# Nitric Oxide Synthesis Inhibition and Anti-Inflammatory Effect of Polypeptide Isolated from Chicken Feather Meal in Lipopolysaccharide-Stimulated RAW 264.7 Macrophages

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## SUMMARY

Nitric oxide (NO) plays a key role in the pathogenesis of inflammation and has been implicated in endotoxin-induced tissue injury. Chicken feather meal is a rich source of amino acids that may serve as a peptide hydrolysate to inhibit NO activity. Anti-inflammatory hydrolysates of chicken feather meal were prepared and fractionated into five samples based on molecular mass. The smallest fraction (<0.65 kDa) exhibited the highest NO inhibitory activity without cytotoxicity towards macrophage RAW 264.7 cells. Further subfractions were sufficient to obtain amino acid sequences by Q-TOF LC-MS/MS ESI analysis. Of these, the SNPSVAGVR (885.97 Da) peptide and its corresponding pure synthetic peptide have inhibitory activity against NO production by RAW 264.7 cells ($IC_{50}$=(55.2±0.2) mM) without cytotoxicity. Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR results revealed that the peptide of the obtained fraction reduced transcript expression levels of the pro-inflammatory cytokines iNOS, TNF-α, COX-2 and IL-6 in lipopolysaccharide-stimulated RAW 264.7 cells. These results suggest that the peptides derived from the chicken feather meal protein could potentially be used as a promising ingredient in functional foods or nutraceuticals against inflammatory diseases.

**Key words:** nitric oxide, anti-inflammatory activity, chicken feather meal, protein hydrolysate, polypeptide, macrophage RAW 264.7

## INTRODUCTION

Inflammation is a host defence mechanism that involves physiological and pathological processes within an organism, and is induced by the invasion of pathogens or tissue injury caused by biological, chemical or physical damage (*1*). The activation of several immune cells (monocytes and macrophages) produces inflammation mediators, such as nitric oxide (NO), cyclooxygenase-2 (COX-2), prostaglandins E2 (PGE2) and other pro-inflammatory cytokines (iNOS), including tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and interleukin-1β (*2*). Macrophages play an important role in inflammation and this cellular response is initiated by bacterial lipopolysaccharide (LPS), which is part of the outer membrane of Gram-negative bacteria, interferon-γ (IFN-γ) and pro-inflammatory cytokines (*3*). Oxidative stress is involved in the pathogenesis of several diseases. Physical and biochemical processes in the human body generate free radicals as byproducts that trigger a wide range of diseases (*4*). The interaction of reactive nitrogen species and reactive oxygen species (ROS), *e.g.* NO, with toxic agents is strongly linked with inflammation, while the uncontrolled production of these species is linked to several diseases, including cancer, coronary heart disease, rheumatoid arthritis, asthma and Alzheimer's (*5*). During inflammatory processes, inflammatory mediators, such as NO and PGE2, are produced *via* the oxidation of L-arginine by inducible nitric oxide synthase (iNOS) and the conversion of arachidonic acid by COX-2. Moreover, NO is a key signalling biological molecule involved in vasodilation, regulation of blood pressure, neurotransmission and the host immune defence system. Inflammation can be regulated by suppression of the pro-inflammatory cytokines and NO production (*6*).

Currently, there is an increasing interest in food proteins and their constituent peptides as potential candidates for use as antioxidant and anti-inflammatory agents (*7*). The production and development of functional food or dietary supplements has increased significantly and these products have been used to aid human health (*8*). Peptides are highly selective, efficient and completely safe for humans (*9*). These peptides are often functionally inactive within native proteins and must be released by hydrolysis using enzymes such as Alcalase, Flavourzyme and Neutrase to achieve their specific bioactive roles (*10*). Recently, significant pharmaceutical research has been undertaken in an effort to use bioactive peptides from plants or animals as potential medicines and to underpin research into drug development (*11*). Currently, bioactive peptides that display anticancer (*12*), antimicrobial (*13*), hypocholesterolemic (*14*), antihypertensive (*15*), and anti-inflammatory properties (*16*) have been identified.

Poultry processing plants and related industries are expanding in many countries, especially in Thailand. Although chicken feather meal represents around 5–7 % of the body mass of chicken, it is a major waste byproduct of poultry processing, with large amounts produced annually, and its substantial accumulation causes potential environmental problems and pollution. Instead, chicken feather meal can be used as an alternative value-added product as animal feed or feed supplement and organic fertilizer due to its high (80–90 %) protein content, as well as being rich in hydrophobic amino acids (*17*). The objective of this study is to prepare peptides from chicken feather meal by enzymatic hydrolysis using microbial proteases and then to determine the *in vitro* anti-inflammatory effect of the isolated peptide samples on macrophage RAW 264.7 cells. The results indicate that chicken feather meal is a suitable source of anti-inflammatory peptides, which can be further developed in the pharmaceutical industry or as an ingredient in cosmetic products on the global market.

## MATERIALS AND METHODS

### Biological materials

The chicken feather meal used in this study was obtained from Betagro Science Center Co., Ltd. (Pathumthani, Thailand) and was ground to small particles and dried at 60 °C overnight. Then it was filtered through a 150-μm sieve to give a more homogenous particle size distribution for better accuracy and consistency of the results.

### Chemicals

Alcalase and Flavourzyme were purchased from Brentag (Mülheim, Germany). Neutrase was purchased from Novozymes (Bagsværd, Denmark). Acetic acid, ethanol and phosphoric acid were purchased from Merck (Gibbstown, NJ, USA). Acetonitrile (ACN), L-α-amino-*n*-butyric acid, bovine serum albumin (BSA), budesonide, curcumin from *Curcuma longa* (turmeric), Coomassie brilliant blue G-250,

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), disodium hydrogen phosphate, Dulbecco's modified Eagle medium (DMEM), foetal bovine serum (FBS), phosphoric acid, formic acid, hydrochloric acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), methanol, monosodium dihydrogen orthophosphate, mouse interferon gamma (IFN-γ), lipopolysaccharides (LPS) from *Escherichia coli*, potassium persulfate, sodium nitrite, (1-naphthyl)ethylenediamine (NED), sodium nitroprusside (SNP), sodium pyruvate, streptomycin sulphate, sulphanilamide, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich, Merck (St. Louis, MO, USA).

### Determination of the chicken feather meal amino acid content

The amino acid content of the chicken feather meal was determined based on the standard AOAC method 994.12 (*18*). One gram of chicken feather meal was dissolved in 5 mL of 6 M HCl in a test tube and then placed into a heating block at 110 °C for 24 h to liberate the amino acids. The internal standard (10 mL 2.5 mM L-α-amino-*n*-butyric acid in 0.1 M HCl) was added to this sample, diluted with deionized water to 250 mL and placed in a heating block at 55 °C for 10 min. Amino acids were analysed by reversed-phase high performance liquid chromatography (RP-HPLC; Waters Corporation, Milford, MA, USA) using a Hypersil GOLD column $C_{18}$ (50 mm×0.5 mm, 5 μm; Thermo Fisher Scientific, San Jose, CA, USA) shielded by a guard column on a Hypersil GOLD (30 mm×0.5 mm, 5 μm $C_{18}$; Thermo Fisher Scientific, San Jose, CA, USA, elution buffer of 20 mM sodium acetate (pH=4.90) and 60 % acetonitrile (ACN) at a flow rate of 0.3 mL/min.

### Preparation of enzymatic hydrolysate from chicken feather meal

The sieved chicken feather meal (5 μg) was mixed with 100 mL of phosphate-buffered saline (PBS; 20 mM phosphate buffer, 0.15 M NaCl, pH=7.2) and stirred at 4 °C overnight. The suspension was then hydrolysed with one of three types of microbial proteases (Alcalase, Flavourzyme (both from Brentag) and Neutrase (Novozymes)) at 0, 1, 2.5 or 5 % (*m/V*) for 4 h at 50 °C and pH=7 (except for Alcalase treatment which was at pH=8) with shaking (180 rpm; model Innova 4330 refrigerated floor incubator shaker; New Brunswick Scientific (UK) Ltd., Hatfield, Herts, UK). After hydrolysis, the mixtures were heated to 90 °C for 10 min to inactivate the enzymes and the samples were clarified by centrifugation (model Kubota 6500; Shimadzu, Kyoto, Japan) at 6440×*g* for 15 min. The supernatant (*i.e.* hydrolysate) was collected and stored at –20 °C until use.

### Protein content determination

The concentration of the chicken feather meal protein hydrolysate was determined according to the Bradford procedure (*19*), using BSA as the standard protein to construct the

calibration curve. For each sample three aliquots (20 µL) were separately mixed with 200 µL of the Bradford working solution (50 mg of Coomassie brilliant blue G-250 in 50 mL of methanol and 100 mL 85 % (*m/V*) phosphoric acid) in a 96-well plate (flat-bottom 96-well; Bio-Rad Laboratories, Inc., Hercules, MA, USA), incubated for 20 min and then the absorbance was measured at $A_{540\,nm}$ with a microplate reader (Multiskan GO; Thermo Fisher Scientific, Waltham, MA, USA). The protein content in each sample was determined from the standard curve derived from the BSA measurements.

### NO radical scavenging assay

The NO radical scavenging assay was slightly modified from that previously reported by Chantaranothai *et al.* (*20*). The peptide hydrolysates (5 µL) were mixed with 10 mM of SNP in PBS and incubated at room temperature for 150 min before the addition of 100 µL of Griess reagent (0.33 % sulfanilamide in 5 % phosphoric acid) and incubation for 5 min. Next, 100 µL NED were added and the samples were incubated for 30 min at room temperature before measurement of the absorbance at 540 nm using a microplate reader (model Multiskan GO, Thermo Fisher Scientific Inc., San Jose, CA, USA). The percentage of NO inhibition (%) and concentration of hydrolysate that inhibited 50 % of the NO radical production ($IC_{50}$) were calculated, the latter using the GraphPad Prism v. 6.01 for Windows (GraphPad Software Inc., San Diego, CA, USA) (*21*). Curcumin was used as the positive control. The NO scavenging activity (%) was calculated from:

$$\text{NO inhibition} = \frac{(A_{control} - A_{blank}) - (A_{sample} - A_{background})}{(A_{control} - A_{blank})} \cdot 100 \qquad /1/$$

where $A_{control}$ is the absorbance of the control (no sample), $A_{sample}$ is the absorbance of the sample, $A_{background}$ is the absorbance of the background (colour of the sample), and $A_{blank}$ is the absorbance of blank (deionized water).

### Fractionation and enrichment of the chicken feather meal peptide hydrolysate

Molecular mass cut-off by ultrafiltration

The chicken feather meal peptide hydrolysate was fractionated through ultrafiltration membranes with molecular mass cut-off (MMCO; Pellicon XL filter; Merck Millipore, Billerica, MA, USA) values of 10, 5, 3 and 0.65 kDa to give five different size fractions of >10, 5–10, 3–5, 0.65–3 and <0.65 kDa. The obtained protein hydrolysate fractions were stored at –20 °C until further use.

### Gel filtration chromatography

The fraction with the highest NO radical scavenging activity from the ultrafiltration step (*i.e.*<0.65 kDa fraction) was loaded onto a preparative Sephacryl S-100 gel filtration column (1.6 cm×60 cm, AKTA™ prime with Hitrap™; Amersham Biosciences, Uppsala, Sweden). The samples were eluted in distilled water at a flow rate of 0.5 mL/min and collected in 5-mL fractions (Fraction Collector Frac-950; Amersham

Biosciences), and the absorbance was monitored at $A_{280\,nm}$ to determine protein concentration. Fractions in each protein peak were pooled and the resulting separate fractions ($F_1$ to $F_4$) were assayed for NO radical scavenging activity.

### RP-HPLC

The protein fraction obtained by gel filtration chromatography with the highest NO radical scavenging activity ($F_2$) was further fractionated by RP-HPLC using a C18 column (250 mm×4.6 mm, Luna 5U; Phenomenex, Torrance, CA, USA). The peptides were eluted at room temperature using a gradient (0–100 % mobile phase B for 20 min) of mobile phase A (0.1 %, *V/V*, trifluoroacetic acid; TFA) and B (70 %, *V/V*, ACN in 0.05 %, *V/V*, TFA) at a flow rate of 0.7 mL/min. Chromatographic analyses were performed using the ChromQuest software (Thermo Fisher Scientific Inc., Waltham, MA, USA) (*22*). Peptides were detected by measuring the absorbance at $A_{280\,nm}$, the principal subfractions ($F_{2-1}$ to $F_{2-5}$) were isolated and collected, and the NO radical scavenging activity of three aliquots was determined while the rest was lyophilized.

### Identification of anti-inflammatory peptides

The principal subfractions (enriched peptides) obtained from the RP-HPLC fractionation that showed a marked NO radical scavenging activity and a sufficient yield ($F_{2-1}$ to $F_{2-3}$ and $F_{2-5}$) were identified by amino acid sequencing using quadrupole time-of-flight (Q-TOF) liquid chromatography-tandem mass spectrometry (Q-TOF LC-MS/MS) coupled with electrospray ionization (ESI; model Amazon SL, Bruker, Bremen, Germany). The MS/MS data were searched against the Swiss-Prot database with the MASCOT package (www.matrixscience.com) (*23*).

### Comparison between the NO radical scavenging activity of the pure synthetic peptide and enriched enzymatic subfraction

The peptide sequences obtained from the Q-TOF LC-MS/MS ESI analysis were synthesized using a 433A Synergy solid phase peptide synthesizer (model ABI 433A; Applied Biosystems, Foster City, CA, USA). The purity of the peptides was verified by analytical mass spectrometry (model Finnigan™ LXQ™; Thermo Fisher Scientific Inc., Waltham) coupled to a Surveyor HPLC. 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 for 50 min, where A was 0.1 % (*V/V*) formic acid in water and B was 100 % ACN. Mass spectral data from 300–1500 *m/z* were collected in positive ionization mode and HyStar v. 3.2 software (Bruker Daltonics Inc., Billerica, MA, USA) (*24*) was used to interface the HPLC and MS systems. All data obtained from LC-MS/MS were analysed using *de novo* sequencing. The NO radical scavenging activity of the pure synthesized peptides was determined in comparison with those obtained from the RP-HPLC fractionation.

## Cell culture

The RAW 264.7 cell line was maintained in complete medium (CM: DMEM supplemented with 10 %, $V/V$, FBS, 100 U/mL penicillin G, 0.4 mg/mL streptomycin sulfate, 1 %, $m/V$, sodium pyruvate and 1 %, $m/V$, HEPES at 37 °C in a humidified atmosphere with 5 % ($V/V$) $CO_2$. For routine maintenance in the culture (passage) (*22*), the cells were seeded in non-tissue culture treated dishes at approx. 10 % confluency and grown to approx. 80 % confluency, which typically took 2 days. The used medium was aspirated, cells were gently rinsed with PBS, then dislodged by gently scraping with a rubber spatula and harvested by centrifugation (Hettich, Tuttlingen, Germany) at 15 000×$g$ and 4 °C for 5 min.

## Pretreatment of macrophage RAW 264.7 cells

The macrophage RAW 264.7 cells were seeded in 96-well plates at $N$=10^4 cells/well in 100 µL CM and incubated overnight at 37 °C in a humidified atmosphere with 5 % $CO_2$. The medium was replaced with CM alone (negative control) or with different concentrations of the chicken feather meal protein hydrolysate (or fractions thereafter), or with budesonide (2.5 µg/mL) as a positive control, and incubated for 1 h. NO production was stimulated by the addition of 100 ng/mL of LPS and incubated for 12 h.

## Measurement of cell viability/proliferation by the MTT assay

The cytotoxicity activity of the $F_2$ and the peptide from $F_{2-1}$ (SNPSVAGVR) were determined according to Saisavoey *et al.* (*22*) with slight modifications. The macrophage RAW 264.7 cells were plated at $N$=10^4 cells/well in a 96-well plate and then incubated at 37 °C with 5 % ($V/V$) $CO_2$ for 24 h. Cells were then treated with different concentrations of the test sample (peptide) and LPS. Subsequently, 100 µL of a 5 mg/mL MTT solution (in PBS) were added to each well. Cells were incubated at 37 °C in a humidified atmosphere with 5 % $CO_2$ for 4 h before the medium was removed and DMSO added at 100 µL/well to solubilise the formed formazan crystals, and subsequently the $A_{540\ nm}$ was measured (model Multiskan GO; Thermo Fisher Scientific Inc.).

## Determination of NO production by macrophage RAW 264.7 cells

The level of NO production was determined by measuring nitrite production in the CM according to the Griess reaction. Macrophage RAW 264.7 cells were incubated with the test sample at various concentrations (control 0 µg/mL). Then 50 µL of sulfanilamide were added to 50 µL of the culture supernatant in a 96-well plate and incubated at room temperature for 10 min before 50 µL of the NED solution were added and incubated for 10 min. Finally, the $A_{540\ nm}$ was measured using a microplate reader (model Multiskan GO; Thermo Fisher Scientific Inc.).

## Detection of iNOS, TNF-α, COX-1, COX-2 and IL-6 mRNA by two-stage reverse transcriptase (RT)-PCR or quantification by two-stage quantitative real time (qrt)-RT-PCR analyses

Macrophage RAW 264.7 cells were pretreated for 1 h with a solvent (negative control), and synthesized peptide at various concentrations or 2.5 µg/mL of budesonide (positive control). The pretreated cells were then stimulated by the addition of 100 ng/mL of LPS and incubated for 12 h before their total RNA was harvested using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre; Lucigen, a part of LGC, Biosearch Technologies, Middleton, WI, USA) according to the manufacturer's instruction. The RNA concentration was measured using a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc.) at 260 nm. Reverse transcription of the total RNA (1 µg) was performed with oligo-dT primers using a Precision nanoScript II Reverse Transcription Kit (PrimerDesign, Camberley, UK) according to the manufacturer's protocol. The PCR was performed using selective primers for the β-actin (5'- ACCAACTGGGACGACATGGAGAA-3' and 5'-GTGGTGGTGAAGCTGTAGCC-3'), iNOS (5'- CCATCATGGAC-CACCACACA-3' and 5'-CCATGCAGACAACCTTGGTG-3'), IL-6 (5'- CATGTTCTCTGGGAAATCGTGG-3' and 5'-AACGCACTAG-GTTTGCCGAGTA-3'), TNF-α (5'-CCTGTAGCCCACGTCGTAGC-3' and 5'-TGACCTCAGCGCTGAGTTG-3'), COX-1 (5'- AGTGCGGTC-CAACCTTATCC-3' and 5'- GGTAAAGCCAGGACCCATCTTTC-3'), and COX-2 (5'- GGAGAGACTATCAAGATAGT-3' and 5'- ATGGT-CAGTAGACTTTTACA-3').

For the (qrt)-RT-PCR analysis, the PCR mixture comprised 1 µL cDNA, 1 µL of each primer (10 mM), 7 µL ultrapure water and 10 µL 2× qPCRBIO SyGreen Mix (PCR Biosystems Ltd, London, UK) to give a final volume of 20 µL. The (qrt)-RT-PCR reactions (performed separately per gene) were amplified using a MyGo Pro® Real time PCR apparatus (IT-IS International Ltd, Stokesley, UK) and thermal cycles at 95 °C for 2 min, followed by 40 cycles at 95 °C for 10 s, 68 °C (or 60 °C for TNF-α and IL-6) for 20 s and 72 °C for 30 s with melting at 55–95 °C for 1 min. The RT-PCR was performed using selective primers for the β-actin (5'-GATCAAGATCATTGCTCCTCCTG-3' and 5'-CGCAGCTCAGTAACAGTCCG-3'), iNOS (5'-CGGCAAA-CATGACTTCAGGC-3' and 5'-TAGGTCGATGCACAACTGGG-3'), IL-6 (5'-CTCTCTGCAAGAGACTTCCATCC-3' and 5'-ACAGGTCT-GTTGGGAGTGGTATC-3'), TNF-α (5'-GGGCAGGTCTACTTTG-GAGTCA-3' and 5'-ACAGATGGGGGCTCTGAGG-3'), COX-1 (5'-AGCTGCTGCTGAGAAGGGAGTT-3' and 5'-GGTAAAGCCAG-GACCCATCTTTC-3'), and COX-2 (5'-CTGACCCCCAAGGCT-CAAAT-3' and 5'-AAGTCCACTCCATGGCCCAG -3'). β-Actin was used as the internal reference gene and the three q-PCR reactions were analysed by relative quantitation. The relative gene expression level was determined using the Ct (threshold cycle) value by calculating as follows:

$$\text{Relative gene expression} = 2^{-\Delta\Delta Ct} \hspace{2cm} /2/$$

where ΔΔCt correlates with the increase in the threshold cycle of the gene (*25*).

## Statistical analysis

Numerical data are shown as the mean value±standard deviation, derived from three independent repeats. The data were subjected to analysis of variance followed by Duncan's multiple range *post hoc* tests, accepting significance at the p<0.05 level. The analysis was performed using the SPSS statistical software (*26*).

## RESULTS AND DISCUSSION

### Amino acid composition of chicken feather meal

The chicken feather meal contains essential and non-essential amino acids (**Table 1**). Glutamic acid, proline, serine, glycine and leucine are present in higher mass fractions than other amino acids. Active anti-inflammatory peptides from *Mytilus coruscus* were reported to consist of glycine, valine, serine, leucine, glutamine and phenylalanine (*5,27*). Moreover, it was reported that peptides consisting of glycine, cysteine, alanine, valine and serine could inhibit NO production, while peptides comprising glycine, cysteine and histidine exhibited anti-inflammatory activity by inhibiting the NF-κB signalling pathway (*28*). Certain amino acids, such as glycine, histidine, cysteine, glutamine and tryptophan, have been reported to possess anti-inflammatory properties (*29*).

### In vitro NO radical scavenging assay

The anti-inflammatory activity of the crude protein hydrolysates of chicken feather meal from the three protease digests was tested using a NO radical scavenging assay. NO radical scavenging activity of hydrolysates obtained with Alcalase and Neutrase increased with the increase of enzyme concentration up to 25 mg/mL, and then at 50 mg/mL the activity decreased. However, a dramatically increased NO radical

**Table 1.** Amino acid profile of chicken feather meal

| Amino acid | *w*/% |
|---|---|
| Alanine (Ala) | 3.80 |
| Arginine (Arg) | 6.30 |
| Glycine (Gly) | 6.82 |
| Aspatic acid (Asp) | 5.70 |
| Cysteine (Cys) | 2.90 |
| Glutamic acid (Glu) | 10.6 |
| Leucine (Leu) | 6.46 |
| Isoleucine (Ile) | 3.94 |
| Histidine (His) | 0.59 |
| Threonine (Thr) | 3.96 |
| Proline (Pro) | 8.37 |
| Lysine (Lys) | 1.45 |
| Methionine (Met) | 0.67 |
| Serine (Ser) | 7.84 |
| Phenylalanine (Phe) | 4.03 |
| Tyrosine (Tyr) | 1.10 |
| Tryptophan (Trp) | 0.20 |
| Valine (Val) | 5.85 |
| Total | 80.58 |

scavenging activity was observed after treatment with 10 mg/mL Flavourzyme (**Table 2**). Overall, the hydrolysate obtained with 10 mg/mL Flavourzyme exhibited the highest NO radical scavenging activity, with an $IC_{50}$=(5.5±1.0) µg/mL. Several studies have suggested that the antioxidant activity of peptides in hydrolysates varies depending on their amino acid composition, sequence and length (*30*). This suggests that enzymatic hydrolysis with different proteolytic enzymes and conditions would lead to the formation of different peptide sequences. In the same way, the anti-inflammatory activity of the Flavourzyme hydrolysate from *M. coruscus* showed a strong inhibition of NO production in macrophage RAW 264.7 cells (*27*). Since the hydrolysate obtained from chicken feather meal with 10 mg/mL Flavourzyme had the highest NO radical scavenging activity, it was selected for further study.

**Table 2.** Effect of enzyme concentration on NO radical scavenging activity of chicken feather meal hydrolysates expressed as concentration of hydrolysate that inhibited 50 % of NO production ($IC_{50}$)

| Enzyme | γ(enzyme)/(µg/mL) | | | |
|---|---|---|---|---|
| | 0 | 10 | 25 | 50 |
| | $IC_{50}$/(µg/mL) | | | |
| Alcalase | (134.0±4.7)[e] | (80.6±5.4)[d] | (30.0±1.8)[a] | (40.2±0.8)[b] |
| Flavourzyme | (158.4±1.5)[E] | (5.5±1.0)[A] | (82.1±7.0)[D] | (68.7±3.6)[C] |
| Neutrase | (175.7±4.0)[ee] | (133.0±5.4)[dd] | (37.0±0.4)[aa] | (78.7±3.9)[cc] |

Data are shown as mean value±standard deviation of triplicates. Different superscripts indicate significant difference (p<0.05). Curcumin was used as positive control ($IC_{50}$=(60.5±3.2) µg/mL)

### Enrichment of NO inhibitory peptides from the chicken feather meal hydrolysate obtained with 10 µg/mL Flavourzyme

The 50 % inhibitory concentrations of the obtained >10, 5–10, 3–5, 0.65–3 and <0.65 kDa fractions were (21.9±0.7), (10.4±0.7), (7.2±0.5), (5.8±0.6) and (3.6±0.3) µg/mL respectively (**Table S1**). Thus, it can be concluded that the inhibitory activity increased with the decrease of peptide fraction size. The molecular mass is an important parameter that correlates with the bioactivity of protein hydrolysates. Previous research has shown that the effective hydrolysates depend on their molecular mass distribution, *e.g.* Li *et al.* (*31*) reported that the peptide fraction with a molecular mass of 200–3000 Da was probably associated with a higher antioxidant activity, Nalinanon *et al.* (*32*) reported that low molecular mass peptides contributed to the antioxidant activity, and Wang *et al.* (*33*) reported that the biological activity of hydrolysates depended on the molecular mass of each peptide.

Arrays of NO inhibitory peptides with low molecular mass fractionated by ultrafiltration were previously shown to be effective in interacting with internal barriers to promote biological activities including antioxidant and anti-inflammatory activities (*34*). In addition, Lee *et al.* (*35*) also identified low molecular mass (<1.3 kDa) anti-inflammatory egg white peptides that were able to attenuate the symptoms of inflammatory bowel
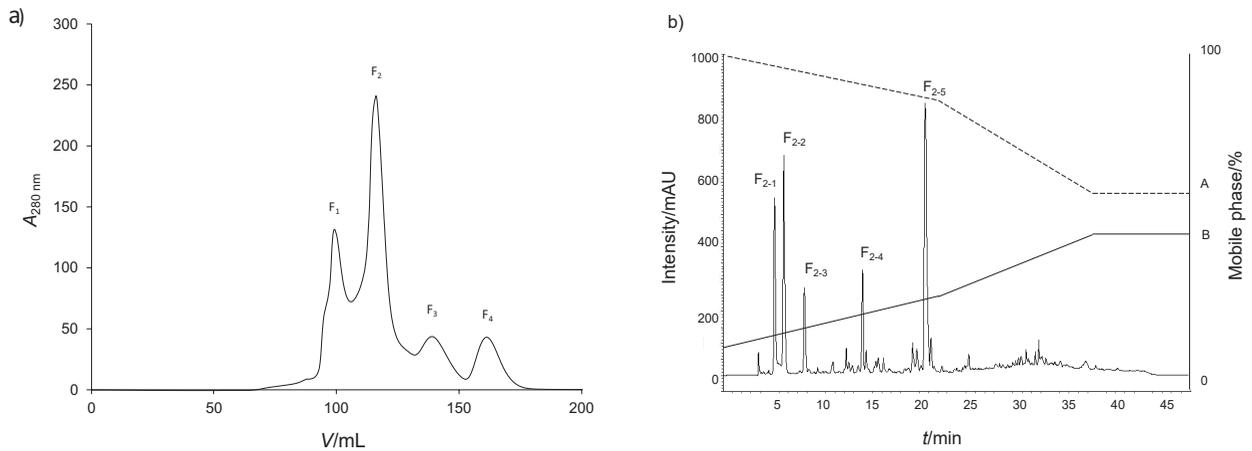
**Fig. 1.** Enrichment of bioactive peptides from the <0.65 kDa fraction of chicken feather meal hydrolysate obtained with 10 mg/mL Flavourzyme: a) the absorbance values of four fractions obtained after gel filtration chromatography of bioactive peptide, and b) RP-HPLC chromatograph of the $F_2$ fractionation into five principal subfractions ($F_{2-1}$ to $F_{2-5}$)

disease. Anti-inflammatory Leu-Asp-Ala-Val-Asn-Arg (683 Da) and Met-Met-Leu-Asp-Phe (655 Da) from *Spirulina maxima* (*11*), Gln-Cys-Gln-Gln-Ala-Val-Gln-Ser-Ala-Val (1061 Da) from *Ruditapes philippinarum* (*5*) and Gln-Cys-Gln-Cys-Ala-Val-Glu-Gly-Gly-Leu (1007 Da) from *Crassostrea gigas* were also isolated from marine organisms (*36*), while the tripeptide Val-Pro-Tyr (377 Da) from soybean exhibited anti-inflammatory effects (*37*). Therefore, the <0.65 kDa fraction from chicken feather meal was selected for further study.

Since peptide length is closely related to biological activities (antioxidant, antihypertensive, antimicrobial and immunomodulatory), gel filtration chromatography is suitable for fractionation, enrichment and concentration of proteins and peptides (**Fig. 1**). It has previously been reported to be useful for improving the anticancer activities of protein hydrolysates (*38*). Thus, further separation of lyophilized active <0.65 kDa fraction yielded four ($F_{1-4}$) protein fractions based upon segregation of the $A_{280\,nm}$ profile (**Fig. 1a**). Measurement of NO inhibitory activity of each of the four fractions revealed that $F_1$ and

$F_2$ had NO inhibitory activity with $IC_{50}$ values of (73.5±2.8) and (34.6±3.4) μg/mL (**Table S2**), respectively, whereas the yields of fractions $F_3$ and $F_4$ were too low to determine their $IC_{50}$ values.

Given the higher inhibitory activity (lower $IC_{50}$ value) of fraction $F_2$, it was selected for further fractionation. The $F_2$ fraction was divided into five principal subfractions ($F_{2-1}$, $F_{2-2}$, $F_{2-3}$, $F_{2-4}$ and $F_{2-5}$) (**Fig. 1b**) all with NO radical scavenging activity. The yield of subfraction $F_{2-4}$ was too low to determine its $IC_{50}$ value, while the $IC_{50}$ values of the remaining four fractions were: (16.8±1.5), (15.3±0.7), (17.4±0.4) and (34.0±1.9) μg/mL (**Table S3**), respectively, and they were subjected to amino acid sequencing by mass spectrometry.

*Identification of peptides by Q-TOF mass spectrometry and comparison of the NO radical scavenging activity between synthetic and enzymatic peptides*

The amino acid sequences and molecular masses of $F_{2-1}$, $F_{2-2}$, $F_{2-3}$ and $F_{2-5}$ are shown in **Table 3**. The *de novo* sequencing

**Table 3.** Amino acid sequences of RP-HPLC subfractions obtained from the chicken feather meal hydrolysate, identified by Q-TOF LC/MS/MS

| Fraction | Amino acid sequence | $M$/Da | Protein name | Organism | Accession number |
|---|---|---|---|---|---|
| $F_{2-1}$ | SNPSVAGVR | 886 | Putative E3 ubiquitin-protein ligase SH3RF2 | *Gallus gallus* | XP_015157384.1 |
| | | | Putative E3 ubiquitin-protein ligase SH3RF2 ioform X2 | *Gallus gallus* | XP_015157384.1 |
| | | | Putative E3 ubiquitin-protein ligase SH3RF2 isoform X1 | *Gallus gallus* | XP_414662.3 |
| | | | PRELI domain containing protein 3B | *Gallus gallus* | NP_001026037.1 |
| | | | Hypothetical protein RCJMB04_17b4, partial | *Gallus gallus* | CAG32065.1 |
| $F_{2-2}$ | SLFLHTHSIVADK | 1468 | Lactate dehydrogenase | *Phodilus badius* | GI629677270 |
| $F_{2-3}$ | AVLKKKVTSTFGR | 1435 | Cystine/glutamate transporter | *Gallus gallus* | XP_426289.3 |
| | | | Dynein heavy chain 1, axonemal | *Gallus gallus* | XP_015148334.1 |
| | | | Unconventional myosin-XVI isoform X2 | *Gallus gallus* | XP_004938593.1 |
| | | | Unconventional myosin-XVI isoform X1 | *Gallus gallus* | XP_416950.3 |
| | | | Transcription initiation factor TFIID subunit 9B | *Gallus gallus* | NP_001264725.1 |
| $F_{2-5}$ | LSPWPVKGV | 982 | Zinc finger matrin-type protein 1-like isoform X1 | *Gallus gallus* | XP_004936323.1 |
| | | | Neogenin isoform X6 | *Gallus gallus* | XP_015134538.1 |
| | | | Neogenin isoform X5 | *Gallus gallus* | XP_015134537.1 |
| | | | Neogenin isoform X4 | *Gallus gallus* | XP_015134536.1 |
| | | | Neogenin isoform X3 | *Gallus gallus* | XP_015134535.1 |

yielded a homology identification of 100 % based on the *Phasianidae* family. Each sequence contained 9–13 amino acid residues. Fraction $F_{2-1}$ was identified as Ser-Asn-Pro-Ser-Val-Ala-Gly-Val-Arg (SNPSVAGVR; 886 Da), $F_{2-2}$ as Ser-Leu-Phe-Leu-His-Thr-His-Ser-Ile-Val-Ala-Asp-Lys (SLFLHTHSIVADK; 1468 Da), $F_{2-3}$ as Ala-Val-Leu-Lys-Lys-Lys-Val-Thr-Ser-Thr-Phe-Gly-Arg (AVLKKKVTSTFGR; 1435 Da) and $F_{2-5}$ as Leu-Ser-Pro-Trp-Pro-Val-Lys-Gly-Val (LSPWPVKGV; 982 Da).

Since $F_{2-1}$, $F_{2-2}$, $F_{2-3}$ and $F_{2-5}$ exhibited NO radical scavenging activity, these obtained peptide sequences were synthesized and evaluated for their NO radical scavenging activity. However, the SNPSVAGVR peptide showed a high NO radical scavenging activity with an $IC_{50}$ value of (55.2±0.2) mM (**Table S4**). Regarding the relationship between the properties and anti-inflammatory activity of the amino acids, all of the peptide sequences contained mainly hydrophobic (leucine, histidine, phenylalanine, proline, tryptophan and valine) and positively charged (arginine and lysine) amino acids. These have been reported previously to have a high antioxidant activity (*39*). Hasegawa *et al.* (*28*) reported the anti-inflammatory activity of glycine and histidine, suggesting that glycine, histidine and cysteine act as anti-inflammatory agents by reducing NF-κB activation and inhibiting the expression of IL-6 in human coronary arterial endothelial cells. The relationships between the structure and the activity of the peptides and their mechanism of anti-inflammatory activities are not yet fully understood. However, the inhibition of NO production (%) by these peptides might be related to their amino acid composition and sequences since hydrophobic amino acid sequences in peptides have been suggested to play important roles in anti-inflammatory activities (*40*).

### Anti-inflammatory effect of the $F_2$ fraction in LPS-induced macrophage RAW 264.7 cells

The $F_{2-1}$ fraction showed no cytotoxicity to LPS-induced RAW 264.7 cells up to the highest tested concentration (120 µg/mL), where a cell viability of (92.6±0.6) % was still observed (**Fig. 2a**). Thus, the $F_{2-1}$ fraction can be used at concentrations up to 120 µg/mL. Likewise, the bioactive peptides of *Lupinus angustifolius* were previously shown not to be cytotoxic against RAW 264.7 cells (*41*). Therefore, the nontoxic $F_{2-1}$ peptide was evaluated further by testing its NO inhibitory activity.

In this study, an inflammation-type response was created in RAW 264.7 cells by treating them with 100 ng/mL LPS. The NO released from the LPS-stimulated RAW 264.7 cells was found to decrease with increasing concentrations of $F_{2-1}$ (2.5-40 µg/mL), where LPS treatment alone was considered as the control (P in **Fig. 2b**). The enriched $F_{2-1}$ peptide significantly inhibited NO production: at the concentration of 5 µg/mL by <15 % and at 20 µg/mL by >85 % (**Fig. 2b**). Peptides derived from marine sources were previously shown to possess NO inhibitory activities, such as the lupine protein hydrolysate from *L. angustifolius* in LPS-stimulated THP-1 cells (*41*) and *R. philippinarum* (*5*) and *M. coruscus* (*27*) in LPS-stimulated RAW264.7 cells. Both NO and proinflammatory cytokines play a critical role in the physiology
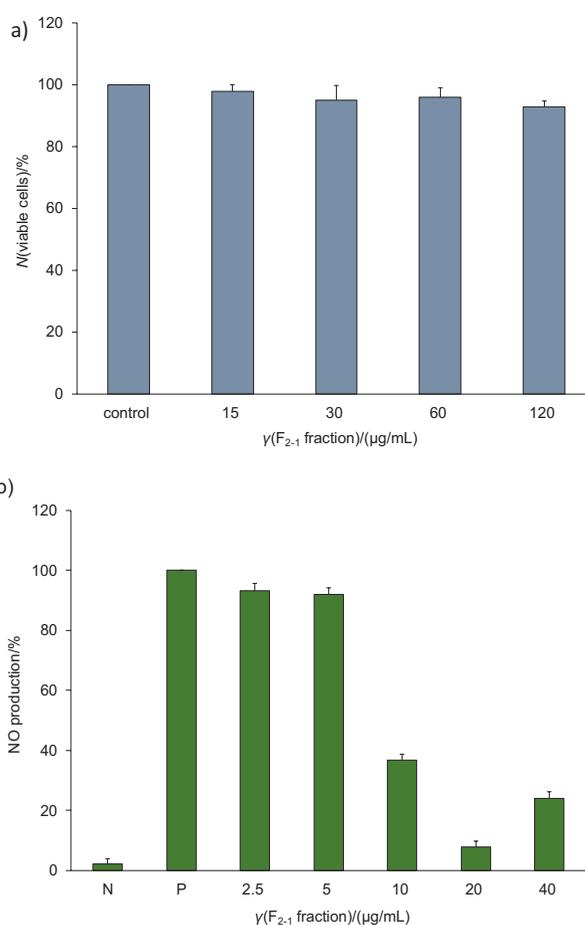


**Fig. 2.** Effect of different concentrations of $F_{2-1}$ fraction on: a) viability of and b) NO production by lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells, where N and P represent the negative (cells alone) and positive (cells+LPS) controls respectively

and pathology of diverse tissues, including the immune system of the body.

**Fig. 3** showed that in unstimulated macrophage RAW 264.7 cells the transcript levels of iNOS, COX-2, TNF-α and IL-6 were undetectable, but they were all expressed after treatment with LPS, and the $F_{2-1}$ fraction significantly inhibited these expression levels without affecting the constitutive expression of COX-1 and β-actin transcripts. Cotreatment of the RAW 264.7 cells with LPS and $F_{2-1}$ fraction still revealed the upregulation of COX-2 and TNF-α, whereas the expression of IL-6 and iNOS transcripts was downregulated. On the other hand, cotreatment with $F_{2-1}$ fraction negated the LPS-induced expression of COX-2 and TNF-α as well as IL-6 and iNOS. It is known that iNOS responds to various proinflammatory cytokines, including INF-γ, TNF-α and IL-6, and mediates several inflammatory responses (*42*). Moreover, it has been reported that the inducible isoforms of NOS and COX-2 are involved in the production of large amounts of NO and $PGE_2$, respectively (*43*). The activation of NF-κB is responsible for the induced expression of iNOS and COX-2 (*44*).

Oxidative stress has been reported to promote inflammation-related diseases through activation of the NF-κB pathway (*45*). Therefore, an antioxidative effect is a possible mechanism of limiting the damage induced by inflammatory reactions,
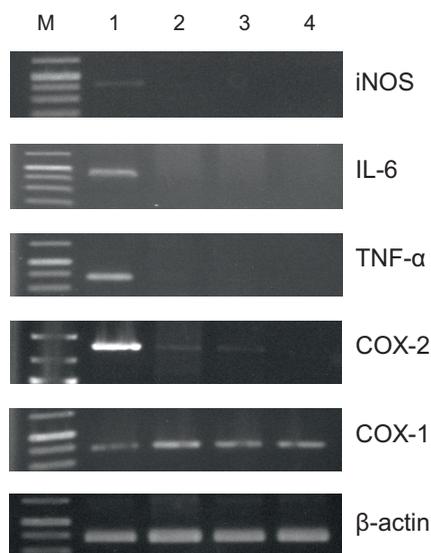
**Fig. 3.** Effect of $F_{2-1}$ fraction on transcript expression levels of iNOS, IL-6, TNF-α, COX-1, COX-2 and β-actin in lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells. Lane 1=LPS, lane 2=LPS+γ($F_{2-1}$ fraction)=40 μg/mL, lane 3=LPS+budesonide; 2.5 μg/mL (positive control), lane 4=no addition of LPS or $F_{2-1}$, and lane M=1000-bp size markers

although a detailed mechanism remains unclear. The NO radical scavenging assays in both *in vitro* and cellular models indicated that all the chicken feather meal hydrolysate-based fractions, especially fraction $F_{2-1}$, can potentially impair oxidative stress. The effects of peptide length or peptide mixture were not observed in the cellular anti-inflammatory assays, and this could be due to complex interactions of the constituents in each hydrolysate with the cell matrices, and the likelihood of further proteolytic processing of the peptides within the cell cultures.

Several studies have reported anti-inflammatory activities of low-molecular-mass peptides derived from food proteins, such as soybean (46), milk (47), soybean flour (48), egg (49) and whey (50). However, a high-molecular-mass peptide lunasin derived from soybean was reported to down-regulate the production of IL-6, IL-1β, NF-κB, iNOS and NO, indicating an anti-inflammatory function (46). Moreover, other factors, such as the peptide sequence and amino acid composition (not evaluated for all the hydrolysates in this study), can also determine the bioactivity of protein hydrolysates. This activity could be due to electron donation by the sulfhydryl group of cysteine leading to dimerization, although this mechanism would be more relevant in free radical quenching than ROS scavenging. Therefore, the structure-function relationships of antioxidative and anti-inflammatory peptides and protein hydrolysates appear complex, especially within physiological matrices.

### Anti-inflammatory activity of the synthesized SNPSVAGVR peptide

The SNPSVAGVR peptide displayed no cytotoxic effect against the RAW 264.7 cells at various concentrations

(15–120 mM), as shown in **Fig. 4**a. Stimulation of RAW264.7 cells with LPS (100 ng/mL) resulted in a significant increase in NO production compared to the unstimulated group, and SNPSVAGVR inhibited this NO production in a dose-dependent manner (**Fig. 4**b). The potential mechanism underlying the anti-inflammatory activity of SNPSVAGVR in LPS-induced RAW 264.7 macrophages was evaluated in terms of the transcript expression level of proinflammatory mediators. As shown in **Fig. 5**, LPS treatment markedly stimulated the expression of iNOS, IL-6, TNF-α and COX-2 (but not COX-1) in RAW 264.7 cells. When added to the cells, 60 mM of SNPS-VAGVR blocked the stimulatory effects of LPS on these genes (at $p<0.05$ and $p<0.001$). It is known that IL-6 and COX-2 have important effects on inflammation, and so it is relevant that SNPSVAGVR at 60 mM clearly decreased the IL-6 and COX-2 transcript expression levels by 96 and 83 % respectively. All of these results support the conclusion that the synthetic peptide had *in vitro* anti-inflammatory effect.
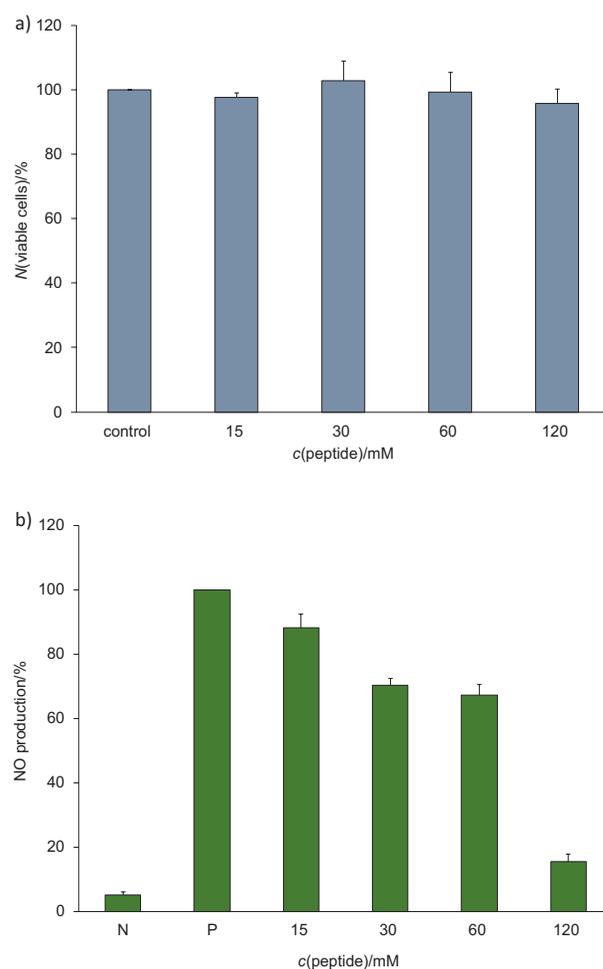


**Fig. 4.** Effect of different concentrations of synthesized SNPSVAGVR peptide on: a) viability of and b) NO production by lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells, where N and P represent the negative (cells alone) and positive (cells+LPS) controls respectively
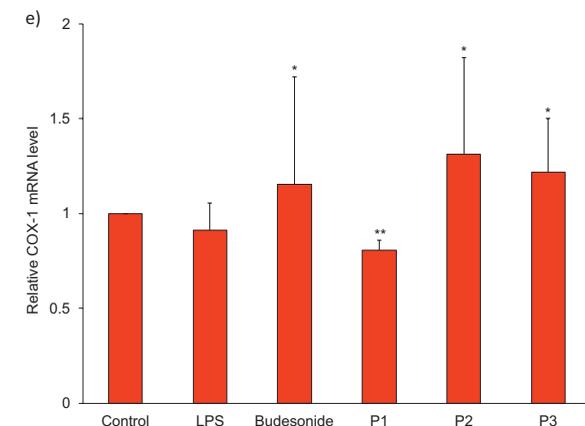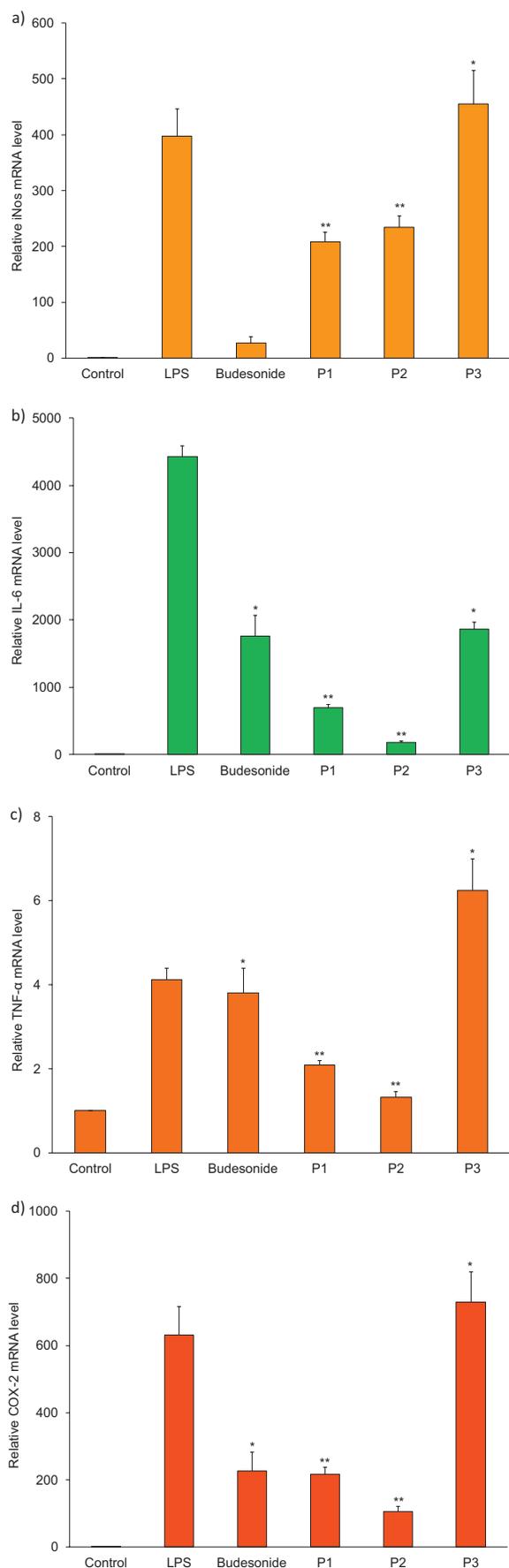
**Fig. 5.** Effects of synthesized SNPSVAGVR peptide on transcript expression levels of: a) inducible nitric oxide synthase (iNOS), b) interleukin 6 (IL-6), c) tumour necrosis factor (TNF-α), d) cyclooxygenase-2 (COX-2) and e) cyclooxygenase-1 (COX-1) in lipopolysaccharide (LPS)-stimulated RAW264.7 cells measured by qrtRT-PCR. Cells were incubated for 12 h with $\gamma$(LPS)=100 ng/mL in the presence of $c$(peptide)=30 ($P_1$), 60 ($P_2$) and 120 ($P_3$) mM, or with $\gamma$(budesonide)=2.5 µg/mL as positive control. Statistical significance is expressed as *$p<0.05$ and **$p<0.001$ compared to LPS

## CONCLUSIONS

Our results show that chicken feather meal peptides obtained by microbial enzyme hydrolysis exhibit anti-inflammatory activity, as evaluated by the NO radical scavenging assay. The chicken feather meal hydrolysate obtained with 10 mg/mL Flavourzyme had the highest NO radical scavenging activity. Peptides with the lowest molecular mass (<0.65 kDa) have the highest NO radical scavenging activity. Moreover, the $F_{2-1}$ fraction and pure SNPSVAGVR peptide are potent inhibitors of the LPS-induced expression of pro-inflammatory cytokines, including iNOS, TNF-α, COX-2 and IL-6. These results suggest that chicken feather meal hydrolysate can potentially be used as a natural anti-inflammatory agent in functional foods or pharmaceutical products.

## CONFLICT OF INTEREST

The authors report no conflict of interest. The authors alone are responsible for the content and writing of this article.

## SUPPLEMENTARY MATERIAL

All supplementary material is available at www.ftb.com.hr.

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