ACE-inhibitory activity assay: IC50

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ABSTRACT

This protocol describes the procedure for the determination of the IC50 in inhibition on angiotensin-I converting enzyme (ACE) activity. ACE is also known as peptidyl dipeptidase A because it removes C-terminal dipeptides from a wide variety of peptide substrates. In the assay described here, the chosen substrate is the intramolecularly quenched fluorescent tripeptide o-aminobenzoylglycyl-p-nitrophenylalanylproline (Abz–Gly–Phe(NO2)–Pro). Hydrolysis of this substrate by the action of ACE generates the fluorescent product o-aminobenzoylglycine (Abz–Gly).

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GUIDELINES

ACE-inhibitory activity is measured by fluorescence using the method of Sentandreu & Toldrá (2006) with some modifications (Coscueta, Campos, Osório, Nerli, & Pintado, 2019). A total of 40 µL of ultrapure water or ACE working solution are added to each microtiter-plate well, then adjusted to 80 µL by adding ultrapure water to blank, control or samples. For direct or 1/2 diluted samples a sample blank is also made. The enzyme reaction is started by adding 160 µL of substrate solution and the mixture is incubated at 37 °C. Serial dilutions are made of each sample, usually 1/1 to 1/32. The fluorescence generated is measured at 30 min using a Multidetection plate reader (Synergy H1, Vermont, USA). The assay is performed in a black 96-well microplate (Nunc, Denmark). Excitation and emission wavelengths are 350 and 420 nm, respectively.

Note: In some cases it is necessary to extend the range to 1/5 or 1/10 serial dilutions.

Note: Generally the most concentrated dilutions are the ones that can fluoresce as the sample itself, so in the example sample blanks (SPLB) are used only for the two most concentrated dilutions. In the case of highly fluorescent samples, SPLB should be used for all dilutions.

CITATION


LINK
https://doi.org/10.1016/j.foodchem.2005.06.006

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MATERIALS
## Equipment

### Synergy H1M

**NAME**

Multidetection plate reader

**TYPE**

BioTek

**BRAND**

679SH1M-SN

**SKU**

Detection modes: UV-Vis absorbance; fluorescence intensity; luminescence

Read methods: endpoint, kinetic, spectral scanning, well area scanning

Microplate types: 6- to 384-well plates

Temperature control: to 45 °C

Shaking: linear, orbital, double orbital

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Angiotensin Converting Enzyme from rabbit lung Sigma – Aldrich Catalog #A6778

Tris Base Fisher Scientific Catalog #BP152-1

or Tris-HCl Sigma Aldrich

Ultrapure Water Contributed by users

ZnCl2 anhydrous Thermo Fisher Scientific Catalog #11497737

Sodium Chloride Fisher Scientific Catalog #S271

Abz-Gly-p-nitro-Phe-Pro-OH Trifluoroacetate bachem Catalog #4003531.0050
BEFORE START INSTRUCTIONS

Prepare the necessary reagents carefully.

- **1 U/mL ACE stock solution**
  - Dissolve the
    - **Angiotensin Converting Enzyme from rabbit lung Sigma – Aldrich Catalog #A6778** (peptidyl-dipeptidase A, EC 3.4.15.1) in a solution of 50% glycerol in ultrapure water, to obtain a final concentration of 1 U/mL.
  - Make aliquots of **200 µL** of the solution and store at **-20 °C**.

- **0.150 Molarity (M) Tris buffer**
  - Dissolve **1.817 g** of Tris base (MW = 121.14) or **2.364 g** Tris-HCl (MW = 157.60) in approx. **90 mL** ultrapure water.
  - Titrate to **8.77** at the lab temperature of **Room temperature** with monovalent strong base or acid as needed.
  - Make up volume to **100 mL** with ultrapure water.
  - Buffer will be **8.3** at **37 °C**.

- **42 mU/mL ACE work solution**
  - Prepare a solution of **1 millimolar (mM) ZnCl2**, dissolving **1.4 mg ZnCl2 (MW = 136.28)** in **10 mL** ultrapure water. Store at **-20 °C**.
  - Prepare **0.1 millimolar (mM) ZnCl2** and **0.150 Molarity (M) Tris buffer**.
**Enzyme buffer.** Dilute 1/10 the previous solution (0.1 millimolar (mM)) and add 25 µL of this solution to 25 mL of 0.150 Molarity (M) Tris buffer 8.3. Store at 4 °C for a maximum of one week, or at -20 °C for a maximum of six months.

- Dilute 1/24 the 1 U/mL ACE stock solution with the Enzyme buffer. Prepare daily.

**0.45 millimolar (mM) Substrate solution**

- Prepare 1.125 Molarity (M) NaCl 0.150 Molarity (M) Tris buffer 8.3 (Substrate buffer). Dissolve 3.2872 g NaCl (MW = 58.44) in 50 mL of 0.150 Molarity (M) Tris buffer 8.3. Store at 4 °C for a maximum of one week, or at -20 °C for a maximum of six months.

- Dissolve 3.6 mg of substrate Abz-Gly-p-nitro-Phe-Pro-OH Trifluoroacetate bachem Catalog #4003531.0050 in 16 mL Substrate buffer (for 96 wells). Prepare at the moment.

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**Analysis**

Microplate outline example for IC₅₀ determination.

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[Protocols.IO](https://dx.doi.org/10.17504/protocols.io.q26q74g5kgwz/v1)  
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1.1 Add 80 µL ultrapure water to blank (BLK)

1.2 Add 40 µL ultrapure water to control (CTL) and sample blank (SPLB)

1.3 Add 40 µL sample dilution to sample (SPL) and sample blank (SPLB)

1.4 Add 40 µL ACE work solution to control (CTL) and sample (SPL)

1.5 Add 160 µL Substrate solution to control all wells

1.6 Incubate at 37 °C 00:30:00 and read fluorescence with 350 nm excitation wavelength and 420 nm emission wavelength

Result treatment

Inhibitory activity is expressed as the peptide concentration required to inhibit the original ACE activity by 50% (IC₅₀). The formula applied to calculate the percentage of ACE-inhibitory is:

\[
iACE(\%) = \left( \frac{F_{CTL} - F_{BLK}}{F_{CTL} - F_{BLK}} \right) \times \frac{100}{F_{CTL} - F_{BLK}}
\]
Non-linear fitting to the data is performed to calculate the IC$_{50}$ values, using the 5 Parameter curve fit method and then Interpolating to 50.

Example of a typical inhibition curve as a function of dilution factor.