Biocatalysis and Agricultural Biotechnology 20 (2019) 101258

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

New ACE-inhibitory peptides derived from α-lactalbumin produced by hydrolysis with *Bromelia antiacantha* peptidases

Carolina Villadóniga[a,*], Ana María B. Cantera[a,b]

[a] Laboratorio de Enzimas Hidrolíticas, Facultad de Ciencias, Universidad de la República, Iguá 4225, 11400, Montevideo, Uruguay
[b] Cátedra de Bioquímica, Facultad de Química, Universidad de la República, Gral. Flores 2124, 11800, Montevideo, Uruguay

ARTICLE INFO

Keywords:
α-lactalbumin
Bioactive peptides
ACE inhibitors
Cysteine peptidases
Whey
Bromelia antiacantha Bertol.

ABSTRACT

Angiotensin converting enzyme (ACE) plays a key role in regulating blood pressure and ACE inhibitors are first-line drugs for treating hypertension. Food protein-derived peptides with ACE inhibitory activity are natural and safe alternatives for both prevention and treatment of hypertension. The second most abundant whey protein, α-lactalbumin (ALA) was hydrolysed with proteases from *Bromelia antiacantha* Bertol., a native plant. Two new peptides with potent ACE-inhibitory activity: TTFHTSGY (IC_{50} = 142 μM) and GYDTQAIVQ (IC_{50} = 1.0 mM) were purified by affinity chromatography and sequences were determined by mass spectrometry. *In silico* analysis indicated that neither of these peptides was toxic (ToxinPred). Inhibition kinetic analysis showed that TTFHTSGY was a fully functional ACE competitive inhibitor, whereas GYDTQAIVQ caused only partial ACE inhibition. Our results indicate that cysteine peptidases from *B. antiacantha* hydrolyse ALA to produce novel ACE inhibitory peptides that could be useful even as functional food ingredients.

1. Introduction

Non-communicable diseases (NCD) are a health priority that results in 71% of global deaths annually (41 million people). Cardiovascular diseases (CVD) are the main cause of NCD resulting in 17.9 million deaths per year (WHO, 2018). Hypertension is a chronic disease but is controllable for CVD, but new strategies for prevention and control are urgently needed. Different systems are involved in regulation of blood pressure in the human body. The renin-angiotensin system is the most important and most studied system, where angiotensin-converting enzyme (ACE) plays a key role. ACE catalyses production of a potent vasoconstrictor peptide (angiotensin II) and, in parallel, the breakdown of bradykinin, a vasodilator peptide. Synthetic ACE inhibitors are used as first-line drugs in the treatment of hypertension, although they have adverse side effects such as dry cought, dysgeusia and angioedema (Akif et al., 2011).

Peptides derived from food protein with ACE inhibitory activity could be a natural and safe alternative for these synthetic drugs. Milk proteins have a reservoir of ACE inhibitory peptides that can be released by enzymatic proteolysis (Iwaniak et al., 2014). Peptidases from animal and bacterial sources are currently used to produce bioactive peptides from whey protein. ACE inhibitory peptides have been produced by enzymatic hydrolysis of milk proteins using gastrointestinal enzymes (Power et al., 2014), microbial enzymes (Hernández-Ledesma et al., 2006) and those involved in fermentation processes (Gobbetti et al., 2002). Plant peptidases have also been useful for generating bioactive peptides. ACE inhibitory peptides have been produced by digestion of whey protein with papain (Silva et al., 2014) as well as with non-commercial peptidase extracts from cardoon flowers (*Cynara cardunculus*) (Tavares and Malcata, 2013), of *Arctium minus* (Cimino et al., 2015) and from fruit of *Maclura pomifera* (Bertucci et al., 2015). The use of other plant proteases opens the possibility of finding other previously undescribed bioactive peptides. We recently reported ACE inhibitory activity in hydrolysates of whey, β-lactoglobulin (BLG) and α-lactalbumin (ALA) using antiacanthain, a mixture of cysteine endopeptidase isoforms extracted from ripe fruit of *Bromelia antiacantha* Bertol. (Villadóniga et al., 2018).

Bromelia antiacantha Bertol. is a native plant spiece of Brazil and Uruguay with endemic distribution in atlantic forest. In traditional medicine, fruits are used to prepare cought syrup (Santos et al., 2009), while leaves infusions are employed to treat mouth and skin ulcers and macerated leaves as anti-thermal and anthelmintic agents (Manetti et al., 2010).

Since such protein hydrolysates are highly complex, steps for purification and identification of peptides responsible for ACE inhibitory activity is difficult, in which distinct purification methods are needed. The objective of this study was to identify and characterise peptides responsible for ACE inhibitory activity produced by hydrolysis of ALA

* Corresponding author.
E-mail address: carolinav@fcien.edu.uy (C. Villadóniga).

https://doi.org/10.1016/j.bcab.2019.101258
Received 3 May 2019; Received in revised form 26 June 2019; Accepted 19 July 2019
Available online 20 July 2019
1878-8181/

with enzymes obtained from *B antiacantha* fruit.

2. Materials and methods

2.1. Materials

ALA from bovine milk, ACE from rabbit lung, o-phthalaldehyde (OPA), bovine serum albumin (BSA), L-leucine, azocasein, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), acetonitrile, hippuryl-L-histidyl-L-leucine (HHL) and N-[3-(2-furyl) acryloyl]-L-phenylalanylglycylglycine (FAPGG) were purchased from Sigma-Aldrich (St.Louis, MO, USA). SP-Sepharose Fast Flow and N-hydroxysuccinimide (NHS) activated Sepharose 4 Fast Flow were obtained from GE Healthcare (Uppsala, Sweden).

2.2. Enzyme preparation

Crude extract (CE) from *B. antiacantha* ripe fruit was prepared as described previously (Villadóniga et al., 2018). An aliquot of 5 mL of CE (5/7 dilution in 10 mM sodium citrate buffer, pH 4.5) was added per 6.2 mL of SP-Sepharose Fast Flow (Amersham Pharmacia, Sweden) previously equilibrated in 10 mM sodium citrate buffer, pH 4.5. Batch cation exchange chromatography was done by rotation end-by-end for 30 min at 4 °C. After washing with the same buffer, the gel was packed into a column (15 × 35 mm) and bound proteins were eluted with 10 mM sodium citrate buffer, pH 4.5, containing 0.4 M NaCl (Vallés and Cantera, 2018). Fractions collected were analysed by UV absorption between 220 and 400 nm using a UV-1800 spectrophotometer (Shimadzu, Japan). Proteolytic activity in fractions was determined using azocasein as substrate according to a modification of the method of Andrews and Asenjo (Fullana et al., 2017). Briefly, reaction mixture containing 0.34 mL enzymatic preparation (1/200 dilution), 0.34 mL 1% (w/v) azocasein solution and 0.34 mL 0.2 M phosphate buffer, pH 7.5, was incubated for 10 min at 37 °C. The reaction was stopped by adding 0.34 mL 10% (w/v) TCA. After centrifugation for 20 min at 20,600 *g* at room temperature, absorbance at 337 nm of supernatants was measured. One enzymatic unit (U) was defined as the amount of enzyme, which produces an increment of one absorbance unit at 337 nm per minute of reaction under assay conditions. Protein concentration was determined by Bradford (1976) using BSA as standard. Active fractions obtained in repeated chromatography were mixed and then concentrated by ultrafiltration using Viva Spin 15R (Sartorius, 2000 MWCO) devices.

2.3. Hydrolysis of ALA

Hydrolysis of ALA (1.3 mg/mL) was performed at pH 9.2 at enzyme-substrate ratio of 1.0 U/mg for 30 min at 50 °C. To stop the reaction, the hydrolysate was incubated 15 min at 95 °C and then centrifuged at 7000 g for 15 min at 4 °C. Subsequently, the ALA hydrolysate (ALAH) was fractionated by ultrafiltration using VivaSpin 15R (2000 MWCO; Sartorius, Germany). Peptide concentration of samples and degree of hydrolysis (DH) were determined by a spectrophotometric assay using OPA and ACE inhibitory activity was measured by Cushman and Cheung (1971), with modification as described previously (Villadóniga et al., 2018). Molecular weight distribution of ALAH and ALAH 2 kDa permeate (ALAHP) were analysed by size exclusion high-performance liquid chromatography (SEC-HPLC) on a Prominence UFLC System with a photodiode array UV–visible detector (SPD-M20A) using LCsolution software (Shimadzu Corporation, Kyoto, Japan). Separations were carried out on a Superdex™ Peptide 10/300 GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at a flow rate of 0.5 mL/min with 0.1% (v/v) TFA, 30% (v/v) acetonitrile in ultrapure water as eluent. The column was calibrated using insulin B chain oxidized (3495.9 Da; standard 1) and standard 2 composed by: angiotensin II (1046.2 Da), Tyr-Gly-Gly-Phe-Leu (555.6 Da) and Gly-Phe (222 Da). ALAHP was further analysed by reverse phase HPLC (RP-HPLC) on the system previously described for SEC-HPLC. Chromatography was run on a Viva C18 column (5 µm, 300 Å, 4.6 × 250 mm; Restek, USA) equilibrated with 0.1% (v/v) TFA in ultrapure water (solvent A) and maintained at 40 °C. Elution was performed with a flow of 1 mL/min using a binary gradient of solvent A and solvent B (0.1% (v/v) TFA in acetonitrile) using a linear gradient from 0 to 60% (v/v) of solvent B in 60 min and from 60 to 100% (v/v) of solvent B in 10 min.

2.4. Immobilisation of ACE onto NHS-Sepharose 4 Fast Flow

An aliquot of 100 µL NHS-Sepharose 4 Fast Flow was placed in a mini-column (Pierce Spin Columns, Thermo Scientific, USA). The matrix was activated with 1.5 mL cold 1 mM HCl and then washed twice with 100 µL 0.5 M NaCl solution in 0.2 M potassium phosphate buffer, pH 7.5 (immobilisation buffer). Immediately, 200 µL ACE (0.9 mg/mL) dissolved in immobilisation buffer was added and incubated overnight at 4 °C. In parallel, a control column was prepared, with immobilisation buffer only, i.e., without ACE. This control column was used to identify and discard peptides that were retained by nonspecific binding. The unretained flow-through fraction (supernatant) of each column was separated by centrifugation at 1000 g and 4 °C. Columns were washed with 200 µL immobilisation buffer six times. Unreactive groups were blocked with 200 µL 0.5 M ethanolamine, pH 8.3 containing 0.5 M NaCl for 2 h at room temperature and by rotation end-by-end agitation. Finally, the columns were washed with 300 µL 0.5 M NaCl solution in 0.1 M sodium acetate buffer pH 4.5, then with a 0.5 M NaCl solution in 0.1 M Tris-HCl buffer, pH 8.5. This alternative washing protocol was repeated three times. The columns were stored in 20% (v/v) ethanol in water until use. Percentage protein immobilisation for columns was determined.

2.5. Purification of ACE inhibitory peptides

ALAHP was concentrated using a Bakerbond™ Spe Octadecyl C18 cartridge (JT Baker, USA). Peptides were eluted with a 30% acetonitrile aqueous solution and after vacuum evaporation samples were dissolved in 0.2 M potassium phosphate buffer, pH 7.0 containing 0.5 M NaCl. Aliquots of 100 µL ALAHP were admitted to the column with immobilised ACE and to the control column, both previously equilibrated in 0.2 M potassium phosphate buffer pH 7.0, 0.5 M NaCl (equilibration buffer). After incubation overnight at 4 °C, unretained components (NR) were separated by centrifugation at 1000 g for 2 min and washed with 200 µL equilibrium buffer (four times). Retained peptides were then eluted with 100 µL 0.2 M potassium phosphate buffer, pH 7.0 containing 1.0 M NaCl and centrifugation at 1000 g for 2 min, this step was repeated three times.

2.6. Identification of potential ACE inhibitors

Eluents of the ACE immobilised column and control column were analysed to identify peptides that specifically bound to immobilised ACE. Samples (200 µL) were concentrated by vacuum evaporation of solvent, dissolved in 15 µL 0.1% (v/v) formic acid and concentrated using microextraction tips C18 Agilent Bond ElutOmix (Agilent Technologies, USA). The solvent was vacuum evaporated and dried samples were dissolved in 0.1% (v/v) formic acid. Samples were then injected into a Poroshell column (Agilent Technologies, USA) connected to an Easy-nLC 1000 system (Thermo Scientific, USA) coupled to a tandem mass spectrometer with electrospray ionization (ESI MS/MS LTQ Thermo Scientific, USA). Mass spectra were analysed and peptide sequences were identified using the Mascot search engine in MS/MS ion search mode, with a mass tolerance of ± 1.5 Da. Search analysis was done with both public and local databases (including the reported bovine ALA sequence, UniProtKB - P00711). Results were compared with those corresponding to those eluted from the control column so to

identify and discard peptides that bound nonspecifically to the ACE affinity column.

2.7. In silico prediction of peptide toxicity, stability, hydrophobicity and solubility

Potential toxicity of peptides was predicted using ToxinPred available at: http://crdd.osdd.net/raghava/toxinpred/(Gupta et al., 2013). Stability and hydropathy (GRAVY = Grand Average of Hydropathy) were predicted using ProtParam Tool (https://web.expasy.org/protparam). Additionally, peptide solubility was evaluated using Innovagen Peptide Solubility Calculator Proteomic Tool, available at https://pepcalc.com/peptide-solubility-calculator.php.

2.8. In silico peptide gastrointestinal digestion

We used Peptide Cutter Proteomic Tool at ExPASy (Gasteiger et al., 2005) to simulate gastrointestinal digestion of peptides. Each peptide was theoretically digested with pepsin and then with trypsin and chymotrypsin. The predicted peptide products were searched on Biopep Database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) to determine its bioactive potential.

2.9. Synthesis of ACE inhibitory peptides

Peptides were prepared by the synthesis service at Peptide 2.0 Inc. (Virginia, USA). Synthetic peptides were used to confirm predicted ACE inhibitory activity, determine their IC_{50} and perform inhibition kinetic studies.

2.10. Inhibition kinetics

Kinetics of ACE inhibition was done by monitoring hydrolysis of FAPGG spectrophotometrically by the method of Buttery and Stuart (1993) with some modification. 490 µL of FAPGG (dissolved in 50 mM sodium borate buffer, pH 8.2, 0.3 M NaCl) was mixed with 10 µL inhibitor and preincubated at 37 °C for 5 min. The reaction was started with the addition of 10 µL of ACE stock (7.0×10^{-7} M) and absorbance decrease at 345 nm was recorded every 0.1 min for 10 min at 37 °C. Initial velocity (v_o) of substrate hydrolysis was calculated from the linear portion of A345 nm graphs as a function of time using the following equation:

$$v_o = \frac{\Delta A_{345nm}/min \times 1\ cm}{0.5343\ mM^{-1}cm^{-1}}$$

where: 0.5343 $mM^{-1}\ cm^{-1}$ is the absorptivity of FAPGG (Buttery and Stuart, 1993).

The v_o was determined for increasing concentrations of each of the inhibitory peptides, in which ACE and substrate concentrations were constant. Partial or total character of inhibition was established from graphs of v_o as a function of concentration of inhibitor.

Hydrolysis of substrate was determined at different substrate concentrations in the absence and in the presence of different concentrations of inhibitor to determine the type of inhibition. Kinetic parameters of ACE for hydrolysis of FAPGG (K_M and V_{Max}) in the absence of inhibitor and the apparent kinetic parameters in the presence of different concentrations of inhibitor were determined from the linear fit of [S]/*vo* versus [S] graph (Hanes-Woolf graph). Once the type of inhibition was established, the inhibition constant (K_i) was determined. The K_i was calculated from linear regression of the graph of [S]/*vo* versus inhibitor concentration (Cornish-Bowden graph).

2.11. Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation. Data were evaluated by one way analysis of variance (ANOVA). Means were considered to be different at a significance level of 0.05.

3. Results and discussion

3.1. Partial purification of B. antiacantha bertol. peptidases

Our previous studies have shown that *B. antiacantha* Bertol. CE has an abundance of proteolytic enzyme activities as has been found in other tropical fruits (Vallés et al., 2007). Significant levels of peptidase make this CE very attractive for protein hydrolysate production. This CE, however, contains a number of non-protein components, including lipids, carbohydrates, phenolic compounds and pigments (Krumreich et al., 2015) that could interfere with proteolysis but mainly with identification of specific hydrolysis products. Cation exchange chromatography (SP-Sepharose) was used to obtain an enzymatic preparation enriched in proteolytic enzymes. Proteolytic activity was detected in fractions eluted with 0.4 M NaCl (E). UV spectra between 220 and 400 nm were measured for chromatography fractions and for CE (Fig. S1). The E fraction spectrum was different from those of CE and NR fraction with disappearance of 300–360 nm absorption, whereas maximum signal at about 280 nm was found. These differences could correspond to elimination of phenolic compounds (hydroxycinnamic acids and flavone derivatives), ascorbic acid and carotenoids present in *B antiacantha* fruits (Santos et al., 2009) and enrichment in protein (absorption maximum at 280 nm).

After repeating chromatography under the same conditions, eluents were pooled and concentrated by ultrafiltration. Finally, a fraction designated ion exchange prepared antiacanthain (IEX antiacanthain) with enzymatic activity of 88 U/mL, protein concentration of 2,5 mg/mL (0,25%, w/v) and specific activity of 35 U/mg was obtained and used to prepare ALAH.

3.2. ALA hydrolysis by IEX antiacanthain

In a previous study, partially purified extract of *B. antiacantha* fruits (termed antiacanthain) was used to hydrolyse whey proteins and reaction parameters were evaluated to achieve extensive degradation of target proteins. Best combination of reaction parameters were seleced to prepare ALAH (DH = 17.3 ± 1.9%) with ACE inhibitory activity (Villadóniga et al., 2018). 3 kDa permeate of ALAH obtained by antiacanthain hydrolysis for 30 min showed the highest ACE inhibitory activity (100.0 ± 0.1%). In this study, ALAH prepared with IEX antiacanthain, under conditions previously difined had slightly lower DH (13.4 ± 1.1%) that could be attributed to lower IEX antiacanthain stability than antiacanthain. ALAH was fractionated by ultrafiltration using 2 kDa cut off membranes to prepare low molecular mass peptides. The 2 kDa ALAHP had ACE inhibitory activity of 90.0 ± 1.0% and a peptide concentration of 0.30 ± 0.02 mg/mL. SEC-HPLC analysis of ALAH showed a broad molecular mass distribution that was reduced by UF and consistent with the ALAHP SEC-HPLC profile (Fig. 1A). The main ALAHP components were of molecular mass around 1000 Da and lower than 550 Da. Although ALAHP was less complex than ALAH, RP-HPLC analysis indicated that ALAHP contained many components of different hydrophobicity and purification of bioactive peptides may require several steps protocol (Fig. 1B).

3.3. Purification of ACE inhibitory peptides

Affinity chromatography was tested as a specific method for purification of ACE inhibitory peptides from ALAHP. Microaffinity column with immobilised ACE was prepared reaching 60% of immobilisation yield (protein basis). Purification of ACE inhibitory peptides from ALAHP was done followed by RP-HPLC-MS/MS. Chromatography with an identical protocol but employing a control column (without immobilised ACE) was used to identify and discard peptides with

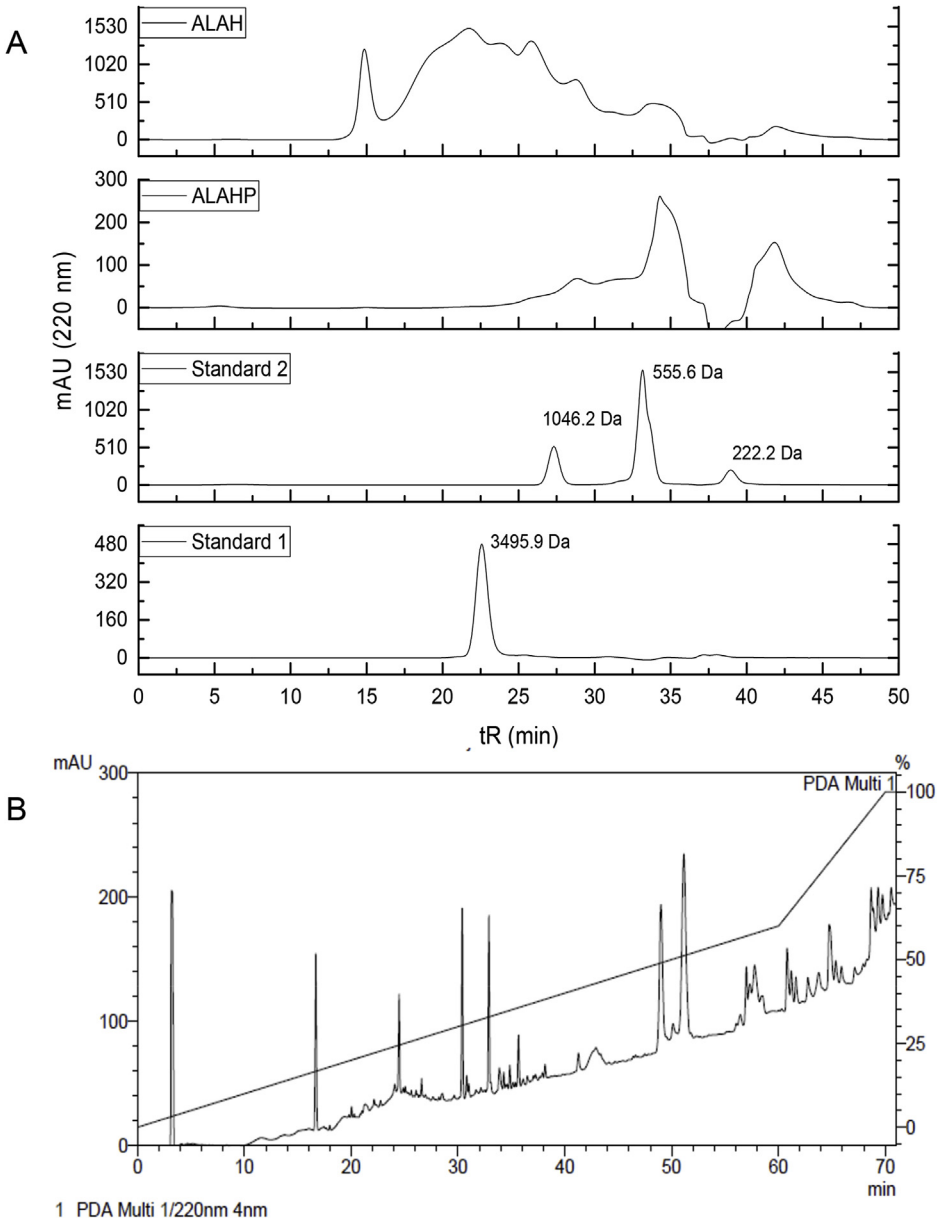

Fig. 1. Analysis of ALAH and ALAHP composition. (A) SEC-HPLC profiles on Superdex Peptide column of ALAH, ALAHP and molecular weight standards; (B) RP-HPLC profile of ALAHP.

Table 1
Peptides identified in eluted fractions of ALAHP by affinity chromatography. Mascot search was performed with a local database including sequence of bovine ALA (UniProtKB-P00711) and with Public Database. Sequences were also identified in fractions eluted from the control column were eliminated from further analysis.

Sequence number	Mr(exp)	Mr(calc)	Delta	Score	ALA Fragment	Peptide sequence	Database
1	912.4132	912.3978	0.0154	57	48–55	C.TTFHTSGY.D	L/P
2	1027.1617	1027.4247	−0.2630	37	48–56	C.TTFHTSGYD.T	L
3	1668.16	1667.77	0.3896	58	48–62	C.TTFHTSGYDTQAIVQ.N	P
4	811.2100	811.3501	−0.1400	41	49–55	T.TFHTSGY.D	L
5	926.7511	926.3770	0.3741	43	49–56	T.TFHTSGYD.T	L
6	825.0261	825.3293	−0.3033	37	50–56	T.FHTSGYD.T	L
7	1319.17	1318.61	0.5625	53	51–62	F.HTSGYDTQAIVQ.N	P
8	1080.83	1080.50	0.3224	58	53–62	T.SGYDTQAIVQ.N	P
9	992.7431	993.4767	−0.7337	53	54–62	S.GYDTQAIVQ.N	L/P

L = local database search; P = public database search.

Table 2
In silico gastrointestinal digestion using Peptide Cutter ExPASy.

Peptides	Enzyme	No of cleavage	Positions of cleavage sites	Fragments released by digestion
TTFHTSGY	Pepsin (pH 1.3)	2	2,3, 8	TT, F, HTSGY
	Pepsin (pH > 2)	4	2, 3, 7, 8	TT, F, HTSG, Y
	Trypsin	0		
	Chymotrypsin	2	3, 8	TTF, HTSGY
HTSGY	Chymotrypsin	2	1, 5	H, TSGY
HTSG	Chymotrypsin	2	1, 4	H, TSG
GYDTQAIVQ	Pepsin (pH 1.3)	0		
	Pepsin (pH > 2)	2	1, 2	G, Y, DTQAIVQ
	Trypsin	0		
	Chymotrypsin	1	2, 9	GY, DTQAIVQ

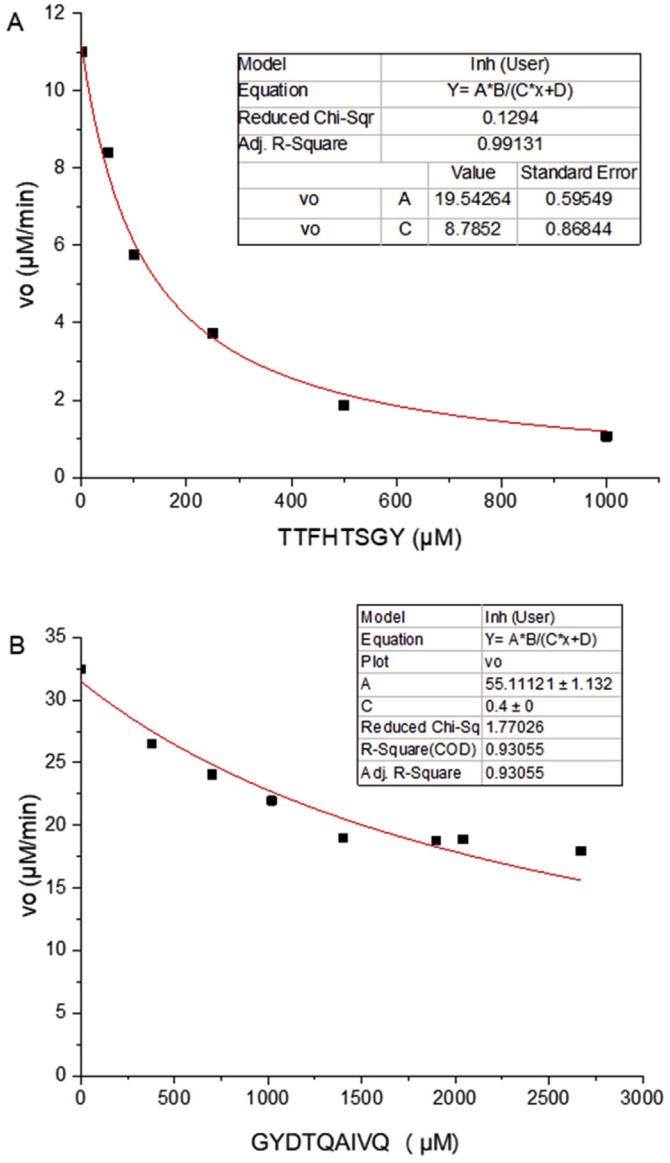

Fig. 2. Initial velocity (vo) versus peptide concentration graphs. (a) TTFHTSGY (0–1 mM); (b) GYDTQAIVQ (0–2.8 mM).

nonspecific interaction with the column resin from further study. Nine peptides with potential ACE inhibitory activity were identified and are listed in Table 1. As far as the authors have been able to establish, these peptides have not been previously reported to be ACE inhibitors. All of these peptides originate from the same region of ALA primary structure

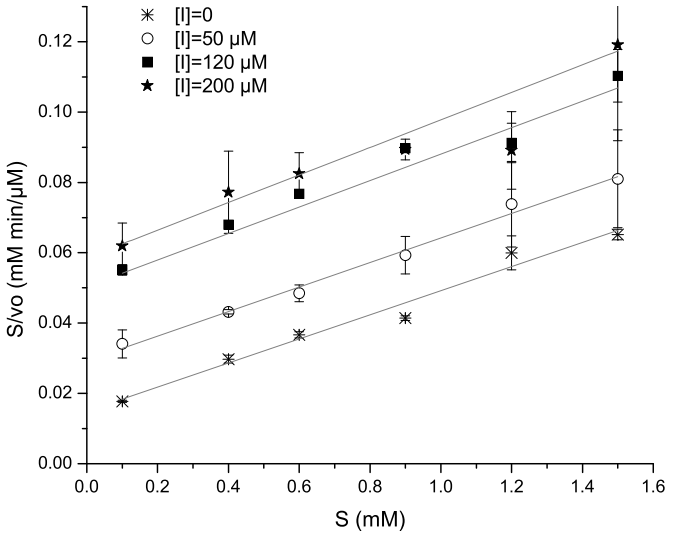

Fig. 3. Hanes-Woolf graphs of ACE (7.0×10^{-7} M), in the absence or presence of TTFHTSGY (0, 50, 120 or 200 µM), with different concentrations of FAPGG (0.5–1.5 mM). All assays were performed by triplicate; vertical bars correspond to SD.

Table 3
Kinetic parameters of ACE catalysed reaction in the absence or presence of inhibitory peptides.

Kinetic parameter	Control	TTFHTSGY		
		50 µM	120 µM	200 µM
V_{Max} (µM/min)	29 ± 2.7	29 ± 1.4	27 ± 3.9	26 ± 8,9
K_M (mM)	0.43 ± 0.12	0.83 ± 0.09	1.34 ± 0.36	1.49 ± 0.83
K_i (µM)		69 ± 5.3		

The results are given as mean ± SD from triplicate determinations.

(sequences number 1 to 9, Table 1).

3.4. Characterization of ACE inhibitory peptides

3.4.1. Prediction of potential toxicity, hydrophobicity and solubility

Potential toxicity of identified peptides was predicted using ToxinPred. *In silico* evaluation predicted that none of the peptides had toxicity (Table S1). Peptide stability, accessed by ProtParam, indicated instability indexes lower than 40 in all cases, which is predicted as stable (Table S1). All peptides showed poor water solubility when evaluated by Innovagen online tool. Hydrophilic character (negative value of GRAVY, Table S1) for all peptides was computed with the ProtParam analysis tool.

Sequences 1 and 9 (Table 1) with the highest ion scores, identified in both local and public database searches, were selected for further evaluation of ACE inhibitory activity.

3.4.2. Dose-response ACE-inhibitory activity

The octapeptide (Thr-Thr-Phe-His-Thr-Ser-Gly-Tyr) and the nonapeptide (Gly-Tyr-Asp-Thr-Gln-Ala-Ile-Val-Gln) were chemically synthesised and purified to 98.97% and 98.03%, respectively. Both synthetic peptides showed dose-dependent ACE-inhibitory activity, TTFHTSGY (IC_{50} = 142 µM) was the most inhibitory followed by GYDTQAIVQ (IC_{50} = 1.0 mM). ACE inhibitory peptides with IC_{50} lower than 1000 µM are considered to be of high inhibitory activity (de Oliveira et al., 2018). According to data from the online Database of Antihypertensive Peptides (AHTPDB) (http://crdd.osdd.net/raghava/ahtpdb) most reported ACE inhibitory peptides have IC50 values in the range of 1–1000 µM.

It has been described that the C-terminal tripeptide is fundamental for ACE inhibitory activity, being hydrophobic residues favourable in any of these three positions (Tavares and Malcata, 2013). The C-terminal dipeptide of TTFHTSGY peptide agrees with previously reported ACE inhibitory peptides IYEGY (IC_{50} = 79.42 μM) (Lassoued et al., 2016), KNGDGY (IC_{50} = 51.63 μM) (Balti et al., 2015) and RYLGY (IC_{50} = 0.71 μM) (Contreras et al., 2009). The IVQ sequence present at the C-terminal of GYDTQAIVQ peptide was previously reported as ACE inhibitor (Wu et al., 2006). The synthetic peptide IVQ has an IC_{50} of 95.5 μM and similar synthetic tripeptide LVQ a lower IC_{50} value (14.13 μM) (Wu et al., 2006).

3.4.3. *In silico* prediction of peptide gastrointestinal stability

In vitro ACE-inhibitory activity does not always correlate with *in vivo* activity, since peptide stability in the gastrointestinal tract may be compromised. In addition, peptide absorption mechanisms and transport to target of action may influence *in vivo* activity. *In silico* gastrointestinal digestion of potential ACE inhibitory peptides was done and results are shown in Table 2. Of the two peptides, neither was predicted to resist the action of pepsin and pancreatic enzymes. TTFHTSGY *in silico* gastrointestinal digestion released ACE inhibitory dipeptides TF and SG (Biopep ID: 8185 and 7618 respectively). TF was shown to have more potent ACE-inhibitory effect (IC_{50} = 18 μM) (Nogata et al., 2009) than original peptide. The same was found for GYDTQAIVQ peptide in that after digestion two known ACE-inhibitory peptides were generated (GY and IVQ). In these case, both GY (IC_{50} = 210 μM) (Cheung et al., 1980) and IVQ (aforementioned in section 3.2) peptides were more efficient ACE inhibitors than original peptide. So, the ACE inhibitory activity of these peptides could be potentiated by *in vivo* gastrointestinal digestion.

3.4.4. Inhibition kinetics

When increasing amounts of TTFHTSGY (0–1.0 mM) peptide were incubated with constant amount of ACE (2.8×10^{-2} M), total inhibition of ACE activity was observed (Fig. 2A). In contrast, GYDTQAIVQ peptide only partially inhibited ACE activity (Fig. 2B) even at a higher concentration (2.6 mM).

Hanes-Woolf graphs of ACE (7.0×10^{-7} M), in the absence or presence of TTFHTSGY at different FAPGG concentrations (0.1–1.5 mM), are shown in Fig. 3. The K_M value of ACE in the absence of inhibitory peptides was 4.3×10^{-1} mM (Table 3), which was slightly higher than previously reported values: 3.5×10^{-1} mM (Holmquist et al., 1979), 3.0×10^{-1} mM (Udenigwe et al., 2009) and 2.9×10^{-1} mM (Hou et al., 2003). These differences could be attributed to assay conditions used that differed in pH and temperature. Results indicated that TTFHTSGY peptide acted as competitive inhibitor, characterised by increase apparent K_M in the presence of inhibitor while having no effect on V_{Max} (Table 3). ACE inhibitory peptides could inhibit ACE via different inhibition mechanisms such as competitive, non-competitive, uncompetitive and mixed type (Aluko, 2015). Results suggest that this competitive inhibitory peptide has affinity for binding to the active site. The inhibition constant (K_i) is a measure of peptide affinity for ACE. According to determined K_i value (Table 3), TTFHTSGY (K_i = 69 ± 5.3 μM) may be considered a classical competitive inhibitor, since Ki value was higher than assay ACE concentration (0.7 μM).

4. Conclusions

Antiacanthain was able to release new ACE inhibitory peptides from ALA. These inhibitors were purified in a single step, using affinity chromatography and were identified by mass spectrometry. Two new sequences with in vitro ACE inhibitory activity were reported. The most potent peptide was TTFHTSGY with IC_{50} of 142 μM and was able to inhibit ACE activity acting as a competitive inhibitor. GYDTQAIVQ peptide acted as a partial inhibitor with lower potency, but *in vivo* activity could be interesting since, by gastrointestinal digestion, two potent ACE inhibitory peptides could be released from its sequence. Further studies are required to evaluate antihypertensive effect of these peptides *in vivo*. Although, *in silico* evaluation of toxicity predicted that TTFHTSGY and GYDTQAIVQ peptides were non-toxic, it is necessary to perform cell cytotoxicity assays to confirm it. The results of this work show the potential of a non-commercial plant enzymatic extract for production of bioactive peptides.

Acknowledgment

The authors thank Ph.D. Rosario Duran (Unidad de Bioquímica y Proteómica Analiticas, Institut Pasteur Montevideo) for mass spectrometry analysis and her valuable technical contribution. We thank Ph.D. Paul Gill for language edition and valuable contributions to the final version of the manuscript. This work was done with support from Comisión Sectorial de Investigación Científica (CSIC, Uruguay) [Grant no. C278-348]. C Villadóniga also received a doctoral fellowship from (Agencia Nacional de Investigación e Innovación ANII), Uruguay [Grant no. POS_NAC_2010_1_2_2438]. A. Cantera is member of Programa de Desarrollo de las Ciencias Básicas (PEDECIBA), Uruguay.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101258.

References

Akif, M., Schwager, S.L., Anthony, C.S., Czarny, B., Beau, F., Dive, V., Sturrock, E.D., Acharya, K.R., 2011. Novel mechanism of inhibition of human angiotensin-I-converting enzyme (ACE) by a highly specific phosphinic tripeptide. Biochem. J. 436, 53–59. https://doi.org/10.1042/BJ20102123.

Aluko, R.E., 2015. Antihypertensive peptides from food proteins. Annu. Rev. Food Sci. Technol. 6, 235–262. https://doi.org/10.1146/annurev-food-022814-015520.

Balti, R., Bougatef, A., Sila, A., Guillochon, D., Dhulster, P., Nedjar-Arroume, N., 2015. Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (Sepia officinalis) muscle protein hydrolysates and antihypertensive effect of the potent active peptide in spontaneously hypertensive rats. Food Chem. 170, 519–525. https://doi.org/10.1016/j.foodchem.2013.03.091.

Bertucci, J.I., Liggieri, C.S., Colombo, M.L., Vairo Cavalli, S.E., Bruno, M.A., 2015. Application of peptidases from Maclura pomifera fruit for the production of active biopeptides from whey protein. LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 64, 157–163. https://doi.org/10.1016/j.lwt.2015.05.041.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Buttery, J.E., Stuart, S., 1993. Assessment and optimization of kinetic methods for angiotensin-converting enzyme in plasma. Clin. Chem. 39, 312–316.

Cheung, H.-S., Wang, F.-L., Ondetti, M.A., Sabo, E.F., Cushman, D.W., 1980. Binding of peptide substrates and inhibitors of angiotensin-converting enzyme importance of the COOH-terminal Dipeptide sequence*. J. Biol. Chem. 255, 401–405.

Cimino, C.V., Colombo, M.L., Liggieri, C.S., Bruno, M.A., Vairo-Cavalli, S.E., 2015. Partial molecular characterization of Arctium minus aspartylendopeptidase and preparation of bioactive peptides by whey protein hydrolysis. J. Med. Food 18, 856–864. https://doi.org/10.1089/jmf.2014.0101.

Contreras, M. del M., Carrón, R., Montero, M.J., Ramos, M., Recio, I., 2009. Novel casein-derived peptides with antihypertensive activity. Int. Dairy J. 19, 566–573. https://doi.org/10.1016/j.idairyj.2009.05.004.

Cushman, D.W., Cheung, H.S., 1971. Spectophotometric assay and properties of the angiotensin I-converting enzyme of rabbit lung. Biochem. Pharmacol. 20, 1637–1648. https://doi.org/10.1016/0006-2952(71)90292-9.

de Oliveira, M.R., Silva, T.J., Barros, E., Guimarães, V.M., Baracat-Pereira, M.C., Eller, M.R., dos Reis Coimbra, J.S., de Oliveira, E.B., 2018. Anti-Hypertensive peptides derived from caseins: mechanism of physiological action, production bioprocesses, and challenges for food applications. Appl. Biochem. Biotechnol. 185, 884–908. https://doi.org/10.1007/s12010-018-2692-8.

Fullana, N., Braña, V., José Marizcurrena, J., Morales, D., Betton, J.-M., Marín, M., Castro-Sowinski, S., 2017. Identification, recombinant production and partial biochemical characterization of an extracellular cold-active serine-metalloprotease from an Antarctic Pseudomonas isolate. AIMS Bioeng. 4, 386–401. https://doi.org/10.3934/bioeng.2017.3.386.

Gasteiger, E., Hoogland, C., Gattiker, A., Duvaud, S., Wilkins, M.R., Appel, R.D., Bairoch, A., 2005. Protein identification and analysis tools on the ExPASy server. In: The Proteomics Protocols Handbook. Humana Press, Totowa, NJ, pp. 571–607. https://doi.org/10.1385/1-59259-890-0:571.

Gobbetti, M., Stepaniak, L., De Angelis, M., Corsetti, A., Di Cagno, R., 2002. Latent

bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. Crit. Rev. Food Sci. Nutr. 42, 223–239. https://doi.org/10.1080/10408690290825538.

Gupta, S., Kapoor, P., Chaudhary, K., Gautam, A., Kumar, R., Raghava, G.P.S., 2013. In silico approach for predicting toxicity of peptides and proteins. PLoS One 8, e73957. https://doi.org/10.1371/journal.pone.0073957.

Hernández-Ledesma, B., Ramos, M., Recio, I., Amigo, L., 2006. Effect of β-lactoglobulin hydrolysis with thermolysin under denaturing temperatures on the release of bioactive peptides. J. Chromatogr. A 1116, 31–37. https://doi.org/10.1016/j.chroma.2006.03.006.

Holmquist, B., Bünning, P., Riordan, J.F., 1979. A continuous spectrophotometric assay for angiotensin converting enzyme. Anal. Biochem. 95, 540–548. https://doi.org/10.1016/0003-2697(79)90769-3.

Hou, W.-C., Chen, H.-J., Lin, Y.-H., 2003. Antioxidant peptides with angiotensin converting enzyme inhibitory activities and applications for angiotensin converting enzyme purification. J. Agric. Food Chem. 51, 1706–1709. https://doi.org/10.1021/jf0260242.

Iwaniak, A., Minkiewicz, P., Darewicz, M., 2014. Food-originating ACE inhibitors, including antihypertensive peptides, as preventive food components in blood pressure reduction. Compr. Rev. Food Sci. Food Saf. 13, 114–134. https://doi.org/10.1111/1541-4337.12051.

Krumreich, F.D., Corrêa, A.P.A., Silva, S.D. da, Zambiazi, R.C., 2015. Composição físico-química e de compostos bioativos em FRUTOS de Bromelia antiacantha Bertol. Rev. Bras. Frutic. 37, 450–456. https://doi.org/10.1590/0100-2945-127/14.

Lassoued, I., Mora, L., Barkia, A., Aristoy, M.C., Nasri, M., Toldrá, F., 2016. Angiotensin I-converting enzyme inhibitory peptides FQPSF and LKYPI identified in Bacillus subtilis A26 hydrolysate of thornback ray muscle. Int. J. Food Sci. Technol. 51, 1604–1609. https://doi.org/10.1111/ijfs.13130.

Manetti, L.M., Turra, A.F., Takemura, O.S., Svidzinski, T.I.E., Laverde, A., 2010. Avaliação das atividades antimicrobiana, citotóxica, moluscicida e antioxidante de Bromelia antiacantha Bertol. (Bromeliaceae). Rev. Bras. Plantas Med. 12, 406–413.

Nogata, Y., Nagamine, T., Yanaka, M., Ohta, H., 2009. Angiotensin I converting enzyme inhibitory peptides produced by autolysis reactions from wheat bran. J. Agric. Food Chem. 57, 6618–6622. https://doi.org/10.1021/jf900857w.

Power, O., Fernández, A., Norris, R., Riera, F.A., FitzGerald, R.J., 2014. Selective enrichment of bioactive properties during ultrafiltration of a tryptic digest of â-lactoglobulin. J. Funct. Foods 9, 38–47. https://doi.org/10.1016/j.jff.2014.04.002.

Santos, V.N.C., De Freitas, R.A., Deschamps, F.C., Biavatti, M.W., 2009. Ripe fruits of Bromelia antiacantha: investigations on the chemical and bioactivity profile. Rev. Bras. Farmacogn. Brazilian J. Pharmacogn. Abr 19, 358–365.

Silva, M.R., Silvestre, M.P.C., Silva, V.D.M., Souza, M.W.S., Lopes Junior, C.O., Afonso, W.O., Lana, F.C., Rodrigues, D.F., 2014. Production of ace-inhibitory whey protein concentrate hydrolysates: use of pancreatin and papain. Int. J. Food Prop. 17, 1002–1012. https://doi.org/10.1080/10942912.2012.685821.

Tavares, T., Malcata, F.X., 2013. Whey proteins as source of bioactive peptides against hypertension. In: Hernández-Ledesma, B., Chia-Chien, H. (Eds.), Bioactive Food Peptides in Health and Disease. InTech Open, Rijeka, pp. 75–114. https://doi.org/10.5772/52680.

Udenigwe, C.C., Lin, Y.S., Hou, W.C., Aluko, R.E., 2009. Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. J. Funct. Foods 1, 199–207. https://doi.org/10.1016/j.jff.2009.01.009.

Vallés, D., Cantera, A.M.B., 2018. Antiacanthain A: new proteases isolated from Bromelia antiacantha Bertol. (Bromeliaceae). Int. J. Biol. Macromol. 113, 916–923. https://doi.org/10.1016/j.ijbiomac.2018.03.025.

Vallés, D., Furtado, S., Cantera, A.M.B., 2007. Characterization of news proteolytic enzymes from ripe fruits of Bromelia antiacantha Bertol. (Bromeliaceae). Enzym. Microb. Technol. 40, 409–413. https://doi.org/10.1016/j.enzmictec.2006.07.011.

Villadóniga, C., Macció, L., Cantera, A.M.B., 2018. Acid whey proteolysis to produce angiotensin-I converting enzyme inhibitory hydrolyzate. Environ. Sustain. 1, 267–278. https://doi.org/10.1007/s42398-018-0027-x.

WHO, 2018. Noncommunicable diseases. [WWW Document]. URL. http://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases accessed 11.6.18.

Wu, J., Aluko, R.E., Nakai, S., 2006. Structural requirements of angiotensin I-converting enzyme inhibitory Peptides : quantitative structure − activity relationship study of di- and tripeptides. J. Agric. Food Chem. 54, 732–738. https://doi.org/10.1021/jf051263l.