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ORIGINAL ARTICLE

Kinetics of Enzyme Inhibition and Antihypertensive Effects of Hemp Seed (*Cannabis sativa* L.) Protein Hydrolysates

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Abstract The aim of this study was to determine the antihypertensive effects of enzymatic hemp seed protein hydrolysate (HPH) and its peptide fractions. Hemp seed protein isolate was digested by the sequential action of pepsin and pancreatin to mimic gastrointestinal digestion in human beings. The resultant HPH was separated by membrane ultrafiltration into peptide fractions with different sizes (<1 and 1–3 kDa). The HPH led to significantly higher ($P < 0.05$) in vitro inhibition of the activities of angiotensin I-converting enzyme (ACE) and renin, the two main enzymes involved in abnormal blood pressure elevation (hypertension). Kinetic studies showed that HPH and peptide fractions inhibited renin and ACE activities in a mixed-type pattern, indicating binding to areas other than the active site. Oral administration of HPH (200 mg/kg body weight) to spontaneously hypertensive rats led to significant reductions ($P < 0.05$) in systolic blood pressure (SBP) that reached a maximum of −30 mmHg after 8 h. In contrast, the hypotensive effects of peptide fractions (<1 and 1–3 kDa) had a maximum value of about −15 mmHg after 6–8 h post oral administration. The results suggest a synergistic antihypertensive effect of the peptides present within HPH; this effect was reduced significantly ($P < 0.05$) upon separation into peptide fractions.

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Introduction

Hypertension is a major global health problem generally affecting 20–45% of the population: 15–20% of all adults and nearly 50–60% of elderly people. Hypertension is one of the main controllable risk factors associated with cardiovascular disease events like myocardial infarction, heart failure, and end-stage diabetes [1]. Hypertension is regulated mainly by the renin-angiotensin system (RAS), which generates a variety of regulatory peptides that modulate blood pressure, fluid and electrolyte balance throughout the human body. Renin and angiotensin I-converting enzyme (ACE), a Zn protease, are the key enzymes controlling the RAS pathway [2]. In the RAS, renin converts angiotensinogen to angiotensin I (AT-I), which in turn is converted by ACE to angiotensin II (AT-II), a vasoconstrictor. In disease conditions, or as a result of genetic and environmental influences, the level of ACE in the body is up-regulated resulting in high levels of AT-II, which promotes undesirable rates of blood vessel contraction that leads to the development of high blood pressure and hypertension. ACE is also known to inactivate bradykinin, a potent vasodilator, further leading to inability of the blood vessels to relax following contraction. The current treatment for hypertension in human beings has involved the use of synthetic ACE inhibitors, such as captopril, enalapril, alacepril and lisinopril. However, some undesirable side effects, such as coughs, taste disturbances and skin rashes have been reported, which limit their use in treating

hypertension [3, 4]. There is a growing interest in the use of naturally occurring renin and ACE-inhibitory peptides as therapeutic agents for the treatment and management of hypertension and other chronic diseases [4]. Food-derived ACE-inhibitory peptides, though less effective as hypotensive agents in vitro when compared to drugs, have no known side effects and have the potential to offer lower healthcare cost [1]. Furthermore, these peptides are observed to have multifunctional properties, are easily absorbed to target organs [5] and possess inherent high nutritional value that contributes to the overall wellbeing of the individual.

Historically, hemp seed (*Cannabis sativa* L.) cultivation for food utilization has been limited due to the presence of the psychoactive compound tetrahydrocannabinol (THC). The availability of low THC hemp plants has increased accessibility to the edible seeds, which are a by-product obtained during the commercial utilization of the valuable hemp plant fibre, and are a rich source of high quality oil and protein [6]. The seed contains over 30% oil (composed primarily of linoleic and α-linolenic acids) and about 25% protein; the storage proteins consist mainly of edestin (globulin) and albumin, with a superior essential amino acid profile and high digestibility [7, 8]. The increased utilization of hemp seed for edible oil production has led to abundant amounts of protein-rich meal, which serves as a suitable raw material for production of peptide products. This is due to the presence of high levels of residues, especially arginine and branched chain amino acids [9], which are desirable components of bioactive peptides. Increasingly, food consumption is considered a source not only of nutrients but also of bioactive compounds such as peptides. Enzymatic hydrolysis could be applied to modify the properties of food proteins but additionally it could impart these proteins with added value, such as potential health effects. Bioactive peptides from natural sources usually contain 3–20 amino acid residues and their activity is based on their amino acid composition and sequence [10]. Peptides that are inactive within the parent protein could be activated by enzymatic hydrolysis to exhibit various health regulatory effects such as antioxidant properties [11] and antihypertensive activities [2]. Several food protein hydrolysates have been shown to possess in vitro inhibition of ACE activity resulting in the lowering of elevated blood pressure in spontaneously hypertensive rats [2] and in hypertensive human beings [12]. However, reports on natural food-based renin inhibitors are scarce. Moreover, it has been hypothesized that the direct inhibition of renin activity provides a more effective control of elevated blood pressure since it is the first and rate-limiting step in the RAS. Renin inhibition prevents the production of AT-I, which could otherwise be converted to appreciable amounts of AT-II in certain organs through an ACE-independent pathway catalyzed by chymase—a product of masts cells in the connective tissues of the heart and blood vessels [13]. Inhibiting renin alone in the RAS does not completely prevent the ACE-catalyzed bradykinin degradation that could also result in vasoconstriction [14]. It has therefore been proposed that a better approach to lowering of elevated blood pressure is to develop natural therapeutic agents that could exhibit multiple effects such as the simultaneous inhibition of renin and ACE activities [13]. Recently, some renin-inhibitory peptides have been reported following the enzymatic hydrolysis of flaxseed and pea proteins [13, 15].

Therefore, the objectives of this study were to determine the antihypertensive properties of hemp seed protein hydrolysate (HPH) and its peptide size-based fractions using the in vitro inhibition of ACE and renin activities, as well as in vivo lowering of systolic blood pressure (SBP) in spontaneously hypertensive rats (SHRs). We also determined the kinetics of renin and ACE inhibition by HPH and its fractions in order to provide a mechanistic basis for the observed inhibitory properties.

Materials and Methods

Materials

Defatted hempseed meal, referred to as hempseed protein powder, was purchased from Manitoba Harvest Fresh Hemp Foods (Winnipeg, MB, Canada). Pepsin (from porcine gastric mucosa, EC 3.4.23.1), pancreatin (from porcine pancreas), trinitrobenzene sulfonic acid (TNBS), sodium dodecyl sulfate, *N*-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), and ACE from rabbit lung (E.C.3.4.15.1) were purchased from Sigma-Aldrich (St. Louis, MO). A Human recombinant Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI). Other analytical grade reagents and ultrafiltration membranes (1 and 3 kDa molecular weight cut-off) were obtained from Fisher Scientific (Oakville, ON, Canada).

Methods

Production of HPH and Membrane Fractions

Hemp seed protein isolate (HPI), hydrolysate (HPH) and membrane fractions were prepared according to procedures described in our previous publication [11]. Briefly, HPH was produced by consecutive treatment of HPI with pepsin (2 h) and pancreatin (4 h) followed by centrifugation to recover soluble peptides. The soluble peptides were first passed through a 1-kDa membrane and the permeate

collected as the <1 kDa sample. The retentate was then passed through a 3-kDa ultrafiltration membrane and the permeate collected as the 1–3 kDa sample. The HPH and membrane permeates were freeze-dried and peptide contents determined by the modified Lowry method [16] using bovine serum albumin as standard. The degree of hydrolysis (DH) of HPH was determined according to the TNBS method [17]. The yields of HPH were determined for samples hydrolyzed with pepsin alone and in combination with pancreatin. The percent yield of HPH was determined as the ratio of peptide weight of lyophilized HPH to the protein weight of unhydrolyzed HPI. Similarly, percent yields of the ultrafiltration membrane fractions were calculated as the ratio of peptide weight of a lyophilized peptide permeate to the peptide weight of the original HPH.

Kinetics of ACE Inhibition

The ability of hemp seed peptide fractions to inhibit the activity of ACE in vitro was measured in triplicate as previously reported [13]. 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 ACE (final activity of 20 mU) and 200 μL HPH in 50 mM Tris–HCl buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature. Tris–HCl buffer was used instead of peptide fraction solutions in the blank experiment. ACE activity was expressed as rate of reaction (ΔA/min) and inhibitory activity was calculated as:

$$\text{ACE inhibition}\,(\%) = \left[\left(\Delta A/\text{min}_{(\text{blank})} - \Delta A/\text{min}_{(\text{sample})}\right)/\Delta A/\text{min}_{(\text{blank})}\right] \times 100$$

where $(\Delta A/\text{min}_{(\text{sample})}$ and $[(\Delta A/\text{min}_{(\text{blank})}$ are ACE activity in the presence and absence of the hemp seed peptide samples, respectively. The concentration of peptide fractions that inhibited ACE activity by 50% (IC_{50}) was calculated for each sample using non-linear regression from a plot of percentage ACE inhibition versus sample concentrations. The kinetics of ACE inhibition was studied with 0.0625, 0.125, 0.25 and 0.5 mM FAPGG. The mode of ACE inhibition was determined from the Lineweaver-Burk plots, while inhibition constant (K_i) was calculated as the *x*-axis intercept from a plot of the slope of the Lineweaver-Burk lines against peptide concentration [18].

Renin Inhibition Assay

In vitro inhibition of the activity of human recombinant renin assay was conducted according to the previously described method [15] using the Renin Inhibitor Screening Assay Kit. Prior to the assay, renin buffer was diluted with 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with assay buffer before use, and the assay buffer was pre-warmed to 37 °C before the reaction was initiated in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) maintained at 37 °C. Before the reaction, (1) 20 μL substrate, 160 μL assay buffer, and 10 μL Milli-Q water were added to the background wells; (2) 20 μL substrate, 150 μL assay buffer, and 10 μL Milli-Q water were added to the control wells; and (3) 20 μL substrate, 150 μL assay buffer, and 10 μL sample were added to the inhibitor wells. The reaction was initiated by adding 10 μL renin to the control and sample wells. The microplate was shaken for 10 s to mix and incubated at 37 °C for 15 min, and the fluorescence intensity (FI) was then recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm. The percentage inhibition was calculated as follows:

$$\text{Inhibition}\% = [(\text{FI of control well} - \text{FI of sample well})/(\text{FI of control well})] \times 100$$

The concentration of protein hydrolysates that inhibited 50% of renin activity was determined and defined as the IC_{50} value. The renin inhibition kinetics studies were conducted using 0.625, 1.25, 2.5, 5 and 10 μM substrate in the absence and presence of peptides.

SHRs and Measurement of Systolic Blood Pressure

Animal experiments were carried out following the Canadian Council on Animal Care ethics guidelines with a protocol approved by the University of Manitoba Animal Protocol and Management Review Committee. SHRs (six rats/treatment, 20-week-old, male, 250–300 g body weight, BW) with tail SBP of over 150 mmHg were purchased from Charles Rivers Laboratories (Montreal, PQ, Canada). SHRs were housed individually in steel cages in a room kept at 25 °C with a relative humidity of 50% and a 12 h light–dark cycle, and fed a standard laboratory diet (chow) with free access to water. HPH and the membrane permeates (<1 and 3 kDa) were dissolved in 1 mL saline at a dose of 200 mg/kg BW. Captopril (an antihypertensive drug) was administered at a dose of 3 mg/kg BW as a positive control while negative control rats received the same volume of saline solution. Following oral administration by syringe gavage, the effects of samples on SBP were compared to that of captopril. SBP was measured by tail-cuff plethysmography at 2, 4, 6, 8 and 24 h (post oral administration of samples) in mildly anesthetized rats according to the method of Aukema et al. [19]. In order to mitigate the SBP depression effect of isofluorane, the gas flow was optimized such that rats became conscious usually within 3–4 min after removal from the chamber, which

provided enough time to perform the blood pressure measurement. Rats were first anesthetized in a chamber (maintained at about 40 °C) with 4% isofluorane for 4 min. They were then removed from the isofluorane chamber and tail-cuff measurement of blood pressure performed in the unconscious state by taking three electronic readings.

Statistical Analysis

Analyses were conducted in replicates as indicated above and analyzed by one-way analysis of variance (ANOVA). Data were reported as mean ± standard deviation. Statistical significance of differences was evaluated by Duncan's multiple range test ($P < 0.05$) using the Statistical Analysis Systems software version 9.2 (SAS, Cary, NC).

Results and Discussion

Yield and DH of HPH and Membrane Permeates

Table 1 shows the DH and yield of HPH and membrane permeate fractions. The DH is usually employed as a proteolysis-monitoring parameter to ascertain the extent to which peptide bonds have been broken down to release short chain peptides [20]. The DH value of 39.1% obtained for the peptic hydrolysate (HPH_{pep}) was significantly ($P < 0.05$) lower than that of the sequential pepsin–pancreatin (HPH) hydrolysate (90.6%). The higher DH exhibited by the HPH could be due to the different domain of cleavage of bonds associated with each of the enzymes used. While pepsin is an endopeptidase that cleaves at specific sites, pancreatin is a mixture of endo- and exopeptidases, which enhances a more extensive hydrolysis of peptide bonds. Moreover, the duration of pancreatin hydrolysis was 4 h in contrast to the 2 h period for pepsin; previous reports have shown that the DH increases with increase in time of hydrolysis [21, 22]. The greater efficiency of hydrolysis observed for the combined enzyme usage could also be because pre-digestion with pepsin led to exposure of susceptible peptide bonds, facilitating optimal hydrolysis by pancreatin. Percent yield is an indication of the efficiency of enzyme hydrolysis process because a higher yield of peptides is the expected outcome for increased protein breakdown. The yield also puts into perspective the economic viability of commercializing the protein hydrolysate as an ingredient to formulate functional foods and nutraceuticals. Higher percent yields are more beneficial to commercial processing and marketing of new products. As expected, the yield (65.7%) of HPH_{pep} was significantly lower ($P < 0.05$) than the value (86.7%) obtained for HPH. The results showed a direct correlation between the extent of hydrolysis and the yield of protein hydrolysate, which is consistent with previously reported works [23, 24]. Size-dependent separation of the HPH by membrane ultrafiltration showed that low molecular weight peptides (<1 and 1–3 kDa) are present in a higher proportion (74%) when compared to the peptides with size >3 kDa (26%). The high content of small peptides in the HPH suggests a high potential for in vivo bioavailability during oral administration, and may contribute to increased physiological efficiency. This is because short-chain peptides have been shown to be usually resistant to gastrointestinal proteolysis and can be absorbed intact into blood circulation whereas long-chain peptides are susceptible to proteolysis [25].

Table 1 Percent yield and degree of hydrolysis (DH) of hemp seed protein hydrolysates (HPH) and membrane ultrafiltration fractions

Sample	Peptide yield (%)	DH (%)
Hydrolysates		
$HPH_{(pep)}$	65.7 ± 3.75	39.1 ± 0.44
HPH	86.7 ± 5.07	90.3 ± 1.15
Peptide fractions		
<1 kDa	41.8 ± 0.67	NA[a]
1–3 kDa	32.8 ± 0.45	NA
Retentate[b]	25.7 ± 0.20	NA

$HPH_{(pep)}$ Unfractionated hydrolysate from pepsin digestion of hemp seed protein isolate (HPI), *HPH* unfractionated hydrolysate from pepsin–pancreatin digestion of HPI, peptide fractions (<1 and 1–3 kDa) were separated from HPH

[a] Not applicable

[b] Peptide fraction >3 kDa

Enzyme Inhibitory Activities of HPH and Membrane Fractions

ACE Inhibition

ACE catalyzes two main reactions responsible for the constriction of blood vessels that leads to blood pressure elevation. The IC_{50} values of HPH (0.67 mg/mL) against ACE activity was significantly lower ($P < 0.05$) than the values obtained for the membrane fractions (1.05 and 1.17 mg/L, respectively, for <1 kDa and 1–3 kDa membrane fractions (Fig. 1, open bars). The IC_{50} values obtained in this study are similar to values (1.03–1.06 mg/mL) reported for ACE-inhibitory bovine milk using tryptic hydrolysates [26]. In contrast, flaxseed protein hydrolysate fractions inhibited ACE activity with lower IC_{50} values of 0.024–0.15 mg/mL [13], while fermented products containing mainly β-casein-derived peptides had IC_{50} values from 0.008–0.0112 mg/mL [27]. Fractionation of the HPH by membrane ultrafiltration resulted in peptide fractions with reduced potency against ACE (Fig. 1), which is in contrast to results obtained

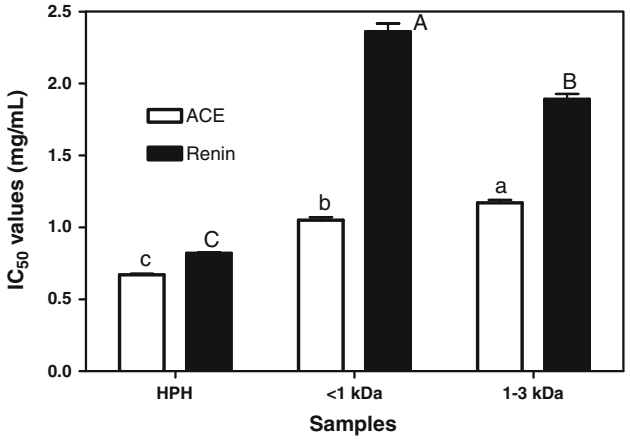

Fig. 1 Hemp seed protein-derived peptide inhibitory concentrations that reduced 50% (IC_{50}) activity of angiotensin converting enzyme (ACE) and renin. HPH is the protein hydrolysate from pepsin–pancreatin digestion of hemp seed protein isolate while <1 and 1–3 kDa are the peptide fractions from membrane ultrafiltration of HPH. *Bars* belonging to the same enzyme but with different alphabets are significantly different at $P < 0.05$

for shrimp protein hydrolysate [24] where the <3 kDa fraction was shown to have higher activity than the original hydrolysate. The <1 kDa peptides had significantly lower ($P < 0.05$) IC_{50} value than the 1–3 kDa peptides, which suggests that smaller size peptides may be more effective as ACE inhibitors when compared to the larger size peptides. The results are similar to reports obtained for cod frame protein hydrolysate [28] and shrimp [24], which showed an increase in ACE-inhibitory activity with decreasing molecular weight (MW) of peptide fractions produced by ultrafiltration. The reduced ACE-inhibitory activities of the ultrafiltration membrane fractions suggest that there was a synergistic effect when the peptides are present in the HPH. ACE prefers to bind to substrates or inhibitors containing hydrophobic (aromatic or branched-chain) amino acid residues at each of the three C-terminal positions; many naturally occurring ACE-inhibitory peptides contain Tyr, Phe, Trp, Pro or Lys at the C-terminus, especially the di and tripeptides [29]. Pro, Trp and Lys are the most effective in increasing the ACE-inhibitory activity [12, 29] while the branched-chain aliphatic amino acids such as Ile, Leu and Val are the most prevalent in highly active peptide inhibitors [2]. Thus, the higher amounts of Phe and Leu in the <1 kDa fraction [11] may have contributed to a higher ACE-inhibitory activity when compared to the 1–3 kDa fraction.

Renin Inhibition

Renin inhibitors produce highly selective inhibition of the RAS, resulting in an improved side-effect profile for therapeutic agents, since renin catalyses hydrolysis of only one naturally occurring substrate: angiotensinogen [30]. Thus, the search for natural renin inhibitors has become a challenge and most approaches to identifying such inhibitors have been focused on peptide modification or synthesis. In this study, the peptide samples exhibited moderate renin inhibitory activities with IC_{50} values of 0.81, 2.52 and 1.89 mg/mL for HPH, <1 and 1–3 kDa fractions, respectively (Fig. 1, solid bars). Similar to the observed effects on ACE activity, the HPH had significantly lower IC_{50} values when compared to the <3 kDa fractions obtained from membrane separation. The results also suggest that the peptides act synergistically to produce more effective renin inhibition when present together in the HPH. Fractionation of the peptides resulted in loss of this synergistic effect, probably due to size-dependent partitioning of active peptides into different groups. It was earlier reported that ACE inhibition is more easily achieved when compared to renin inhibition, thus, there are more ACE-inhibitory peptides than food-protein-derived peptide inhibitors of renin [13]. Renin inhibition by food protein hydrolysates are rare because of the difficulty associated with the down regulation of the activities of renin both in vitro and in vivo. Currently, there are only a few food protein-derived peptide inhibitors of renin [13, 15]. Udenigwe et al. [13] reported that flaxseed protein hydrolysate fractions moderately inhibit human recombinant renin activity, which was also dependent on the type of enzyme used to hydrolyze the proteins. The IC_{50} values for renin inhibition by HPH, <1 and 1–3 kDa fractions are higher than those for ACE inhibition, which is in agreement with the fact that inhibition of ACE is achieved easily compared to renin inhibition.

Kinetics of Enzyme Inhibition

Based on the inhibitory results of the peptides used in this experiment, the mode of inhibition of ACE and renin activities was investigated via kinetic studies in the absence and presence of the HPH and membrane fractions. Kinetic parameters are vital to interpreting the effectiveness of peptides in eliciting their inhibitory potential against the activities of enzymes. Furthermore, kinetic plots give a rough estimate of the amount of substrate or peptides (inhibitor) required to accelerate the reaction or inhibit the activities of the enzymes as reflected by the affinity to bind to the active site of the enzyme. K_i is the dissociation constant, which defines inhibitor binding ability to the enzyme to form the enzyme-inhibitor complex. Lineweaver–Burk plots of the ACE reaction with and without peptide inhibitors at two concentrations are shown in Fig. 2a–c. The pattern of inhibition displayed was mixed-type tilting more towards noncompetitive inhibition, which means that the peptide can combine with an enzyme molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound to the enzyme active site or

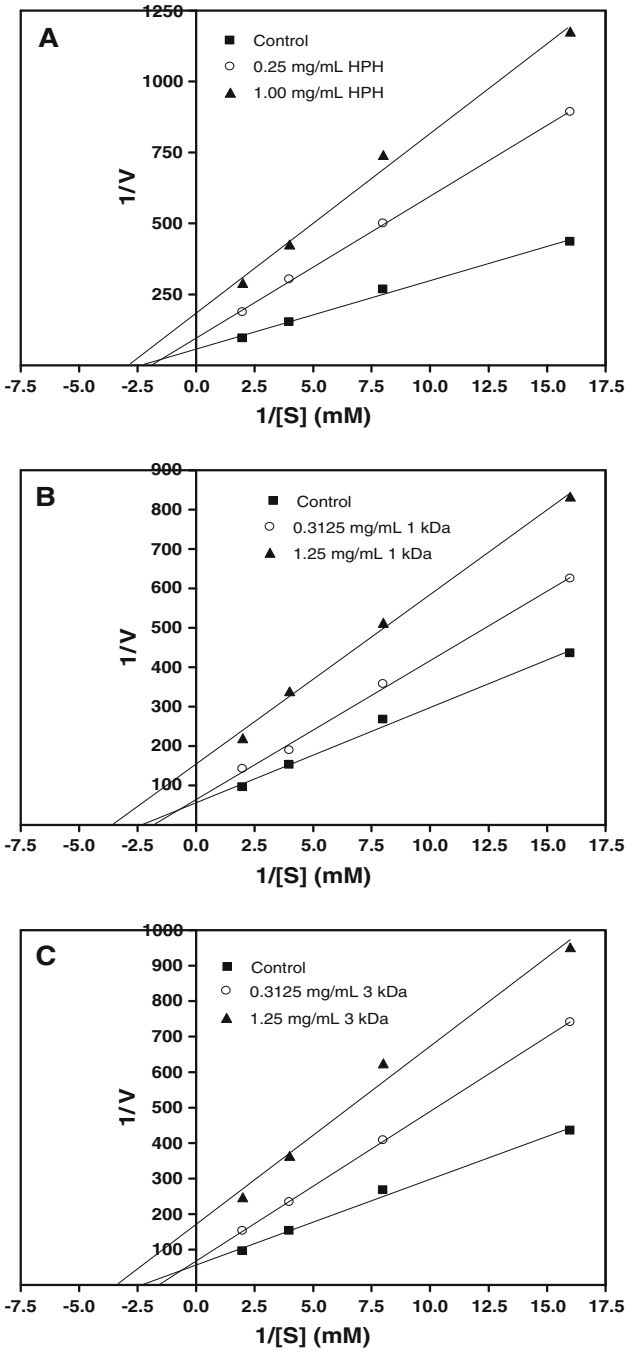

Fig. 2 Lineweaver-Burk plots of ACE inhibition by different concentrations of: **a** unfractionated protein hydrolysate (HPH) from pepsin–pancreatin digestion of hemp seed protein isolate; **b** <1 kDa and **c** 1–3 kDa peptide fractions (from membrane ultrafiltration of HPH) at varying substrate concentrations (0.0625–0.5 mM). *V* Initial rate of reaction (ΔA_{345} nm/min)

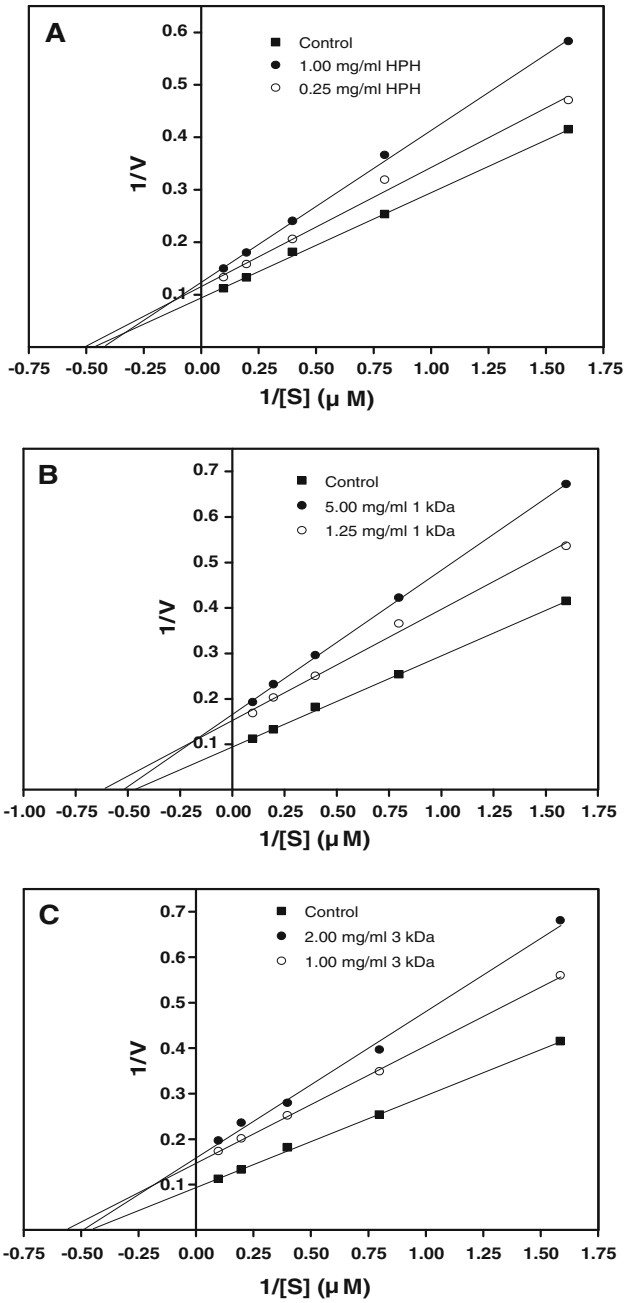

Fig. 3 Lineweaver-Burk plots of the inhibition of human recombinant renin by different concentrations of: **a** unfractionated protein hydrolysate (HPH) from pepsin–pancreatin digestion of hemp seed protein isolate; **b** <1 kDa; and **c** 1–3 kDa peptide fractions (from membrane ultrafiltration of HPH) at varying substrate concentrations (0.625–10 μM); *V* initial rate of reaction (change in fluorescence intensity/min)

not. This also implies that the peptide binds at a different site from the substrate and hence act as ACE inhibitors by forming enzyme–substrate–inhibitor and enzyme–inhibitor complexes, which will reduce efficiency of enzyme catalysis. The K_i values of 2.55, 3.96 and 4.74 mg/mL for ACE inhibition by HPH, <1 and 1–3 kDa peptide fractions, respectively, are directly correlated with the in vitro ACE-inhibitory effects of the samples. Thus, the HPH binds more effectively to ACE and could explain the higher inhibitory effects when compared to the <1 and 1–3 kDa peptides, which have higher values of K_i.

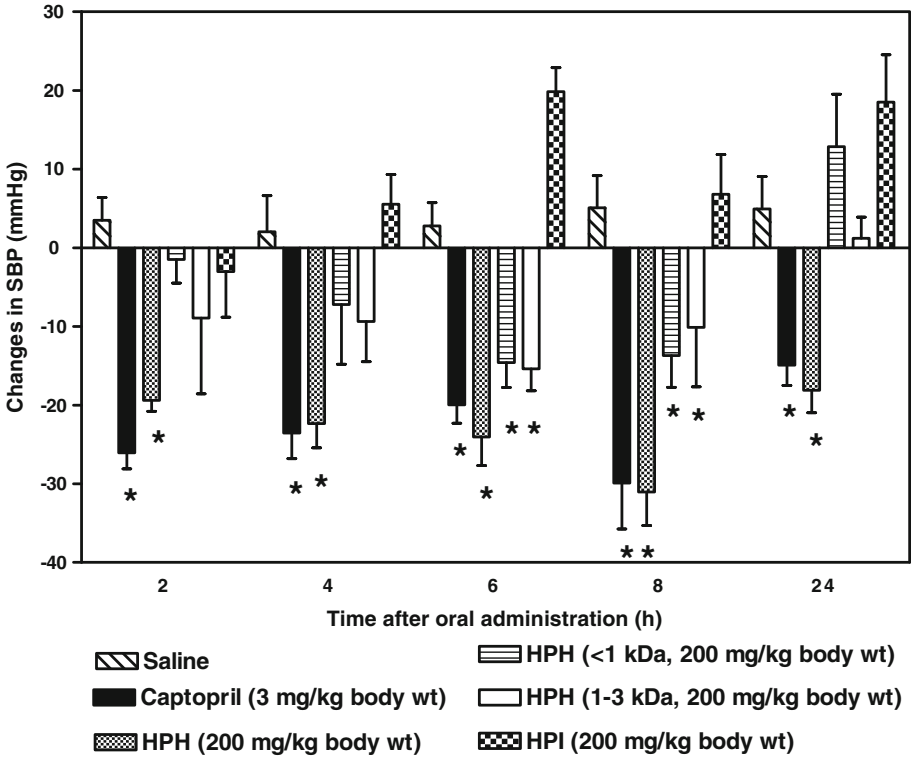

Fig. 4 Time-dependent changes in systolic blood pressure (SBP) of spontaneously hypertensive rats (SHRs) after oral administration of 200 mg/kg body weight each of hemp seed protein isolate (HPI), unfractionated protein hydrolysate (HPH) from pepsin–pancreatin digestion of HPI and membrane ultrafiltration fractions of HPH (<1 and 1–3 kDa) in comparison to 3 mg/kg body weight of captopril. *Bars with asterisks* have mean values that are significantly lower ($P < 0.05$) than the mean values for saline and HPI bars

Figure 3a–c shows the double reciprocal plots for the inhibition of human recombinant renin by HPH and the 1 and 3 kDa peptide fractions. These peptides all exhibited mixed type non-competitive mode of renin inhibition. The K_i values are directly correlated to IC_{50} values obtained for renin inhibition by HPH and peptide fractions. For instance, HPH had a lower K_i of 2.45 mg/mL compared to the <1 and 1–3 kDa with 11.25 and 3.02 mg/mL, respectively, which implies that a lower amount of HPH is required for enzyme inhibition than the amount of the <1 or 1–3 kDa fractions required to achieve the same effects.

Hypotensive Effects of Protein Hydrolysates in SHRs

Figure 4 shows the SBP-lowering effects of hemp seed protein and peptide samples in SHRs when compared to the antihypertensive drug, captopril. A single oral administration of HPI, HPH, fractions (<1 and 3 kDa) and captopril to SHRs revealed that HPH significantly ($P < 0.05$) decreased SBP (−20 mmHg) after only 2 h of oral administration and a maximum effect of −30 mmHg reduction in SBP was achieved after 8 h. By the 4 h mark, the antihypertensive effect of the HPH was already comparable to the effect obtained for captopril and remained similar up to 24 h. In contrast, the hypotensive effects of the <1 and 3 kDa peptide fractions was significantly different ($P < 0.05$) from the control rats at 6–8 h, but were significantly lower ($P < 0.05$) than the effect of HPH. The results suggest that the HPH is faster acting and more potent than the peptide fractions, which may be indicative of synergistic effects of peptides present in the HPH; this synergy was lost upon peptide fractionation. The degree of hypotensive effect observed for the HPH and peptide fractions was directly correlated with observed in vitro inhibition of ACE and renin activities. This is because the ultrafiltration fractions (<1 and 1–3 kDa) showed milder SBP lowering effects, which is in agreement with their in vitro inhibition effects against ACE and renin, which were lower than that of HPH. Moreover, unlike HPH, the hypotensive effects of the <1 and 1–3 kDa peptide fractions was eventually lost by 24 h. The HPI and saline solution had negative effects, elevating rather than suppressing SBP in rats, which suggests that it is the in vitro hydrolysis of hemp seed proteins that led to the production of bioactive peptides. Similar blood pressure-reducing effects have been reported by other researchers using hydrolysates from tuna muscle [31], tuna frame [32] and oyster [33]. Results from the current study have demonstrated that HPH can lower SBP in SHRs and could be used as an antihypertensive agent for the prevention or management of hypertension.

Conclusions

The HPH and <3 kDa peptide fractions produced from pepsin–pancreatin hydrolysis of hemp seed proteins

possessed inhibitory activities towards ACE and renin under in vitro conditions. The high yield of HPH coupled with its hypotensive effects in SHRs indicates a potential for commercial utility, especially as an ingredient to formulate antihypertensive agents. Since HPH was more bioactive than the peptide fractions, there will also be no additional cost associated with peptide purification, which is normally required to enhance potency. The positive correlation between in vitro and in vivo data with respect to enzyme (ACE and renin) inhibition and hypotensive effect supports efforts aimed at using the less expensive in vitro approach to predict the potential bioactive properties of food protein-derived peptides.

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References

1. Hong F, Ming L, Yi S, Zhanxia L, Yongquan W, Chi L (2008) The antihypertensive effect of peptides: A novel alternative to drugs? Peptides 29:1062–1071
2. Aluko RE (2008) Antihypertensive properties of plant-derived inhibitors of angiotensin I-converting enzyme activity: a review. Recent Prog Med Plants 22:541–561
3. Atkinson AB, Robertson JIS (1979) Captopril in the treatment of clinical hypertension and cardiac. Lancet 314:836–839
4. Lahogue V, Rehel K, Taupin L, Haras D, Allaume P (2010) A HPLC-UV method for the determination of angiotensin I-converting enzyme (ACE) inhibitory activity. Food Chem 118:870–875
5. Erdmann K, Cheung BLY, Schroder H (2008) The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. J Nutr Biochem 19:643–654
6. Wang XS, Tang CH, Chen L, Yang XQ (2009) Characterization and antioxidant properties of hemp protein hydrolysate obtained with neutrase. Food Technol Biotechnol 47:428–434
7. Tang CH, Ten Z, Wang XS, Yang XQ (2006) Physicochemial and functional properties of hemp (*Cannabis sativa* L.) protein isolate. J Agric Food Chem 54:8945–8950
8. Wang XS, Tang CH, Yang XQ, Gao WR (2008) Characterization amino acid composition and in vitro digestibility of hemp (*Cannabis sativa* L.) proteins. Food Chem 107:11–18
9. House JD, Neufeld J, Leson G (2010) Evaluating the quality of protein from hemp seed (*Cannabis sativa* L.) products through the use of the protein digestibility-corrected amino acid score method. J Agric Food Chem 58:11801–11807
10. Pihlanto LA, Koskinen P, Piilola K, Tupasela T, Korhonen H (2000) Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. J Diary Res 67:53–64
11. Girgih, AT, Udenigwe CC, Aluko RE (2011) In vitro antioxidant properties of hemp seed (*Cannabis sativa* L.) protein hydrolysate fractions. J Am Oil Chem Soc 88:381–389
12. FitzGerald RJ, Murray BA, Walsh DJ (2004) Hypotensive peptides from milk proteins. J Nutr 134:9805–9885
13. Udenigwe CC, Lin YS, Hou WC, Aluko RE (2009) Kinetics of the inhibition of renin and angiotensin I-converting by flaxseed protein hydrolysate fractions. J Funct Foods 1:199–207
14. Segall L, Covic A, Goldsmith DJA (2007) Direct renin inhibitors: the dawn of a new era, or just a variation on the theme? Nephrol. Dial Transplant 22:2435–2439
15. Li H, Aluko RE (2010) Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. J Agric Food Chem 58:11471–11476
16. Markwell MAC, Haas SM, Biebar LL, Tolbert NE (1978) A modification of lowry procedure to simplify protein determination in membrane and protein samples. Anal Biochem 87:206–211
17. Adler-Nissen J (1979) Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. J Agric Food Chem 27:1256–1262
18. Li H, Aluko RE (2005) Kinetics of the inhibition of calcium/calmodulin-dependent protein kinase II by pea protein-derived peptides. J Nutr Biochem 16:656–662
19. Aukema HM, Gauthier J, Jia Y, Roy M, Li H, Aluko RE (2011). Distinctive effects of plant protein sources on renal disease progression and associated cardiac hypertrophy in experimental kidney disease. Mol Nutr Food Res. doi: 10.1002/mnfr.201000558
20. Giménez B, Alemán A, Montero P, Gómez-Guillén MC (2009) Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid. Food Chem 114:976–983
21. Yin SW, Tang CH, Cao JS, Wen HuEK, QB YangXQ (2008) Effects of limited enzymatic hydrolysis with trypsin on functional properties of hemp (*Cannabis sativa* L.) protein isolate. Food Chem 106:1004–1013
22. Barbana C, Boye JI (2010) Angiotensin I-converting enzyme inhibitory activity of chickpea and pea protein hydrolysates. Food Res Int 43:1642–1649
23. Shahidi F, Han X-Q, Synowiecki J (1995) Production and characteristics of protein hydrolysates from capelin (*Mallotus villosus*). Food Chem 53:285–293
24. Benjakul S, Morrissey MT (1997) Protein hydrolysates from pacific whiting solid wastes. J Agric Food Chem 45:3423–3430
25. Roberts PR, Burney JD, Black KW, Zaloga GP (1999) Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. Digestion 60:332–337
26. Haque E, Chand R (2008) Antihypertensive and antimicrobial bioactive peptides from milk proteins. Eur Food Res Technol 277:7–15
27. Belem MAF, Gibbs BF, Lee BH (1999) Proposing sequences for peptides derived from whey fermentation with potential bioactive sites. J Diary Sci 82:486–493
28. Jeon Y-J, Byun H-G, Kim S-K (1999) Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. Process Biochem 35:471–478
29. Wu J, Aluko RE, Nakai S (2006) Structural requirements of angiotensin I-converting enzyme inhibitory peptides: quantitative structure-and-activity relationship study of di- and tri-peptides. J Agric Food Chem 54:732–738
30. Stanton A (2003) Potential of renin inhibition in cardiovascular disease. J Renin Angiotensin Aldosterone Syst 4:6–10
31. Qian ZJ, Je JY, Kim SK (2007) Antihypertensive effect of angiotensin I-converting enzyme-inhibitory peptide from hydrolysates of bigeye tuna dark muscle, *Thunnus obesus*. J Agric Food Chem 55:8398–8403
32. Lee S-H, Qian Z-J, Kim S-K (2010) A novel angiotensin I converting enzyme inhibitory peptide from tuna frame protein hydrolysate and its antihypertensive effect in spontaneously hypertensive rats. Food Chem 118:96–102
33. Wang J, Hu J, Cui J, Bai X, Du Y, Miyaguchi Y, Lin B (2008) Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the antihypertensive effect of hydrolysate in spontaneously hypertensive rats. Food Chem 111:302–308