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ORIGINAL CONTRIBUTION

Preventive and treatment effects of a hemp seed (*Cannabis sativa* L.) meal protein hydrolysate against high blood pressure in spontaneously hypertensive rats

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Abstract
Purpose This work determined the ability of hemp seed meal protein hydrolysate (HMH)-containing diets to attenuate elevated blood pressure (hypertension) development in spontaneously hypertensive rats (SHRs). Effects of diets on plasma levels of renin and angiotensin I-converting enzyme (ACE) in the SHRs were also determined.
Methods Defatted hemp seed protein meal was hydrolyzed using simulated gastrointestinal tract digestion with pepsin followed by pancreatin, and the resulting HMH used as a source of antihypertensive peptides. The HMH was substituted for casein at 0.5 and 1.0 % levels and fed to young growing rats for 8 weeks (preventive phase) or adult rats for 4 weeks (treatment phase).
Results Feeding of young growing SHRs with HMH resulted in attenuation of the normal increases in systolic blood pressure (SBP) with an average value of ~120 mmHg when compared to the casein-only group of rats (control) with a maximum of 158 mm Hg ($p < 0.05$). Feeding adult rats (SBP ~145 mmHg) with same diets during a 4-week period led to significant ($p < 0.05$) reduction in SBP to ~119 mmHg in comparison with 150 mmHg for the control rats. Plasma ACE activity was significantly ($p < 0.05$) suppressed (0.047–0.059 U/mL) in HMH-fed rats when compared to control rats (0.123 U/mL). Plasma renin level was also decreased for HMH-fed rats (0.040–0.054 μg/mL) when compared to control rats that were fed only with casein (0.151 μg/mL).
Conclusions The results suggest that HMH with strong hypotensive effects in SHRs could be used as a therapeutic agent for both the prevention and treatment of hypertension.

Keywords Hemp seed meal · Protein hydrolysate · Spontaneously hypertensive rats · Systolic blood pressure · Plasma ACE activity · Plasma renin activity

Introduction

Hypertension or elevated blood pressure (BP), defined as systolic blood pressure (SBP) >140 mmHg or diastolic blood pressure (DBP) >90 mmHg, forms an important risk factor for the development of cardiovascular diseases [1, 2]. Hypertension is a major public health problem, and its global prevalence is increasing at an alarming rate affecting over 20 % of the adult population [3]. Worldwide prevalence of hypertension is estimated to affect as much as one billion individuals with approximately 7.1 million associated deaths per year [4]. BP is regulated by several mechanisms, but the most significant and widely studied is the renin–angiotensin–aldosterone system (RAAS). In the RAAS, kidney-secreted renin cleaves angiotensinogen to produce an inactive decapeptide called angiotensin 1 (AT-I). AT-I is then hydrolyzed by angiotensin I-converting enzyme (ACE) to produce a potent vasoconstrictor octapeptide called angiotensin II (AT-II). ACE also breaks

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down bradykinin (a vasodilator) to produce inactive fragments leading to increases in arterial BP [5]. Independent of ACE, chymase is an enzyme that also converts AT-I–AT-II, and these combined enzyme actions ultimately are responsible for regulating BP. Excessive activities of these enzymes could lead to BP elevation that leads to hypertension if left untreated and may progress into cardiovascular complications that sometimes result in death. Bradykinin achieves its vasodilation properties by binding to the β-receptor with an eventual increase in Ca^{2+} levels. The binding of bradykinin to β-receptors and the increase in Ca^{2+} level stimulate nitric oxide synthase (NOS) to convert L-arginine to nitric oxide (NO), another potent vasodilator. Therefore, the hydrolytic action of ACE on bradykinin to produce inactive fragments indirectly inhibits the production of NO.

Elevated BP is routinely treated using a combined therapy of antihypertensive drugs, such as captopril (C), lisinopril, enalapril, etc. [6]. However, these synthetic drugs are believed to have certain side effects, such as cough, taste disturbances, skin rashes, or angioneurotic edema, which limit their use in some patients such as pregnant women and the elderly who are easily susceptible to health complications. While there are many commercially available synthetic ACE inhibitors, only one known commercial renin inhibitor (Aliskiren) is available for human therapy [7]. Renin, an aspartyl protease, has been found to produce highly selective inhibition in RAAS by catalyzing the first and rate-limiting step that converts angiotensinogen to angiotensin I. This selective behavior makes renin a very difficult enzyme to inhibit due to its high substrate specificity [8]. Therefore, it has been suggested that research and development to find safer, innovative, and economical ACE and renin inhibitors from food-based sources are necessary for expanding hypertension treatment and prevention strategies [6]. Research has shown that some food proteins possess the ability to release both ACE and renin inhibitory peptides after enzymatic hydrolysis, exhibiting multifunctional properties [9]. Such peptides may serve as ingredients for functional foods or nutraceuticals and could be used as alternative or complementary treatment tools for reducing high BP. Therefore, bioactive antihypertensive peptides of food origin are increasingly gaining recognition as alternatives or compliments to synthetic drugs in hypertension therapy. Preliminary in vitro studies have shown that industrial hemp seed peptides possess both antioxidant [10–12] and antihypertensive properties [13]. The antioxidant and antihypertensive activities may be due to the presence of high levels of negatively charged amino acids for electron donation to reactive oxygen species and arginine for the production of NO, a vasodilating agent, respectively. The presence of superior amino acid profile in hemp seed proteins (principally identified as edestin and albumin) and high digestibility promotes their efficacy as a source of health-enhancing bioactive peptides [14]. Hemp seed proteins are currently sold for food product formulation in Canada mostly in the form of protein concentrates or hemp seed protein powders. Short-term (24 h) oral administration (200 mg/kg body weight) of hemp seed protein hydrolysate (HPH) to spontaneously hypertensive rats (SHRs) was shown to reduce SBP (−30 mmHg after 8 h) and was positively correlated with the in vitro ACE and renin inhibitions [13]. Having previously established the ability of a hemp seed hydrolysate to reduce SBP on a short-term basis, the primary objective of this study was to evaluate the ability of a hemp seed hydrolysate to attenuate hypertension (prevention) in growing SHRs during an 8-week feeding experiment. We also determined the BP-lowering effect (treatment) of the hydrolysate in SHRs with established hypertension during a 4-week secondary study while normotensive rats were used for comparison.

Materials and methods

Materials

Defatted coarse hemp seed protein meal (HPM, 25 % protein content) was a gift from Hemp Oil Canada (Ste. Agathe, Manitoba, Canada). Briefly, the hemp seed is mechanically pressed to extract oil, and the resulting product is the defatted hemp seed cake, which is then milled in a classifier milling system to the desired particle size. The milled powder is sifted using various screens to obtain products sold as high-value protein powders. The by-product that does not pass through the sieves is the HPM, which is normally considered a waste product. Renin enzyme and renin substrate were purchased from Cayman (Cayman Chemical, Ann Arbor, MI). Pepsin (from porcine gastric mucosa, EC 3.4.23.1 with activity ≥250 U/mg solid), pancreatin (from porcine pancreas; digests not less than 25 times its weight of casein in 60 min at pH 7.5 and 40 °C), *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), captopril, and rabbit lung ACE (E.C.3.4.15.1) were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of hemp seed protein isolate (HPI) and hemp seed meal hydrolysate (HMH)

HPI was prepared according to a previously described protocol [10]. Briefly, the HPM was extracted for 2 h at 37 °C with alkaline water (pH 10) followed by centrifugation (7,000*g* for 1 h at 4 °C). The supernatant was adjusted to pH 5.0 with 2 M HCl, centrifuged, and the precipitate was neutralized to pH 7.0 with 2 M NaOH

followed by freeze-drying to produce the HPI. The HPI powder was decolorized as previously described using acetone to remove residual fat and phenolics [10]. To prepare HMH, the HPM was directly hydrolyzed sequentially, first with 4 % (w/w, HPM protein basis) pepsin, (pH 2.0, 37 °C, and 2 h) and second with pancreatin (pH 7.5, 37 °C, and 4 h) to simulate gastrointestinal tract (GIT) digestion [13]. The digest was centrifuged, and the residue was discarded while the supernatant was lyophilized to produce HMH, which was stored at −20 °C until needed for further analysis. Protein content/peptide concentration was determined using the modified Lowry method [15].

Proximate composition

Proximate composition of HPI and HMH was analyzed according to the appropriate standard methods [16]. Crude protein content was determined as nitrogen content multiplied by 6.25.

Amino acid composition analysis

The amino acid profiles of the samples were determined using an HPLC Pico-Tag method, after samples were hydrolyzed with 6 M HCl according to the method of Bidlingmeyer et al. [17]. The cysteine and methionine contents were determined after performic acid oxidation [18], while the tryptophan content was determined after alkaline hydrolysis [19].

Experiment animals and feeding protocols

All rat experiments were performed according to protocols approved by the University of Manitoba Animal Care Protocol and Management Review Committee. The rat-feeding experiments were carried out as follows using SHRs or normotensive Wistar–Kyoto rats (NTRs) purchased from Charles River Laboratories (Montreal, PQ, Canada). In the first feeding experiment, 32 male SHRs (6-week old) were housed in the Animal Facility at the Richardson Centre for Functional Foods and Nutraceuticals under a 12-h day and night cycle at 22 °C. The rats were acclimatized by feeding ad libitum with a regular chow diet and tap water for 2 weeks. For feeding trial I, SHRs (now 8-week old) were randomly divided into 4 groups (similar average body weight and BP) of 8 rats each. The rat groups were given similar feed but with addition of hydrolyzed (HMH) and unhydrolyzed (HPI) hemp seed products to determine the ability of each diet to attenuate elevated BP development during the rapid growth phase (preventive effect). Table 1 shows the composition of the various diets. Baseline SBP was measured at the beginning of the feeding trial using the tail-cuff method as previously described [13]. The rats were then fed with their respective diets ad libitum for 8 weeks during which SBP, feed consumption, and body weight were measured weekly. At the end of the 8 weeks of feeding, 4 rats from each group were terminated followed by harvesting and weighing of body organs while blood was collected into heparinized tubes. The blood was centrifuged at 1,500*g* for 10 min to obtain plasma, which was then stored at −80 °C until needed for further analysis. All the remaining SHRs were then switched to the regular chow diet for 4 weeks to serve as a washout period and allow establishment of hypertension (SBP >140 mmHg). After the 4-week washout period, the rats (now 20-week old) were randomized (4 each) to the four diet groups described above to perform feeding trial II. Feeding (ad libitum) was conducted for 4 weeks with weekly SBP and body weight measurements to determine the ability to reduce high BP in established hypertension (treatment effect). At the end of the 4-week feeding period, all rats were terminated for blood and organ collection as described above. Feeding trial III involved the use of NTRs (20-week old), which were randomly assigned to the following 3 protein treatment groups with 6 rats per group: control diet (20 %, w/w casein); 1 % HMH diet (19 % casein + 1 % HMH); or 1 % HPI diet (19 % casein + 1 % HPI). Only one HMH dose was used for trial III because from the initial SHR experiment, the 1 % HMH was a more effective dose than the 0.5 % HMH in lowering SBP. The NTRs were allowed ad libitum access to their respective group feeds for 4 weeks during which body weight and SBP were measured weekly. The NTRs were also terminated at the end of 4 weeks with blood and organs collected and processed as indicated for the SHRs.

Table 1 Composition of experimental diets showing inclusion levels of hemp seed meal hydrolysate (HMH) and hemp seed protein isolate (HPI)

Ingredients (g)	Diet group			
	Control	0.5 % HMH[a]	1 % HMH[a]	1 % HPI[a]
Cornstarch	40.00	40.00	40.00	40.00
Casein	20.00	19.5	19.00	19.00
HMH	0	0.50	1.00	0
HPI	0	0	0	1.00
Maltodextrin	13.20	13.20	13.20	13.20
Sucrose	10.00	10.00	10.00	10.00
Soy oil + TBHQ	7.00	7.00	7.00	7.00
Fiber	5.00	5.00	5.00	5.00
Min mix	3.50	3.50	3.50	3.50
Vit mix	1.00	1.00	1.00	1.00
L-Cys	0.30	0.30	0.30	0.30

[a] Protein weight basis and results are presented on dry weight basis

Determination of in vitro ACE and renin inhibitory activities of HMH

The ability of HMH to inhibit in vitro ACE activity was determined by a spectrophotometric method using FAPGG as substrate [13], while renin inhibition was determined using the fluorescence spectrometry method [8].

Determination of plasma ACE activity

The ACE activity in the plasma was measured according to the spectrophotometric method using FAPGG as substrate [13] with slight modifications. Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5) was mixed with 20 µL plasma or ACE (final enzyme concentrations were 0.0313, 0.0625, 0.125, 0.25, 0.5 U/mL), and 200 µL of 50 mM Tris–HCl buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at 23 °C, and the result was expressed as $\Delta A\ min^{-1}$, which was plotted against ACE enzyme concentration to obtain a standard curve. Plasma ACE activity (U/mL) was obtained by linear regression using the standard curve.

Determination of plasma renin concentration

The plasma renin activity (concentration) was measured using the fluorometric method as previously reported [8] but with slight modifications as follows. Prior to the assay, renin was diluted with 50 mM Tris–HCl (pH 8.0), containing 100 mM NaCl (assay buffer) to give different concentrations (4.15, 8.3, 16.5, 33, 66, 132, and 250 µg protein/mL). Before the reaction, 20 µL of renin substrate and 160 µL assay buffer were added to the wells. The reaction was initiated by adding 10 µL plasma or each diluted renin solution to the wells of a 96-well microplate, which was shaken for 10 s to ensure proper mixing and then incubated at 37 °C for 15 min in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA). The fluorescence intensity (FI) was then recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm, and the results were expressed as $\Delta FI\ min^{-1}$. A standard curve was obtained by using linear regression from a plot of $\Delta FI\ min^{-1}$ versus renin concentrations. The $\Delta FI\ min^{-1}$ obtained for each plasma was used to calculate plasma renin concentration (µg/mL) from the regression equation.

Statistical analysis

All in vitro data were collected in duplicate or triplicate, while in vivo data were based on the number of rats used for each experiment per group. Data were subjected to one-way analysis of variance using Statistical Analysis System Software (SAS version 9.2, SAS Institute, Cary, NC). Significant differences were determined by Duncan's multiple range test and accepted at $p < 0.05$.

Results

Proximate and amino acid composition of HMH and HPI

Table 2 shows that the major differences in proximate composition were the higher protein content of HPI and higher ash (NaCl) content of HMH. Therefore, the diet protein was formulated by including HPI and HMH based

Table 2 Proximate compositions (as is basis) of hemp seed protein isolate (HPI) and hemp seed meal hydrolysate (HMH)

Parameter (%)	HPI	HMH
Moisture	4.67 ± 0.01	14.24 ± 0.23
Dry matter	95.33 ± 0.01	85.77 ± 0.23
Crude protein	95.22 ± 0.83	55.19 ± 0.10
Crude fiber	0.295 ± 0.23	0.42 ± 0.40
Fat	0.00 ± 0.00	4.03 ± 0.14
Ash	5.24 ± 0.33	30.31 ± 0.04
Sodium chloride	0.94 ± 0.06	22.35 ± 0.43

Results are presented as mean ± standard deviation of duplicate determinations

Table 3 Amino acid composition of hemp seed meal hydrolysate (HMH) and hemp seed protein isolate (HPI)

Amino acid	HPI	HMH
Asx	11.81	10.79
Thr	3.54	3.70
Ser	4.78	5.43
Glx	22.39	18.12
Pro	4.13	4.72
Gly	4.38	4.50
Ala	4.14	3.95
Cys	1.49	1.20
Val	4.14	5.17
Met	2.36	2.03
Ile	3.67	4.01
Leu	5.51	6.78
Tyr	3.09	3.61
Phe	3.66	4.50
His	2.65	2.96
Lys	2.96	3.93
Arg	13.91	2.11
Trp	1.39	12.56

on their respective protein contents such that total protein content (including casein) was 20 % for all the diets. Similarly, since the HMH had higher salt content, NaCl was added to the control and HPI diets in amounts that ensured equal concentrations across the diets. Table 3 shows the amino acid profiles of hemp seed products (HMH and HPI) used in this work with the major differences being the contents of arginine and tryptophan. Overall, the HMH had substantially higher total contents of hydrophobic (HAA) and aromatic (AAA) amino acids when compared to HPI.

HMH inhibition of ACE and renin activities

Prior to using the HMH for in vivo experiments, its inhibitory effects against ACE and renin enzymes were tested in vitro. Figure 1 shows that ACE was inhibited by 70 % in contrast to 35 % inhibition observed for renin at 1 mg/mL peptide concentration.

Antihypertensive effects of HMH

Figure 2a shows that the normal SBP of growing rats (feeding trial I) was maintained or significantly ($p < 0.05$) reduced by hemp seed protein (HPI) or protein hydrolysate (HMH) containing diets when compared to the casein-only diet during an 8-week feeding experiment. All the rats had similar SBP values one week after the beginning of the feeding experiment. However, by the third week, the rats on diet containing 1 % HMH had significantly ($p < 0.05$) reduced SBP when compared to the other diet groups. In the fourth week, SBP of the casein-only (control) group rose to 144 ± 1.25 mmHg and continued its increase up to a peak of

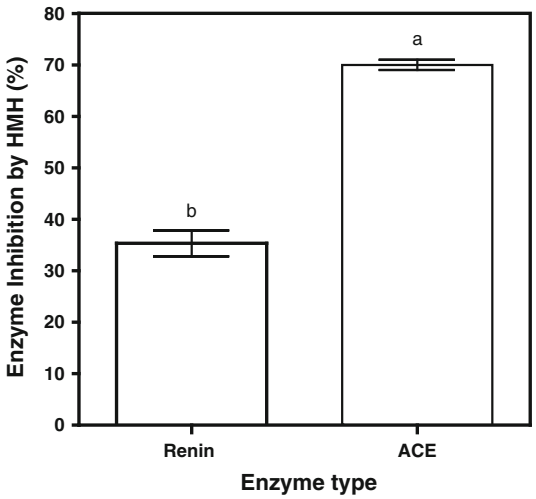

Fig. 1 In vitro ACE- and renin inhibitory activities of hemp seed meal hydrolysate (HMH) determined at 1 mg/mL peptide concentration. *Bars* with *different letters* have mean values that are significantly different ($p < 0.05$)

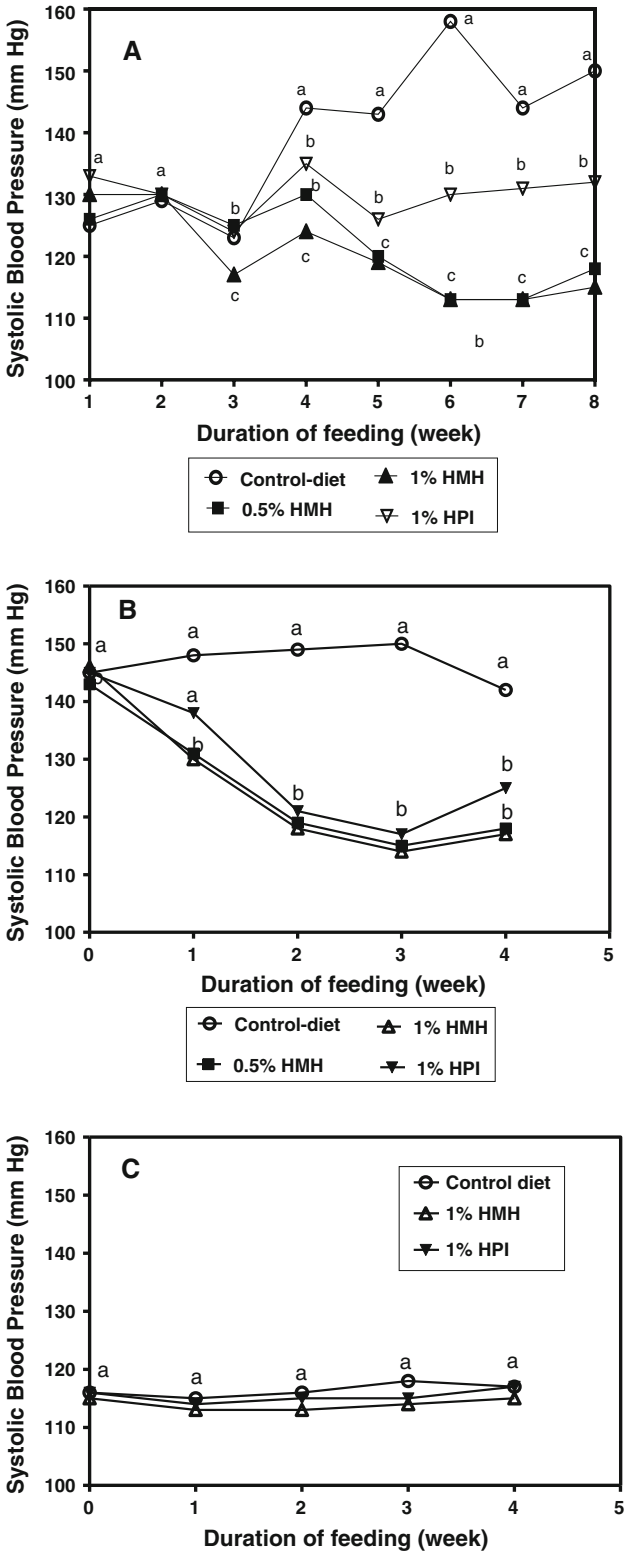

Fig. 2 Effects of casein-only diet or casein diet that contained hemp seed products on the systolic blood pressure (SBP) of **a** young growing spontaneously hypertensive rats (SHRs) from feeding trial I, **b** adult SHRs with established hypertension from feeding trial II, and **c** normotensive rats from feeding trial III. At each time point, differences in *letters* indicate that mean values are significantly different ($p < 0.05$)

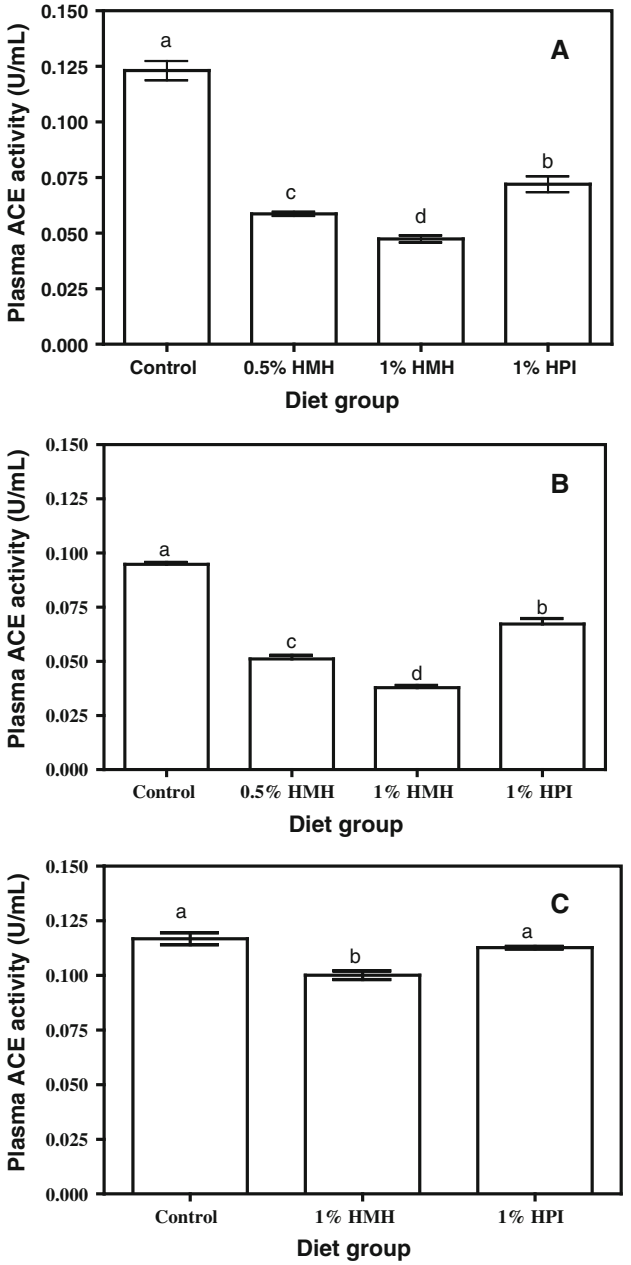

Fig. 3 Effect of the long-term feeding of **a** young growing spontaneously hypertensive rats (SHRs) (trial I), **b** adult SHRs with established hypertension (trial II), and **c** normotensive rats (trial III) with casein-only diet or casein diet that contained hemp seed products on plasma ACE activity. *Bars* with *different letters* have mean values that are significantly different ($p < 0.05$). Values are means ($n = 4$) ± SD

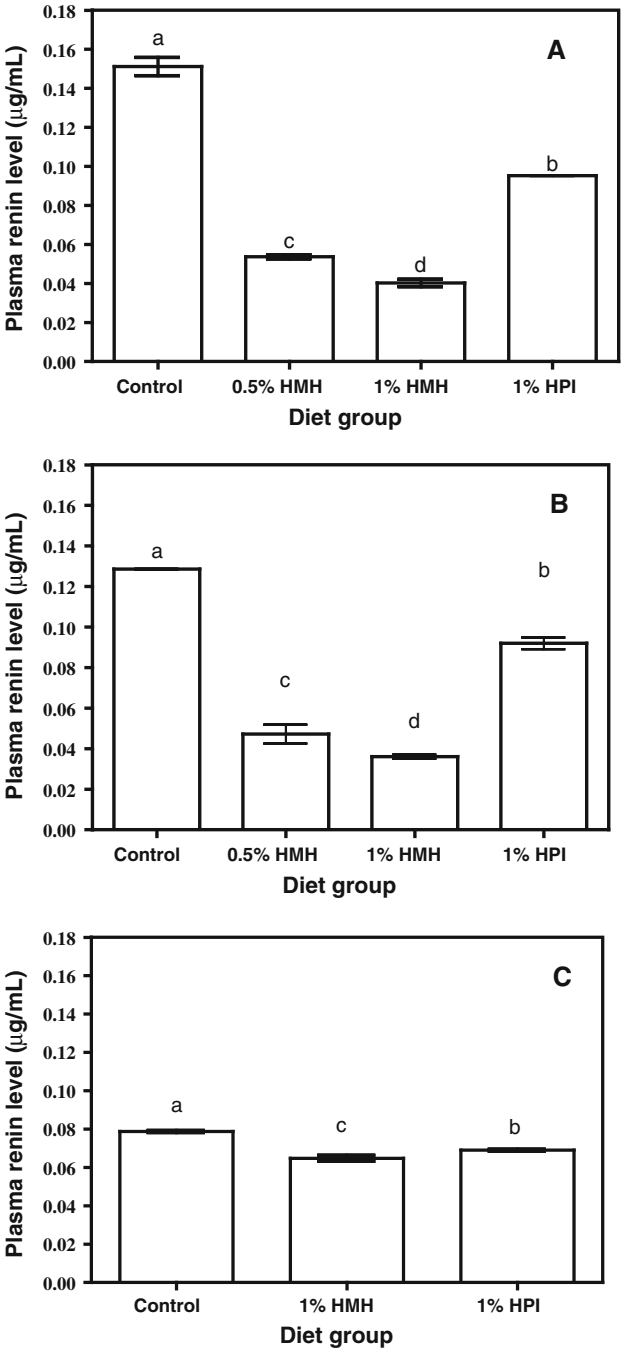

Fig. 4 Effect of the long-term feeding of **a** young growing spontaneously hypertensive rats (SHRs) (trial I), **b** adult SHRs with established hypertension (trial II), and **c** normotensive rats (trial III) with casein-only diet or casein diet that contained hemp seed products on plasma renin level. *Bars* with *different letters* have mean values that are significantly different ($p < 0.05$). Values are means ($n = 4$) ± SD

158 ± 1.09 mmHg, which corresponded to an 18 % increase in SBP from week one. The 1 % HMH diet lowered SBP to 117 ± 2.11 mmHg, which represented a 10 % decrease (−17 mmHg) from week one. In weeks 2–4, there were no significant ($p > 0.05$) differences in SBP-lowering effects of the HPI and the 0.5 % HMH-containing diet. However, from weeks 5–8, the diets containing HMH significantly ($p < 0.05$) reduced SBP more than the HPI.

After a 4-week washout period, the remaining rats were then randomized to the four diets and their SBPs were

monitored weekly for 4 weeks (feeding trial II). Figure 2b shows that the hemp seed products-containing diets produced the lowest SBP ($\sim$119 $\pm$ 2.21 mmHg) by week 3, which was about −31 mmHg less than the value obtained for rats on the casein-only diet (150 $\pm$ 0.13 mmHg). The 1 % HPI had similar reduction in SBP (−28 mmHg) as the HMH diets but only after 2 weeks. Figure 2c shows that unlike the data obtained for SHRs, the diets had no significant ($p > 0.05$) effect on SBP of NTRs over a 4-week period (feeding trial III).

Plasma ACE activity in SHRs and NTRs

The plasma ACE activity of SHRs from feeding trials I–III is shown in Fig. 3a–c, respectively. Plasma ACE activity in the SHRs was significantly ($p < 0.05$) lower (0.047 and 0.059 U/mL, for 0.5 and 1 %, respectively) after the 8-week HMH feeding trial when compared to HPI (0.072 U/mL) and the casein-only (0.123 U/mL) diets (Fig. 3a). In turn, the HPI-containing diet produced significantly ($p < 0.05$) lower SBP and plasma ACE activity when compared to rats fed with casein-only diet. After the 8-week study, the rats were placed on regular chow diet for 4 weeks (washout period) during which SBP increased to $\sim$145 mmHg before the rats were then used for the 4-week feeding trial II. A similar pattern of reduced plasma ACE activity as obtained during 8-week feeding period (prevention) was observed after the subsequent 4-week feeding period, which served as an intervention (treatment) to reduce elevated BP (Fig. 3b). The plasma ACE activity for the feeding trial II in SHRs fed with 0.5 and 1 % HMH was 0.038 and 0.051 U/mL, respectively, while the values were 0.067 and 0.095 U/mL, respectively, for HPI and casein-only fed rats. The plasma ACE activity in NTRs as shown in Fig. 3c was similar in magnitude for all diet groups; however, value was slightly lower in the 1 % HMH-fed rats (0.100 U/mL) in comparison with the 1 % HPI (0.112 U/mL) or casein-only group of rats (0.117 U/mL).

Plasma renin level in SHRs and NTRs

Figure 4a–c shows that the renin level in the plasma of SHRs is directly related to the antihypertensive effects of the diets. In the growing SHRs from feeding trial I (Fig. 4a), 8 weeks of feeding with experimental diets resulted in significant ($p < 0.05$) and dose-dependent suppression of plasma renin level in the HMH rat groups with values of 0.040 and 0.054 μg/mL, for 1 and 0.5 % diets, respectively. In contrast, significantly ($p < 0.05$) higher plasma renin level was obtained for rats that consumed the diets having 1 % HPI (0.095 μg/mL) or casein-only (0.151 μg/mL) diets. Similar pattern of results was obtained in feeding trial II during the 4-week post-washout feeding period (Fig. 4b) that tested the treatment potential of hemp seed products. Normotensive rats fed with 1 % HMH and HPI had significantly ($p < 0.05$) lower plasma renin levels (0.065–0.069 μg/mL) when compared to the casein-only rat group with a renin level of 0.079 μg/mL. The results also showed that plasma renin concentration in untreated SHRs (0.151 μg/mL) was significantly ($p < 0.05$) higher than the values obtained for untreated NTRs (0.079 μg/mL), suggesting a direct relationship of renin level with BP. However, as observed for plasma ACE levels, the changes in plasma renin level did not have any influence on SBP of NTRs.

Discussion

The functionality and activity of any peptide under in vitro or in vivo conditions are dependent on the type of proteases and the extent to which the protein is hydrolyzed. Other factors that may influence peptide activity include amino acid composition/sequences and molecular weight [20]. Protein hydrolysates such as HMH normally contain high sodium contents due to the addition of alkali (NaOH) during digestion to neutralize liberated protons and maintain pH at optimally set value for protease activity. The amino acid composition data suggest that because pepsin and chymotrypsin preferentially hydrolyze peptide bonds that involve aromatic amino acids, most of the bonds hydrolyzed involved tryptophan, hence higher content of tryptophan in HMH. In contrast, it seems that most of the arginine-containing sequences were resistant to protease digestion, hence the low content of arginine in HMH when compared to HPI. High level of hydrophobicity as evident in the high amounts of aromatic amino acids (AAA) and hydrophobic amino acids (HAA) can enhance peptide uptake through the cellular lipid bilayer, which could have contributed to the observed enhanced bioactivity of HMH peptides. The arginine (13.91 %) and sulfur-containing amino acid (3.85 %) contents obtained for HPI in this work are higher than the values of 9.91 and 1.55 %, respectively, that were reported by Wang et al. [21]. The differences between amino acid composition of HPI used in this work and the HPI reported by Wang et al. [21] could be due to variations in the source of hemp seed meals used to prepare the protein isolate.

Food-derived peptides that have shown in vitro ability to modulate the renin–angiotensin system (RAS) by inhibiting ACE and renin enzymatic activities could be used in animal and subsequently in human clinical intervention experiments against hypertension and associated cardiovascular diseases [22]. Therefore, initial screening of protein hydrolysates for their in vitro inhibition of enzyme activities could be used to select active samples that can be

evaluated for in vivo effects. The level of ACE inhibition by HMH was greater than inhibition of renin, which is in agreement with the previous reports showing that ACE is easily inhibited than renin, in vitro [13, 23]. Previous works have also shown similar high [24] or lower inhibitions [25] of ACE activity by food protein hydrolysates at 1 mg/mL concentration, when compared to results obtained in this work. Reports of renin inhibition by food protein hydrolysates are not very common, but a papain protein hydrolysate from macroalgae was shown to have $\sim$42 % inhibitory activity [26], which is higher than the $\sim$35 % inhibition obtained in this work. Rapeseed protein hydrolysates produced using alcalase, proteinase K, pepsin + pancreatin, and thermolysin also had higher renin inhibitory properties while inhibition by the flavourzyme hydrolysate was similar [27] when compared to values obtained in this work.

SHRs are considered one of the best experimental models for evaluating antihypertensive drugs or food-based inhibitors [28]. Therefore, the demonstrated better BP-lowering ability of the HMH when compared to HPI suggests that the former peptides were either more active in nature or had higher absorption characteristics than peptides produced from HPI within the GIT of the rats. The hemp seed products-containing diets maintained or reduced SBP compared to baseline values during rat growth, suggesting their potential use in preventing hypertension development. The results obtained in present work are similar to those reported for soybean protein hydrolysate (same diet inclusion values of 0.5 and 1.0 %), which was also shown to attenuate hypertension development in SHRs [29]. However, the attenuation effect obtained in present work for the hemp seed diets (SBP $\leq$120 mmHg after 8 weeks) is greater because after 8 weeks, the soybean hydrolysate-fed rats had SBP >120 mmHg. The initial lag time for HPI may be due to the need for extensive enzyme hydrolysis within the GIT to produce bioactive peptides in sufficient quantities, whereas the HMH diets already contained predigested peptides.

SHRs and NTRs are generally compared in terms of their cardiovascular phenotype as models for studying hypertension. These strains have shown some genetic variations in markers of hypertension, which suggests that ACE activity in the plasma may be dependent on genetic, environmental, experimental, hormonal, and age-related factors [30]. The lower plasma ACE level during attenuation of SBP increase in growing SHRs is consistent with the earlier work of Yang et al. [29]. The ACE values tended to be positively related to SBP changes, which confirm previous works that have shown that the plasma level of ACE could be used as a hypertension marker in SHRs [31]. The result is further supported by the fact that rats fed with the HMH-containing diets had significantly ($p < 0.05$) lower SBP and plasma ACE levels when compared to the rats that consumed the HPI-containing diet. Thus, the results further support the role of high plasma ACE levels in maintaining hypertension conditions and the use of ACE level-modulating agents as effective antihypertensive agents. The slightly lower plasma ACE levels observed during feeding trial II when compared to trial I suggest that some of the rats did not regain 100 % of enzyme level during the washout period. In contrast to data obtained in this work, a previous report showed no changes in plasma ACE of SHRs after a 30-day feeding period with corn protein-derived peptides [32], which suggests that peptides from different sources may produce antihypertensive effects through different mechanisms. The slight reduction in plasma ACE level for the 1 % HMH-treated NTRs was not translated to SBP reduction, showing that the protein hydrolysate had no BP-reducing effect under normotensive conditions. The lack of diet effect on SBP of the NTRs indicates that the BP-reducing effects of HMH observed for SHRs are peculiar to hypertension conditions and should not cause hypotension if consumed under normal BP conditions. The reduced plasma renin and ACE levels in SHRs could be confirmed as one of the main mechanisms involved in the antihypertensive effects of the HMH and HPI. Inhibition of renin is a very important physiological target for modulation of the RAAS in order to control hypertension because the enzyme catalyzes the rate-limiting step in a cascade of reactions that lead to vasoconstriction. To the best of our knowledge, this work is the first to show modulation of plasma renin level in SHRs that have been fed with diets containing food proteins or peptides.

Conclusion

Long-term studies of feeding growing SHRs with different levels (0.5 and 1 %) of HMH peptides were used to confirm SBP attenuation, which suggest absorption of bioactive peptides from the diets. The HMH diet but not the HPI diet was able to maintain the SHRs at normal blood pressure. The delayed and reduced antihypertensive effect of unhydrolyzed hemp seed proteins (HPI diet) suggests that predigestion in the form of protein hydrolysates can provide a more rapid means of reducing blood pressure during diet intervention. Thus, the HMH could prevent hypertension development in SHRs. In SHRs with established hypertension, diets containing hemp seed peptides or proteins were also effective in reducing blood pressure when compared to a casein-only diet. The bioactive products did not change SBP of NTRs, which indicates lack of potency under normal BP conditions. SBP reductions were directly related to plasma levels of ACE and renin, which suggest

the in vivo mechanism of action. The higher level of plasma renin in SHRs when compared to NTRs confirms the role of this enzyme in maintaining hypertension and that the SHR is a suitable model to study efficacy of antihypertensive compounds. The results confirm the potential of HMH as a useful ingredient that can be used to formulate functional foods and nutraceuticals for the prevention and treatment of hypertension. Future experiments will be directed toward purification and identification of active peptide sequences that are responsible for the antihypertensive effects of HMH.

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Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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