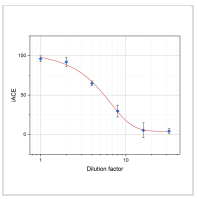

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ACE-inhibitory activity assay: IC50

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ABSTRACT

This protocol describes the procedure for the determination of the IC_{50} 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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KEYWORDS

ACE, angiotensin-converting enzyme, antihypertensive, IC50, bioactivity, ngiotensin-I converting enzyme

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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.

Sentandreu, M. Á., & Toldrá, F. (2006). A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme. Food Chemistry. https://doi.org/10.1016/j.foodchem.2005.06.006

Coscueta, E. R., Campos, D. A., Osório, H., Nerli, B. B., & Pintado, M. (2019). Enzymatic soy protein hydrolysis: A tool for biofunctional food ingredient production. Food Chemistry: X. https://doi.org/10.1016/j.fochx.2019.100006

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MATERIALS TEXT

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**

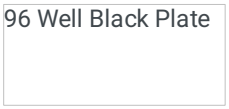
ZnCl2 anhydrous **Thermo Fisher Scientific Catalog #11497737**

Sodium Chloride **Fisher Scientific Catalog #S271**

Abz-Gly-p-nitro-Phe-Pro-OH Trifluoroacetate **bachem Catalog #4003531.0050**

Synergy H1M
Multidetection plate reader
BioTek **679SH1M-SN**
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

96 Well Black Plate
microplate
Nunc **237108**
96-Well black polystyrene microplate, flat bottom, 50 to 250 µL, non-sterile, non-treated



BEFORE STARTING

Prepare the necessary reagents carefully.

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[M]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** .

[M]0.150 Molarity (M) Tris buffer **pH8.3**

- 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 **pH8.77** 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 **pH8.3** at **37 °C** .

[M]42 mU/mL ACE work solution

- Prepare a solution of **[M]1 millimolar (mM) ZnCl2** , dissolving
  1.4 mg ZnCl2 (MW = 136.28) in **10 mL ultrapure water** . Store at **-20 °C** .
- Prepare **[M]0.1 millimolar (mM) ZnCl2** **[M]0.150 Molarity (M) Tris buffer** **pH8.3** (Enzyme buffer). Dilute 1/10 the previous solution (**[M]0.1 millimolar (mM)**) and add **25 µL** of this solution to **25 mL** of **[M]0.150 Molarity (M) Tris buffer** **pH8.3** . Store at **4 °C** for a maximum of one week, or at **-20 °C** for a maximum of six months.
- Dilute 1/24 the **[M]1 U/mL ACE stock solution** with the Enzyme buffer. Prepare daily.

[M]0.45 millimolar (mM) Substrate solution

- Prepare **[M]1.125 Molarity (M) NaCl** **[M]0.150 Molarity (M) Tris buffer** **pH8.3** (Substrate buffer). Dissolve **3.2872 g NaCl (MW = 58.44)** in **50 mL** of **[M]0.150 Molarity (M) Tris buffer** **pH8.3** . 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.

Analysis 30m

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1

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	BLK	SPLB1:1 1	SPLB1:1 1	SPLB2:1 1	SPLB2:1 1	SPLB3:1 1	SPLB3:1 1	SPLB4:1 1	SPLB4:1 1	
B	CTL	CTL	CTL	SPLB1:2 2	SPLB1:2 2	SPLB2:2 2	SPLB2:2 2	SPLB3:2 2	SPLB3:2 2	SPLB4:2 2	SPLB4:2 2	
C	SPL1:1 1	SPL1:1 1	SPL1:1 1	SPL2:1 1	SPL2:1 1	SPL2:1 1	SPL3:1 1	SPL3:1 1	SPL3:1 1	SPL4:1 1	SPL4:1 1	SPL4:1 1
D	SPL1:2 2	SPL1:2 2	SPL1:2 2	SPL2:2 2	SPL2:2 2	SPL2:2 2	SPL3:2 2	SPL3:2 2	SPL3:2 2	SPL4:2 2	SPL4:2 2	SPL4:2 2
E	SPL1:3 4	SPL1:3 4	SPL1:3 4	SPL2:3 4	SPL2:3 4	SPL2:3 4	SPL3:3 4	SPL3:3 4	SPL3:3 4	SPL4:3 4	SPL4:3 4	SPL4:3 4
F	SPL1:4 8	SPL1:4 8	SPL1:4 8	SPL2:4 8	SPL2:4 8	SPL2:4 8	SPL3:4 8	SPL3:4 8	SPL3:4 8	SPL4:4 8	SPL4:4 8	SPL4:4 8
G	SPL1:5 16	SPL1:5 16	SPL1:5 16	SPL2:5 16	SPL2:5 16	SPL2:5 16	SPL3:5 16	SPL3:5 16	SPL3:5 16	SPL4:5 16	SPL4:5 16	SPL4:5 16
H	SPL1:6 32	SPL1:6 32	SPL1:6 32	SPL2:6 32	SPL2:6 32	SPL2:6 32	SPL3:6 32	SPL3:6 32	SPL3:6 32	SPL4:6 32	SPL4:6 32	SPL4:6 32

BLK Blank CTL Control SPLB Sample blank SPL Sample

Microplate outline example for IC_{50} determination.

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** 30m and read fluorescence with **350 nm excitation** wavelength and **420 nm emission** wavelength

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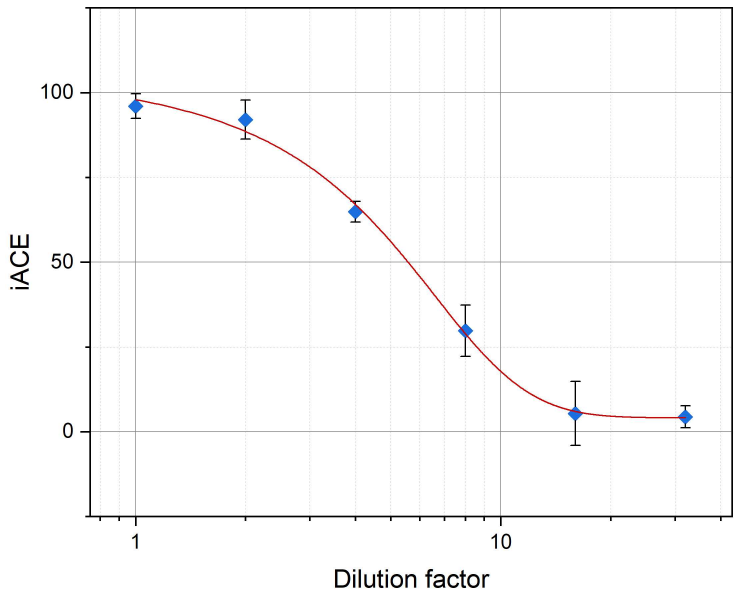
Result treatment

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

$$iACE\ (\%) = \left((F_{CTL} - F_{BLK}) - (F_{SPL} - F_{SPLB})\right) * \frac{100}{F_{CTL} - F_{BLK}}$$

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.

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