Evidence for an antihypertensive effect of a land snail (Helix aspersa) by-product hydrolysate – Identification of involved peptides

Benoit Cudennec a,*, Nicolas Violle b, Gabrielle Chataigné a, Pascal Drevet c, Jean-François Bisson b, Pascal Dhulster a, Rozenn Ravallec a

a Univ. Lille, EA 7394, USC 1281 – ICV – Institut Charles Viollette, F-59000 Lille, France
b ETAP-Lab, Health-Nutrition, F-54500 Vandoeuvre-lès-Nancy, France
c HPE Ingrédients, F-14650 Carpiquet, France

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ABSTRACT
The antihypertensive potential of a land snail by-product hydrolysate (SBH), obtained after an industrial treatment of the raw material, was studied in vitro and in vivo. The ACE inhibitory activity of SBH was characterised by an IC₅₀ value of 23 μg·mL⁻¹, which was not affected by in vitro digestion. SBH enhanced the Caco-2 intestinal cell metabolic activity and did not induce any toxicity in Wistar rats. The partial purification of SBH led to the obtainment of an active fraction characterised by an IC₅₀ of 0.007 μg·mL⁻¹. The sequences of the 17 most abundant peptides of the fraction were identified by LC/MS/MS analysis. Seven of them (YG, YA, VY, SF, FG, GF and VW) are known ACE inhibitory peptides. Finally, in vivo study on SHR rats showed that SBH significantly reduced systolic blood pressure. SBH represents therefore a new candidate as an ingredient for the design of functional foods against hypertension.

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1. Introduction
Hypertension is the leading cause of cardiovascular mortality and is the most common chronic disease in the world (Wolf-Maier et al., 2003). Blood pressure is determined by the cardiac flow and is principally regulated by two pathways: sympathetic nervous system and renin–angiotensin system. The angiotensin-converting enzyme (ACE) is a non-specific dipeptidyl carboxypeptidase associated with the blood pressure regulating renin–angiotensin system. This enzyme increases blood pressure by converting the decapeptide angiotensin I into the potent vasoconstrictor octapeptide angiotensin II. This peptide displays several central effects all leading to a further increase in blood pressure. Moreover, ACE is a multifunctional enzyme that also catalyses the degradation of bradykinin, a blood pressure-lowering nonapeptide. Therefore, inhibition of the ACE results in an overall antihypertensive effect (Aronow, 2015). Synthetic ACE inhibitors are one group of drugs in the treatment of hypertension. However,
these synthetic drugs can have significant side effects. So, a number of methods are used to produce peptides with this specific biological activity. These peptides may be present in food, or may be released during protein hydrolysis by digestive enzymes such as trypsin, chymotrypsin or pepsin. Lately, these “biologically active peptides” from partial enzymatic hydrolysates of food proteins have received greater attention than ever before because they could be a healthier and natural alternative for ACE inhibitory drugs. A large number of ACE inhibitory peptides were identified from proteins of diverse taxonomic origins like from plant by-products, such as for example rubisco from alfalfa (Kapel, Rahhou, Lecouturier, Guillochon, & Dhuylle, 2006), mammals protein by-product such as egg proteins (Majumder et al., 2015), fish by-products such as sardine by-products (Bougafet et al., 2008), bivalve molluscs such as oyster protein by-products (Wang et al., 2008), or also from cephalopod molluscs such as cuttlefish protein by-products (Balit et al., 2015). For review see the works of Harnedy and Fitzgerald (2012), He, Liu, and Ma (2013) and Martínez-Maqueda, MiraIles, Recio, and Hernandez-Ledesma (2012). ACE inhibitory peptides usually contain only a few amino acid residues and have to reach their target under active form after drastic proteolytic conditions of gastrointestinal digestion to exert their physiological actions. Protein hydrolysates could be obtained by treatment with bacterial or gastrointestinal enzymes. The resultant hydrolysates possess higher functional properties than protein crude extracts partially due to the presence of specific peptides able to be absorbed by intestinal cells or to exert some biological specific effects (Cudennec, Caradec, Catiau, & Ravallec, 2012) High added value protein hydrolysates can be commercially produced from agro alimentary by-products through simple engineering and have been used as food supplements, animal feed or fertilizer (Ravallec-Ple & Van Wormhoudt, 2003).

The land snail is a gastropod, a soft-bodied type of mollusc that is basically a head with a flattened foot. Because they crawl over a variety of potentially contaminated surfaces, the snail foot is the primary site of entry for pathogens and parasites. Due to this fact, land snails could be used as a biomarker of material or gastrointestinal enzymes. The resultant hydrolysates contain only a few amino acid residues and have to reach their target under active form after drastic proteolytic conditions of gastrointestinal digestion to exert their physiological actions. Protein hydrolysates could be obtained by treatment with bacterial or gastrointestinal enzymes. The resultant hydrolysates possess higher functional properties than protein crude extracts partially due to the presence of specific peptides able to be absorbed by intestinal cells or to exert some biological specific effects (Cudennec, Caradec, Catiau, & Ravallec, 2012). High added value protein hydrolysates can be commercially produced from agro alimentary by-products through simple engineering and have been used as food supplements, animal feed or fertilizer (Ravallec-Ple & Van Wormhoudt, 2003). The presence of some endogenous opioid peptides in the nervous tissue of periesophageal ganglia of the land snail was also highlighted (León-Olea et al., 2013). In addition, numerous neuropeptides were identified in the gastropod family (Cosmo & Polese, 2013; Morishita et al., 2015) and also excitatory peptide toxins from the venom of snails (Imperial et al., 2014). Three myomodulin-related peptides, involved in the feeding behaviour of the animals, have also been purified and sequenced from extracts of land snails (Helix aspersa) (Cosmo & Polese, 2013). H. aspersa is one of the easiest species to breed in snail farming, exhibiting a high potential for growth and fertility. It particularly adapts well to different types of climate and environment and as a consequence is significantly produced mainly in Europe and all over the world (Segade, García-Estévez, Arias, & Iglesias, 2013). It is prepared for human consumption by removing the gland, the hepatopancreas, generating an important raw material that can for example be valorised by enzymatic digestion. However, at the current time, this by-product is not used, and the search for bioactive peptides represents an innovative way of upgrading this as ingredient for functional food.

In the present work, the ACE inhibitory activity of an industrially produced land snail by-product hydrolysate (SBH) was first studied in vitro. SBH and the non-hydrolysed by-product (used as control) were then submitted to an in vitro simulated human gastrointestinal digestion during which their ACE inhibitory activities were followed. The key bioactive peptide sequences involved in the biological activity were then obtained by LC/MS/MS analysis. In a second experiment, the antihypertensive effects of SBH on SHR rats were evaluated. In parallel, the cytotoxicity of SBH was assessed in vitro on human intestinal cells, and its acute toxicity was evaluated in Wistar rats according to the OECD Test Guideline 423 (OECD, 2001).

2. Materials and Methods

2.1. Materials and chemicals

Angiotensin Converting Enzyme from rabbit lung, N-Hippuryl-His-Leu, Captopril, porcine pepsin, porcine pancreatin, albumin, cytochrome C, aprotinin, vitamin B12, and glutathione were obtained from Sigma Aldrich (Saint-Quentin-Fallavier, France). Alcalase 2–4 L was obtained from Novozyme (Bagsvaerd, Denmark). Foetal calf serum, Dulbecco’s modified Eagle’s Medium (DMEM), and all other cell culture reagents were purchased from PAN-Biotech GmbH (Aidenbach, Germany). All other chemicals were of reagent grade.

2.2. Obtainment of snail by-product hydrolysate (SBH)

Technical details of the SBH are limited as it was industrially prepared. Briefly, the shells of animals were removed and hepatopancreas (digestive gland) were collected before being washed with distilled water. Hepatopancreases were then hydrolysed with alcalase (1%, w/w) under controlled hydrolysis conditions (pH, temperature, and stirring speed) according to the pH-stat method (Diniz & Martin, 1996). Enzymatic inactivation was performed by heating at 90 °C for 20 min. The hydrolysate was then sieved and centrifuged (7000 × g for 30 min) and the supernatant was further ultra-filtered on a polysulphone membrane of 10,000 Da molecular weight cut-off. Permeate obtained was then freeze-dried and kept at −20 °C for further experiments. The characterisation of the active components of the SBH and more particularly the ACE inhibitory peptides is described later in the text.

2.3. ACE inhibitory activity

The method described by Wu, Aluko, and Muir (2002) with some modifications was used to determine the ACE inhibitory activity of hydrolysates. The samples were diluted in 30 μL borate buffer (Na2B4O7 100 mM, NaCl 300 mM, pH 8.3) at increasing concentrations and pre-incubated with 100 μL of hippuryl-histidyl-leucine (HHL) at 2.17 mM and 37 °C for 10 min. For the control, the sample was replaced by 30 μL of the same borate buffer. Then, the reaction was initiated by the addition of the ACE (30 μL
at 55 mU·mL⁻¹). All the reagents were diluted in borate buffer. The reaction was stopped after 1 h at 37 °C with the addition of 100 μL HCl (1 mol·L⁻¹). Reaction medium separation was performed using a C18 150 mm × 4.60 mm, kinetex, 2.6 μ 100 Å, reverse-phase column (Phenomenex, Le Pecq, France) at a flow rate of 0.5 mL·min⁻¹. The liquid chromatographic system (Waters, Milford, CT, USA) consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Millennium software was used to plot, acquire and analyse chromatographic data. Online UV absorbance was performed at 228 nm during the 10 minutes of isocratic elution. The solvent was made of 0.1% TFA and 25% acetonitrile in ultrapure water. The peak area of the hippuric acid (HA) produced during the reaction was used to calculate the ACE inhibition rate as below:

\[
\% \text{ of ACE inhibition} = 100 \times \left[1 - \left(\frac{\text{Inhibitor area}}{\text{Control area}}\right)\right]
\]

where Inhibitor area and Control area are HA peak areas obtained for inhibitor and control samples, respectively.

The IC₅₀ was represented by the concentration of hydrolysate necessary to obtain 50% of ACE activity inhibition. IC₅₀ values were calculated with the linear regression parameters obtained after the plotting of the natural logarithm (ln) of the hydrolysate concentrations assayed versus the ACE % of inhibition. Captopril, a well-known ACE inhibitor, was used as reference inhibitor.

### 2.4 Human in vitro gastrointestinal digestion

A static mono-compartmental digestion protocol was set up to reproduce as close as possible the whole gastrointestinal environment. The method previously described by Versantvoort, Oomen, Van de Kamp, Rompelberg, and Sips (2005) was used with some modifications. The first steps of digestion were simulated, which means mouth, stomach and duodenum. Three simulated fluids were prepared to mimic the physiological conditions of each step. Each fluid was prepared using a C18 150 mm × 4.60 mm, kinetex, 2.6 μ 100 Å, reverse-phase column (Phenomenex, Le Pecq, France) at a flow rate of 0.5 mL·min⁻¹. The liquid chromatographic system (Waters, Milford, CT, USA) consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Millennium software was used to plot, acquire and analyse chromatographic data. Online UV absorbance was performed at 228 nm during the 10 minutes of isocratic elution. The solvent was made of 0.1% TFA and 25% acetonitrile in ultrapure water. The peak area of the hippuric acid (HA) produced during the reaction was used to calculate the ACE inhibition rate as below:

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### 2.5 Effects of SBH on cell viability

Effects of hydrolysate on Caco-2 human intestinal cells’ proliferation and viability were measured using CCK-8 assay based on the reduction of tetrazolium salt by active mitochondria (Dojindo Molecular Technologies, Japan). Cells were seeded on a 96 well plate at 8000 cells per well in 100 μL culture media and cultivated during approximately 48 h at 37 °C, 5% CO₂ upon reaching confluence. Then, 50 μL media in the presence or in the absence of hydrolysates at different concentrations were then added in each well. After 24 h at 37 °C, 5% CO₂ atmosphere, media were removed and cells were washed twice with phosphate saline buffer. A volume of 150 μL DMEM containing 50 μL CCK-8 reagent was added in each well and cells were incubated for 4 h. Plates were then read at 450 nm using a microplate reader spectrophotometer (Xenius, Safas, Monaco). Results were expressed in percentage of basal growth observed with non-treated cells.

### 2.6 Size exclusion chromatography

Size exclusion chromatography was used to study peptide apparent molecular weight distribution of hydrolysates. Separation was performed at room temperature using a Superdex Peptide 10/300 GL column (GE Healthcare, Uppsala, Sweden).
Acetonitrile–H2O–TFA (30:70:0.1) was used as mobile phase. The flow rate was fixed at 0.5 mL·min⁻¹ and the absorbance was monitored at 214 nm using an AKTA purifier high performance liquid chromatography system (GE Healthcare, Uppsala, Sweden). Freeze-dried hydrolysates were diluted in bi-distilled water at 20 mg·mL⁻¹, filtered (0.22 μm) before being injected (40 μL). The column was calibrated using standard molecules (albumin: 60,000 Da; cytochrome C: 12,400 Da; aprotinin: 6500 Da; vitamin B12: 1355 Da; glutathione: 307 Da). The relationship between the Log of molecular weight standards and the elution volume was used to perform the molecular weight profiles.

2.7. Identification of ACE inhibitory peptides

2.7.1. Size fractionation of SBH

The same dispositive as previously described (section 2.6) was used for the first step of purification to obtain five different fractions of SBH. Forty microlitres of hydrolysate diluted at 50 mg·mL⁻¹ was used for injections. Ten successive injections were performed and the same fractions of each injection were pooled before being evaporated and freeze-dried. Obtained fractions were then submitted to ACE inhibitory assay at similar concentrations.

2.7.2. HPLC-MS/MS

The analyses were performed on an Accela UHPLC system acquired from Fisher Scientific (Thermo Fisher Scientific, Bremen, Germany) that was composed of an auto-sampler equipped with a column oven, a tray compartment cooler and a quaternary pump with a built-in solvent degasser, all piloted by Xcalibur software. The chromatographic separation was performed with a Vydac C18, 250 × 3 mm 5 μm column (Grace Discovery Sciences, Deerfield, IL, USA). The separations were performed with a constant flow rate of 600 μL·min⁻¹. The eluent was composed of Solvent A (water, 0.1% formic acid) and Solvent B (acetonitrile, 0.1% formic acid). The following gradient was used: 0–5 min: B 0%; 5–55 min: B 0% to 60%; 55–65 min: B 60% to 100%; 65–70 min: B 100%. The Accela UHPLC system was hyphenated with a vantage mass spectrometry system (Thermo Fisher Scientific, Bremen, Germany). A flow divider was used as the output rate of HPLC was higher than that tolerated by the mass spectrometer. The flow was split to give approx. 200 μL·min⁻¹ directed into mass spectrometer via the electro-spray interface. The system was equipped with an ESI interface that was used in positive ionization mode with the following conditions: capillary temperature and voltage at 275 °C and 9 V respectively, ion spray voltage at 3.3 kV and tube lens voltage at 95 V. Nitrogen (N2) was used as the sheath gas and helium (He) as auxiliary gas with a flow rate of 25 and 5 arbitrary units.

For the MS experiments, full MS scan was applied, for which the spectra were recorded in the range of m/z 100–2000 with a resolution of 30,000. An external calibration of the equipment for mass accuracy was carried out the day before the analysis according to the manufacturer’s guidelines.

De novo sequencing by MS/MS spectrometry was performed to determine the amino acid sequence of majority peptides contained in the hydrolysate active fraction. This approach correlated the mass tandem data with the peptide sequencing. Thus, the MS/MS spectrum contained fragment ions reported by Biemann (1990). This fragmentation rule allows deducing a partial or total amino sequencing of each unknown peptide without protein data base (Biemann & Martin, 1987).

2.8. In vivo studies

2.8.1. Animals

Six Wistar female rats (Crl:WI(Han)), 6-week old (120–130 g), were used for the acute toxicity study. Thirty-two SHR male rats (SHR/NCr), 11-week old, were used to assess SBH antihypertensive properties. All rats were purchased from Charles River Laboratories (L’Arbresle, France).

Wistar rats were housed 3 per cage and SHR rats 2 per cage in a regulated environment (20 ± 2 °C; humidity 50 ± 20%) under a reversed light/dark cycle (light on from 8:00 p.m. to 8:00 a.m.) and subjected to a one-week acclimatisation period before starting studies. Food pellets (Special Diets Services, Witham, UK) and tap water were provided ad libitum. All rats were handled in the same way, under the same conditions, and were randomly allocated to treatment groups. All procedures were in compliance with the rules provided by the European Communities Council Directive of 22 September 2010 (2010/63/UE).

The study received the approval by the French Ministry of Higher Education and Research (agreement no. 00836.02 from January 2015).

2.8.2. Acute toxicity study

A single oral administration of SBH, at the limit dose of 2 g·kg⁻¹ in an administration volume of 10 mL per kg of body weight, was performed in fasted rats according to the Annex 2d of the OECD Test Guideline 423 using 2 sets of 3 rats each at weekly interval. Rats were followed up during 14 days after the single oral administration with daily observation and regular weighing. They were then sacrificed and a macroscopic observation of all organs was performed.

2.8.3. Antihypertensive study

Systolic blood pressure (SBP) of SHR rats was measured in awake rats, using the tail-cuff method with an automated multichannel device (BP2000, Visitech Systems, Apex, NC, USA). Habituation of SHR rats to the measurement procedure was performed 2 days before SBH testing. SBP measurements were performed in a quiet silent room under a red dim light. During both habituations and test, each rat was gently handled and placed in a slightly heated restrainer. After a 10-minute restraint period, 20 SBP measurements were performed and the 10 last measurements were recorded for SBP evaluation. For each rat, the overall procedure lasted around 25 minutes.

On the day of testing, animals received a single oral administration of SBH at the doses of 400 or 800 mg·kg⁻¹ (n = 8 per group) 3 hours before SBP measurements. The ACE-inhibitor captopril (Captopril Mylan, Mylan, Saint-Priest, France) was used at a dose of 70 mg·kg⁻¹ as a reference drug (n = 8). Freeze-dried SBH and Captopril were suspended in a 0.5% methylcellulose solution. A vehicle group received the same volume of methylcellulose solution in the same conditions (n = 8). All treatments were administered at a volume of 10 mL per kg of body weight, using blunt-ended stainless steel feeding needles.
Table 2 – Apparent molecular weight distribution of SBH.

<table>
<thead>
<tr>
<th>Apparent molecular weight (Da)</th>
<th>% of chromatogram total area</th>
</tr>
</thead>
<tbody>
<tr>
<td>7000–4000</td>
<td>0.6</td>
</tr>
<tr>
<td>4000–2000</td>
<td>4.9</td>
</tr>
<tr>
<td>2000–1000</td>
<td>14.1</td>
</tr>
<tr>
<td>1000–300</td>
<td>41.6</td>
</tr>
<tr>
<td>&gt;300</td>
<td>38.8</td>
</tr>
</tbody>
</table>

(Popper and Sons, Inc., New Hyde Park, NY, USA). Habitation of animals to the oral administration procedure was performed 3 days before testing with methylcellulose solution. At the end of the in vivo study, each SBP raw signal was visually checked to eliminate recordings with artefacts due to excessive movements of rats or reduced signal. A rat was included if at least 3 SBP measurements were achieved. Mean SBP was then calculated for each rat. All the experimental procedures were performed blindly.

2.9. Statistical analysis

For in vitro experiment, data were expressed as mean values with their standard deviations. SigmaPlot 11.0 software package (Germany) was used to carry out statistical analysis on in vitro data. All tests of significance between groups were performed using the ANOVA one way with Tukey test. For in vivo experiments, no statistical analysis was performed for the acute toxicity study. For the antihypertensive experiment, data were expressed as mean ± SEM. Effects of treatment regimen on SBP was analysed using one-way analysis of variance (one-way ANOVA). Dunnett’s post-hoc test was used to compare the groups of treatment with the vehicle group. Statistical analyses were carried out using the StatView 5 software package (SAS Institute, Cary, NC, USA). For all statistical tests, differences between means were considered significant when p < 0.05.

3. Results

3.1. Characterisation and ACE inhibitory capacity of SBH

First, the ACE inhibitory activity of hydrolysates obtained before and after an ultrafiltration step with a 10 kDa membrane cut-off was assessed. The IC50 obtained were of 98.3 μg·mL⁻¹ and 23 μg·mL⁻¹, respectively, showing a 4.2 fold increase in ACE inhibitory capacity after the ultrafiltration step. In accordance with these results, this step was therefore adopted in the process to obtain the snail by-product hydrolysate, SBH. The SBH apparent molecular weight profile obtained after size exclusion chromatography showed that the hydrolysate was composed of molecules whose apparent molecular weight (MW) was smaller than 7000 Da and that only 20% of them had an apparent MW higher than 1000 Da. Moreover, 38.8% of these molecules had an apparent MW below 300 Da (Table 2).

3.2. Effects of SBH on human intestinal cell viability

The effects of SBH at 0.2%, 0.5% and 1.0% (w/v) on the mitochondrial activity of Caco-2 cells were measured using CCK-8 assay (Fig. 1). Results showed that SBH was not toxic for intestinal cells at the doses used. Moreover, the mitochondrial activity of cells exposed to hydrolysate during 24 h was significantly enhanced for all the concentrations used. Thus, the viability of intestinal cells was of 1.1, 1.2 and 1.2 fold higher than for untreated cells for 0.2%, 0.5% and 1.0% SBH respectively.

3.3. SBH and Snail By-product (SB) submitted to a gastrointestinal digestion: following the ACE inhibitory activity

The objective of this stage of the work was twofold: on one hand, to study the subsistence of the ACE inhibitory capacity of SBH in the different compartments of the GI tract; on the other, to compare the ACE inhibitory capacities of the SBH and the crude by-product (which has not undergone any prior hydrolysis) during an in vitro simulated digestion. The results obtained, presented in Fig. 2, showed that the SBH inhibitory activity of ACE was maintained throughout the gastrointestinal digestion. Indeed, despite a slight increase along the digestion process, no significant difference of the ACE inhibition rate (around 90% at 0.47 mg·mL⁻¹) was observed between the different compartments. The SB submitted to gastrointestinal digestion showed no ACE inhibitory activity in the mouth before proteases act. In the stomach, the activity significantly increased to reach a maximum inhibition rate of 56.3 ± 0.3% after 125 minutes of digestion. Then, the inhibitory capacity of SB decreased in the intestine to finally reach 38.8 ± 0.4% after 245 minutes of digestion. Indeed, at the end of the digestive process, the IC50 of SBH and SB were 25.2 μg·mL⁻¹ and 414.9 μg·mL⁻¹ respectively.

3.4. Identification of ACE inhibitory peptides involved

3.4.1. Size exclusion chromatography

The first separation dimension used to identify peptides involved in the ACE inhibitory activity observed for SBH was size...
exclusion chromatography using as Superdex Peptide 10/300 GL column. Five fractions were designed as illustrated in the chromatogram presented in Fig. 3. The ACE-inhibitory activities of the fractions were then assessed and their respective IC_{50} were determined. Results showed that at a concentration of 71.4 μg·mL^{-1}, only F2, F3 and F4 fractions exerted an ACE-inhibitory activity. At the concentration assayed, F3 and F4 fractions only showed significantly higher activity than SBH before separation with 70.9 ± 1.5% and 76.6 ± 1.8% of inhibition, respectively. The IC_{50} value obtained for the fraction F4 was 0.007 μg·mL^{-1} (Table 3). The fraction F4 represented 6.5% of the total area under the absorbance curve (Fig. 3). Regarding the obtained results, fraction F4 was then submitted to LC/MSMS analysis.

3.4.2. Identification of ACE-inhibitory peptides
The base peak profile of the F4 fraction obtained in positive RP-HPLC/ESI-MS is presented in Fig. 4A. De novo sequencing by MS/MS spectrometry was performed to determine the amino acid sequence of majority peptides. Fig. 4B presents an example of MS/MS spectrum obtained for Val-Tyr peptide identified in the F4 fraction. This fraction contained a large majority of di- and tri-peptides. Using this methodology, the 17 most relative abundant peptides (representing around 50% of base peak area of the chromatogram) were identified and presented in Table 4.

3.5. In vivo experiments
3.5.1. Acute toxicity study
No toxicity (no death, no body weight loss and no abnormal behaviour) and no abnormality of organs at autopsy (form, size and colour) were observed after the single oral administration of SBH at the limit dose of 2 g·kg^{-1} (data not shown).

3.5.2. Antihypertensive activity
Only 2 out of the 32 SHR rats were excluded because of unachieved measurements. ANOVA showed a significant effect of treatments on SBP of SHR rats (F[3;26] = 8.29; P = 0.0005). Captopril at the tested dose of 70 mg·kg^{-1} and SBH, at both doses used, induced significant decreases of SBP. The maximum antihypertensive effect was observed with Captopril, with a mean SBP difference of 38 mm Hg in comparison with the vehicle. When administered at the doses of 400 or 800 mg·kg^{-1}, SBH had similar significant effects on SBP with respective mean decrease of 19 and 23 mm Hg in comparison with the vehicle (Fig. 5).

4. Discussion
Here we report for the first time that a land snail (H. aspersa) by-product hydrolysate obtained from hepatopancreas (SBH) was able to highly inhibit in vitro ACE activity with an IC_{50} of.
23 μg·mL⁻¹. We demonstrated that this activity was maintained after in vitro human gastrointestinal digestion simulation, and further, that SBH was not only safe for intestinal cells but also stimulated their metabolism. Very interestingly, we demonstrated that at the end of the gastrointestinal digestion, the ACE inhibitory capacity of the snail by-product used as control that did not undergo prior hydrolysis was lower than for SBH with respective IC₅₀ of 414.9 and 25.2 μg·mL⁻¹. This observation highlights the huge impact of the hydrolysis process of SBH on the ACE inhibitory activity of the final peptides reaching the gut after gastrointestinal digestion. The SBH fractionation evidenced that almost all the ACE inhibitory activities were recovered in the F4 fraction with an IC₅₀ value of 0.007 μg·mL⁻¹, representing a 3000 fold decrease compared to SBH IC₅₀ obtained before size exclusion, which was very close to that of Captopril (0.005 μg·mL⁻¹). The LC/MS/MS analysis allowed the identification of the 17 most abundant peptides of the fraction. Interestingly, 7 of them are known ACE inhibitory peptides (YG, YA, VY, SF, FG, GF and VW). The most active are VY and VW characterised by an IC₅₀ of 7.1 and 1.4 μM, respectively (Saito, Wanezaki, Kawato, & Imayasu, 1994). Moreover, the aptitude of Val-Tyr as natural dipeptide to be absorbed into the human circulatory blood system was demonstrated (Matsui et al., 2002).

In vivo studies showed that SBH reduced hypertension by 20 mm Hg in SHR rats after a single oral administration of both doses of 400 and 800 mg·kg⁻¹. Among the very abundant literature on antihypertensive effects of peptides derived from dietary and by-product proteins, few studies are interested in the protein raw material from invertebrates and more especially from molluscs. However, some studies have investigated...
antihypertensive effects of hydrolysates obtained from bivalve protein. Thus, Wang et al. (2008) evidenced antihypertensive peptide from oyster (Crassostrea talienwahensis Crosse), which reduced SBP in SHR rats. In the same way, Shiozaki et al. (2010) identified hypotensive peptide from oyster (Crassostrea gigas) hydrolysate. Moreover, Balti et al. (2015) evidenced nine peptides with ACE inhibitory activity from a mollusc cephalopod (Sepia officinalis) hydrolysate. Our results are also in accordance with previous studies that showed, in the same strain of SHR rats, that hydrolysates derived from milk or egg proteins, with ACE IC₅₀ ranging from 30 to 99 µg·mL⁻¹, produce antihypertensive effect in SHR ranging between −20 and −40 mm Hg after a single oral administration at such high doses (Contreras, Carrón, Montero, Ramos, & Recio, 2009; Miguel, Contreras, Recio, & Aleixandre, 2009; Miguel, Lopez-Fandino, Ramos, & Aleixandre, 2005). Interestingly, Yamada et al. (2013) showed that a single oral administration of a hydrolysate from bovine caseins, with an ACE IC₅₀ of 74 µg·mL⁻¹, induced antihypertensive effects in SHR rats with lower doses ranging from 5 to 100 mg·kg⁻¹. On the contrary, Miguel et al. (2005) showed that an egg hydrolysate with an ACE IC₅₀ of 55 µg·mL⁻¹ induced significant antihypertensive effects in SHR rats only from the dose of 150 mg·kg⁻¹. Taken together, these reports underline the difficulty of predicting an in vivo minimal effective dose from ACE IC₅₀ values. This could be first explained by the variable bioavailability of the peptides involved in the bioactivity. Thus, ACE inhibitory peptides have to be resistant to gastrointestinal, microbial and intestinal brush barrier enzymes present in the gut and have to cross the intestinal barrier, which involves different mechanisms, to reach their target via the circulation. Three main pathways for intestinal absorption of bioactive peptides were described and obviously participated in bioavailability. Thus, the PEPT1 transporter is implicated in the main route for di- and tri-peptides, which represents a large proportion of ACE inhibitory peptides. For larger peptides, transcytosis and paracellular passive diffusion are the two pathways mainly implicated in the passage of the gut barrier (Daniel, 2004; Wada & Lönnerdal, 2014). Furthermore, other mechanisms than ACE inhibition could be the cause of the reduction of SBP observed. For example, Yamada et al. (2010) identified a tripeptide called rapakinin derived from rapeseed and exerting antihypertensive effect through ACE inhibitory activity but also through the dilatation of mesenteric artery of SHR rats via the prostaglandin IP receptor followed by CCK1 receptor.

In the present work, both doses used produced similar decreases of blood pressure, suggesting that the SBH may present some efficacy at lower doses. Moreover, the systolic blood pressure was measured 3 hours after oral treatment. However, several studies with SHR rats observed a progressive increase of the antihypertensive effect over a few hours after treatment with protein hydrolysates to reach a maximum effect after 4 to 6 hours. This phenomenon was observed by Kapel et al. (2006) with an alfalfa (Medicago sativa) white protein concentrate hydrolysate, which significantly lowered blood pressure of SHR rats at doses ranging from 100 to 700 mg·kg⁻¹. The maximum of inhibition (~29 mm Hg) was observed after 4 hours after a single oral administration of 500 mg·kg⁻¹ (Kapel et al., 2006). Thus, it is possible that the maximum antihypertensive effect of SBH for a given dose appears later than 3 hours after oral treatment. Our preliminary results are to be completed to fully explore the pharmacodynamics of SBH on hypertension in the goal to further valorise it as ingredient in functional food to prevent or fight hypertension.

Table 4 – Most abundant peptides identified by ESI-MS/MS analysis.

<table>
<thead>
<tr>
<th>Retention time (min)</th>
<th>Peptide mass (Da)</th>
<th>Sequence</th>
<th>Relative abundance (% of base peak area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.32</td>
<td>288.4</td>
<td>RL</td>
<td>2</td>
</tr>
<tr>
<td>6.61</td>
<td>269.4</td>
<td>YS</td>
<td>4</td>
</tr>
<tr>
<td>6.89</td>
<td>239.2</td>
<td>YG</td>
<td>4</td>
</tr>
<tr>
<td>6.89</td>
<td>253.3</td>
<td>YA</td>
<td>3</td>
</tr>
<tr>
<td>7.74</td>
<td>166.0</td>
<td>F</td>
<td>1</td>
</tr>
<tr>
<td>7.74</td>
<td>219.2</td>
<td>LS</td>
<td>4</td>
</tr>
<tr>
<td>9.57</td>
<td>189.3</td>
<td>LG</td>
<td>3</td>
</tr>
<tr>
<td>11.57</td>
<td>281.2</td>
<td>VY</td>
<td>2</td>
</tr>
<tr>
<td>12.35</td>
<td>253.3</td>
<td>SF</td>
<td>6</td>
</tr>
<tr>
<td>13.06</td>
<td>223.3</td>
<td>FG/GF</td>
<td>5</td>
</tr>
<tr>
<td>13.94</td>
<td>281.3</td>
<td>DF</td>
<td>1</td>
</tr>
<tr>
<td>1394</td>
<td>313.3</td>
<td>YM</td>
<td>1</td>
</tr>
<tr>
<td>19.75</td>
<td>304.3</td>
<td>VW</td>
<td>4</td>
</tr>
<tr>
<td>19.75</td>
<td>329.5</td>
<td>YF</td>
<td>2</td>
</tr>
<tr>
<td>22.52</td>
<td>279.4</td>
<td>FL</td>
<td>2</td>
</tr>
<tr>
<td>24.11</td>
<td>313.5</td>
<td>FF</td>
<td>2</td>
</tr>
<tr>
<td>24.11</td>
<td>318.4</td>
<td>LDA</td>
<td>2</td>
</tr>
</tbody>
</table>

Sequences that appear in bold are known as ACE inhibitory peptides.

Fig. 5 – Effects of Captopril (70 mg·kg⁻¹ body weight) and SBH (400 and 800 mg·kg⁻¹ body weight) on SBP in SHR rats, 3 hours after a single oral administration. Data are presented as means ± SEM. *p < 0.05: Significant difference with vehicle group (Dunnett’s test).

5. Conclusion

This work evidenced the antihypertensive effect of a hydrolysate obtained from a land snail by-product hydrolysate in SHR rats. This effect could be partially explained by the high capacity of this hydrolysate to inhibit ACE activity in vitro, which was maintained after gastrointestinal digestion. The
fractionation of this hydrolysate revealed that the peptides involved in ACE inhibitory activity are predominately di-peptides. This observation was sustained by LC/MS/MS analysis, which led to the identification of 7 known ACE-inhibitory peptides such as Val-Tyr and Val-Trp. Moreover, SBH stimulates human epithelial intestinal cell metabolism in vitro and is not toxic for Wistar rats. Thus, SBH appears to be very promising as a new ingredient for functional foods for the prevention and/or the treatment of hypertension. Moreover, these findings show evidence of a new way to valorise the processing waste of H. aspersa that represents an important raw material mainly in European countries.

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REFERENCES


