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Antihypertensive, ACE-inhibitory and vasodilator properties of an egg white hydrolysate: Effect of a simulated intestinal digestion

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Abstract

In previous studies we showed that the egg white hydrolysate with pepsin (HEW) and its fraction with molecular mass lower than 3000 Da (HEW < 3000) have ACE-inhibitory activity *in vitro* and exert antihypertensive effects after single-oral and long-term administrations to spontaneously hypertensive rats (SHR). In this paper we have simulated an intestinal digestion on HEW and HEW < 3000 and addressed its effect on their ACE-inhibitory and vasodilator activities *in vitro* and antihypertensive activity *in vivo*. The results showed that the ACE-inhibitory activity of HEW and HEW < 3000 was maintained after the simulated intestinal digestion. HEW also exerted vasodilator activity in isolated aortic rings before and after the digestion. Both activities may explain the antihypertensive effects of these products in SHR. The peptides RADHP and YPI, which were detected by RP–HPLC–MS in the hydrolysates after the action of the pancreatic enzymes, could be responsible for the antihypertensive effects. In conclusion, HEW or HEW < 3000 could be useful in the prevention and/or treatment of hypertension and other associated disorders.

Keywords: Egg white hydrolysate; Intestinal digestion; Antihypertensive activity; ACE-inhibition; Vasodilator effects

1. Introduction

Cardiovascular diseases represent the first cause of morbidity and mortality in Western countries, with hypertension affecting about 20% of the world's adult population. Lifestyle modifications and diet therapy are two of the most important tools in the prevention and treatment of hypertension (Hermansen, 2000). In this context, milk protein hydrolysates containing bioactive peptides were proved to reduce significantly the blood pressure of moderately hypertensive subjects (López-Fandiño, Otte, & van Camp, 2006).

The most common mechanism underlying the blood pressure-lowering effect of food peptides seems to be the inhibition of the activity of angiotensin-I-converting enzyme (ACE) and thus, the search for ACE inhibitory activity *in vitro* is a widespread strategy in the selection of antihypertensive hydrolysates and peptides (Li, Le, Shi, & Shrestha, 2004). The *in vivo* effects are usually tested in spontaneously hypertensive rats (SHR) which constitute an accepted model for human essential hypertension (Fitz-Gerald, Murray, & Walsh, 2004). In general terms, the results of those tests have highlighted an important lack of correlation between the *in vitro* ACE inhibitory activity and the *in vivo* action, because the former does not take into consideration the physiological transformations that determine the bioavailability of the peptides and because there might be other mechanisms of action than ACE

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inhibition (Vermeirssen, Van Camp, & Verstraete, 2004). In this respect, despite endothelial dysfunction being an important factor in the pathogenesis of atherosclerosis, hypertension, and heart failure (Ross, 1999), there are only very few studies dealing with the effects of peptides or hydrolysates derived from food proteins on endothelial function (Fujita, Usui, Kurahashi, & Yoshikawa, 1995; Matoba, Usui, Fujita, & Yoshikawa, 1999).

In previous reports we showed that a hydrolysate of egg white with pepsin (HEW) and its fraction with molecular mass lower than 3000 Da (HEW < 3000) had ACE-inhibitory activity *in vitro* and exerted antihypertensive effects after single oral administrations and long-term administrations to SHR (Miguel, López-Fandiño, Ramos, & Aleixandre, 2005, 2006; Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004). Furthermore, several peptide sequences with ACE-inhibitory activity and blood pressure lowering effects in SHR were identified in the hydrolysates (Miguel et al., 2004).

These results suggest that HEW or HEW < 3000 could be used as functional food ingredients with potential therapeutic benefits in the treatment of hypertension. In an attempt to go deeper into the factors that determine the antihypertensive activity of these products, we have addressed the effect of a simulated intestinal digestion on the *in vitro* ACE-inhibitory activity and *in vivo* antihypertensive activity of HEW or HEW < 3000. We also tried to clarify whether another mechanism, different from ACE inhibition, could be involved in their antihypertensive action.

2. Materials and methods

2.1. Preparation of the hydrolysate of egg white proteins

Crude egg white was obtained in our laboratory from hen fresh eggs, dissolved in Milli-Q water at a concentration of 100 mg/ml, and adjusted to pH 2.0 with 1 M HCl. Hydrolysis with pepsin (EC 3.4.23.1 type A, 10,000 U/mg from pork stomach obtained from Sigma Chemical Co., St. Louis, MO.; E/S 1/100, wt/wt) was carried out at 37 °C for 3 h. Inactivation of pepsin was achieved by increasing the pH to 7.0 with 1 M NaOH. The hydrolysate was then centrifuged at 4500*g* for 15 min (HEW) and ultrafiltered through a hydrophilic 3000 Da cut-off membrane (Centriprep, Amicon Inc, Beverly, MA, USA) by centrifugation at 1900*g* for 40 min to yield HEW < 3000 (Miguel et al., 2004).

2.2. Simulated intestinal digestion

The usual procedure to mimic the *in vivo* digestion process consists of a two-stage hydrolysis process that simulates the passage through the stomach, using pepsin at pH 1.5–2.0 for 0.5–3 h, followed by hydrolysis by pancreatic enzymes at the pH of the small intestine (Alting, Meijer, & Van Beresteijn, 1997). Since, in the production of HEW and HEW < 3000, egg white proteins had already been treated with pepsin under conditions similar to the physiological situation in the stomach (please see above), we directly simulated intestinal digestion using Corolase PP®, a proteolytic enzyme preparation from the pig pancreatic gland, that in addition to trypsin and chymotrypsin, contains numerous amino and carboxypeptidase activities. Hydrolysis of HEW and HEW < 3000 with Corolase PP® (Röhm, Darmstadt, Germany) was conducted at an enzyme substrate ratio of 1:50 w/w at pH 7.0–8.0 and 37 °C for 240 min, under continuous stirring (Unitron, Infors AG, Bottmingen, Switzerland). The reaction was stopped by heating at 95 °C for 10 min in a water bath, followed by cooling to room temperature. Each sample was stored at −20 °C until further analysis.

2.3. Analysis by RP–HPLC–MS/MS

Identification of peptides in the hydrolysates, obtained before and after simulation of the intestinal digestion, was performed on an Agilent HPLC system (Agilent Technologies, Waldbronn, Germany) connected on line to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany). The HPLC was equipped with a quaternary gradient pumping system, an in-line degasser, a variable wavelength absorbance detector set at 214 nm, and an automatic injector (all 1100 Series, Agilent Technologies). The column used in these experiments was a 250 mm × 4.6 mm Widepore C_{18} column (Bio-Rad, Richmond, CA, USA). The injection volume was 50 µl. Solvent A was 0.37 ml/l TFA in milli-Q water and solvent B 0.27 ml/l TFA in acetonitrile. Peptides were eluted with a linear gradient of solvent B in A, from 2% to 10% in 15 min, 10% to 20% in 35 min and 20% to 30% in 20 min. The flow rate was 0.8 ml/min. The flow was split post detector by placing a T-piece (Valco, Houston, TX, USA) connected with a 75 µm ID PEEK outlet tube of an adjusted length to give, approximately, 20 µl/min of flow directed into the mass spectrometer via the electrospray interface. Nitrogen was used as nebulizing and drying gas and operated with an estimated helium pressure of 5×10^{-3} bar. The capillary was held at 4 kV. Spectra were recorded over the mass/charge (*m*/*z*) range 100–1500. About 25 spectra were averaged in the MS analyses and about 5 spectra in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 5000 and the precursor ions were isolated within a range of 4.0 *m*/*z* and fragmented with a voltage ramp going from 0.35 to 1.4 V. Using Data Analysis™ (version 3.0; Bruker Daltoniks), the *m*/*z* spectral data were processed and transformed to spectra representing mass values. BioTools (version 2.1; Bruker Daltoniks) was used to process the MS(n) spectra and to perform peptide sequencing (Gómez-Ruiz, Ramos, & Recio, 2004).

2.4. ACE-inhibitory activity

ACE-inhibitory activity was measured *in vitro* using the spectrophotometric assay described by Cushman and

Cheung (1971) with some modifications (Miguel et al., 2004). The substrate, hippuryl-histidil-leucine (HHL) and angiotensin converting enzyme (ACE) from rabbit lung (EC 3.4.15.1) were purchased from Sigma. Testing solutions (40 μl) were incubated with 100 μl of 0.1 M borate buffer (pH 8.3) containing 5 mM HHL and 0.3 M NaCl and with 20 μl of ACE (2 mU) at 37 °C for 30 min. The reaction was stopped with 150 μl of 1 M HCl. The hippuric acid formed was extracted with ethyl-acetate (1000 μl), centrifuged at 1500*g* for 10 min and 750 μl of the organic phase were evaporated. The residue was dissolved in 800 μl of distilled water and the absorbance was measured at 228 nm. Triplicate tests were performed for each sample. Inhibitory activity was expressed as the protein concentration-determined by the bicinchoninic acid assay (Pierce, Rockford, IL, USA) using bovine serum albumin as standard- needed to inhibit 50% of ACE activity (IC_{50}).

2.5. Antihypertensive activity in spontaneously hypertensive rats

Male spontaneously hypertensive rats (SHR), 17–20 weeks-old, weighing 300–350 g (Charles River Laboratories Spain S.A.) were used in this study. The animals were maintained at a temperature of 23 °C with 12 h light/dark cycles, and consumed tap water and a standard diet for rats (A04 Panlab, Barcelona, Spain) *ad libitum* during the experiments. The products were orally administered by gastric intubation, between 9 and 10 a.m. Distilled water served as the negative control, and captopril (50 mg/kg) (Sigma), a known ACE inhibitor, served as the positive control. All the products were administered in 1 ml of water. The systolic blood pressure (SBP) of the rats was measured by the tail cuff method (Buñag, 1973), before administration and also 2, 4, 6, 8, and 24 h post-administration using LE 5001 equipment (Letica, Hospitalet, Barcelona, Spain). Before the measurement, the rats were kept at 37 °C for 10 min to make the pulsations of the tail artery detectable. In order to guarantee the reliability of the measurements, the rats were accustomed to the procedure two weeks before beginning the experiments. Changes in SBP were calculated as the difference between the mean values of three measurements obtained before and after the administration. The results, expressed as mean values ± SEM for a minimum of 5 rats, were analyzed by one-way ANOVA. Differences between the groups were assessed by the Bonferroni test and were considered significant when $P < 0.05$.

2.6. In vitro studies in aortic rings

Three-month old male Sprague–Dawley rats weighing 250–300 g were used for the rat aorta experiments. These animals were obtained from the Animals Quarters of Facultad de Medicina of the Universidad Autónoma de Madrid. They were euthanized by decapitation, and the thoracic aorta was removed and placed in Krebs–Henseleit solution (KHS, in mM: 115 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 11.1 glucose and 0.01 Na_2EDTA) at 4 °C.

Segments of thoracic aorta (3 mm in length), free of fat and connective tissue, were mounted between two steel hooks in isolated tissue chambers and were incubated in 5 ml of KHS at 37 °C, continuously bubbled with a 95%O_2–5%CO_2 mixture, which gave a pH of 7.4. An optimal resting tension of 1.5 g was applied to all aortic segments. This tension was adjusted every 15 min during a 45-min equilibration period before adding drugs. Isometric tension was recorded by using an isometric force displacement transducer connected to an acquisition system (MacLab 8/S, ADInstruments Pty Ltd, Castle Hill, Australia).

After the equilibration period, segments were initially exposed twice to 75 mM KCl to test their functionality and its maximum contractility. The integrity and the presence of endothelium were confirmed by the relaxing effect of 10 μM acetylcholine on segments contracted with phenylephrine at a concentration that produces close to 50% of the contraction induced by 75 mM KCl. A relaxation equal to or greater than 68% was considered as evidence of the functional integrity of the endothelium and the absence of relaxation induced by acetylcholine as absence of the endothelium.

After 60 min of washout, the relaxations produced by single doses of HEW before and after simulated intestinal digestion (1 mg/ml), were studied on segments contracted with phenylephrine at a concentration that produces close to 50% of the contraction induced by 75 mM KCl. In every experiment, we carried out a control to evaluate the possible loss of vascular tone in the aortic rings due to the duration of the experiment. The small loss of vascular tone observed was corrected in the relaxation produced by the hydrolysates. Results are expressed as means ± SEM of the number of rats indicated in each case. Differences between HEW and HEW treated with Corolase PP® were analysed by Student's *t*-test. A probability value of less than 5% ($P < 0.05$) was considered significant.

The investigations with animals conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996) and complies with the current Spanish and European laws (RD 223/88 MAPA and 609/86).

3. Results and discussion

3.1. Effect of a simulated intestinal digestion in the peptide pattern of the egg white hydrolysate

HEW and HEW < 3000 were analysed by RP–HPLC–MS before and after the treatment with Corolase PP®. The RP–HPLC chromatograms of HEW and HEW < 3000 revealed a complex peak pattern (Figs. 1a and 2a), but most of the peptides were effectively hydrolysed on treatment with the pancreatic extract, yielding almost

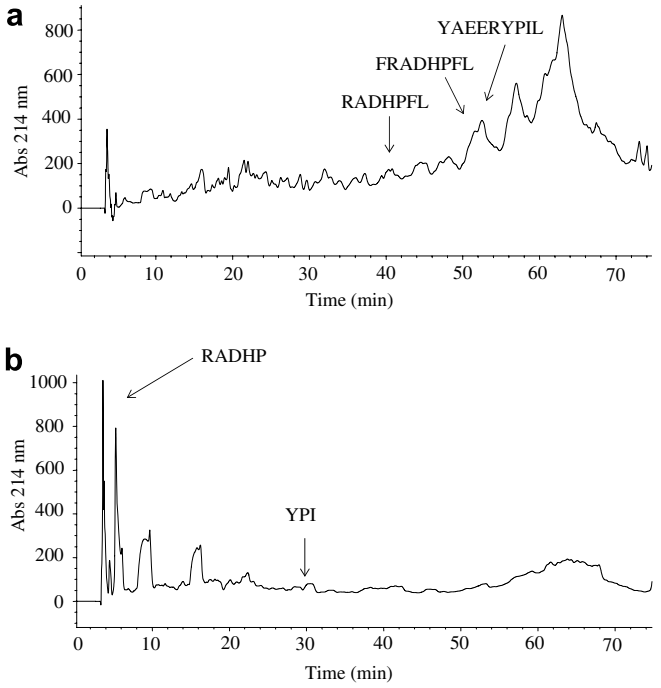

Fig. 1. RP–HPLC chromatograms of the hydrolysate of egg white with pepsin (HEW) before (a) and after (b) a simulated intestinal digestion.

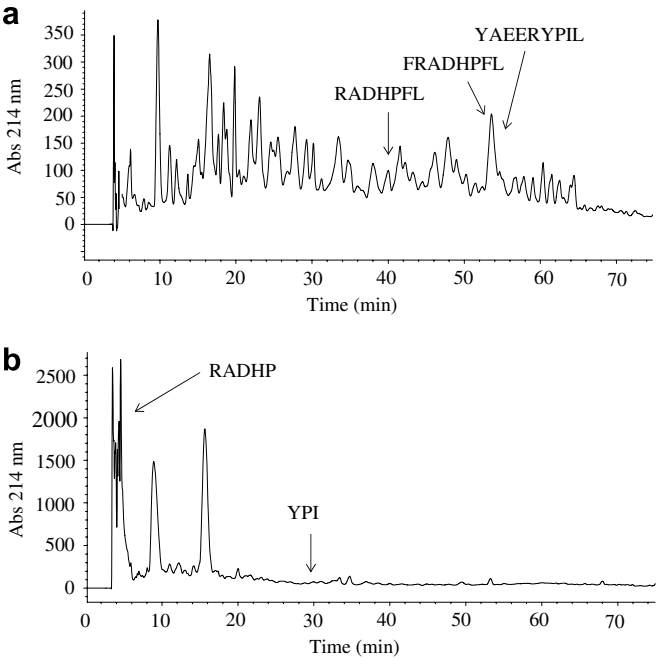

Fig. 2. RP–HPLC chromatograms of the fraction with molecular mass lower than 3000 Da of the hydrolysate of egg white with pepsin (HEW < 3000) before (a) and after (b) a simulated intestinal digestion.

identical profiles for the hydrolysate and its low molecular mass fraction (Figs. 1b and 2b). We investigated the presence of previously identified sequences with ACE-inhibitory and antihypertensive activity, such as FRADHPFL, RADHPFL and YAEERYPIL (Fujita et al., 1995; Miguel et al., 2005, 2004), by analysis by RP–HPLC–MS and extraction of the characteristic ions of the peptides of interest at the same retention time of the pure peptides. As expected, HEW and HEW < 3000 contained the sequences FRADHPFL, RADHPFL and YAEERYPIL, but these disappeared after hydrolysis with Corolase PP® (Figs. 1 and 2).

We had previously shown that the bioactive peptides present in HEW, FRADHPFL and RADHPFL, are susceptible to *in vitro* gastrointestinal digestion, giving rise to the shortest sequence RADHP. The active peptide YAEERYPIL is also hydrolysed to the fragments YAEER and YPI (Miguel, Ramos, Aleixandre, & López-Fandiño, 2006). The digestion products, RADHP and YPI, present low ACE-inhibitory activity *in vitro*, but exhibit strong antihypertensive effects when given orally to SHR (Miguel, Ramos, et al., 2006). The ions corresponding to the sequences RADHP and YPI were also found in the hydrolysates of HEW and HEW < 3000 with Corolase PP (Figs. 1b and 2b), showing that they were released from FRADHPFL or RADHPFL and YAEERYPIL by the pancreatic enzymes, not only when the pure peptides were used but also when they were part of a complex peptide mixture. The peak corresponding to RADHP was particularly prominent even when using the UV detector at 214 nm (Figs. 1b and 2b). This strongly suggests that the active sequences RADHP and YPI could be end products of the digestion of HEW and HEW < 3000 in the small intestine.

3.2. ACE-inhibitory and antihypertensive activity of the egg white hydrolysate upon simulated intestinal digestion

The ACE-inhibitory activity was measured in HEW and HEW < 3000 before and after hydrolysis with Corolase PP®. The IC_{50} values increased from 44.00 ± 1.18 μg/ml to 67.98 ± 2.08 μg/ml and from 20.48 ± 0.75 μg/ml to 74.40 ± 1.16 μg/ml ml in HEW and HEW < 3000, respectively. Even though the ACE inhibitory activity decreased, probably due to the degradation of some ACE-inhibitory peptides during the simulated pancreatic digestion (see Figs. 1 and 2), the final IC_{50} values were still indicative of a strong ACE inhibition. According to Matsui et al. (2000), the ACE-inhibitory activity of a wheat germ hydrolysate increased by 27% after a combined digestion, suggesting that new active peptides were produced by the action of trypsin. However, in other studies, the simulated digestion reduced the activity. Thus, the α_{S1}-casein-derived peptide, YKVPEL, with strong ACE-inhibitory activity, failed to exert any antihypertensive effect after pancreatin degradation (Maeno, Yamamoto, & Takano, 1996).

The antihypertensive activity of HEW < 3000 was evaluated in SHR, at a dose of 100 mg/kg, before and after treatment with Corolase PP® (Fig. 3). Prior to the administration, the SBP value of the SHR was 217.4 ± 3.1 mm Hg. The administration of distilled water to SHR did not change the values of SBP, while the maximum decreases in the SBP were observed with 50 mg/kg of captopril, an ACE-inhibitor prototype with an IC_{50} value of 0.02 μM

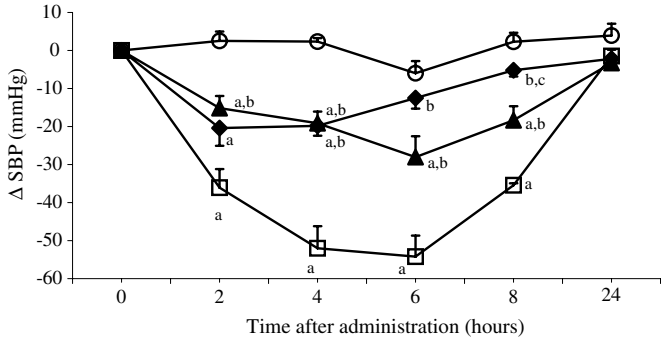

Fig. 3. Decreases in SBP after the administration to spontaneously hypertensive rats (SHR) of: 1 ml water (○, $n = 8$), 50 mg/kg of captopril. (□, $n = 8$), 100 mg/kg of HEW < 3000 before (▲, $n = 5$) and after (♦, $n = 5$) simulated intestinal digestion. Data are expressed as mean ± SEM for a minimum of five animals. $^{a}P < 0.05$ versus Water, $^{b}P < 0.05$ vs Captopril; $^{c}P < 0.05$ vs HEW < 3000 before simulated intestinal digestion.

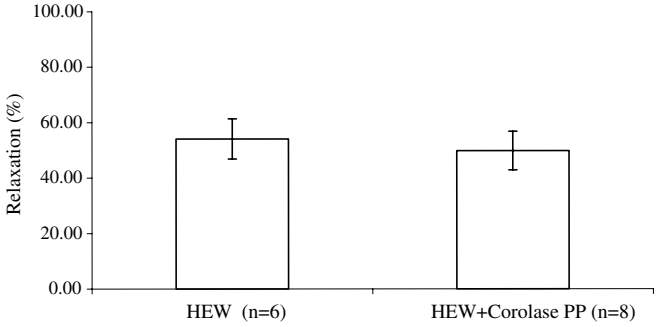

Fig. 4. Relaxation produced by a single dose (1 mg/ml) of HEW before and after a simulated intestinal digestion in SD aortic segments. Results (mean ± SEM) are expressed as a percentage of the contraction previously induced by phenylephrine.

(Fujita & Yoshikawa, 1999). HEW < 3000 and HEW < 3000 treated with Corolase PP® produced a clear antihypertensive effect in these animals. No significant differences ($P > 0.05$) were observed between the effects of both products at 2, 4 and 6 h after the administration and the SBP returned to baseline after 24 h.

It should be mentioned that, in agreement with previous studies, the maximum reduction in SBP was observed 6 h after the administration of HEW < 3000 (~28 mm Hg) (Miguel et al., 2005). However, when HEW < 3000 was treated with Corolase PP, the maximum antihypertensive effect (~20 mm Hg) appeared earlier, 2–4 h after the administration. This is probably because, in the latter case the active peptide sequences were already accessible to enter peripheral blood and exert systemic effects, or to produce local effects in the gastrointestinal tract (Yoshikawa et al., 2000). It is likely that RADHP and YPI were responsible for the antihypertensive effect observed after the oral administration of the hydrolysate treated with Corolase PP® to SHR. In previous studies, we have shown that both peptides, YPI and RADHP, significantly decrease SBP when administered to SHR at doses of 2 mg/kg. This decrease is maximal 2–4 h after administration, while in the case of the longer sequences, YAEERYPIL and RADHPFL, the maximum SBP reductions are achieved 6 h after the oral administration (Miguel et al., 2005; Miguel, Ramos, et al., 2006).

3.3. In vitro studies in aortic rings of the egg white hydrolysate upon simulated intestinal digestion

In order to establish the existence of additional mechanisms of antihypertensive activity in addition to ACE inhibition, we investigated the vascular effects of HEW in aorta of SD rats, before and after treatment with Corolase PP. As shown in Fig. 4, HEW (1 mg/ml) relaxed the endothelium-intact aortic segments pre-contracted by phenylephrine. After the simulated intestinal digestion, HEW also showed vasodilator activity. No differences were observed between HEW and HEW treated with Corolase PP®, with degrees of relaxation close to 50% (54.1% ± 7.2 and 49.9% ± 6.9, respectively).

As already mentioned, HEW possesses angiotensin converting enzyme (ACE) inhibitory activity (Miguel et al., 2004), one of the most often studied mechanisms underlying the blood pressure-lowering effect of food derived peptides. In addition, peptides that influence the vascular function *in vitro* via several mechanisms and control hypertension by exerting vasodilator effects have been found in the primary sequence of egg proteins and other food proteins (Fujita et al., 1996; Fujita et al., 1995; Kuono, Hirano, Kuboki, Kasai, & Hatae, 2005; Matoba et al., 1999; Sipola et al., 2002). On the other hand, HEW also possesses radical scavenging activities (Dávalos, Miguel, Bartolomé, & López-Fandiño, 2004). The pathogenesis of hypertension is associated with oxidative stress and thus antioxidant-rich diets reduce the arterial blood pressure in SHR and may modulate the endothelial function (Akpaffiong & Taylor, 1998; Rodríguez-Iturbe, Zhan, Quiroz, Sindhu, & Vaziri, 2003). Therefore, in the antihypertensive effect of HEW, different mechanisms may be involved, such as ACE inhibition, vascular relaxation and antioxidant activities.

4. Conclusions

The present results show that HEW and HEW < 3000 possess ACE-inhibitory and vasodilator activity *in vitro* before and after simulated intestinal digestion that may explain their antihypertensive effect in SHR. It is likely that these effects are due to the presence of the antihypertensive peptides RADHP and YPI, which arise from the digestion with pancreatic enzymes of FRADHPFL and/or RADHPFL and YAEERYPIL, respectively. In particular, RADHP was present in considerable amounts after the simulated intestinal digestion, despite the thorough proteolytic degradation undergone by the hydrolysates. The combination of ACE-inhibitory activity and vascular relaxation properties, in addition to antioxidant effects, could provide superior cardiovascular protection, due to an additive

effect, suggesting that HEW or HEW < 3000 could be useful in the prevention and/or treatment of hypertension and other associated disorders.

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