 7.0):

Protease (milli-Units/mL) = 117 x Abs. (440 nm) + 3.0; R = 0.99
Linear absorbance range = 0.1 to 1.0.

Protease activity is defined in Tyrosine Units. One Unit of protease activity is the amount of enzyme required to produce one μmole of solubilised (in TCA) tyrosine equivalents per minute from casein under standard assay conditions (pH 7.0 or 8.0 and 40°C). This method is available on request.

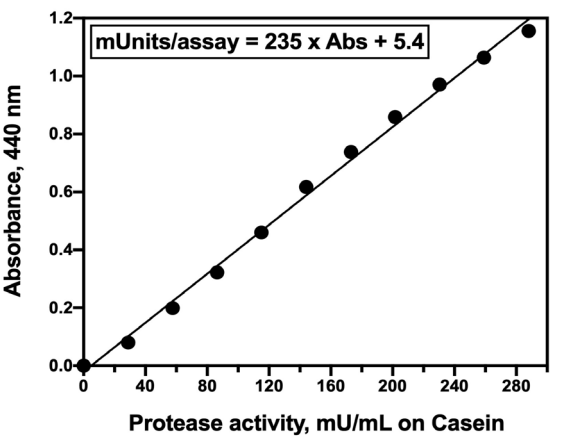

Figure 1. Papain standard curve on Azocasein (Lot 110602).

Calculation of Activity:

Protease activity is determined by reference to a standard curve (e.g. Figure 1) or to a Regression Equation to convert absorbance values to mUnits of protease activity per assay (i.e. per 1.0 mL) and then calculated as follows:

Units/mL of Original Preparation:

$$= \text{mUnits/per assay} \times 50 \times \frac{1}{1000} \times \text{Dilution}$$

where:

mUnits per assay (i.e. per1.0 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 per 50 mL or 1 mL of enzyme added to 49 mL of buffer) (the ***Enzyme Extract***).

1/1000 = conversion from mUnits to Units.

Dilution = further dilution of the ***Enzyme Extract***.

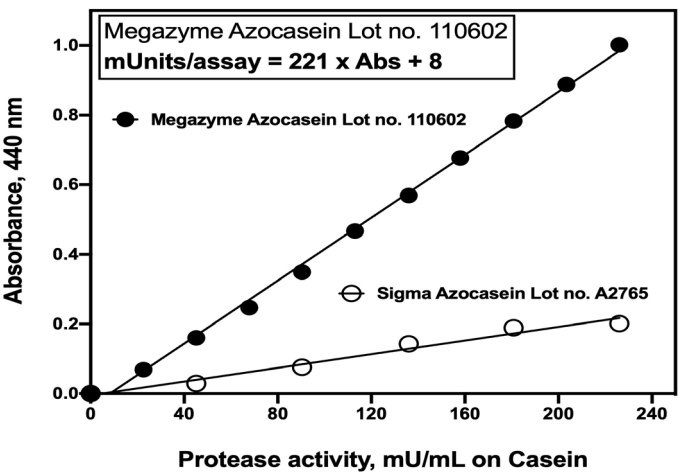

Figure 2. Bromelain Standard Curve on Azocasein (Lot 110602).

In the assay of bromelain, Megazyme Azocasein Lot no. 110602 is approx. 5-times more sensitive than Sigma Azocasein Lot no. A2765. Furthermore, with Megazyme Azocasein, the standard curve is linear up to an absorbance of 1.00. With Sigma Azocasein, poor linearity is obtained, especially at absorbance values above 0.2.

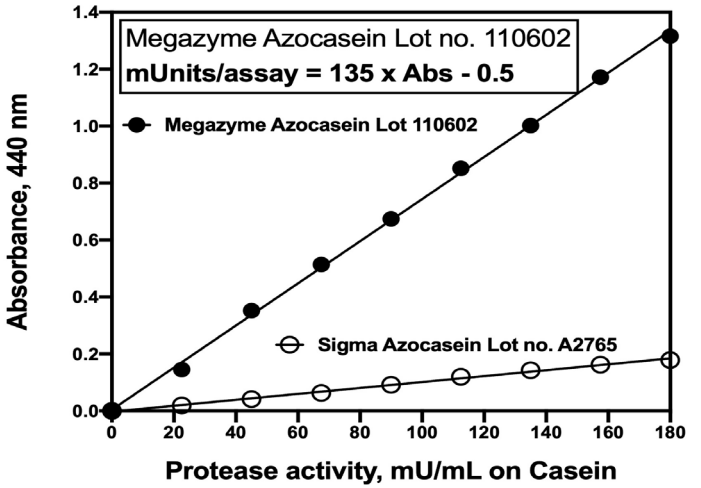

Figure 3. Subtilisin A Standard Curve on Azocasein (Lot 110602).

In the assay of Subtilisin A, Megazyme Azocasein Lot no. 110602 is approx. 6-times more sensitive than Sigma Azocasein Lot no. A2765. Furthermore, with Megazyme Azocasein, the standard curve is linear up to an absorbance of 1.00. With Sigma Azocasein, poor linearity is obtained, especially at absorbance values above 0.2.

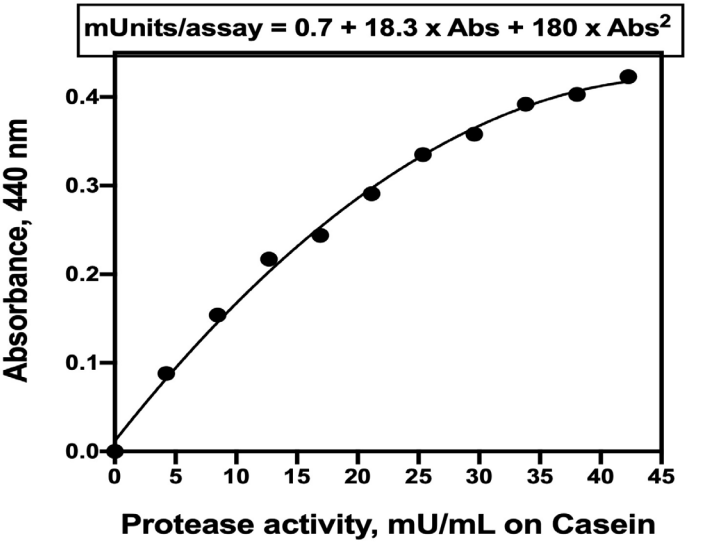

Figure 4. Trypsin Standard Curve on Azocasein (Lot 110602).

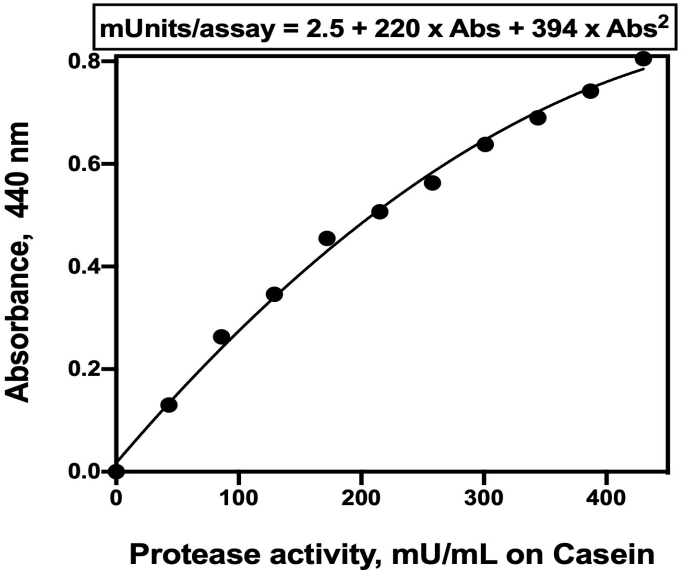

Figure 5. Chymotrypsin Standard Curve on Azocasein (Lot 110602).

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