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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · AUGUST 2013

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Glycinyl-Histidinyl-Serine (GHS), a Novel Rapeseed Protein-Derived Peptide Has Blood Pressure-Lowering Effect in Spontaneously Hypertensive Rats

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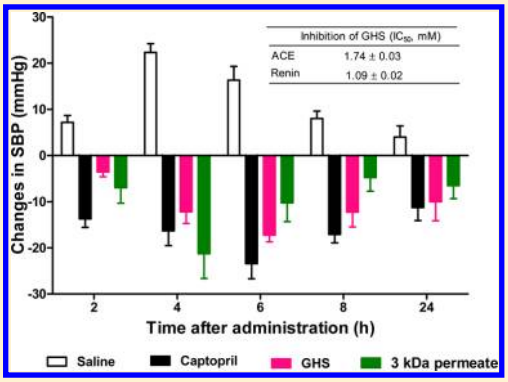
ABSTRACT: A novel antihypertensive peptide (Gly-His-Ser or *GHS*) with dual inhibition of angiotensin I-converting enzyme (ACE) and renin activities was isolated from the 3 kDa membrane ultrafiltration permeate of a pepsin +pancreatin rapeseed protein digest. The IC_{50} values of *GHS* were 0.52 ± 0.01 mg/mL and 0.32 ± 0.01 mg/mL for ACE and renin inhibitions, respectively, which are 1.5 times the ACE inhibition and 3.5 times the renin inhibition of the 3 kDa permeate. Oral administration (30 mg/kg body weight) to spontaneously hypertensive rats showed *GHS* to be an effective hypotensive agent with maximum blood pressure reduction of −17.29 ± 2.47 mmHg after 6 h. In contrast, the 3 kDa permeate exhibited a maximum of −21.29 ± 9.29 mmHg after 4 h, although at a relatively higher dose of 100 mg/kg body weight). *GHS* inhibited ACE and renin activities noncompetitively, but the renin inhibition became uncompetitive at a higher peptide concentration.



KEYWORDS: *rapeseed protein isolate, angiotensin I-converting enzyme, renin, antihypertensive peptide, spontaneously hypertensive rats*

INTRODUCTION

Hypertension (high blood pressure) is a major global threat to human health because it constitutes a high risk factor for the development of cardiovascular diseases, stroke, and end-stage renal disease.[1] Blood pressure is regulated by the renin-angiotensin system (RAS), which consists of two key enzymes, renin and angiotensin-I converting enzyme (ACE).[2] Renin catalyzes conversion of angiotensinogen to angiotensin I, which is the rate-determining step. ACE then converts angiotensin I to angiotensin II, which is a potent vasoconstrictor. Excessive physiological levels of angiotensin II contribute directly to the development and maintenance of hypertension because of the increased rate of blood vessel contraction accompanied by a reduced rate of relaxation. Therefore, inhibition of renin and/or ACE is considered to be a major strategy for hypertension treatment. Currently, aliskiren is the only pharmacological agent approved as a renin inhibitor, while several drugs (captopril, enalapril, lisinopril, etc.) are available as effective ACE inhibitors. Other drugs called angiotensin receptor blockers (ARB) are also available which prevent binding of angiotensin II to specific cellular receptors and are used as blood pressure-lowering agents. Therefore, there is an increased interest in the use of compounds that provide dual blockade of the RAS; for example, a combination therapy that consists of aliskiren and an ACE inhibitor or ARB for the management of congestive heart failure and hypertension.[3] The opportunity to block the RAS through a multiple enzyme inhibition approach has a compelling biological rationale because of the increased chance of successful control of blood pressure.[4] However, the safety of this type of drug combination treatment, including negative side effects such as dry cough and edema that are associated with use of antihypertensive drugs, is of great concern.[4]

Food protein-derived peptides, in addition to their nutritional value, have been shown to significantly reduce blood pressure following intravenous or oral administration, and are considered safer (reduced risk of negative side effects) than drugs for the treatment of hypertension.[5−7] For example, the famous milk protein-derived tripeptides VPP and IPP have been shown to produce significant decreases in systolic blood pressure (SBP) and diastolic blood pressure (DBP) at doses between 3 and 52 mg per day.[8,9] Moreover, oral administration of 5-fold excess of casein protein-derived peptides to hypertensive and normotensive people over a 4-week period produced an average of −8 mmHg reduction in SBP of hypertensives only but there was no adverse effect in all of the groups.[10] More recently, several products containing ACE-

Received: February 25, 2013
Revised: July 30, 2013
Accepted: August 6, 2013
Published: August 6, 2013

dx.doi.org/10.1021/jf400865m | J. Agric. Food Chem. 2013, 61, 8396−8402

inhibitory peptides such as Calpis (VPP and IPP), Biozate (whey peptide), and Lowpet (RYLGY and AYFYPEL) have been patented and are available on the market.[11] Therefore, currently available food derived antihypertensive peptides are established ACE inhibitors but with no demonstrated inhibition of renin. However, dual renin and ACE-inhibitory peptides could provide more effective blood pressure reductions than peptides that inhibit only ACE activity.[4,12] Because peptides are highly susceptible to structural degradation and loss of activity during passage through the gastrointestinal tract (GIT) or upon interaction with ACE, careful selection of the digestion method to release the resistant amino acid sequences is very important for producing bioactive peptides. For example, antihypertensive peptides IRW and LKP that were purified from egg ovotransferrin hydrolysate were degraded into inactive dipeptides IR and KP, respectively, by pancreatin and mucosal peptidase.[13] Thus, a suitable approach to generate bioactive peptides that are resistant to loss of activity during oral administration is to use simulated GIT digestion with pepsin and pancreatin (PP) as proteases for hydrolysis of the native proteins.

Rapeseed proteins can be extracted from rapeseed meal and are considered suitable ingredients for the release of bioactive peptides with antioxidant,[14] antitumor,[15] and ACE-inhibitory activities.[16] Our previous work has shown that rapeseed protein hydrolysate (RPH) obtained by Alcalase hydrolysis has effective SBP reduction,[17] and the dual ACE and renin inhibitory peptides LY, TF, and RALP purified from this hydrolysate evidence their contributions to the hypotensive effect.[18] In addition, we reported that the PP RPH was also effective in reducing SBP up to 24 h after oral administration, but the active peptide was not identified.[17] Moreover, the 3 kDa permeate from the PP RPH showed higher ACE and renin inhibitory activities when compared to the other ultrafiltration fractions.[17] In this study, the aim was to perform peptide separation and purification protocols with the 3 kDa permeate of the PP RPH in order to identify a new and novel peptide with dual in vitro inhibitions of ACE and renin activities. The kinetics of peptide inhibition of ACE and renin were also determined in addition to the measurement of the blood pressure-lowering effect in SHR.

MATERIALS AND METHODS

Chemicals. A Renin inhibitor screening assay kit was purchased from Cayman Chemicals (Ann Arbor, MI). Pepsin, pancreatin, rabbit lung ACE, and *N*-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG), were purchased from Sigma-Aldrich (St. Louis, MO). Other analytical grade reagents and ultrafiltration membranes were obtained from Fisher Scientific (Oakville, ON, Canada).

Materials. The defatted rapeseed meal (DRM) was supplied by COFCO Eastocean Oil & Grains Industries Co., Ltd. (Zhang Jiagang, P.R. China).

Preparation of Rapeseed Protein Hydrolysate. Rapeseed protein isolate (RPI) was produced by alkali extraction and acid precipitation from DRM according to a previously described method.[17] RPI (5%, w/v, protein basis) was suspended in deionized water in a reaction vessel equipped with a stirrer, heated to 37 °C, and adjusted to pH 2.0 or 7.5 prior to the addition of the pepsin or pancreatin, respectively. Each enzyme was added to the slurry at an enzyme−substrate ratio (E/S) of 1:25 based on the protein content of the RPI. Digestion was performed at the above temperature and pH conditions first with pepsin (2 h) followed by pancreatin (4 h); reaction mixture pH was kept constant by addition of 2 M NaOH when necessary. After digestion, the enzymes were inactivated by immersing the reaction vessel in boiling water for 10 min, cooled to room temperature and undigested proteins precipitated by centrifugation at 8000*g* for 60 min. The supernatant containing target peptides was passed through an ultrafiltration membrane with a molecular weight cutoff (MWCO) of 3 kDa in an Amicon stirred ultrafiltration cell. The permeate fraction was collected, lyophilized, and stored at −20 °C until needed for further analysis. The protein content of the freeze-dried permeate (3 kDa permeate) was determined using the modified Lowry method.[19]

Purification of Renin and ACE-Inhibitory Peptide. The 3 kDa permeate was dissolved in 50 mM phosphate buffer (pH 7.0, containing 0.15 M NaCl) at a concentration of 10 mg/mL. An aliquot (5 mL) of the sample was loaded onto a gel-permeation Hiload 26/600 Superdex 30 Prep grade column (600 × 26 mm) attached to an ÄKTA explorer 10 system (GE Healthsciences, Montreal, PQ). The column was calibrated with cytochrome *c* (12 384 Da), aprotinin (6512 Da), vitamin B12 (1855 Da), and Gly (75 Da), which were used to estimate molecular weight (MW) of eluted fractions. An isocratic elution was conducted at a flow rate of 3.0 mL/min using the phosphate buffer solution; fractions were collected, dialyzed against water using a 100 Da MWCO membrane (Spectra/Por, Spectrum Laboratories, Houston), lyophilized, and analyzed for ACE and renin inhibitions. The fraction with the highest inhibitions of both ACE and renin activities was dissolved in distilled water (containing 0.1% TFA) at a concentration of 10 mg/mL and 1 mL of the fraction loaded on a Jupiter 4u Proteo 90A C12 preparative reverse-phase column (250 × 21.10 mm, 5 μm, Phenomenex, Inc., Torrance, CA) attached to a Varian 940 semipreparative HPLC system. The column was eluted with a gradient from 5 to 80% methanol (containing 0.1% TFA) within 60 min at a flow rate of 5 mL/min, and six fractions were collected and lyophilized; absorbance of eluted peptides was monitored at 215 nm. The lyophilized fraction with the highest activity against ACE and renin was further purified with a C18 protein and peptide column (4.6 × 250 mm, 5 μm, VYDAC, CA) on above HPLC system, and the elution was conducted with a gradient from 25% to 35% methanol (containing 0.1% TFA) within 30 min at a flow rate of 0.5 mL/min; two fraction were collected, lyophilized, and analyzed for ACE and renin inhibitions.

Identification of the Purified Peptide. The most active fraction from the second round of HPLC separation (C18 column) was dissolved in deionized water (containing 0.1% formic acid) and then loaded onto an Eclipse Plus C18 RRHD columns (2.1 × 100 mm, 1.8 μm, ZORBAX, U.S.) on an ACQUITY UPLC system (Waters, Milford, MA) with an injection volume of 5 μL at flow rate of 0.2 mL/min. The separation was performed with a linear gradient of 30% acetonitrile in 6 min. The elution was monitored with a Quadrupole Mass Spectrometer (Micromass, Waters, U.S.) coupled with electrospray ionization (ESI) source using 30 V collision energy, 1.5 kV capillary voltage, 20 V cone voltage, and 150 °C capillary temperature. The determined peptide sequence was synthesized (>95% purity) by GenWay Biotech (GenWay Biotech Inc. San Diego, CA).

ACE Inhibition Assay. The ability of sample to inhibit in vitro activity of ACE was measured according to the spectrophotometric method described by Holmquist et al.[20] 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 as the FAPGG. 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 blank experiment. ACE activity was expressed as rate of reaction (Δ*A*/min) and inhibitory activity was calculated as follows:

$$\text{ACE inhibition (\%)} = \left[1 - \Delta A\cdot\text{min}^{-1}_{(\text{sample})} / \Delta A\cdot\text{min}^{-1}_{(\text{blank})}\right] \times 100$$

where $\Delta A\cdot\text{min}^{-1}_{(\text{sample})}$ and $\Delta A\cdot\text{min}^{-1}_{(\text{blank})}$ are ACE activity in the presence and absence of inhibitory peptides, respectively. Final tested sample concentrations for the gel-permeation and RP-HPLC purification fractions were 1 mg peptide/mL and 0.5 mg peptide/mL, respectively. The concentration of peptide that inhibited 50%

ACE activity (IC_{50}) was calculated by nonlinear regression from a plot of percentage ACE inhibition versus four peptide concentrations (0.125, 0.25, 0.5, and 1.0 mg/mL). 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 kinetic parameters (V_{max} and K_m) were estimated from nonlinear regression fit of the data to the Michaelis–Menten equation using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, U.S.). Inhibition constant (K_i) was calculated as the x-axis intercept from a plot of the slope of the Lineweaver–Burk lines against two sample concentrations (0.25 and 0.5 mg/mL). The catalytic efficiency (CE) was calculated from V_{max}/K_m ratio.

Renin Inhibition Assay. In vitro inhibitory activity of human recombinant renin assay was conducted using Renin Inhibitor Screening Assay Kit according to the method previously described by Girgih et al.[21] Briefly, the sample was diluted in Tris-HCl buffer (50 mM, pH 8.0, containing 100 mM NaCl), and prewarmed to 37 °C prior to initiating the reaction. Before the reaction, (1) 20 μL substrate, 160 μL assay buffer, and 10 μL double distilled water (DDW) were added to the background wells; (2) 20 μL substrate, 150 μL assay buffer, and 10 μL DDW were added to the blank wells; and (3) 20 μL substrate, 150 μL assay buffer, and 10 μL sample were added to the inhibitor (sample) 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; fluorescence intensity (FI) was then recorded at excitation wavelength of 340 nm and emission wavelength of 490 nm using a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnyvale, CA). The percentage renin inhibition was calculated as follows:

$$\text{Renin inhibition (\%)} = \left[1 - \Delta\text{FI}\cdot\text{min}^{-1}_{(\text{sample})} / \Delta\text{FI}\cdot\text{min}^{-1}_{(\text{blank})}\right] \times 100$$

where $\Delta\text{FI}\cdot\text{min}^{-1}_{(\text{sample})}$ and $\Delta\text{FI}\cdot\text{min}^{-1}_{(\text{blank})}$ are renin activity in the presence and absence of inhibitory peptides, respectively. The final tested sample concentrations for the gel-permeation and RP-HPLC purification fractions were 1 mg peptide/mL and 0.5 mg peptide/mL, respectively, while IC_{50} was calculated by nonlinear regression from a plot of percentage renin inhibition versus peptide concentrations (0.125, 0.25, 0.5, and 1.0 mg/mL). The renin inhibition kinetics was conducted using 1.25, 2.5, 5, and 10 μM substrate in the absence and presence of sample, while kinetic parameters were calculated as described above for ACE.

Evaluation of Antihypertensive Activity in SHR. 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 SHR (Charles River Laboratories, Montreal, PQ) with 350–390 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 °C and fed regular diet and tap water. The rats were divided into four groups with 4 rats per group: 3 kDa permeate, *GHS*, captopril, and phosphate buffered saline (PBS, pH 7.4). The 3 kDa permeate (100 mg/kg bw), *GHS* (30 mg/kg bw), and captopril (10 mg/kg bw) were dissolved in PBS buffer and administered to the SHRs by oral gavage followed by measurement of SBP at 2, 4, 6, 8, and 24 h using the tail-cuff method in slightly anesthetized rats as previously described.[21] Prior to oral gavage, the baseline (time zero) SBP was determined. The change in SBP (ΔSBP, mm Hg) was determined by subtracting the baseline data from the data obtained at different time points.

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 $p < 0.05$.

RESULTS AND DISCUSSION

Purification and Identification of ACE and Renin Inhibitory Peptides from Rapeseed Protein Hydrolysate. RPI was hydrolyzed consecutively with PP, and the digest was first separated by ultrafiltration membrane with the MWCO of 3 kDa. The 3 permeate, with ACE and renin inhibition values (at 1 mg/mL peptide concentration) of 61.52 ± 1.77% and 42.06 ± 1.32%, respectively, was then applied to a preparative gel permeation column. Figure 1A shows that most of the

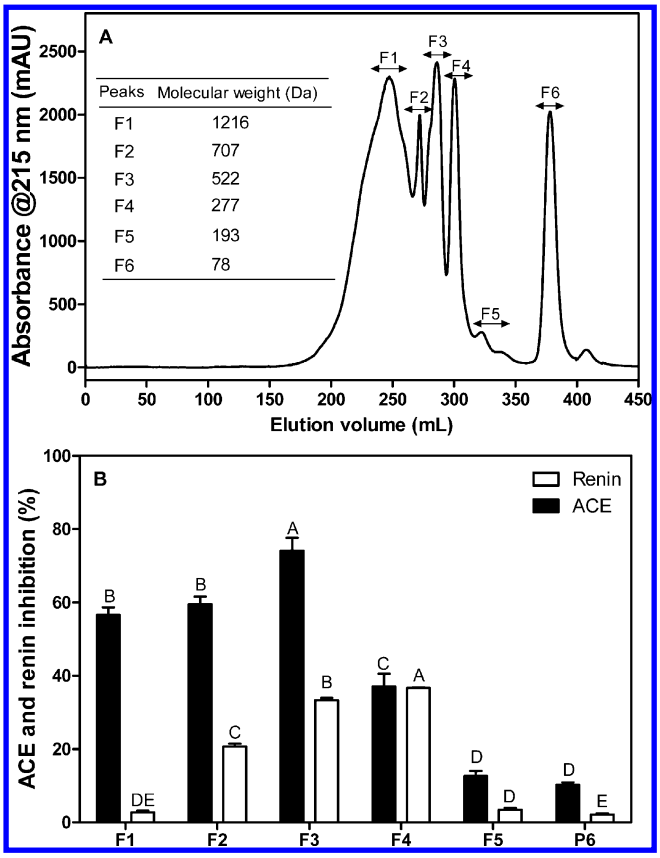


Figure 1. (A) Gel permeation chromatography of the 3 kDa membrane permeate from pepsin+pancreatin hydrolysate on a Hiload 26/600 Superdex 30 Prep grade column. (B) ACE and renin inhibitory activities of fractions from the gel permeation chromatography separation. Final peptide concentration was 1.0 mg/mL for renin and ACE assays. Bars (mean ± standard deviation, $n = 3$) with different letters have mean values that are significantly different ($p < 0.05$).

eluted peptides can be divided into six main fractions (F1–F6). F3 had significantly highest ($p < 0.05$) ACE inhibition of 74.15 ± 3.45%, while F4 exhibited highest renin inhibition with 36.70 ± 0.14% (Figure 1B). From the estimated MW values, it is reasonable to suggest that F5 (193 Da) and F6 (78 Da) are not peptides but free amino acids and other small size compounds. The nonpeptidic nature of F5 and F6 may be responsible for the observed low inhibitory values against ACE and renin activities (Figure 1B). The results are consistent with those reported for apricot kernel protein hydrolysate where the highest ACE-inhibitory activity was attributed to low MW peptide fractions ranging from 200 to 900 Da.[22] The F4 fraction likely consists of mostly dipeptides, which have been shown through quantitative structure–activity relationship modeling to be strong renin inhibitors.[23]

On the basis of results from the gel permeation chromatography, F3 had the highest combined ACE and renin inhibitions; this fraction was then subjected to semi-preparative RP-HPLC column separation to give six fractions (Figure 2A). Although the F3-D fraction was the dominant peak, it showed relatively lower ACE and renin inhibitions when compared to the other fractions (Figure 2B). Mass spectrometry analysis showed that F3-D was mainly composed of phenylalanine as free amino acids (data not shown), which may be responsible for the low ACE and renin inhibitory values. However, a relatively less abundant F3-C showed the highest ($p < 0.05$) dual inhibition of ACE and renin activities with values of 56.86 ± 1.38% and 41.67 ± 2.18%, respectively. Therefore, F3-C was used for final peptide purification by an analytical RP-HPLC column to yield 2 fractions (F3-C-I and F3-C-II) as shown in Figure 3. F3-C-I had a higher ACE and renin inhibitory activity when compared to F3-C-II (inset table in Figure 3); therefore, F3-C-I was selected for peptide identification and amino acid sequence analysis.

The amino acid sequence of F3-C-I was identified using UPLC coupled to online triple-quadruple linear ion trap mass spectrometry; *Y*-type or *b*-type are the common fragmenting peptide ions at low energy collision-induced dissociation (CID).[24] F3-C-I was separated on a C18 column to yield only one peak in its total ion chromatogram (data not shown).

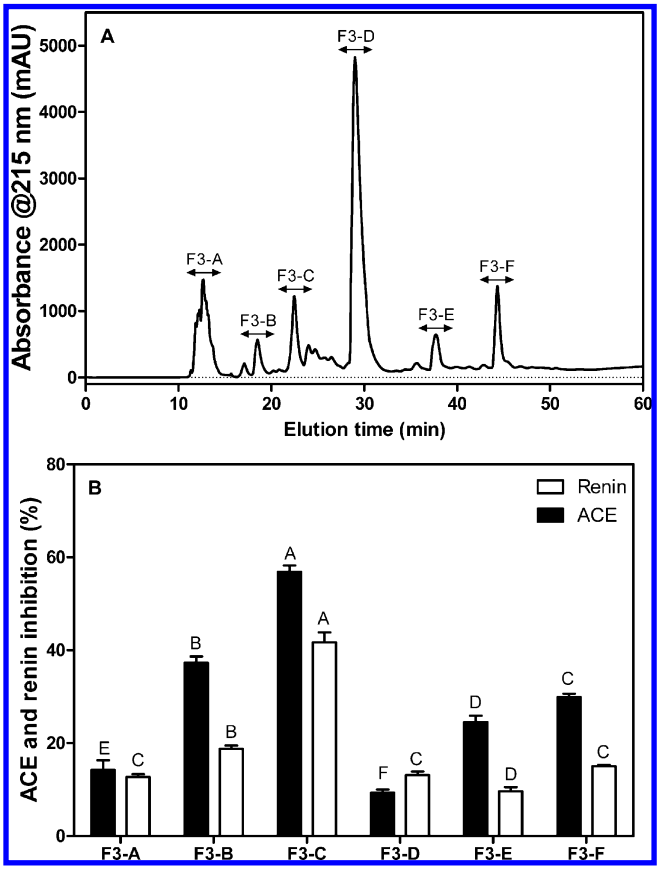


Figure 2. (A) Separation of fraction F3 on a Jupiter 4u Proteo 90A C12 preparative RP-HPLC column with a flow rate of 5 mL/min and a linear gradient width of 5−80% methanol in 60 min. (B) ACE and renin inhibitory activities of F3 fractions. Final peptide concentration was 0.5 mg/mL for renin and ACE assays. Bars (mean ± standard deviation, $n = 3$) with different letters have mean values that are significantly different ($p < 0.05$).

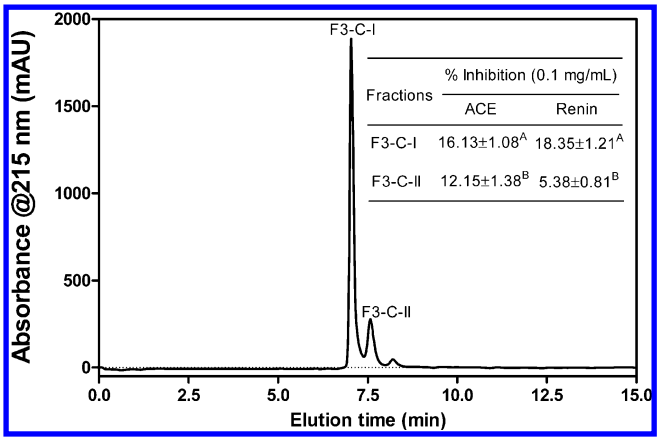


Figure 3. Separation of fraction F3-C on a C18 analytical RP-HPLC column a flow rate of 0.5 mL/min and a linear gradient width of 25−35% methanol within 30 min. Inserted data are the ACE and renin inhibitory activities of isolated fractions. Results are presented as mean ± standard deviation ($n = 3$), and values with different letters within the same column are significantly different ($p < 0.05$).

The MS/MS spectrum of a single charged ion peak (299.8 *m*/*z*) is shown in Figure 4, indicating a dominant *b*-type fragment ion peak 194.7 *m*/*z*. The low MW region corresponds to the C-terminal *y*-type fragment ion 88.0 *m*/*z* (y_1−18, *Ser*). Similarly, according to *b*-type fragment ion 57.3 *m*/*z* (b_1) and *y*-type fragment ion 241.6 *m*/*z* (y_2), *Gly* was identified as an N-terminal amino acid, while the 137.2 *m*/*z* is a typical fragment ion of *His*. Therefore, F3-C-I was identified as a tripeptide with the amino acid sequence of *Gly-His-Ser* (*GHS*). This is the first report of the identification of *GHS* as a peptide product from enzymatic hydrolysis of food proteins. While our previous work had identified other antihypertensive peptides such as *LY*, *TF*, and *RALP* from an Alcalase digest of rapeseed proteins,[18] the present work provides identity of a new (never previously reported) antihypertensive peptide obtained from sequential digestion with PP. Due to the low yield from analytical column purification, GHS was synthesized for use during in vitro and in vivo studies.

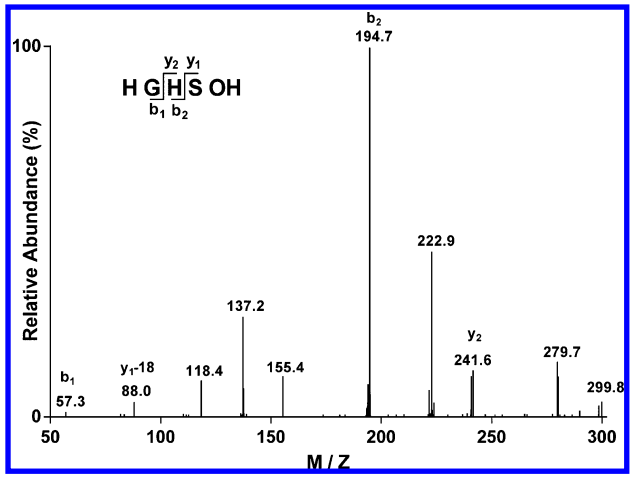


Figure 4. ESI-MS/MS spectrum of fraction F3-C-I containing a 299.8 *m*/*z* peptide. The quadrupole mass spectrometer was coupled with an electrospray ionization (ESI) source using collision energy of 30 V, capillary voltage 1.5 kV, cone voltage 20 V, and capillary temperature of 150 °C.

dx.doi.org/10.1021/jf400865m | J. Agric. Food Chem. 2013, 61, 8396−8402

In Vitro and In Vivo Activities Of *GHS*. The Ic_{50} values (from regression equation) of GHS against ACE (0.52 ± 0.01 mg/mL) and Renin (0.32 ± 0.01 mg/mL) are summarized in mM as inserted data in Figure 5, and show slightly higher

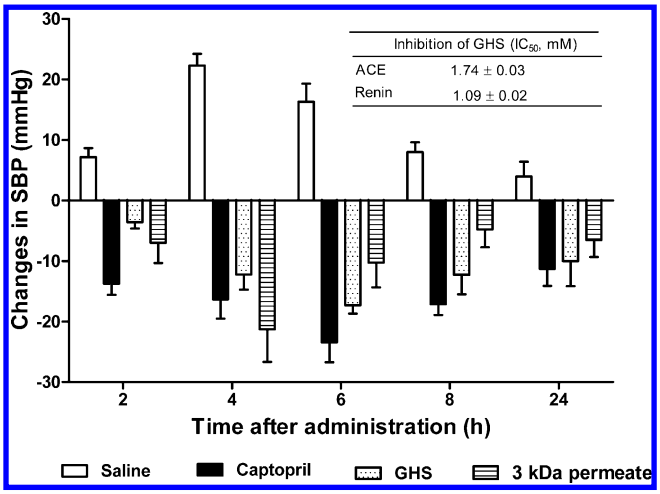


Figure 5. Effects of *GHS* and the 3 kDa permeate on systolic blood pressure (SBP) of spontaneously hypertensive rats after oral gavage. *GHS* and the 3 kDa permeate peptides were administered using 30 and 100 mg/kg rat body weight (bw) doses, respectively, while captopril was given at 10 mg/kg bw. Insert data are ACE and renin inhibitory activities (IC_{50} values) of *GHS*.

potency against renin than ACE. From the regression line, the ACE and renin inhibitory values of the synthetic *GHS* at 0.1 mg/mL peptide concentration were 20 and 25%, respectively; these values are close to the 16 and 18% obtained for F3-C-I (Figure 3). In comparison to the original source of the peptide, i.e., the 3 kDa permeate with IC_{50} values of 0.79 ± 0.01 mg/mL and 1.19 ± 0.01 mg/mL for ACE and renin inhibition, respectively, the dual inhibitory peptide *GHS* showed 1.5 times potency against ACE but 3.5 times against renin. The ACE-inhibitory IC_{50} value (1.74 mM or 0.52 mg/mL) of *GHS* in present study is higher (reduced potency) than the previously reported values for rapeseed protein-derived peptides, such as *VW* (0.0016 mM), *VWIS* (0.030 mM), *IY* (0.037 mM), *RIY* (0.028 mM), *LY* (0.11 mM), *TF* (0.81 mM), and *RALP* (0.65 mM).[18,25] In contrast to other tripeptides, *GHS* is poorer ACE inhibitor when compared to *GTG* (5.54 μM) that was isolated from shrimp[26] or *GPM* (17.13 μM) and *GPL* (2.65 μM) that were isolated from fish skin.[27] The *GHS* is also a less potent ACE inhibitor when compared to *NAQRP* obtained from arachin hydrolysate (IC_{50} of 32 μM) and *VAP* obtained from grass carp protein hydrolysate (IC_{50} of 0.00534 mg/mL),[28,29] but has higher potency than several other food protein-derived peptides.[28,29] In comparison to ACE inhibition, *GHS* has a lower IC_{50} value (1.09 mM) against renin, which is similar to that of *RALP* (0.97 mM), another peptide isolated from rapeseed proteins.[25] *GHS* is more active (lower IC_{50} value) than other reported renin-inhibitory peptides from pea protein hydrolysate (*KF*, 17.84 mM; *IR*, 9.2 mM; *EF*, 22.66 mM),[30] QSAR modeling of renin-inhibitory peptides (IW, 2.32 mM),[22] and rapeseed proteins (*LY*, 1.87 mM; *TF*, 3.06 mM).[25] While *GHS* does not contain C-terminal hydrophobic or aromatic residues, the presence of an aliphatic *Gly* at the N-terminal and a positively charged amino acid (*His*) in the middle may contribute to ACE-inhibitory activity.[31,32] *His* may also contribute to the high renin-inhibitory activity of *GHS* by acting as a hydrogen bond acceptor based on the 3D quantitative structure−activity relationship pharmacophore model.[33]

In order to confirm antihypertensive effects, *GHS* and the 3 kDa permeate were evaluated in vivo using oral administration to SHR. As shown in Figure 5, administration of *GHS* (at 30 mg/kg bw) led to significant decreases in SBP when compared to the saline treatment. The maximum SBP decrease (−17.29 ± 2.47 mmHg) was obtained at 6 h, and a significant ($p < 0.05$) hypotensive effect (in comparison to saline and 3 kDa permeate) was maintained until 24 h (−10 ± 7.15 mmHg) postadministration. On the basis of the 30 mg/kg body weight administered to the rats, a 70 kg person would need to consume ∼2g of the *GHS* on a daily basis to maintain sustained SBP decrease. At a higher dose of 100 mg/kg bw, the 3 kDa permeate exerted a maximum decrease in SBP of −21.29 ± 9.29 mmHg at 4 h after gavage, and the effect decreased rapidly thereafter when compared to *GHS*. The higher initial hypotensive effect of the 3 kDa permeate could have been due to higher dosage and potential synergistic effects of the peptides. However, the longer persistence of the hypotensive effect of *GHS* at lower concentrations indicates that *GHS* is much more resistant to structural inactivation during passage through the GIT or within the blood circulatory system when compared to the 3 kDa permeate. The antihypertensive effects obtained for the peptide and 3 kDa permeate were lower than that of the commercial antihypertensive drug, captopril, which at a much lower concentration (10 mg/kg bw) had higher or similar effects as the peptide or hydrolysate. The *GHS* had lower blood pressure-reducing effect when compared to the reported −40 mmHg decrease in SBP at 6 h after oral administrations of 30 mg/kg bw of *TGVY*, a peptide identified from rice protein hydrolysate.[34] Overall, these results demonstrate that both *GHS* and the 3 kDa permeate can exert in vivo antihypertensive activity after a single oral administration to SHR, and may be used as potential ingredients for the formulation of hypotensive functional foods and nutraceuticals.

ACE and Renin Inhibition Kinetics. As shown in Figure 6A, convergence of the lines on the *x*-axis indicates that the mechanism of ACE inhibition by *GHS* was mostly non-competitive. This means that *GHS* exhibited its activity possibly by binding to ACE protein molecule both in the free and substrate (FAPGG)-bound forms. The data also indicate that *GHS* could bind to ACE at other sites apart from the substrate (active) binding site, which will inhibit enzyme activity by causing conformational changes that reduce ACE-substrate interactions. The result is similar to the inhibition mode reported for *VECYGPNRPQF* from algae protein and *IFL*, *WL* from fermented Soybean.[35,36] Although noncompetitive ACE inhibitors have been most frequently reported, the inhibition sites on the ACE protein structure are not yet properly understood.[37] In addition, the similar K_m values tend to support a noncompetitive inhibition of ACE by *GHS* while reduced CE (Table 1) indicates less conversion of substrate because of a strong interference from the inhibitor.

Similar to the ACE inhibition, Figure 6B shows that *GHS* exhibited a noncompetitive renin inhibition at 0.25 mg/mL, which indicates initial binding to the free renin protein or to the renin-substrate complex. However, at a higher concentration of 0.5 mg/mL, *GHS* inhibition of renin became uncompetitive, which suggests that the presence of more *GHS* molecules led to

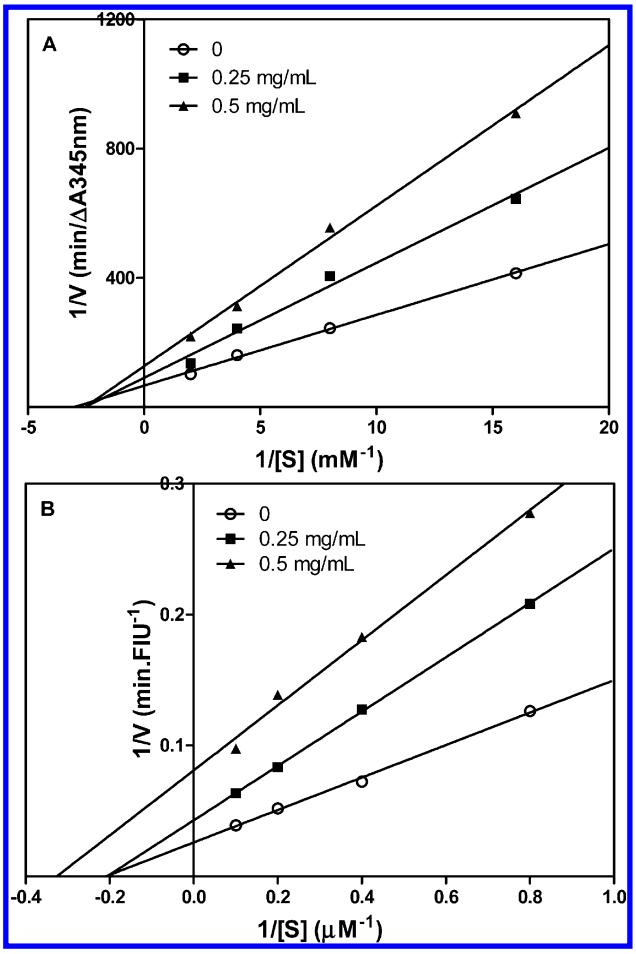


Figure 6. Lineweaver–Burk plots of the inhibition of ACE (A) and renin (B) by different concentrations of *GHS*. *V* is rate of reaction; [S] is the concentration of substrate: ACE substrate (0.0625–0.5 mM); renin substrate (1.25–10 μM).

predominant binding to the renin-substrate complex and hence a decrease in renin activity.[38] The renin-inhibitory effect is further confirmed as decreases in V_{max} from 38.58 FIU/min for the uninhibited reaction to 23.35 and 12.34 FIU/min at the concentration of 0.25 and 0.5 mg/mL of *GHS*, respectively (Table 1). Unlike ACE-inhibitory peptides, there is little information on inhibition kinetics for food protein-derived peptides, although previous work with flaxseed protein hydrolysate has also shown an uncompetitive type of renin inhibition.[39] However, Girgih et al. indicated a mixed-type inhibition of renin activity by hempseed protein hydrolysate.[20] Interaction of *GHS* with renin, leading to downregulation of enzyme activity, was further confirmed from the reduced CE as the peptide concentration was increased (Table 1). Table 1 also shows that the inhibition constant (K_i) is less for ACE inhibition than that for renin inhibition, which suggests that the interaction of *GHS* with ACE is stronger than that with renin. The K_i results are consistent with the kinetic plot showing a noncompetitve ACE inhibition (mainly involves interaction of *GHS* with free ACE molecules), whereas the renin inhibitory pattern also involves binding of *GHS* to the renin-substrate complex (less binding to free renin molecules), especially at a higher inhibitor concentration. Overall, this work has shown the possibility of food-derived peptides with dual inhibitions of renin and ACE, which could contribute to our knowledge of the mechanism of action of multifunctional peptides. Future work is necessary to determine if the observed in vitro activities of *GHS* are reflected in vivo as reduced plasma activities of renin and ACE.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

Funding for this work was provided through the National Key Technology Research and Development Program of Jiangsu Province, China (Project No. SBE201130495), the Jiangsu Province Agriculture Independent Innovation Program (Project No. CX (10)444), and the research program of R.E.A. is funded by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery Grant.

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Table 1. Kinetics Constants for GHS-Inhibited Angiotensin Converting Enzyme (ACE) and Renin Activities[a]

	ACE			Renin		
	peptide concentration (mg/mL)					
catalytic parameter	0	0.25	0.5	0	0.25	0.5
V_{max}	0.015	0.011	0.008	38.580	23.348	12.349
K_m	0.331	0.392	0.399	0.005	0.005	0.003
CE	0.045	0.028	0.020	7716	4670	4116
K_i		0.383			1.016	

[a] K_m is Michaelis constants (mM); V_{max} is maximum reaction velocities, and the units are respectively ΔA/min and fluorescence intensity units/min for ACE and renin; CE is the catalytic efficiency; K_i is the enzyme-inhibitor dissociation constant (mg/mL).

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