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Journal: Food Chemistry

ISSN: 0308-8146 **Year:** 2013 **Pages:** 153 - 159

Volume: 141 **Issue:** 1

Abstract: In this study, rapeseed protein isolate (RPI) was digested with various proteases to produce rapeseed protein hydrolysates (RPHs), which were then separated into different peptide fractions (<1, 1¿3, 3¿5, and 5¿10 kDa) by membrane ultrafiltration. Membrane fractionation showed that peptides with sizes <3 kDa had significantly ($p < 0.05$) reduced surface hydrophobicity when compared to the RPHs and peptide fractions with sizes >3 kDa. In contrast, the <3 kDa peptides showed significantly ($p < 0.05$ ...

Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates

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Abstract

In this study, rapeseed protein hydrolysates (RPHs) were produced using various proteases and then separated into different peptide fractions (<1, 1-3, 3-5, and 5-10 kDa) by membrane ultrafiltration. Membrane fractionation showed that peptides with sizes <3 kDa had significantly reduced ($p<0.05$) surface hydrophobicity when compared to the RPHs and peptide fractions with sizes >3 kDa. In contrast, the low molecular weight peptide fractions (<3 kDa) showed significantly higher ($p<0.05$) oxygen radical scavenging ability when compared to the >3 kDa peptides and RPHs. *In vitro* inhibition of angiotensin converting enzyme (ACE) was significantly greater ($p<0.05$) for the Thermolysin, Proteinase K and Alcalase RPHs when compared to the (P+P) and Flavourzyme RPHs. The Alcalase RPH had significantly highest ($p<0.05$) renin inhibition among the RPHs, while with the exception of Thermolysin, the 5-10 kDa peptide fraction had the least renin-inhibitory ability when compared to the <5 kDa peptide fractions. Oral administration (100 mg/kg body weight) of the RPHs to spontaneously hypertensive rats showed the Alcalase RPH to be the most effective at blood pressure (BP) reduction (~24 mmHg) while Proteinase K RPH was the least effective (~5 mmHg) after 8 h. However, the P+P RPH had the most prolonged effect with BP reduction of ~20 mmHg after 24 h of oral administration. We concluded that dual inhibition of ACE and renin may provide greater BP reducing effects than single inhibition since Alcalase (highest BP reducing effect) had the highest *in vitro* inhibition of both enzymes.

Keyword: Rapeseed protein hydrolysate; Antihypertensive properties; Angiotensin converting enzyme; Renin; Oxygen radical absorbance capacity; Spontaneously hypertensive rat; Membrane ultrafiltration

1. Introduction

Hypertension or high blood pressure, which is estimated to affect one third of the worldwide population, is one of the primary risk factors associated with cardiovascular disease events including myocardial infarction, heart failure, stroke, and vascular dementia (Sharp et al., 2011). The renin angiotensin system (RAS) plays a vital role in blood pressure regulation with renin and angiotensin-converting enzyme (ACE) as the main regulators that control the RAS pathway (Daien et al., 2012). Blood pressure (BP) regulation is based on the fact that renin can convert angiotensinogen to angiotensin I (AT-I), which in turn is converted by ACE to angiotensin II (Ang-II). Ang-II is a potent vasoconstrictor that also induces the release of aldosterone and therefore, increases sodium concentration and blood pressure. Besides, ACE is also known to hydrolyze bradykinin (Yang, Erdos, & Levin, 1970), which is a potent vasodilator, thus leading to the inability of the blood vessels to relax following contraction. Therefore, by inhibiting ACE activity, formation of angiotensin II and destruction of bradykinin will be reduced, which can contribute to lowering of blood pressure. Another strategy is to inhibit renin activity directly, which could provide a more complete blockade of the RAS, since renin catalyzes the rate-determining step in RAS (Fitzgerald, 2011). In addition, oxidative stress is both a cause and a consequence of hypertension, which induces cardiovascular and renal damage with associated increase in blood pressure. For example, it has been shown that Ang II-dependent hypertension is particularly sensitive to NAD(P)H oxidase derived reactive oxygen species (ROS) (Uzuner, Tokay, Cetin, & Yesilkaya, 2010). Therefore, some therapies based on scavenging of ROS could have potential effects in the management of diseases associated with vascular damage, including hypertension.

It has been recognized that nutritional factors play a significant role in the prevention or treatment of hypertension, and food derived bioactive peptides are attracting increasing interest because of their safety and multi-functionality (Udenigwe & Aluko, 2012). Recently, various ACE-inhibitory peptides were obtained from rice, soybean and peanut (Jimsheena & Gowda, 2010; Kang, Kim, Ahn, & Lee,

2012; Rho, Lee, Chung, Kim, & Lee, 2009), showing that plant proteins are good sources of ACE-inhibitory peptides. In addition to ACE inhibition, some studies have also demonstrated that food-derived peptides could inhibit the activity of renin (Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011; Li & Aluko, 2010), and possess free radical scavenging activities (Huang, Majumder, & Wu, 2010). Accordingly, it is believed that simultaneous inhibition of ACE and renin during antihypertensive therapy by food-derived peptides could potentially provide better BP-lowering effects than inhibiting ACE activity alone (Udenigwe & Aluko, 2012).

Rapeseed is an important global commodity, and is a good source of edible oil preceded only by soybean in the world. Rapeseed meal, which is a byproduct of oil extraction, is considered to be a potential alternative source of plant protein containing up to 50% protein on a dry weight basis. Rapeseed protein has a well-balanced amino acid composition (including high levels of lysine, cysteine and methionine) that is comparable to that of other commonly consumed legumes, and can been used in the development of numerous products for the therapeutic, functional foods, and nutraceuticals industries (Yoshie-Stark, Wada, Schott, & Wasche, 2006). Recently, the nutritional and functional properties of rapeseed protein isolate (RPI) was evaluated and compared with those of milk protein isolate and soy protein isolate. The results emphasize ability of RPI to induce particular metabolic effects at the tissue level in healthy individuals during the postprandial period (Dong, et al., 2011). Another study also explained the properties of RPI as an edible film ingredient in food packaging (Jang, Lim, & Bin Song, 2011). In addition, rapeseed protein hydrolysates (RPHs) produced by Alcalase has been reported to possess ACE-inhibitory activity (Mäkinen, Johannson, Gerd, Pihlava, & Pihlanto, 2012), but the work did not provide information on the relationships between ACE inhibition and molecular weight of peptides obtained from different proteinase hydrolysates, or ability of the RPHs to inhibit renin activity.

Since the structure and activity of antihypertensive peptides could be affected by the method of production, there is need to evaluate the efficiency of proteases in releasing antihypertensive peptides

from RPI. Therefore, the first objective of the study was to determine the ability of several proteases to produce antihypertensive RPHs from RPI, evaluated as *in vitro* inhibitions of both ACE and renin activities. Since high oxidative stress may also contribute to blood pressure elevation, the antioxidant ability of the RPHs was determined using the oxygen radical absorption capacity (ORAC) method. The second objective was to determine actual BP-lowering effects of the RPHs in spontaneously hypertensive rats (SHRs) and relate the observed *in vivo* effects to *in vitro* ACE and renin inhibitions.

2. Materials and methods

2.1 Materials

The defatted rapeseed meal (RPM) was supplied by COFCO Eastocean Oil & grains industries Co., Ltd., (Zhangjiagang, China). The meal was milled and passed through a 15 mm screen. Alcalase, Proteinase K, Pepsin, Pancreatin, Thermolysin, Flavourzyme, Angiotensin I-converting enzyme (ACE), N-(3-[2-furyl]acryloyl)-phenylalanylglycylglycine (FAPGG), 8-Anilino-1-naphthalenesulfonic acid ammonium salt (ANS), Captopril, L-glutathione reduced (GSH), and 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), were purchased from Sigma-Aldrich (St. Louis, MO). Renin Inhibitor Screening Assay Kit was purchased from Cayman Chemicals (Ann Arbor, MI). Ultrafiltration membranes, fluorescein free acid, trolox and other analytical grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

2.2. Preparation of rapeseed protein isolate

RPI was produced from RPM according to a previously reported method (Yoshie-Stark, Wada, & Wasche, 2008) with slight modifications. RPM was dispersed in deionized water (1:15, w/v), adjusted to pH 10.0 with 1 M NaOH, mixed at 45^0C for 2 h and the slurry centrifuged at 10000×*g* for 30 min. The supernatant was recovered, adjusted to pH 4.5 with 1 M HCl for 1 h and centrifuged at 10000×*g* for 30 min. The recovered precipitated proteins were washed with anhydrous ethyl alcohol (to remove the polyphenolic components), re-dispersed in deionized water, adjusted to pH 7.0 with 1

M NaOH and freeze-dried to produce RPI. Protein content of the RPI was determined by the modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978).

2.3. Preparation of enzymatic rapeseed protein hydrolysates and membrane fractions

Hydrolysis of the RPI was conducted using each of the following enzyme and reaction conditions as previously reported (He, Girgih, Malomo, Ju, & Aluko, 2012): Alcalase (50^{o}C, pH 8.0); Proteinase K (37^{o}C, pH 7.5); Thermolysin (50^{o}C, pH 8.0); Flavourzyme (50^{o}C, pH 6.5); and Pepsin (37^{o}C, pH 2.0) + Pancreatin (37^{o}C, pH 7.5). RPI (5%, w/v, protein basis) was suspended in deionized water in a reaction vessel equipped with a stirrer, heated to the appropriate temperature and adjusted to the appropriate pH value prior to addition of the proteolytic enzyme. Each protease was added to the RPI slurry at an enzyme–substrate ratio (E/S) of 4:100, based on the protein content of the RPI. Digestion was performed for 4 h (pH maintained constant by addition of NaOH) after which the enzymes were inactivated by adjusting to pH 4.0 with 2 M HCl followed by immersing the reaction vessel in boiling water bath for 10 min. The undigested proteins were precipitated by centrifugation at 8000x*g* for 60 min. The supernatant containing target peptides were passed through ultrafiltration membranes with molecular weight cut-off (MWCO) of 1, 3, 5, 10 kDa in an Amicon stirred ultrafiltration cell. Supernatant was first passed through the 1 kDa membrane and the retentate passed through 3 kDa. The 3 kDa retentate was passed through a 5 kDa membrane whose retentate was then passed through a 10 kDa membrane (He et al., 2012). The permeate from each MWCO membrane (<1, 1-3, 3-5, and 5-10 kDa, respectively) were collected, lyophilized, and stored at -20^{o}C until needed for further analysis. The protein contents of the freeze dried rapeseed protein hydrolysates (RPH) fractions were also determined using the modified Lowry method (Markwell et al., 1978).

2.4. Analysis of molecular weight distribution

Molecular weight distribution of RPH peptides was determined using an AKTA FPLC system (GE Healthcare, Montreal, PQ) equipped with a Superdex Peptide12 10/300 GL column (10×300 mm), and UV detector (λ＝214 nm). An aliquot (100 μL) of the sample (5 mg/mL in 50 mM phosphate

buffer, pH 7.0 containing 0.15 M NaCl) was loaded onto the column and elution performed at room temperature using the phosphate buffer at a flow rate of 0.5 mL/min.

2.5. Surface hydrophobicity (*So*)

So was determined using an aromatic hydrophobicity fluorescence probe (ANS) according to the method described by Wu, Hettiarachchy, & Qi (1998) with some modifications. RPH and ultrafiltration membrane permeates were serially diluted to a final contraction of 50 - 250 µg/mL in 0.01 M phosphate buffer (pH 7.0). Then 20 µL ANS (8.0 mM in 0.01 M phosphate buffer, pH 7.0) was added to 4 mL of each dilution and fluorescence intensity (FI) of the mixture measured with a JASCO FP-6300 fluorescence spectrophotometer (JASCO, Tokyo, Japan) at excitation and emission wavelengths of 390 nm and 470 nm respectively. The initial slope of the FI versus sample concentration plot (calculated by linear regression analysis) was used as an index of *So*.

2.6. Oxygen radical absorbance capacity

ORAC assay was performed according to the method of You, Udenigwe, Aluko, & Wu (2010) with some modifications. Briefly, 120 µL fluorescein (0.008 µM) and 20 µL antioxidant [Trolox (6.25-50 µM) as a standard or sample (50 µg/mL)] in 75 mM phosphate buffer (pH 7.4) were transferred into a 96-well microplate (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) and incubated at 37 ^{o}C for 20 min. After mixing with 60 µL of AAPH (150 mM), the fluorescence was recorded under constant shaking at 1 min intervals for 60 min (λ_{ex}=485 nm, λ_{em}=530 nm). Fluorescence measurements were normalized to the curve of the blank (no antioxidant). ORAC values of the peptide samples were calculated as previously reported (You et al., 2010) and were expressed as µM Trolox equivalent (TE) per gram of sample.

2.7. ACE inhibition assay

The ability of RPH and membrane permeates to inhibit *in vitro* activity of ACE was measured according to the spectrophotometric method described by Holmquist, Bünning, & Riordan (1979) using FAPGG as substrate. 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 (1 U/mL, final activity of 20 mU) and 200 µL sample dissolved in same buffer. The rate of decrease in absorbance at 345 nm was recorded for 2 min at room temperature. The buffer was used instead of sample solutions in the control experiment. ACE activity was expressed as rate of reaction (ΔA/min) and inhibitory activity was calculated as:

ACE inhibition (%) = $1-[\Delta A.min^{-1}_{(sample)} / \Delta A.min^{-1}_{(control)}] \times 100$

Where $\Delta A.min^{-1}_{(sample)}$ and $\Delta A.min^{-1}_{(control)}$ represent ACE activity in the presence and absence of the RPH or peptide fractions, respectively.

2.8. Renin inhibition assay

In vitro inhibition of human recombinant renin activity was conducted using the Renin Inhibitor Screening Assay Kit according to the method previously described by Girgih et al. (2011). Prior to the assay, renin buffer was diluted in 50 mM Tris–HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20 times with the assay buffer before use and pre-warmed to 37 ºC prior to initiating the reaction in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA) maintained at 37 ºC. Before the reaction, (i) 20 µL substrate, 160 µL assay buffer, and 10 µL Milli-Q water were added to the background wells; (ii) 20 µL substrate, 150 µL assay buffer, and 10 µL Milli-Q water were added to the control wells; and (iii) 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 10s to mix, incubated at 37 ºC for 15 min, and the fluorescence intensity (FI) recorded using an excitation and emission wavelengths of 340 and 490 nm, respectively. The percentage renin inhibition was calculated as follows:

$$\text{Renin inhibition (\%)} = \frac{\text{FI (control well)} - \text{FI (inhibitor well)}}{\text{FI (control well)}}$$

2.9. Evaluation of antihypertensive activity in SHRs

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. The male SHRs, 270-320 g body weight (bw) were kept in the Animal Housing Facility at the Richardson Centre for Functional Foods and Nutraceuticals, under a 12-h day and night cycle at 21 ^{o}C and were fed regular diet and tap water. Six rats were used for each of the three groups; sample, captopril and phosphate buffered saline (PBS, 0.1 M, pH 7.4). RPHs (each at 100 mg/kg bw) and captopril (3 mg/kg BW) were dissolved in PBS. The PBS, captopril and samples were administered to the SHR by oral gavage followed by measurement of systolic blood pressure (SBP) at 2, 4, 6, 8 and 24 h using the tail-cuff method in slightly anesthetized rats as previously described (Girgih, et al., 2011). Prior to sample administration, the baseline (time zero) SBP was determined. The change in SBP (mmHg) was determined by subtracting the baseline data from the data obtained at different time points.

2.10. Statistical Analysis

All assays were conducted in triplicate and analyzed by one-way analysis of variance (ANOVA). The means were compared using Duncan's multiple range test and significant differences accepted at $p<0.05$.

3. Results and discussion

3.1. Molecular size distribution of RPHs

The structure (peptide size) and activity of peptides could be affected by the method of production, and there is a need to evaluate the efficiency of proteases in releasing antihypertensive peptides from RPI. The size exclusion chromatogram of RPHs illustrates differences between the proteases used in this work (Fig. 1). The chromatographic data indicated that RPI was not completely hydrolyzed by Flavourzyme or Pepsin+Pancreatin (P+P) as evident in the high molecular weight (MW) peak labeled as 'A', which is absent in the chromatograms for Alcalase, Proteinase K and Thermolysin. The exoprotease activity of Flavourzyme and P+P are reflected in the chromatogram as shown by the

high peak intensity (B and C) of low MW (≤143 Da) compounds, which are probably amino acids (Fig. 1). In contrast, peaks B and C were not observed in the chromatogram of RPH from Alcalase, Proteinase K and Thermolysin. With respect to peptide peak intensity, Alcalase, Thermolysin and Proteinase K hydrolysates contained more peptides in the MW range of 143-2639 Da when compared to those of Flavourzyme and P+P. The results suggest that Alcalase, Thermolysin and Proteinase K had greater endoprotease activity than Flavourzyme and P+P. It was reported that low MW peptides possessed a stronger effect on ACE-inhibitory activity of protein hydrolysate (Terashima, Oe, Ogura, & Matsumura, 2011) when compared to high MW peptides; therefore, Alcalase, Thermolysin and Proteinase K RPHs may have high potential for use as ingredients to formulate antihypertensive products.

3.2. So of RPHs and membrane fractions

It has been reported that hydrophobic and steric properties of ACE-inhibitory peptide sequences played important roles in their bioactivities, and novel peptide sequences could be designed based on these properties of amino acid residues (Shu, et al., 2011). Moreover, hydrophobic residue at the N terminus was also shown to be a preferred structural arrangement for renin inhibition (Li & Aluko, 2010). Fig. 2 shows that *So* was significantly ($p<0.5$) directly related to peptide size because the values were higher as peptide size increased from <1 kDa to 5-10 kDa. It has been known that in native protein molecules, hydrophobic groups are buried inside the core of the folded structure, but after partial hydrolysis, some of these groups would be exposed, resulting in the increased *So* (Paraman, Hettiarachchy, Schaefer, & Beck, 2007). However, as enzyme hydrolysis progresses, the hydrophobic patches are disrupted and *So* will decrease with increased enzyme hydrolysis or reduced peptide size. From Fig. 2, it could be seen that the *So* of RPH obtained from Alcalase, Proteinase K, P+P and Thermolysin were 776.2±5.19, 651.66±2.88, 699.6±11.2 and 694.52±3.05 respectively, which were significantly lower ($p<0.05$) than that of Flavourzyme with a *So* of 1106.33±8.62. The results suggest that the Flavourzyme hydrolysate contained higher molecular weight peptides than the other

hydrolysates. This may be due to the exoprotease activity in Flavourzyme that limits rapid depolymerization of proteins when compared to enzymes with predominant endoprotease activity.

3.3. ORAC capacity of RPHs and membrane fractions

The ORAC values of RPH and ultrafiltration fractions obtained from various proteinases are shown in Fig. 3. There is a notable inverse relationship between ORAC values and peptide molecular weight because the values were highest for the <1 kDa but decreased as peptide size increased to 5-10 kDa. The Alcalase <1 kDa fraction had significantly highest ($p<0.5$) ORAC value (2493.71±134.95 μM TE/mg protein) when compared to the other peptides fractions, RPHs or GSH. Among the RPHs, Flavourzyme had least ORAC value, which may also be due to the presence of large-size peptides. This is because results for the fractionated peptides showed higher ORAC values for small peptides, suggesting that large-size peptides may not interact efficiently with the free radical. The results demonstrate that rapeseed hydrolysates have potentially high antioxidant potency as compared to other hydrolysates found in literature. For example, soy protein hydrolysates with Alcalase yielded ORAC values between 23 and 83.8 μM TE/g protein (Zhang, Li, & Zhou, 2010) while amaranth protein hydrolysate produced by P+P had a value of about 75 μM TE/g protein (Orsini Delgado, Tironi, & Cristina Anon, 2011), which are substantially lower than values obtained in this work.

3.4. ACE-inhibitory activities of RPHs and membrane fractions

The ACE-inhibitory activities of RPHs and membrane fractions showed different trends as evident in Fig. 4. The activity of peptide fractions from Proteinase K hydrolysate were clearly MW dependent; the <1 kDa fraction had the highest ACE-inhibitory activity of 86.94±1.14%, which then declined significantly ($p<0.05$) as peptide size increased to 5-10 kDa. However, for P+P, there was an increase in ACE-inhibitory activity from <1 kDa to the 1-3 kDa but activity decreased for the 3-5 and 5-10 kDa peptides. For Thermolysin, ACE-inhibitory activity increased significantly ($p<0.05$) from <1 kDa and up to the 3-5 kDa but decreased for the 5-10 kDa peptides. In contrast, the ACE-inhibitory trend for Alcalase and Flavourzyme ultrafiltration fractions was independent of peptide MW (Fig. 4).

Overall, the results showed that the 5-10 kDa peptides had significantly lowest ($p<0.05$) ACE-inhibitory properties in comparison with lower (<5 kDa) MW peptides. For the unfractionated RPHs, P+P and Flavourzyme had significantly lowest ACE-inhibitory activities when compared to Alcalase, Proteinase K and Thermolysin. Results for the RPHs suggest that the presence of high MW peptides in P+P and Flavourzyme RPHs may have contributed to the decreased ACE-inhibitory activity; thus low MW peptides seem more active at inhibiting ACE activity when compared to high MW peptides. The results agree with studies carried out by Zhu, Qiu, & Yi (2010), which indicated that the best ACE-inhibitory activity was attributed to low MW peptide fractions; similar ACE-inhibitory activity behavior of peptides was also reported by Segura Campos et al. (2010). Thus the different antihypertensive potentials observed among the RPHs were dependent mainly on the protease used for hydrolysis. The results are consistent with the work of Gao, Chang, Li, & Cao (2010), who also showed that ACE-inhibitory activity of cottonseed protein hydrolysates was dependent on type of protease with papain hydrolysate being the most active among the six proteases used.

3.5. Renin inhibitory activities of RPHs and membrane fractions

Renin inhibition was significantly highest ($p<0.05$) for Alcalase RPH with when compared to RPHs from the other enzymes; however, the ultrafiltration peptide fractions did not show a similar trend to the unfractionated hydrolysates (Fig. 5). For the RPHs, the results were similar to those obtained for ACE inhibition: P+P and Flavourzyme hydrolysates had significantly ($p<0.05$) least renin-inhibitory properties when compared to Alcalase, Thermolysin and Proteinase K hydrolysates. Therefore, the results suggest that smaller peptide sizes which are more abundant in the Alcalase, Thermolysin and Proteinase K hydrolysates than the P+P and Flavourzyme hydrolysates (Fig. 1) contribute to enhanced inhibition of ACE and renin activities. For ultrafiltration fractions, the Proteinase K small peptides (<3 kDa) showed higher renin inhibitions when compared to other peptide fractions from different enzymes. The renin-inhibitory potency of the 5-10 kDa peptide fraction was significantly lowest ($p<0.05$) among the fractionated samples, which also supports the suggestion that

large size peptides have weaker inhibitory activity towards renin when compared to small size peptides. Some of the renin inhibition values obtained in this work using 1 mg/mL peptide concentration are higher than those previously reported for flaxseed protein hydrolysate fraction (Udenigwe et al., 2012) which showed a maximum of 44.5% renin inhibition at 7.5 mg/ml.

3.6. Antihypertensive activity of RPHs in SHRs

Figure 6 shows that all the samples displayed higher BP-reducing effects after 6 h of oral administration when compared to the PBS. Alcalase and P+P RPHs produced significantly highest ($p < 0.05$) decreases in SBP by about -24 and -21 mmHg, respectively after 8 and 24 h of oral gavage. The Alcalase RPH demonstrated higher activity with a good correlation between the *in vivo* and *in vitro* test, and gradually decreased SBP of SHRs up to 24 h (-8.83 mmHg). The observed activity could be due to the higher *in vitro* renin inhibition by Alcalase when compared to the other hydrolysates. In contrast, though Proteinase K and Thermolysin RPH possessed high *in vitro* inhibitions of ACE and renin, the maximum decreases in SBP were only -5 and -9 mm Hg, respectively after 8 h. However, Flavourzyme RPH with relatively lower *in vitro* inhibitions of ACE and renin activities exhibited greater lowering of SBP in the SHR with a maximum of -17 mmHg after 6 h. These inconsistencies between *in vitro* and *in vivo* activities of some of the RPHs could be due to structural degradation of peptides in the rats, which can lead to two outcomes. First, the *in vitro* active peptides could have become inactivated as a result of structural degradation during transit through the digestive tract, which may explain why the Proteinase K and thermolysin hydrolysates had weak reductions of SBP despite strong *in vitro* ACE and renin inhibitions. These types of peptides that have *in vitro* but not *in vivo* activity are classified as substrates (Fujita, Yokoyama, & Yoshikawa, 2000). Secondly, the peptides could be degraded in the rat to form peptide products with greater activity than parent peptide; this could explain the strong SBP-reducing effect of the Flavourzyme hydrolysate despite weak *in vitro* ACE inhibition. These second group of peptides are classified as either true inhibitors or pro-drugs (Fujita et al., 2000). Moreover, the antihypertensive effect of P+P RPH was observed to last longer

and were significantly better (p<0.05) than that of the other hydrolysates and captopril after 24 h. This indicates a more efficient absorption and/or stronger *in vivo* activity of the peptides that are present P+P RPH. Similar results had been obtained for the enzymatic hydrolysates of hemp seed proteins (200 mg/kg bw) with SBP decreases of ~20 and 32 mmHg, respectively at 6 and 8 h after oral administration to SHRs (Girgih et al., 2011). Ishiguro et al. (2012) also reported SBP reduction of about -20 mmHg after 8 h of oral administration of 100 mg/kg bw dose of a sweet potato protein hydrolysate. However, the BP-reducing effects of RPHs used in this work are weaker than those reported for whey protein hydrolysates (80 mg/kg bw), which reduced SBP of SHRs by about -20 and -40 mmHg, respectively at 6 and 8 h (Wang et al., 2012). But the P+P RPH used in this work had a longer lasting effect withabout -21 mmHg SBP reduction after 24 h when compared to <15 mmHg for whey protein hydrolysate and almost zero for sweet potato protein hydrolysate.

4. Conclusions

The *in vitro* and *in vivo* activities of RPHs were dependent on type of protease used for protein digestion and also on molecular size of peptide fractions. The Alcalase and Proteinase K RPHs had better ability to inhibit the *in vitro* activities of ACE and renin in addition to high levels of free radical scavenging. Molecular size of peptide fractions were directly related to *So* but inversely related to free radical scavenging as inhibitory effects towards ACE an renin. Therefore, it is possible that small-sized peptides are better able to penetrate enzyme protein structure to cause reduction in enzyme activity. Alcalase RPH had effective inhibitions of ACE and renin, which could have contributed to the observed highest BP reduction when compared to the other RPHs. However, on a long-term basis, the P+P RPH may provide better effectiveness in reducing BP because of its higher BP-reducing effects after 24 h of oral administration. Thermolysin and Proteinase K RPHs had comparatively weak BP-reducing effects despite strong *in vitro* inhibitions of renin and ACE activities, which suggest structural inactivation of constituent peptides during passage through the gastrointestinal tract (GIT).

In contrast, the Flavourzyme RPH had weaker *in vitro* activities but stronger BP-reducing effects than Thermolysin and Proteinase K RPHs, which suggests that most of the bioactive peptides in Flavourzyme RPH were effectively absorbed or converted into more active peptides in the GIT. Results from this study indicate that RPHs, especially those produced through Alcalase and P+P hydrolysis may be used as peptide ingredients to formulate functional foods and nutraceutical products that can lower blood pressure.

Acknowledgement

Funding for this work was provided through the Ministry of science and technology of agriculture Lee technical achievements transformation Fund project. (Project No. 2011GB2C100012), the Jiangsu province agriculture independent innovation program (Project No.CX (10)444), and the research program of Dr. R.E. Aluko is funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant.

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Figure legends

Fig. 1. Gel-permeation chromatograms of enzymatic rapeseed protein hydrolysates after passage through a Superdex Peptide12 10/300 GL column. Column was calibrated with Cytochrome C (12,384 Da), Aprotinin (6,512 Da), Vitamin B12 (1,855 Da), and Glycine (75 Da). Peak A represents polypeptides with molecular weight >2.6 kDa while B and C are small molecules (<143 Da).

Fig. 2. Surface hydrophobicity (*So*) of enzymatic rapeseed protein hydrolysates and membrane ultrafiltration fractions. Bars (mean ± standard deviation, n=3) with different alphabets have mean

values that are significantly different ($p < 0.05$).

Fig. 3. Oxygen radical absorbance capacity of enzymatic rapeseed protein hydrolysate and membrane ultrafiltration fractions. Bars (mean ± standard deviation, n=3) with different alphabets have mean values that are significantly different ($p < 0.05$).

Fig. 4. Inhibition of angiotensin converting enzyme (ACE) by enzymatic rapeseed protein hydrolysates and membrane ultrafiltration fractions at a concentration of 1 mg/ml. Bars (mean ± standard deviation, n=3) with different alphabets have mean values that are significantly different ($p < 0.05$).

Fig. 5. Inhibition of renin by enzymatic rapeseed protein hydrolysate and membrane ultrafiltration fractions at a concentration of 1 mg/ml. Bars (mean ± standard deviation, n=3) with different alphabets have mean values that are significantly different ($p < 0.05$).

Fig. 6. Effects of enzymatic rapeseed protein hydrolysates (RPHs) on systolic blood pressure (SBP) of spontaneously hypertensive rats after oral gavage. RPHs were each administered using a dose of 100 mg/kg rat body weight (bw) while captopril was given at 3 mg/kg bw.

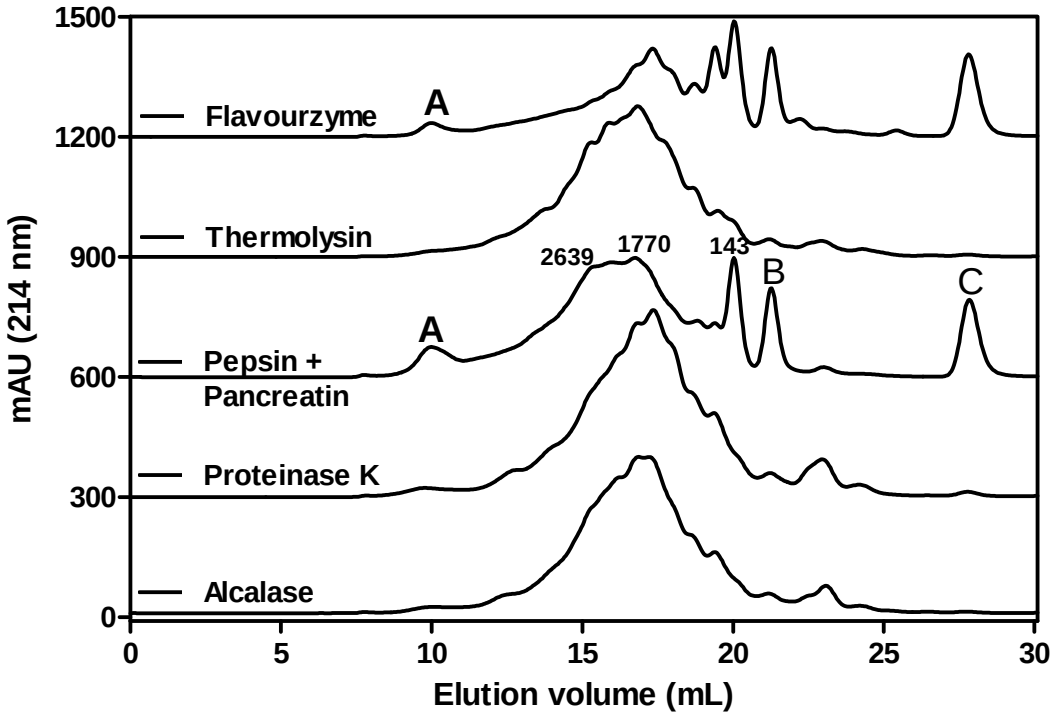
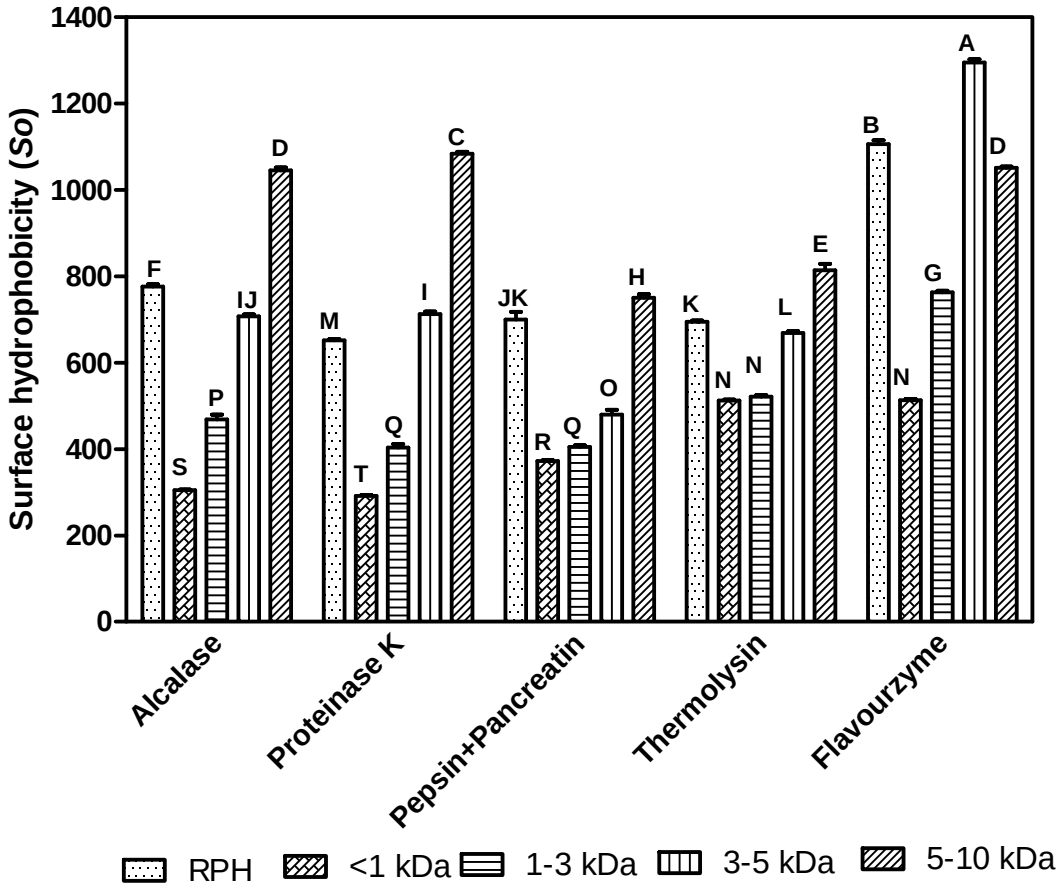

1500
1200
900
600
300
0
mAU (214 nm)
Flavourzyme
Thermolysin
Pepsin +
Pancreatin
Proteinase K
Alcalase
A
A
2639
1770
143
B
C
0
5
10
15
20
25
30
Elution volume (mL)

Fig. 1.


Surface hydrophobicity (So)
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1200
1000
800
600
400
200
0
F
S
P
IJ
D
M
T
Q
I
C
JK
R
Q
O
H
K
N
N
L
E
B
N
G
A
D
Alcalase
Proteinase K
Pepsin+Pancreatin
Thermolysin
Flavourzyme
RPH
<1 kDa
1-3 kDa
3-5 kDa
5-10 kDa

Fig. 2.

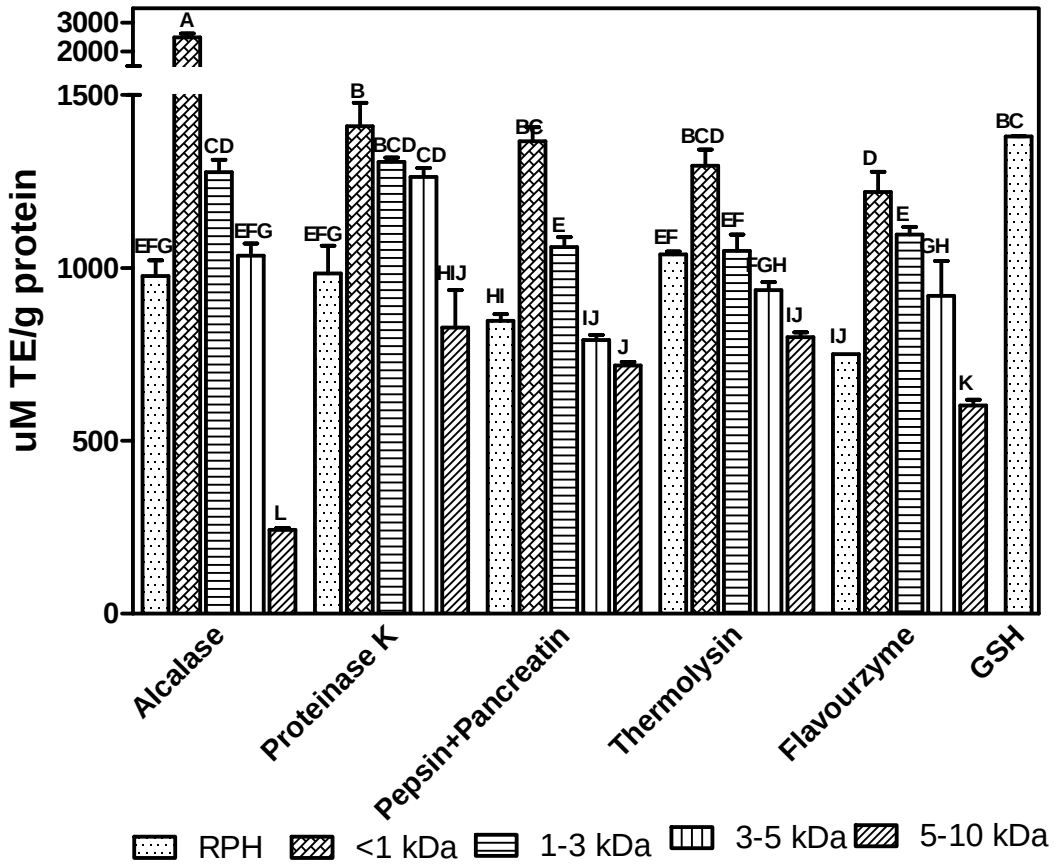
uM TE/g protein
3000
2000
1500
1000
500
0
A
CD
EFG
L
B
BCD
CD
HIJ
HI
BC
E
IJ
J
BCD
EF
FGH
IJ
D
E
GH
K
BC
Alcalase
Proteinase K
Pepsin+Pancreatin
Thermolysin
Flavourzyme
GSH
RPH
<1 kDa
1-3 kDa
3-5 kDa
5-10 kDa

Fig. 3.

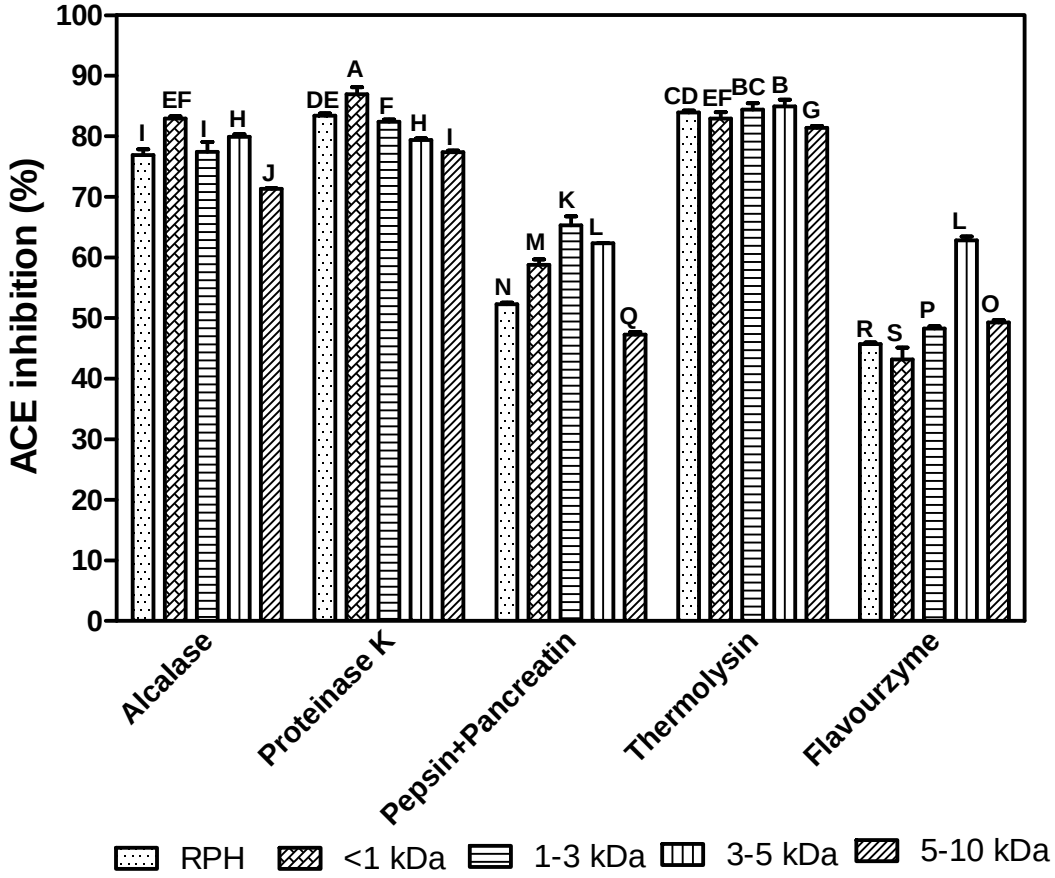
ACE inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
I
EF
I
H
J
DE
A
F
H
I
N
M
K
L
Q
CD
EF
BC
B
G
R
S
P
L
O
Alcalase
Proteinase K
Pepsin+Pancreatin
Thermolysin
Flavourzyme
RPH
<1 kDa
1-3 kDa
3-5 kDa
5-10 kDa

Fig. 4.

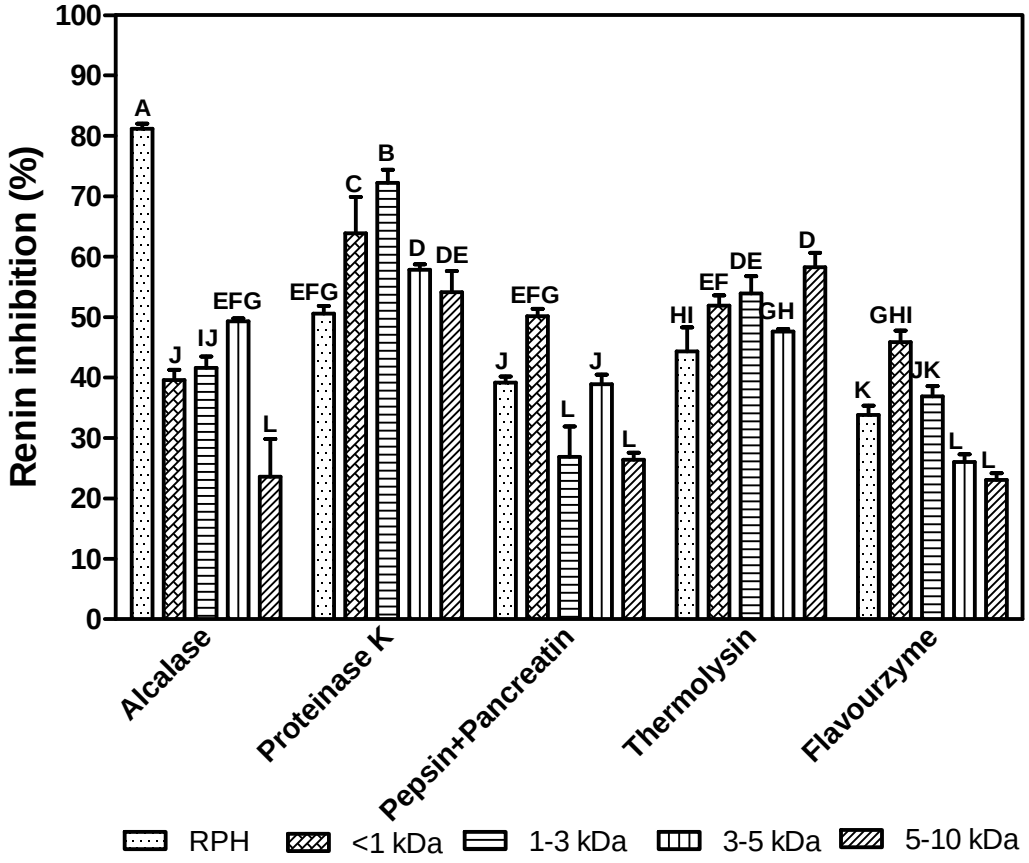
Renin inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
A
J
IJ
EFG
L
EFG
C
B
D
DE
J
EFG
L
J
L
HI
EF
DE
GH
D
K
GHI
JK
L
L
Alcalase
Proteinase K
Pepsin+Pancreatin
Thermolysin
Flavourzyme
RPH
<1 kDa
1-3 kDa
3-5 kDa
5-10 kDa

Fig. 5.

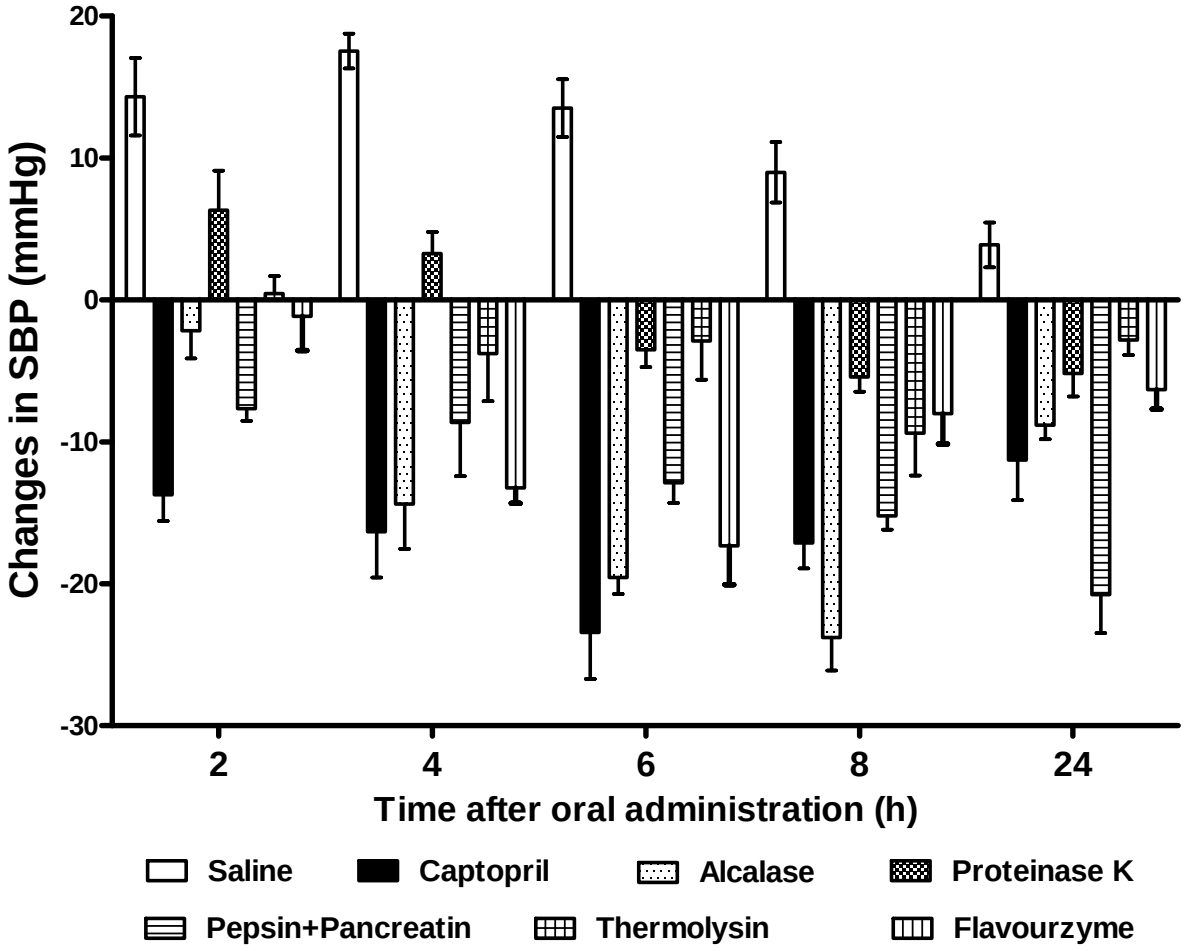
Changes in SBP (mmHg)
20
10
0
-10
-20
-30
2
4
6
8
24
Time after oral administration (h)
Saline
Captopril
Alcalase
Proteinase K
Pepsin+Pancreatin
Thermolysin
Flavourzyme

Fig. 6.