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High angiotensin-I converting enzyme (ACE) inhibitory activity of Alcalase-digested green soybean (*Glycine max*) hydrolysates

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ABSTRACT

As a protein-rich, underutilized crop, green soybean could be exploited to produce hydrolysates containing angiotensin-I converting enzyme (ACE) inhibitory peptides. Defatted green soybean was hydrolyzed using four different food-grade proteases (Alcalase, Papain, Flavourzyme and Bromelain) and their ACE inhibitory activities were evaluated. The Alcalase-generated green soybean hydrolysate showed the highest ACE inhibitory activity (IC_{50}: 0.14 mg/mL at 6 h hydrolysis time) followed by Papain (IC_{50}: 0.20 mg/mL at 5 h hydrolysis time), Bromelain (IC_{50}: 0.36 mg/mL at 6 h hydrolysis time) and Flavourzyme (IC_{50}: 1.14 mg/mL at 6 h hydrolysis time) hydrolysates. The Alcalase-generated hydrolysate was profiled based on its hydrophobicity and isoelectric point using reversed phase high performance liquid chromatography (RP-HPLC) and isoelectric point focusing (IEF) fractionators. The Alcalase-generated green soybean hydrolysate comprising of peptides EAQRLLF, PSLRSYLAE, PDRSIHGRQLAE, FITAFR and RGQVLS, revealed the highest ACE inhibitory activity of 94.19%, 99.31%, 92.92%, 101.51% and 90.40%, respectively, while their IC_{50} values were 878 μM, 532 μM, 1552 μM, 1342 μM and 993 μM, respectively. It can be concluded that Alcalase-digested green soybean hydrolysates could be exploited as a source of peptides to be incorporated into functional foods with antihypertensive activity.

1. Introduction

Hypertension is a major health problem, affecting people from all walks of life, around the world. It is considered a major threat to public health as it's a leading risk factor for mortality (Ezzati, Lopez, Rodgers, Vander, & Murray, 2002) and is expected to affect 1.56 billion people by the year 2025 (Kearney et al., 2005). Currently, treatment for hypertension involves synthetic drugs such as captopril, enalapril, and lisinopril. However, these drugs often cause unwelcome side-effects such as angioedema, persistent dry coughs, and fetopathy (Bicket, 2002; Messerli, 1999; Roberts, 2014). The drugs target a key enzyme in the renin-angiotensin system, the angiotensin I-converting enzyme (ACE), which regulates blood pressure by converting angiotensin I into the potent vasoconstricting peptide, angiotensin II, while also inactivating a vasodilator, bradykinin (Iwaniak, Minkiewicz, & Darewicz, 2014). Therefore, inhibition of ACE would result in a reduction of blood pressure.

Natural ACE inhibitors are considered to be a safe alternative, unlike the synthetic drugs. Peptides with in vitro ACE inhibitory activity have been shown to affect positively blood pressure in spontaneously hypertensive rats (SHR) and humans, without the accompanying adverse side effects (Lafarga & Hayes, 2016). Hence, the search for raw materials which could serve as a potential source of ACE inhibitory peptides for the development of functional foods. A diverse range of ACE inhibitory peptides have been identified from an equally diverse selection of enzyme hydrolysates of different food proteins such as casein and whey proteins (Corrons, Liggieri, Trejo, & Bruno, 2017; Lacroix, Meng, Cheung, & Li-Chan, 2016), fish proteins (Ko et al., 2016; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017), cereals (Shamloo, Eck, & Beta, 2015), common beans (Luna-Vital, Mojica, de Mejía, Mendoza, & Loarca-Piña, 2015), and soybean protein (Sanjukta & Rai, 2016; Wu & Ding, 2002). The development of novel functional foods incorporated with ACE inhibitory peptides obtained from food sources has the potential to be an important component in the fight against hypertension.

Exponential increase of the human population is expected to drive food protein demand in the near future. Both animal- and plant-based protein play important roles in serving our dietary protein needs. However, the increasing demand and consumption of animal-based protein is not expected to be sustainable in the long-term while also being linked with health risks (Wu et al., 2014). Protein from plant

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sources presents a sustainable option for global consumption, as well as mitigating the impact of meat-based protein from an environmental and health point of view (Sabaté & Soret, 2014). Green soybean or vegetable soybean (*Glycine max*) are harvested when the seeds are immature (R6 stage), and have expanded to fill almost the entire pod width (Mimura, Coyne, Bambuck, & Lumpkin, 2007). Worldwide, it is a minor crop but is popular in East Asia especially in Japan and China. As with regular soybeans, green soybean varieties are rich in protein and highly nutritious (Redondo-Cuenca, Villanueva-Suarez, Rodriguez-Sevilla, & Mateos-Aparicio, 2006). The high protein content of green soybean could yield various peptide sequences able to inhibit ACE thus controlling hypertension. To the best of our knowledge, green soybean protein has not been appraised for its ACE inhibitory activity. In the present study, defatted green soybean was hydrolyzed by four different proteases, and the resulting hydrolysates' ACE inhibitory activity was evaluated. Finally, the enzyme-generated hydrolysate with higher ACE inhibitory activity was profiled and subjected to peptide sequence identification and analysis.

2. Materials and methods

2.1. Materials

Green soybean was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. Samples were stored at 4 °C before analysis. Flavourzyme 500 MG and Alcalase 2.4 L FG were purchased from Novozymes A/S, (Bagsvaerd, Denmark). Papain, Bromelain, trifluoroacetic acid and acetonitrile (ACN) of HPLC grade were purchased from Acros Organics (New Jersey, USA). Hippuryl-his-leu (HHL), angiotensin-I converting enzyme (ACE) from rabbit lung (2 units/mg protein), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade and obtained from Merck Co. (Darmstadt, Germany).

2.2. Determination of crude protein content

The crude protein content of green soybean was determined from the total nitrogen content using the AOAC method (AOAC, 2005) with 6.25 as the conversion factor.

2.3. Preparation of defatted green soybean meal

Defatting of green soybean meal was performed using the method described by L'hocine, Boye, and Arcand (2006) with some modifications. The full-fat meal was prepared using a centrifugal grinding mill (Retsch ZM 200, Germany) equipped with a 0.5 mm ring sieve. The resulting green soybean meal was defatted by petroleum ether in a 1:3 ratio (w/w) with the mixture agitated using a magnetic bar for 30 min. The mixture was decanted, removing the solvent layer. The process was repeated another 2 times to ensure optimal defatting. Samples were then air-dried at room temperature under a fume hood overnight to remove the solvent residues.

2.4. Amino acid composition

The amino acid composition was determined as described by Forghani et al. (2012). Green soybean meal was hydrolyzed by 6 N HCl for 24 h at 110 °C. Briefly, 10 µL of samples are dried under vacuum (37 °C, 20 mm Hg) in a vial for 30 min. Dried samples were dissolved in a solution of methanol:water:triethylamine (2:2:1 v/v) and was vacuum-dried for 30 min upon mixing. Afterwards, samples were derivatized and mixed with 20 µL of methanol:triethylamine:water:phenyl isothiocyanate (7:1:1:1 v/v). The samples were then left at room temperature for 20 min before being vacuum-dried for 30 min. The derivatized sample was injected into a C18 column (Thermo Scientific Hypersil GOLD, 5 µm, 250 × 4.6 mm) coupled to a JASCO HPLC system equipped with a photodiode array detector (MD-2010 Plus, JASCO International Co. Ltd., Tokyo, Japan). The mobile phase, comprising of buffer A containing 0.1 M ammonium acetate (pH 6.5) and buffer B containing 0.1 M ammonium acetate:ACN:methanol, at a ratio of 44:46:10 (v/v/v), [pH 6.5], eluted along a linear gradient at a flow rate of 1 mL/min. The derivatized amino acids were detected at 254 nm.

2.5. Enzymatic hydrolysis of green soybean

Defatted green soybean meal was hydrolyzed in a jacketed vessel under controlled conditions using the pH stat method according to Wan Mohtar, Abdul-Hamid, Abd-Aziz, Muhamad, and Saari (2014). Defatted green soybean samples were dispersed in distilled water at a ratio of 1:25 (w/v). Samples were separately hydrolyzed for 10 h by four different proteases according to their respective optimum conditions using an enzyme/substrate ratio of 1:50 (w/w, based on the protein content of defatted green soybean meal) (Alcalase, pH 8.0, 60 °C; Bromelain, pH 6.5, 45 °C; Flavourzyme, pH 8.0, 55 °C; Papain, pH 6.5, 70 °C). The pH of the mixture during hydrolysis was maintained by adding NaOH (1 N) using an autotitrator (902 Titrando, Metrohm, Herisau, Switzerland). The volume of NaOH used was noted by the system to determine the degree of hydrolysis (DH). Samples were withdrawn from the proteolytic mixture initially at 0.5 h and at 1 h intervals thereafter until 10 h, which were then immediately boiled in a water bath for 10 min followed by centrifugation at 10,000 × *g* for 20 min at 4 °C (Sigma 3–18 k Sartorius, Germany). The supernatant was collected and lyophilized before being stored at −40 °C, pending further analyses.

2.6. Determination of degree of hydrolysis using pH stat method

The degree of hydrolysis (DH) was determined based on the amount of NaOH added to maintain constant pH during the course of hydrolysis, monitored by the Tiamo software Version 2.2 (Metrohm, Herisau, Switzerland), using the following equation (Adler-Nissen, 1986):

$$\mathrm{DH}\ (\%) = \frac{\mathrm{h}}{\mathrm{htot}} \times 100 = \frac{\mathrm{B} \times \mathrm{Nb}}{\mathrm{MP}} \times \frac{1}{\alpha} \times \frac{1}{\mathrm{htot}} \times 100$$

B is the amount of alkaline solution added (mL), Nb is the normality of the alkaline solution, htot is the total amount of peptide bonds per gram of protein, MP is the mass (g) of protein (N × 6.25), and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as:

$$\alpha = \frac{10^{\mathrm{pH}-\mathrm{pK}}}{1 + 10^{\mathrm{pH}-\mathrm{pK}}},$$

where pH is the value at which the hydrolysis was conducted. The pK values were calculated using the equation below:

$$\mathrm{pK} = 7.8 + \frac{298 - \mathrm{T}}{298\ \mathrm{T}} \times 2400$$

where, T is the hydrolysis temperature in Kelvin.

2.7. Determination of ACE inhibitory activity

The ACE inhibitory assay was performed following the method as described by Jimsheena and Gowda (2009) with some modifications. Fifteen µL of samples (10 mg/mL of hydrolysates; 0.1 mg/mL of hydrolysate fractions; 10 mM of identified peptides) was pre-incubated with 10 µL (100 mU/mL) of ACE for 10 min at 37 °C. The mixture was then incubated with 50 µL of the substrate solution containing 5 mM HHL, 100 mM sodium borate buffer (pH 8.3), and 300 mM sodium chloride for 60 min at 37 °C followed by the addition of 75 µL (1 M) HCl. Afterwards, 150 µL of pyridine was added followed by 75 µL of benzene sulphonyl chloride. The solution was then vortexed for 1 min and immediately cooled in an ice bath. Afterwards, 200 µL was transferred into