Enzymatic Assay of Trypsin Inhibition

Neilier Junior

1Universidade Federal de Viçosa

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Neilier Junior
Universidade Federal de Viçosa

ABSTRACT

This protocol has been standardized for analysis of protease inhibitors in soybean leaf extract, but can be easily adjusted for other biological samples.

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Prepare the solutions and the workspace

1 Preparation of solutions

Trypsin solution:
Dilute 1.25 mg of bovine trypsin in 1 mL of water pH 3.0 (adjust with HCl).

L-BApNA stock solution:
Dilute 130.47 mg L-BApNA in 5 mL DMSO (concentration: 6.0 $10^{-2}$ M). Store at -20 °C and protected from light.

L-BApNA usage solution (freshly prepared):
Dilute 200 mL of stock solution in 10 mL of 100 mM Tris-HCl buffer, pH 8.2 and 20 mM CaCl$_2$. Protect from light.

2 Separate three microtubes and name them "blank", "control (uninhibited test)" and "test (inhibited test)".
Pipette the following reagents.

**Blank**: 500 µL BApNA usage solution and 500 µL buffer.

**Control**: 100 µL trypsin solution and 400 µL buffer.

**Test**: 100 µL enzyme, 100 µL leaf extract (source of inhibitors) and 300 µL buffer.

3 Mix the three microtubes by inversion and equilibrate to 25°C for 5 min

Zero spectrophotometer with blank content at 410 nm

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4. To the control microtube, add 500 µL of the BA\(p\)NA usage solution. Immediately mix by inversion and mark the time and pour the contents into a cuvette. After 30 s of reaction onset, monitor readings at 410 nm for 120 s.

5. Add 500 µL of the BA\(p\)NA usage solution to the test tube. Immediately mix by inversion and mark the time and pour the contents into a cuvette. After 30 s of reaction onset, monitor readings at 410 nm for 120 s.

6. **Calculations**

\[
\text{‰ Inhibition} = \frac{(\Delta A_{410 \text{ nm control}} - \Delta A_{410 \text{ nm test}}) \times 100}{(\Delta A_{410 \text{ nm control}} - A_{410 \text{ nm blank}})}
\]

or

\[
\text{Trypsin Inhibitor Units / mL} = \frac{(\Delta A_{410 \text{ nm control}} - \Delta A_{410 \text{ nm test}})}{(8800 \times \text{time} \times \text{leaf extract volume})}
\]

\(A_{410 \text{ nm blank}} = \text{Absorbance in the blank at 410 nm}
\]

\(\Delta A_{410 \text{ nm control}} = \text{Absorbance variation in the control sample at 410 nm within 120 seconds}
\]

\(\Delta A_{410 \text{ nm test}} = \text{Absorbance variation in the test sample at 410 nm within 120 seconds}
\]

8800 = extinction coefficient of \(p\)-nitroanilide at 410 nm

Time = 120 seconds

Leaf extract volume = Volume of inhibitor source used (in milliliters)

The presence of trypsin inhibitors in the leaf extract decreases the enzymatic activity and this inhibition can be represented in the Lineweaver-Burk graph, where, as the inhibitor concentration increases, the slope of the line also increases.

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Lineweaver-Burk plot analysis of the inhibitory activity of soybean leaf extract toward trypsin. In blue, the kinetics in the absence of inhibitors (control). The kinetics in the presence of inhibitors (test) at 0.5 $K_i$ and $K_i$ are represented in orange and gray, respectively.

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