รายงานวิจัยฉบับสมบูรณ์

เรื่อง

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โดยเพปไทด์จากเกสรผึ้งพันธุ์ *Apis mellifera*

นำเสนอโดย

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ABSTRACT (THAI)

งานวิจัยนี้เตรียมโปรตีนไฮโดรไลเสตจากเกสรผึ้งพันธุ์ ที่ได้จากปฏิกิริยาย่อยสลายด้วยเอนไซม์ 3 ชนิด ได้แก่ แอลคาเลส ฟลาโวไซม์ และนิวเทรส พบว่าเมื่อใช้นิวเทรสในอัตราส่วนของเอนไซม์ต่อสับสเตรต 1:1 (NH1) โดยปริมาตร จะให้แสดงค่าการยับยั้งการสร้างอนุมูลอิสระด้วยวิธีไนตริกออกไซด์ได้ดีที่สุด คัดแยกเพปไทด์ตามขนาดโมเลกุลด้วยเทคนิคอัลตราฟิลเตรชัน 10, 5, 3 และ 0.65 กิโลดาลตัน ตามลำดับ พบว่าเพปไทด์ที่มีขนาดโมเลกุลต่ำกว่า 0.65 กิโลดาลตัน (MW1) มีฤทธิ์ในการขจัดอนุมูลอิสระไนตริกออกไซด์ได้ดีที่สุด จากนั้นได้ทำการตรวจสอบความเป็นพิษของ MW1 ด้วยวิธี MTT และตรวจสอบผลการยับยั้งการสร้างไนตริกออกไซด์ในเซลล์แมคโครฟาจ RAW 264.7ที่ถูกกระตุ้นด้วยไลโปโพลิแซคคาไรด์ พบว่า MW1 ไม่มีความเป็นพิษต่อเซลล์ และมีฤทธิ์ในการยับยั้งการสร้างไนตริกออกไซด์ ผลการแสดงออกของยีนที่เกี่ยวของกับการอักเสบ พบว่า MW1 มีฤทธิ์ในการยับยั้งการแสดงออกของยีน inducible nitric oxide synthase (iNOS) Cyclooxygenase-2 (COX-2) Interleukin-6 (IL-6) และ Tumor necrosis factor alpha (TNF-α) จากนั้นนำ MW1 ไปทำบริสุทธิ์ด้วยเทคนิคโครมาโตกราฟีของเหลวสมรรถนะสูง สามารถแยกเพปไทด์ได้ทั้งหมด 6 พีค (H1–6) โดยที่ H2, H3 และ H4 แสดงค่าการยับยั้งการสร้างอนุมูลอิสระด้วยวิธีไนตริกออกไซด์ได้ดีที่สุด และนำเพปไทด์ที่ได้ไปพิสูจน์เอกลักษณ์ด้วยเทคนิคแมสสเปกโตรเมตรีพบเพปไทด์ทั้งหมด 7 สาย จากผลการศึกษาดังกล่าวแสดงให้เห็นว่าฤทธิ์ต้านการอักเสบของเพปไทด์จากเกสรผึ้งพันธุ์ สามารถนำไปประยุกต์ใช้ในอุตสาหกรรมทางการแพทย์ เภสัชกรรมและผลิตภัณฑ์เครื่องสำอางต่อไป

คำสำคัญ: ฤทธิ์ต้านการอักเสบ; เกสรผึ้ง; *ผึ้งพันธุ์*; โปรตีนไฮโดรไลเสต; เซลล์แมคโครฟาจ RAW 264.7

ABSTRACT (ENGLISH)

Bee pollen protein was hydrolyzed using the commercial Alcalase, Flavourzyme and Neutrase enzymes. The Neutrase hydrolysate formed from a 1:1 (v/v) enzyme/substrate ratio (NH1) showed the highest nitric oxide (NO) radical scavenging activity. The NH1 was further separated into five fractions based on molecular weight (MW1–5) and MW1, the smallest weight fraction (< 0.65 kDa), possessed the highest NO inhibitory activity. The effects of MW1 on the production of NO were assessed by incubating with lipopolysaccharide-stimulated RAW264.7 macrophage cells. NO levels from the culture supernatants were determined by the Griess reaction. The results showed that NH1 inhibiting the lipopolysaccharide-induced NO production and upregulation of cyclooxygenase-2, inducible nitric oxide synthase, tumor interleukin-6 and necrosis factor transcript expression in RAW264.7 macrophage cells. Thus, the MW1 fraction was fractionated using reversed-phase high-performance liquid chromatography into six principal fractions (H1–6), where H2, H3 and H4 showed strong NO inhibitory activity. Seven peptide sequences were obtained by quadrupole time-of-flight mass spectrometry, three of which displayed potent anti-inflammatory activity and may be useful ingredients in functional food and pharmaceutical drugs.

Keywords: anti-inflammatory, bee pollen, *Apis mellifera*, protein hydrolysate, Macrophage RAW 264.7

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FULL TEXT

Introduction

The therapeutic applications of bioactive peptides from natural sources have received increasing attention for their or their derivatives potential role as safer alternatives, either as functional foods or nutraceuticals (Chakrabarti, Jahandideh, & Wu, 2014). More specifically, researchers have focused on the generation, separation, purification and identification of novel peptides from various protein sources, as it has been shown that short peptides (3–20 amino acids) from protein hydrolysis have a higher nutrition value and are utilized more efficiently than an equivalent mixture of free amino acids (Grimble, Keohane, Higgins, Kaminski, & Silk, 1986; Kitts & Weiler, 2003).

The production of protein hydrolysates involves the use of digestive proteolytic enzymes from food grade enzymes obtained from microorganisms, including Alcalase, Flavourzyme and Neutrase, and are regarded as safe for human nutrition (Schaafsma, 2009). Many of these protein peptides have demonstrated anti-inflammatory properties under experimental conditions (Hartmann & Meisel, 2007) and are being used as an alternative treatment for various inflammatory diseases and autoimmune conditions (Gonzalez-Rey, Anderson, & Delgado, 2007).

The ability of these peptides to inhibit nitric oxide (NO) and the anti-inflammatory response has been the subject of numerous experiments based on *in vitro* studies performed in RAW264.7 macrophage cells stimulated with lipopolysaccharide (LPS). This cell culture system offers a fast, economically feasible and reproducible in vitro assay for the analysis and potential validation of the effects of peptides on a wide range of inflammatory markers (Rutledge et al., 2012). Peptides derived from rich sources of protein, such as bovine milk, egg, fish and soybean, have been reported for their potential anti-inflammatory effects (Majumder, Mine, & Wu, 2015). However, alternative protein sources are required to overcome some of the limitations and

challenges for anti-inflammatory peptides before they are sold for commercial use for functional foods or nutraceutical ingredients.

Bee pollen is comprised of floral pollen collected by honeybees, such as *Apis mellifera*, to produce bee bread (bee pollen with added honey), a product rich in protein (Eraslan, G., Kanbur, Silici, & Liman, 2009; Komosinska-Vassev, Olczyk, Kazmierczak, Mencner, & Olczyk, 2015). Pollen and bee bread is used in the human diet as a food supplement (Kieliszek et al., 2018). In recent decades, a growing number of authors have reported that bee pollen exhibits antifungal, antimicrobial, antiviral, anti-inflammatory and immunostimulant properties (Kroyer & Hegedus, 2001). Although the flavonoids and phenolic acids inside bee pollen are reported to be mainly responsible for these properties (Pascoal, Rodrigues, Teixeira, Fe´as, & Estevinho, 2014), the protein components of bee pollen have been reported to contribute to the balancing and continuation of free radical scavenging effects, but knowledge of their protein hydrolysates remains insufficient (Campos et al., 2008).

The present study aimed to investigate the anti-inflammatory properties of bee pollen peptides. The results revealed that these peptides exhibit high levels of NO radical scavenging activity and suppress LPS-induced inflammatory responses in RAW264.7 macrophage cells.

Material and methods

Materials

Bee pollen from *Apis mellifera* hive was obtained from the Phatthanakit Bee Farm Limited Partnership (Chiangmai, Thailand). The results of the compositional analysis are reported as the percentage of weight by weight (w/w). Purchases were made as follows: ethanol and phosphoric acid from Merck (New Jersey, USA); Alcalase and Flavourzyme d from Brentag (Mülheim, Germany); Neutrase from Novozymes (Bagsvaerd, Denmark); 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), bovine serum

albumin (BSA), budesonide (BUD), curcumin, dimethyl sulfoxide (DMSO), Dulbecco's modified Eagle medium (DMEM), ethidium bromide, fetal bovine serum (FBS), LPS from *Escherichia coli*, N-(1-naphthyl)ethylenediamine (NED), penicillin, potassium persulfate, sodium nitroprusside (SNP), sulfanilamide, streptomycin and trifluoric acetic acid (TFA) from Sigma-Aldrich (Missouri, USA); acetonitrile (ACN) and methanol from Thermo Fisher Scientific (Massachusetts, USA); β-actin, cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS) and tumor necrosis factor (TNF-α) primers from LGC Biosearch (California, USA).

Determination of amino acid content

Amino acid content of the bee pollen was determined based on the standard AOAC 994.12 acid hydrolysis method. In brief, 1 g of bee pollen was dissolved in 5 mL of 6 N HCl in a test tube, and placed in a heating block at 110 °C for 24 h to liberate the amino acids. Then, the internal standard (10 mL of 2.5 mM L-α-amino-n-butyric acid in 0.1 M hydrochloric acid [HCl]) was added, diluted with deionized water to 250 mL and placed in a heating block at 55 °C for 10 min. Amino acid identity and quantity was determined by reverse-phase high performance liquid chromatography (RP-HPLC) analysis on a Hypersil GOLD column C_{18} (4.6 mm × 150 mm, 3 μM), eluted with sodium acetate buffer pH 4.90 and 60% (v/v) ACN at 0.3 mL/min. These amino acid contents were obtained from ALS Laboratory Group (Thailand) Co., Ltd., Suan Luang, Bangkok, Thailand.

Preparation of bee pollen protein hydrolysates

To prepare protein hydrolysates, bee pollen was hydrolyzed as reported (Saisavoey, Sangtanoo, Reamtong, & Karnchanatat, 2016) with some modifications. Fifty grams of bee pollen (substrate) was pre-treated with 1,000 mL of 20 mM phosphate buffer solution (pH 7.2) at 4 °C for 24 h, and then hydrolyzed by Alcalase, Flavourzyme or Neutrase

(separately) at three different enzyme/substrate (E/S) ratios (1:5, 1:2 and 1:1) at 50 °C for 4 h at pH 7 (except pH 8 for Alcalase). After 4 h the enzyme activity was stopped by heating in a boiling water bath at 80 °C for 20 min, cooling and clarified by centrifugation at 5,000 ×*g* for 15 min. The supernatant was collected and stored at -20 °C until used.

Fractionation of bee pollen protein hydrolysates by ultrafiltration

The selected hydrolysate (Neutrase) was fractionated using 0.65, 3, 5 and 10 kDa molecular weight cut-off Pellicon XL filter membranes (Merck). The five protein fractions obtained were designated by their assumed size accordingly as <0.65 kDa, 0.65–3 kDa, 3–5 kDa, 5–10 kDa and >10 kDa peptide fractions, respectively. All fractions were stored at -20 °C prior to further analysis.

Purification of anti-inflammatory peptides by RP-HPLC

The peptide fraction from the ultrafiltration that exhibited the highest NO scavenging activity (NH1) was then filtered through 0.45 μm filters (Whatman, GE, Buckinghamshire, UK) and separated using a RP-HPLC system (Spectra System, Thermo Fisher Scientific) equipped with a reverse phase C18 column (250 × 4.6 mm, Luna 5U, Phenomenex, California, USA). Peptides were eluted using a linear gradient, where A was 0.1% (v/v) TFA and B was 70% (v/v) ACN in 0.05% (v/v) TFA, at a flow rate of 0.7 mL/min. The eluate was monitored by absorbance at 280 nm. Chromatographic analyses were completed with ChromQuest Software (Thermo Fisher Scientific). Six fractions (H1–6) were isolated, collected and lyophilized.

Identification of anti-inflammatory peptides

The accurate molecular mass and amino acid sequence of each purified peptide was determined using a quadruple time-of-flight mass spectrometry (Q-TOF-MS) coupled with an electrospray ionization source (ESI) mass spectrometer (Model Amazon SL, Bruker, Germany). Ionization was performed in the positive mode. The separation was performed at a flow rate of 100 m/min under a linear gradient of 5–80% B over 50 min, where A was 0.1% (v/v) formic acid in water and B was 100% ACN. Mass spectral data from 300–1,500 m/z were collected in positive ionization mode and Hystar software was used to interface the HPLC and MS systems. All data obtained from LC/MS/MS were analyzed using *de novo* sequencing.

Determination of NO scavenging assay

The peptide sample (25 µL) and 25 µL of 10 mM SNP were mixed and incubated for 2.5 h, then 100 µL of 0.33% (w/v) sulfanilamide (in 5% (v/v) phosphoric acid) was added and incubated for 5 min. Following this, 100 µL of 0.1% (w/v) NED was added and incubated for a further 30 min. The absorbance at 540 nm (A_{540}) was measured using a microplate reader (Multiskan GO, Thermo Fisher Scientific). Curcumin was used as positive control. A control containing SNP without the sample was also prepared.

Calculation of the IC_{50}

The percentage radical scavenging was calculated as follows from Eq. (1);

$$\frac{[(Abs\ \text{control} - Abs\ \text{blank}) - (Abs\ \text{sample} - Abs\ \text{background})]}{(Abs\ \text{control} - Abs\ \text{blank})} \times 100, \qquad (1)$$

where *Abs* control is the A_{540} of the control (no sample), *Abs* sample is the A_{540} of the tested bee pollen hydrolysate, *Abs* background is the A_{540} of the bee pollen hydrolysate without the reagents, and *Abs* blank is the A_{540} of deionized water. The IC_{50} (the

concentration of bee pollen hydrolysate that inhibits 50% of the NO radical scavenging) was calculated using the GraphPad Prism vs. 6.01 for windows software (GraphPad Software Inc., California, USA).

Measurement of protein content

The concentration of the protein hydrolysate was determined as reported (Bradford, 1976), using BSA as the standard. The respective sample (20 μL) was mixed with 200 μL Bradford working buffer, incubated for 2 min and then the absorbance was determined at 595 nm (A_{595}) using a microplate reader.

RAW 264.7 macrophage cell culture and cytotoxicity assay

The RAW264.7 macrophage cells were grown in complete medium (CM; DMEM supplemented with 10% (v/v) FBS, 100 mg/mL streptomycin and 100 units/mL penicillin) at 37 °C in a 5% CO_2 water-jacketed incubator (Model 3111, Forma Series II, Thermo Fisher Scientific).

RAW264.7 cells (2 x 10^5 cells/mL) in 90 μL CM were plated in a 96-well plate with 10 μL of the respective hydrolysate at the selected concentration dissolved in sterile distilled water. The cells were then incubated for 24 h at 37 °C in a 5% (v/v) CO_2 incubator before 10 μL of MTT stock solution (2 mg/mL) was added to each well to a total reaction volume of 110 μL and incubated for 4 h. The supernatants were removed and replaced with 150 μL of DMSO for dissolution of the formazan crystals prior to measuring the A_{540} by microplate reader.

Determination of NO production in RAW264.7 macrophage cells

After pre-incubation of RAW264.7 macrophage cells (2 x 10^5 cells/mL in CM) with 2.5 μg/mL LPS and aliquots of the hydrolysates in sterile distilled water, the production of

NO in culture medium was measured. The CM was mixed at 1:1 (v/v) with Griess reagent (1% (w/v) sulfanilamide and 0.1% (w/v) NED in 5% (v/v) phosphoric acid) and incubated at room temperature for 10 min prior to measuring the A_{540} by microplate reader. Culture medium without LPS was used as a blank in every experiment. Budesonide (BUD), an anti-inflammatory drug, was used as positive control.

Total RNA isolation from RAW 264.7 macrophage cells

RAW 264.7 macrophage cells (2×10^5 cells/mL in CM) were treated with solvent (negative control), the desired concentrations (IC_{50} value) of peptide fraction or BUD (positive control), and then seeded in a cell culture flask and allowed to adhere overnight at 37 °C and 5% (v/v) CO_2 for 24 h prior to the addition of 2.5 µg/mL LPS, culturing for 12 h and then harvesting. Cells were washed twice with sterile water and the debris was pelleted by centrifugation at 8,000 ×g at 4 °C for 10 min. The total cellular RNA was isolated using a MasterPure™ RNA purification kit (Epicentre, Wisconsin, USA), according to the manufacturer's instructions.

Detection of COX-2, IL-6, iNOS and TNF-α mRNA by reverse transcription

The total RNA (1 µg) was reverse transcribed (RT) from each sample using 10 mM dNTP, oligo $(dT)_{18}$ and reverse transcriptase using a Primerdesign Ltd *Precision*™ nanoScript 2 reverse Transcription kit (PrimerDesign Ltd., UK), according to the manufacturer's instructions. PCR analyses were performed on aliquots of the cDNA preparations to detect COX-2, IL-6, iNOS and TNF-α (using β-actin and COX-1 as an internal standard) gene expression using a thermal cycler T100 (Bio-Rad, California, USA). Reactions were performed in a volume of 25 µL containing ultra buffer, 15 mM $MgCl_2$, 1.25 units Taq polymerase, 1 µL of template DNA (1 µg), PCR grade dH_2O and 10 µM of the 5′ and 3′ primers. Thermal cycling was performed at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 50 °C (except 60 and 65 °C for COX-1 and

iNOS, respectively) for 30 s and 72 °C for 1 min. The PCR amplicons, 380 base pairs (bp) β-actin, 382 bp COX-1, 861 bp COX-2, 417 bp IL-6, 423 bpiNOS and 375 bp TNF-αDNA fragments, were separated by 1.2% (w/v) agarose gel electrophoresis, stained by ethidium bromide staining for 10 min and observed under ultraviolet transillumination using a gel documentation system (Bio-Rad). The PCR was performed using selective primers for the β-actin (5'- ACCAACTGGGACGACATGGAGAA-3' and 5'-GTGGTGGTGAAGCTGTAGCC-3'), iNOS (5'-CCATCATGGACCACCACACA-3' and 5'- CCATGCAGACAACCTTGGTG-3'), IL-6 (5'- CATGTTCTCTGGGAAATCGTGG-3' and 5'-AACGCACTAGGTTTGCCGAGTA-3'), TNF-α (5'-CCTGTAGCCCACGTCGTAGC-3' and 5'-TTGACCTCAGCGCTGAGTTG-3'), COX-1 (5'-AGTGCGGTCCAACCTTATCC-3' and 5'-CCGCAGGTGATACTGTCGTT-3'), and COX-2 (5'-GGAGAGACTATCAAGATAGT-3' and 5'-ATGGTCAGTAGACTTTTACA-3').

Statistical analysis

All data were performed in triplicate and are shown as the mean ± one standard error of the mean. Statistical comparisons of the mean values were performed by analysis of variance (ANOVA), followed by Duncan's multiple-range test using the SPSS software version 22 (IBM, New York, USA). A value of $P < 0.05$ was considered statistically significant. Regression analysis was performed using GraphPad Prism Version 6.01 for Windows (GraphPad Software Inc., California, USA).

Results

Amino acid composition of bee pollen

To determine the amino acid composition of the bee pollen, the dried bee pollen was analyzed according to the AOAC994.12 method. The amino acid composition of the bee

pollen is summarized in Table 1. The total amino acid of bee pollen was 22.0% (w/w), revealing it to be an excellent protein source, in accord with a previous report (Komosinska-Vassev, Olczyk, Kazmierczak, Mencner, & Olczyk, 2015). The most abundant amino acid in the bee pollen was Asp at 2.72% (w/w), followed by Pro and Glu, which constituted over 2% (w/w) each, while Trp at 0.23% (w/w) was present in the lowest amount. Hydrophobic (Iso, Leu, Met, Phe, Try, Tyr and Val) and positively charged (Lys, Arg and His) amino acids in bee pollen accounted for 27.9% and 16.2% of the total amino acids, respectively. These hydrophobic and polar residues were reported to be major components of anti-inflammatory peptides (Vogel et al., 2002; Nan et al., 2007; Zhao, Wanga, Zhanga, & Xie, 2016)

Table 1 Amino acid composition in bee pollen protein

Amino acids	% Content (w/w)
Alanine (Ala, A)	1.27
Arginine (Arg, R)	1.32
Aspartic acid (Asp, D)	2.72
Cysteine (Cys, C)	0.50
Glutamic acid (Glu, E)	2.26
Glycine (Gly, G)	0.91
Histidine (His, H)	0.65
Isoleucine (Ile, I)	0.88
Leucine (Leu, L)	1.76
Lysine (Lys, K)	1.67
Methionine (Met, M)	0.39
Phenylalanine (Phe, F)	0.98
Proline (Pro, P)	2.28
Serine (Ser, S)	1.23
Threonine (Thr, T)	1.02
Tryptophan (Trp, W)	0.23
Tyrosine (Tyr, Y)	0.84
Valine (Val, V)	1.11
Total	22.02

NO scavenging activity of bee pollen protein hydrolysates

To prepare bee pollen hydrolysates, bee pollen was hydrolyzed using the proteases Alcalase, Flavourzyme and Neutrase at different E/S ratios to produce the potential anti-inflammatory peptides. To verify the anti-inflammatory activity of the bee pollen protein hydrolysates, the NO radical scavenging assay was conducted, with the derived IC_{50}

values shown in Table 2. The method for the determination of NO scavenging could be a useful tool for the screening of anti-inflammatory peptides (Chakrabarti, Jahandideh, & Wu, 2014).

Table 2 IC_{50} values of bee pollen protein hydrolysates from different E/S ratios of proteases against NO.

E/S ratio (w/w)	IC_{50} (μg/mL)		
	Alcalase	**Flavourzyme**	**Neutrase**
0:5	108.10 ± 4.70^{g}	86.64 ± 0.67^{f}	86.64 ± 0.67^{f}
1:5	35.05 ± 1.23^{d}	15.91 ± 0.69^{b}	23.45 ± 1.10^{c}
1:2	19.80 ± 0.75^{bc}	21.43 ± 0.97^{c}	15.80 ± 0.80^{b}
1:1	31.48 ± 0.74^{d}	10.01 ± 1.08^{a}	9.08 ± 0.83^{a}

$^{a\text{-}f}$ Means with a different superscript letter are significantly different ($P < 0.05$). Positive control (curcumin), $IC_{50} = 60.53 \pm 1.84^{e}$

The NO scavenging activity of the pollen protein hydrolysates ranged from IC_{50} values of 19.8 ± 0.8 to 35.1 ± 1.2 μg/mL (Alcalase), 10.0 ± 1.1 to 21.4 ± 1.0 μg/mL (Flavourzyme) and 9.1 ± 0.8 to 23.5 ± 1.1 μg/mL (Neutrase), which were all significantly stronger than that for the the negative control (bee pollen without hydrolysis) at 86.6 ± 0.7 to 108.1 ± 4.7 μg/mL and the IC_{50} of curcumin (positive control) at 60.5 ± 1.8 μg/mL. Thus, the NO radical scavenging activity of bee pollen protein was increased by the enzymatic hydrolysis.

Fractionation, enrichment and characterization of the anti-inflammatory peptides in the NH1 fraction of bee pollen

To fractionate the protein hydrolysate, the NH1 was filtered through 10, 5, 3 and 0.65 kDa molecular weight cut-off membrane filters. The NO scavenging activity of the different molecular weight fractions is summarized in Table 3, where the peptide fractions with molecular weights lower than 5 kDa had a significantly higher NO scavenging capacity than the higher molecular weight fractions. The < 0.65 kDa molecular weight fraction (MW1) exhibited the best numerical NO scavenging activity (although there was no significant difference between MW1–3) compared to the other fractions. Peptide sequences between 5 and 22 amino acids in length are best suited for quantification purpose (Demeure, Duriez, Domon, & Niclou, 2014) and therefore MW1 was selected for further experiment.

Table 3 IC_{50} values of ultrafiltration fractions of NH1 against NO.

Fractions	IC_{50} (µg/mL)
< 0.65 kDa	4.00 ± 0.03^a
0.65–3 kDa	4.83 ± 0.25^a
3–5 kDa	4.68 ± 0.29^a
5–10 kDa	12.32 ± 0.55^c
> 10 kDa	7.70 ± 0.32^b

$^{a\text{-}c}$ Means with a different superscript letter are significantly different ($P < 0.05$).

The high activity against free radicals is perhaps due to the small size of peptides, while more peptide sequences will lead to a dilution effect of the peptides exhibiting a free radical scavenging activity as described by Zou, He, Li, Tang, & Xia (2016). These authors also reported that peptides of an appropriately low molecular weight can exert a significant effect on their free radical scavenging capacities. Moreover, Wang et al. (2013) suggested that low molecular weight peptides enriched by ultrafiltration could easily cross the intestinal cell barrier to exert biological effects and interact with targets. Thus, the MW1 fraction was selected for further study.

To purify the anti-inflammatory peptides, the lyophilized MW1 fraction was further separated by RP-HPLC and the elution was divided into six principal fractions (H1–6) as shown in Fig. 1. The NO inhibitory activities at 50% (IC_{50}) of H2, H3 and H4 were 3.55 ± 0.09, 1.57 ± 0.11 and 5.64 ± 0.28 μg/mL, respectively. However, the H1, H5 and H6 fractions exhibited less than 50% inhibition at 50 μg/mL.

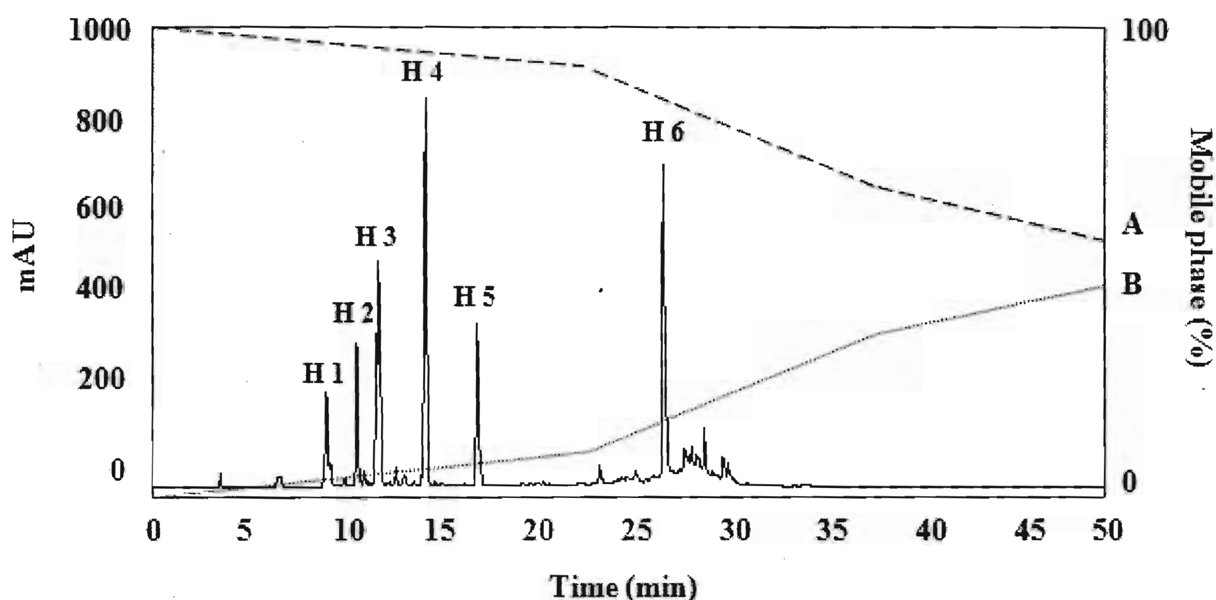

Figure 1. RP-HPLC separation profile of peptides from the MW1 (< 0.65 kDa) fraction.

To characterize the anti-inflammatory peptides, the amino acid sequences of the active fractions were identified by Q-TOF-MS (*de novo* peptide sequencing) and were searched against the NCBI database for 100% sequence identity with sequences from within the *Mimosa* genus, since this was reported to be an important pollen source of *A. mellifera* (Ramalho, Kleinert-Giovannini, & Imperatriz-Fonseca, 1990; Santos do Nascimento, Marchini, Lopes de Carvalho, Araújo, & Antonia da Silveira, 2015). From the peptide sequences (H2-H4) obtained, seven were matched with proteins from three *Mimosa* species (*M. pudica*, *M. spegazzinii* and *M. strigillosa*), as shown in Table 4.

Table 4 Amino acid sequences of anti-inflammatory peptides obtained from bee pollen

Fractions	NO scavenging activity (IC_{50}, μg/mL)	Sequences	Mass (m/z)	Organisms	Query cover (%)	Identity (%)	Accession
H2	3.55 ± 0.09^{b}	VLAKNAPP	809	Hypothetical protein TSUD_31550 (*Trifolium subterraneum*)	100	88	GAU29349.1
				Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, partial (chloroplast) (*Mimosa diplotricha*)	50	100	AZC11473.1
		RLNTAEAGH	969	Hypothetical protein PHAVU_007G105300g (*Phaseolus vulgaris*)	100	78	XP_007143831.1
				Apyrase (*Mimosa pudica*)	44	100	BAK78982.1
H3	1.57 ± 0.11^{a}	VTAHSATVLPR	1152	Hypothetical protein L195_g031431 (*Trifolium pretense*)	63	100	PNX75494.1
				Mimosinase (*Mimosa pudica*)	36	100	BAN57423.1
		KNKKWPAEAAH	1280	Hypothetical protein LR48_Vigan627s001300 (*Vigna angularis*)	63	86	KOM28964.1
				NADH dehydrogenase-like protein, partial (chloroplast) (*Mimosa foetida*)	54	67	ACJ05100.1
		KLRSRNLLHPT	1335	Uncharacterized protein LOC106773448 (*Vigna radiata* var. radiate)	72	100	XP_014515627.1
				NADH dehydrogenase-like protein, partial (chloroplast) (*Mimosa foetida*)	63	71	ACJ05100.1
		TNRLLSGHSAKKH	1449	PREDICTED: uncharacterized protein LOC109358471 isoform X2 (*Lupinus angustifolius*)	53	100	XP_019458257.1
				Cystathionine beta-lyase (*Mimosa pudica*)	38	80	BAS32612.1
H4	5.64 ± 0.28^{c}	TPVPWEAPRLN	1280	Nuclear pore complex protein NUP133 (*Glycine max*)	90	90	XP_003519660.1
				Actin isoform C, partial (*Mimosa pudica*)	54	83	BAA89215.1

$^{a\text{-}c}$ Means with a different superscript letter are significantly different ($P < 0.05$).

Effect of the MW1 fraction on LPS-induced NO production and their expression of COX-2, IL-6, iNOS and TNF-α

To estimate the effect of the MW1 fraction on NO production in LPS-induced RAW264.7 macrophage cells and the regulation of inflammatory genes, the cytotoxicity of MW1 fraction was first determined using the MTT viability assay (Saisavoey, Sangtanoo, Reamtong, & Karnchanatat, 2018) to ascertain the appropriate concentrations for use in cell treatments. The viability of RAW264.7 macrophage cells was not reduced by the MW1 fraction at concentrations up to 12.0 μg/mL (data not shown), and so RAW264.7 macrophage cells were treated with MW1 at 0 (negative control), 1.5, 3.0 and 6.0 μg/mL, or with 3.0 μg/mL BUD as a positive control, for 24 h, followed by 2.5 μg/mL LPS stimulation for 12 h prior to evaluating the transcript expression levels of COX-2, IL-6, iNOS and TNF-α. The transcript levels of β-actin and COX-1, as housekeeping genes, were also evaluated and used as the internal standard and reference point for the analysis of the expression levels of the above target genes (Crofford, 1997; Lin & Redies, 2012).

The expression of COX-2, IL-6, iNOS and TNF-α were all increased by LPS-stimulation compared to the un-stimulated control (Fig. 2). LPS is known to be an endotoxin released by Gram-negative bacteria that can activate endothelial cells in an *in vitro* assay of inflammation mechanisms, which promotes the secretion of NO and pro-inflammatory cytokines (Rietschel et al., 1994; Zhang & An, 2007; Nakamura, Hayashi, & Kubokawa, 2015). Co-treatment of the RAW264.7 cells with LPS and 1.5 μg/mL MW1 fraction still revealed the up-regulation of COX-2 and TNF-α, whereas the expression of IL-6 and iNOS transcripts were down-regulated, whereas co-treatment with 3.0 or 6.0 μg/mL of MW1 negated the LPS-induced expression of COX-2 and TNF-α as well as IL-6 and iNOS.

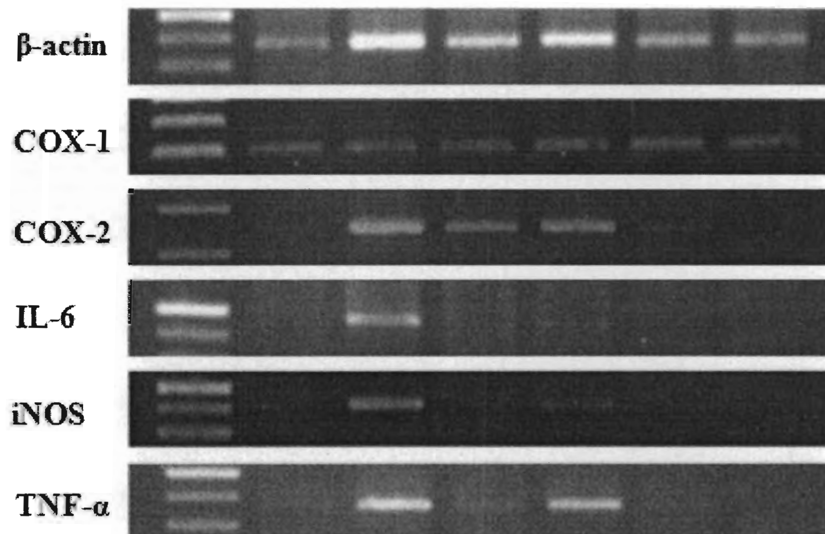

Figure 2. Effect of the MW1 fraction on COX-2, IL-6, iNOS and TNF-α transcript expression levels in LPS (2.5 μg/mL) stimulated RAW 264.7 cells. β-actin (380 bp), COX-1 (382 bp), COX-2 (861 bp), IL-6 (417 bp), iNOS (423 bp), and TNF-α (375 bp). For Lane M: 1 Kb plus, Lane 1: No addition, Lane 2: LPS, Lane 3: 3.0 μg/mL BUD (positive control), Lane 4: LPS and 1.5 μg/mL MW1, Lane 5: LPS and 3.0 μg/mL MW1, and Lane 6: LPS and 6.0 μg/mL MW1.

Discussion

The bee pollen sample from *Apis mellifera*, presumed to be mainly from *Mimosa pigra*, was collected from an *A. mellifera* farm in Chiangmai Province, Thailand. *Mimosa pigra* has been shown to be the major source of pollen utilized by *A. mellifera* within Thailand (Kongpitak, Akratanakul, & Amornsak, 1990). This plant blooms all year round and

provides an inexhaustible floral source (Santos do Nascimento, Marchini, Lopes de Carvalho, Araújo, & Antonia da Silveira, 2015). Usually, bee pollen consists of the pollen from one plant (Komosinska-Vassev, Olczyk, Kazmierczak, Mencner, & Olczyk, 2015). In addition, pollen of this genus was also reported in the honey of *A. mellifera* (Ramalho & Kleinert-Giovannini, 1990). However, the pollen identification is still needed to confirm principally one species by PCR amplification and sequencing of suitable DNA barcoding genes.

Employing similar methodology, comparable results have been seen with soy protein hydrolysates prepared from enzymatic hydrolysis, which had greater bioactive potential than un-hydrolysed soy protein isolate (Peña-Ramos & Xiong, 2002), while peptide fragments are often functionally inactive within the native proteins and must be released by enzymatic hydrolysis to achieve their bioactivities (Chakrabarti, Jahandideh, & Wu, 2014).

Flavourzyme and Neutrase hydrolysates showed a broadly similar activity, but a higher activity than the Alcalase hydrolysates. The higher activity from Flavourzyme and Neutrase hydrolysates could be attributed to the lower pH (pH 7.0) required for the hydrolysis being suitable for anti-inflammatory peptide production. At a lower pH, the peptide is positively charged and is reported to have a major influence on the anti-inflammatory activities of peptides (Zhao, Wanga, Zhanga, & Xie, 2016). The Neutrase hydrolysate produced from an E/S ratio of 1:1 (NH1) had the highest NO radical scavenging activity (IC_{50} of 9.1 ± 0.8 μg/mL) compared to other hydrolysates or E/S ratios ($P < 0.05$) and was also some 6.67-fold more potent than the positive control (curcumin with an IC_{50} of 60.5 ± 1.8 μg/mL). Previous studies have already shown that the active peptides from Neutrase hydrolysates of blue mussel and rice endosperm exhibited a higher free radical scavenging activity than the parent form (Zhang et al., 2010; Wang et al., 2013). Therefore, NH1 was selected to enrich the anti-inflammatory peptides.

The Arg-Leu-Asn-Thr-Ala-Glu-Ala-Gly-His peptide in the H2 fraction matched to apyrase, an enzyme found to alleviate airway inflammation (Li, Cao, Chen, Wang, &

Yang, 2014). The H4 fraction showed the highest NO scavenging activity compared to the other fractions, which might be due to the Leu and Lys amino acids present in these peptides since a potent anti-inflammatory peptide was developed from Leu/Lys-rich fragments (Nan et al., 2007). In addition, Leu, Ser, Tyr and Arg residues were reported to be dominant in anti-inflammatory peptides (Gupta, Sharma, Shastri, Madhu & Sharma, 2017). Moreover, the Lys-Leu-Arg-Ser-Arg-Asn-Leu-Leu-His-Pro-Thr peptide was identified as from glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which has an anti-inflammatory function in preventing LPS-induced, sepsis-related severe acute lung injury in mice (Takaoka et al., 2014), while the Thr-Asn-Arg-Leu-Leu-Ser-Gly-His-Ser-Ala-Lys-Lys-His peptide matched to a ribosomal protein, which has the potential to be a repressor of inflammatory signaling (Zhou, Liao, Liao, Liao, & Lu, 2015).

COX-2 is an enzyme responsible for inflammation and pain that is significantly and markedly up-regulated in colorectal breast, pancreatic and lung cancers (Hla & Neilson, 1992; Sobolewski, Cerella, Dicato, Ghibelli, & Diederich, 2010). Furthermore, iNOS is an enzyme that produces NO and stimulates pro-inflammatory cytokines, the inappropriate expression of which results in many inflammatory diseases (Green et al., 1994; Cassini-Vieira et al., 2015). Understanding of the COX-2 and iNOS enzyme inhibition is important for the development of anti-inflammatory agents and treating inflammatory diseases (Chuang, Kardosh, Gaffney, Petasis, & Schönthal, 2008; Delker, Xue, Li, Jamal, Silverman, & Poulos, 2010).

The inflammatory cytokines IL-6 and TNF-α are known to be secreted by LPS-stimulation in RAW264.7 macrophage cells (Nakamura, Hayashi, & Kubokawa, 2015). IL-6 has been reported to be involved in inflammatory and auto-immune processes in many diseases, including atherosclerosis, Alzheimer's disease, asthma, diabetes, prostate cancer and rheumatoid arthritis (Smith, Hobisch, Lin, Culig, & Keller, 2001; Kristiansen & Mandrup-Poulsen, 2005; Nishimoto, 2006; Dubiński & Zdrojewicz, 2007; Swardfager et al., 2010; Peters et al., 2016). TNF-α was also associated with Alzheimer's disease and cancers, as well as other human diseases such as inflammatory bowel disease and psoriasis (Locksley, Killeen, & Lenardo, 2001; Brynskov et al., 2002; Victor & Gottlieb,

2002). Although the MW1 fraction could suppress the LPS-upregulated transcript levels of these pro-inflammatory cytokines, the important mechanism, as proposed by Dinarello (2000), is that the reduction in the biological activity of these cytokines is necessary to reduce inflammation, promote healing and provide treatment.

The IC_{50} of the MW1 fraction (2.03 ± 0.28 μg/mL) against NO production in RAW264.7 macrophage cells was some 1.56-fold stronger than that BUD (3.16 ± 0.06 μg/mL), a synthetic steroid that has potent anti-inflammatory effects for the treatment of asthma, allergic rhinitis and inflammatory bowel disease (Todd et al., 2002; Stanaland, 2004; Kuenzig et al., 2014). This result suggested that anti-inflammatory peptides from bee pollen might be promising substances for use against inflammatory diseases, subject to then being absorbed and distributed in an efficient manner.

In conclusion, this preliminary study examined the potential anti-inflammatory peptides from *A. mellifera* bee pollen. The NO scavenging activity of the enzymatic hydrolysates from bee pollen was determined, and the resulting anti-inflammatory peptides from the Neutrase hydrolysate at an E/S ratio of 1:1 were enriched using fractionation and chromatographic methods. Finally, the *de novo* amino acid sequences of anti-inflammatory peptides were obtained by Q-TOF-MS. The smallest peptide fraction (< 0.65 kDa, MW1) inhibited NO production in LPS-stimulated RAW264.7 macrophage cells, and negated the LPS-induced expression of COX-2, IL-6, iNOS and TNF-α transcripts. Thus, the enzymatic hydrolysis of bee pollen improves improves its anti-inflammatory activity, while bee pollen hydrolysates may be useful for application in functional foods and pharmaceutical peptide drugs against inflammatory diseases. However, more research about *in vivo* inflammatory stress is needed to confirm the potential health benefits of purified bee pollen peptides.

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วท.ม.	ชีวเคมี	จุฬาลงกรณ์มหาวิทยาลัย	2544
วท.ด.	เทคโนโลยีชีวภาพ	จุฬาลงกรณ์มหาวิทยาลัย	2549

6. สาขาวิชาการที่มีความชำนาญพิเศษ (แตกต่างจากวุฒิการศึกษา) ระบุสาขาวิชาการ
   เทคโนโลยีชีวภาพของเอนไซม์ (Enzyme Biotechnology), เคมีของโปรตีน: โครงสร้าง และหน้าที่ (Protein Chemistry: Structure and Function, เคมีของผลิตภัณฑ์ธรรมชาติ (Natural Products), ชีววิธีการฟื้นฟูสภาพแวดล้อมโดยเชื้อรา (fungal Bioremediation)
7. ประสบการณ์ที่เกี่ยวข้องกับการบริหารงานวิจัยทั้งภายในและภายนอกประเทศ
   7.1 งานวิจัยที่ผ่านมา (ย้อนหลัง 5 ปี)

ลำดับที่	หัวข้อเรื่อง	แหล่งทุน	ปีที่ได้รับทุน	ปีที่แล้วเสร็จ
1	เอนไซม์สลายไฟบรินจากเพรียงทราย *Perinereis nuntia* (Fibrinolytic enzyme from *Perinereis nuntia*)	สำนักงานคณะกรรมการวิจัยแห่งชาติ	2555	2556
2	ฤทธิ์ยับยั้งไทโรซิเนสของโปรตีนไฮโดรไลเสตจากเมล็ดผลไม้ไทย (*Tyrosinase* inhibitory activity of the protein hydrolysate from the seeds of Thai fruits)	สำนักงานกองทุนสนับสนุนงานวิจัย (โครงการเชื่อมโยงภาคการผลิตกับงานวิจัย ทุน สกว. – อุตสาหกรรม (MAG Window I)	2555	2557
3	พอลิเมอร์ชีวภาพฉลาดจากพืชสมุนไพรไทยเพื่อการบำบัดโรค	โครงการพัฒนามหาวิทยาลัยวิจัย	2553	2556

	(Smart biopolymer from Thai medicinal plants for therapeutic use)	แห่งชาติ คลัสเตอร์วัสดุขั้นสูง		-
4	สารออกฤทธิ์ชีวภาพในการป้องกันและรักษาโรคกระดูกพรุนและโรคหลอดเลือดแข็งตัวในผู้สูงอายุ (Bioactive compounds for prevention and treatment of osteoporosis and arthrosclerosis in aging person)	โครงการพัฒนามหาวิทยาลัยวิจัยแห่งชาติ คลัสเตอร์สังคมผู้สูงวัย	2553	2556
5	ฤทธิ์ยับยั้งไทโรซิเนสของโปรตีนไฮโดรไลเสตจากเมล็ดผลไม้ไทย (Tyrosinase inhibitory activity of the protein hydrolysate from the seeds of Thai fruits))	โครงการทุนวิจัยมหาบัณฑิต สกว. สาขาวิทยาศาสตร์และเทคโนโลยี ภายใต้โครงการเชื่อมโยงภาคการผลิตกับงานวิจัย ทุน สกว.-อุตสาหกรรม	2555	2557
6	เรื่อง "การพัฒนาสารต้นแบบสำหรับรักษาโรคจากโปรตีนไฮโดรไลเสต: กรณีศึกษาเมล็ดผลไม้ไทย (Development of therapeutic leads from protein hydrolysate: A case study of Thai fruit seeds)	โครงการทุนช่วยเหลือทางด้านวิจัยวิทยาศาสตร์และเทคโนโลยี (Thailand Toray Science Foundation) ครั้งที่ 20 พ.ศ. 2556	2556	2557
7	ฤทธิ์ยับยั้งไกลเคชั่นของสารชีวภาพจากสมุนไพรไทย (Anti-glycation of biomaterials from Thai herbs)	โครงการส่งเสริมการทำงานวิจัยเชิงลึกในสาขาวิชาที่มีศักยภาพสูง ศูนย์นวัตกรรมสหศาสตร์ โครงการในแผนพัฒนาวิชาการ จุฬาลงกรณ์มหาวิทยาลัย	2556	2557
8	การพัฒนาสารต้นแบบสำหรับการรักษาโรคอัลซัยเมอร์และโรคมะเร็งจากพืชสมุนไทยไทยและราเอนโดไฟต์ที่แยกได้ (Development of therapeutic leads for alzheimer's disease and cancer from Thai medicinal plants and their endophytic fungi)	โครงการวิจัยต่อเนื่อง 7 คลัสเตอร์ คลัสเตอร์สังคม ผู้สูงวัย	2556	2558
9	โปรตีนไฮโดรไลเสตจากเมล็ดผลไม้ไทยเพื่อการบำบัดโรค (Protein hydrolysate from Thai fruit seeds for therapeutic use)	โครงการวิจัยต่อเนื่อง 7 คลัสเตอร์ คลัสเตอร์สังคมผู้สูงวัย	2556	2558
10	การการเตรียมโปรตีนไฮโดรไลเสตจากขนไก่ป่นเพื่อการประยุกต์ใช้ในผลิตภัณฑ์เพื่อสุขภาพและเครื่องสำอาง (Preparation of protein hydrolysate from chicken feather meal for applications in health products and cosmetics)	สำนักงานกองทุนสนับสนุนงานวิจัย (โครงการเชื่อมโยงภาคการผลิตกับงานวิจัย ทุน สกว. - อุตสาหกรรม(MAG Window I)	2557	2559
11	การพัฒนาสารต้นแบบสำหรับรักษาโรคมะเร็ง: กรณีศึกษาเพปไทด์ที่มีฤทธิ์ทางชีวภาพจากหอยหวาน (Development of therapeutic leads for cancer treatment: A case study of bioactive peptides from Spotted Babylon)	สำนักงานกองทุนสนับสนุนงานวิจัย (โครงการเชื่อมโยงภาคการผลิตกับงานวิจัย ทุน สกว. - อุตสาหกรรม(MAG Window I)	2557	2559.
12	การใช้แอลคาไลน์โปรติเอสในการผลิตโปรตีนไฮโดรไลเสตที่มีฤทธิ์ทางชีวภาพจากขนไก่ป่น (The use of alkaline protease to produced protein hydrolysate with biological activities from chicken feather meal)	สำนักงานกองทุนสนับสนุนงานวิจัย (โครงการเชื่อมโยงภาคการผลิตกับงานวิจัย ทุน สกว. - อุตสาหกรรม(MAG Window I)	2557	2559
13	ฤทธิ์ต้านการอักเสบของเพปไทด์จากขนไก่ป่นในเซลล์เพาะเลี้ยงแมคโครฟาจ RAW 264.7 (Anti-inflammatory activity of peptide from chicken feather meal in Macrophage RAW 264.7)	สำนักงานกองทุนสนับสนุนงานวิจัย (โครงการเชื่อมโยงภาคการผลิตกับงานวิจัย ทุน สกว. - อุตสาหกรรม(MAG Window I)	2559	2560
14	ฤทธิ์ขจัดอนุมูลอิสระและยับยั้งการเพิ่มจำนวนเซลล์มะเร็งของ	สำนักงานกองทุนสนับสนุนงานวิจัย	2559	2560

	เพปไทด์จากขนไก่ป่น (Free radical scavenging and antiproliferative of peptide from chicken feather meal)	(โครงการเชื่อมโยงภาคการผลิตกับงานวิจัย ทุน สกว. - อุตสาหกรรม(MAG Window I)		
15	การเพิ่มมูลค่าผลิตภัณฑ์พลอยได้จากอุตสาหกรรมอาหารทะเลแช่แข็ง: กรณีศึกษากลไกการออกฤทธิ์ต้านอักเสบของเพปไทด์จากกระดูกปลาแซลมอนในเซลล์เพาะเลี้ยงแมคโครฟาจ (RAW 264.7) (Value added products from by-products of frozen seafood processing: A case study of anti-inflammatory mechanisms of peptide from salmon bones in macrophage cell line (RAW 264.7)	โครงการทุนยุทธศาสตร์การวิจัยเชิงลึก ประจำปีงบประมาณ 2559 งบเชื่อมโยงงานวิจัยระดับคลัสเตอร์	2559	2560

7.2 ผลงานวิจัยที่พิมพ์เผยแพร่ (ย้อนหลัง 5 ปี)

7.2.1 Chantaranothai, C., Palaga, T., **Karnchanatat, A.**, and Sangvanich, P.* (2013) Inhibition of nitric oxide production in the Macrophage-like Raw 264.7 cell line by protein from the rhizomes of Zingberaceae plants. *Preparative biochemistry and biotechnology* 43: 60-78.

7.2.2 Rungsaeng, P., Sangvanich, P., and **Karnchanatat, A.*** (2013) Zingipain, a ginger protease with acetylcholinesterase inhibitory activity. *Applied Biochemistry and Biotechnology* 170: 934-950.

7.2.3 **Karnchanatat, A.***, Sihanonth, P., Piapukiew, J., and Sangvanich, P. (2013) An antioxidation and antiproliferation of polysaccharide-protein complex extracted from *Phaeogyroporus portentosus* (Berk. & Broome) McNabb. *African Journal of Microbiology Research* 7: 1668-1680.

7.2.4 Saisavoey, T., Thongchul, N., Sangvanich, P. and **Karnchanatat, A.*** (2014) Effect of methyl jasmonate on isoflavonoid accumulation and antioxidant enzymes in *Pueraria mirifica* cell suspension culture. *Journal of Medicinal Plants Research* 8: 401-407.

7.2.5 Niyomploy, P., Srisomsap, C., Chokchaichamnankit, D., Vinayavekhin, N., **Karnchanatat, A.**, and Sangvanich, P.* (2014) Superoxide dismutase isozyme detection using two-dimensional gel electrophoresis zymograms. *Journal of Pharmaceutical and Biomedical Analysis* 90: 72-77.

7.2.6 Niyomploy, P., Boonsombat, R., **Karnchanatat, A.**, and Sangvanich, P.* (2014) A Superoxide dismutase purified from the roots *Stemona tuberosa*. *Preparative Biochemistry and Biotechnology* 44: 663-679.

7.2.7 Saisavoey, T., Palaga, T., Malaivijitnond, S., Jaroenporn, S., Thongchul, N., Sangvanich, P. and **Karnchanatat, A.*** (2014) Anti-osteoclastogenic,

estrogenic and antioxidant activities of cell suspension culture and tuber roots extract from *Pueraria mirifica*. *Food Science and Biotechnology* 23: 1253-1259.

7.2.8 Srinieng, K., Saisavoey T., and **Karnchanatat, A.*** (2015) Effect of salinity stress on antioxidative enzyme activities in tomato cultured *in vitro*. *Pakistan Journal of Botany* 47: 1-10.

7.2.9 Songserm, P., Thitiprasert, S., Tolieng, V., Piluk, J., Tanasupawat, S., Assabumrungrat, S., Yang, S.T., **Karnchanatat, A.,** Thongchul N6. Regulating pyruvate carboxylase in the living culture of *Aspergillus Terreus* NRRL 1960 by L-aspartate for enhanced itaconic acid production. *Applied Biochemistry and Biotechnology* 177: 595-609.

7.2.10 Saisavoey T., Sangtanoo, P., Reamtong, R., and **Karnchanatat, A.*** (2016) Antioxidation and anti-inflammatory effects of defatted rice bran (*Oryza sativa* L.) protein hydrolysate on RAW 264.7 Macrophage cells. *Journal of Food Biochemistry* 40: 731-740.

7.2.11 Inthuwanarud, K., Sangvanich, P., Puthong, S., and **Karnchanatat, A.*** (2016) Antioxidant and antiproliferative activities of protein hydrolysate from the rhizomes of Zingiberaceae plants. *Pakistan Journal of Pharmaceutical Sciences* 29: 1893-1900.

7.2.12 Pongkai, P., Saisavoey T., Sangtanoo, P., Sangvanich, P., and **Karnchanatat, A.*** (2017) Effects of protein hydrolysate from chicken feather meal on tyrosinase activity and melanin formation in B16F10 murine melanoma cells. *Food Science and Biotechnology* 26: 1199–1208.

7.2.13 Saisavoey T., Sangtanoo, P., Reamtong, R., and **Karnchanatat, A.*** (2018) Anti-inflammatory effects of lychee (*Litchi chinensis* Sonn.) seed peptide hydrolysate on RAW 264.7 macrophage cells. *Food Biotechnology* 32: 79–94.

7.2.14 Prakot, P., Chaitanawisuti, N., Sangtanoo, P., Saisavoey,T., and **Karnchanatat, A.*** (2018) Inhibitory activities of protein hydrolysates from Spotted Babylonsnails on tyrosinase and melanogenesis. *Journal of Aquatic Food Product Technology* https://doi.org/10.1080/10498850.2018.1499687.

7.2.15 Thongyoo, S., Phakham, T., Khongchareonporn, N., Reamtong, O., **Karnchanatat, A.**, Phutong, S., Wichai, T., Noitang, S., and Sooksai, S. (2019) Expression, purification and biological activity of monomeric insulin precursors from methylotrophic yeasts. *Protein Expression* and *Purification* https://doi: 10.1016/j.pep.2018.08.002.

7.2.16 Srimongkol, P., Thongchul, N., Phunpruch, S., and **Karnchanatat, A.*** (2019) Ability of marine cyanobacterium *Synechococcus* sp. VDW to remove ammonium from brackish aquaculture wastewater. *Agricultural Water Management* 212: 155-161.

7.2.17 Srimongkol, P., Thongchul, N., Phunpruch, S., and **Karnchanatat, A.*** (2019) Optimization of *Synechococcus* sp. VDW cultivation with artificially prepared shrimp wastewater for ammonium removal and its potential for use as a biofuel feedstock. *Journal of Oleo Science* (accepted manuscript)

7.3 งานวิจัยที่กำลังดำเนินการ

7.3.1 โครงการวิจัย ศูนย์ความเป็นเลิศด้านเทคโนโลยีชีวภาพทางการแพทย์ (ศทพ.) ประจำปีงบประมาณ 2560 เรื่อง “นวัตกรรมเพปไทด์ที่มีฤทธิ์ทางชีวภาพซึ่งเป็นผลพลอยได้จากอุตสาหกรรมสัตว์ปีก (Innovative bioactive peptides derived from by-product in poultry industry)” (หัวหน้าโครงการ) ระยะเวลา 2 ปี (พ.ศ. 2560-2561)

7.3.2 โครงการวิจัยงบประมาณของสำนักงานคณะกรรมการวิจัยแห่งชาติประจำปีงบประมาณ 2561 สำนักงานพัฒนาการวิจัยการเกษตร (องค์การมหาชน) เรื่อง “นวัตกรรมเพปไทด์ที่มีฤทธิ์ยับยั้งการสังเคราะห์เม็ดสีเมลานินจากรำข้าวสกัดน้ำมัน (Innovative peptides with melanogenesis inhibition by defatted rice bran)” (หัวหน้าโครงการ) ระยะเวลา 2 ปี (พ.ศ. 2561-2562)