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PURIFICATION AND CHARACTERISATION OF ANGIOTENSIN I CONVERTING ENZYME (ACE) INHIBITORY PEPTIDE FROM BLOOD COCKLE (*Anadara granosa*) MEAT HYDROLYSATE

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ABSTRACT

Blood cockle (*Anadara granosa*) is the most abundant and available bivalves in Malaysia. Blood cockles meat has high protein content and has potential to generate bioactive peptides. To date, no study has been reported on purification and identification of angiotensin I converting enzyme (ACE) inhibitory peptides from blood cockle meat. Thus, the objectives of this study were to purify and characterize ACE inhibitory peptide from blood cockle meat hydrolysate. ACE inhibitory peptides from blood cockle meat hydrolysate (CMH) were prepared by enzymatic protein hydrolysis using Protamex®. Crude CMH was characterized for its stability against gastrointestinal proteases, at varying pH (2–11) and temperature (4–90°C). Next, crude CMH was purified by ultrafiltration, ion exchange chromatography and reverse-phase chromatography and its amino acid sequence was identified. It was found that crude CMH was highly stable at low pH and temperature, and was resistant to gastrointestinal proteases (pepsin and trypsin). A three-step purification increased the inhibitory activity of CMH, reducing its IC_{50} from 0.35 mg/ml to 0.0094 mg/ml. The amino acid sequence of the purified peptide was determined as VNDLLSGSFKHFLY, with a molecular weight of 1621.88 Da. This study suggested the potential of ACE inhibitory peptide derived from cockle meat as a nutraceutical ingredient in functional food.

Key words: Blood cockle, angiotensin converting enzyme, peptide, hydrolysis

INTRODUCTION

Bioactive peptides offer potential physiological benefits to human gastrointestinal, nervous, cardiovascular and immune systems (Korhonen & Pihlanto, 2006). For this reason, the purification and isolation of bioactive peptides from foods has aroused much interest in recent years. One such bioactive peptide is the angiotensin I converting enzyme (ACE) inhibitory peptide officially identified as angiotensin I converting enzyme (ACE, peptidyl dipeptide hydrolase, EC 3.4.15.1) inhibitor (Korhonen & Pihlanto, 2006). ACE occurs naturally in cardiovascular system and it causes high blood pressure. ACE inhibitory peptide catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II, inactivating the vasodilator bradykinin and consequently reducing the blood pressure. Since the first discovery of an ACE inhibitor in snake venom, it has been extensively studied for pharmaceutical purposes, and synthetic variants such as captopril and enalapril, have become available (Ondetti, 1977). However, these synthetic ACE inhibitors incur adverse side effects such as mild azotemia at the start of therapy, hypotension, dizziness, renal dysfunction, non-productive cough, angioedema, hyperkalemia and renal insufficiency (Mann & Chakinala, 2012). ACE inhibitors from natural food-derived protein sources are considered milder and safer than synthetically derived ACE inhibitors (Lee *et al.*, 2010; Wu *et al.*, 2015).

ACE inhibitory peptides are inactive within their parent proteins, but become active when liberated by enzymatic hydrolysis, fermentation or autolysis (García *et al.*, 2013). ACE inhibitory

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peptides are commonly liberated by enzymatic hydrolysis, which is more easily controlled, requires a milder hydrolysis condition and yields a more uniform quality of hydrolysate than other methods (Liaset *et al.*, 2000; He *et al.*, 2013). The resulting peptides must resist complete hydrolysis by gastrointestinal proteases, and must pass through the intestinal wall while preserving their biological activity (Tavares *et al.*, 2011). For application in food systems, functional peptides must also be robust to the various adversaries of food processing, such as alkaline, neutral or acidic conditions, and different ranges of temperature and storage time (Sánchez-Rivera *et al.*, 2014). ACE inhibitory peptides are purified through a series of chromatographic separations, and typically contain 2–20 amino acid residues when fully isolated (Korhonen, 2009). Among the identified peptides are LKPNM from fish, LKP from oysters (Je *et al.*, 2005), VLP and VLL from fresh water clams (Tsai *et al.*, 2006) and VAP from grass carp (Chen *et al.*, 2012).

Blood cockle (*Anadara granosa*) is an edible marine bivalve that is commercially cultured in the tidal mudflats of Southeast Asian region and known as a cheap protein source in Malaysia. The production of blood cockle dominates 87% of shellfish production, which account for 16,642 metric tonnes for the year of 2018 in Malaysia (Department of Fisheries Malaysia, 2018). After enzymatic hydrolysis in the lyophilized form, the protein content of raw cockle meat increased from 14% to 74% (Nurnadia *et al.*, 2011). To date, studies on blood cockle hydrolysates have been limited to optimization of the enzymatic hydrolysis conditions of blood cockle meat for maximum degree of hydrolysis (Amiza & Masitah, 2012), maximum ACE inhibitory activity (Aishah *et al.*, 2017) and its wash water meat precipitate (Haslaniza *et al.*, 2013). The purification and identification of ACE inhibitory peptides from blood cockle has not been reported. Thus, the present study aimed to purify and characterize ACE inhibitory peptides from blood cockle meat hydrolysate as a natural alternative to synthetic hypertension agents.

MATERIALS AND METHODS

Materials

Blood cockles (*Anadara granosa*) were sampled from a cultivated farm at Sungai Kerang, Perak, Malaysia. The whole cockles were cleaned, washed with tap water to remove mud and impurities, steamed (30 min, 100°C) and manually deshelled to extract the meat. After homogenization in a food processor, the cockle meat was sealed in polyethylene bags and immediately frozen at –40°C until required. Determination of crude protein content of blood cockle meat using Kjeldahl method found that it contained 14.58% crude protein. Protamex™ was purchased from Novo Nordisk A/S Co. (Bagsvaerd, Denmark). Analytical-grade angiotensin I converting enzyme (EC 3.4.15.1) from rabbit lung, hippuryl–histidyl–leucine (HHL), and other proteases and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of ACE inhibitory peptides from blood cockle meat hydrolysate (CMH)

CMH was prepared by enzymatic hydrolysis using commercial food grade proteinase Protamex™, as described by Aishah *et al.* (2017). The hydrolysis mixture was then centrifuged at 4°C and 4000 rpm for 20 min. The resulting supernatant was lyophilized and stored in an airtight container at –20°C for future use.

ACE inhibitory activity assay

ACE inhibitory activity was determined by the method of Cushman and Cheung (1971), which measures the concentration of hippuric acid liberated from HHL. The detailed assay was described by Aishah *et al.* (2017). The ACE inhibitory activity (%) was calculated as $(A_c - A_s)/(A_c - A_b) \times 100$, where A_c, A_s and A_b are the absorbances of the control, sample and blank, respectively. The half maximal inhibitory concentration (IC_{50}) was calculated as the concentration of the hydrolysate that provided 50% inhibition of ACE activity under the assay conditions. It can be determined by regression analysis of ACE inhibitory activity (%) versus hydrolysate concentration (mg/ml) (Tsai *et al.*, 2006).

Effect of temperature, pH and gastrointestinal proteases on ACE inhibitory activity in crude CMH

To determine the effect of temperature on ACE inhibitory activity in crude CMH, it was incubated at different temperatures (4, 25, 37 and 90°C) and stored for various times (0.5–3.5 h). The effect of pH on ACE inhibitory activity in crude CMH was assessed by adjusting the peptide solution to pH 2, pH 8 or pH 11 with 1 N HCl or NaOH, and incubating at 25°C for various times (0.5–3.5 h) (Qu *et al.*, 2010).

To simulate the enzymatic conditions in the human stomach and intestines, crude CMH was treated with pepsin and trypsin enzymes. Control sample (T0) was crude CMH solution (10 mg/ml) without any enzyme treatment. T1 sample was crude CMH solution (10 mg/ml) after treatment with pepsin (1:50 w/w) (pH 2.0, 37°C, 2 h) whereas T2 represent crude CMH solution (10 mg/ml) after treatment with pepsin (1:50 w/w (pH 2.0, 37°C, 2 h) followed by treatment with trypsin (1:25 w/w)

(pH 8.0, 37°C, 4 h). Finally, the sample was inactivated at 95°C and cooled to room temperature prior to determining its ACE inhibitory activity (Quirós *et al.*, 2009).

Purification of crude CMH by ultrafiltration, ion exchange and RP–HPLC

Purification of ACE inhibitory peptide from crude CMH was purified as described by Ko *et al*. (2012).

Ultrafiltration

Crude CMH (50 mg/ml) with IC_{50} of 0.35 mg/ml was fractionated through a series of ultrafiltration membranes (Sartorious Stedim Biotech GmbH, Germany) with molecular weight cut-offs (MWCOs) of 10 kDa, 5 kDa and 3 kDa. The four resulting peptide fractions (<10 kDa, 10–5 kDa, 3–5 kDa and <3kDa) were lyophilized and assayed for their ACE inhibitory activity.

Ion exchange chromatography

The ultrafiltration fraction with the highest ACE inhibitory activity was subjected to further purification through a HiPrep 16/10 DEAE FF anion exchange column (GE Healthcare) using an AKTA purifier system (GE Healthcare, Uppsala, Sweden) at a concentration of 20 mg/ml. The column was equilibrated with 5 column volumes of 20 mM sodium acetate buffer (pH 4). The sample was then injected and eluted at a linear gradient of 2 M NaCl (0–30%) in the same buffer at a flow rate of 60 ml/h. The absorbance eluent was monitored at 280 nm. The fraction was collected to a volume of 10 ml, desalted and lyophilized before its ACE inhibitory activity was determined.

Reverse-phase high performance liquid chromatography (RP–HPLC)

The peptide fraction (10 mg/ml) exhibiting the highest ACE inhibitory activity was further purified by RP–HPLC on a Gemini-NX 10 µm C_{18} column (21.2 × 250 mm, Phenomenex Co. Ltd., Torrance, CA, USA). The column was pre-equilibrated with Milli-Q water for 10 min and the sample was eluted under a linear gradient of acetonitrile (0–50%), applied for 20 min at a flow rate of 2 ml/min (monitored at 215 nm). The active peak representing the highest ACE inhibitory activity was pooled and lyophilized immediately.

Identification of amino acid sequence and molecular mass of purified peptide

The molecular mass and amino acid sequence of the purified peptide was analysed by electrospray ionization mass spectrometry using the Shimadzu Prominence nano HPLC system coupled to a 5600 Triple TOF mass spectrometer (AB Sciex). Peptide sample was loaded onto an Agilent Zorbax 300SB-C18, 3.5 µm (Agilent Technologies) and separated with a linear gradient of water/ acetonitrile/0.1% formic acid (v/v). The sequence information from the resulting MS/MS spectra was analysed using PEAKS Studio Version 4.5 SP2 (Bioinformatic Solutions) and manual interpretation.

RESULTS AND DISCUSSION

Effect of temperature, pH and gastrointestinal proteases on ACE inhibitory activity in crude CMH

The stability of ACE inhibitory activity in crude CMH was tested under different temperatures and pH values, and in the presence of various gastrointestinal proteases at various storage times (Figure 1(a)–(c)).

It was found that CMH maintained high ACE inhibitory activity under all temperature treatments for 30–60 min, and at 4°C and 25°C for the maximum tested storage time (3.5 h). CMH also retained more than 50% of its initial activity at 37°C, but its activity significantly degraded at 90°C, indicating that CMH was more stable at low temperatures than at higher temperatures. Reduction in ACE inhibitory activity after heat treatment could be due to hydrolysis of peptide bonds, alteration of peptide sequences resulting in changes of hydrolysate properties, hydrophobic interaction and aggregation of their structure (Meisel, 1998; La Fuente *et al.*, 2002). Peptides from *P. yezeonsis* seaweed tested at similar storage times exhibited the same trend i.e: high ACE inhibitory activity at 4°C and 25°C, but reduced activity at higher temperatures and longer storage times. After 3.5 h storage at 90°C, the *P. yezeonsis* peptides had lost approximately 53.4% of their initial activity. Peptides from tuna cooking juice (Hwang, 2010) and soy protein (Wu & Ding, 2002) were more stable to high temperature whereby peptides from both samples were strongly resistant to 2 h storage at 20–100°C.

The effect of pH was also evaluated over the same storage times (0.5–3.5 h) (Figure 1(b)). At pH 2 and 8, CMH remained highly stable after 1.5 h. The ACE inhibitory activity of the peptides from blood cockle was better preserved in acidic than in alkaline environments. Similar finding has been reported for ACE inhibitory activity from *P. yezeonsis* exposed to pH levels of 2.0, 8.0 and 11.0 reduced from its initial value by 9.5%, 29.5% and 59.7%, respectively. The high stability of peptides under different processing conditions is important from a practical viewpoint, because the target food systems are usually processed with many steps with different pH, temperatures and durations. Stable peptides should retain their bioactivity during these

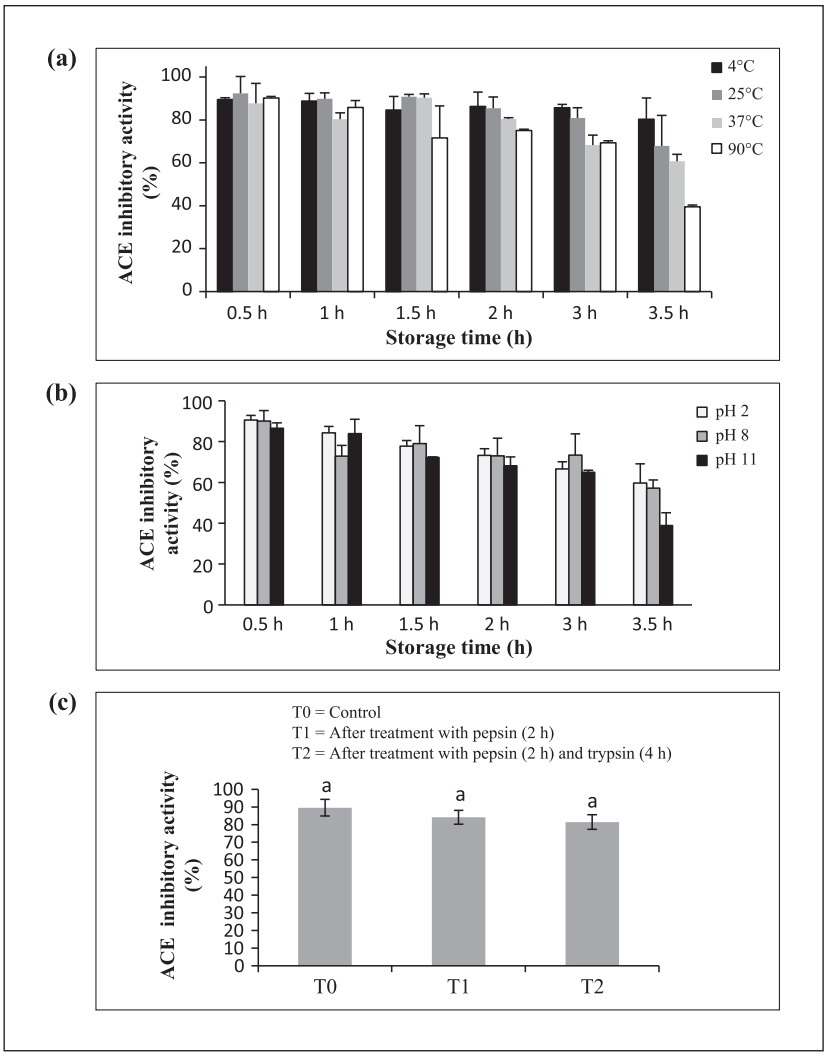

Fig. 1. Stability of ACE inhibitory activity of CMH **(a)** at various storage times and temperatures, **(b)** at various storage times and pH and **(c)** following enzymatic treatments with gastrointestinal proteases.

processes. Besides the processing conditions, the bioactivity of a peptide depends on the composition of the food matrix and the bioactive peptide structure (López-Fandiño *et al.*, 2006).

Figure 1(c) shows the effect of gastrointestinal proteases on the ACE inhibitory activity of CMH. The ACE inhibitory activities under the control treatment (without proteases) and treatments T1 and T2 were 89.56%, 84.09% and 81.44%, respectively. These results indicate that CMH was relatively stable against gastrointestinal proteases (the differences among treatments were insignificant; $p > 0.05$). ACE inhibitory peptides from other protein sources such as algae (Qu *et al.*, 2010) and dry-cured ham (Escudero *et al.*, 2014) are also stable to digestion with gastrointestinal proteases. Human gastrointestinal digestion proceeds by physical and chemical processes involving proteases at appropriate pH before absorption occurs (Langerholc *et al.*, 2011).

Purification of ACE inhibitory peptide from crude CMH using ultrafiltration, ion exchange and RP–HPLC

Blood cockle meat was subjected to enzymatic hydrolysis by Protamex® and fractionated through a series of ultrafiltration membranes resulting in 4 fractions with different molecular weight cut-offs (>10 kDa, 10–5 kDa, 5–3 kDa and <3 kDa). The lyophilized fraction with <3 kDa MWCO gave the highest percentage of weight as compared to other UF fractions (36.92%), indicating the highest peptide recovery in that fraction. The lyophilized fraction with >10kDa MWCO gave the second highest percentage of weight (12.18%), followed by lyophilized fraction with 5-10 kDa MWCO (0.68%) and finally lyophilized fraction with 3-5 kDa MWCO (0.46%). This finding is similar to other studies whereby < 3 kDa UF hydrolysate fraction contained highest ACE inhibitory activity (Du *et al.*, 2013; Rho *et al.*, 2009). Moreover, it exhibited the

Table 1. Recovery, IC_{50} and ACE inhibitory activities of ultrafiltration fractions from blood cockle hydrolysate

CMH fractions	Recovery (%)	IC_{50} value (mg/ml)	ACE inhibitory activity (%)
<3kDa	36.92%	0.27 ± 0.027	91.75 ± 7.81
3–5 kDa	5.64%	0.46 ± 0.020	82.29 ± 0.54
5–10 kDa	6.34%	0.68 ± 0.013	71.74 ± 4.22
>10 kDa	12.18%	0.60 ± 0.007	81.92 ± 2.33

highest ACE inhibitory activity among the fractions, with an IC_{50} of 0.27 mg/ml (Table 1). These results confirmed the presence of peptides with high ACE inhibitory activity; moreover, these peptides were concentrated in the low molecular weight fraction as reported by Ko *et al.* (2012) for *Styela clava* hydrolysate, Lee *et al.* (2010) for frame protein hydrolysate, Tsai *et al.* (2006) for freshwater clam hydrolysate. High ACE inhibitory activity by low molecular weight peptide fractions (< 10 kDa) could be due to the presence of a high amount of hydrophobic amino acids (i.e. Gly, Val, Ala, Pro and Leu) that concentrate in these fractions which have the binding ability to both N-terminal and C-terminal of ACE, thus better for inhibition ACE activity (Korhonen & Pihlanto, 2003; Ketnawa & Rawdkuen, 2013).

Peptides (3 kDa) were purified on a HiPrep16/10 anion exchange column with a linear gradient of NaCl (0–2 M) and separated into a non-adsorptive portion of an unbound protein followed by three adsorptive portions of bound proteins (Fr-1, Fr-2 and Fr-3), as shown in Fig. 2(a). The three bound fractions (Fr-1, Fr-2 and Fr-3) were eluted from 0.2–0.3 M NaCl and assayed for ACE inhibitory activity (refer Fig. 2(b)). The highest activity was exhibited by Fr-2 (86.45% at 0.1 mg/ml). Ion exchange chromatography selectively isolates ACE inhibitory peptide based on charge (Ketnawa & Rawdkuen, 2013). This technique is widely used to purify ACE inhibitory peptides from food protein sources such as tuna frame (Lee *et al.*, 2010) and the skin gelatine of Pacific cod (Moskowitz, 2003). The reported ACE inhibitory activities of tuna frame protein and Pacific cod skin gelatine were 76.4% at 2 mg/ml and 71.81% at 0.1 mg/ml, respectively. In both studies, all potent peptides were eluted in the range 0.2–0.6 M NaCl. Other studies have sorted ACE inhibitory peptides by molecular size through gel filtration columns using distilled water for example the oyster sauce (Je *et al.,* 2005). Alternatively, Jung *et al.* (2006) filtered the frame protein from yellowfin sole through an SP-Sephadex C-25 ion exchange column and they eluted the potent peaks in the range of 1.4–1.5 M NaCl.

After further purification of Fr-2 on an HPLC column, a single peak was obtained at 18% acetonitrile. This peak was isolated and tested for ACE inhibitory activity (Fig. 2(c)). The isolated peak exhibited higher activity than the isolates separated in the previous steps (ultrafiltration and ion exchange chromatography), with an IC_{50} of 0.0094 ± 0.0006 mg/ml. The refined isolate was characterized by LC–MS/MS. ACE inhibitory peptides have been isolated from other food protein sources, such as fermented oyster sauce with an IC_{50} of 0.0874 mg/ml (Je *et al.*, 2005), yellowfin sole frame protein with an IC_{50} of 0.0287 mg/ml (Jung *et al.* 2006) and from sea bream scales (Fahmi *et al.*, 2004), salmon skin (Gu *et al.*, 2011) and fermented anchovy, sardine and bonito sauce (Ichimura *et al.*, 2003) with IC_{50}s ranging from 0.003 to 0.332 mg/ml. In previous studies, the purified peptides were usually eluted from 5%–20% acetonitrile, depending on the precise distribution of the hydrophobic residues in each species and the preliminary purification steps (ultrafiltration, ion exchange or gel filtration) (Lee *et al.*, 2010; Himaya *et al.*, 2012; Jung *et al*., 2006; Qian *et al.*, 2007; Tsai *et al.*, 2008). As the acetonitrile concentration increased, each analyte reached its critical value, desorbed from the hydrophobic stationary-phase surface and eluted from the column into the flowing mobile phase (Segura-Campos *et al.*, 2011).

Identification of purified CMH using LC–MS/MS

The RP-HPLC purified fraction was determined using LC-MS/MS analysis coupled with database-assisted sequence matching. Figure 3 shows relative intensity (%) versus mass. The deduced sequence of the peptides was presented as b and y ions. From the figure, the RP–HPLC purified fraction was composed of 14 amino acid residues: Val-Asn-Asp-Leu-Leu-Ser-Gly-Ser-Phe-Lys-His-Phe-Leu-Tyr (VNDLLSGSFKHFLY; MW 1621.88 Da) with an aliphatic amino acid (valine) at the N-terminal, and hydrophobic residues (leucine, valine) and aromatic amino acids (tyrosine, phenylalanine) at the C-terminal. This sequence was the ACE inhibitory peptides with various sequences have been derived

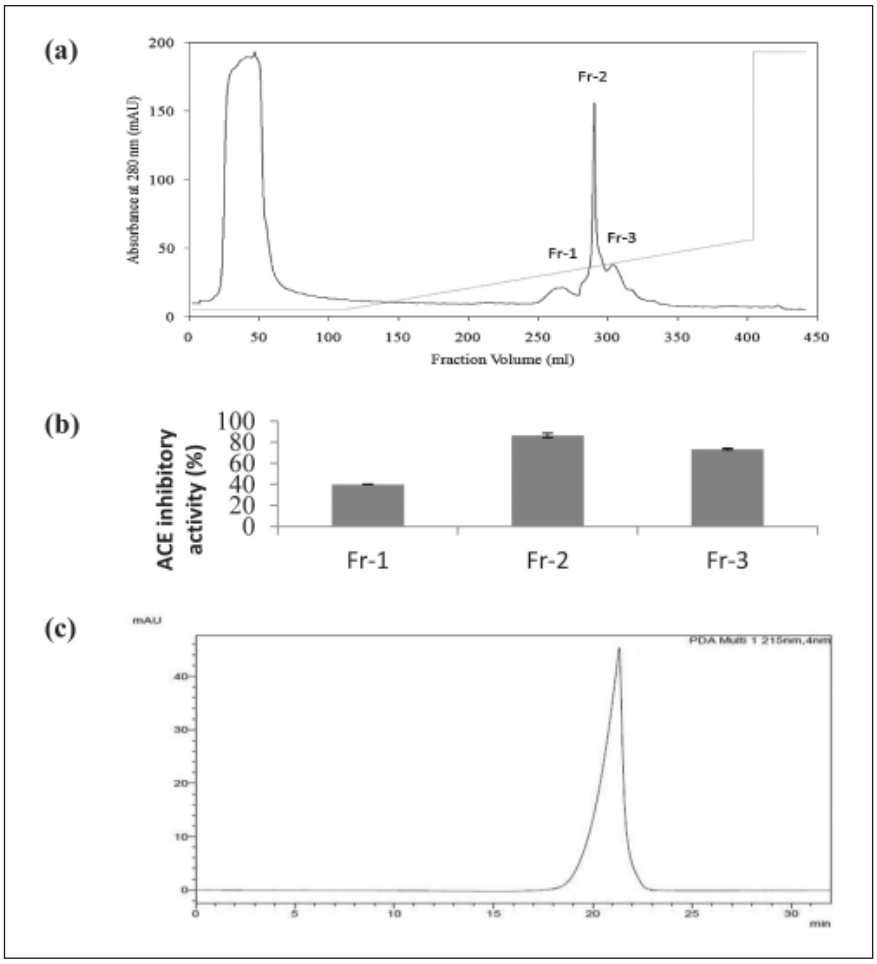

Fig. 2. (a) HiPrep 16/10 DEAE FF anion exchange chromatogram of the <3 kDa fraction separated under a linear gradient of NaCl **(b)** ACE inhibitory activity of the fraction separated by an ion exchange column **(c)** RP–HPLC chromatogram after eluting Fr-2 with a linear gradient of acetonitrile.

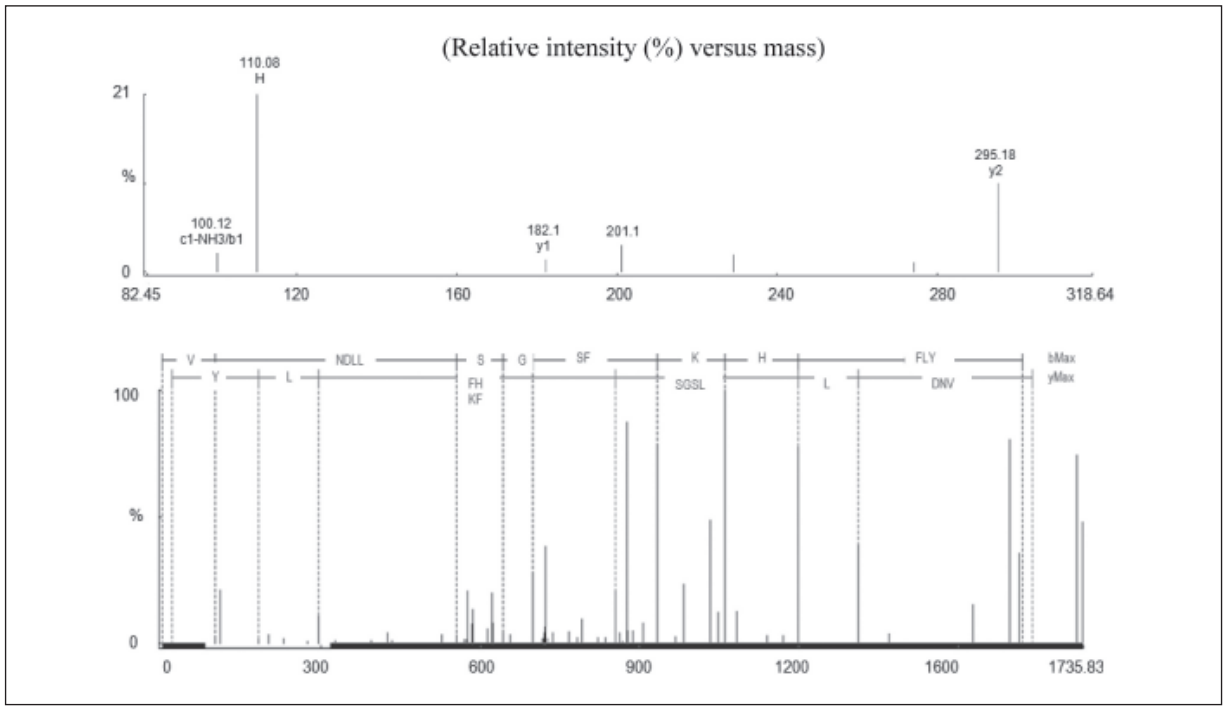

Fig. 3. *De novo* spectra of purified ACE inhibitory peptide from crude CMH characterized by LC–MS/MS at m/z 820.42.

from food proteins, but to our knowledge, have not been previously isolated from blood cockle. Among the previous reports of isolated ACE inhibitory peptides are LKPNM and LKP from oyster (Je, 2005), MIFGAGGPEL from yellowfin sole frame (Jung *et al.*, 2006), VLP and VLL from fresh water clams (Tsai *et al.*, 2008), VAP from grass carp (Chen *et al.*, 2012) and LPGRPPIKPWPL from walnut (Wang *et al.*, 2014). Most of the reported ACEI peptides are short peptides composed of 3–20 amino acid residues (Qian *et al.*, 2007; Tsai *et al.*, 2008). According to Pihlanto (2000), the most potent and specific peptide inhibitors have similar structures and their ACE activities are strongly influenced by the C-terminal sequence, which generally possesses a proline or aromatic residue. Conversely, their N-terminals usually contain hydrophobic amino acid residues. Among the N-terminal amino acids, the branched-chain aliphatic amino acids such as Ile and Val predominate (Cheung *et al.*, 1980).

In this study, the isolated peptide from blood cockle meat is composed of 14 amino acid residues (VNDLLSGSFKHFLY). This peptide is considered long where its structural feature is important in determining ACE inhibitory potency. The presence of Tyr at the C-terminal of the isolated ACE inhibitory peptide from blood cockle meat could be the potential contributor to high ACE inhibitory activity (Aluko, 2012). According to Iwaniak *et al.* (2014), long chain ACE inhibitory peptides other than dipeptide and tripeptide usually possess of basic amino acids (Arg, Lys) at the C-terminal end. For long peptides, which consist of more than 4 amino acid residues, the type and arrangement of the last four amino acid residues at the C-terminal play role in determining their ACE inhibitory potency. The potential contributor to their ACE inhibitory potency are the presence of tyrosine and cysteine at the C-terminal position or histidine, tryptophan and methionine at the penultimate C-terminal position, the presence of isoleucine, leucine, valine and methionine at the C3 position or tryptophan at the C4 position of long chain ACE inhibitory peptides (Aluko, 2012). A run on BIOPEP database showed that no amino acid sequence has been reported before similar to this cockle-derived ACE inhibitory peptide. It was also found that four dipeptide sequence with potential ACE inhibitory activity was encrypted in the amino acid sequence of cockle-derived ACE inhibitory peptide, namely LY, GS, SG and SF. Aluko (2012) also reported a long chain ACE inhibitory peptide with 21 amino acid residues (GDLGKTTTVSNWSPPKYKDTP) from tuna frame hydrolysate.

CONCLUSION

Crude CMH was found to be stable against various gastrointestinal proteases at low pH (pH 2) and temperature (4°C), but was sensitive to higher pH, higher temperatures and longer storage times. After a three-step purification, a potent ACE inhibitory peptide (VNDLLSGSFKHFLY) was isolated from blood cockle. The IC_{50} was improved from 0.35 mg/ml in the crude sample to 0.0094 mg/ml in the pure isolate. This finding suggests that blood cockle meat hydrolysate is a potent source of ACE inhibitory peptides.

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