Food Chemistry 141 (2013) 3435–3442

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Extraction of antioxidative and antihypertensive bioactive peptides from *Parkia speciosa* seeds

Hwee-Leng Siow [a,b], Chee-Yuen Gan [b,*]

[a] Bioprocess Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia
[b] Centre for Advanced Analytical Toxicology Services, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

ARTICLE INFO

Article history:
Received 29 March 2013
Received in revised form 3 June 2013
Accepted 6 June 2013
Available online 15 June 2013

Keywords:
Antioxidative
Antihypertensive
Bioactive peptide
Parkia speciosa

ABSTRACT

Antioxidative and antihypertensive bioactive peptides were successfully derived from *Parkia speciosa* seed using alcalase. The effects of temperature (25 and 50 °C), substrate-to-enzyme ratio (S/E ratio, 20 and 50), and incubation time (0.5, 1, 2 and 5 h) were evaluated based on 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and angiotensin-converting enzyme (ACE) assays. Bioactive peptide extracted at a hydrolysis condition of: temperature = 50 °C, S/E ratio = 50 and incubation time = 2 h, exhibited the highest DPPH radical scavenging activity (2.9 mg GAE/g), reducing power (11.7 mM) and %ACE-inhibitory activity (80.2%). The sample was subsequently subjected to fractionation and the peptide fraction of <10 kDa showed the strongest bioactivities. A total of 29 peptide sequences from peptide fraction of <10 kDa were identified as the most potent contributors to the bioactivities. These novel bioactive peptides were suggested to be beneficial to nutraceutical and food industries.

1. Introduction

Today, diet and a healthy lifestyle play a significant role in the quality of our lives. The increasing awareness of the importance of a healthy diet leads to the development of new, safe and healthy foods. Natural food-derived peptides with specific bioactivity were therefore aroused a great deal of interest among researchers. Food-derived bioactive peptides was defined as the specific protein fragments that show beneficial pharmacological properties in the human body beyond the normal and adequate nutrition (Hartmann & Meisel, 2007). In recent years, it was acknowledged that the dietary protein is a good source of bioactive peptides with a broad spectrum of biological activities, including antioxidant, antihypertensive, anti-inflammatory, opioids and immunostimulants (Hartmann & Meisel, 2007). The precursor of bioactive peptides can be categorised according to animal origin, marine origin as well as plant origin. Bioactive peptides derived from the animal source (milk, egg and meat muscle) and the marine source (fish, salmon, oyster and seahorse) have been investigated in association with their pharmacological properties (Udenigwe & Aluko, 2012). Nevertheless, plant proteins are emerging as an important food ingredient to be used for the improvement of modern foods in the aspect of nutrition, processing technology and contribution to health (Duranti, 2006). In view of this, plant-derived bioactive peptides have been attracting a great deal of interest in the scientific community. Among all plant sources, legumes (the second largest food crops after cereals for world agriculture) is a well known source for the generation of bioactive role of peptides and are believed to provide nutritional benefits as they are rich in high quality of protein (Duranti, 2006).

Parkia speciosa, a Southeast Asian legume of the Mimosae subfamily, grows wild in the lowland tropical forests and is often cultivated in Malay villages. The tree bears long, flat, edible bean pods with bright green plump almond shaped seeds that have a unique Shitake mushroom-like flavour. The seeds of *P. speciosa* are flattened and elliptical in shape with a nutty and firm texture. Besides that, *P. speciosa* has earned its nickname "stink bean" due to its strong and pungent odor, which could cause the body to excrete a distinct smell through the skin, urine and faeces. *P. speciosa* seeds have always been a popular ingredient in cooking and usually served with sambal, dried shrimp and chili pepper, as a popular local delicacy in Malaysia. Besides culinary uses, it is reported to contain crucial chemical medicinal compounds which exhibit potential biological activity such as anticancer (Ali, Houghton, & Soumyanath, 2006), antibacterial (Sakunpak & Panichayupakaranant, 2012), antioxidant (Aisha, Abu-Salah, Alrokayan, Ismail, & Abdul Majid, 2012), antiangiogenic (Aisha et al., 2012) as well as hemagglutinating activity (Chankhamjon, Petsom, Sawasdipuksa, & Sangvanich, 2010). However, the potential roles of *P. speciosa* seed-derived bioactive peptides with a biofunctional activity have not been investigated.

* Corresponding author. Address: Bioprocess Division, School of Industrial Technology, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia. Tel.: +60 4 6595606x2461; fax: +60 4 656 9869.
E-mail address: cygan@usm.my (C.-Y. Gan).

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http://dx.doi.org/10.1016/j.foodchem.2013.06.030

Peptides are inactive when it is encrypted in the parental protein but can be released with various biological activities (Korhonen & Pihlanto, 2006). The production, characterisation and in vitro or in vivo evaluation in food has been extensively reviewed in recent years. Enzymatic hydrolysis is found to be the most common way to produce bioactive peptides using proteolytic enzymes such as pepsin, trypsin, alcalase, and pancreatin (Korhonen & Pihlanto, 2006). The main objective of this project was to extract antioxidative and antihypertensive bioactive peptide from *P. speciosa* seed using enzyme alcalase. Following, the identification of the extracted bioactive peptide using mass spectrometry approach was performed.

2. Materials and methods

2.1. Materials

P. speciosa seeds were purchased from local markets (i.e. Air Itam market, Jelutong market and Apollo market), Penang. The seeds were lyophilised using Labconco Freeze-dryer (Fisher Scientific, USA) and ground into powder (average diameter of 30 mesh screen size) prior to extraction of bioactive peptide. Alcalase with activity of 2.4 AU-A/g was purchased from Novoenzyme A/S, Denmark. All other chemicals and reagents used in the experiment were of analytical grade purchased from Sigma–Aldrich (Malaysia) company or otherwise mentioned.

2.2. Extraction of bioactive peptides from P. speciosa seeds

P. speciosa seed flour (1 g) was suspended in 10 ml of phosphate buffer at pH 8.0 and alcalase was added into the suspension with substrate-to-enzyme (S/E) ratio of 20 or 50. The hydrolysis was performed via incubation at 25 or 50 °C with constant shaking at 200 rpm for 0.5, 1, 2, and 5 h. The hydrolysis reaction was then terminated by heating the sample at 95 °C for 30 min in a water bath (Memmerit, Germany), followed by centrifugation (10,000×*g*) at 4 °C for 30 min. The supernatant was collected and stored at −80 °C for further analysis. Non-hydrolysed sample was extracted according to the method aforementioned without the presence of enzyme.

2.3. Amino acid analysis

A 0.1 g hydrolysed samples were heated at 110 °C for 24 h after adding with 5 ml of 6 **N** HCl and purged with nitrogen. The samples were derivatised and then analysed using Waters-HPLC-System (USA) coupled with Waters 24675 Multi-λ Fluorescence Detector (Zarkadas et al., 2007). Methionine and cysteine were determined separately according to the performic acid procedure of Moore (1963). Each analysis was performed in three replicates.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE analysis of the non-hydrolysed sample and protein hydrolysate extracted from *P. speciosa* seed was performed using 15% resolving gel and 4% stacking gel. Samples (10 μg of protein) were added with 10 μl of Laemmli sample buffer and 1 μl of 2-mercaptoethanol. The samples were then heated at 95 °C for 5 min using heating block (Grant, Fisher Scientific, UK). Samples were loaded into the well and ran the electrophoresis at a constant voltage of 80 V for 10 min, followed by 120 V for 120 min using Mini Protean III Cell (Bio-Rad, USA). The gel was then stained for 2 h using 0.1% Bio-Rad Coomassie Brilliant Blue R-250 in 40% (v/v) methanol and 10% acetic acid, followed by destaining with solution containing 40% methanol and 10% acetic acid. The image was subsequently captured using Fujifilm luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan). Multi Gauge Version 3.0 software (Fujifilm, Tokyo, Japan) was used to analyse the electrophoresis pattern. Bio-Rad's prestained SDS–PAGE standard with broad range molecular weight (MW 6.0–202.4 kDa) was used as standard for comparison.

2.5. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity

The antioxidant activity of non-hydrolysed sample and protein hydrolysate (at a concentration of 0.5 mg/ml) extracted from *P. speciosa* seed was determined using the DPPH free radical scavenging assay, as described by Lee, Kwon, Shin, and Yang (2009). Prior to analysis, the samples were pre-diluted at a factor of 5 and DPPH stock solution (0.1 mM) in ethanol was prepared. A 500 μl DPPH stock solution was added to 16.65 μl sample and incubated at 30 °C for 30 min in the dark. After incubation, the sample was centrifuged at 14,000×*g* for 5 min. The absorbance of the sample was then measured at 517 using a spectrophotometer (Spectramax M5, Molecular Devices, USA). A control sample (16.65 μl of methanol and 500 μl DDPH solution) was prepared as mentioned above. The antioxidant activity was expressed as percentage of DPPH free radical scavenging activity (%$DPPH_{sc}$) and calculated using formula:

$$\%DPPH_{sc} = (A_{control} - A_{sample})/A_{control} \times 100\% \quad (1)$$

where A_{sample} is the absorbance of sample at t = 30 min and $A_{control}$ is the absorbance of control sample. Garlic acid at a concentration range of 0–60 μg/ml was used as standard.

2.6. Ferric reducing antioxidant power (FRAP) assay

The reducing abilities of samples (at a concentration of 0.5 mg/ml) towards ferric ions were determined according to the method of Benzie and Strain (1996) with some modifications. The working FRAP reagent contained 10 mM 2,4,6-tri(2′-pyridyl)-s-triazine (TPTZ) solution in 40 mM HCl, 20 mM $FeCl_3{\cdot}6H_20$ and 0.3 M acetate buffer at pH 3 at a ratio of 1:1:10. The reagent was pre-warmed at 37 °C and the samples were pre-diluted at a factor of 5 prior to analysis. The pre-diluted sample (2.7 μl) was then added to 200 μl of FRAP reagent and mixed thoroughly. The sample was subsequently incubated at 37 °C for 1 h and the absorbance was measured at 593 nm using a spectrophotometer (Spectramax M5, Molecular Devices, USA). Iron (II) sulphate heptahydrate ($FeSO_4{\cdot}7H_2O$) at a concentration range of 0–2 mM was prepared as standard. The ability of ferric reducing antioxidant potential was expressed in mM $FeSO_4$.

2.7. Angiotensin-converting-enzyme (ACE) inhibitory activities

The analysis of antihypertensive activities was determined as outlined by Cushman and Cheung (1971) with some modifications. Pre-diluted sample (50 μl, at a concentration of 0.5 mg/ml) was mixed with 50 μl ACE solution (50 mU/ml). The mixtures were pre-incubated at 37 °C for 10 min in a heating block (Grant, Fisher Scientific, UK). A 150 μl of 4.15 mM substrate (i.e. hippuryl-histidyl-leucine in borate buffer containing 0.3 M NaCl, pH 8.3) was then added into the sample and incubated at 37 °C for 30 min. The reaction was subsequently stopped by adding 500 μl of 1 M HCl. Ethyl acetate (1.5 ml) was then added to extract the hippuric acid. The resulting mixture was vortex for 1 min and stood for 5 min. An 800 μl of ethyl acetate layer was then transferred into 2 ml microcentrifuge tube and vacuum dried in a vacuum concentrator (Concentrator 5301, Eppendorf, Germany) at 45 °C for