Peptide Inhibitors of Angiotensin-I Converting Enzyme (ACE) Produced From Legumes Subjected to Hydrothermal Treatment

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ABSTRACT: The objective of this study was to evaluate the effect of Angiotensin-I Converting Enzyme (ACE) inhibitory peptides derived from five legumes (*Vigna unguiculata, Cicer arietinum, Lens culinaris, Vicia faba,* and *Phaseolus vulgaris*) after undergoing a hydrothermal treatment. The seeds were divided into two groups: one was subjected to drying and grinding, and the other one to cooking for 2 hours (100 °C), followed by drying and grinding. The flours obtained from the different processes were subjected to digestion with pepsin-pancreatin, and the resulting peptides were evaluated for their ACE inhibitory activity. The obtained results were subjected to analysis of variance and Tukey's test. The group consisting of the treatments *V. unguiculata* (82.63%), *V. unguiculata* cooked (96.41%), and *C. arietinum* (80.84%) showed the highest percentages of ACE inhibition, while the group comprising *V. faba* (6.0%), cooked *L. culinaris* (3.03%), and *P. vulgaris* (4.0%) exhibited the lowest inhibition percentages. These results demonstrate that *V. unguiculata, V. faba*, and *C. arietinum* can undergo hydrothermal treatment while still retaining their bioactive potential, ensuring a cooked flour, ready for formulating a multifunctional food.

Keywords: ACE inhibition, *Cicer arietinum, Lens culinaris, Phaseolus vulgaris, Vigna unguiculata, Vicia faba*

INTRODUCTION

According to the Pan American Health Organization, systemic arterial hypertension (SAH) is the leading cause of death associated with cardiovascular problems. It has been reported that in the Americas, hypertension affects approximately 250 million people, representing 20-40% of the adult population in the region. Annually, about 1.6 million people die from cardiovascular events, with approximately half a million being under 70 years old (PAHO, 2023).

Physiologically, the renin-angiotensin-aldosterone system (RAAS) is responsible for maintaining the homeostasis of the internal environment and blood pressure (Mauer *et al.,* 2001). The increase in blood pressure occurs in a proteolytic cascade-connected to a signaling transduction system, in which renin is secreted and stored in the juxtaglomerular apparatus located in the afferent arteriole of the renal glomerulus (Mauer *et al.,* 2001). Renin catalyzes angiotensinogen present in the blood circulation (synthesized in the liver) into angiotensin I (inactive decapeptide) which, when passing through the lungs, is catalyzed by angiotensin-converting enzyme (ACE) into angiotensin II (biologically active octapeptide), this compound has different functions, such as a vasoconstrictor, stimulates the secretion of ADH, Aldosterone and the activity of the sympathetic nervous system (Matoba *et al.,* 2001; Liu *et al.,* 2018). ACE (EC 3.4.15.1), also known as convertase or kinase II, is a metalloprotease that cleaves in the RAAS system. Its major activity occurs at the luminal endothelial edge of the pulmonary vascular bed. Three isoforms have been described: somatic ACE, testicular or germinal ACE, and plasma ACE (Yu *et al.,* 2017).

Currently, research in the nutraceutical field is focused on finding functional foods with antihypertensive properties. Proteins in foods not only serve as a source of essential amino acids for metabolism but can also generate low-molecular-weight peptides with beneficial bioactive functions for health (Daliri *et al.,* 2017). These peptides can act as antioxidants, antimicrobials, antithrombotics, antihypertensives, and immunomodulators, among others (Chew *et al.,* 2019). The potential of certain foods to reduce blood pressure has been described in numerous studies. Research indicates that foods from various sources, both plant and animal, can generate ACE inhibitory peptides during digestion (Kaur *et al.,* 2021;

Xue *et al.,* 2021). Studies on legume seeds, such as those by Torruco-Uco (2002) with *Phaseolus lunatus* and *Phaseolus vulgaris*, Tagliazucchi *et al.* (2015) with *Phaseolus vulgaris*, and Cú-Cañetas (2015) with *Vigna unguiculata*, have demonstrated the presence of peptides with ACE inhibitory activity in the hydrolysates of these seeds.

To be suitable for consumption, beans must undergo a cooking process. This hydrothermal process affects the three-dimensional structures of the proteins present in the grain, which improves the access of digestive enzymes, favoring their hydrolysis and the production of low molecular weight bioactive peptides. This statement is supported by studies such as the one carried out by Pertiwi (2020), who found that the fermentation and boiling process of beans (*Phaseolus lunatus* L.) improved the release of ACE-inhibiting peptides. Likewise, Sangsukiam and Duangmal (2022) reported similar results for *Vigna angularis*.

This demonstrates that foods are a potential alternative for modulating ACE activity. However, it is essential to consider that in the case of seeds, many of them contain antinutritional compounds such as trypsin and chymotrypsin inhibitors (Muñoz-Llandes *et al.,* 2021), α-amylase, α-glucosidase, pancreatic lipase, vicine, and convicine (Rizzello *et al*., 2016), which are harmful to the body. The usual way to inactivate these negative molecules is through thermal or hydrothermal processes. There is evidence in the literature that thermal treatment applied to legumes enhances protein availability and eliminates antinutritional factors present in the seed (Filipiak-Florkiewicz *et al*., 2014; Vashishth *et al.,* 2021).

This research aimed to evaluate the effect of hydrothermal treatment (cooking) of various legumes on ACE activity to determine whether the cooking treatment affects the availability of legume peptides and limits their potential as antihypertensive agents.

MATERIALS AND METHOD

Biological materials

Seeds origin: Five seeds, namely *Phaseolus vulgaris* (black variety), *Cicer arietinum*, *Vigna unguiculata* L. Walp, *Lens culinaris*, and *Vicia faba*, were used as sources for obtaining bioactive peptides of plant origin. All were acquired from a local market in Maracay, Venezuela.

Animal origin: The enzyme (ACE) was extracted from the lung of rabbit *Oryctolagus cuniculus*, obtained from a supermarket in Maracay, Venezuela.

Analytical grade chemicals: Analytical grade chemical compounds produced by commercial companies were used for biological activity testing. Hipuril-L-histidyl-L-leucine (HHL) Sigma-Aldrich (207386-83-2) was used as the ACE substrate, Captopril Sigma-Aldrich (62571-86-2) was employed as a control drug, and Porcine origin pepsin Sigma-Aldrich (9001-75-6) and porcine origin pancreatin Sigma-Aldrich (8049-47-6) were used for enzymatic protein digestion.

Extraction of ACE extract

For the extraction of ACE enzymatic extract, the method described by Makoto *et al.,* (1978) with modifications was utilized. Rabbit lung tissue was washed with 10 mM potassium phosphate buffer at pH 8.3 (cold), cut into pieces, and homogenized using a blade disruptor (blender). The homogenized mixture was centrifuged at 5000 g for 10 minutes in a centrifuge (Becton Dickinson, MD 21152), and the supernatant was dialyzed (1000 kDa cut-off membrane) for 24 hours using the same buffer at 4 °C. The obtained dialysate was used as the source of ACE for the assays.

Preparation of flours

Flours from Raw Legumes: The seeds of all legumes were cleaned separately to remove all impurities. To obtain flour from raw seeds, the seeds were directly dried in an oven at 60 °C until a constant weight was reached, and then they were ground using an electric seed pulverizer mill (ALIXI, 45Y3G5HX) to obtain uniformly sized particles. This flour was stored at 4 °C for later use.

Flours from cooked: The seeds were hydrated with water in a 1:1 volume ratio for 12 hours. Subsequently, they were subjected to hydrothermal treatment at 100 °C for 2 hours (conditions in which these beans are cooked in Venezuela). After this time, the water was removed, and the seeds were dehydrated in an oven at 60 °C until a constant weight was reached. Then, they were ground using an electric seed pulverizer mill (ALIXI, 45Y3G5HX) to obtain particles of uniform size. This flour was stored at 4 °C for later use.

Protein hydrolysate preparation

The prepared flours were subjected to the sequential pepsin-pancreatin hydrolysis method described by Li *et al.*, (2005) with modifications. An amount of the flour was subjected to enzymatic hydrolysis at acidic pH with

a solution of pepsin (0.2 mg/mL) and 0.1 N HCl to reach a pH = 2. The mixture was incubated at 37 °C for 2 hours in an orbital shaking incubator (Jisico, J-NS10). After the time elapsed, the mixture was neutralized with a 1M NaOH solution to reach a pH = 8. Then, pancreatin (0.5 mg/mL) was added and incubated at 37 °C for 2 hours in an incubator with orbital shaking. The hydrolysis was stopped by inactivating the protease through heating at 85 °C for 20 minutes. The hydrolysates were centrifuged at 6000 x g for 45 minutes at 4 °C (BECKMAN J6M), and the supernatants were frozen at -4 °C until analysis.

Protein concentration determination

The protein concentrations of the protein hydrolysates obtained from pepsin-pancreatin digestion were quantified using the Hartree-Lowry method. A volume of the supernatant was combined with an equal volume of reagent A (0.5 N sodium hydroxide, 10% anhydrous sodium carbonate, 0.2% sodium potassium tartrate), and it was incubated at 50 °C for 10 minutes. Reagent B (0.1 N sodium hydroxide, 1% copper sulfate pentahydrate, 2% sodium potassium tartrate) was then added. The reaction was stopped by adding reagent C (Folin-Ciocalteau phenol reagent: water 2:15 v/v). Absorbances were determined using a spectrophotometer (Beckman DU) at 650 nm (Flores & Ruiz, 2017).

Determination of ACE inhibitory activity

The methodology proposed by Cú-Cañetas *et al.,* (2015) with modifications was employed to determine ACE inhibitory activity. The ACE extract, a volume of HHL, and a volume of the digestion supernatant were mixed at a concentration of 1mg/mL, and they were then incubated at 37 °C for 30 minutes. The reaction was stopped by adding 2N HCl, resulting in L-leucine-L-histidine and hippuric acid as products. The concentration of hippuric acid was determined using the methodology of Brizuela and Jiménez (2010) through visible absorption spectrophotometry, Method 8300 (NIOSH, 1994).

To obtain the percentage inhibition of ACE for each sample, the following formula, as established by Asoodeh (2016), was used:

$$\%ACE\ Inhibition = \left(\frac{CAH\ Control - CAH\ Inhibitor}{CAH\ Control} \right) \times 100\%$$

Where:

CAH: The concentration of hippuric acid that was quantified in each assay.

Control test with commercial ACE inhibitor (Captopril): To determine if the analysis system proposed in this study

was reliable, the inhibitory effect of a commercial ACE inhibitor, captopril, was determined using its IC_{50} concentration (0.091 µg/mL).

Statistical analysis

The hippuric acid concentration values were grouped and subjected to mean and standard deviation tests. A trial with 10 treatments and three repetitions was conducted under a completely randomized design. The treatments consisted of both raw and cooked forms in each case: *Phaseolus vulgaris* (black variety), *Cicer arietinum*, *Vigna unguiculata* L. Walp, *Lens culinaris*, and *Vicia faba*, to measure the percentage of ACE inhibition. Compliance with homogeneity of variances was verified using the Bartlett test, and the normality of residuals was verified using the Ryan-Joyner test. One-way analysis of variance (ANOVA) was applied to verify differences between the treatments. Additionally, mean comparisons were made using Tukey's honestly significant difference multiple comparison test. The significance level was 5%, so a result was considered statistically significant if $p \leq 0.05$. The data were processed using the Minitab 18.0 statistical software for Windows.

To facilitate the comprehension of the methodology employed in this study, refer to Figure 1.
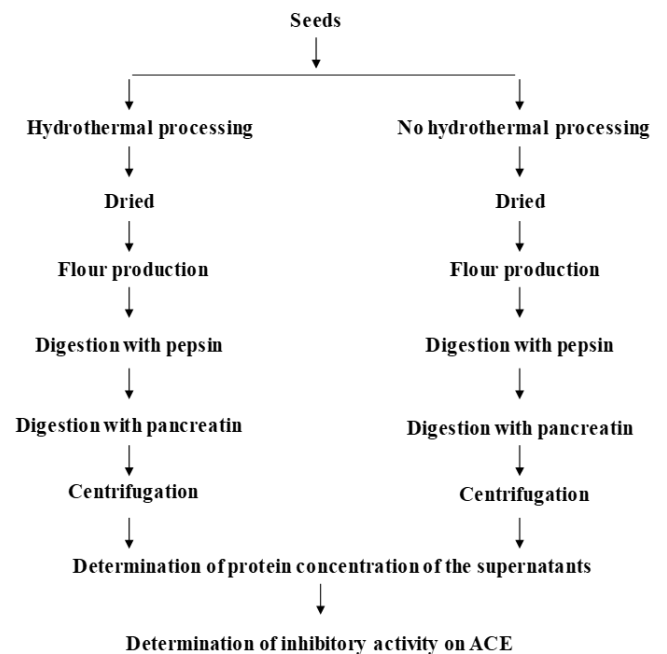

Figure 1. Diagram of the methodology applied in the study

RESULT

In view of the fact that most of the articles that use HHL as a substrate for ECA evaluate the hippuric acid
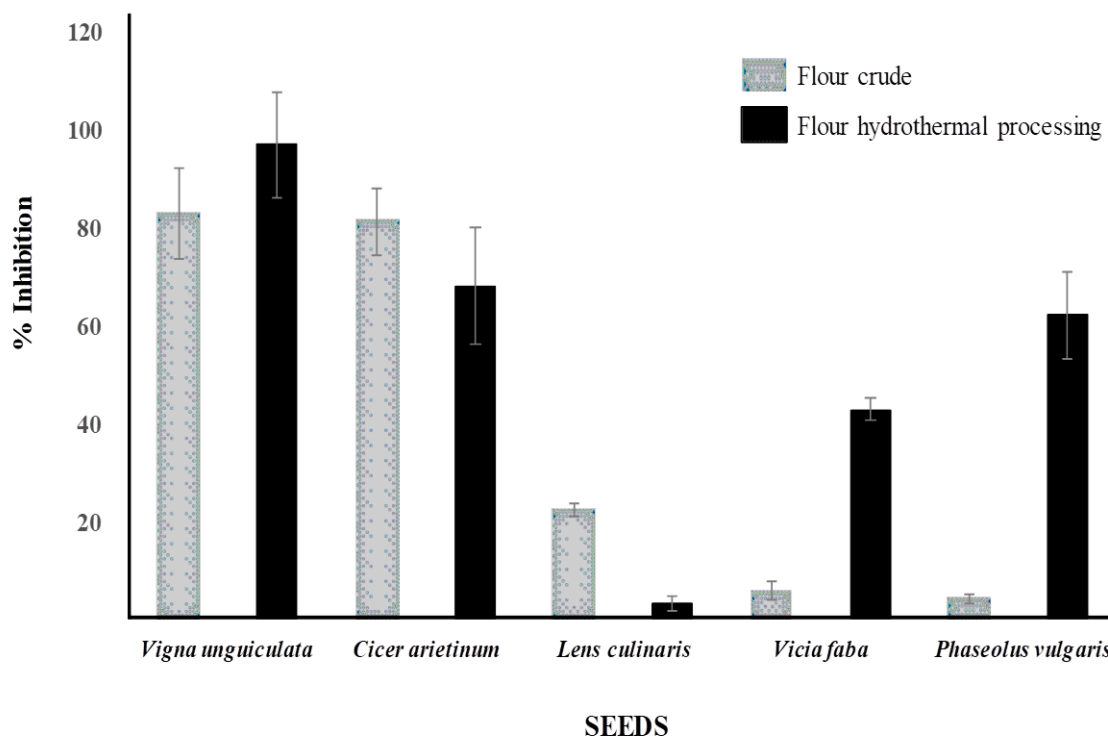
Figure 2. Inhibitory Effect of Bioactive Peptides on ACE Activity of Evaluated Seeds with and without Hydrothermal Treatment.

concentration (product of the reaction) by HPLC, it was proposed in this study to determine this metabolite by a colorimetric method (simple and economical). The first thing that was done was to validate the proposed methodology using an ACE inhibitor of synthetic origin. The inhibitory effect of a known concentration of captopril (0.091 µg/mL), corresponding to its IC_{50}, was determined. The results obtained from this test showed an inhibition of 42 ± 4%, which could be considered an acceptable result and validates the methodology used in the study.

Once the methodology was standardized to quantify ACE activity, we determined the inhibitory effect of the peptides generated by each seed after the different applied treatments. The values were grouped for descriptive statistics to determine the mean and standard deviation. The results are presented in the following figure.

As seen in Figure 2., both *V. unguiculata* and *C. arietinum* showed few representative changes after hydrothermal processing, while *V. faba* and *P. vulgaris* showed an increase in inhibitory activity, implying that in these species, temperature favored the production of bioactive peptides. In the case of *L. culinaris*, the low activity observed for the raw flour was significantly reduced after processing, suggesting that its proteins are more thermolabile than those of the other seeds.

The obtained data were subjected to analysis of variance (ANOVA) to determine if there were significant differences in the inhibitory effect among the different substrates studied. The Levene test for homogeneity of variances indicated that the variances of the treatments were statistically equal (B = 4.77; p = 0.989), and the Ryan-Joyner normality test indicated that the residuals were normally distributed (RJ = 0.981; p > 0.100), allowing for the application of one-way ANOVA.

The one-way ANOVA indicated statistically significant differences for the ACE inhibition percentage among the considered treatments (F = 16.13; p < 0.001), demonstrating that the inhibition percentage depends on these treatments. In this regard, the Tukey multiple comparisons test established 5 groups of homogeneous means, which can be seen in Table 1. Group A, comprised of peptides obtained from *V. unguiculata* flour, cooked *V. unguiculata*, and *C. arietinum*, exhibited the highest ACE inhibition percentages, with the highest inhibition percentages observed in peptides from cooked *V. unguiculata* (96.41%) and *C. arietinum* (80.84%). A second transitional group, AB, included peptides from cooked *C. arietinum* and *P. vulgaris flours*. A third transitional group, ABC, consisted of peptides from cooked *V. faba* flours. The fourth group, BC, included peptides from cooked *L. culinaris* flour. Finally, Group C

exhibited the lowest inhibition percentages and comprised peptides obtained from *V. faba*, cooked L. *culinaris*, and *P. vulgaris* flours.

Table 1. Homogeneous mean groups according to the Tukey multiple comparisons test.

Treatment	Inhibition mean ± SD	Agrupation
V. ungiculata	82.63 ± 9.32	A
V. ungiculata cooked	96.41 ± 10.16	A
C. arietinum	80.84 ± 6.78	A
C. arietinum cooked	67.66 ± 11.86	A B
L. culinaris	22.10 ± 18.60	B C
L. culinaris cooked	3.03 ± 1.7	C
V. faba	6.0 ± 2.1	C
V. faba cooked	42.51 ± 2.32	A B C
P. vulgaris	4.0 ± 1.06	C
P. vulgaris cooked	61.70 ± 8.60	A B

Note: Means with the same letter do not present statistically significant differences at a 5% level.

DISCUSSION

The inhibitory effect observed for *V. unguiculata*, both raw (82.63%) and cooked (96.41%), differ from those reported by Segura (2010), who observed an inhibition percentage of 50% using a concentration range between 44.7 and 112 μg/mL using the pepsin-pancreatin system. However, our results are very similar to those observed by Cú-Cañetas *et al.* (2015), who reported a 50% inhibition at a concentration of 402.23 μg/mL, which is almost half of the concentration used in this study (1000 μg/mL). This difference observed between the literature and the results of this study may be associated with the presence of greater numbers of bioactive peptides per micrograms of protein. The thermal effect on the production of bioactive peptides was studied by Drago *et al.* (2016), who included *V. unguiculata* protein hydrolysates in a pasta product and determined the residual activity after different thermal treatments. The researchers demonstrated that the pasta maintained its ACE inhibitory capacity after cooking. All these findings align with the results obtained in this study.

In the case of *C. arietinum*, the slight loss of activity after the cooking process may be associated with partial inactivation of the proteins present in *C. arietinum*. These results resemble the findings of Hidalgo *et al.* (2018), who demonstrated that cooking affects the protein content of chickpeas. Regarding the inhibition percentage, our results are similar to those obtained by Chávez-Ontiveros

et al. (2022), who evaluated the effect of extrusion on protein hydrolysates from this seed subjected to digestion in an enzymatic system (α-amylase-pepsin-pancreatin), finding 50% inhibition at a concentration of 0.4 mg/mL (close to half of the concentration used in the study, 1 mg/mL), for both the hydrolysates from extrusion and the group that did not receive it, demonstrating that thermal treatment has a minor effect on the production of bioactive peptides.

One of the seeds most affected by hydrothermal treatment was *Lens culinaris*. The loss of activity after the cooking process may be associated with complete denaturation of the bioactive peptides or a loss in the supernatant after cooking. The crude extract's inhibitory effect was lower than the findings of García-Mora *et al.* (2017), who found high inhibition values for peptides from this legume digested by Savinase®. This inhibitory performance for *L. culinaris* peptides was further improved when the hydrolysate underwent cross-linking or sonication, as demonstrated in the study by Rezvankah (2023). Regarding *V. faba*, it can be observed that hydrothermal treatment improved the availability of bioactive peptides, resulting in an increase in ACE activity inhibition. However, despite the improvement in inhibitory capacity, it was lower than the other seeds. The result for raw *V. faba* is similar to that described by Hernández-Rodríguez *et al.* (2020), who found 50% inhibition at concentrations between 7.48 ± 1.15 mg/mL and 12.58 ± 0.99 mg/mL for seeds subjected to fermentation with *A. niger* and enzymatic hydrolysis (Flavourzyme), respectively. Felix *et al.* (2019) demonstrated that ACE inhibition capacity improved after hydrolytic treatment mediated by the

pepsin-corolase PP system. Consequently, the cooking treatment undoubtedly favored the functionality of the peptides.

P. vulgaris (black variety) showed a significant increase in inhibitory activity after the cooking treatment. These results differ from those described by Mojica *et al.* (2014), who evaluated the effect of pre-cooking on the production of bioactive peptides from various types of *Phaseolus* beans (including the black variety) and found that thermal processing did not affect the bioactive properties of the released peptides. This difference could be associated with the cooking time, which in the present research was longer than that of Mojica *et al.* (2014), who only used between 10 and 15 minutes at 100 °C. Tagliazucchi *et al.* (2015) observed the effect of hydrothermal treatment on *P. vulgaris* beans (pinto variety) regarding ACE inhibitory activity, finding high inhibition at 105.6 ± 2.1 μg/mL. Unfortunately, this study had the weakness of not comparing the activity before and after cooking, so it cannot be said that the inhibitory capacity was affected.

Although there is a wealth of scientific literature on the effect of cooking on legumes, specifically the *Phaseolus* genus, few studies evaluate the effect of this process on the production of ACE inhibitory peptides. Consequently, the results of this research constitute an important finding and could serve as a basis for future *in vivo* investigations that confirm that these flours are safe and maintain their nutraceutical potential, which could be the basis for future functional food formulations.

CONCLUSION

The hydrothermal treatment did not significantly affect the ACE inhibitory activity of the peptides produced by *Vigna unguiculata* L. Walp and *Cicer arietinum*. In the case of *Vicia faba* and *Phaseolus vulgaris*, cooking favored an increase in the presence of peptides with inhibitory activity, while the peptides of *Lens culinaris* lost the activity, as mentioned earlier, after processing. These results are important for the food industry since most of the anti-nutritional factors present in the seeds evaluated are inactivated by temperature. This would allow the production of bioactive peptides devoid of many molecules harmful to health.

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