

Almond protein hydrolysate fraction modulates the expression of proinflammatory cytokines and enzymes in activated macrophages

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Simulated gastrointestinal treatment of almond proteins with pepsin and pancreatic proteases resulting in 16.6% degree of hydrolysis or 1.33 milliequivalent leucine per g protein yielded a hydrolysate that modulated excessive nitric oxide production in lipopolysaccharide-activated RAW264.7 macrophages. After fractionation, a resulting fraction of molecular size > 5 kDa retained the nitric oxide modulatory effect observed initially in the crude hydrolysate. The high molecular size fraction was found to modulate levels of proinflammatory cytokines, interleukin (IL)-6, IL-1 β , and tumour necrosis factor (TNF)- α in the activated cells. Immunoblotting analysis indicated that the hydrolysate fraction decreased the expression levels of inflammatory enzyme indicators, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 in the activated cells. RT-PCR analysis showed that treatment of the activated cells with the hydrolysate fraction resulted in the inhibition of relative gene expressions of proinflammatory IL-6, IL-1 β , TNF- α , iNOS and COX-2. These results indicate a potential application of almond protein hydrolysates against inflammatory conditions, and will contribute to delineating the possible contributions of proteins to health benefits attributed to almond consumption.

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1 Introduction

The consumption of nuts has been associated with positive health functions^{1,2} given their demonstrated effects in reducing blood lipid levels and markers of oxidative stress in hyperlipidaemic subjects,³ and in lowering postprandial glycaemia, insulinaemia and oxidative damage in healthy subjects.⁴ Inflammation is recognized as a major risk factor for the pathogenesis of coronary heart disease⁵ and the consumption of nuts has been linked to reducing inflammatory markers in humans.⁶ Incorporation of almonds into diets has resulted in the reduction of inflammatory acute phase C-reactive proteins, E-selectin, interleukin (IL)-6, tumour necrosis factor (TNF)- α and plasma protein carbonyl in human subjects, and has been attributed to the unsaturated fatty acids although other dietary components may have contributed to the effects in the “portfolio diets” containing a variety of foods with potential health benefits.^{7–9} The almond nut contains an excellent nutritional profile with zero cholesterol, 50% fats comprised mostly (about 86%) of mono- and polyunsaturated fatty acids, 21% proteins, 21%

carbohydrates comprised of 57% dietary fibre,¹⁰ and phytochemicals that are concentrated mostly in the seed pellicle.¹¹

To date, the potential contribution of proteins to the health benefits of almonds has not been extensively studied. The almond seeds contain 8 protein allergens including Pru du 6 also known as amandin or almonds' major protein.¹² Amandin belongs to the 11 S legumin-type globulin family and comprises two major proteins, Prunin (Pru) 1 and Pru 2.¹² Pru 1 exists as a hexamer comprising two trimers¹³ and both Pru 1 and Pru 2 are held together by disulphide bonds to form amandin.¹² These proteins are potential precursors of bioactive peptides, which can be liberated from the parent proteins by enzymatic hydrolysis.¹⁴ Bioactive peptides derived from food proteins have exhibited scores of beneficial biological functions.^{14,15} Bioinformatics analysis of the almond proteins using the BIOPEP software (<http://www.uwm.edu.pl/biochemia/index.php/pl/biopep>) indicated that Pru 1 (UniProtKB accession # Q43607) and Pru 2 (accession # Q43608) contain a myriad of bioactive peptide sequences within their primary structures, and *in silico* proteolysis of the almond proteins with gastrointestinal enzymes (pepsin, trypsin and chymotrypsin) yielded 41 (Pru 1) and 29 (Pru 2) low-molecular size peptides with potential anti-hypertensive and antioxidant properties. Moreover, peptides derived from food proteins have demonstrated notable modulatory effects against inflammatory reactions in cultured cells and animal models by inhibiting the production of proinflammatory markers such as nitric oxide, IL-1 β , IL-6, tumour necrosis factor (TNF)- α , inducible nitric oxide synthase (iNOS)

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and cyclooxygenase (COX)-2.^{16–18} Therefore, the physiological anti-inflammatory properties of whole almonds may partly be due to bioactive peptides released during gastrointestinal digestion of the nut proteins. The present study evaluated the modulatory effects of almond protein hydrolysates produced with gastrointestinal proteases on overexpressed inflammatory cytokines and enzymes in cultured macrophages under inflammatory conditions.

2 Materials and methods

2.1 Materials

California ground blanched almonds or almond flour containing 50% fats, 21.4% proteins and 21.4% carbohydrates were purchased from Bulk Barn (Guelph, ON, Canada). Dulbecco's modified Eagle's minimal essential medium (DMEM), trypsin-EDTA, penicillin/streptomycin and fetal bovine serum (FBS) were purchased from Gibco BRL Life Technologies (Grand Island, NY). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Amersham Pharmacia Biosciences (Piscataway, NJ), respectively. All solvents were of analytical grade.

2.2 Almond protein extraction and SDS-PAGE analysis

The almond flour was first defatted with hexane: 10% (w/v) of the flour in hexane was stirred at 4 °C for 2 h and the solvent was removed by decanting; the process was repeated after stirring overnight and the resulting defatted flour was then air-dried in a fume hood for 5 h. The protein components of the defatted almond flour were extracted by alkaline solubilisation: 10% (w/v) of the defatted flour was suspended in distilled water and adjusted to pH 10 using 1 M NaOH followed by continuous stirring for 1 h at a constant pH. Thereafter, the suspension was centrifuged at 15 000g for 30 min, and the supernatant was recovered and stored at –20 °C until further use. The protein content of the almond protein extract (AP) was determined with the Bio-Rad protein assay kit using a bovine serum albumin standard curve, and profiled by SDS-PAGE as follows: AP solution was mixed with sample buffer containing SDS and β -mercaptoethanol, and the mixture was heated at 50 °C for 10 min followed by centrifugation at 14 000g for 5 min. The mixture was loaded onto a 12% polyacrylamide SDS gel and electrophoresis was conducted at 200 V for 50 min in a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, CA, USA). The gel was then washed three times with water, stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL, USA) and visualized using a Chemi Genius 2 Syngene Bio imaging system.

2.3 Peptic and pancreatic hydrolysis of almond proteins

A portion of AP was diluted to 1% protein (w/v) and adjusted to 37 °C and pH 2.0 using 1 M HCl, followed by the addition of porcine stomach mucosa pepsin (475 units) at E/S 1 : 100 (protein basis) to initiate proteolysis. The hydrolytic reaction progressed for 90 min and the pH of the resulting solution was adjusted to 7.5 with 1 M NaOH. Thereafter, porcine pancreatin was added at E/S 1 : 100 (protein basis) and the reaction was

continued for another 2 h at constant temperature and pH. 1 mL aliquot was removed from the AP hydrolysate (APH) every 30 min during the peptic and pancreatic reactions and the degree of hydrolysis (DH) of AP was determined using the TNBS method;¹⁹ results were expressed as degree of hydrolysis and milliequivalent leucine (meq. Leu) per g protein. After hydrolysis, the solution was heated to 95–98 °C in a boiling water bath for 15 min to completely inactivate the enzymes and terminate proteolysis. The resulting solution was centrifuged at 15 000g for 30 min to remove undigested materials and then subjected to ultrafiltration.

2.4 Membrane ultrafiltration of the hydrolysate

APH was subjected to ultrafiltration with an Amicon stirred cell using membranes of different molecular weight cut-offs (MWCO) and a pressure of 40 psi. The hydrolysate was first passed through a membrane of MWCO 5 kDa; the resulting permeate and retentate were labelled as APH < 5 and APH > 5 kDa, respectively. Fractionation was also conducted with a 1 kDa MWCO membrane to produce APH < 1 and APH > 1 kDa fractions. APH > 1 kDa was then passed through a 5 kDa MWCO membrane to produce the APH1-5 kDa permeate fraction. The ultrafiltration fractions were collected on ice for 8 h and then frozen at –80 °C prior to freeze-drying. The freeze-dried samples were stored at –20 °C for further studies.

2.5 Determination of amino acid profile

Amino acid analysis of APH and its <1, 1–5, <5 and >5 kDa fractions was performed by the Advanced Protein Technology Centre, The Sick Kids Hospital, Toronto, Canada using a Waters Pico-Tag System after hydrolysis with 6 N HCl, pre-column derivatization with phenylisothiocyanate and reverse-phase UPLC analysis with peak detection at 254 nm.

2.6 Macrophage culture and treatments

The RAW264.7 macrophages were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 $\mu\text{g mL}^{-1}$ of streptomycin and 100 U mL^{-1} of penicillin. For the duration of the experiments, the macrophages were incubated at 37 °C in a humidified atmosphere with 5% CO₂. RAW264.7 cells were activated by treatment with 1 $\mu\text{g mL}^{-1}$ lipopolysaccharide (LPS). The effect of APH and its fractions in modulating the inflammatory mediators was evaluated in the presence of LPS.

2.7 Measurement of inflammatory mediators

2.7.1 Nitrite formation. After macrophage activation and sample treatment, the culture medium was collected for measurement of inflammatory mediators. Nitrite formation was used as an indicator of nitric oxide production in the cells and was determined with Griess reagent as reported earlier.²⁰ Briefly, 100 μL of the culture medium was mixed with 100 μL of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride). The mixture

was incubated for 15 min followed by measurement of absorbance at 550 nm using a microplate reader. The nitrite formed in the cell culture media was calculated using a sodium nitrite (dissolved in DMEM) standard curve.

2.7.2 Enzyme-linked immunosorbent assay (ELISA) for TNF- α , IL-6 and IL-1 β . An aliquot of the macrophage cell culture medium was withdrawn for each treatment and the levels of proinflammatory cytokines, IL-6, IL-1 β and TNF- α , released into the culture medium were quantified using Biotrak™ ELISA kits (Amersham Pharmacia Biosciences) according to the manufacturer's instructions.

2.8 Western blot analysis for COX-2 and iNOS

The macrophage cell lysates from the treatments (each containing 30 μ g of proteins) were analysed on an 8–12% SDS-PAGE gel gradient.²⁰ The proteins were transferred onto an immunoblot PVDF membrane and blocked with 5% skim milk in TBS containing 0.1% Tween 20 (TBST) for 1 h. Thereafter, primary monoclonal antibodies were added to the TBST (1 : 1000 dilution) and incubated for 1 h. Binding of the proteins were measured with the secondary antibody conjugated to horseradish peroxidase and enhanced using a chemiluminescence ECL assay kit (Amersham Pharmacia Biosciences, England, UK) according to the manufacturer's instructions. Visualization of the protein bands was conducted on a FUJIFILM LAS-3000 system (Tokyo, Japan). The basal levels of the proteins were normalized to the level of β -actin in the cell lysates.

2.9 Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted from the macrophages using Trizol reagent and RT-PCR was used to quantify the mRNA for IL-6, IL-1 β , TNF- α , iNOS and COX-2 as reported earlier.²⁰ Briefly, total RNA (2 μ g) was converted to single stranded cDNA using a reverse transcription system (Promega, Madison, WI, USA). The genes of interest were designed using the primer3 program, and their cDNA were amplified using the following primers: forward 5'-GGGCCTCAAAG-GAAAGAATC-3' and reverse 5'-TACCAGTTGGGGAACCTCTGC-3' for IL-1 β ; forward 5'-AGCCCCAGTCTGTATCCTT-3' and reverse 5'-CATTTCGAGGCTCCAGTGAAT-3' for TNF- α ; forward 5'-AGTTGCCTTCTGGGACTGA-3' and reverse 5'-CAGAATTGCCATTGCACAAC-3' for IL-6; forward 5'-TTCCAGAATCCCTGGACAAG-3' and reverse 5'-TGGTCAAACCTCTGGGGTTC-3' for iNOS; forward 5'-AGAAG GAAATGGCTGCAGAA-3' and reverse 5'-GCTCGGCTTCCAGTATT GAG-3' for COX-2; forward 5'-GAGTCAACGGATTTGGTCGT-3' and reverse 5'-GACAAGCTTCCCGTTCTCAG-3' for GAPDH. Each amplification cycle was conducted at 95 °C for 45 s, 60 °C for 1 min and 72 °C for 45 s. After 35 cycles, the amplified PCR products were analysed by agarose gel (1.5%) electrophoresis at 100 V for 30 min. Thereafter, gels were stained with 1 mg mL⁻¹ ethidium bromide and visualized under UV light using AlphaEase® gel image analysis software (Alpha Innotech., San Leandro, CA).

2.10 Statistical analysis

The cell culture experiments were conducted in triplicate and each value was expressed as means \pm standard deviation.

Where applicable, the statistical significance of difference between treatments was analysed by one-way analysis of variance followed by a Holm-Sidak multiple comparison test using SigmaPlot 11.0 (Systat Software, San Jose, CA, USA).

3 Results and discussion

3.1 Almond protein hydrolysis

Results from SDS-PAGE under reducing conditions indicated that AP contained the polypeptides from Pru 1 and Pru 2 with major protein bands corresponding to the α (~40 and 35 kDa) and β subunits (~22 and 21 kDa) of the amandin proteins, respectively.^{12,21} Sequential *in vitro* hydrolysis of the AP with gastrointestinal pepsin and pancreatin resulted in a hydrolysate with 6.5% and 16.6% DH, equivalent to 0.52 and 1.33 meq. Leu g⁻¹ proteins, respectively. The maximum DH was attained after 180 min of the pancreatin reaction. The resulting hydrolysate was separated into fractions of $M_w < 1$, 1–5, < 5 and >5 kDa in order to evaluate the impact of the molecular size range on bioactivity. The bioactivity of peptides has been shown to be dependent on various factors including nature of amino acid residues and molecular weight.¹⁴ The amino acid profile, as shown in Table 1, indicates that crude APH and the >5 kDa fraction contain the highest amounts of total sulphur-containing amino acid residues, especially for cysteine, whereas no particular trend was observed for other amino acid groups. If resistant to further physiological hydrolysis, it is expected that peptides in the APH fractions will be differentially absorbed to exert their physiological effects in different tissues. There is

Table 1 Amino acid composition (g per 100 g protein) of the almond protein hydrolysates (APH) and their ultrafiltration fractions^a

	APH	<1 kDa	1–5 kDa	<5 kDa	>5 kDa
Asx	11.7	13.0	13.3	12.7	11.9
Glx	28.8	25.3	30.7	25.3	31.3
Ser	3.87	3.96	3.69	4.02	3.78
Gly	4.72	4.26	4.94	4.31	5.07
His	2.43	2.41	2.40	2.42	2.43
Arg	13.3	13.5	12.1	13.2	12.3
Thr	2.41	2.31	2.22	2.57	2.33
Ala	3.87	4.33	3.84	4.37	3.58
Pro	3.82	3.95	4.29	3.95	3.92
Tyr	3.05	3.46	2.08	3.37	2.44
Val	3.68	4.11	3.72	4.20	3.54
Met	0.58	0.37	0.40	0.37	0.71
Ile	3.40	3.77	3.53	3.82	3.29
Leu	5.24	6.05	4.65	6.08	4.59
Phe	5.57	6.54	4.81	6.60	4.63
Lys	2.27	2.06	1.88	1.94	2.36
Cys	1.05	0.31	0.92	0.51	1.49
Trp	0.09	0.21	0.39	0.12	0.17
HAA	30.3	33.1	28.6	33.3	28.3
CAA	18.0	18.0	16.4	17.6	17.1
SCAA	1.63	0.68	1.32	0.88	2.20
AAA	8.71	10.2	7.28	10.1	7.24

^a Asx = Asp + Asn; Glx = Glu + Gln; HAA, total hydrophobic acids; CAA, total cationic amino acids; SCAA, total sulphur-containing amino acids; AAA, total aromatic amino acids.

considerable evidence that di- and tripeptides have the highest chance of crossing the enterocytes into circulation through dedicated peptide transporters²² whereas the larger unabsorbed peptides, if intact, will likely be transported to the lower intestine for further metabolism. Moreover, lunasin, a 43-amino acid residue peptide, has been reported to be absorbed intact into the blood stream of humans after consumption of soybeans,²⁴ indicating the possibility of direct or mediated transepithelial transport of larger peptides in human enterocytes. In this study, the low molecular weight peptides are contained in APH < 1 and < 5 whereas APH > 5 contained components of the highest molecular weights.

3.2 Effects of almond protein hydrolysate fractions on nitric oxide production in the macrophages

Endogenous nitric oxide plays important physiological roles in mediating cellular signal transduction but its overproduction in macrophages during inflammation can lead to deteriorating effects primarily due to its reaction with superoxide radicals to produce highly reactive oxidant peroxynitrite and subsequently hydroxyl radicals.²³ Therefore, the suppression of mitogen-induced nitric oxide production in macrophages constitutes a strategy for protecting cellular constituents from oxidative damage during inflammation. In the present study, activation of the RAW264.7 macrophages with LPS resulted in over 6-fold increase in nitric oxide in the culture supernatant. As shown in Fig. 1, treatment of the activated cells with crude APH and its < 1, 1–5, < 5 and > 5 kDa fractions at 500 $\mu\text{g mL}^{-1}$ resulted in the inhibition of nitric oxide production by 51%, 14%, 19%, 30% and 51%, respectively and 12–27% at twice lower concentration. These activities are moderate compared to the effect of a pea protein hydrolysate that inhibited nitric oxide production by 20% at 20-times lower concentration in LPS/interferon- γ activated RAW264.7 NO(-) macrophages.¹⁶ APH > 5 kDa exhibited the best activity of the fractions, with dose-dependence, in comparison to the crude hydrolysate. All the samples inhibited

nitric oxide production at 2-times lower concentration to levels comparable to 20% inhibitory activity reported for yak protein hydrolysates in LPS-stimulated murine peritoneal macrophages.¹⁷ The inhibition of nitric oxide can be due to direct scavenging of the reactive oxygen species or modulation of cellular inflammatory pathways. The sulfhydryl group of Cys can react with nitric oxide under aerobic conditions to form an *S*-nitrosothiol adduct.²⁵ This implies that the lower nitrite concentration in cells treated with APH and > 5 kDa fraction could be attributed to their higher Cys composition compared to the other fractions (Table 1). However, this may not be a plausible mechanism due to the potential dissociation of the *S*-nitrosothiol adduct, which will lead to the release of nitric oxide into the cell media for nitrite formation. Based on the observed activity, the APH > 5 fraction, which contained higher molecular weight peptides, was selected to further evaluate its effects on proinflammatory proteins and gene expressions in the activated macrophages.

3.3 Modulation of cellular production of proinflammatory IL-6, IL-1 β and TNF- α

The effect of APH > 5 on proinflammatory cytokines was evaluated in the RAW264.7 macrophages in the presence of LPS. Incubation of the cells with LPS alone for 24 h resulted in increased secretion of cytokines IL-6, IL-1 β and TNF- α by 8, 7 and 5-folds, respectively, as measured by ELISA. As shown in Fig. 2, treatment of the activated cells with APH > 5 dose-dependently decreased ($P < 0.05$) the secreted levels of the proinflammatory cytokines compared to the LPS-only treatment. Moreover, the modulatory effects induced by APH > 5 was more pronounced for IL-1 β , which was inhibited by up to 75% (from 546 to 136 $\text{pg IL-1}\beta \text{ mL}^{-1}$) at 200 $\mu\text{g mL}^{-1}$ of the peptide sample in the activated cells; a similar sample amount did not inhibit the cellular secretion of IL-6 and TNF- α by up to 50%. A recent study reported that a similar concentration of yak protein hydrolysates inhibited the secretion of IL-6 by 61%, and IL-1 β and TNF- α by about 50% in activated murine peritoneal macrophages.¹⁷ Moreover, pea protein hydrolysates produced with thermolysin exhibited more promising effects in cultured macrophages by inducing similar decreases in IL-6 (by 80%) and TNF- α (by 35%) at a lower concentration.¹⁶ Proinflammatory cytokines play important roles in upregulating inflammatory processes in macrophages. TNF- α , an important mediator of inflammation, binds its receptors to trigger downstream activation of inflammatory gene expression. Thus, inactivation of TNF- α has become a major target towards the treatment and management of inflammatory conditions.²⁶ The present study has demonstrated that an almond protein hydrolysate fraction can inhibit TNF- α secretion and cellular levels of downstream proinflammatory IL-6 and IL-1 β in activated macrophages.

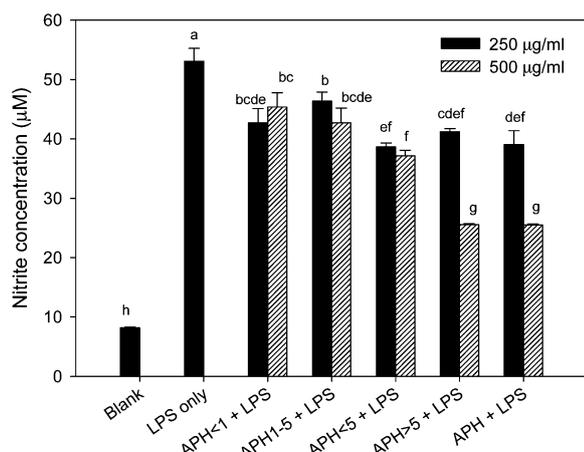


Fig. 1 Effects of almond protein hydrolysate and its ultrafiltration fractions on nitric oxide production in activated RAW264.7 macrophages; bars with different alphabets in each chart represent significantly different mean values with $P < 0.05$.

3.4 Effects of almond protein hydrolysate fraction on iNOS and COX-2 enzymes

iNOS catalyses the production of NO from arginine and molecular oxygen in the macrophages whereas COX-2 activity

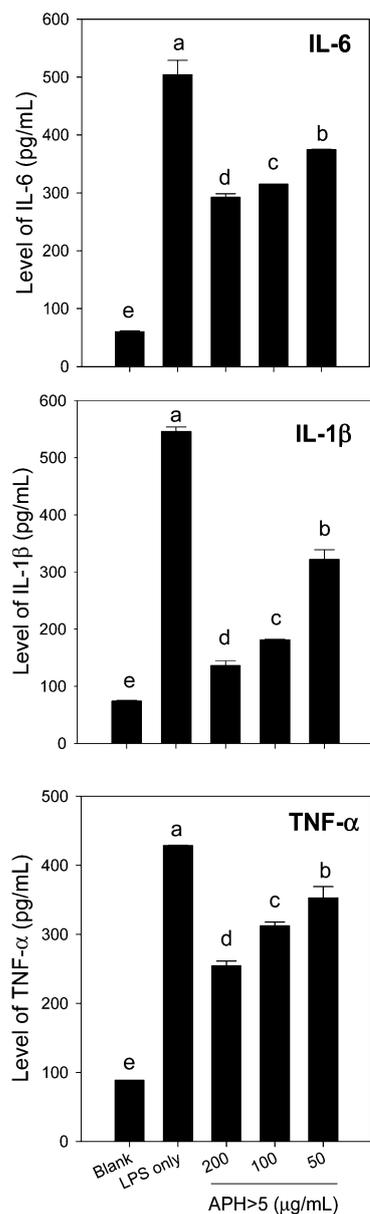


Fig. 2 Modulatory effect of almond protein hydrolysate fraction with molecular weight >5 kDa (APH > 5) on secreted proinflammatory cytokines as evaluated by immunoassays. RAW264.7 cells were treated with three concentrations of APH > 5 and incubated for 1 h followed by LPS treatment and further 24 h incubation prior to immunoassays; bars with different alphabets in each chart represent significantly different mean values with $P < 0.05$.

leads to the conversion of arachidonic acid to prostaglandin (PG)-H₂, a precursor of PGE₂, which contributes to pathogenesis during inflammatory diseases.^{23,27} The expression of iNOS and COX-2 is induced by reactive oxygen species, bacterial LPS, TNF- α and IL-1 β during inflammation through the nuclear factor- κ B signalling pathway.^{23,27} Thus, it is expected that downregulation of the inflammatory cytokines will decrease endogenous levels of iNOS and COX-2 and associated pathogenesis during inflammation. Fig. 3 shows that the presence of APH > 5 in the LPS-treated macrophages induced a lowering of the translational levels of iNOS and COX-2 relative to β -actin based on

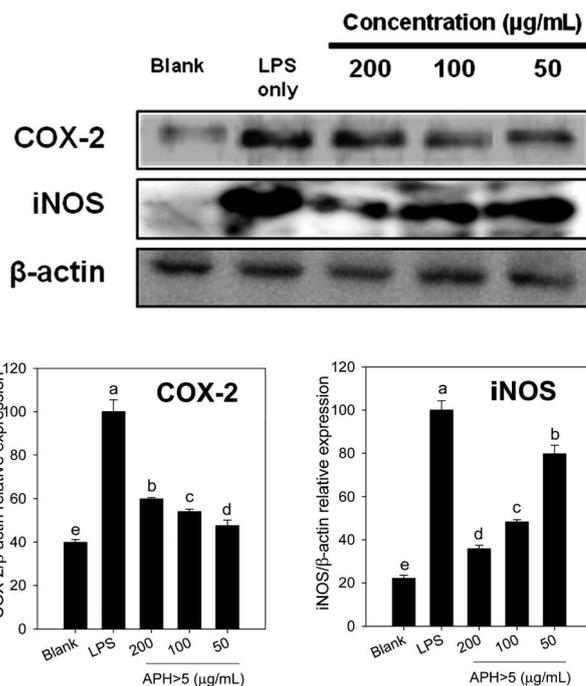


Fig. 3 Effect of APH > 5 on the expression of COX-2 and iNOS proteins relative to β -actin in LPS-activated macrophages as evaluated by Western blot analysis; bars with different alphabets in each chart represent significantly different mean values with $P < 0.05$.

Western blot analysis. The modulatory activity of the sample on relative expression of iNOS was dose-dependent with the maximum inhibitory effect of 64% at 200 $\mu\text{g mL}^{-1}$ APH > 5. However, a 4-fold lower amount of APH > 5 induced over 52% inhibition of the relative COX-2 expression; subsequent increases in sample amounts did not enhance the effect but resulted in a dose-dependent decrease in activity *via* an unknown mechanism.

3.5 Effects on mRNA expressions of IL-6, IL-1 β , TNF- α , iNOS and COX-2

To verify whether the observed modulatory activities were exerted at the gene (transcriptional) or protein (translational) levels, the effect of APH > 5 kDa fraction on gene expression of proinflammatory cytokines and enzymes was evaluated. The macrophages were treated with APH > 5 and LPS, and following incubation the total mRNA were extracted, amplified with specific primers and quantified by RT-PCR. Treatment of the macrophages with LPS-only elevated the mRNA expression of IL-6, IL-1 β , TNF- α , iNOS and COX-2 by about 2-folds relative to the blank treatment without LPS (Fig. 4). These proinflammatory gene expressions were inhibited to various degrees in the presence of 50, 100 and 200 $\mu\text{g mL}^{-1}$ APH > 5 as shown in Fig. 4 and the observed effects were dose-dependent except for TNF- α based on RT-PCR quantification. The expression of the house-keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was not affected by LPS and APH > 5 treatments. Based on the RT-PCR results, it is possible that the modulatory effects of APH > 5 on the inflammatory mediators at the protein

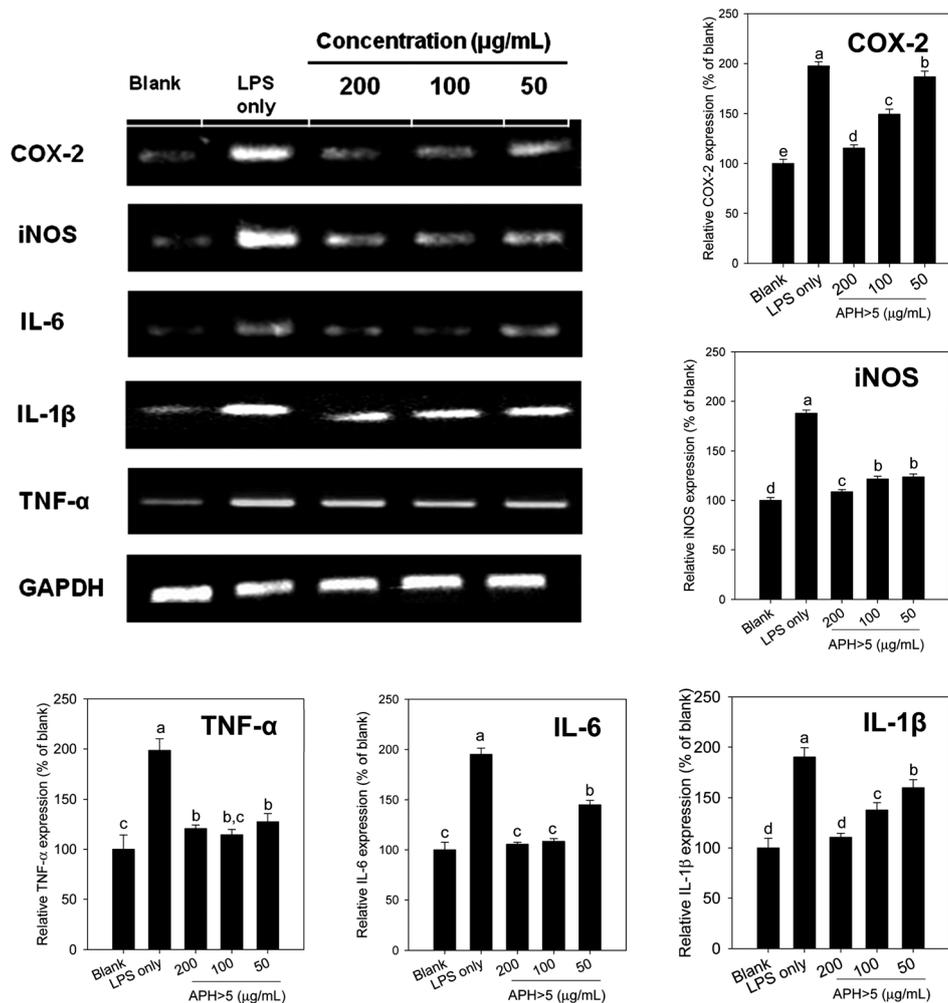


Fig. 4 Effects of various concentrations of APH > 5 on mRNA expression of IL-6, IL-1 β , TNF- α , iNOS and COX-2 in LPS-activated macrophages; bars with different alphabets in each chart represent significantly different mean values with $P < 0.05$.

level (Fig. 2 and 3) were directly related to the effects observed at the gene transcriptional level. Similarly, previous studies have demonstrated that inhibition of these cytokines are associated with the anti-inflammatory activities of soy and pea protein hydrolysates and peptides in cultured mammalian cells and animal models of inflammation.^{16,18,28} However, there is scarcity of information on the structural requirements of food protein-derived peptides for potent anti-inflammatory functions.

In conclusion, a protein hydrolysate fraction of molecular weight >5 kDa, derived from simulated gastrointestinal digestion of almond proteins, exhibited modulatory effects on LPS-activated overexpression of proinflammatory mediators in cultured macrophages. Due to the relationship between inflammation and cardiovascular disease, these results will contribute to understanding the possible roles of proteins in the health benefits attributed to whole almond consumption. However, the hydrolysate fraction concentrations that showed anti-inflammatory effects in this study may not represent the final doses attained by the constituent bioactive molecules in biological fluids. Therefore, further work is needed to evaluate

the absorption and bioavailability of the hydrolysate fraction constituents in target tissues, and reassess their anti-inflammatory functions at physiological doses. Moreover, the positive effects of the almond protein hydrolysate fraction can be further explored towards the discovery of natural agents for formulating functional foods and nutraceutical products that can be used to modulate adverse inflammatory reactions in humans. The high molecular size range of the almond protein hydrolysate fraction may preclude increased absorption of intact peptide components after ingestion for cardiovascular health promotion. However, if conserved within the colon and based on observed inhibition of TNF- α expression, there is a possibility that the components of the hydrolysate fraction can modulate inflammatory reactions in the gastrointestinal tract especially during inflammatory bowel disease. Finally, based on the results of this study, further work is needed to substantiate the anti-inflammatory functions of APH > 5 kDa fraction using appropriate animal models, and to identify the active peptide sequences in order to obtain a better understanding of structure–function relationships.

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