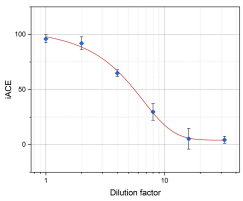
Aug 19, 2022

ACE-inhibitory activity assay: IC50



DOI
https://dx.doi.org/10.17504/protocols.io.q26g74q5kgwz/v1

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Protocol Citation: Ezequiel Coscueta, & Maria Manuela Pintado 2022. ACE-inhibitory activity assay: IC50. **protocols.io** https://dx.doi.org/10.17504/protocols.io.q26g74q5kgwz/v1

Manuscript citation:
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, 1(100006). https://doi.org/10.1016/j.fochx.2019.100006

Protocol status: Working
We use this protocol and it's working

Created: August 17, 2022

Last Modified: August 19, 2022

Protocol Integer ID: 68779

Keywords: ACE, angiotensin-converting enzyme, antihypertensive, IC50, bioactivity, ngiotensin-I converting enzyme, angiotensin, inhibitory activity assay, ic50 in inhibition, nitrophenylalanylproline, enzyme, terminal dipeptide, peptide substrate, action of ace, converting enzyme, assay, ace

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).

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

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 LINK

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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 LINK

Materials

Angiotensin Converting Enzyme from rabbit lung **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A6778

Tris Base **Fisher Scientific Catalog #**BP152-1 or Tris-HCl **Merck MilliporeSigma (Sigma-Aldrich)**

Ultrapure Water

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

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	SPECIFICATIONS

Equipment

96 Well Black Plate	NAME
microplate	TYPE
Nunc	BRAND
237108	SKU
96-Well black polystyrene microplate, flat bottom, 50 to 250 µL, non-sterile, non-treated	SPECIFICATIONS

Before start

Prepare the necessary reagents carefully.

[M] 1 U/mL ACE stock solution

- Dissolve the
  Angiotensin Converting Enzyme from rabbit lung **Merck MilliporeSigma (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 Mass Percent Tris buffer pH 8.3

- 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 pH 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 pH 8.3 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 Mass Percent Tris buffer pH 8.3 (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 Mass Percent Tris buffer pH 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 [M] 1 U/mL ACE stock solution with the Enzyme buffer. Prepare daily.

[M] 0.45 millimolar (mM) Substrate solution

- Prepare [M] 1.125 Mass Percent NaCl [M] 0.150 Mass Percent Tris buffer pH 8.3 (Substrate buffer). Dissolve 3.2872 g NaCl (MW = 58.44) in 50 mL of [M] 0.150 Mass Percent Tris buffer pH 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.

Analysis

30m

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

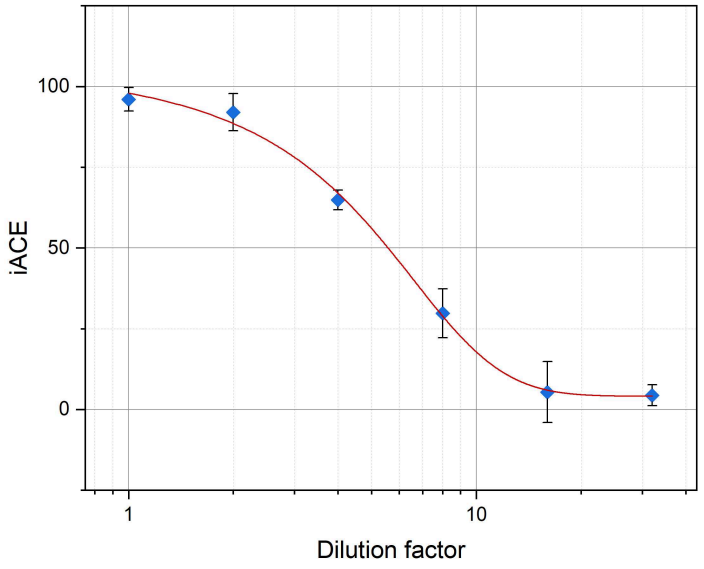
30m

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.

Citations

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

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