Food Bioscience 49 (2022) 101860

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Differential effects of oilseed protein hydrolysates in attenuating inflammation in murine macrophages

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ARTICLE INFO

Keywords:
Oilseeds
Ultrafiltration
Protein hydrolysate
Bioactive peptide
Inflammation
Anti-inflammatory properties
LPS binding

ABSTRACT

Proteins from underutilized defatted oilseed meals have been proposed as promising sources of bioactive peptides. This study was conducted to determine the anti-inflammatory activities of five oilseed proteins (flaxseed, rapeseed, sunflower, sesame and soybean) hydrolysed via alcalase, pepsin (at pH 1.3 and pH 2.1), respectively, and to compare these against two dairy proteins (whey, casein). The potential of protein hydrolysates of three different molecular weight fractions (M_w > 10 kDa, 3–10 kDa, < 3 kDa) to modulate nuclear factor kappa B (NF-κB) signalling was screened via RAW-Blue™ reporter cells. Fractions with M_w < 3 kDa of pepsin (pH 1.3)-treated protein hydrolysates were subsequently selected to validate anti-inflammatory properties in RAW 264.7 macrophages. Rapeseed fractions showed greatest potency to attenuate inflammation, via efficiently down-regulating the expression of IL-6 (−49.1%), IL-1β (−58.6%), iNOS (−41.9%) and COX-2 (−58.7%) and up-regulating the IL-10 (+47.2%) mRNA level at 2000 μg peptides/mL. Rapeseed, sesame and casein demonstrated marked repression of NF-κB pathway, through down-regulating NF-κB1, p65 and/or IκBα mRNA levels. In addition, rapeseed, sesame and soybean reduced the expression of TLR4 and/or CD14 associated with attenuated LPS recognition. In addition, it was confirmed that rapeseed and soybean hydrolysates showed capabilities to bind 43.9 and 52.4% of LPS in solution, thereby weakening inflammatory response; an effect that could at least partially be related to the presence of hydrophobic amino acids. To summarize, current data demonstrate differing capacity of plant protein hydrolysates to interact with inflammatory signalling, indicating the need for further research into the molecular mechanisms of peptide action.

1. Introduction

Oilseed crops are widely utilized in agricultural industry mainly due to their high oil content. Oilseeds, such as rapeseed and soybean, are of significant economic importance because they are not only profitable in yielding edible vegetable oils with low cholesterol content but can also be utilized as biofuel. Thus, they contribute significantly to the incomes of farmers (Adeleke & Babalola, 2020; Jaeger & Siegel, 2008). The remainders of these oil industries, defatted oilseed cake, contain approximately 20–25% protein (Moure et al., 2006), which is most frequently utilized either as organic fertilizer or cheap livestock feed. At present, there is an increasing interest in plant proteins, including oilseed proteins, for their functional properties and potential health benefits, some of which are linked to bioactive peptides. Bioactive peptides are small protein fragments, that are increasingly recognized as an important group of compounds with a range of properties, such as antioxidant (He et al., 2013; Sánchez-Velázquez et al., 2021), antihypertensive (Aondona et al., 2021), antidiabetic (Nongonierma & FitzGerald, 2015) and anti-inflammatory (He et al., 2019) activities. They can be released from the parent proteins via hydrolysis or fermentation and usually comprise 2 to 20 amino acid residues.

Inflammation is a complex and innate defensive immune response that involves various cells i.e., macrophages and a range of signalling molecules; and can be activated by injury, infection or other harmful stimuli. Acute inflammation is essential for initiating, maintaining and regulating the immune response to infection and tissue damage, whilst uncontrolled prolonged inflammation is closely linked to the pathogenesis of several chronic diseases, including metabolic disorders, atherosclerosis and cancers (Hotamisligil, 2006). Lipopolysaccharide (LPS), an endotoxin secreted by gram-negative bacteria, is widely used

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https://doi.org/10.1016/j.fbio.2022.101860
Received 30 December 2021; Received in revised form 24 April 2022; Accepted 16 June 2022
Available online 19 June 2022

as a stimulus to evoke the multiple downstream intracellular signalling cascades, including activation of nuclear factor-kappa B (NF-κB) signalling, in various cell types to study inflammatory response. Dysregulation of NF-κB activation generates inflammatory profiles and promotes the increased synthesis of pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-1β (IL-1β), tumour necrosis factor (TNF-α); as well as inducible nitric oxide synthases (iNOS) and cyclooxygenase-2 (COX-2) that generate nitric oxide (NO) and prostaglandins (PGs), respectively (Tornatore et al., 2012). Therefore, targeting NF-κB signalling can be a crucial and effective strategy for characterizing natural anti-inflammatory agents.

Anti-inflammatory activities of oilseed derived peptides and protein hydrolysates have been reported in several studies. According to Udenigwe et al. (2009), low M_w fractions of pepsin-, ficin- or papain-treated flaxseed protein hydrolysates significantly reduced the generation of NO in RAW 264.7 macrophages, with IC_{50} values of 0.250, 0.504 and 0.215 mg protein/mL, respectively. Using the same cell line, He et al. (2019) identified three rapeseed peptides, LY, RALP and GHS, that markedly suppressed expression of iNOS, IL-6 and TNF-α and the secretion of NO. They further confirmed that LY, RALP and GHS inhibited IL-6 and TNF-α levels *in vivo* using a spontaneously hypertensive rat (SHR) model. In a different study, four novel sunflower-derived peptides (YFVP, SGRDP, MVWGP and TGSYTEGWS) were confirmed as blunting the activation of NF-κB signalling in human monocytic leukemia THP-1 cells (Velliquette et al., 2020). As well, soybean is widely reported as a noticeable source of anti-inflammatory peptides, such as the tripeptide LSW (Lin, Q. et al., 2017; González-Montoya et al., 2018; Hao et al., 2020). Whilst evidence on anti-inflammatory peptides/hydrolysates from several different protein sources is available, there are gaps in our knowledge regarding the molecular mechanisms underlying their cellular actions. Further, in view of the heterogeneity of enzymatic hydrolysis conditions applied to different protein samples, there is a huge range of variation of bioactive peptide profiles that can be generated from the same proteins.

Therefore, our study aimed to directly compare the anti-inflammatory properties of five alcalase- or pepsin-treated oilseed protein hydrolysates (flaxseed, rapeseed, sunflower, sesame and soybean), against hydrolysates from two dairy proteins, whey and casein, under the same well-controlled hydrolysis conditions. As bovine milk proteins, whey and casein show substantial differences to plant proteins, and have been documented as good precursors of bioactive peptides (Brandelli et al., 2015; Phelan et al., 2009). Therefore, these were selected for the purpose of comparison. The present study investigated the potency of crude protein hydrolysates and three different M_w fractions ($M_w > 10$, 3–10 and < 3 kDa) to attenuate NF-κB signalling via RAW Blue™ reporter cells. Wild-type RAW264.7 macrophages were used to confirm the effects of selected fractions of pepsin (pH 1.3)-treated protein hydrolysates on the expression of pro- and anti-inflammatory cytokines, mediators and transcription factors. In addition, this study investigated the potential of peptide-containing samples to bind and immobilize LPS, as well as hydrophobicity, which was explored as an indicator of anti-inflammatory activity.

2. Materials and methods

2.1. Materials and reagents

Pepsin from porcine gastric mucosa, alcalase from *Bacillus licheniformis*, LPS from *Escherichia coli* O111:B4, and LPS from *E coli* O111:B4 FITC conjugate, neutral red, casein and o-Phenylenediamine (OPD), Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum, penicillin and streptomycin were purchased from Sigma (Dorset, UK). Primers were obtained from Invitrogen™ (Inchinnan, UK). RAW-Blue™ cell line (mouse macrophage reporter cell line) and QUANTI-Blue™ reagent were purchased from InvivoGen (Toulouse, France). Trisure and SensiFAST™ SYBR® Hi-ROX Kit were purchased from Bioline (Nottingham, UK) and iScript reagent kit was from Bio-Rad (Watford, UK). RAW 264.7 macrophages were obtained from the European Collection of Authenticated Cells (Salisbury, UK). Commercial food grade oilseeds and dairy proteins were purchased from a local supermarket (Leeds, UK).

2.2. Preparation of protein hydrolysates and their fractions

Sample preparation and enzyme hydrolysis procedures were performed as recently described (Han et al., 2021). Briefly, oilseed and dairy proteins were hydrolysed at 60 °C for 6 h with alcalase enzyme using an Enzyme/Protein (E/P) ratio of 1.5:25 at pH 8. For pepsin hydrolysates, proteins were digested at 37 °C for 6 h using pepsin with a 1:25 E/P ratio at pH 1.3 or 2.1. Three different M_w ($M_w > 10$, 3–10 and < 3 kDa) fractions were prepared for each sample through ultrafiltration using 3 and 10 kDa M_w cut-off membranes (Ultracel®, regenerated cellulose, 76 mm diameter).

2.3. Cell culture and treatments

RAW-BLUE™ cells and RAW 264.7 macrophages were both cultivated in high glucose DMEM supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were incubated at 37 °C in a humidified atmosphere (5% CO_2).

2.4. Measurement of cell viability

The cytotoxicity of protein hydrolysates towards RAW-Blue™ and RAW 264.7 macrophages was determined using Neutral Red assay according to Repetto et al. (2008) and Perez-Hernandez et al. (2020) with slight modifications. Briefly, RAW-BLUE™ and RAW 264.7 cells were seeded into 24-well plates at a density of 200,000 cells per well and grown to reach 70–80% confluence. Then, protein hydrolysates or low Mw fractions were diluted 1:10 from a stock solution in PBS into the medium for final concentrations of 250, 1000 and 2000 μg protein/mL, according to soluble protein concentration, determined via BCA protein assay. After incubation for 24 h, the medium was removed and replaced by medium containing neutral red (40 μg/mL). After 3 h of incubation at 37 °C, the neutral red containing medium was removed, the cells washed with PBS and bleach solution (50% ethanol, 49% deionized H_20, 1% glacial acetic acid) added to dissolve the accumulated neutral red dye. The absorbance was recorded at 540 nm using a Spark10M plate reader (Tecan, Männedorf, Switzerland) against bleach solution as blank and calculated in percent of untreated control cells (medium only).

2.5. NF-κB reporter assay

RAW-Blue™ cells are a commercially available NF-κB reporter cell line derived from murine macrophages, with secreted embryonic alkaline phosphatase (SEAP) reporter construct chromosomally integrated. Activation of inflammatory signalling via NF-κB and AP-1 leads to increased secretion of SEAP into the cell culture medium. RAW-Blue cells grown in 24-well plates, were incubated with protein hydrolysates and their different Mw fractions (250 and 1000 μg protein/mL) for 1 h and then stimulated with LPS (100 ng/mL). After 24 h stimulation, the medium was removed and mixed 1:4 with QUANTI-Blue™ substrate, in duplicate for each sample, and incubated for 1 h at 37 °C. SEAP activity, indicating the magnitude of transcriptional activation, was measured via absorbance at 620 nm using a Spark10M plate reader. Absorbance of non-stimulated control samples was subtracted from absorbance values of stimulated samples. The results were expressed as inhibition in percent of the sample treated with LPS only.

2.6. Real-time quantitative PCR

The mRNA expression levels of IL-6, TNF-α, IL-1β, iNOS and COX-2, nuclear factor κ B subunit 1 (NF-κB1), p65, nuclear factor of κ light

polypeptide gene enhancer in B cells inhibitor, α (IκBα), toll-like receptor 4 (TLR4) and cluster of differentiation 14 (CD14) were determined using quantitative RT-PCR. To this end, RAW 264.7 macrophages were stimulated with 100 ng/mL LPS in the absence or presence of protein hydrolysate samples (2000 μg protein/mL) for 6 h, a concentration that was determined through preliminary experiments (data not shown). Total RNA was isolated using Trisure reagent according to the manufacturer's instructions, and the quality and quantity of RNA was determined using Nanoquant plate (Spark plate reader). Following cDNA synthesis using iScript kit, amplification of target gene mRNA levels was performed using SensiFast SybrGreen reagent on a StepOne cycler (ABI). Primers were designed using NCBI tools, the sequence information is shown in Table 1. The β-actin gene was used as housekeeping gene to normalize the target gene expression. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

2.7. LPS-binding assay

The LPS-binding capacity in peptide-containing samples was determined via immunoassay according to Martínez-Sernández et al. (2016) with slight modifications. Briefly, wells of a 96-well plate were coated with 200 μL of casein hydrolysate solutions (protein concentration 12.5 μg/mL) covered with adhesive film and incubated at 4 °C overnight. The following day, 60 μL of LPS O111:B4-FITC (15 μg/mL) was mixed with the same volume of each of the seven low M_w fractions of pepsin (pH 1.3)-treated protein hydrolysates (0–256 μg protein/well) and incubated for 1 h at room temperature. The contents of the wells in the 96-well plate were aspirated and the wells washed three times with PBS using plate washer (Tecan). To each well, 100 μL of the preincubated solution was transferred and subsequently incubated at room temperature for 30 min. Afterwards, the plate was washed five times with 200 μL of PBS-T (0.05% Tween 20) per well. Then 100 μL of 1/4000 diluted sheep anti-FITC HRP was added and incubated for a further 30 min at room temperature at 750 rpm on a rocking platform. Following a washing step, OPD solution (100 μL) was added to each well and incubated for 20 min in the dark. After that the reaction was stopped via adding 25 μL of 3N H_2SO_4. Finally, the absorbance was read at 492 nm.

2.8. Surface tension measurement

The surface tension of protein hydrolysates at 1 mg/mL (dissolved in 0.05 M PBS, pH 7) was monitored for 12 min using Tensiometer (KRÜSS, Hamburg, Germany) in triplicate. All the experiments were conducted at 25.0 ± 0.9 °C and the surface tension of pure water was 72.2 ± 0.1 mN/m.

2.9. Statistical analysis

Depending on group number, data were analysed via student's t-test, multiple *t*-test and two-way analysis of variance (ANOVA) with post hoc analysis using GraphPad Prism 9.0. Differences were considered significant when p-value < 0.05. All experiments were performed in triplicate and data were expressed as mean ± standard error of the mean (SEM).

Table 1
Mouse primer pairs used for RT-PCR.

Gene	Forward sequence (5′-3′)	Reverse sequence (5' -3′)
β-actin	CCTCTATGCCAACACAGTGC	CCTGCTTGCTGATCCACATC
IL-6	AGTTGCCTTCTTGGGACTGA	CAGAATTGCCATTGCACAAC
TNF-α	GTGCCTATGTCTCAGCCTCT	AGTTGGTTGTCTTTGAGATCCA
IL-1β	CAGGCAGGCAGTATCACTCA	CAGGCAGGCAGTATCACTCA
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
iNOS	GCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
COX-2	TTCAACACACTCCTATCACTGGC	AGAAGCGTTTGCGGTACTCAT
NF-κB1	ATGGCAGACGATGATCCCTAC	TCTTCACAGTGGTATTTCTGGTG
p65	AGGCTTCTGGGCCTTATGTG	TGCTTCTCTCGCCAGGAATAC
IκB-α	TGAAGGACGAGGAGTACGAGC	TTCGTGGATGATTGCCAAGTG
TLR 4	AGGCAGCAGGTGGAATTGTATC	GGTCCAAGTTGCCGTTTCTT
CD14	CTCTGTCCTTAAAGCGGCTTAC	GTTGCGGAGGTTCAAGATGTT

3. Results and discussion

3.1. Inhibition of SEAP secretion via peptide fractions

NF-κB is an inducible transcription factor which plays a pivotal role in regulating the cellular inflammatory response. The activation of NF-κB pathway directly promotes the expression of pro-inflammatory target genes, including cytokines and chemokines (Liu et al., 2017). Interruption of NF-κB signalling has been demonstrated by many anti-inflammatory agents and is being applied as part of therapeutic approaches to modulate inflammation and its progression (Liu et al., 2017). In this study, the potency of protein hydrolysates and their three different M_w ($M_w > 10$, 3–10 and < 3 kDa) fractions to modulate the activation of NF-κB signalling was evaluated. RAW Blue™ reporter cells were used as a macrophage-based screening model responsive to (anti-) inflammatory triggers which can be quantified according to secreted alkaline phosphatase (SEAP) levels.

As shown in Table 2, the inhibition of SEAP secretion of oilseed and dairy peptides were tested at 250 and 1000 μg/mL. At both concentrations, no significant reduction of cell viability was found by any of the samples (data not shown). Apart from alcalase-treated rapeseed ($M_w >$ 10 kDa) and whey ($M_w < 3$ kDa); pepsin (pH 1.3)-treated flaxseed (whole); pepsin (pH 2.1)-treated rapeseed ($M_w > 10$ kDa), sesame (M_w > 10 kDa) and casein ($M_w > 10$ kDa), inflammation-inhibitory effects were significantly improved when the concentration of protein hydrolysate fractions was increased from 250 μg/mL to 1000 μg/mL. The M_w > 10 kDa fractions presented overall lowest effectiveness in reducing SEAP secretion, in contrast to the low M_w fraction (<3 kDa) which exhibited the strongest effects to inhibit SEAP secretion. These findings were in line with Vo et al. (2013) and Sangtanoo et al. (2020), who demonstrated that the low M_w (<3 kDa) protein fractions obtained from edible microalgae (*Spirulina maxima*) and peanut worm protein exerted the most promising anti-inflammatory potential, compared with other fractions (e.g. M_w 3–10 kDa or >10 kDa). In contrast, Sandoval-Sicairos et al. (2021) claimed that higher M_w (>10 and 3–10 kDa) fractions from amaranth protein hydrolysates tended to exert more promising anti-inflammatory responses, compared with the $M_w < 3$ kDa fraction. In the current study, the fractions with M_w 3–10 kDa presented the strongest inhibitory properties only in alcalase-treated sesame (84.5%) and rapeseed (61.5%), both at 1000 μg/mL, a finding that might be related to the specific amino acid composition and the presence of specific features, such as the occurrence of certain hydrophobic/positive charged amino acid residues in the peptide sequences (especially at carboxyl- and amino-terminals) (Guha & Majumder, 2019). However, in most cases, low M_w ($M_w < 3$ kDa) fractions tended to exert the most promising anti-inflammatory potential compared with the other two fractions ($M_w > 10$ kDa and 3–10 kDa).

Utilization of RAW-Blue™ cells to evaluate the anti-inflammatory potential of bio-compounds has also been reported via Li, L.-H. et al. (2017). In their work, GW-A2, a peptide with sequence GAKYAKIIY-NYLKKIANALW, dose-dependently suppressed the activation of NF-κB transcriptional activities in the reporter cell line, which was directly linked to downregulation of pro-inflammatory markers iNOS, COX-2, TNF-α, IL-6 and NO in LPS-activated RAW 264.7 macrophage cells.

Taken together, as evident from the reporter assay, sources of protein and types of proteases contributed to the large variations observed in anti-inflammatory capabilities of protein hydrolysates and their fractions. With the low M_w ($M_w < 3$ kDa) fractions of pepsin (pH 1.3)-treated rapeseed, sesame and soybean protein hydrolysates presenting the best potential of suppressing activation of NF-κB, samples from the low M_w fraction were selected for validation of anti-inflammatory properties in wild type macrophages.

Table 2
Anti-inflammatory properties (% inhibition) of whole oilseed and diary protein hydrolysates and different M_w fractions as determined using SEAP reporter assay in LPS (100 ng/mL) stimulated RAW-Blue™ cells.

Conc.	Condition	Whole		>10 kDa		3–10 kDa		<3 kDa	
		250 μg/mL	1000 μg/mL	250 μg/mL	1000 μg/mL	250 μg/mL	1000 μg/mL	250 μg/mL	1000 μg/mL
Flaxseed	Alcalase	7.2 ± 0.5^{Ab}	$16.3 \pm 1.5^{ABCDb*}$	n/a	n/a	15.2 ± 0.2^{BCc}	$13.4 \pm 0.2^{ABb*}$	33.6 ± 3.2^{EFGHa}	$81.5 \pm 1.8^{IJKa*}$
	Pepsin pH 1.3	18.3 ± 2.1^{Bb}	23.8 ± 1.9^{DEa}	6.1 ± 0.4^{ABCa}	n/a	4.7 ± 1.0^{Aa}	$17.1 \pm 1.1^{Aba*}$	n/a	$17.3 \pm 2.5^{Aba*}$
	Pepsin pH 2.1	6.8 ± 0.9^{Aa}	$22.3 \pm 0.9^{CDEc*}$	0.6 ± 0.1^{Ab}	$7.3 \pm 1.3^{Aba*}$	n/a	n/a	3.0 ± 0.3^{Aa}	$11.1 \pm 0.4^{Ab*}$
Rapeseed	Alcalase	n/a	$10.5 \pm 0.4^{Aba*}$	1.3 ± 0.1^{ABa}	1.4 ± 0.1^{Ab}	n/a	$8.1 \pm 1.4^{A*}$	n/a	$19.6 \pm 2.9^{ABCa*}$
	Pepsin pH 1.3	13.1 ± 1.8^{ABb}	$31.3 \pm 1.9^{EFb*}$	10.1 ± 1.1^{ABCDb}	$24.8 \pm 0.8^{CDc*}$	n/a	$43.8 \pm 2.6^{EFb*}$	48.3 ± 2.4^{IJa}	$86.2 \pm 2.0^{JKa*}$
	Pepsin pH 2.1	n/a	$8.6 \pm 2.2^{Ac*}$	14.4 ± 2.7^{DEa}	15.0 ± 4.3^{BCc}	40.6 ± 4.3^{Fb}	$61.5 \pm 4.5^{GHb*}$	18.9 ± 1.5^{BCDa}	$44.9 \pm 2.3^{DEFa*}$
Sunflower	Alcalase	n/a	$21.9 \pm 4.9^{BCDEb*}$	n/a	$21.1 \pm 2.5^{CDb*}$	n/a	n/a	30.4 ± 2.6^{CDEFGa}	$68.6 \pm 4.3^{GHIa*}$
	Pepsin pH 1.3	8.1 ± 2.3^{Ab}	$37.6 \pm 2.2^{Fab*}$	23.5 ± 1.7^{EFa}	$49.7 \pm 3.8^{Fb*}$	n/a	$29.2 \pm 4.1^{CDa*}$	n/a	$32.3 \pm 5.9^{CDEa*}$
	Pepsin pH 2.1	n/a	$51.8 \pm 1.6^{Gd*}$	3.7 ± 0.7^{ABCb}	$10.9 \pm 0.6^{ABCc*}$	5.5 ± 0.3^{Ab}	$16.3 \pm 0.5^{ABb*}$	17.2 ± 1.9^{BCa}	$38.6 \pm 2.7^{DEa*}$
Sesame	Alcalase	n/a	$26.6 \pm 1.5^{DEFc*}$	n/a	$39.0 \pm 2.3^{Ea*}$	44.2 ± 2.8^{Fb}	$84.5 \pm 2.7^{Ib*}$	21.3 ± 0.9^{BCDEa}	$46.8 \pm 2.1^{EFa*}$
	Pepsin pH 1.3	17.6 ± 3.5^{Bc}	20.6 ± 4.2^{DEFc}	n/a	$39.1 \pm 1.6^{Eb*}$	28.4 ± 2.3^{DEb}	$42.5 \pm 1.8^{EFb*}$	38.2 ± 2.4^{GHIa}	$88.7 \pm 1.6^{Ka*}$
	Pepsin pH 2.1	n/a	$10.4 \pm 2.4^{Abd*}$	29.1 ± 4.1^{Fb}	29.6 ± 3.1^{DEc}	31.2 ± 2.0^{Eb}	$71.5 \pm 4.4^{HIb*}$	52.5 ± 1.9^{Ja}	$96.1 \pm 1.8^{Ka*}$
Soybean	Alcalase	n/a	$7.1 \pm 1.9^{Ac*}$	n/a	n/a	n/a	$18.2 \pm 1.3^{ABCb*}$	25.5 ± 3.8^{BCDEFa}	$82.0 \pm 5.7^{IJKa*}$
	Pepsin pH 1.3	n/a	n/a	n/a	n/a	7.1 ± 0.1^{ABb}	$19.2 \pm 0.5^{BCb*}$	26.5 ± 1.9^{BCDEFGa}	$93.0 \pm 2.1^{Ka*}$
	Pepsin pH 2.1	8.1 ± 0.9^{Ab}	$20.8 \pm 0.7^{BCDEc*}$	n/a	$8.4 \pm 1.5^{Abb*}$	n/a	$9.3 \pm 1.3^{ABb*}$	19.3 ± 2.4^{BCDa}	$72.9 \pm 1.4^{HIJa*}$
Whey	Alcalase	8.5 ± 1.1^{Ab}	$25.8 \pm 1.2^{DEcd*}$	10.5 ± 2.2^{BCDb}	$20.5 \pm 1.4^{CDcd*}$	10.0 ± 0.2^{ABb}	$15.5 \pm 0.3^{ABbd*}$	20.0 ± 4.8^{BCDa}	31.2 ± 4.2^{BCDac}
	Pepsin pH 1.3	9.1 ± 0.7^{Ab}	$30.7 \pm 0.9^{EFb*}$	n/a	$9.2 \pm 1.0^{ABc*}$	n/a	$35.8 \pm 1.1^{DEb*}$	37.3 ± 2.0^{FGHIa}	$90.2 \pm 3.0^{Ka*}$
	Pepsin pH 2.1	n/a	$51.3 \pm 2.0^{Ga*}$	n/a	n/a	12.4 ± 1.6^{ABa}	$36.7 \pm 2.3^{DEb*}$	16.4 ± 2.0^{Ba}	$62.0 \pm 1.6^{Ga*}$
Casein	Alcalase	n/a	$11.7 \pm 2.1^{ABCb*}$	n/a	n/a	9.3 ± 0.5^{ABb}	$16.5 \pm 1.1^{ABb*}$	26.9 ± 1.5^{BCDEFGa}	$55.5 \pm 1.5^{FGa*}$
	Pepsin pH 1.3^{AB}	12.8 ± 0.7^{ABb}	$23.5 \pm 2.0^{DEc*}$	1.8 ± 0.3^{ABCa}	n/a	15.2 ± 1.1^{BCb}	$34.9 \pm 1.7^{DEb*}$	30.5 ± 1.7^{DEFGa}	$73.8 \pm 1.0^{HIJa*}$
	Pepsin pH 2.1^{AB}	10.8 ± 1.6^{ABc}	$17.3 \pm 1.4^{ABCDd*}$	8.0 ± 2.0^{ABCDc}	9.2 ± 0.5^{ABc}	22.0 ± 0.3^{CDb}	$52.8 \pm 1.0^{FGb*}$	45.4 ± 4.0^{HIJa}	$89.2 \pm 2.9^{Ka*}$

Data are expressed as mean with SEM of three independent experiments performed in duplicate. Different capital or small letters within the same column or row indicate significant differences, respectively ($p < 0.05$). * means significant difference between 250 and 1000 μg protein/mL ($p < 0.05$). n/a means no inhibition.

3.2. Effect of protein hydrolysates on cell viability in RAW 264.7 macrophages

Prior to the evaluation of anti-inflammatory activity in RAW 264.7 macrophages, the potential cytotoxicity of low M_w fraction of pepsin (pH 1.3)-treated protein hydrolysates was examined via the Neutral Red assay. As shown in Fig. 1, none of the samples tested exerted any negative effects on the viability of RAW 264.7 macrophages at any concentration. In contrast, flaxseed, rapeseed, sunflower, sesame and casein samples showed enhanced cell viability values at 2000 μg protein/mL, in the range of 9.4–25.6% above the medium control. These enhanced control values in % cell viability may indicate an increase in cell proliferation which has also been observed by others. For example, He et al. (2019) reported that three rapeseed derived peptides, LY, RALP and GHS, increased cell viability by 13.4%, 20.8% and 24.1% at 1 mM, respectively. Further, Zhao et al. (2016) demonstrated that velvet antler protein hydrolysates increased cell viability by 24.3% and 31.7% (at 200 and 500 μg peptides/mL). The enhancement of proliferation and survival of macrophages indicated positive immunostimulatory effects (Li, E.W. and Mine, 2004; Girón-Calle et al., 2010). Based on the cell viability data, concentrations of 2000 μg protein/mL were selected for further experiments.

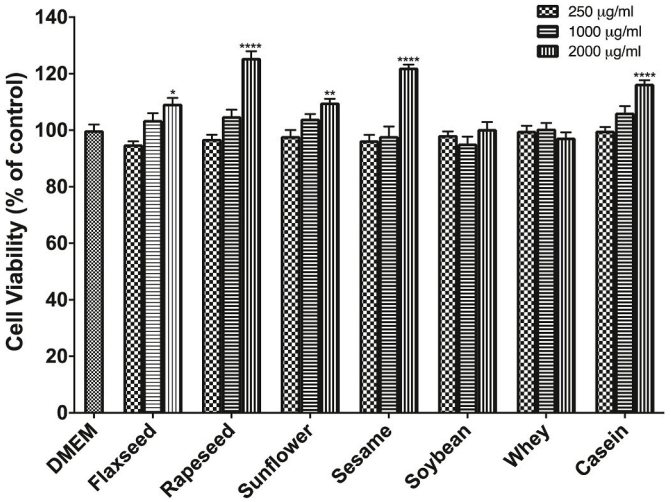

Fig. 1. Effects of low M_w fraction of pepsin (pH 1.3)-treated protein hydrolysates (250, 1000 and 2000 μg protein/mL) on cell viability in RAW 264.7 macrophages. Data are mean with SEM of three independent experiments performed in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with the value of cell viability of RAW 264.7 macrophages treated with medium only.

3.3. Protein hydrolysates modulate the expression of cytokines and mediators

Attenuating the NF-κB pathway is associated with modulation of pro- and anti-inflammatory gene expression; to this end, IL-6, TNF-α, IL-1β and IL-10 were chosen as representative cytokine targets. As shown in Fig. 2a and b, mRNA levels of IL-6 and TNF-α were significantly increased after LPS stimulation when compared with the negative control. However, only rapeseed significantly reduced IL-6 mRNA levels, by 37.5%, whilst no marked difference was observed with regards to TNF-α mRNA levels. Ren et al. (2018) also claimed that LDAPGHR peptide (at 50 μM), derived from hazelnut, decreased IL-6 mRNA levels by 66.1%. Meanwhile, results showed that rapeseed, sesame and casein significantly inhibited the expression of pro-inflammatory IL-1β by 67.7%, 51.8% and 50.1%, respectively (Fig. 2c). In addition, our data demonstrated that LPS-induced inflammation led to a significant increase in anti-inflammatory IL-10 gene expression, which is consistent with other studies (Salkowski et al., 1997; Sun, H. et al., 2017; Ghalem et al., 2018), and emphasizes the importance of IL10 immuno-regulatory function. Apart from soybean and whey, all samples showed up-regulation of IL-10 mRNA levels at 2000 μg protein/mL in the range of 44.5%–57.3% (Fig. 2d). Coincidentally, cell viability with these five samples was also significantly increased.

In addition to cytokines, iNOS and COX-2 are two major inflammatory proteins regulated via NF-κB. The enzyme iNOS converts NO from arginine whereas COX-2 synthesizes PGE2 using arachidonic acid (Aktan, 2004; Nasry et al., 2018); and persistent generation of both NO and PGE2, is directly associated with inflammatory disease such as rheumatoid arthritis. Whilst undetectable in unstimulated macrophages, iNOS and COX-2 strongly respond to the LPS inflammatory trigger. Therefore, the ability of peptides to modulate iNOS and COX-2 gene expression was analysed in order to evaluate further their anti-inflammatory potential. As shown in Fig. 2e and f, only rapeseed efficiently reduced (to 47.2%) iNOS expression after 6 h LPS stimulation. Rapeseed was also associated with the reduction of COX-2 mRNA level to 44.5%, similar to casein (56.5%).

Taken together, our results suggest that rapeseed was the most promising source of anti-inflammatory peptides, since it efficiently

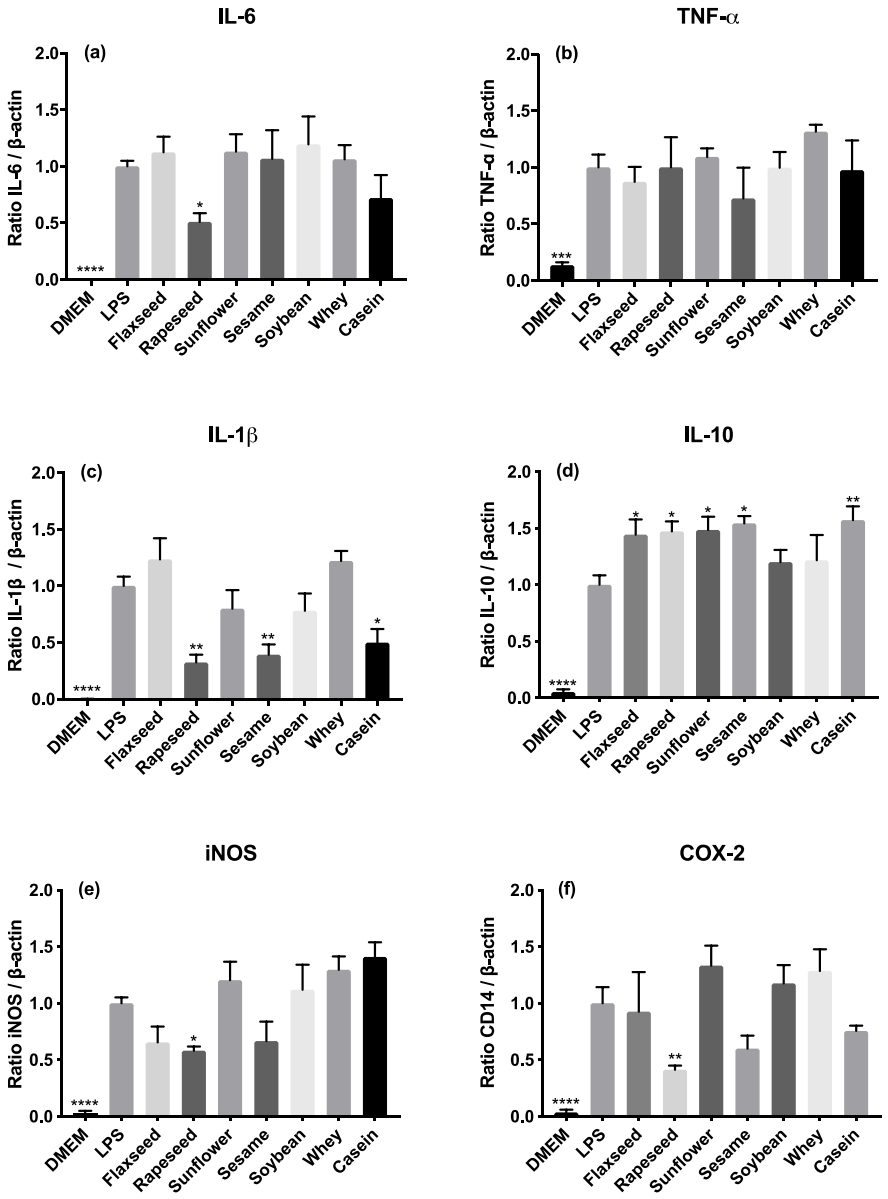

Fig. 2. Effects of low M_w fractions (2000 μg protein/mL) of pepsin (pH 1.3)-treated hydrolysates on mRNA levels of pro-inflammatory cytokines, a) IL-6, b) TNF-α and c) IL-1β, d) anti-inflammatory cytokine IL-10, and pro-inflammatory mediators e) iNOS and f) COX-2 in LPS (0.1 μg/mL)-stimulated RAW 264.7 macrophages. Data are mean with SEM of three independent experiments performed in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with the positive control (LPS-stimulated cells).

down-regulated IL-6, IL-1β, iNOS and COX-2 and up-regulated IL-10 transcriptional activities. Apart from soybean, other protein hydrolysates, especially sesame and casein, tended to modulate one or few cytokines, indicating some potential for lowering inflammatory response.

3.4. Attenuation of NF-κB activation in LPS-stimulated RAW 264.7 macrophages

To further explore the changes on markers of NF-κB pathway, transcriptional changes on NF-κB1 (p50), p65 and IκBα were determined. NF-κB1 and p65 are the two most common subunits of NF-κB/Rel proteins, which act as transcriptional regulators and therefore determine inflammatory-related gene expression to a large extent. After 6 h LPS stimulation, the mRNA levels of NF-κB1 and p65 were significantly up-regulated in RAW 264.7 macrophages. As shown in Fig. 3a and b, sesame significantly reduced expression of NF-κB1 by 50.7%, while rapeseed (73.9%) and casein (58.1%) down-regulated the expression of p65. Similarly, Ye et al. (2019) and Gasparrini et al. (2018) reported that chlojaponilactone B (2.5–10 μM) and manuka honey (8 mg/mL) attenuated LPS-induced increases in NF-κB1 and p65 gene expression, respectively. IκBα, functioning as an inhibitor of NF-κB transcriptional activation in the cytoplasm, prevents NF-κB activation and binding to the DNA (Jacobs & Harrison, 1998). As shown in Fig. 3c, the LPS-induced increase in IκBα gene expression showed a strong decrease (59.1%) upon co-incubation with rapeseed. Meanwhile, casein also downregulated IκBα expression by 47.8%. Since IκBα directly controls NF-κB activation (via phosphorylation) it is also a target of NF-κB signalling and therefore shows an increase under inflammatory conditions. In support or our findings, Sun, S.-C. et al. (1993) reported that induction of IκBα gene expression (by 140 U/mL TNF-α or 50 ng/mL of phorbol 12-myristate 13-acetate) was efficiently attenuated in the presence of 0.2 mM pyrrolidinedithiocarbamate (PDTC, NF-κB

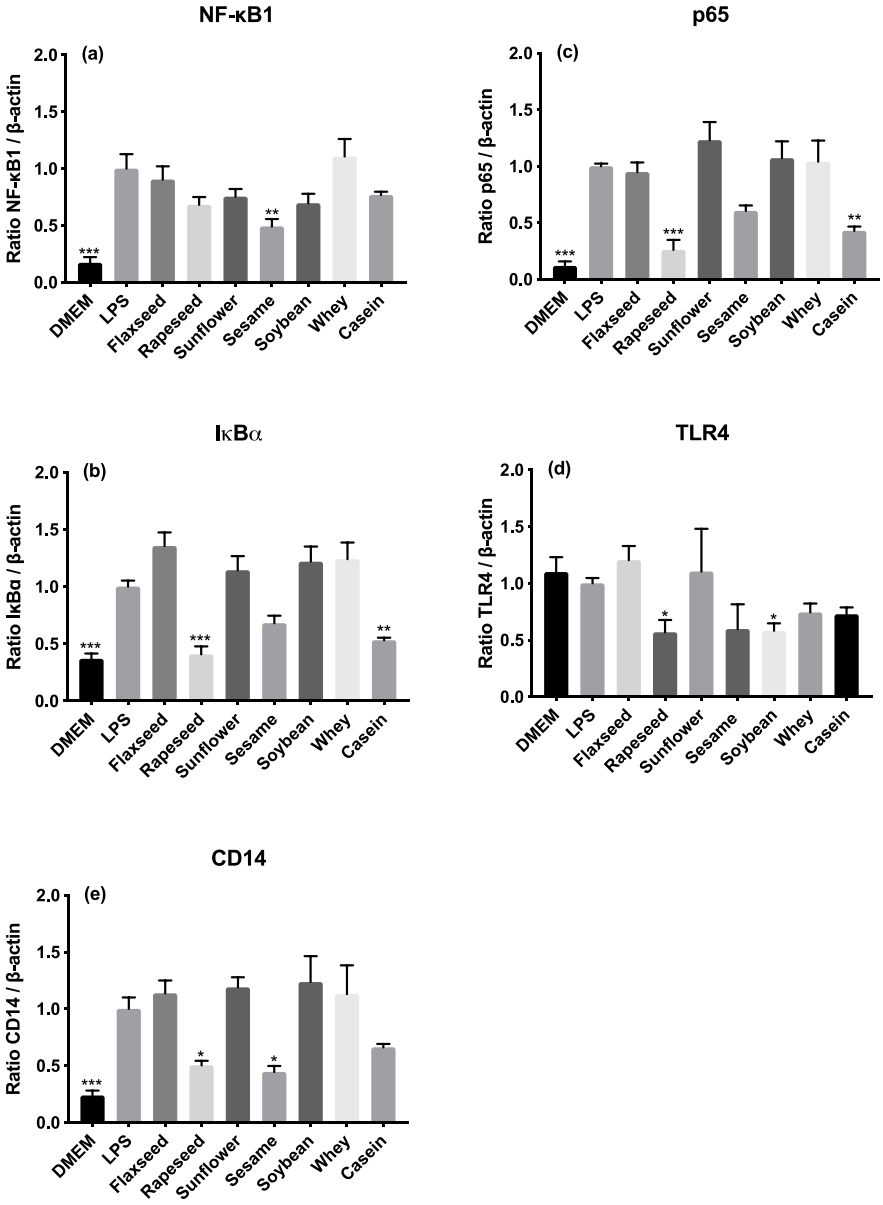

Fig. 3. Effect of low M_w fractions (2000 μg protein/mL) of pepsin (pH 1.3)-treated protein hydrolysates on the expression of a) NF-**k**B1, b) p65, c) IκBα, d) TLR 4 and e) CD14 in LPS-stimulated raw 264.7 macrophages. Data are shown as mean with SEM of three independent experiments preformed in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with the positive control (LPS-stimulated cells).

inhibitor). In addition, Fu et al. (2019) showed that 100 μM of QNZ (a NF-κB inhibitor) significantly impaired IκBα expression in mice splenic cells.

TLR 4 is a transmembrane protein receptor primarily known for its specificity to bind LPS. Binding of LPS to TLR4 leads to activation of inflammatory signalling and subsequently increased pro-inflammatory cytokine expression. In this study, the potential modulation of TLR4 mRNA levels was assessed. As shown in Fig. 3d, TLR4 mRNA levels were detectable in macrophages, whilst no significant change was observed after 6 h LPS stimulation, which is in line with Matsuguchi et al. (2000) who also showed unaltered TLR4 gene expression after 2 or 8 h LPS stimulation in macrophages. When incubated with protein hydrolysate samples, rapeseed and soybean demonstrated significantly decreased TLR4 mRNA levels, by 43.0% and 41.8% at 2000 μg protein/mL, respectively. As a further target, the expression of CD14, a receptor that is anchored on the cell surface and jointly acts with TLR4 to initiate LPS/TLR4/NF-κB signalling, was determined. Unlike TLR4, a 2-fold increase of CD14 expression was found after LPS stimulation, compared to the unstimulated control (Fig. 3e). Rapeseed and sesame exerted similar suppression of CD14 expression, which were 49.5% and 54.4%, respectively.

Taken together, suppression of NF-κB signalling was demonstrated by rapeseed, sesame and casein in RAW 264.7 macrophages, whilst soybean and whey were unable to suppress pro-inflammatory gene expression. Rapeseed, sesame and soybean also demonstrated reduced expression for TLR4 and/or CD14, thereby potentially contributing to delayed LPS recognition. In the current study, all target genes were NF-κB targets, and there is some expectation for correlation between changes at mRNA levels with protein levels for individual targets. Whilst this has been confirmed in some studies (Ma et al., 2016; Ren et al., 2018; Wang et al., 2017), the correlation overall between mRNA levels and the associated protein expression is only approximately 40%, affected via transcription, translation and stability (mRNA and protein) (Vogel & Marcotte, 2012).

3.5. LPS binding capacity of peptides

Apart from suppressing the activation of the NF-κB pathway,

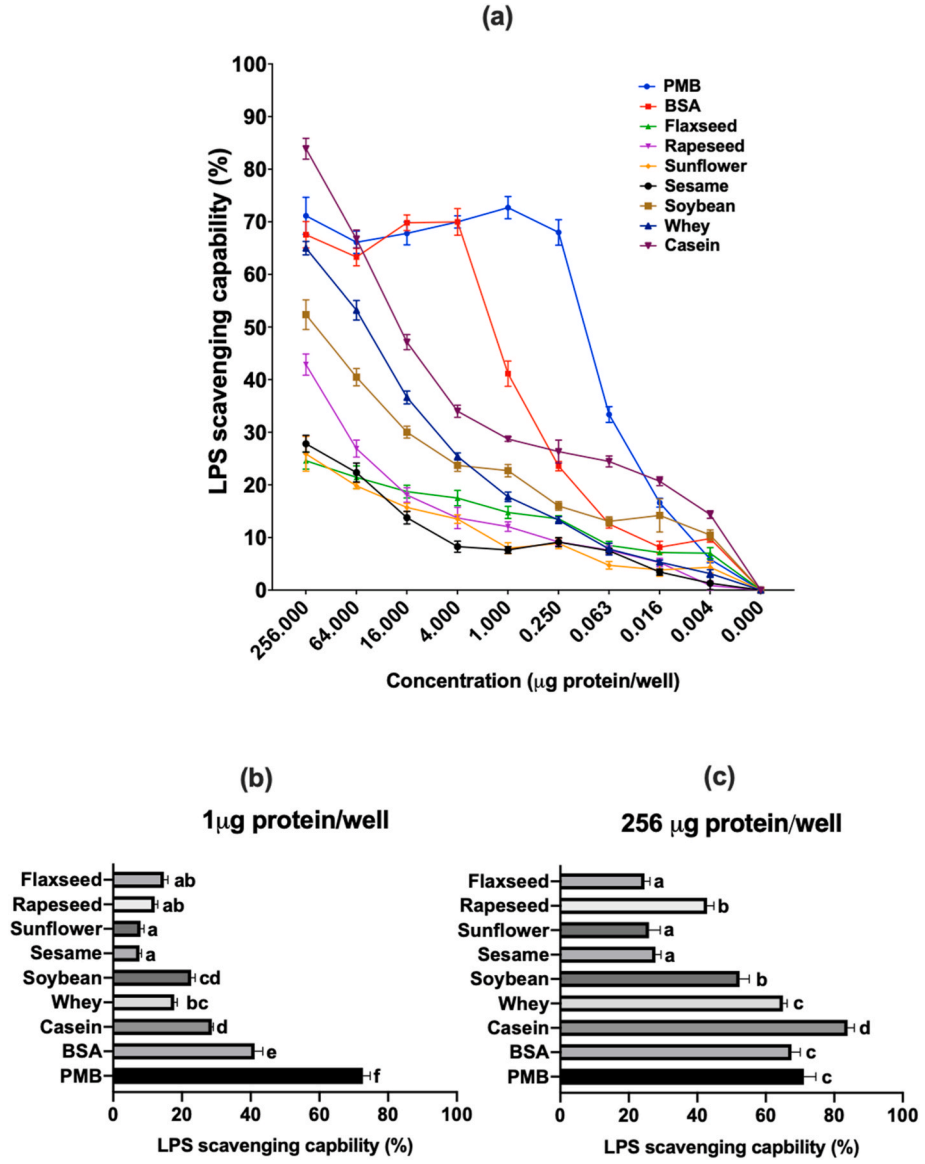

Fig. 4. Concentration-dependent effects of pepsin (pH 1,3)-treated protein hydrolysates (MM_w < 3 kDa fractions) on LPS-binding shown over (a) a range of concentrations (0.004–256 μg protein per well) as well as selected concentrations: (b) 1 and (c) 256 μg protein per well. Data are shown as mean with SEM of three independent experiments performed in duplicate. Different letters indicate significant differences at p-value < 0.05. BSA, bovine serum albumin; PMB, polymyxin B.

protein/peptide-LPS binding may also contribute to the modulation of inflammation via preventing the binding of LPS with its receptors on the cell surface. In order to determine the affinity of peptides to LPS, all the low M_w fractions of pepsin (pH 1.3)-treated protein hydrolysates were evaluated for their scavenging capabilities towards LPS. As a positive control, polymyxin B (PMB), an antibiotic with the function of binding to Lipid A and located in the outer leaflet of the LPS membrane, was used (Domingues et al., 2012; Morrison & Jacobs, 1976; Steimle et al., 2016).

As shown in Fig. 4a, the peptides exerted a dose-dependent increase of LPS binding (from 0.004 to 256 μg peptides/per well). At 0.004 and 0.016 μg protein per well, casein demonstrated the highest binding capability, scavenging 14.4 and 20.7% of LPS, respectively. However, when the concentration of peptides/protein reached 0.063 μg protein per well, PMB showed the most robust binding capability (33.5%), 8.9% higher than casein. The inhibition by PMB increased to 72.7% at 1 μg per well, while casein and soybean presented similar affinities for LPS with 28.7% and 22.7%, respectively (Fig. 4b). At the highest concentration tested (256 μg protein per well, shown in Fig. 4c), 83.9% of LPS bound with casein fractions. Among oilseed proteins, rapeseed and soybean scavenged 42.9% and 52.4% of LPS, respectively, i.e., not significantly different values. In contrast, whey was more promising as it reduced LPS binding by 65%. Whilst studies are lacking to report on plant protein derived peptide effects on LPS binding, there is some evidence from mammalian peptides, derived from serum Amyloid P with IC_{50} values 0.06 μM–4.41 μM and CM4 peptide suppressing LPS binding at 5–10 μM (de Haas et al. (1999); (Lin, Q.-P. et al., 2008).

As shown above, the LPS binding capabilities of rapeseed and soybean may reduce the available amount of LPS that could bind to its receptors on the cell surface. Meanwhile, BSA also presented marked LPS binding activity, which increased to 70% at 4.0 μg/per well, superior to all protein hydrolysates tested. However, there is no evidence elsewhere to suggest that BSA can act as an anti-inflammatory agent. This might be because the moiety of LPS that most contributes to its pathophysiological effects is the hydrophobic lipid A, located in the membrane-anchoring region. It is possible that BSA (and peptides) may bind to other parts of LPS but fail to block the lipid A recognized via the receptors. Therefore, these peptides cannot efficiently block the LPS biological activity and avoid triggering the cascade that results in the NF-κB activation. Rosenfeld et al. (2006) also claimed direct interaction of peptides with LPS in solution that did not directly block the LPS biological activity. It was shown that only peptides with LPS-detoxifying function (neutralization) could dissociate of LPS aggregating to the receptors.

3.6. Surface tension measurement

The surface tension of hydrolysates was also determined in order to explore the general hydrophobicity and therefore potential membrane affinity of the low M_w fraction protein hydrolysates. A higher content of hydrophobic amino acids is generally present in the majority of anti-inflammatory peptides. (Guha & Majumder, 2019). Casein presented the lowest value of surface tension (59.0 mN/m), not significantly different from rapeseed (60.0 mN/m) and whey (60.8 mN/m), shown in Table 3. Besides, the surface tension of other oilseed protein hydrolysates ranged from 64.0 mN/m to 66.0 mN/m, much higher compared to dairy and rapeseed protein hydrolysates. Taghizadeh et al. (2020) measured surface tension of protein hydrolysates obtained from three medicinal plants (*C.retica*, *Z.clinopodioides* and *M.chamomilla*) after *in vitro* gastrointestinal digestion with values being much lower than those in our study, ranging from 48.4 mN/m to 59.6 mN/m). In addition, do Evangelho et al. (2017) reported the surface tension of pepsin-treated black bean protein hydrolysates to be below 54.0 mN/m. However, in these other studies, longer hydrolysis times and removal of larger peptides ($M_w > 3$ kDa) may have resulted in enrichment of smaller peptides, thus explaining these lower surface tension values.

Reduced surface tension is directly associated a decrease in hydrophilic-lipophilic balance (HLB), and lower HLB is correlated with higher hydrophobicity of amphiphilic protein hydrolysates (Amrhein et al., 2015). Therefore, rapeseed, together with whey and casein fractions, appear to contain more polar and hydrophobic amino acids, compared with the other oilseeds. Indeed, a higher molar ratio of hydrophobic to total amino acids has been reported in rapeseed protein isolates (0.34), compared with those in soybean, sunflower and *Jatropha* seed (0.30–0.33) (Fleddermann et al., 2013; Sari et al., 2016; Tranchino et al., 1983). A common occurrence of hydrophobic amino acids is a typical structural feature of anti-inflammatory peptides, therefore, based on this analysis, rapeseed, whey and casein fractions may present stronger anti-inflammatory properties (Guha & Majumder, 2019). In fact, apart from the whey fraction, the rapeseed and casein fractions efficiently modulated LPS induced inflammation in RAW-Blue™ reporter cells as well as in RAW 264.7 macrophages.

Table 3
Surface tension (mN/m) of low M_w ($M_w < 3$ kDa) fractions of pepsin (pH 1.3)-treated protein hydrolysates at 1 mg protein/mL.

	Surface tension (mN/m)
0.05 M PBS	70.0 ± 0.5^{a}
Flaxseed	64.9 ± 0.6^{bc}
Rapeseed	60.0 ± 0.2^{d}
Sunflower	66.0 ± 0.2^{b}
Sesame	64.2 ± 0.4^{bc}
Soybean	64.0 ± 0.3^{c}
Whey	60.8 ± 0.2^{d}
Casein	59.0 ± 0.1^{d}

Data are shown as mean with SEM of three independent experiments preformed in triplicates. Different letters indicate significant differences at p-value < 0.05.

4. Conclusion

In summary, our data presented several potential mechanisms by which oilseed protein hydrolysates/peptides could contribute to attenuate inflammation. These as well as findings from the literature on peptide interaction with NF- κB signalling are summarized in Fig. 5. Most hydrolysates derived from oilseed proteins effectively inhibited SEAP secretion in RAW-blue™ reporter cells. Apart from flaxseed and sunflower, low M_w ($M_w < 3$ kDa) fractions derived from pepsin (pH 1.3) hydrolysis presented the most promising anti-inflammatory potential in reporter cells with inhibition ranging from 73.8% to 93%. The rapeseed sample was most consistent in lowering expression of pro-inflammatory cytokines and mediators in RAW 264.7 macrophages, followed by the sesame and casein fractions. In addition, noticeable LPS binding capabilities were detected with rapeseed and soybean, although these were much lower compared with casein and whey, indicating distinct differences depending on the peptide profile. Furthermore, higher surface activity was determined in rapeseed, whey and casein fractions, which in case of rapeseed and casein agrees with their more marked anti-inflammatory properties.

Whilst evidence is presented on the potential of bioactive peptide-enriched fractions to lower inflammatory response, further research efforts should be directed towards the purification and characterization of bioactive peptide fractions derived from the different plant protein sources. This will enable better understanding of the contribution of individual peptides and/or their synergistic and antagonistic actions in modulating inflammatory processes and their mechanisms. Consequently, bioactive peptides could be effectively incorporated into formulations that target inflammatory response *in vivo*.

Credit author statement

Ruixan Han: Conceptualization, Methodology, Investigation, Formal

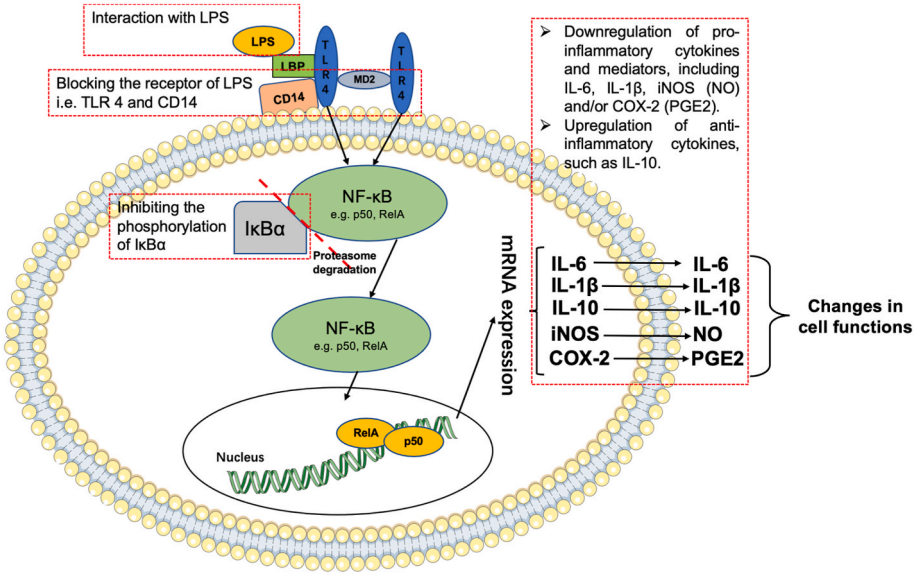

Fig. 5. The potential anti-inflammatory mechanisms of hydrolysates in LPS-induced RAW 264.7 macrophages.

Analysis, Writing - Original Draft. Alan Hernández Álvarez: Conceptualization, Writing - Review & Editing, Supervision. Joanne Maycock: Conceptualization, Methodology. Brent Murray: Conceptualization, Writing - Review & Editing, Supervision.Christine Boesch: Conceptualization, Writing - Review & Editing, Supervision.

Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the work described in this manuscript.

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