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Novel antioxidant and anti-inflammatory peptides from the Siamese crocodile *(Crocodylus siamensis)* hemoglobin hydrolysate

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Running title: Bioactive peptides from *C. siamensis* hydrolysates

Abstract Novel antioxidant and anti-inflammatory peptides were isolated from hydrolysates of Siamese crocodile (*Crocodylus siamensis*) hemoglobin. *Crocodylus siamensis* hemoglobin hydrolysates (CHHs) were obtained by pepsin digestion at different incubation times (2, 4, 6 and 8 H) at 37 °C and subjected to antioxidant and anti-inflammatory activity assessment. CHH obtained by 2-H hydrolysis (2H-CHH) showed the highest anti-inflammatory activity with respect to decreasing nitric oxide (NO) production, while the strongest antioxidant activity was found for 6-H hydrolysis (6H-CHH) against nitric oxide radicals. To evaluate the anti-inflammatory and antioxidant activity of individual peptide components, 2H-CHH and 6H-CHH were purified by semi-preparative HPLC. Peptide fraction P57 isolated from 6H-CHH was found to exhibit the highest nitric oxide radical inhibition activity (32.0%). Moreover, purification of 2H-CHH yielded peptide fraction P16, which displayed a high efficacy in decreasing NO production of macrophage RAW 264.7 cells (83.2%) and significantly reduced pro-inflammatory cytokines and inflammatory mediators interleukin-6 (IL-6), interleukin-1 beta (IL-1β) and prostaglandin-E2 (PGE_2) production to about 2.0, 0.3 and 1.9 ng/mL, respectively. Using LTQ orbitrap XL mass spectrometry, active peptide sequences were identified as antioxidant KIYFPHF (KF7), anti-inflammatory SAFNPHEKQ (SQ9) and IIHNEKVQAHGKKVL (IL15). Additionally, CHHs simulated gastric and intestinal *in vitro* digestion positively contributed to antioxidant and anti-inflammatory activity. Taken collectively, the results of this work demonstrate that CHHs contain several peptides with anti-inflammatory and antioxidant properties which may prove valuable as treatment or supplement against diseases associated with inflammation and oxidative stress.

Keywords: *Crocodylus siamensis, enzymatic hydrolysis, hemoglobin hydrolysate, inflammation, oxidative stress, purification and identification*

Abbreviations: *CHHs, Crocodylus siamensis hemoglobin hydrolysates; cHb, C. siamensis hemoglobin; COX-2, cyclooxygenase-2; DH, degree of hydrolysis; IL-1β, Interleukin 1 beta; IL-6, Interleukin 6; NO, Nitric oxide;* PGE_2*, Prostaglandin E2; TNF-α, Tumor necrosis factor alpha.*

1. Introduction

Inflammation, oxidative stress and free radical damage have recently been acknowledged as a global threat for human health. Albeit being common by-products of a broad range of processes ensuring cellular homeostasis, excessive generation of radical metabolites is unambiguously associated with a variety of serious disorders such as cancer, heart disease, strokes, Alzheimer's, and premature aging [1]. Similarly, the inflammatory response constitutes an important natural reaction of the host defense system to injury and the invasion of toxins or infectious particles. In the case of pathogenic infection, inflammation contributes to combatting the disease and protecting vital parts of the body, while suspending the normal immune response and shunting certain metabolic pathways at the same time. Specific pro-inflammatory mediators and cytokines, such as NO, PGE_2, TNF-α, IL-1β and IL-6, comprise vital parts of the inflammatory cascade and are generated to affect immune cell function as well as proliferation activity [2]. In the long term, however, inflammatory processes are known to result in progressive damage and are usually associated with excessive free radical release and oxidative stress [3].

The latter is defined as an inequality between the free radicals production and reactive metabolites resulting in damage to cells and important biomolecules which severely impacts all organisms [4]. Free radicals, which account for the majority of both reactive nitrogen species (RNS) and reactive oxygen species (ROS), are defined as molecular species possessing one or more non-bonding paired electrons in spatially different orbitals of atoms or molecules [5]. Also, they have high potential to either donate an electron to or accept an electron from other molecules, consequently behaving as oxidants [6]. The latter process is capable of inflicting severe damage to the membranes and nuclei of cells by oxidizing important biochemical molecules such as proteins, lipids, carbohydrates, and DNA [7]. During the course of the inflammation high amounts of free radicals are produced within the inflamed tissue, in particular NO, which acts as a major pro-inflammatory mediator in the human body and is generated from macrophage in response to the inflammation [8]. In this context NO is generally regarded as the key mediator correlating inflammation and oxidative stress. The discovery and development of substances capable of inhibiting either the activity or production of NO has therefore gained considerable attention within the recent years as they may be utilized to reduce the detrimental effects of both inflammation and oxidative damage.

Crocodylus siamensis, commonly called Siamese crocodile, is a small freshwater crocodilian populating parts of Southeast Asia, including Thailand. Recently, several components of *C. siamensis* blood, i.e. plasma, serum, white blood cells and hemoglobin have been reported to possess a broad spectrum of biological properties, mainly attributed to abundance of a number of biologically active peptides and proteins. Among

these, hemoglobin constitutes the most abundant component and has been shown to exhibit antimicrobial [9], antioxidant [10, 11] and anti-inflammatory activity [11, 12].

In addition to the direct extraction of specific proteins with desired biological activity, enzymatic hydrolysis of protein sources has been established as a convenient method to generate novel protein fragments with enhanced biological properties. A variety of animal proteins was shown to be applicable for producing bioactive peptides via protein hydrolysis within recent decades, granting access with specific properties of interest such as inhibitory activity on angiotensin I converting enzyme [13-16], antibacterial activity [17], antioxidant activity [18, 19] and anti-inflammatory activity [20]. In this context, it is anticipated that the biological properties of *C. siamensis* blood proteins and their potent antioxidant and anti-inflammatory activity may be further enhanced by protein hydrolysis.

As a consequence, this study aimed to investigate the antioxidant and anti-inflammatory peptides from *C. siamensis* hemoglobin hydrolysates (CHHs) derived by pepsin digestion. NO scavenging, linoleic peroxidation and ferric reducing power assays were conducted to determine the antioxidant activity of the CHHs, whereas MTT, NO (of macrophage RAW 264.7 cells), IL-6, IL-1β and PGE_2 assays were used to investigate the anti-inflammatory activity. *In vitro* digestion and hemolytic activity were used to determine the application uses. Furthermore, the identity of the antioxidant and anti-inflammatory peptides was elucidated via amino acid sequence determination using ultra-high performance liquid chromatography-LTQ orbitrap XL mass spectrometry.

2. Materials and Methods

2.1. Materials and reagents

RPMI 1640 medium, antibiotic/antimycotic (penicillin/streptomycin/amphotericin B), trypsin–EDTA and fetal bovine serum (FBS) were purchased from Gibco (USA). Pepsin from porcine gastric mucosa, picrylsulfonic acid solution (TNBS), sodium sulfite, L-leucine, ammonium thiocyanate, ferrous chloride, potassium hexacyanoferrate, ferric chloride, Linoleic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonic acid (Trolox), glutathione (GSH), 2,20-azobis-(2-amidinopropane)-dihydrochloride (ABAP), dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), NaCl, HCl, PBS, bile salts solution, pancreatin solution, $CaCl_2$, Triton X-100 were purchased from Sigma-Aldrich (Germany). Sodium nitroprusside was purchased from Merck (Germany). 3-(4,5-diamethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Eugene (USA).

2.2. Crocodile hemoglobin preparation

Crocodile blood was collected from a slaughterhouse (Srirachamoda Co., Ltd.) in Thailand, and the extraction of hemoglobin from red blood cells was performed following the method of Srihongthong et al. [9]. Blood was collected and transferred to 15 mL sterile tubes containing 0.08 g of EDTA. Blood samples were stored at 4 °C overnight to allow blood cells to settle. Red blood cells (bottom layer) were collected in sterile tubes after elimination of plasma (top layer) and white blood cells (middle layer). Isolated red blood cells were washed three times with phosphate buffer saline (PBS), pH 7.0, and centrifuged at 3,000*g* for 5 min at 4 °C. Ice-cold distilled water of five-fold volume was added to the RBC pellet, followed by vigorous mixing and allowing the mixture to settle for 10 min. After centrifugation at 10,000*g* for 20 min at 4 °C, the supernatant was collected for lyophilization and then stored at −70 °C.

2.3. Enzymatic hydrolysis

Enzymatic hydrolysis was performed according to the method of Yu et al. [21]. Shortly, the hemoglobin solution was digested by pepsin with a ratio of enzyme to substrate of 1:100 (w/w) at 37 °C for 2, 4, 6 and 8 H and boiled at 95 °C for 10 min to quench the reaction by inactivating the enzyme. The hydrolysis condition of hemoglobin by pepsin was performed at pH 2.0 (adjusted with 1 M HCl), followed by removal of insoluble components by centrifugation at 7,168*g* for 20 min. The supernatant was collected and adjusted to pH 7.0 by addition of 1 M HCl or 1 M NaOH. Finally, the supernatants of the crocodile hemoglobin hydrolysates (CHHs) were lyophilized and stored at −20 °C.

2.4. Degree of hydrolysis

The degree of hydrolysis was determined following the method of Benjakul et al. [22]. Briefly, 125 µL of CHHs were added to 2.0 mL of 0.21 M sodium phosphate buffer, pH 8.2, followed by addition of 1 mL of 0.01% TNBS solution. The mixture was incubated in a water bath at 50 °C for 30 min in the dark, and 2 mL of 0.1 M sodium sulfite was added to stop the reaction. The mixture was then allowed to cool for 15 min. The absorbance was measured at 420 nm and the α-amino acid content expressed in terms of L-leucine. The percentage of the degree hydrolysis was calculated using the formula:

$$DH = [(L_t - L_0)/(L_{max} - L_0)] \times 100$$

where L_0 determines the amount of α-amino acid expressed in the sample. L_t corresponds to the amount of α-amino acid released at time t. L_{max} determines the maximum amount of α-amino acid after hydrolysis by 5 M HCl at 100 °C for 24 H.

2.5. Nitric oxide (NO) scavenging assay

Nitric oxide (NO) was generated from sodium nitroprusside and measured using Griess reagent. The assay was conducted according to the method of Yen et al. [23] with modifications. For the experiment, 250 µL sodium nitroprusside (10 mM) in PBS were mixed with 15, 31, 62, 125, 250 and 500 µg/mL of CHHs and incubated at 25 °C for 2 H. An aliquot of the incubated solution (100 µL) was added to 100 µL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) and incubated in the dark for 10 min before measuring the absorbance at 540 nm. Butylated hydroxytoluene (BHT) was used as a positive control. All samples were analyzed in quadruplicate.The percentage of inhibition was calculated using the formula:% inhibition = $[(Abs_{cont} - Abs_{test})/Abs_{cont}] \times 100$.

2.6. Linoleic peroxidation assay

This assay was performed following the method of Ledesma et al. [24] with modifications. Briefly, 50 µL of CHHs (0.005, 0.05, 0.5, 5, 50 and 500 µg/mL) were added to 50 µL of linoleic acid (0.05% v/v) solution. 0.07 M ABAP (10 µL) was added and the solution mixed for 10 min. After addition of 150 µL 20% (v/v) acetic acid, the mixture was incubated at 70 °C for 1 H. Twenty microliter of each solution was then added to a 96-well plate already containing 75% ethanol (160 µL), 15% ammonium thiocyanate (10 µL) and 10 mM ferrous chloride (10 µL). The new solution was mixed and incubated at for 3 min. The absorbance was measured at 500 nm and Trolox used as positive control. All samples were analyzed in sextuplicate. The antioxidant activity was analyzed and the percentage of antioxidant inhibition (% AI) of each sample was calculated using the following formula:

% AI (antioxidant inhibition) = $100 \times [Abs_{cont} - Abs_{test})/Abs_{cont}]$

2.7. Ferric reducing power assay

This assay was modified from the method of Girgih et al. [25]. Briefly, 250 µL of CHHs (0.005, 0.05, 0.5, 5, 50 and 500 µg/mL) and positive control (glutathione) were added to 250 µL of 0.2 M phosphate buffer, pH 6.6. 250 µL of 1% (w/v) potassium hexacyanoferrate solution were added, vortexed and the mixture incubated at 50 °C

for 20 min. The reaction was quenched by adding 250 µL of 10% TCA and incubated for 10 min before centrifugation at 800*g* for 10 min. Aliquots of each solution (30 µL) were then transferred to a 96-well plate already containing double distilled water (160 µL) and 0.1% (w/v) ferric chloride (10 µL). The reaction mixture was homogenized and incubated at 25 °C for 10 min, followed by measuring the absorbance at 700 nm. All samples were analyzed in quadruplicate. The antioxidant activity for each sample was expressed as Trolox (mM) equivalents and calculated using the equation of the standard curve of the positive control.

2.8. Cell culture

A murine macrophage cell line (RAW 264.7) was purchased from the American Type Culture Collection (American Type Culture Collection [ATCC], USA) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/mL of streptomycin, 100 U/mL of penicillin, 25 µg/mL amphotericin B and incubated at 37 °C in a 5% CO_2 humidified atmosphere.

2.9. Measurement of nitric oxide

This assay was conducted according to the method of Phosri et al. [11]. CHHs (62, 125, 250 and 500 µg/mL) were mixed with LPS (100 ng/mL), and the resulting solution incubated with RAW 264.7 cells. Another incubation was set up between LPS and RAW 264.7 cells at 37 °C in a 5% CO_2 humidified atmosphere for 24 H. 100 µL of culture medium from each CHH sample were slightly mixed with 100 µL of Griess reagent and incubated at 25 °C for 10 min. The absorbance was measured at 540 nm using a microplate reader (BioRad, Model 680, USA). Nitric oxide (NO) production was calculated as percentage of control. All samples were analyzed in quadruplicate.

2.10. Cell viability

Cell viability determination was performed using the MTT assay according to the method of Phosri et al. [11]. RAW 264.7 cells (1×10^5 cells/mL) were cultured on a 96-well plate overnight. Lipopolysaccharides (LPS) were co-incubated with CHHs in defined concentrations (62, 125, 250 and 500 µg/mL) and 100 ng/mL of LPS at 37 °C in a 5% CO_2 humidified atmosphere. After incubation for 24 H the medium was discarded. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (0.5 mg/mL) was added to RAW 264.7 cells and incubated at 37 °C in a 5% CO_2 humidified atmosphere for 30 min before the medium was discarded. DMSO was added and the reaction mixture incubated at 25 °C for 30 min. The absorbance was measured at 570 nm and

209 the cell viability evaluated by comparing the absorbance with that of the control for each sample. All samples
210 were analyzed in quadruplicate.
211
212 **2.11. Measurement of IL-6, IL-1β and PGE_2**
213 Aliquots of culture medium employed in the NO assay were further used for determination of IL-6, IL-1β and
214 PGE_2 expression using the ELISA kit and following the instructions in the manufacturer's manual (R&D,
215 Minneapolis, MN, USA).
216
217 **2.12. Peptide purification from crocodile hemoglobin hydrolysates**
218 The purification of CHH was performed according to the method of Srihongthong et al. [9] with modifications.
219 A HPLC system connected to a C-18 reverse phase column (10 × 150 mm; Sunfire^{Tm} prep 5 µm) was employed
220 in the purification process. Fractions were separated using a mobile phase system consisting of mobile phase A
221 (0.1% TFA in deionized water) and mobile phase B (60% acetonitrile in 0.1% TFA) with a flow rate of 1.0 mL/
222 min. A gradient of 0-25% (v/v) B over 15 min, then 25-100% (v/v) B over 95 min was applied. The elution
223 peaks were collected by monitoring the absorption at 220 nm. The antioxidant and anti-inflammatory activity of
224 all peaks were assayed, followed by determination of the amino acid sequences for each peak showing
225 significant biological activity.
226
227 **2.13. Amino acid sequence analysis**
228 The active fractions P16 of 2H-CHH and P57 of 6H-CHH were selected and the peptides identified using LTQ
229 orbitrap XL Mass spectrometry employing the following search parameters: non-specified enzymatic cleavage
230 with three possible missed cleavages, +/−0.8 Da mass tolerances for MS and MS/MS, a peptide mass tolerance
231 of +/−5 ppm, methionine oxidation and Gln->pyro-Glu (N-term Q) variable modification, and monoisotopic
232 mass. Data were additionally processed at the Mascot Server (http://www.matrixscience.com/) using MS/MS
233 ion searches against SwissProt (current release).
234
235 **2.14. *In vitro* simulated gastric and intestinal digestion**
236 *In vitro* digestion models were used to simulate the condition of human stomach and intestine for observing
237 remaining activity of CHHs as described by Cheong et al. [26] with modification. The ratio of sample: simulated
238 gastric: simulated intestinal was 1: 1.5: 2. Simulated gastric solution was prepared by dissolving 2 g of NaCl, 7

mL of HCl and 3.2 g pepsin with DI up to 1 L. The pH was adjusted to 1.6 using 1 M HCl and maintained at 37 °C in a temperature-controlled water bath (Julabo, Germany). Each CHHs were mixed and pH was adjusted to 2.5 using 1 M HCl then incubated at 37 °C for 1 H. The simulated intestinal solution was prepared by mixing 160 mL bile salts solution (5 mg/mL in PBS), 100 mL of pancreatin solution (4.8 mg/mL in PBS) and 40 mL of CaCl2 solution (110 mg/mL in PBS). The pH was adjusted to 7 and maintained at 37 °C in a temperature-controlled water bath. After the gastric digestion, samples were immediately adjusted to pH 7 with 1 M NaOH. Digested samples were added to simulated intestinal solution and incubated at 37 °C for 2 H. Samples were adjusted to pH 9 to ensure enzyme inactivation.

2.15. Hemolytic activity

The hemolytic activity was determined according to the method of Pata et al. [27]. After isolation of the erythrocytes by centrifugation at 1,000*g* for 5 min, human red blood cells (hRBCs) were washed three times with PBS, pH 7.4 and then adjusted to 2% (v/v). Ten microliter CHHs were added separately to the 100 μL reaction solution, incubated for 1 H at 37 °C and centrifuged at 1,000*g* for 5 min. 100 μL of supernatant was then transferred into 96-well plate. The absorbance was measured at 415 nm. 1% (v/v) Triton X-100 and DI used as positive and negative control respectively. All samples were analyzed in quadruplicate.

2.16. Statistical analysis

Statistical analysis was performed using ANOVA and followed by Dunnett's test (Prism 5.0, GraphPad Inc., San Diego, CA, USA). Data are presented as mean ± SEM. A value of $P < 0.05$ was accepted to be significant (*$P <$ 0.05, ** $P < 0.01$, ***$P < 0.001$).

3. Results and Discussion

3.1. Preparation of crocodile hemoglobin hydrolysates (CHHs) and degree of hydrolysis (DH)

In the present study, crocodile (*C. siamensis*) hemoglobin was extracted from red blood cells. To obtain active fragments, crocodile hemoglobin was hydrolyzed by pepsin digestion at different reaction times. The extent of enzymatic protein degradation was evaluated by degree of hydrolysis (DH) which was 20.4%, 23.1%, 25.0% and 35.9% for 2 H, 4 H, 6 H and 8 H of incubation, respectively (Fig. 1). Results indicated that cleavage of peptide bonds was higher in longer enzymatic hydrolysis.

3.2. Antioxidant activity, purification and amino acid sequence identification of CHHs

NO is a very unstable radical which produces highly reactive molecules [28]. Intact crocodile Hb and all investigated Hb hydrolysates inhibited NO radicals (Fig. 2a). Notably, the hydrolysates had superior antioxidant activity compared to intact Hb at all concentrations (15-500 µg/mL). The NO-scavenging activity of the intact protein (500 µg/mL) was 75.7%, while the inhibitory ability of the hydrolysates, including 2H-CHH, 4H-CHH, 6H-CHH and 8H-CHH, showed inhibition percentages of 77.4%, 78.2%, 88.3% and 83.1%, respectively. Moreover, BHT employed as positive standard displayed 77.8% NO radical inhibition. These results indicate that CHHs obtained by pepsin digestion possess pronounced antioxidant activity against nitric oxide radicals. From the report of Kabbua et al. [29] found that recombinant α-globin from cHb can act as a heme-nitric oxide and/or oxygen binding (H-NOX) hemoprotein. Furthermore, the value of NO inhibition activity of CHH was higher than yellowfin tuna hydrolysate [30].

Lipid peroxidation is considered to be a free radical process involving a source of secondary free radical, which can act as secondary messenger or directly react with other biomolecules, enhancing biochemical lesions. The results of linoleic peroxidation assay reveal that 2H-CHH, 4H-CHH, 6H-CHH and 8H-CHH (at concentrations of 0.005-500 µg/mL) effected significant inhibition of linoleic peroxidation in a concentration dependent manner when compared with Trolox. The inhibitory ability of all hydrolysates, including 2H-CHH, 4H-CHH, 6H-CHH and 8H-CHH were 94.2%, 79.0%, 97.0% and 82.2%, respectively. Moreover, Trolox (500 µg/mL) showed 94.4% of linoleic peroxidation inhibition and intact Hb showed 89.1% inhibition (Fig. 2b). Furthermore, the linoleic peroxidation inhibition of protein hydrolysates obtained from smooth hound has been investigated. As shown by the results, the value of this activity was lower than that obtained in this study [31].

2H-CHH, 4H-CHh, 6H-CHH and 8H-CHH at the highest concentration (500 µg/mL) displayed significant ferric ion reducing power. 4H-CHH displayed the highest reduction activity equivalent to Trolox of about 2.1 mM, while intact Hb, 2H-CHH, 6H-CHH and 8H-CHH displayed reduction equivalents of 0.8, 1.2, 1.4 and 0.6 mM Trolox (Fig. 2c). Moreover, 500 µg/mL glutathione (positive control) affected significant ferric ion reduction equivalent to Trolox at 18.60 mM (data not shown). It has been reported that hydrolysates with high reducing power show a great ability to donate electrons to form stable compounds and thereby interrupt the free radical chain reactions and showing antioxidant activity [32].

Due to displaying the highest antioxidant activity in prior experiments, 6H-CHH was subjected to purification by reverse phase C-18 column semi-preparative HPLC to identify the contained active peptide components. As shown in Fig. 3a, 61 individual fractions were eluted. All elution peaks were normalized to a concentration of 78

299 μg/mL and screened with respect to antioxidant activity by nitric oxide scavenging assay. After the activity
300 screening, several active fragments including P2, P39, P40, P43, P45, P46 and P57 were associated with nitric
301 oxide radical inhibition rates of 19.5%, 21.2%, 17.3%, 16.1%, 18.7%, 18.1% and 32.0%, respectively. The
302 highest activity in inhibiting nitric oxide radicals was found for P57 (32.0%), while the positive control (BHT)
303 showed 68.1% nitric oxide radical inhibition (Fig. 3b).
304 The primary structures of the purified antioxidant peptides were elucidated using LTQ orbitrap XL mass
305 spectrometry (Fig. 4). Amino acid sequence of P57 was KIYFPHF (KF7) with molecular mass of 476.26 Da. As
306 shown in Table 1, antioxidant peptide KF7 showed 42% hydrophobicity and a +1 net charge. Alignment of the
307 amino acid sequences of the peptide fragments with cHb indicated the antioxidant peptides originated from the
308 α-subunit, this observation is in excellent agreement with results of Srihongthong et al. [9], who reported that
309 the α-subunit of cHb exhibited higher antioxidant activity than the β-subunit. Peptide fractions from acid-
310 digested cHb with a molecular mass of 180 to 3,000 Da are reported to exhibit antioxidant activity [33].
311 Notably, mass spectrometric analysis further revealed an apparent correlation of peptide length with biological
312 activity as antioxidant peptides were found to be smaller and shorter molecules. In a previous study, Peña-
313 Ramos et al. [34] reported that the presence of particular amino acids including His, Tyr, Met, Lys, Trp and Pro
314 correlates with increased antioxidant potency of most food-derived peptides. Likewise, the presence of Leu, Ile,
315 His, Met, Tyr, Lys and Trp is assumed to contribute to the reducing power of protein hydrolysates [35]. In
316 addition, peptides containing His residues have been documented to exhibit protective effects against lipid
317 peroxidation. An imidazole ring of His has been implicated in the donation of hydrogen and trapping of lipid
318 radicals [36]. Trp, Tyr and Met exhibited the highest antioxidant properties, followed by Phe, Cys and His [37].
319 Nitric oxide, however, is also scavenged by CHH, presumably due to the presence of reactive thiol groups [38].
320

321 **3.3. Anti-inflammatory activity, purification and amino acid sequences identification of CHHs**
322 The anti-inflammatory property of intact Hb and CHHs was evaluated on the basis of NO production and cell
323 viability against macrophage RAW 264.7 cells (Fig. 5). After induction of inflammation in RAW 264.7 cells by
324 LPS for 24 H, the percentage of nitric oxide production was defined as 100% when treated with LPS. Fig. 5a,
325 2H-CHH, 4H-CHH, 6H-CHH and 8H-CHH (at concentrations of 62-500 μg/mL) show nitric oxide production
326 decrease in a concentration dependent manner. Intact Hb, 2H-CHH, 4H-CHH, 6H-CHH and 8H-CHH at a
327 concentration of 500 μg/mL show nitric oxide production at 56.5%, 52.6%, 68.6%, 62.0% and 60.9%,
328 respectively. In order to evaluate potential cytotoxic effects of CHHs, the viability of RAW 264.7 cells treated

with defined concentrations of CHHs was determined (Fig. 5b). The results showed that intact Hb, 2H-CHH, 4H-CHH, 6H-CHH and 8H-CHH at concentrations of 62-500 µg/mL had no observable effect on cell viability while the viability percentage of cells treated with LPS only was defined as 100%. This result indicates that CHHs show high efficacy to reduce nitric oxide production and are essentially non-toxic to macrophage RAW 264.7 cells, these results correlate with that from the report of Phosri et al. [11] who reported that cHb provides anti-inflammatory activity via the suppression of nitric oxide synthase (NOS), which inhibits the NO production. Jangpromma et al. [39] reported that cHb significantly decreased the production of NO in LPS-stimulated RAW 264.7 cells and decreased inducible nitric oxide synthase (iNOS). In addition, results showed that CHHs have higher anti-inflammatory activity than gastropod (*Harpa ventricosa*) hydrolysate [40].

Since 2H-CHH was found to possess the highest anti-inflammatory activity, its peptide constituents were purified by reverse phase C-18 column semi-preparative HPLC. The resulting chromatogram depicted in Fig. 6a consists of 59 fraction peaks which were diluted to a concentration of 31 µg/mL prior to anti-inflammatory activity determination. The assay revealed 6 peaks (P16, P17, P28, P29, P30 and P38) which show nitric oxide production at 83.2%, 86.0%, 87.9%, 88.8%, 86.3% and 83.6% (Fig. 6b) with no effect on the viability of RAW 264.7 cells (Fig. 6c). Moreover, these fractions were also selected to determine the influence on the expression levels of IL 6, IL-1β and PGE_2 (Table 2). The results indicate that P16, P17, P28, P29, P30 and P38 are able to significantly decrease IL-6 and PGE_2 production, whereas P16 and P17 further inhibited IL-1β production. Notably, P16 showed the highest activity to decrease IL-6 levels (approximately 2.0 ng/mL) compared to LPS and effectively decreased the IL-1β level to about 0.3 ng/mL. P16 showed the highest activity to reduce PGE_2 levels (approximately 1.9 ng/mL) when compared to LPS. Furthermore, P16 was found to reduce the production of pro-inflammatory cytokines and inflammatory mediators IL-6, IL-1β and PGE_2, which are associated with the inflammation-related in neuropathological diseases, for example Alzheimer's, multiple sclerosis and cerebral ischemia [41, 42]. IL-6 that acts as a multifunctional cytokine was up-regulated by lipopolysaccharide (LPS). In inflammation, trauma and autoimmune diseases were found high expression levels of IL-1β and IL-6 [43]. Furthermore, IL-1β induced IL-6 production is mediated predominantly through the p38 MAPK/NF-κB pathway [44]. cHb was found to exhibit anti-inflammatory effects in the cotton pellet model and RAW 264.7 cells by reducing expression levels of IL-1β, TNF-α, COX-2 and IL-6 mRNA [39, 45]. The collected results in this work indicate that the anti-inflammatory activity of sample might be related to an interaction with the MAPK/NF-κB pathway by their ability to decrease pro-inflammatory cytokine and inflammatory mediator production.

The primary structures of the purified anti-inflammatory peptides were elucidated using LTQ orbitrap XL mass spectrometry (Fig. 4). Amino acid sequences of P16 were determined as SAFNPHEKQ (SQ9) and IIHNEKVQAHGKKVL (IL15) corresponding to molecular mass of 529.26 Da and 857.51 Da, respectively. As shown in Table 1, anti-inflammatory peptides SQ9 and IL15 had hydrophobicity values of about 22% and 40%, together with net charges of 0 and +2, respectively. The sequence of the anti-inflammatory peptide showed strong correlation with the β-chain of cHb. Likewise, the anti-inflammatory activity of SQ9 and IL15 was traced back to the presence of specific amino acids. According to reports, anti-inflammatory peptides can be found in a wide molecular weight range [20, 46]. Hydrophobic amino acid side chains (e.g. Leu, Phe, Val, Ile, and Trp) as well as positively charged amino acids (Lys, Arg and His) were documented to have a major influence on the anti-inflammatory activity of peptides [47-52].

3.4. *In vitro* simulated gastric and intestinal digestion of CHHs and its activity

Simulated gastric and intestinal digestion (*in vitro* digestion model) was used as the conditions of human stomach and intestine. The degree of hydrolysis of 2H-CHH and 6H-CHH after intestinal digestion was increased about 3.7% and 8.8%, respectively. From the results, it is inferred that CHH tended to be more hydrolyzed by intestinal digestion. Gastrointestinal tract is known to be a major oxidation site where various free radicals are generated in the digestion process [53]. As shown in Fig. 7a, 6H-CHH after intestinal digestion was inhibited nitric oxide (42.5%, the concentration of 125 µg/mL) and exhibited higher reducing power (2.8 mM Trolox equivalent, the concentration of 500 µg/mL) than 6H-CHH before intestinal digestion (Fig. 7b). With excellent agreement with Moure et al. [36] who reported that the reducing power increased with higher degree of hydrolysis. The anti-inflammatory activity of 2H-CHH after intestinal digestion showed NO production (19.8%, the concentration of 125 µg/mL) without toxicity against macrophage RAW 264.7 cells (Figs. 8a and 8b). During the course of the inflammation high amounts of free radicals are produced within the inflamed tissue, in particular NO, which acts as a major pro-inflammatory mediator in the human body and is generated from macrophage in response to the inflammation [8]. In this context, NO is generally regarded as the key mediator correlating inflammation and oxidative stress. The capability of NO production has decreased resulting in the reduction of detrimental effects of both inflammation and oxidative damage. The hemolytic activity was performed to determine the toxicity against human red blood cells and the results showed that both 2H-CHH and 6H-CHH at all concentrations were not toxic against human red blood cells (Figs. 9a and 9b). So far, only a few bioactive peptides including peptides in this study displaying activities *in vitro* that have been

proven effective *in vivo*. Results indicated that both 2H-CHH and 6H-CHH with intestinal digestion have potential to be used as an alternative source of antioxidant and anti-inflammatory agents with safety.

4. Conclusions

A number of novel antioxidant and anti-inflammatory peptides were derived from pepsin hydrolysis of cHb. Experimental evidence collected in this study indicates the antioxidant fragment is a comparatively small peptide, consisting of only 7 amino acid residues with slightly positive charge and low hydrophobicity. Similarly, the peptides with anti-inflammatory activity were found to comprise 9 and 15 amino acid residues, respectively, and are also characterized by positive charge and low hydrophobicity. This is the first report to identify antioxidant and anti-inflammatory peptides from pepsin digested cHb. Due to their remarkable biological activity, ease of production and absence of cytotoxicity, these naturally-derived peptides are believed to bear a great potential for a future application as health promoting supplements and therapeutic agents against inflammation and oxidative stress-related conditions.

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TABLE 1 ***Structural characteristics of the anti-inflammatory peptides (P16) from 2H-CHH and antioxidant peptide (P57) from 6H-CHH***

Properties	*Peptide sequences*	*Hydrolysates*	*% hydrophobicity*	*Net charge*	*Sequence alignment*
Anti-inflammatory	SAFNPHEKQ (SQ9)	2H-CHH	22%	0	cHb β-subunit (position 2-10)
Anti-inflammatory	IIHNEKVQAHGKKVL (IL15)	2H-CHH	40%	+2	cHb β-subunit (position 55-69)
Antioxidant	KIYFPHF (KF7)	6H-CHH	42%	+1	cHb α-subunit (position 41-47)

TABLE 2 ***IL-6, IL-1β and PGE_2 production in LPS-stimulated RAW 264.7 cells incubated with purified fractions of 2H-CHH at a concentration of 31 μg/mL***

Fraction peaks (P)	*The production concentration (ng/mL)*		
	IL-6	*IL-1β*	*PGE_2*
LPS	10.647 ± 0.213	0.360 ± 0.002	6.486 ± 0.163
P16	2.019*** ± 0.000	0.315*** ± 0.002	1.871*** ± 0.068
P17	7.287*** ± 0.447	0.338 ± 0.001	1.877*** ± 0.042
P28	7.967*** ± 0.110	0.420*** ± 0.002	3.046*** ± 0.220
P29	9.340* ± 0.155	0.412*** ± 0.001	3.578*** ± 0.035
P30	9.407* ± 0.116	0.430*** ± 0.002	2.880*** ± 0.205
P38	4.707*** ± 0.173	0.538*** ± 0.011	3.181*** ± 0.377

*denotes $P < 0.05$ and *** denotes $P < 0.001$. Data expressed as a mean ± SEM of 3 independent experiments. Significance was measured using ANOVA followed by Dunnett's test.

Figure captions

FIG. 1 *Degree of hydrolysis (% DH) of CHH after pepsin digestion for 2, 4, 6 and 8 H. Hemoglobin hydrolyzed enzymatically by pepsin displayed a direct correlation between the rate of hydrolysis (DH) and the time of incubation (H).*

FIG. 2 *(a) Nitric oxide scavenging activity of CHHs at concentrations of 15-500 µg/mL. Each bar displays the mean ± SEM of four demonstrations. (** $P < 0.01$ and *** $P < 0.001$) probability levels compared with BHT. (b) Linoleic peroxidation activity of CHHs at concentration of 0.005-500 µg/mL. Each bar displays the mean ± SEM of six demonstrations. (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$) probability levels compared with Trolox and (c) reducing power of CHHs at concentration of 0.005-500 µg/mL expressed in Trolox equivalents. Each bar displays the mean ± SEM of four demonstrations. (***$P < 0.001$) probability levels compared with glutathione.*

FIG. 3 *Reverse phase C-18 column semi-preparative HPLC profile of (a) 6H-CHH. 0.1% Trifluoroacetic acid (TFA) in deionized water and 60% acetonitrile in 0.1% Trifluoroacetic acid (TFA) were chosen as mobile phase A and B, respectively. CHH was filtered through 25 mm PES filters and 700 µg (4 mL) were injected at 1.0 mL/ min of flow rate. The linear gradient was 0-25% (v/v) mobile phase B over 15 min, then 25-100% (v/v) mobile phase B over 95 min. (b) The antioxidant activity of purified peaks from 6H-CHH (78 µg/mL) determined by the nitric oxide scavenging assay. Each bar displays the mean ± SEM of four demonstrations (*** $P < 0.001$).*

FIG. 4 *Mass spectrogram of anti-inflammatory peptides and antioxidant peptide from P16 (2H-CHH) and P57 (6H-CHH), respectively, determined by LTQ orbitrap XL mass spectrometry. The following sequence interpretation was concluded: anti-inflammatory peptides were identified as (a) Ser-Ala-Phe-Asn-Pro-His-Glu-Lys-Gln (SAFNPHEKQ) and (b) Ile-Ile-His-Asn-Glu-Lys-Val-Gln-Ala-His-Gly-Lys-Lys-Val-Leu (IIHNEKVQAHGKKVL) and (c) the antioxidant peptide was identified as Lys-Ile-Tyr-Phe-Pro-His-Phe (KIYFPHF).*

FIG. 5 *The effect of CHHs on (a) NO production in LPS-activated macrophage RAW 264.7 cells and (b) the cytotoxicity (cell viability) of CHHs on macrophage RAW 264.7 cells determined by the MTT assay. CHHs (62-500 µg/mL) were then incubated with macrophage RAW 264.7 cells and another overnight incubation was set between LPS and macrophage RAW 264.7 cells. The media were further used to measure the nitrite level (NO assay). Each bar displays the mean ± SEM of four demonstrations (* P < 0.05, ** P < 0.01 and *** P < 0.001).*

FIG. 6 *Reverse phase C-18 column semi-preparative HPLC profile of (a) 2H-CHH. 0.1% Trifluoroacetic acid (TFA) in deionized water and 60% acetonitrile in 0.1% Trifluoroacetic acid (TFA) were chosen as mobile phase A and B, respectively. CHH was filtered through 25 mm PES filters and 700 µg (4 mL) were injected at 1.0 mL/ min of flow rate. The linear gradient was 0-25% (v/v) mobile phase B over 15 min, then 25-100% (v/v) mobile phase B over 95 min. (b) The anti-inflammatory property of purified peaks from 2H-CHH (31 µg/mL) against NO production determined in LPS-activated macrophage RAW 264.7 cells. (c) The cytotoxicity (cell viability) of purified peaks from 2H-CHH (31 µg/mL) on macrophage RAW 264.7 cells determined by the MTT assay. Each bar displays the mean ± SEM of four demonstrations (* P < 0.05, ** P < 0.01 and *** P < 0.001).*

FIG. 7 *(a) Nitric oxide scavenging activity of 6H-CHHs after intestinal digestion at concentrations of 15-125 µg/ mL. Each bar displays the mean ± SEM of four demonstrations. (* P < 0.05 and *** P < 0.001) probability levels compared with BHT. (b) Reducing power at concentration of 0.005-500 µg/mL expressed in Trolox equivalents. Each bar displays the mean ± SEM of four demonstrations. (***P < 0.001) probability levels compared with glutathione.*

FIG. 8 *The effect of 2H-CHHs after intestinal digestion on (a) NO production in LPS-activated macrophage RAW 264.7 cells and (b) the cytotoxicity (cell viability) on macrophage RAW 264.7 cells determined by the MTT assay. 2H-CHHs (31-125 µg/mL) were then incubated with macrophage RAW 264.7 cells and another overnight incubation was set between LPS and macrophage RAW 264.7 cells. The media were further used to measure the nitrite level (NO assay). Each bar displays the mean ± SEM of four demonstrations (* P < 0.05, ** P < 0.01 and *** P < 0.001).*

588 **FIG. 9** *The hemolytic activity of (a) 2H-CHH after intestinal digestion and (b) 6H-CHH after intestinal*
589 *digestion against human red blood cells. Each bar displays the mean ± SEM of four demonstrations*
590 *(*** $P < 0.001$).*

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