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Purification and hypotensive activity of rapeseed protein-derived renin and angiotensin converting enzyme inhibitory peptides

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ABSTRACT

Rapeseed protein isolate (RPI) was hydrolyzed with Alcalase followed by reverse-phase high performance liquid chromatography (RP-HPLC) purification of bioactive peptides. The rapeseed protein hydrolysate (RPH) obtained after 4 h digestion with Alcalase had a degree of hydrolysis (DH) of ~11%. Gel permeation chromatography separation showed high contents of low molecular weight peptides in the RPH when compared to the RPI. After preparative and analytical RP-HPLC separations, three peptides (**LY**, **TF** and **RALP**) were purified and amino acid sequence determined by tandem mass spectrometry. **LY** (IC_{50}, 0.11 mM) was the most potent ($p < 0.05$) against ACE activity when compared to **TF** (IC_{50}, 0.81 mM) and **RALP** (IC_{50}, 0.65 mM). However, **RALP** (IC_{50}, 0.97 mM) was the most potent ($p < 0.05$) against renin activity when compared to **LY** (IC_{50}, 1.87 mM) and **TF** (IC_{50}, 3.1 mM). Single oral administration (30 mg/kg body weight) to spontaneously hypertensive rats showed **LY** and **RALP** to be the more effective hypotensive agents with maximum blood pressure reduction of −26 and 16 mmHg, respectively when compared to TF (−12 mmHg). The results suggest that the higher number of hydrophobic amino acid residues **LY** and **RALP** contributed to their higher *in vitro* and *in vivo* activities when compared to **TF**.

1. Introduction

The renin-angiotensin system (RAS) is a major regulator of blood pressure and fluid homeostasis; dysregulation of the RAS can lead to blood pressure elevation with ensuing cardiovascular disease, chronic kidney disease and diabetes (Crowley & Coffman, 2012). Within RAS, the aspartyl protease renin cleaves 10 amino acids from the N-terminus of angiotensinogen to release the inactive angiotensin I, which is subsequently converted to the pro-hypertensive angiotensin II by angiotensin converting enzyme (ACE). Therefore, renin and ACE are two key enzymes that regulate RAS operation. Studies have shown that long-term therapy with ACE-inhibitory antihypertensive drugs leads to angiotensin I accumulation, which can activate the alternative pathway (e.g., chymase) and convert angiotensin I to angiotensin II, resulting in the

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failure of hypertension treatment (Borer, 2007). In contrast, direct renin inhibition is an attractive target for physiological intervention due to the high degree of enzyme substrate specificity, which can reduce unwanted interactions and negative side effects (Nishiyama, 2012; Taylor & Pool, 2012). Moreover, blocking the renin-catalyzed rate-limiting step that initiates RAS operation can produce effective decreases in angiotensin I and angiotensin II concentrations (Nishiyama, 2012; Taylor & Pool, 2012). Recently, investigators have suggested that a combination of renin inhibitor with ACE inhibitor is more promising in reducing blood pressure or protecting target organs when compared to single use therapy (Harel et al., 2012). Consequently, identification of antihypertensive agents or bioactive substances that possess both renin and ACE-inhibitory activities has become an important research focus.

Food protein-derived bioactive peptides are attracting increasing interest due to their safety and multi-functionality (Martinez-Maqueda, Miralles, Recio, & Hernandez-Ledesma, 2012). Several of the peptides released from enzymatic hydrolysis of plant proteins such as soybean, pea, and peanut peptide have shown ACE-inhibitory activities, (Jimsheena & Gowda, 2011; Li & Aluko, 2010; Rho, Lee, Chung, Kim, & Lee, 2009). In addition, renin-inhibitory peptides have been produced from enzymatic hydrolysis of hemp seed (Girgih, Udenigwe, Li, Adebiyi, & Aluko, 2011) and flaxseed proteins (Udenigwe and Aluko, 2012; Udenigwe, Lin, Hou, & Aluko, 2009); antihypertensive effects of the protein hydrolysates were also demonstrated. Another recent work has also shown the isolation and structural characterization of renin-inhibitory peptides from the red seaweed (*Palmaria palmate*) protein (Fitzgerald et al., 2012). Rapeseed meal is an important raw material for development of bioactive peptides because it is cheap and contains high level of proteins (Yoshie-Stark, Wada, Schott, & Wasche, 2006). Some researchers have suggested that rapeseed proteins contain antioxidant (Cumby, Zhong, Naczk, & Shahidi, 2008; He, Girgih, Malomo, Ju, & Aluko, 2013; Makinen, Johannson, Gerd, Pihlava, & Pihlanto, 2012; Zhang, Wang, Xu, & Gao, 2009), antitumor (Xue, Liu, Wu, Zhuang, & Yu, 2010), and ACE-inhibitory (Yamada et al., 2010) peptide sequences. However, there is scanty report on the enzymatic hydrolysis of rapeseed proteins to release renin-inhibitory peptides, especially evaluation of *in vivo* antihypertensive properties. Therefore, the objectives of this study were to purify, identify, characterize enzyme-inhibitory properties and determine the blood pressure-lowering effects of renin- and ACE-inhibitory peptides present in Alcalase-digested rapeseed enzymatic hydrolysate.

2. Materials and methods

2.1. Materials

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. Renin inhibitor screening assay kit was purchased from Cayman Chemicals (Ann Arbor, MI). Alcalase (Protease from *Bacillus licheniformis*, EC 3.4.21.14), ACE (from rabbit lung, E.C.3.4.15.1), and N-[3-(2-furyl) acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG), were purchased from Sigma–Aldrich (St. Louis, MO). Other analytical-grade reagents were obtained from Fisher Scientific (Oakville, ON, Canada).

2.2. Preparation of rapeseed protein isolate (RPI)

RPI was produced from RPM according to a previously reported method (Yoshie-Stark, Wada, Schott, & Wasche, 2006) 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 °C for 2 h and the slurry centrifuged at 10,000*g* for 30 min. The supernatant was recovered, adjusted to pH 4.5 with 1 M HCl for 1 h and centrifuged at 10,000*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 rapeseed protein hydrolysate (RPH)

Hydrolysis of the RPI was conducted with Alcalase using a pH-Stat method. RPI (5%, w/v, protein basis) was suspended in deionized water in a reaction vessel equipped with a stirrer, heated to 50 °C and adjusted to pH 8.0 prior to the addition of the Alcalase. The enzyme was added to the slurry at an enzyme-substrate ratio (E/S) of 4:100, based on the protein content of RPI and digestion carried out for 4 h. The reaction mixture pH was kept constant using 2 M NaOH. After digestion, the enzyme was inactivated by immersing the reaction vessel in a boiling water bath for 10 min. The undigested proteins were precipitated by centrifugation at 8000*g* for 60 min. The supernatant containing the target peptides was collected, lyophilized, and stored at −20 °C until needed for further analysis. The protein contents of the freeze dried RPH was determined using the modified Lowry method (Markwell et al., 1978).

2.4. Degree of hydrolysis (DH)

The time-dependent change in DH during enzymatic digestion of RPI was determined by the pH-stat method (Adler-Nissen, 1986). The DH was calculated according to the following equation:

$$\mathrm{DH}(\%) = \frac{BxN_b}{M_P} \times \frac{1}{a} \times \frac{1}{h_{tot}} \times 100$$

where B, N_b, Mp, α, h_{tot} are the volume (mL) of NaOH added, the concentration of NaOH, the amount of protein (g), the average degree of dissociation of hydrolyzing the α-NH_2, and the total number of peptide bonds in the protein, respectively. For RPI, $1/\alpha$ is 2.27, and h_{tot} is 8.04.

2.5. Molecular weight distribution

Molecular weight distribution of RPH was determined by using the GE AKTA system with a Superdex Peptide 12 10/300 GL column (10 × 300 mm, GE, USA), in combination with a UV detector (λ = 214 nm). An aliquot (100 μL) of the sample (5 mg/mL in 50 mM phosphate buffer, pH 7.0 containing