AZOCASEIN

PRODUCT INSTRUCTIONS

SKU: 700005044
S-AZCAS

04/25

ASSAY OF *endo*-PROTEASE

10 g

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SUBSTRATE:

Azocasein (Azo-casein) is prepared by dyeing casein with sulphanilic acid. The dyeing level is carefully controlled to produce a substrate which has high sensitivity 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 (approx. 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 4 weeks at 4°C

Alternatively, aliquots (approx. 50 mL) can be stored frozen in 120 mL polypropylene containers for 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 Neogen 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 Neogen.

SAFETY:

Safety data sheets are available for all products at www.megazyme.com.

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 ($Na_2HPO_4 \bullet 2H_2O$; 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) and 5.6 g of ethylenediaminetetraacetic acid (EDTA; MW = 372.2 g/mol) 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 2 days.

Buffer C - Extraction and Dilution (*Tris-HCl, 100 mM, pH 8)* Dissolve 24.2 g of Tris buffer salt (Neogen cat. no. **B-TRIS500**, **700004162**) 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. Store 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 (MW = 163.39 g/mol) in 950 mL of distilled water and adjust to volume. Store the solution in a well-sealed glass duran bottle.
Store at room temperature.

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 15,000 g or in a bench centrifuge at ~ 4,000 g 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.

ASSAY PROCEDURE:

1. Add 1.0 mL of Azocasein solution (2% w/v) to a glass test tubes (16 x 160 mm) 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 ~ 4,000 g for 10 min (in this case, some floating particles 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 241257) 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.

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 milli-Units of protease activity per assay (i.e. per 1.0 mL) and then calculated as follows:

Units/mL of Original Preparation:

$$= \text{milli-Units/assay} \times 50 \times \frac{1}{1000} \times \text{Dilution}$$

where:

milli-Units/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 milli-Units to Units.

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

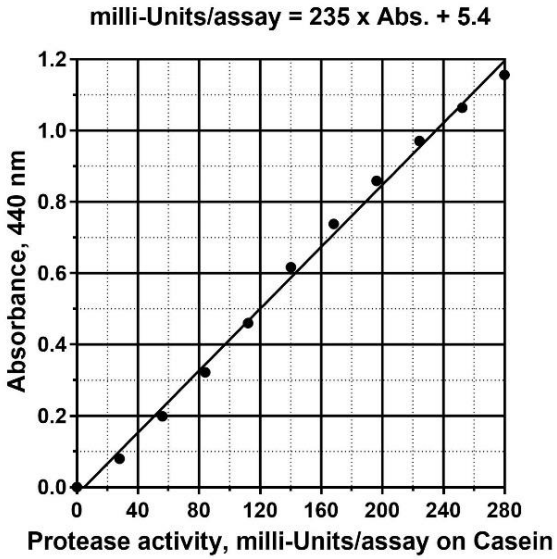

Figure 1. Papain standard curve on Azocasein (Lot 241257).

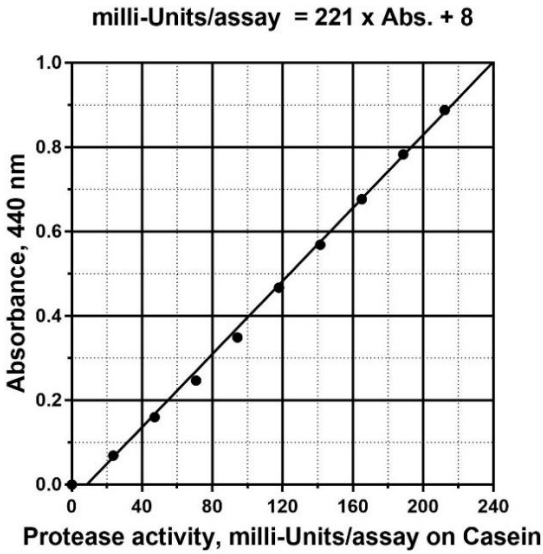

Figure 2. Bromelain Standard Curve on Azocasein (Lot 241257).

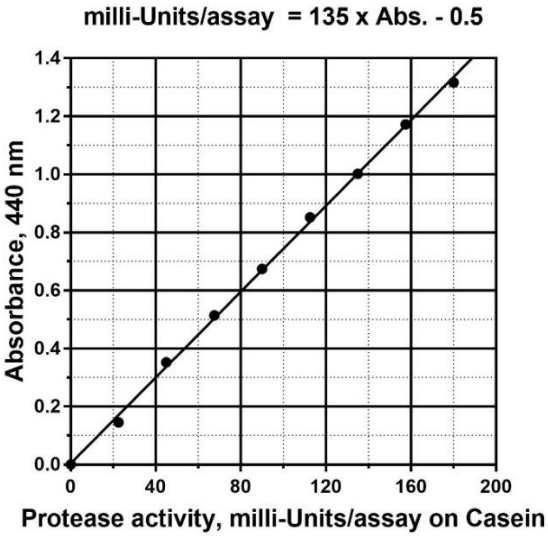

Figure 3. Subtilisin A Standard Curve on Azocasein (Lot 241257).

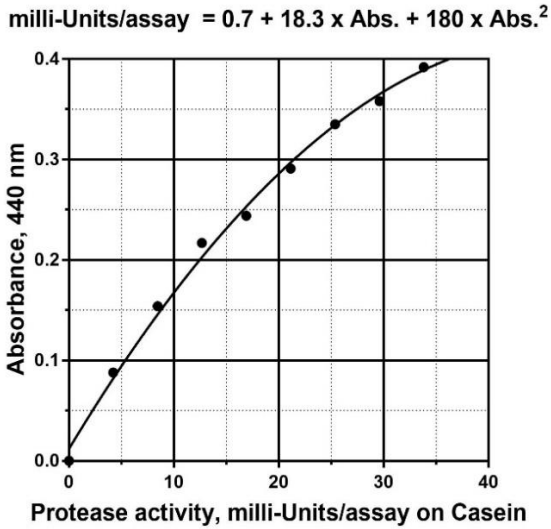

Figure 4. Trypsin Standard Curve on Azocasein (Lot 241257).

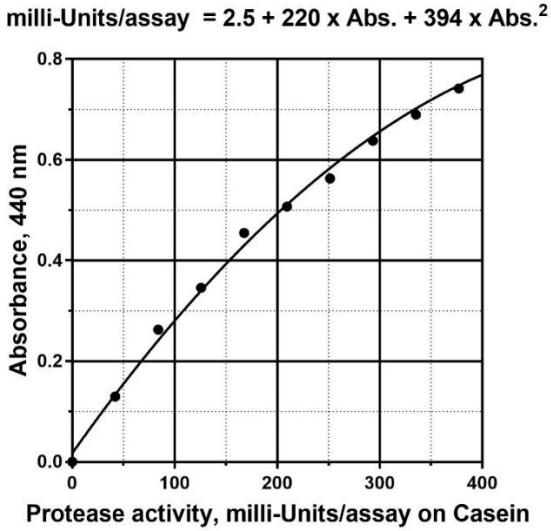

Figure 5. Chymotrypsin Standard Curve on Azocasein (Lot 241257).

Contact us for more information: neogen.com/contact

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