



Purification and characterization of angiotensin converting enzyme-inhibitory derived from crocodile blood hydrolysates

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Abstract

Various enzyme types were used to hydrolyze crocodile blood peptides showing an Angiotensin I-converting enzyme (ACE) inhibitory activity. Alcalase hydrolysates (ALH) and Protease G6 hydrolysates (PG6H) showed the highest degree of hydrolysis ($P < 0.05$). However, PG6H was significantly observed to have an effective ACE-inhibitory (ACE-I) activity (94.23%) with an IC_{50} of 0.021 ± 0.02 mg/mL. An unbound fraction of PG6H showed the highest ACE-I activity and was then subjected to two steps RP-HPLC process. The potent fractions including RC1 and RC2 exhibiting the highest ACE-I activity (88.33 & 84.54%, respectively) were identified using LC-MS/MS. Two novel ACE-inhibitory peptides were identified as GVAAN (431.25 Da) and LHALLL (679.52 Da), and characterized by GRAVY were 60 and 83%, respectively. The crocodile blood hydrolysate obtained by Protease G6 could serve as a source of ACE-inhibitory activity for physiological benefits.

Keywords: ACE inhibitory peptides; antihypertension; crocodile blood hydrolysate.

Practical Application: The crocodile blood hydrolysate was derived from a byproduct exhibiting antihypertensive activity. Not only Protease G6 can be produced from crocodile blood hydrolysates from low-cost sources but also these peptides can effectively inhibit ACE activity as natural ACE inhibitors.

1 Introduction

Hypertension can seriously cause a risk factor of cardiovascular disease (Collins & MacMahon, 1994). Activity of Angiotensin I-converting enzyme (ACE) is a key observation in the high blood pressure or hypertension through two different reactions including the renin-angiotensin-aldosterone system (RAAS) and the kinin nitric oxide system (KNOS). These are treated with many synthetic ACE inhibitors (captopril, fosinopril, enalapril, ramipril, & lisinopril), conversely, these synthetic inhibitors have some side effects (Agostoni & Cicardi, 2001; Chen et al., 2013). Thus, one of the major challenges to today's world healthcare sectors is to identify ACE inhibitors from natural resources such as various meat and fish proteins (Ryan et al., 2011). In addition, a more sustainable alternative providing high-value, including trimmed-meat, bone, and blood has gained much interest in recent years, and may provide a commercial opportunity for many companies (Lalor & Wall, 2011). Nevertheless, only 30% of the blood generated in slaughterhouses is used in the food industry (Bah et al., 2013). Blood proteins are not only used in the food industry as ingredients in numerous foods, but also used in the pharmaceutical industry (Bah et al., 2013; Xie et al., 2012).

Recent studies have been performed observing antioxidant and anti-inflammatory activities on substances extracted from animal blood (Sivaperumal et al., 2013; Jandaruang et al., 2012). In studies of the blood from Siamese crocodiles, many biological activities were observed (Jandaruang et al., 2012; Srihongthong et al., 2012; Kommanee et al., 2014; Patathananone et al., 2015;

Pakdeesuwan et al., 2017; Maraming et al., 2018), however, there is limited information of angiotensin I converting enzymes (ACE) inhibitory activity. Therefore, the purpose of this study was to investigate the effects of crocodile blood hydrolysates on ACE inhibitory activity affecting different enzymes and also to purify and identify the major ACE inhibitory peptides.

2 Material and methods

Angiotensin-converting enzyme (ACE, EC 3.4.15.1 from rabbit lung) was purchased from Sigma-Aldrich, USA. Alcalase 2.4 L[®], Protease G6, Protease GN, Flavourzyme and Protamex were purchased from Siam Victory Company, Thailand (Novozymes agency). Hippuric acid was purchased from Carlo Erba, France.

2.1 Crocodile blood preparation and extraction

Crocodiles (*Crocodylus siamensis*) were captured and housed at the local Srirachamoda Farm, Chonburi, Thailand. Each crocodile (age ranging from 1-3 years) was housed in a single tank, and treated with electric shock. The crocodile blood was collected into sterilized bottles and then kept at -20 °C until used. The crocodile blood was extracted with 300 ml of 50 mM Tris-HCl, pH 7.0 buffers (1:3 v/v) using a homogenizer at 1,000 rpm for 10 min. The homogenate was centrifuged at $10,000 \times g$ for 20 min and the supernatant was stored at -4 °C before further analysis.

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2.2 Crocodile blood hydrolysates

The crude crocodile blood extract (CBE) was pre-incubated at 52.5 °C, prior to adjusting the pH to 7.0. The enzymatic hydrolysis of CBE was varied in different proteases including Alcalase, Flavourzyme, Protease G6, Protease GN and Protamex (Table 1). The enzyme-to-substrate (E/S) ratio was set to 1:1 w/w for each enzyme with a reaction time of 12 hours under a temperature-controlled water bath (TAITEC-EX Thermo minder, Saitama, Japan). The mixture reactions were stopped by boiling water for 30 min and cooled immediately in an ice bath. Then reactions were centrifuged at 10,000 x g for 20 min (Hettich ROTINA 380R, German). The supernatants as protein hydrolysates were collected and stored at -4 °C until used for analysis.

2.3 Degree of hydrolysis

The degree of hydrolysis (DH) was analysed by the method using *o*-phthaldialdehyde (OPA) according to Nielsen et al. (2001). The DH (%) was calculated by their equation and used the h_{tot} value ~7.6.

2.4 Angiotensin I-converting enzyme inhibitory (ACE-I) activity

The method used was a HPLC method based on the assay modified from Lahogue et al. (2010). The substrate HHL (Hippuryl-His-Leu) was dissolved (5 mM) in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl. The assay was performed by mixing 50 µL of substrate solution with 20 µL of each protein hydrolysate (or borate buffer as control). After 10 min of incubation at 37 °C, 10 µL of ACE solution (100 mU/mL) were added and the sample was further incubated at 37 °C for 30 min. The reaction was stopped by the addition of 100 µL of 1 M HCl and the solution was filtered through a 0.22 µm nylon syringe filter before being analyzed by reversed-phase HPLC. The HPLC analysis was performed on a Jupiter C18 column (4.6×250 mm), particle size 5 µm (Phenomenex, U.S.A) with a Varian chromatographic system and analyses were detected at the wavelength of $\lambda = 228$ nm. The mobile phase comprised an isocratic system consisting of 12.5% (v/v) HPLC-grade acetonitrile in deionized water, and its pH was adjusted to 3.0 by adding glacial acetic acid (Pegg et al., 2007). The column was eluted at a flow rate of 1 mL/min with an isocratic elution system for 16 min. The IC_{50} value of ACE-I activity versus protein in the concentration range of 5-30 µg/mL was determined.

2.5 Purification of ACE-inhibitory peptides

The separation and purification of a protein hydrolysate sample was performed with a chromatography column containing 40 mL CM Sepharose cation-exchange resin (BIO RAD BioLogic LP, USA). Before packing the column, the resin was pretreated with 2 M NaCl and thoroughly washed with deionized water to eliminate interstitial solution. The hydrolysate solution was then loaded. The unbound fraction was washed off the resin with excess 50 mM Tris-HCl buffer (pH 7.0) at a flow rate of 0.5 mL/min, while the bound fractions were eluted by 2 M NaCl dissolved in 50 mM Tris-HCl buffer (pH 7.0). Elution curves were obtained by measuring absorbance at 220 nm using a UV detector. The fractions that showed high ACE-inhibitory activity were collected and then concentrated using a speed vacuum concentrator (Savant Instrument, U.S.A) for 5 h. The most active fraction was separated by RP-HPLC. Purified fractions from ion exchange chromatography were filtered with a 0.22 µm filter. The filtered fraction (4 mL) was loaded onto a SunFire C18 Prep column (10×150 mm, 10 µm, Waters Co., Milford, MA, USA) with mobile phases 0.1% (v/v) trifluoroacetic acid (TFA) in deionized water (A) and 0.1% (v/v) TFA in 60% acetonitrile (B). A linear gradient at flow rate of 1 mL/min: 15 min at 93% A; 5-60 min at 7-45%B; 65-68 min at 55-45%B; 68-75 min at 30-70%B; 75-80 min at 100%B; and 80-90 min at 93-7%B was performed. The UV absorbance of the eluent was monitored at 220 nm. The fractions were concentrated using a speed vacuum concentrator for 5 h to further evaluate the ACE-I activity and continually rechromatographed in the same RP-HPLC column expect that different gradient elution condition. The mobile phase was water as eluent A and 100% acetonitrile as eluent B. The gradient applied was eluted by eluent A for 10 min with the following eluent B concentrations: 0-5 min 100-0% (v/v), 5-40 min, 75-25% (v/v), 50-65 min 0-100% (v/v) and then 65-70 min, 100-0% (v/v).

2.6 Identification of ACE-inhibitory peptides

The molecular weight and amino acid sequence of the target peptides were determined by using a liquid chromatography coupled to mass spectrometry (LC-MS/MS) analysis and performed on nano-LC (Easy-nLC II, Bruker Daltonics) directly connected to a mass spectrometer hybrid quadrupole-time-of-flight (MicroTOF-Q II, Bruker Daltonics) with captive spray ionization (Bruker Daltonics). Three microliters (~1000 ng) of sample volume were injected and separated by an analytical column at flow rate of 300 nL/min. The gradient mobile phases consisted

Table 1. Details of commercial enzymes from manufacturers.

Enzymes	Temp. range (°C)	pH range	Source	Type of proteinase	Preferential specificity	Enz. Conc.
Protease G6 ¹	55-70	7.0-10.0	<i>Bacillus licheniformis</i>	Alkaline serine endopeptidase	Broad specificity mainly hydrophobic-COOH	174,000 Du/g
Alcalase 2.4 L ²	55-70	6.5-8.5	<i>Bacillus licheniformis</i>	Endoprotease	Broad specificity mainly hydrophobic-COOH	0.7 AU-A/g
Protease GN ¹	40-60	6.0-8.0	<i>Bacillus amyloliquefaciens</i>	Metallo neutral endopeptidase	Leu, Phe-NH3 & others	480 AU-A/g
Flavourzyme ²	50-55	5.5-7.5	<i>Aspergillus oryzae</i>	Endoprotease/ exopeptidase	Arg, Lys-COOH	150 LAPU/g
Protamex ²	35-60	5.5-7.5	<i>Bacillus protease complex</i>	Endoprotease/ exopeptidase	-	1.5 AU/g

¹ Data from Genencor International, Inc., USA; ² Data from Novozymes A/S, Denmark.

of (A) water containing 0.1% formic acid and (B) acetonitrile containing 0.1% formic acid. The gradient elution mode was as follows: 0-20 min, 30%-60% B; 20-30 min, 60%-80% B. The instrument was operated with positive ions using a range of 50-3000 m/z and capillary voltage of the ion source, flow rate of dry gas, and dry temperature were set to 1500 V, 3.0 L/min, and 160 °C, respectively.

The MS/MS measurements were based on collision induced dissociation (CID). The precursor ions were selected in the quadrupole collision cell with the collision energy between 15 eV and 35 eV (Data Analysis 4.0 software, Bruker Daltonics). Data was processed using the *de novo* analysis module of the BioTools protein sequence analysis package (Version 2.2). In addition, the peptide sequence was computed the parameters with a ProtParam tool on the ExPASy server including molecular weight, theoretical pI (isoelectric point) and grand average of hydrophobicity (GRAVY).

2.7 Statistical analysis

The mean values and S.D. from triplicated parameters were calculated. A one-way ANOVA analysis was carried out using SPSS® version 19 with a Duncan comparison function to present the treatments which shows the different significance in their treatments.

3 Results and discussion

3.1 Effect of enzyme types and degree of hydrolysis on ACE-inhibitory activity of blood protein hydrolysates

The CBE was digested by commercial proteases including Alcalase, Flavourzyme, Protease G6, Protease GN and Protamex. The degree of hydrolysis (DH) are shown in Table 2. The highest DH was found in CBE hydrolyzed by Protease G6 and Alcalase ($P < 0.05$). While the CBE hydrolyzed by Flavourzyme and Protamex showed low DH (6.7 & 9.9%, respectively). This result indicates that the endoprotease might be the potential DH of crocodile blood hydrolysates but the mixtures of endo-exopeptidase were not possible to show high DH (Table 1). The differences in DH values obtained may be due to the different specificity of the enzymes used for the proteolysis (Ktari et al., 2013). Moreover, protease types affected the variations in the hydrolysis reaction, which would have expressively affected the resulting peptide

Table 2. Degree of hydrolysis (DH) and ACE-inhibitory (ACE-I) activity of CBE protein hydrolysates prepared by treatment with various bacterial proteases.

Samples ¹	DH (%)	ACE-I activity (%)
ALH	56.10 ± 1.13 ^a	7.37 ± 1.60 ^c
FLH	6.17 ± 0.19 ^d	55.49 ± 1.25 ^b
PTH	9.89 ± 0.29 ^c	7.45 ± 0.42 ^c
PG6H	58.50 ± 2.13 ^a	94.23 ± 0.52 ^a
PGNH	47.06 ± 3.01 ^b	13.68 ± 8.33 ^c

¹CBE hydrolyzed by various enzyme types including Alcalase (ALH), Flavourzyme (FLH), Protamex (PTH), Protease G6 (PG6H), and Protease GN (PGNH). Different letters within each column indicate a significant difference ($P < 0.05$) among treatments. Values are mean ± standard error of triplicate measurements.

compositions and therefore, the ACE-inhibitory activity (Wu et al., 2009).

The inhibition rate of CBE hydrolysates towards ACE involved in the regulation of blood pressure (Li et al., 2015) were determine, All hydrolysates at 10 µg/mL showing different DH, the CBE hydrolyzed by Protease G6 (PG6H) had significantly higher ACE-I activity (94.22%) than other hydrolysates. On the other hand, CBE hydrolyzed by Alcalase (ALH) and Protamex (PTH) showed very low antihypertensive activity ($P < 0.05$; Table 2). The PG6H and ALH have similar DH values, however, these are significant differences in ACE-I activity. This result is in agreement with other studies that reported different enzymes with almost the same DH did not show the same ACE inhibitory potency. According to Barzideh et al. (2014), collagen hydrolysates (ribbon jellyfish) hydrolyzed by Alcalase and Trypsin resulted in similar DH values (26 & 25%, respectively), but different ACE-I activity (59 & 89%, respectively). Thus, the protein sequence of the substrate or protease activity would be the most important factor of hydrolysates exhibiting ACE-I activity (Cheung & Li-Chan, 2010).

Surprisingly, the IC_{50} value of PG6H (0.021±0.02 mg/mL) obtained after logarithmic linearization was higher than those of hydrolysates from the pepsin of porcine hemoglobin hydrolysate ($IC_{50} = 1.53 ± 0.03$ mg/mL; Deng et al., 2014) and the Alcalase hydrolysate of albumin ($IC_{50} = 0.56$ mg/mL; Hyun & Shin, 2000), and the Alcalase hydrolysate of sarcoplasmic protein ($IC_{50} = 0.038$ mg/mL; Ghassem et al., 2014). These differences reveal that the use of Protease G6 was efficient in releasing peptides with ACE inhibitory activity from crude crocodile blood extract. Therefore, the PG6H was chosen for further purification and characterization.

3.2 Purification of ACE inhibitory peptide

ACE inhibitory peptide from Protease G6 hydrolysate was purified using cation ion exchange chromatography. As shown in Figure 1a, there are two main fractions which are unbound and bound fractions at 220 nm. The ACE-I activity was widely observed in all fractions compared to crude hydrolysate, but the unbound fraction exhibited the highest activity (Figure 1b) which was 36%.

The unbound fraction was further purified by RP-HPLC. The elution profiles of the peptides are shown in Figure 2a. Five major peaks showing ACE-I activity were obtained, however, the P3 peak showed the highest ACE-I activity (31.06%, Figure 2b). Thus, the P3 was further purified in the same RP-HPLC conditions with different gradients. After rechromatography, two relatively single pure peptides were isolated and designated as fractions RC1 and RC2 (Figure 2c), respectively. These peptides were identified by LC-MS/MS studies. The RC1 fraction was identified to be GVAAN (431.25 Da) and its ACE-I activity was 88.33%. The RC2 contained Leu-His-Ala-Leu-Leu-Leu and the molecular weight was 679.52 Da, with an ACE-I activity of 84.54%. These peptides showed high ACE-I activity due to their sequences and amino acid composition which might interact with ACE ligands. Previously studies reported that improving the ACE-inhibitory activity of peptides would contain phenylalanine, proline, tryptophan,

tyrosine, lysine, leucine, isoleucine, and valine residues in the C-terminal of peptides (Guang & Phillips, 2009; Ngo et al., 2014; Iwaniak et al., 2014). In addition, the hydrophobic ratio of RC1 and RC2 peptides showed strong hydrophobic ratios of 60 and 83%, respectively. This result is consistent with a previous result that the amino acid composition of purified peptides consist of

hydrophobic amino acids with a total percentage of 44.4% and 55.5% for LL-9 (Leu-Pro-Glu-Ser-Val-His-Leu-Asp-Lys) and VL-9 (Val-Leu-Ser-Thr-Ser-Phe-Pro-Pro-Lys) peptides, respectively. This result suggests that the peptides effectively interacted with the ACE active site, and maybe explain the stronger inhibition activity of VL-9 (Mirzaei et al., 2017). In this study, the ratio of

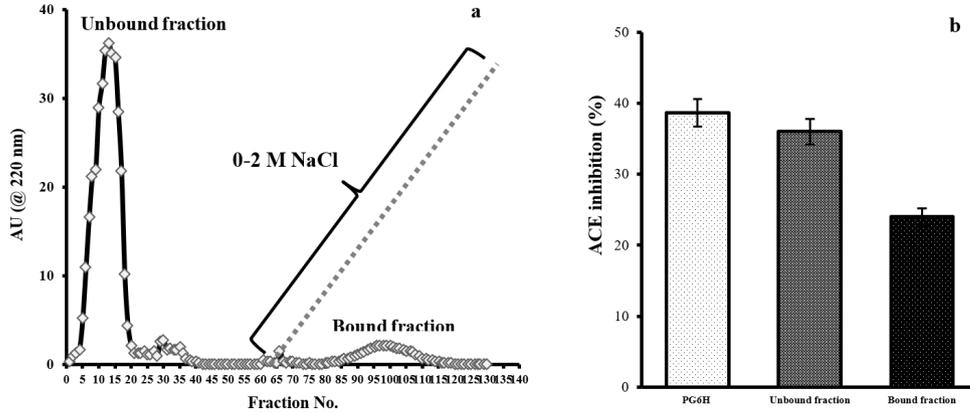


Figure 1. Elution profile of the Protease G6 hydrolysate separated on a CM-Sepharose cation exchange column (a). The ACE inhibitory activity of peptide fractions (b).

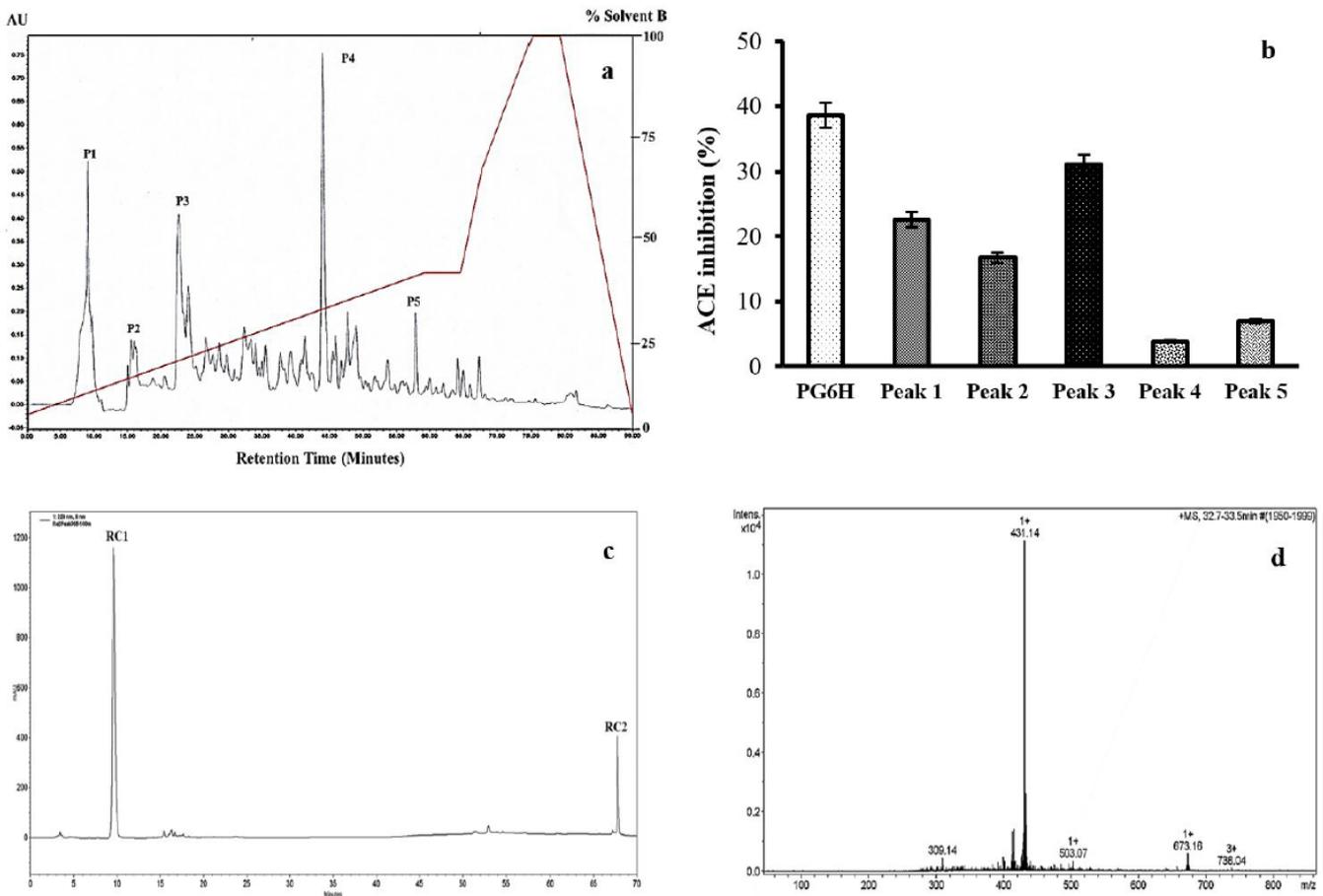


Figure 2. Preparative RP-HPLC chromatograms of unbound fractions isolated from CM- sepharose Fast Flow (a). The ACE inhibitory activity of five fractions (b). Rechromatogram of peak 3 from Preparative RP-HPLC (c). Identification of the molecular mass and amino acid sequence of the peptide from RC1 using LC-MS/MS (d).

hydrophobic amino acids is higher than in the previous studies indicating two peptides can effectively inhibit the ACE activity.

Furthermore, our results showed that the two peptides did not match the sequence database. These results might show that there are two novel ACE-inhibitory peptides from crocodile blood hydrolysates. However, further studies are required to investigate their ACE-inhibitory activity (in vivo) and their synthetic analogues in order to determine the structure needed for mechanical inhibition of ACE activity.

4 Conclusion

ACE-inhibitory protein hydrolysates were successfully produced from crocodile blood by Protease G6 (PG6H). The ACE-inhibitor peptides were efficiently purified from crude PG6H through multi-step chromatographic purification comprising of ion exchange chromatography followed by two-step RP-HPLC, and their sequences were analyzed by LC-MS/MS. This work could be applied to produce antihypertensive peptides from low-cost blood proteins and in an environmental sustainable manner. Furthermore, PG6H could potentially be used to formulate therapeutic food products for the prevention or treatment of hypertension.

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