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Blood pressure lowering effects of Australian canola protein hydrolysates in spontaneously hypertensive rats.

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Abstract

The *in vitro* and *in vivo* antihypertensive activities of canola protein hydrolysates and ultrafiltration membrane fractions (<1, 1-3, 3-5, & 5-10 kDa) were examined in this study. The hydrolysates were obtained after 4 h enzyme hydrolysis of canola protein isolate (CPI) using each of Alcalase, chymotrypsin, pepsin, trypsin and pancreatin. The hydrolysates had significantly ($p<0.05$) reduced (35-70%) surface hydrophobicity when compared to the CPI. Alcalase hydrolysate contained the highest level of low molecular weight peptides and produced highest ($p<0.05$) *in vitro* inhibition of angiotensin converting enzyme (ACE) activity. However, pancreatin hydrolysate was the most effective (63.2%) *in vitro* renin inhibitor. Membrane fractionation of pancreatin hydrolysate led to a 15% reduction in renin inhibition by the 1-3 kDa peptide fraction. In contrast, ACE and renin inhibitions were significantly ($p<0.05$) increased by 10-20% after membrane ultrafiltration fractionation of the trypsin hydrolysate. Trypsin hydrolysate was ineffective at reducing hypertension in spontaneously hypertensive rats after oral administration (200 mg/kg body weight). However, Alcalase and pepsin hydrolysates showed appreciable antihypertensive effects, with Alcalase hydrolysate producing the greatest (-34 mmHg) and fastest (4 h) decrease in systolic blood pressure (SBP). CPI had the most prolonged (24 h) SBP-reducing effect, which is attributable to the extensive protein hydrolysis in the GIT. We conclude that the Alcalase and pepsin hydrolysates may serve as useful ingredients to formulate antihypertensive functional foods and nutraceuticals.

Keywords: Canola protein isolate; protein hydrolysates; hydrophobicity; antihypertensive properties, angiotensin converting enzyme; renin; spontaneously hypertensive rats

1. Introduction

Plant peptides with bioactive properties obtained via enzymatic hydrolysis have continued to gain attention in food and nutraceutical research. Studies on plant protein hydrolysates obtained from canola, soybean, sunflower and peas have led to various findings involving the use of their bioactive peptides for short or long term treatment of hypertension. (Chen, Yang, Suetsuna, & Chao, 2004; Wu, Aluko, & Muir, 2008; Megías et al., 2009; Li et al., 2011; Barbana & Boye, 2011). Dual health benefits of bioactive peptides have also been proposed because peptides may have the ability to modulate more than one physiological pathway. A lot of attention has also been recently placed on the potential of alternative or complementary approaches to regulating the renin-angiotensin-system (RAS) through renin inhibition, in addition to angiotensin converting enzyme (ACE) inhibition, as a means of regulating blood pressure (Segall, Covic, & Goldsmith, 2007).

Blood pressure is physiologically controlled by two major pathways, the RAS and the kinin-nitric oxide system (KNOS) (Martínez-Maqueda, Recio & Hernández-Ledesma, 2012). The RAS involves hydrolysis of angiotensinogen by the proteolytic action of renin (an aspartyl protease), which converts it to angiotensin I (AT I). AT I is then cleaved at the histidyl residue from the C-terminus by the activity of ACE (a glycoprotein peptidyldipeptide hydrolase) to produce angiotensin II (AT II), which is the major end product of the RAS (Segall et al., 2007). AT II is a powerful vasoconstrictor that functions by binding to receptors located in tissues all over the body, eliciting reaction flows that initiate blood vessel contractions to maintain blood pressure (Montani & Vliet, 2004). However, excessive production of AT II may result in severe blood vessel contractions and limited relaxation, which can lead to high blood pressure (hypertension) development (Udenigwe & Aluko, 2012). The KNOS is involved in the production of bradykinin which exerts its antihypertensive effects by eliciting reactions that increase intracellular Ca^{2+} concentration,

leading to the activation of nitric oxide synthase (NOS)-dependent production of nitric oxide, a powerful vasodilator (Griendling, Garret, & FitzGerald, 2003). However, ACE is also able to degrade bradykinin to inactive peptide fragments and thus increased levels of ACE lead to the prevention of vasodilation and the promotion of vasoconstriction. Hence the inhibition of ACE and more importantly, renin could have a profound effect on blood pressure and provide for better management of hypertension (Segall et al., 2007).

Canola meal is a by-product of canola oil production and is generally used as an animal feed because it contains up to 38% protein and a well balanced amino acid profile, which is better than the FAO/WHO reference values (Tan, Mailer, Blanchard, & Agboola, 2011; Dong et al., 2011). Utilizing canola meal protein in human health applications could increase meal value and improve profitability of the canola processing industry. Antihypertensive properties of canola meal and rapeseed protein hydrolysates have been studied, with remarkable outcomes, based on the inhibition of ACE activity (Marczak et al., 2003; Yoshie-Stark, Wada, & Wäsche, 2008; Wu et al., 2008). However, studies on canola hydrolysates which focus on both the inhibition of ACE and renin and *in vivo* antihypertensive activities are limited. Recent work on rapeseed protein hydrolysates showed the effect of both the inhibition of renin and ACE in reducing blood pressure in hypertensive rats (He et al., 2013a).Therefore, the objective of this study was to determine the ACE- and renin-inhibitory activities of protein hydrolysates prepared through enzymatic hydrolysis of Australian canola protein isolate using five proteases (Alcalase, chymotrypsin, trypsin, pepsin and pancreatin). The blood pressure-lowering ability of the protein hydrolysates was evaluated using spontaneously hypertensive rats (SHRs).

2. Materials and methods

2.1. Materials

Canola meal was provided by Cootamundra Oilseeds Pty (Cootamundra NSW, Australia), Pepsin was purchased from Worthington Biochemical Corp. (Lakewood, NJ), while human recombinant renin inhibitor screening assay kit was purchased from Cayman (Cayman Chemical, Ann Arbor MI, USA). Rabbit lung ACE, Alcalase 2.4L (protease from *Bacillus licheniformis*), Pancreatin, Trypsin S II, Chymotrypsin, and N-[3-(2-furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) were purchased from Sigma Chemicals (St. Louis, MO) Ultrafiltration membranes with 1, 3, 5 and 10 kDa molecular weight cut-off (MWCO) sizes were purchased from Fisher Scientific (Oakville, ON, Canada).

2.2 Preparation of canola proteins, hydrolysates, and fractions

2.2.1. Extraction of canola protein isolate (CPI)

Defatted canola meal (20 g) was suspended in 200 ml of 0.1 M NaOH pH 12.0, stirred for 1 h and then centrifuged at 18°C and 3000 *g* for 10 min. Two additional extractions of the residue from the centrifugation process were carried out with the same volume of 0.1 M NaOH and the supernatants pooled. The pH of the supernatant was adjusted to the isoelectric point (pH 4.0) using 0.1 M HCl solution; the precipitate formed was recovered by centrifugation as described above. The precipitate was washed with distilled water, adjusted to pH 7.0 using 0.1 M NaOH, freeze-dried and the protein isolate was stored at -20°C until required for further analysis.

2.2.2. Enzymatic hydrolysis of CPI

CPI was hydrolyzed for 4 h using each of five food grade enzymes (Alcalase, chymotrypsin, pepsin trypsin and pancreatin) at an enzyme: substrate ratio of 1:20 to obtain canola protein hydrolysates (CPHs). The optimum conditions used for each enzyme are outlined in Table 1.

The pH was maintained for each hydrolysis process accordingly using either 1 M NaOH or 1 M HCl. The enzymes were inactivated by heating at 85°C for 10 min. The resulting hydrolysates were then centrifuged and the supernatant lyophilized as the CPHs were stored at -20°C for further analysis.

2.2.3. Determination of degree of hydrolysis

The degree of hydrolysis (DH) of canola protein hydrolysates was estimated using the trichloroacetic acid (TCA) soluble free amino group detection method described by McKellar (1981), with slight modifications. Briefly, 4 mL of 12% TCA solution was added to 0.5 g of canola protein hydrolysate samples in a test tube, mixed and incubated for 20 min at room temperature. This solution was filtered and 0.2 mL of the aliquot was added to 2 mL of 1 M potassium tetraborate buffer (pH 9.2) and 0.8 mL of 5 mM picrylsulfonic acid solution. The resulting solution was incubated in the dark at room temperature. After 30 min, 0.8 mL of 2 M dihydrogen orthophosphate solution, containing 18 mM Sodium hydrosulfide was added and the absorbance read at 420 nm. Glycine (1-10 mM) and blank (0.5 mL deionized water) were treated the same as the samples above and the absorbance obtained from glycine was used to plot a standard curve. The absorbance of samples was converted to mg free amino groups (glycine) per gram of protein/hydrolysate from the standard curve.

2.2.4. Membrane fractionation

Each CPH was fractionated by membrane ultrafiltration stirred cell Amicon® 8400 (Millipore Corp. Billerica, MA, USA), using a sequence of membranes with molecular weight cut-off (MWCO) values of 1, 3, 5 and 10 kDa respectively. The retentate from 1 kDa was passed through a 3 kDa membrane whose retentate was passed through a 5 kDa membrane. Finally the retentate from 5 kDa membrane was then passed through a 10 kDa membrane. Permeate collected from each membrane (<1, 1-3, 3-5, or 5-10, respectively) was lyophilized and stored at -20°C for further use. Percentage yield was calculated as the ratio of the hydrolysate

and ultrafiltration processed fractions on a protein equivalent basis to that of the unhydrolyzed CPI and CPHs, respectively. Protein content of samples was determined by the modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978).

2.3. Determination of surface hydrophobicity (H_o)

H_o was determined using a hydrophobicity fluorescence probe, 1-anilino-8-naphtalene-sulfonate (ANS) as described by Kato & Nakai (1980) with some modifications. Hydrolysates were serially diluted to a final concentration of 0.005-0.025% (w/v) in 0.01 M phosphate buffer, pH 7.0. A 20 µL aliquot of ANS (8.0 mM in phosphate buffer) was added to 4 ml of each sample concentration and fluorescence intensity (FI) of the mixture was measured with a JASCO FP-6300 spectrofluorimeter (JASCO Corp., Tokyo, Japan) at excitation and emission wavelengths of 390 and 470 nm, respectively. The initial slope of the FI versus hydrolysate concentration (µg/mL) plot (calculated by linear regression analysis) was used as an index of H_o.

2.4. Determination of molecular weight (MW) distribution of the hydrolysates

A 1 mL aliquot of sample (5 mg/mL) was analyzed on an AKTA purifier FPLC system, equipped with a Superdex 10/300GL column (GE HealthSciences, Montreal, PQ, Canada). Peptides were separated using phosphate buffer (pH 7.2) at a flow rate of 0.5 mL/min. The column was calibrated with cytochrome C (12.384 kDa), aprotinin (6.512 kDa), vitamin B12 (1.855 kDa), FAPGG (0.3994 kDa) and glycine (0.075 kDa).

2.5. Determination of ACE and renin inhibitory activities

2.5.1. ACE inhibition

The *in vitro* inhibition of ACE was measured using the method described by (Udenigwe, Lin, Hou, & Aluko, 2009). Briefly, 1 mL of 0.5 mM FAPGG (dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 7.5, and kept at 37 °C) was mixed with 20 µL of ACE (final activity of 20 mU) and 200 µL of CPH or CPH membrane fractions dissolved in the Tris-HCl buffer (1 or 0.5 mg/mL final concentration). The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature. Tris–HCl buffer was used as the assay blank. ACE activity was expressed as the change in the rate of reaction (ΔA/min) and inhibitory activity was calculated using the equation below:

ACE inhibition (%) =

$$\left(\frac{(\Delta A/\min)_{(blank)} - (\Delta A/\min)_{(sample)}}{(\Delta A/\min)_{(blank)}}\right) * 100$$

Where $(\Delta A/\min)_{(blank)}$ and $(\Delta A/\min)_{(sample)}$ are ACE activities in the absence and presence of samples, respectively.

2.5.2. Renin inhibition assay

The renin inhibition assay kit was used to measure the *in vitro* inhibition of human recombinant renin activity as previously described (Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011). Renin buffer, CPH and CPH fractions were diluted using 50 mM Tris-HCl, pH 8.0 containing 100 mM NaCl. The renin protein was diluted at a ratio of 1:20 using the assay buffer. Before the reaction, (1) 20 µL of renin substrate, 160 µL of assay buffer and 10 µL Milli-Q water were added to the background wells; (2) 20 µL of renin substrate, 150 µL of assay buffer and 10 µL Milli-Q water were added to the blank wells; and (3) 20 µl of renin substrate, 150 µL of assay buffer and 10 µL samples (1 or 0.5 mg/mL final concentration) were added to the inhibitor wells. The reaction was initiated by adding 10 µL of renin to the blank and sample wells. The microplate was shaken and incubated at 37°C for 15 min and the

FI was measured using a spectrofluorimeter plate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) at an excitation and emission wavelengths of 340 and 490 nm, respectively. Percentage inhibition of renin activity was calculated as follows:

$$\text{Inhibition of renin (\%)} = \left(\frac{\text{(FI of blank well} - \text{FI of sample well)}}{\text{(FI of blank well)}}\right) * 100$$

2.6. In vivo studies

Animal experiments were conducted according to protocols approved by the University of Manitoba Protocol Management Review Committee. Male SHRs aged 4-5 months and weighing 350 ± 20 g were housed in individual environmentally enriched cages with controlled lighting (12 h light/12 h dark) and room temperature (22-23°C) and humidity (50-55%). The SHRs were fed with a standard rat diet and water *ad libitum*. To test each sample, the SHRs were randomly divided into 3 groups: saline, sample and captopril (a pharmaceutical ACE inhibitor) of similar weight with 6 rats used for each group. Each group of rats was administered one of the following treatments using oral gavage: saline, 1 mL of phosphate buffered saline (PBS); sample, 1 mL containing 200 mg/kg body weight of CPHs or CPI dissolved in the PBS; captopril, 1 mL containing 10 mg/kg body weight. The baseline systolic blood pressure (SBP) measurements were performed prior to the oral gavage and then at 2, 4, 6, 8 and 24 h after oral gavage on slightly anesthetized rats (Girgih et al., 2011) using the Mouse Rat Tail Cuff Blood Pressure System (IITC Life Science, Woodland Hills, CA). The SBP value obtained at each measurement point was subtracted from the baseline value to determine time-dependent changes in blood pressure.

2.7. Statistical analysis

Data are expressed as means of three analysis ± SD while one way analysis of variance (ANOVA) was used to evaluate the differences between treatment groups. Statistical significance (p<0.05) between data was determined by the Duncan's multiple range test using the SAS® system for Windows® V8 (TS M1).

3. Results and discussion

3.1. Surface hydrophobicity (H_o)

H_o has been reported to be influenced by the specificity of the enzyme used to hydrolyze proteins and changes in H_o could influence the functional properties of protein hydrolysates (Panyam & Kilara, 1996). The H_o of canola proteins analyzed using the ANS probe method showed that the different enzymes used to hydrolyze the proteins decreased the H_o of canola proteins significantly ($p<0.05$) to various extents (Fig. 1). CPI had the highest H_o (350), which decreased in the following order of treatments: pancreatin > pepsin > chymotrypsin > trypsin > Alcalase, after 4 h of hydrolysis. Similar results have also been reported for hydrolyzed soy protein fractions (Calderón de la Barca , Ruiz-Salazar, & Jara-Marini, 2000). The relationship between the DH and the H_o of proteins has also been reported (Tang, Wang, & Yang, 2009). In this study, a decrease in H_o was observed as DH (Table 1) increased for all the enzymes employed. In addition, the H_o of proteins has been widely reported in relation to its interfacial properties in food systems (Damodaran, 1997; Agboola, Ng, & Mills, 2005). However, the mechanism of action with respect to bioactive functionality is not well understood. Studies based on peptide structures have suggested that ACE prefers substrates containing branched amino acid residues at the N- terminal position and hydrophobic amino acids residues (aromatic or branched chains) at the C- terminal position (Byun & Kim, 2002; Sheih, Fang, & Wu, 2009; He et al., 2013). Hydrophobic residues at the N-terminus of peptides were also preferred structural arrangements for renin (Li & Aluko, 2010). The amino

acid profile of CPI and CPHs have been reported (Alashi, et al., 2014), with high hydrophobic and branched chain amino acid values (about 40 and 18% respectively). Therefore, present results suggest that the relatively high H_o of the pancreatin hydrolysate may enhance ACE-inhibitory activity.

3.2. Peptide size distribution in protein hydrolysates

The MW of peptides has a large influence on their bioactivities because of reported effects on solubility and hence uptake of peptides and absorption from the gastrointestinal tract (Udenigwe et al., 2009). The size exclusion chromatograms of CPHs (Fig. 2) show variations in the size of protein hydrolysate components. As reported by Wu et al. (2008), canola protein hydrolysates are comprised mostly of peptides below 1.3 kDa. In this study, the MW (kDa) range estimates obtained are as follows: Alcalase (6.47-0.027), chymotrypsin (0.711-0.027), pepsin (2.45-0.052), trypsin (6.75-0.347) and pancreatin (7.98-0.049). Alcalase produced a higher peak of smaller MW peptides, probably because it was more efficient in hydrolyzing canola proteins, when compared to the other endoproteases used in this study. The intensity of peptide elution up to 14 min (high molecular weight peptides) was very low for Alcalase while the intensity of peaks eluted between 15 and 19 min (low molecular weight peptides) was higher when compared to the other protein hydrolysates. Similar results have been reported for rapeseed hydrolysates (He et al., 2013a) and these data suggest that CPI was more susceptible to Alcalase-induced proteolysis producing smaller sized peptides when compared to pancreatin, trypsin, pepsin and chymotrypsin.

3.3. Inhibition of ACE and renin by CPI and hydrolysates

Hypertension is a silent killer linked to other degenerative health issues such as renal risks, diabetes and cardiovascular diseases. ACE and RAS blockers are known effective agents that exert blood pressure lowering action via different mechanisms at different levels (Verdecchia,

Gentile, Angeli, & Reboldi, 2012). Hydrophobic amino acid contents appeared to influence antihypertensive properties of the hydrolysates *in vitro* as Alcalase and chymotrypsin derived hydrolysates, which contained higher levels of hydrophobic amino acids (Alashi et. al, 2014), exhibited higher ACE properties of 90.0 and 88.8% respectively. These results are similar to those obtained from Alcalase hydrolysates of rapeseed (Mäkinen, Johannson, Vegarud Gerd, Pihlava, & Pihlanto 2012; He et al., 2013a), and peanut protein isolate and its Alcalase hydrolysates at different degrees of hydrolysis (Jamdar et al., 2010). Pepsin, trypsin and pancreatin fractions exhibited ACE- inhibitory values of 81.2, 84.8 and 67.3%, respectively. These values are higher than those reported for both thermolysin-hydrolyzed pea isolate and rapeseed isolate as well as its pepsin hydrolysates (Yoshie-Stark, Wada, Schott, & Wäsche, 2006; Li et al., 2011). A strong correlation was observed between H_o and percentage ACE inhibition (R^2=0.96), but there was an inverse relationship between the H_o and the DH (Table 1). The results suggest that peptide conformation that exposed hydrophobic groups was also a major determinant for ACE inhibition in this study. This agrees with previous studies that showed the presence of hydrophobic groups plays an integral role during ACE inhibition (Girgih et al.. 2011; Mäkinen et al., 2012).

Wide variation was observed in the percentage renin inhibition between the hydrolysates, with pancreatin having the highest value at 63.2% ($p<0.05$) while chymotrypsin had the lowest value at 10.1% (Fig. 3B). The results agree with the previous report of Udenigwe et al., (2009) who showed that renin activity was also dependent on the type of proteolytic treatment of the substrate protein. The pancreatin hydrolysate also had the highest H_o among the hydrolysates, which could have contributed to the observed stronger renin inhibition, when compared to the other hydrolysates. However, there was no direct relationship between H_o and renin inhibition by the canola protein hydrolysate samples.

The inhibition of renin by alcalase, chymotrypsin and pepsin hydrolysates was below 20%; however these values are comparable to those reported for alkaline extracts of *Palmaria palmata* (red macroalga) protein hydrolysates from Alcalase and flavourzyme by Harnedy & FitzGerald (2013). These authors reported a loss in the renin inhibition activities of these hydrolysates when compared to the unhydrolyzed samples. This was not the case for canola hydrolysates in this study as there was significant increase in the percentage inhibition of all the hydrolysates over that of the protein isolate, probably because the enzymes produced peptides that were better competitors with the renin substrate as described by Udenigwe et al. (2009). Canola protein hydrolysates also had higher or comparable renin inhibitory activities depending on the enzyme type when compared to those of chicken breast skin and chicken thigh skin protein hydrolysates obtained at varying enzyme concentrations (Onuh, Girgih, Aluko, & Aliani, 2013). Overall, trypsin and pancreatin hydrolysates had higher ACE and renin inhibitory activities (Fig. 3); therefore these two hydrolysates were subjected to membrane fractionation. All the protein hydrolysates had significantly ($p<0.05$) higher (except chymotrypsin) renin-inhibitory activities than the unhydrolyzed CPI. The results suggest that shorter-chain peptides released during enzymatic hydrolysis of proteins are more effective renin-inhibitory agents when compared to the unhydrolyzed proteins.

3.4. Inhibition of ACE and renin canola hydrolysate membrane fractions

Membrane fractionation into the <1 and 1-3 kDa peptides significantly ($p<0.05$) improved the ACE-inhibitory activity of trypsin hydrolysates whereas activity of the pancreatin peptides was decreased (Fig. 4A). The results suggest that smaller sized (1-3 kDa) peptides from trypsin hydrolysates are more effective ACE inhibitors as their isolation resulted in higher potency when compared to the larger (3-10 kDa) peptides (Fig. 4A). In contrast, the results for pancreatin suggest a synergistic effect of peptides present in the hydrolysate; separation into different peptide sizes probably led to loss of this synergy and

hence the lower ACE-inhibitory activity of peptide fractions. Membrane fractionation also led to a significant ($p<0.05$) increase in the renin inhibition of trypsin hydrolysate (Fig. 4B), which was better than those reported for cationic peptides of flaxseed peptides (Udenigwe, et al., 2012a). However, in contrast to the observed trend of ACE inhibition, larger sized peptides (3-10 kDa) were more effective renin inhibitors than the <1 kDa permeate peptides. Renin inhibition of the pancreatin hydrolysate was significantly ($p<0.05$) reduced only for the 1-3 kDa permeate peptides but not the other membrane fractions. Similar trends in renin inhibition were observed for Alcalase hydrolyzed chicken thigh skin protein hydrolysate fractions (Onuh et al., 2013). Therefore, unlike ACE inhibition, the pancreatin hydrolysate did not seem to contain peptides that have synergistic effects against renin activity. This is because separation of the pancreatin hydrolysate into different peptide size fractions did produce substantial decreases (except the 1-3 kDa peptides) in renin activity.

3.5. In vivo activity of canola protein isolate and hydrolysates in SHRs

Synthetic drugs available for the treatment of hypertension include diuretic, adrenergic inhibitors such as α - and β – blockers; direct vasodilators; calcium channel blockers; angiotensin II (Ang II) receptor blockers; and ACE inhibitors such as captopril, lisinopril, enalapril, and idrapril (Norris & FitzGerald, 2013; Subissi, Criscuoli, Sardelli, Guelfi, & Giachetti, 1992). Although these synthetic inhibitors show significant effects in treating hypertension, they also cause unpleasant side effects, such as coughing, allergic reactions, taste disturbances and skin rashes (Subissi et al., 1992). Thus, the development of safe and natural ACE inhibitors is necessary for potential treatment and prevention of hypertension (Jao, Huang, & Hsu, 2012). Canola protein peptides exhibited very good antihypertensive properties *in vivo* when administered to SHRs, which is consistent with the fact that ACE and renin activities were inhibited during *in vitro* assays. The changes in SBP that resulted from

the administration of canola hydrolysates are shown in Fig. 5. Pancreatin hydrolysates which had the highest combined *in vitro* ACE and renin inhibitions produced SBP changes of -7.4 and -7.7 mmHg after 2 and 4 h, respectively, but increased to -15.1 after 6 h. Pancreatin and pepsin hydrolysates also had high SBP changes 24 h after oral administration of the hydrolysates. Thus it is possible that peptides present in the pepsin and pancreatin hydrolysates produced a longer-lasting effect either because of slow absorption from the gastrointestinal tract (GIT) or because they have higher resistance to proteolytic inactivation after absorption. Pepsin hydrolysates showed the lowest *in vitro* ACE-inhibitory activity but had very good *in vivo* antihypertensive properties, with SBP changes of up to -11.3 and -23.7 mmHg after 2 and 4 h, respectively. Similar results were obtained for pepsin + papain hydrolyzed jelly fish administered at 400 mg/kg body weight to SHRs (Liu, Zhang, Zhang, & Liu, 2012). It is also possible that some of the pepsin hydrolysate peptides are converted into more active peptides by enzymes within the GIT and belong to the pro-drug group of ACE inhibitors as previously classified by Fujita, Yokoyama, & Yoshikawa (2000). Despite showing high *in vitro* ACE and renin inhibitions, trypsin hydrolysates did not produce any significant ($p<0.05$) SBP changes. Therefore, the results suggest that the trypsin hydrolysate contains peptides that can be classified as substrates (Fujita et al., 2000); these are peptides that inhibit high levels of ACE inhibition during *in vitro* tests but become inactivated by digestive proteases during passage through the GIT. Chymotrypsin had its highest SBP change (-16.3 mmHg) 6 h after administration; this was maintained for up to 8 h, but had returned to the base line value after 24 h. Alcalase however, showed good *in vivo* activity with a change in SBP of -34.5 mmHg 4 h after oral administration; SBP was maintained at -11.3 mmHg after 8 h and at -3.1 mmHg 24 h after administration. The fact that the Alcalase hydrolysate produced the greatest decrease in SBP within the shortest period of time (4 h) could be attributed to the presence of higher amounts of low molecular weight peptides (Fig.

2) and DH (Table 1). These peptides may have facilitated a greater and faster rate of absorption from the GIT into the blood circulatory system. The CPI also produced SBP changes, which increased with time and was more effective than the hydrolysates 24 h after oral administration. The SBP-reducing effect of the CPI is in agreement with the fact that both the pepsin (simulated stomach digestion) and pancreatin (simulated intestinal digestion) hydrolysates were also active. However, the pre-digested pepsin and pancreatin hydrolysates reduced SBP faster (after 4 h), which is consistent with the presence of readily absorbable peptides when compared to the CPI that requires prolonged digestion within the GIT before active peptides can be released. The prolonged digestion of CPI within the GIT will also explain the longer-lasting effect (up to 24 h) when compared to the hydrolysates. The results obtained in this study are different from those obtained for pea and hemp seed protein isolates, which failed to show hypotensive activities when administered to SHRs at the same concentration (Li et al., 2011; Girgih et al., 2011). Thus, the canola proteins may be more susceptible to GIT-dependent release of blood pressure lowering peptides when compared to the pea and hemp seed proteins.

4. Conclusions

Canola protein hydrolysates inhibited *in vitro* activities of renin and ACE in addition to *in vivo* lowering of SBP in SHRs. The presence of higher levels of smaller sized peptides seemed to contribute to a faster rate of SBP decrease as exhibited by Alcalase hydrolysate. However, SBP-lowering activity did not have a direct relationship with *in vitro* ACE or renin inhibition, which is an indication that the peptides had different susceptibility to GIT-dependent enzymatic processing or absorption into the blood circulatory system. The SBP-reducing effects of canola proteins and hydrolysates suggest a potential as viable ingredients

for the formulation of hypotensive nutraceutical products. Further studies are needed to characterize the structural properties of the active peptides and elucidate their mode of action.

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References

Agboola, S., Ng, D., & Mills, D. (2005). Characterisation and functional properties of Australian rice protein isolates. *Journal of Cereal Science, 41*, 283-290.

Alashi, A. M., Blanchard, C. L., Mailer, R. J., Agboola, S. O., Mawson, A. J., He, R., Girgih, A., & Aluko, R. E. (2014). Antioxidant properties of Australian canola meal protein hydrolysates. *Food Chemistry, 146*, 500-506.

Barbana, C., & Boye, J. I. (2011). Angiotensin I converting enzyme inhibitory properties of lentil protein hydrolysates: Determination of the kinetics of inhibition. *Food Chemistry, 127*, 94-101.

Byun, H. G., & Kim, S. K. (2002). Structure and activity of angiotensin I converting enzyme inhibitory peptides derived from Alaskan pollack skin. *Journal of Biochemistry and Molecular Biology, 35*, 239-243.

Calderón de la Barca , A. M., Ruiz-Salazar, R. A., & Jara-Marini, M. E. (2000). Enzymatic hydrolysis and synthesis of soy protein to improve its amino acid composition and functional properties. *Journal of Food Science, 65*, 246-253.

Chen, J.-R., Yang, S.-C., Suetsuna, K., & Chao, J. C. J. (2004). Soybean protein-derived hydrolysate affects blood pressure in spontaneously hypertensive rats. *Journal of Food Biochemistry, 28*, 61-73.

Damodaran S. (1997). Food proteins: an overview. In: S. Damodaran, & A. Paraf (Eds). *Food proteins and their applications*. (pp. 1–24). New York: Marcel Dekker.

Dong, X.-Y., Guo, L.-L., Wei, F., Li, J.-F., Jiang, M.-L., Li, G.-M., Zhao, Yuan-Di., Chen, Hong (2011). Some characteristics and functional properties of rapeseed protein prepared by ultrasonication, ultrafiltration and isoelectric precipitation. *Journal of the Science of Food and Agriculture, 91*, 1488-1498.

Fujita, H., Yokoyama, K., & Yoshikawa, M. (2000). Classification and antihypertensive activity of angiotensin I converting enzyme inhibitory peptides derived from food proteins. *Journal of Food Science, 65*, 564-569.

Girgih, A., Udenigwe, C., & Aluko, R. (2011). In vitro antioxidant properties of hemp seed (*Cannabis sativa* L.) protein hydrolysate fractions. *Journal of the American Oil Chemists' Society, 88*, 381-389

Girgih, A., Udenigwe, C., Li, H., Adebiyi, A., & Aluko, R. (2011). Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (*Cannabis sativa* L.) protein hydrolysates. *Journal of the American Oil Chemists' Society, 88*, 1767-1774.

Griendling K., K., Garret A., & FitzGerald, M., D. (2003). Oxidative stress and cardiovascular injury part II: Animal and human studies. *Circulation, 108*, 2034-2040.

Harnedy, P. A., & FitzGerald, R. J. (2013). *In vitro* assessment of the cardioprotective, anti-diabetic and antioxidant potential of *Palmaria palmata* protein hydrolysates. *Journal of Applied Phycology, 1-11*.

He, R., Alashi, A., Malomo, S. A., Girgih, A. T., Chao, D., Ju, X., & Aluko, R. E. (2013a). Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chemistry, 141*, 153-159.

He, R., Malomo, S. A., Alashi, A., Girgih, A. T., Ju, X., & Aluko, R. E. (2013). Purification and hypotensive activity of rapeseed protein-derived renin and angiotensin converting enzyme inhibitory peptides. *Journal of Functional Foods, 5*, 781-789.

Jamdar, S. N., Rajalakshmi, V., Pednekar, M. D., Juan, F., Yardi, V., & Sharma, A. (2010). Influence of degree of hydrolysis on functional properties, antioxidant activity and ACE inhibitory activity of peanut protein hydrolysate. *Food Chemistry, 121*, 178-184.

Jao, C.-L., Huang, S.-L., & Hsu, K.-C. (2012). Angiotensin I converting enzyme inhibitory peptides: Inhibition mode, bioavailability, and antihypertensive effects. *BioMedicine, 2*, 130-136.

Kato, A., & Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. *Biochimica et Biophysica Acta - Protein Structure, 624*, 13-20.

Li, H., & Aluko, R. E. (2010). Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. *Journal of Agricultural and Food Chemistry, 58*, 11471-11476.

Li, H., Prairie, N., Udenigwe, C. C., Adebiyi, A. P., Tappia, P. S., Aukema, H. M., Jones, Peter J. H., Aluko, Rotimi E (2011). Blood pressure lowering effect of a pea protein hydrolysate in hypertensive rats and humans. *Journal of Agricultural and Food Chemistry, 59*, 9854-9860.

Liu, X., Zhang, M., Zhang, C., & Liu, C. (2012). Angiotensin converting enzyme (ACE) inhibitory, antihypertensive and antihyperlipidaemic activities of protein hydrolysates from *Rhopilema esculentum*. *Food Chemistry, 134*, 2134-2140.

Mäkinen, S., Johannson, T., Vegarud Gerd, E., Pihlava, J. M., & Pihlanto, A. (2012). Angiotensin I converting enzyme inhibitory and antioxidant properties of rapeseed hydrolysates. *Journal of Functional Foods, 4*, 575-583.

Marczak, E. D., Usui, H., Fujita, H., Yang, Y., Yokoo, M., Lipkowski, A. W., & Yoshikawa, M. (2003). New antihypertensive peptides isolated from rapeseed. *Peptides, 24*, 791-798.

Markwell, M. A. K., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry, 87*, 206-210.

Martínez-Maqueda D, M. B., Recio I, & Hernández-Ledesma B. (2012). Antihypertensive peptides from food proteins: A review. *Food & Function, 3,*350-361.

McKellar, R. C. (1981). Development of Off-Flavors in Ultra-High Temperature and Pasteurized Milk as a Function of Proteolysis. *Journal of Dairy Science, 64*, 2138-2145.

Megías, C., Pedroche, J., Yust, M. d. M., Alaiz, M., Girón-Calle, J., Millán, F., & Vioque, J. (2009). Purification of angiotensin converting enzyme inhibitory peptides from sunflower protein hydrolysates by reverse-phase chromatography following affinity purification. *LWT - Food Science and Technology, 42*, 228-232.

Montani, J. P., & Vliet, B. N. (2004). General physiology and pathophysiology of the renin-angiotensin system. In Unger, T. & Schölkens, B. (Eds.), *Angiotensin vol. I* (Vol. 163 / 1, pp. 3-29): Springer; Berlin, Heidelberg.

Norris, R & FitzGerald, R. J. (2013). Antihypertensive Peptides from Food Proteins, Bioactive Food Peptides in Health and Disease, Dr. Blanca Hernández-Ledesma (Ed.), ISBN: 978-953-51-0964-8, InTech, DOI: 10.5772/51710. Available from: http://www.intechopen.com/books/bioactive-food-peptides-in-health-and-disease/antihypertensive-peptides-from-food-proteins.

Onuh, J. O., Girgih, A. T., Aluko, R. E., & Aliani, M. (2013). Inhibitions of renin and angiotensin converting enzyme activities by enzymatic chicken skin protein hydrolysates. *Food Research International, 53*, 260-267.

Panyam, D., & Kilara, A. (1996). Enhancing the functionality of food proteins by enzymatic modification. *Trends in Food Science & Technology, 7*, 120-125.

Segall, L., Covic, A., & Goldsmith, D. J. A. (2007). Direct renin inhibitors: The dawn of a new era, or just a variation on a theme? *Nephrolology Dialysis Transplantion, 22*, 2435-2439.

Sheih, I. C., Fang, T. J., & Wu, T.-K. (2009). Isolation and characterisation of a novel angiotensin I converting enzyme (ACE) inhibitory peptide from the algae protein waste. *Food Chemistry, 115*, 279-284.

Subissi, A., Criscuoli, M., Sardelli, G., Guelfi, M., & Giachetti, A. (1992). Pharmacology of idrapril: A new class of angiotensin converting enzyme inhibitors. *Journal of Cardiovascular Pharmacology, 20*, 139-146.

Tan, S. H., Mailer, R. J., Blanchard, C. L., & Agboola, S. O. (2011). Extraction and characterisation of proteins fractions from Australian canola meals. *Food Research International, 44*, 1075-1082.

Tang, C.-H., Wang, X.-S., & Yang, X.-Q. (2009). Enzymatic hydrolysis of hemp (*Cannabis sativa* l.) protein isolate by various proteases and antioxidant properties of the resulting hydrolysates. *Food Chemistry, 114*, 1484-1490

Udenigwe, C. C., Adebiyi, A. P., Doyen, A., Li, H., Bazinet, L., & Aluko, R. E. (2012a). Low molecular weight flaxseed protein-derived arginine-containing peptides reduced blood pressure of spontaneously hypertensive rats faster than amino acid form of arginine and native flaxseed protein. *Food Chemistry, 132*, 468-475.

Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: Production, processing, and potential health benefits. *Journal of Food Science, 77*, R11-R24.

Udenigwe, C. C., Lin, Y.-S., Hou, W.-C., & Aluko, R. E. (2009). Kinetics of the inhibition of renin and angiotensin I converting enzyme by flaxseed protein hydrolysate fractions. *Journal of Functional Foods, 1*, 199-207.

Verdecchia, P., Gentile, G., Angeli, F., & Reboldi, G. (2012). Beyond blood pressure: Evidence for cardiovascular, cerebrovascular, and renal protective effects of renin–

angiotensin system blockers. *Therapeutic Advances in Cardiovascular Diseases, 6*, 81-91.

Wu, J., Aluko, R. E., & Muir, A. D. (2008). Purification of angiotensin I converting enzyme-inhibitory peptides from the enzymatic hydrolysate of defatted canola meal. *Food Chemistry, 111*, 942-950.

Yoshie-Stark, Y., Wada, Y., Schott, M., & Wäsche, A. (2006). Functional and bioactive properties of rapeseed protein concentrates and sensory analysis of food application with rapeseed protein concentrates. *LWT - Food Science and Technology, 39*, 503-512.

Yoshie-Stark, Y., Wada, Y., & Wäsche, A. (2008). Chemical composition, functional properties, and bioactivities of rapeseed protein isolates. *Food Chemistry, 107*, 32-39.

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Fig. 1. Surface hydrophobicity (H_o) of canola protein isolate (CPI) and hydrolysates (Alcalase (AH), chymotrypsin (CH), pepsin (PH), trypsin (TH) and pancreatin (PcH)) determined by the ANS probe method. Data were expressed as mean ± standard deviation (n=3). Bars with different alphabets have mean values that are significantly different ($p < 0.05$).

Fig. 2. Gel-permeation chromatograms of canola protein hydrolysates after passage through a Superdex Peptide 12 10/300 GL column. Molecular weights of hydrolysate peaks A (> 7.69 kDa), B (7.69 - 0.13 kDa) and C (0.094-0.049 kDa), corresponding to elution times between 15-40 min

Fig. 3. Percentage inhibition of A: angiotensin converting enzyme (ACE) and B: renin by canola protein isolate (CPI) and enzymatic protein hydrolysates (Alcalase (AH), chymotrypsin (CH), pepsin (PH), trypsin (TH) and pancreatin (PcH)) at a concentration of 1 mg protein/ml. Data were expressed as mean ± standard deviation (n=3). Bars with different alphabets have mean values that are significantly different ($p < 0.05$).

Fig. 4. Percentage inhibition of A: angiotensin converting enzyme (ACE) and B: renin by membrane ultrafiltration fractions of pancreatin (PcH) and trypsin (TH) hydrolysates at a concentration of 0.5 mg protein/ml. Data were expressed as mean ± standard deviation (n=3). Bars with different alphabets have mean values that are significantly different ($p < 0.05$).

Fig. 5. Changes in systolic blood pressure (SBP) of spontaneously hypertensive rats (SHRs) administered with canola protein isolate and hydrolysates at 200 mg protein/kg body weight or captopril (10 mg/kg body weight) (n=6). Each bar represents the means ± SD. Bars with different alphabets have mean values that are significantly different ($p<0.05$)

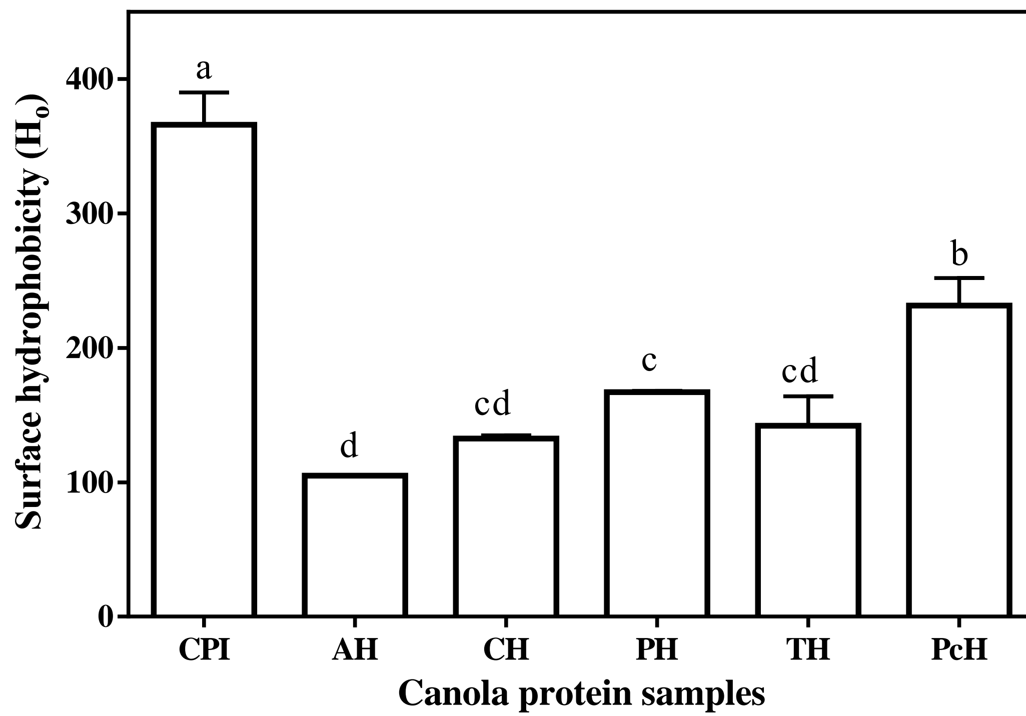


Fig. 1

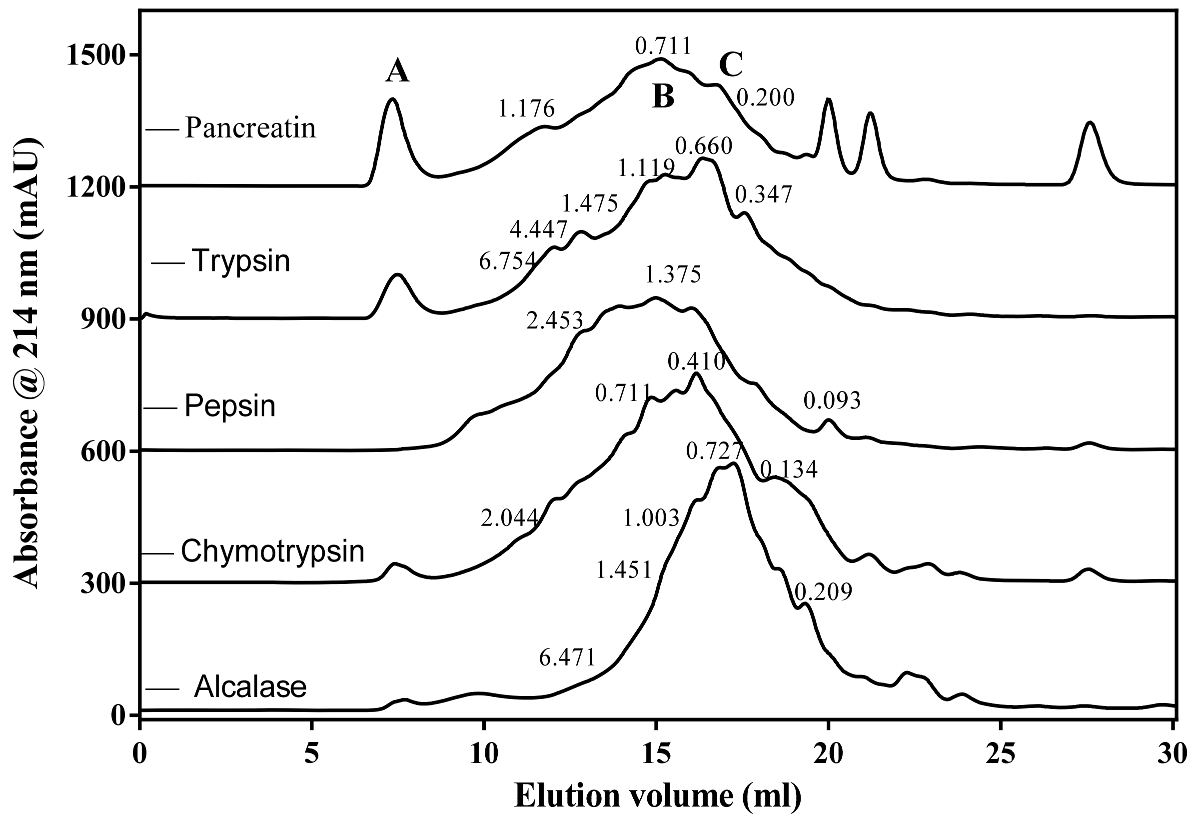
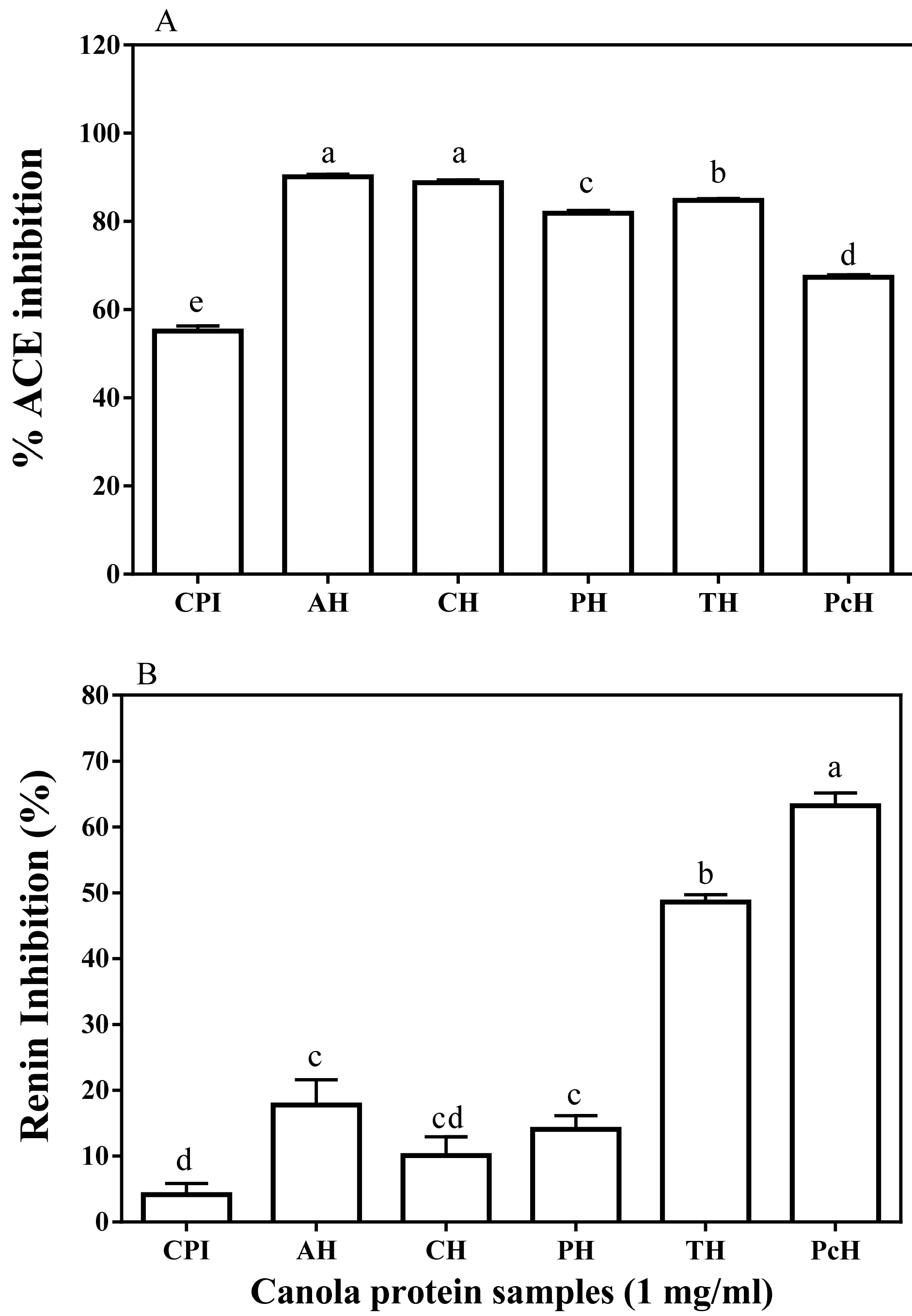


Fig.2



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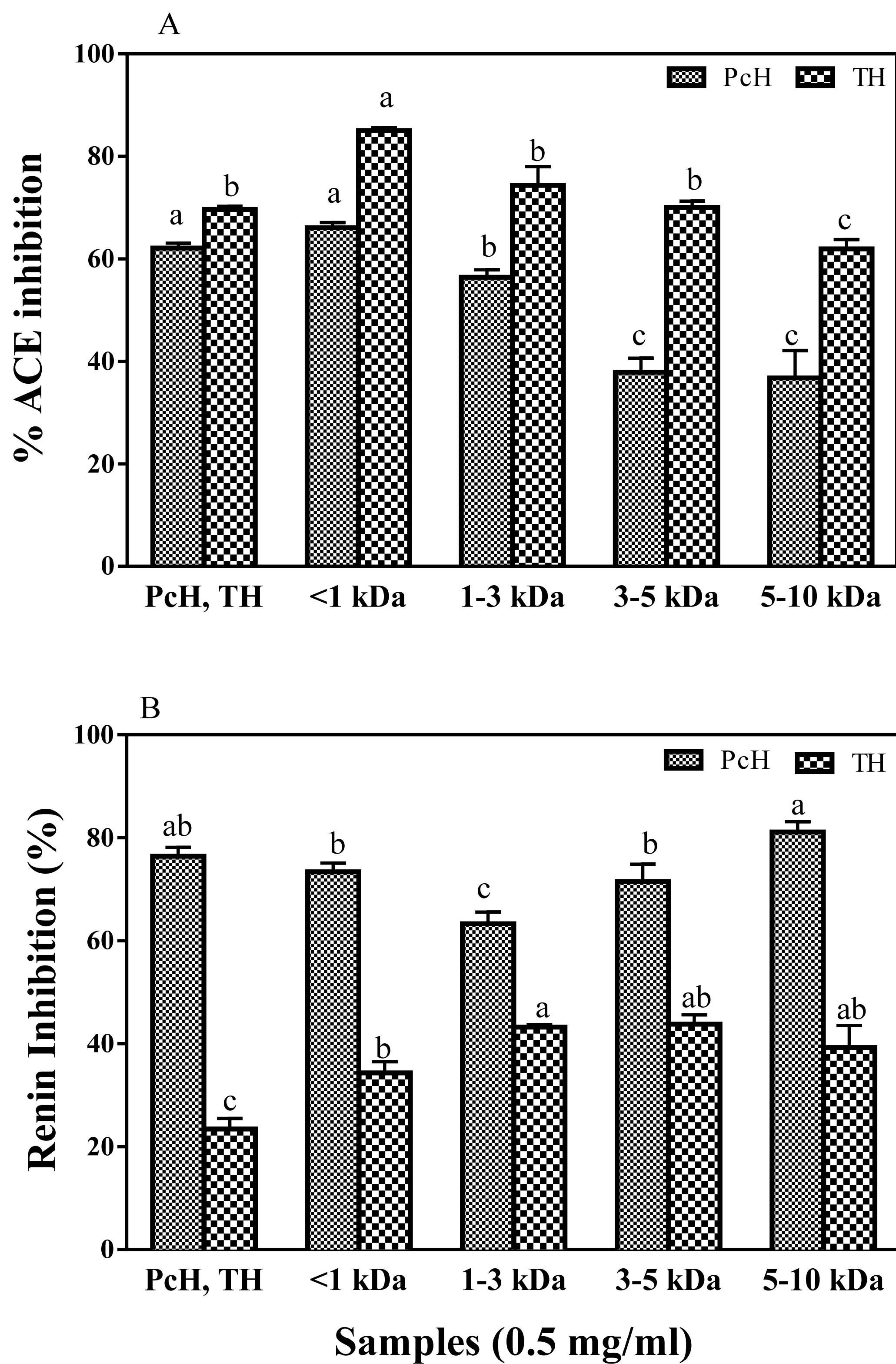

Fig. 4

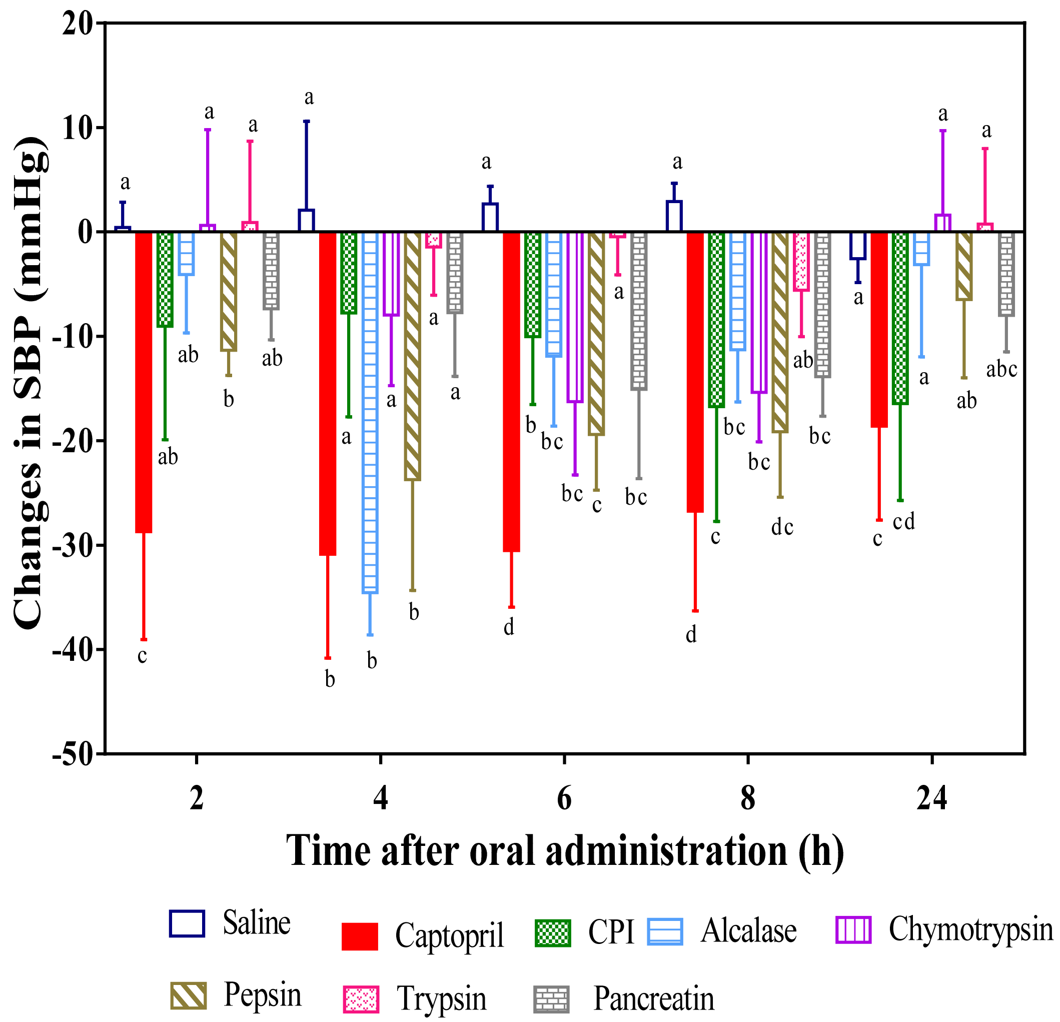

Fig.5

Table 1: Hydrolysis conditions for canola protein hydrolysates

Enzyme	Abbreviation	Hydrolysis conditions (4 h duration)		Degree of hydrolysis (free amino group, mg/g of hydrolysate protein)*
		pH	Temp (°C)	
Alcalase	AH	8.0	60	65.9±0.3[a]
Chymotrypsin	CH	8.0	37	48.5±0.2[b]
Pepsin	PH	3.0	37	45.2±0.3[b]
Trypsin	TH	8.0	37	46.0±0.4[b]
Pancreatin	PcH	8.0	40	44.8±0.2[b]

*CPI: 2.3±0.2 [c] free amino group (mg/g of protein)