Novel bioactive peptides with antioxidant and antihypertension activities from ginger rhizome

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ABSTRACT

Nowadays, human has long life spans while health problem on non-communicable diseases, such as diabetes, hypertension and heart disease increase. For this reason, customers are interested in consuming more functional food for their health benefit. Bioactive peptide (BP) is one of acceptable functional foods due to its safety as well as their beneficial bioactivities. Bioactive peptides are derived from protein digestion by enzymes from either digestive system or microbes. BP consists of 2-20 amino acids in length. Each BP has different properties, for instance, antimicrobial, antihypertensive and antioxidant. Some BPs may have more than one activity. Most of bioactive peptides available in market are derived from foods with high protein content such as meat, soybean and milk. In the contrary, there are few researches on bioactive peptides that are derived from herbs especially edible rhizomes, such as ginger, turmeric and white turmeric. These rhizomes are commonly sold in any markets and are used as important ingredients in many Asian recipes. This study focused on searching for novel BPs with antihypertension and antioxidant activities from ginger. Proteins were extracted and digested using pepsin and trypsin. The crude hydrolysate was fractionated by molecular weight cut-off and each fraction was analyzed for angiotensin-converting enzyme (ACE) inhibitory and antioxidant activities. Fractions with molecular mass less than 1 kDa and having high biological activity were purified using RP-HPLC. The results showed that one of RP-HPLC purified fractions had both ACE inhibitory (30.74%) and antioxidant (51.53%) activities.

KEYWORDS: Bioactive peptides; Functional food; Ginger; Angiotensin-converting enzyme (ACE)

INTRODUCTION

Bioactive peptides have been defined as specific protein fragments that provide a good impact on body functions and may ultimately influence health (Pihlanto & Korhonen, 2003). A variety of protein source can provide bioactive peptides such as milk, meat, eggs, fish, salmon, oyster and cereals (Remond *et al*., 2016). However, plants also have proteins that can be used for the improvement of nutraceutical and can beneficially contribute to health (Duranti, 2006). Zingiber officinale (Zingiberaceae), commonly known as ginger, is used in many cooking recipes. Due to its medicinal properties, it is also used worldwide as therapy against hypertension and several cardiovascular diseases (Srinivasan, 2017).

In addition to their role as nutritional source, peptides also possess several activities such as antihypertension, antioxidant and antimicrobial (Neves *et al*., 2017). Angiotensin converting enzyme (ACE) is a key enzyme which plays an important role in high blood pressure or hypertension (Lee & Hur, 2017). Many bioactive peptides are reported to be inhibitory against ACE. Whereas, antioxidant peptides have amino acid that can donate proton to free radical (Toldra *et al*., 2017)

The aims of this study were search for novel bioactive peptides derived from proteins of ginger rhizome and to study biological and chemical properties of bioactive peptides, such as antioxidant and antihypertensive activities.

MATERIALS AND METHODS

Materials

Ginger rhizomes were purchased from Mahanak market, Phayathai, Thailand. Pepsin, trypsin, captopril, blue dextran, butylated hydroxyanisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent was from Merck KGaA (Darmstadt, Germany). Sephadex G-25 was bought from Wako Pure Chemical Industry (Japan). ACE-kit WST was purchased from Dojindo molecular technology Inc. (Kumamoto, Japan). HPLC grade of acetonitrile (ACN) was bought from RCI Labscan Limited (Thailand). All chemicals are of analytical grade.

Extraction of protein

Ginger rhizomes (1,000 g) were mixed with 200 ml of Tris-HCl buffer (pH 8.3) containing vitamin C (1 g) and homogenized (Huang *et al*., 2006). The final concentration of Tris-HCl was 50 mM. After centrifugation, the supernatant was mixed with four volumes of cold acetone and stirred at 4°C using magnetic stirrer for overnight. The precipitated proteins were collected by centrifugation at 8,000 rpm for 30 min and acetone was removed using rotary evaporator. The proteins were further dried by lyophilization.

Protein digestion by gastrointestinal enzymes

Sample (5 g) was mixed with distilled water (45 ml) and pH was adjusted to 2 using 6 M HCl and proteins were digested by pepsin (0.4 % w/v). Reaction mixture was incubated at 37 °C for 2 hrs. The pH of protein hydrolysate was then adjusted to 7.5 using 6 M NaOH and further digested with trypsin (0.4 % w/v). After incubation at 37 °C for 2 h, the reaction was stopped by boiling at 95 °C for 10 min. The supernatant was collected by centrifugation and filtrated through 0.4 µ membrane and stored at -20 °C.

Protein analysis using Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (Tricine SDS-PAGE)

Tricine SDS-PAGE of non-hydrolyzed and enzyme-hydrolyzed samples was performed using 16% separating gel and 4% stacking gel. The protein sample (18 µl) was mixed with 3 µl of 8X SDS loading buffer [125 mM Tris-HCl pH 6.7, 2% (v/v) β-mercaptoethanol, 10% (v/v) glycerol and 0.01 % (w/v) bromophenol blue] and boiled for 5 min. The boiled sample was centrifuged before loading the sample around 15 µl on gel. The gel was run at 70 V for 30 min, and adjusted to 110 V for 60 min at room temperature using Mini-Protein®II. Then, the gel was washed with distilled water and stained with 0.25% Coomassie brilliant blue R250 (Biorad, USA) for 15 min, and destained with solution containing 10% (v/v) methanol and 10% (v/v) acetic acid.

Peptide sized-selection using molecular weight cut-off (MWCO) ultrafiltration

Crude hydrolysates were fractionated using Amicon ultra (Millipore, USA) with different pore sizes (30, 10, 5, 3 and 1 kDa) of molecular weight cut-off membrane. The fractionation was performed step by step using larger pore size membrane followed by smaller size membranes. The filtration was performed under nitrogen gas pressure at 60 psi.

Gel filtration chromatography

The fraction with molecular mass less than 1 kDa was loaded onto column (1.5 × 40 cm) containing Sephadex G-25 and peptides were eluted with deionized water at a flow rate of 1.0 ml/min. The fractions were tested for ACE inhibitory (Lam *et al*., 2008) and antioxidant activities (Wu *et al*., 2013) for determining the fractions with bioactivity.

Determination of antihypertensive activity using ACE kit-WST

To measure ACE inhibitory activity, 10 µl each of sample, substrate buffer and enzyme mixture provided in the kit were mixed together. Deionized water (10 µl) was used to replace peptide sample and represented a positive control. The reactions were incubated in 96-well plate at 37 °C for 1 hr. Finally, 100 µl of indicator buffer is added to generate color, and then further incubated at room temperature for 10 min before measuring ACE inhibition at 450 nm. The ACE inhibitory activity is calculated using the following equation:

$$\text{Inhibitory activity (\%)} = [(A_{\text{without a sample}} - A_{\text{with a sample}}) / (A_{\text{without a sample}} - A_{\text{blank}})] \times 100$$

Determination of antioxidant activity

The DPPH (1,1-diphenyl-1-picrylhydrazyl) is generally used for measurement of nitrogen radical which has an absorption at 517 nm. The DPPH was dissolved in absolute ethanol to obtain a concentration of 0.1 mM and mixed with sample in the ratio of 1:1, and incubated for 30 min. The rate limiting steps of DPPH radical scavenging assay is a fast electron transfer from antioxidant to DPPH, while the hydrogen atom transfer occurs very slowly in strong hydrogen accepting solvent such as methanol and ethanol (Bondet *et al*., 1997; Huang *et al*., 2005). Butylated hydroxyanisol (BHA) was used as a standard antioxidant.

The scavenging activity was calculated as [(Ab - As) / (Ab - Ac)] × 100, when As is the absorbance generated from DPPH in the presence of peptide sample, Ab is the absorbance of DPPH without peptide sample, and Ac is the absorbance in the presence of BHA.

Reverse phase high performance chromatography (RP-HPLC)

The active fraction was subjected to reverse-phase HPLC (RP-HPLC) on XTerra® RP18 5 µm (4.6×250 mm) with a linear gradient of acetonitrile (5-30% in 35 minutes) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 ml/min. The elution peak was detected at 210 nm. The fractions of peptides which showed activities as antioxidant or ACE inhibition was lyophilized and stored at -20 °C. The fractions with highest activities were further analyzed with mass spectrometry for their molecular mass and amino acid sequences.

RESULTS AND DISCUSSION

Extraction of protein

From fresh ginger of 1,000 g, 50 g of dried proteins were obtained.

Protein analysis by Tricine SDS-PAGE

Crude proteins of ginger rhizome before and after digestion were analysed by Tricine SDS-PAGE. Before digestion densed protein bands of 26 kDa and 14-17 kDa were observed. After digestion the hydrolyzed protein were observed as smeared bands and the dense protein bands of 4-14 kDa were disappeared.

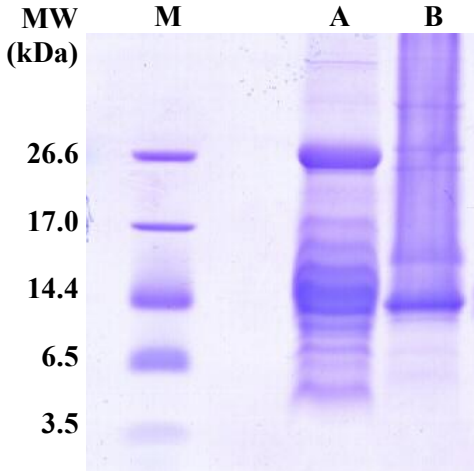

Figure 1. Tricine SDS-PAGE analysis of ginger proteins and their hydrolysates (M) Marker (A) Crude protein extract from ginger rhizome (B) Crude protein hydrolysate from ginger rhizome. Same amount of protein (3.47 µg) was loaded in each lane.

Peptide fractionation and quantification

The fractions with different molecular masses were collected after ultrafiltration, and the peptide fraction with molecular mass less than or equal to 1 kDa was collected. This peptide fraction was further fractionated using Sephadex G-25 gel filtration. Sixty fractions (1 ml/fraction) were collected at a flow rate of 1 ml/min and Angiotensin I-converting enzyme (ACE) inhibitory activity assay (Figure 2.) and antioxidant assay (Figure 3.) were monitored. Fractions 10-50 showed anti-ACE activity, whereas fractions 15-60 showed anti-oxidant activity.

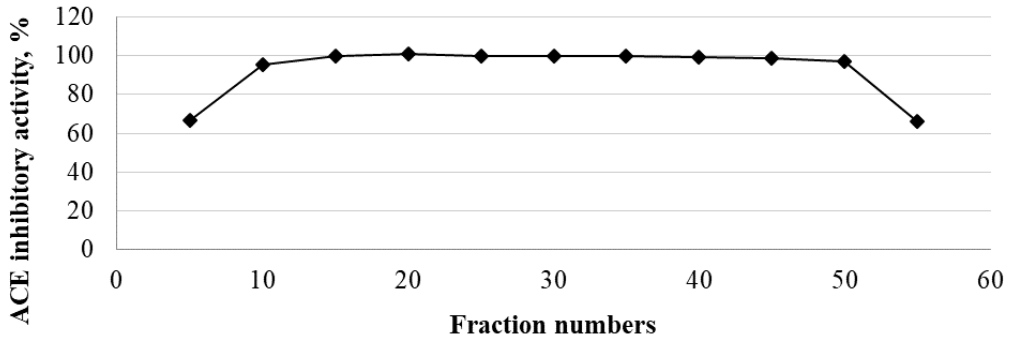

Figure 2. ACE inhibitory activity (%) of peptide fractions (G-25 gel filtration) from ginger proteins. Only one out of every five fractions was assayed.

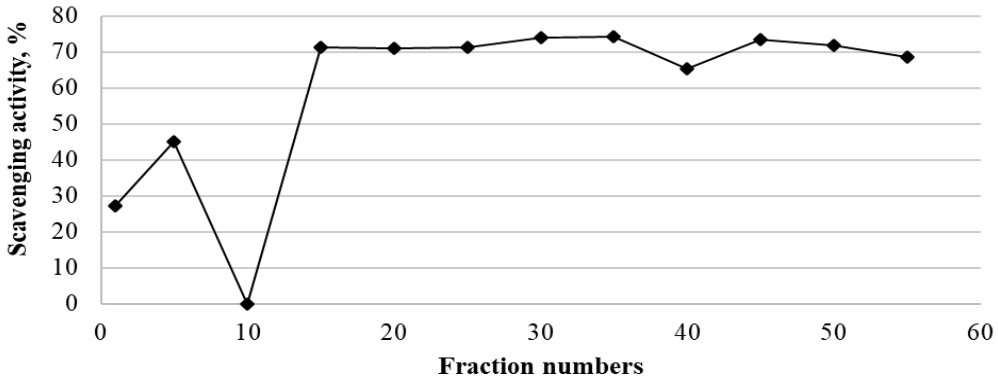

Figure 3. Scavenging inhibitory activity (%) of peptide fractions (G-25 gel filtration) from ginger. Only one in every five fractions was assayed.

Reverse phase high performance chromatography (RP-HPLC) profile of pooled active fraction

The active fractions (pooled fractions 42-44) obtained from size exclusion chromatography with both ACE inhibitory and antioxidant activities were analyzed using reverse-phase HPLC (RP-HPLC) in XTerra® C18. The RP-HPLC profile showed many peaks of peptides which could be grouped into 4 fractions (I-IV) as shown in Figure 4. The peptides from each fraction were measured for ACE inhibitory and antioxidant activities.

The highest ACE inhibition was found in Fraction IV which showed its value at 64.81%, Fractions I, II and III contained many minor peaks, and exhibited their ACE inhibition values at 30.74%, 28.92% and 27.23%, respectively as shown in Table 2. Therefore, Fraction IV which showed a potent ACE inhibition was further purified by RP-HPLC and a single peak was obtained (Figure 5).

The highest antioxidant activity was also found in fraction IV which showed its value at 75.44%, Fractions I, II and III exhibited their antioxidant values at 10.09%, 35.01 and 3.25%, respectively as shown in Table 2. Therefore, Fraction IV which showed a potent activity in both antioxidant and anti-ACE was further purified and collected as shown in Figure 5. The sequences of peptides were determined by mass spectrometry. Measurement of both anti-ACE and anti-oxidant was performed and compared based on fixed volumes (10 µl) of collected fractions.

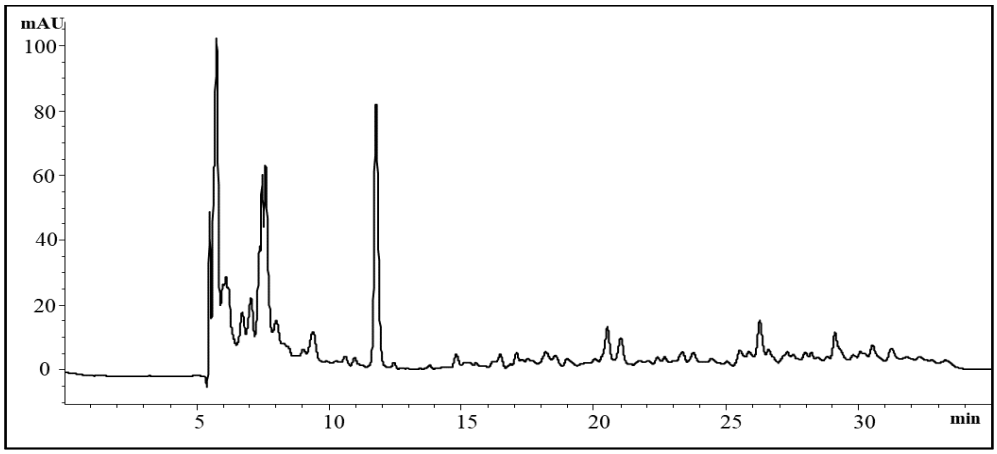

Figure 4. RP-HPLC profile of the active fraction (Pooled fraction 42-44 from gel filtration)

Table 2. ACE inhibitory and antioxidant activities of the active peptide fraction from ginger proteins.

RP-HPLC fractions	ACE inhibitory activity (%)	Scavenging inhibitory activity (%)
I	30.74	10.09
II	28.92	35.01
III	27.23	3.25
IV	64.81	75.44

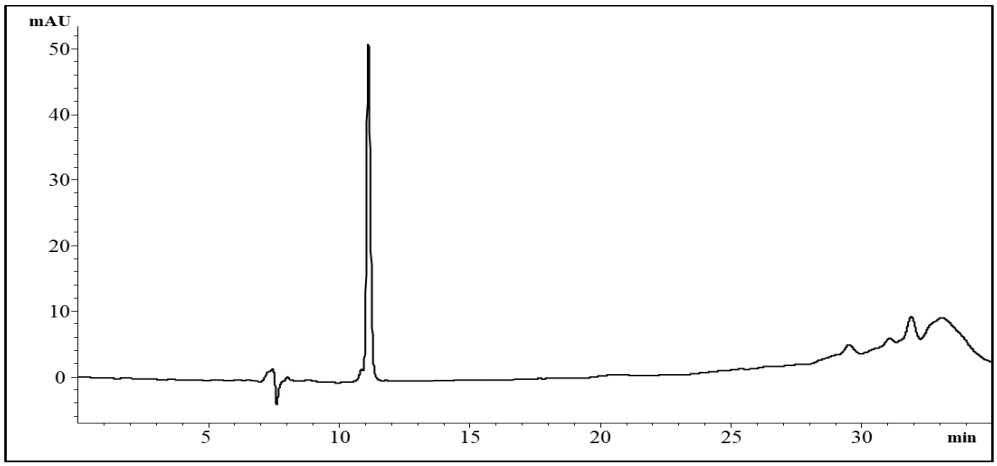

Figure 5. RP-HPLC profile of purified Fraction IV

CONCLUSION

Proteins were extracted from ginger rhizome and were digested by pepsin and trypsin. Peptides with molecular mass less than 1 kDa were collected and were fractionated by gel filtration. Several fractions with antioxidant and antihypertension were obtained and further purified by RP-HPLC. The RP-HPLC purified fraction (Fraction IV) had high biological activities. The ACE inhibitory and antioxidant activities is 30.74% and 51.53%, respectively. The amino acid sequences of selected peptides with

bioactivities were analysed by mass spectrometry and their activity was confirmed using synthetic peptides. The results obtained from this study demonstrated that peptides from proteins of ginger rhizome have a beneficial effect on health.

ACKNOWLEDGEMENTS

This work was partially supported by a research grant from Thailand Research Fund (IRG5980001). KS is a recipient of a scholarship under Institutional Strengthening Program, from Faculty of Science, Mahidol university.

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