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Production, in continuous enzymatic membrane reactor, of an anti-hypertensive hydrolysate from an industrial alfalfa white protein concentrate exhibiting ACE inhibitory and opioid activities

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Abstract

A few hydrolysates of food proteins were reported to exert an antihypertensive effect in vivo due to angiotensin I converting enzyme (ACE) inhibitory peptides or opioid peptides. An industrial alfalfa white protein concentrate (AWPC) hydrolysate, produced on a pilot plant scale, strongly inhibited ACE with an IC50 value of 8.8 μg/ml. Furthermore, the AWPC hydrolysate inhibited the spontaneous contraction of rabbit ileum in a dose-dependent and naloxone-blocked manner, indicating the presence of opioid peptides. The AWPC hydrolysate significantly lowered blood pressure of spontaneously hypertensive rats (SHR) more than 6 h after a single oral administration. The maximum reduction of systolic blood pressure (-29.9 ± 2.0 mmHg) was observed 4 h after an oral administration of 0.5 g/kg hydrolysate dosage. The minimal anti-hypertensive effect was observed for 0.1 g/kg dosage. The AWPC hydrolysate may then be particularly suitable as nutraceutical for functional food useful in the prevention of hypertension.

Keywords: Anti-hypertensive effect; Opioid activity; ACE inhibitory activity; Food protein hydrolysate; Alfalfa; Continuous membrane enzymatic reactor

1. Introduction

During the past decade, many bioactive peptides derived from various food proteins were characterized. Biological activities reported were immunomodulating (Mercier, Gauthier, & Fliss, 2004), opioid-like (Schlimme & Meisel, 1995), mineral carrier (Sato, Naguchi, & Naito, 1986) diazepam like (Bernet, Montel, Noël, & Dupouy, 2000), antimicrobial (Tossi, Sandri, & Giangaspero, 2000) or anti-hypertensive activities (Marczak et al., 2003). These bioactive peptides are latent in the primary sequences of proteins and may be released in the course of gastro-intestinal-like proteolysis (Tauzin, Miclo, & Gaillard, 2002), food processing such as cheese ripening (Ryhänen, Pihlanto-Leppälä, & Pahkala, 2001) and fermentation (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000). These peptides may act locally on different target sites present in the gastrointestinal

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tract, or be absorbed intact, to produce systemic effects. In this last case, peptides must contain only a few amino acid residues to cross the small intestine epithelium (Meisel, 1997). Interestingly, hydrolysates containing bioactive peptides may also be generated by controlled food protein hydrolysis carried out in an enzymatic reactor, to produce nutraceuticals, suitable as ingredients in functional food (Byun & Kim, 2001)

Hypertension is a major public health problem since it causes cardiovascular diseases and hard renal failure. Recently, hydrolysates derived from rapeseed (Marczak et al., 2003) and bonito (Fujita, Yokoyama, Yasumoto, & Yoshikawa, 1995) were reported to reduce the systolic blood pressure of spontaneously hypertensive rats. Furthermore, clinical studies showed that milk-derived protein hydrolysates exert significant blood pressure-lowering effects on hypertensive humans (Seppo, Jauhiainen, Poussa, & Korpela, 2003). The anti-hypertensive effect of those hydrolysates in vivo is often explained by the presence of angiotensin I converting enzyme (ACE) inhibitory peptides (Fujita et al., 1995; Seppo et al., 2003) since ACE both converts inactive angiotensin I into potent vasoconstrictor octapeptide angiotensin II and degrades the vasodilatator peptide bradykinin. Alternatively, anti-hypertensive effect can also be achieved by means of a mechanism involving peptide fixation to blood vessel opioid receptor. Indeed, α-lactorphin, an opioid like peptide derived from α-lactalbumin, was reported to have a naloxone reversible anti-hypertensive effect in spontaneously hypertensive rats (Nurminen et al., 2000).

Alfalfa (*Medicago sativa*), a plant abundantly cultivated in Europe, produces around 2600 kg of protein per hectar (Mauriès, 1994). This constitutes the greatest protein yield observed beyond classical cultivated plants, such as wheat, corn or soy. Alfalfa leaf proteins consist of around 50% "white" hydrophilic proteins and 50% "green" lipophilic proteins. White proteins are very valuable in the field of human nutrition because of their high digestibility, equilibrated aminogram, great functional properties and vegetal origin. Alfalfa leaf white proteins are constituted of about 65% ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, E.C. 4.1.1.39), commonly considered as the most abundant protein in the world (Ellis, 1979). Recently, two opioid-like peptide sequences were characterized from spinach RuBisCO large subunit (Yang et al., 2001) and numerous ACE inhibitor sequences have been identified.

We have recently reported a continuous proteolysis of an industrial alfalfa white protein concentrate (AWPC) in a membrane enzymatic reactor on a pilot plant scale (Prevot-D'Alvise et al., 2004). In the present work, the industrial AWPC and pilot plant scale AWPC hydrolysate were characterized. The presence of ACE inhibitory peptides and opioid peptides were then investigated in the AWPC hydrolysate in vitro before assaying its anti-hypertensive effect in vivo in spontaneously hypertensive rats (SHR).

2. Materials and methods

2.1. Materials

All common chemicals were of analytical grade from commercial sources. Delvolase®, provided from DSM food specialties (Seclin, France), is an industrial protease mixture, from *Bacillus licheniformis*, exhibiting a Subtilisin Carlsberg-type activity (E.C 3.4.21.62). The opioid receptor blocker naloxone, angiotensin I converting enzyme (ACE, E.C 3.4.15.1) extracted from rabbit lung and the ACE synthetic substrate histidyl-hippuryl-leucine (HHL) were purchased from Sigma Chemicals. Acetonitrile was of HPLC grade. Water was obtained from a Culligan system; the resistivity was approximately 18 MΩ. The industrial AWPC was a gift from Viridis (Aulnay aux Planches, France).

2.2. Pilot plant scale AWPC hydrolysate production

The pilot plant scale AWPC hydrolysate was produced according to the process previously described with slight modifications (Prevot-D'Alvise et al., 2004). 3% (w/v) AWPC hydrolysis was carried out in a 32-l continuous reactor, thermostatted at 40 °C, coupled with two tubular ultrafiltration modules containing 10 kDa nominal molecular weight cut-off mineral ZrO_2 membranes (6 mm inner diameter, 1.2 m long, 0.16 m^2 filtering area, Carbosep M5). Volume, pH and temperature were kept at 32 l, 9.5 and 40 °C, respectively, by means of an automat (Setric genie industriel, France). The space time of 8 h, chosen to get small peptides, was obtained by holding the filtrate flow at 4 l/h. The filtrate was then eluted at pH 5.3 at a 0.33-l/min flow rate on a pilot plant scale column of BPG 100/950 (Amersham biosciences, Uppsala, Sweden) fitted with an anion-exchanger polystyrene resin (Amberlite IRA 900 Cl Rhöm & Haas, Germany) to remove polyphenolic coumponds. The discoloured filtrate was then desalted by electrodialysis (EUR2D-5P11, Eurodia industrie S.A, Wissous, France) at a constant 70 V voltage and spray-dried (Niro atomizer minor production type, Rueil, France) at a 10-l/h flow rate with a 170 °C inlet temperature and a 90 °C outlet temperature.

2.3. Moisture

About 1.5 g of spray dried AWPC and spray dried AWPC hydrolysate were placed in an infra-red automatic desiccator balance and warmed at 110 °C. The moisture was noted after weight stabilization. The result was the weight ratio, after and before desiccation,

expressed as a percentage. Each measure was carried out three times.

2.4. Protein content

Protein content, in the spray-dried AWPC and AWPC hydrolysate, was expressed as total nitrogen determined by the Kjeldahl procedure (Gerhardt Vapodest 3 automatic). The 6.25 factor was used to convert nitrogen into protein as commonly seen for alfalfa proteins. Each measure was carried out three times.

2.5. Molecular weight distribution

Spray-dried hydrolysate weight distribution was determined by fast pressure liquid chromatography (FPLC) on a Superdex Peptide HR 10/300 (10×300 mm) column (Amersham biosciences, Upsalla, Sweden). 100 μl of hydrolysate at peptidic concentration of 10 g/l in 30% acetonitrile, 0.1% TFA, were eluted with the same solution on the column connected to a FPLC pump (Pharmacia LKB P-500) at a flow rate set to 0.2 ml/min. The absorbance was recorded on line at 226 nm by means of a UV detector (Pharmacia LKB Uvicord II).

2.6. In vitro ACE inhibition assay

The enzymatic inhibition assay used was reported by Nakamura et al. (1995). 200 μl 5 mM HHL in 100 mM sodium borate, 300 mM NaCl, pH 8.3, were mixed with 80 μl of inhibitors in the same buffer and preincubated at 37 °C for 3 min. The reaction was then initiated with 20 μl of 0.1 U/ml ACE in the same buffer and incubated for 30 min at 37 °C. The reaction was stopped by addition of 250 μl 0.1 M HCl. Released hippuric acid was quantified by RP-HPLC onto a Vydac C18 column connected to a system constituted of a Waters TM 600 automated gradient controller pump module, a Water Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 446 computer. Millennium$^{3.2}$ software was used to plot, acquire and analyse chromatographic data.

2.7. In vitro morphinomimetic effect assay

Albino rabbits ($n = 3$) weighing from 1.5 to 2.0 kg were killed. Segments of the ileum were immediately removed and placed in a Tyrode solution of the following composition (mM): NaCl (137), KCl (2.6), $MgSO_4$ (1.05), NaH_2PO_4 (0.47), $NaHCO_3$ (11.9), $CaCl_2$ and glucose (5.5). The tissues were cleared of intestinal content, cut into 1.5–2 cm segments and attached so as to leave the lumen open to the bathing solution. They were then suspended in an organ bath (60 ml) containing oxygenated (95% O_2 and 5% CO_2) Tyrode solution kept at 37 °C. The fragment were stretched to a sufficient tension and equilibrated for at least 30 min before starting experiments. The changes of tension due to natural peristaltism were recorded by a Biopac computerized system (NORTEK, France). The concentration-response to hydrolysate was achieved by adding 500 μl of hydrolysate solutions to get concentrations of 0.67, 1.0, 1.34, 2.0 and 2.67 mg/ml. After each assay, tissues were washed with fresh Tyrode and equilibrated for around 10 min. The fixation to opioid receptors was assayed by adding hydrolysate at a 2-mg/ml concentration to an organ bath containing 1 μM naloxone.

The statistical analysis of results was carried out using ANOVA, followed by Dunnett's test. The unpaired Student's *t*-test was also used when appropriate. $P < 0.05$ was considered significant.

2.8. In vivo antihypertensive effect in spontaneously hypertensive rats (SHR)

The protocol to study the in vivo antihypertensive effect in SHR was previously described by Matsui et al. (2002). 5 month-old male SHRs, weighing around 400 g, were grown up in a light-controlled room (light period from 7 a.m. to 7 p.m.) with rat chow and tap water available ad libitum. SHRs were randomly divided into two groups of six rats. The assay group received a daily oral ingestion of 500 μl peptidic hydrolysate in distilled water at a 0.5-g/kg dose at 9 a.m. The control group received 500 μl of distilled water under the same conditions. The systolic blood pressure (SBP) was measured by means of the tail cuff method, using a LE5001 blood pressure meter (LETICA, France), 0, 2, 4 and 6 h after the oral administration. Before each measure, SHRs were placed in a 40 °C thermoregulated chamber for 10 min. Manipulation of the animals (rats and rabbits) was performed following the principles of laboratory animal care published by the French Ethical Commitee and the rules of the European Union Normative (86/609/EEC).

3. Results and discussion

3.1. Characterization of the industrial alfalfa white protein concentrate

Alfalfa is harvested, ground and pressed to get a heterogeneous green juice containing "green" lipophilic protein, associated with cellular membrane and the "white" hydrophilic protein. This green juice is heat-treated to precipitate the green protein fraction. The green protein precipitate is then separated by decantation and clarification steps, giving a brown juice principally composed of white proteins, minerals, phenolic

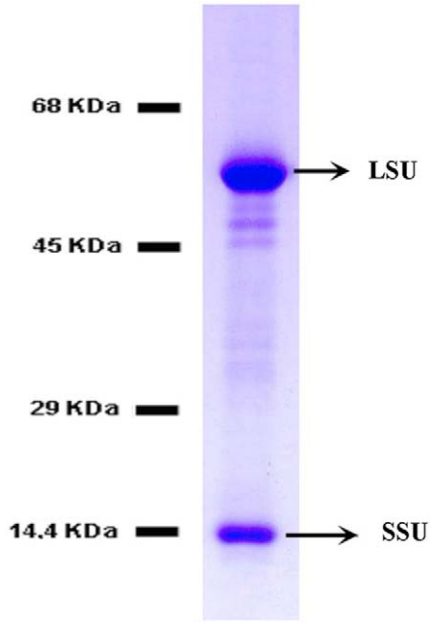

Fig. 1. 10%–25% acrylamide gradient SDS-PAGE profile of alfalfa white protein concentrate. This concentrate was obtained after the industrial process described in Section 2. LSU and SSU mean Rubisco large and small subunits, respectively. This profile is representative of several others that were carried out. Molecular weight markers are shown in the margin.

compounds and sugars. White proteins are then precipitated around pH 4, concentrated, diafiltered with water by tangential microfiltration and spray-dried. The final product is a (92% (w/w) moisture) powder containing 82% (w/w) protein (determined by nitrogen dosage with the Kjeldahl analysis). The SDS-PAGE profile of AWPC presented in Fig. 1 indicates the presence of two massive bands at 14,500 and 55,000 Da, corresponding to small and large RuBisCO subunits, respectively, and a few light bands around 50,000 Da, that may be identified as other white proteins. RuBisCO is reported to constitute 65% of the total white proteins in alfalfa leaf juice (Ellis, 1979). Interestingly, the figure shows that this ratio is significantly higher in the industrial AWPC. This could be due to the heat treatment step of the industrial process, since RuBisCO was reported to be more resistant to thermal denaturation than other white proteins (Mangan & Jones, 1976).

3.2. Characterization of the pilot plant scale AWPC hydrolysate

The AWPC preparation was hydrolyzed at 37 °C, pH 9.5, by Delvolase® at an *E/S* ratio of 1/50 in a pilot plant scale continuous enzymatic membrane reactor, as previously reported (D'Alvise, Lesueur-Lambert, Fertin, Dhulster, & Guillochon, 2000). In our case, the space time used was 8 h, against 3 h in the previous study, in order to get smaller peptides in the size range of bioactive peptides. Polyphenolic compounds remaining were eliminated from ultrafiltered hydrolysate by elution on an exchange ion Amberlite column. The discoloured hydrolysate was then desalted by electrodialysis and spray-dried according to a pilot plant scale method previously described (Prevot-D'Alvise et al., 2004). The AWPC hydrolysate is a (92% (w/w) moisture) powder containing 87% (w/w) peptides (determined by nitrogen dosage with the Kjeldahl analysis). The molecular mass of peptides was evaluated by fast protein liquid chromatography on an exclusion size Superdex peptide HR 10/30 column. The elution chromatogram at 226 nm presented in Fig. 2 shows that the hydrolysate is composed of 6 peptidic fractions evaluated after calibration around 3000, 1400, 700, 450 and 300 Da. Most of the AWPC hydrolysate peptides are then small peptides consisting of 2–15 amino acids. This is in agreement with the poor specificity of enzyme (Subtilisin Carlsberg-like activity) and the large space time used. The opioid peptides and ACE inhibitor peptides, known to be responsible for in vivo anti-hypertensive effect, are in the size range of 2–15 amino acids. Furthermore, two opioid-like sequences, existing in alfalfa RuBisCO large subunit, were reported in spinach RuBisCO large subunit (Yang et al., 2001) and numerous ACE inhibitor sequences have been identified in

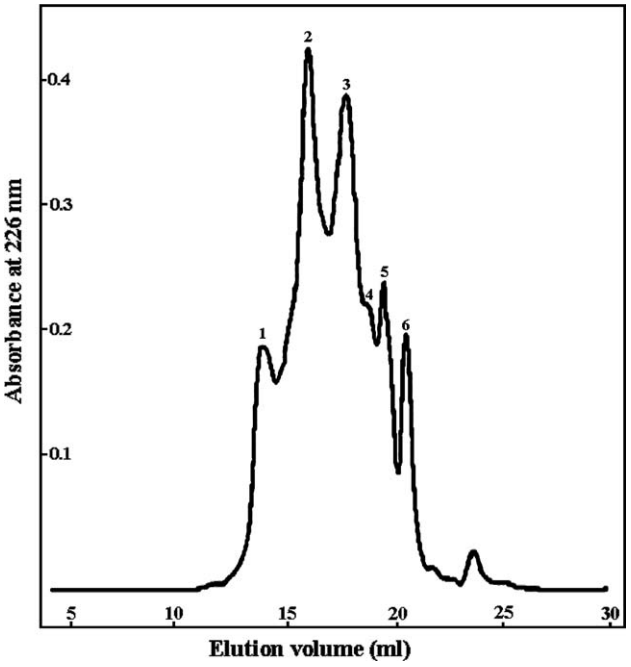

Fig. 2. FPLC gel filtration on Superdex peptide 10/30 column of industrial AWPC hydrolysate. 100 µl of hydrolysate sample at 1% (w/v) in acetonitrile 30% (v/v), 0.1% TFA (v/v), were eluted with the same eluent at a flow rate of 0.2 ml/min. The hydrolysate is obtained by treatment with Delvolase® in a continuous membrane reactor, decolourisation on exchange ion resin and desalting by electrodialysis. ACE inhibition and opioid activity were investigated with this hydrolysate.

both alfalfa RuBisCO subunits sequences. The AWPC hydrolysate was then assayed in vitro for these two biological activities.

3.3. In vitro ACE inhibition and opioid activity of AWPC hydrolysate

The methodology used was validated with the known ACE inhibitor, captopril. The IC50 value obtained after logarithmic linearisation (0.006 μM) was in agreement with the value of 0.007 μM previously published (Pihlanto-Leppälä, Rokka, & Korhonen, 1998). The ACE inhibition was quantified for 2.67, 5.3, 13.3 and 26.6 μg/ml hydrolysate peptidic concentrations. The ACE inhibition ratios for these concentrations were 32.2% ± 1.6, 41.4% ± 4.0, 54.5% ± 0.6 and 69.2% ± 0.3 ($n = 3$), respectively. As seen in Fig. 3, the IC50 value of the total hydrolysate obtained after logarithmic linearisation was 8.8 μg/ml. Such a strong ACE inhibition activity has never been previously reported for a food protein hydrolysate. Indeed, the greatest activities were observed for royal jelly (Matsui et al., 2002), rapeseed (Marczak et al., 2003), soy (Shin et al., 2001) or chickpea (Yust et al., 2003) protein hydrolysates with IC50 values of 99, 160, 180 and 276 μg/ml, respectively. We have then demonstrated, as expected after RuBisCO subunits sequence analysis, that numerous and/or strong ACE inhibitory peptides were released from AWPC in the course of Delvolase® hydrolysis on the pilot plant scale. After demonstrating, in vitro, the presence of a strong ACE inhibition, an opioid activity was also investigated. Indeed, as ACE inhibition, an opioid-like activity was also reported to exert an antihypertensive effect in vivo.

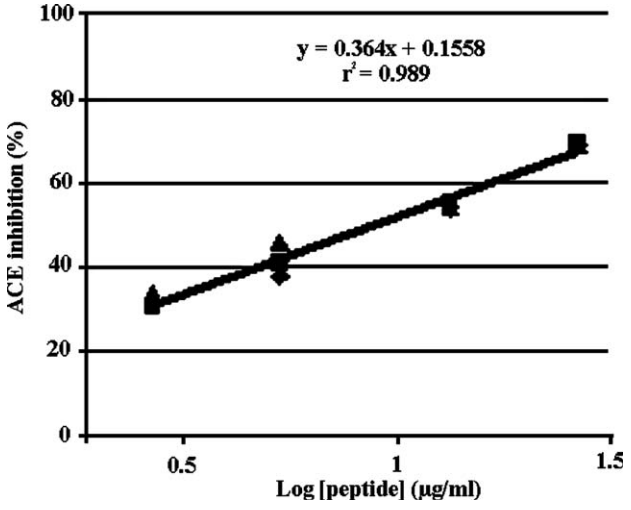

Fig. 3. IC50 value determination of the industrial AWPC hydrolysate obtained after pilot plant scale hydrolysis in a continuous membrane reactor, decolourisation and desalting. IC50 value was obtained after logarithmic linearisation. Each hydrolysate concentration was tested three times.

It is well known that rabbit ileum shows strong and stable spontaneous contraction activities. The spontaneous activity is no longer present when an opioid receptor agonist ligand, such as dynorphine, is added to the organ bath. Moreover, this relaxant effect can be antagonized by naloxone, an opioid receptor antagonist (Pheng, Calo, Guerrini, & Regoli, 2000). As depicted in Fig. 4, the AWPC hydrolysate, at a concentration of 2 mg/ml, shows a significant relaxant effect on rabbit ileum spontaneous contractions. Furthermore, this effect does not occur after preincubation with naloxone, indicating an interaction of compounds with opioid receptors. The hydrolysate was then assayed at different concentrations to determine a dose effect. Fig. 5 shows inhibition of spontaneous contraction intensity of 10.7% ± 2.88; 47.6% ± 11.2; 77.8% ± 4; 86.3% ± 0.49 and 90.6% ± 1.13 ($n = 4$) for hydrolysate concentrations

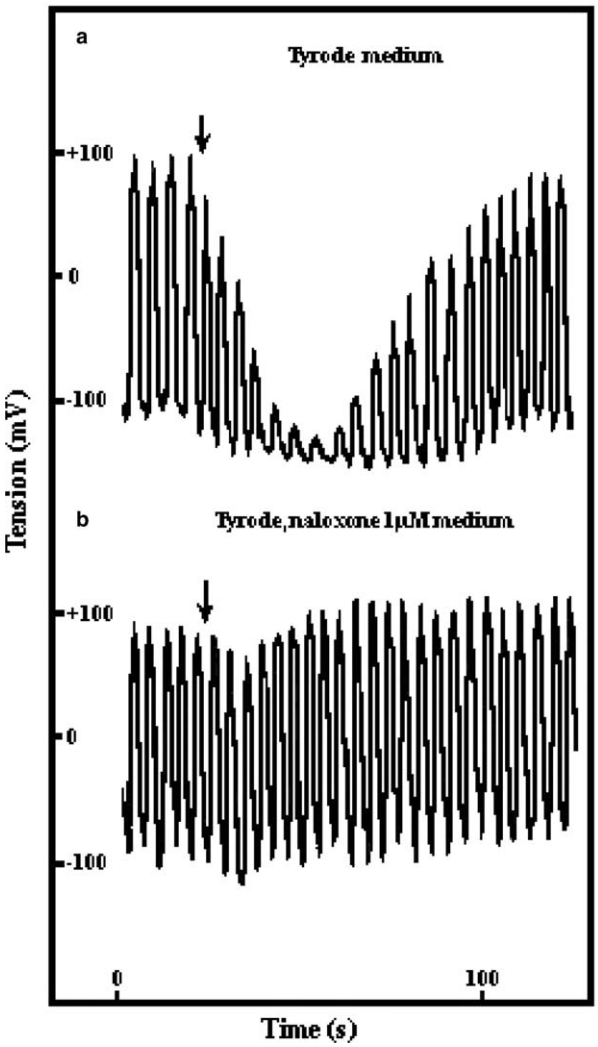

Fig. 4. Effect of the AWPC hydrolysate, at a concentration of 2 mg/ml, on spontaneous rabbit ileum contractions without (a) and with (b) preincubation with the opioid receptor blocker naloxone in the tyrode medium. The arrow indicates the addition of AWPC hydrolysate.

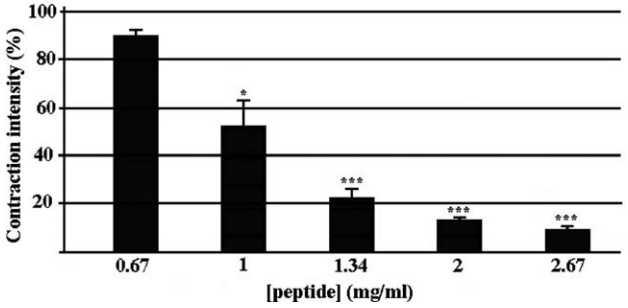

Fig. 5. Effects of different AWPC hydrolysate concentrations on spontaneous contractions of rabbit ileum expressed as percent of changes of contraction intensity of control (spontaneous contractions in clear tyrode medium). Values are means ± SEM ($n = 4$). $^{***}P < 0.001$, $^{*}P < 0.05$ indicate significant differences versus control.

of 0.67, 1, 1.34, 2 and 2.34 mg/ml, respectively, indicating a dose effect of hydrolysate. All together, these investigations clearly indicate the presence of opioid peptides in the AWPC hydrolysate.

We have then demonstrated that AWPC hydrolysate contained opioid and ACE inhibitory peptides well known to potentially exert anti-hypertensive in vivo. An anti-hypertensive effect of hydrolysate in vivo may then be expected. Furthermore, the hydrolysate was produced on a pilot plant scale without limiting amount. The anti-hypertensive effect in vivo was then investigated in spontaneously hypertensive rats (SHR).

3.4. In vivo effects of hydrolysate in SHRs

Anti-hypertensive activities of AWPC hydrolysate were investigated following an oral administration in SHR rats. As shown in Fig. 6, AWPC hydrolysate, administrated at an effective dose of 0.5 g/kg, lowered systolic blood pressure (SBP) for 6 h. Maximal reduction of blood pressure occurred 4 h after the oral administration. Four hours after administration, the reduction

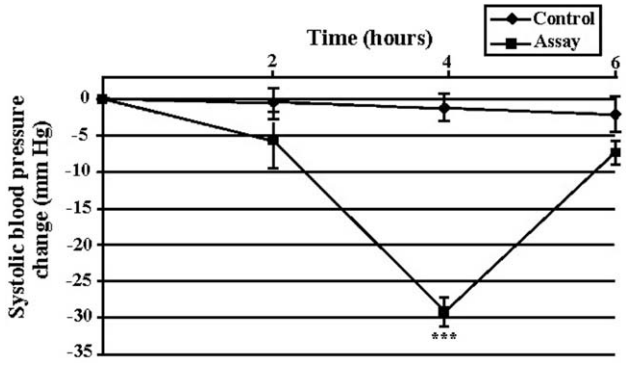

Fig. 6. Anti-hypertensive effect of the industrial AWPC hydrolysate after an oral administration of 0.5 g/kg to 20-week-old SHR. Changes of systolic blood pressure after administration are expressed as means ± SEM. $^{***}P < 0.001$ ($n = 6$) indicate significant differences versus controls.

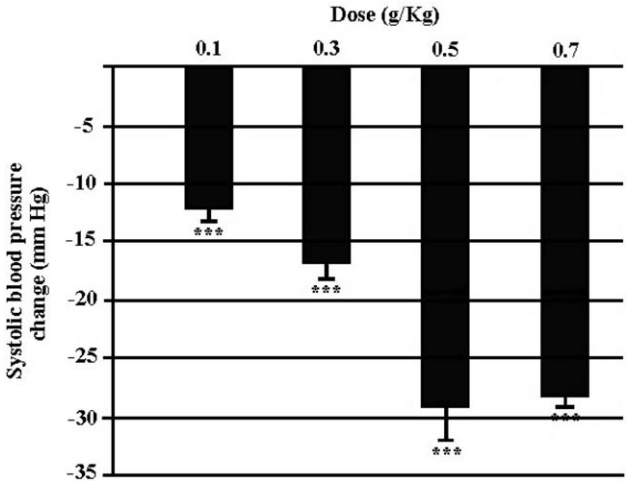

Fig. 7. Anti-hypertensive effects of different AWPC hydrolysate dosages, 4 h after the oral administration, expressed as percent of changes of systolic blood pressure of control SHRs. Data are expressed as means ± SEM. $^{***}P < 0.001$ ($n = 6$) indicate significant differences versus controls.

of SBP observed reached the value of 29.10 mmHg ± 2.06 ($P < 0.001$). In comparison, at the same dose of a rapeseed hydrolysate (Marczak et al., 2003), the reduction of SBP was only of 15.5 mmHg. For a higher dose of royal jelly hydrolysate (1 g/kg), the reduction was 22.7 mmHg (Matsui et al., 2002). The anti-hypertensive effect of our hydrolysate is greater than numerous other hydrolysates previously described. Then, the reduction of SBP was investigated 4 h after administering doses of 0.1, 0.3, 0.5 and 0.7 g/kg. Fig. 7 shows that AWPC hydrolysate displayed a dose-dependent anti-hypertensive effect. Moreover, this effect is significant, even at 0.1 g/kg dosage (reduction of 12 mmHg ± 1.1, $P < 0.001$). The maximum effect was obtained with a 0.5-g/kg dosage.

4. Conclusion

Alfalfa is a source of vegetal protein that is particularly attractive in human nutrition. Indeed, alfalfa white proteins offer some attractive functional and nutritional properties. Furthermore, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), known to constitute around 65% (w/w) of the total alfalfa white proteins contains opioid and ACE inhibitory sequences, considered to potentially generate anti-hypertensive effects in vivo. In this work, the massive presence of RuBisCO (up to 65%) was demonstrated in an alfalfa white protein concentrate (AWPC) produced on an industrial scale. We carried out a controlled pilot plant scale process of industrial AWPC hydrolysis adapted to the production of peptides in the size range of ACE inhibition and opioid activity. These two biological activities were tested in vitro in the total AWPC hydrolysate.

Our results revealed the presence of opioid peptide and a potent ACE inhibition activity with an IC_{50} value of 8.8 μg/ml. The anti-hypertensive effect of the industrial AWPC in vivo was then investigated in spontaneously hypertensive rats (SHR). The results showed an anti-hypertensive effect, significantly much more potent than those of other anti-hypertensive hydrolysates of food proteins. Furthermore, the AWPC hydrolysate was reported in a previous study (Prevot-D'Alvise et al., 2004) to offer some good nutritional properties as equilibrated aminogram. Thus, the AWPC hydrolysate appeared to be particularly suitable as a nutraceutical for functional food in the prevention of hypertension. The next step of our investigations will be to extract and characterize the bioactive peptides responsible for ACE inhibition and opioid activity.

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