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Peptides and protein hydrolysates exhibiting anti-inflammatory activity: sources, structural features and modulation mechanisms†

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Inflammation is the response of the immune system to harmful stimuli such as tissue injury, infection or toxic chemicals, which has the aim of eliminating irritants or pathogenic microorganisms and enhancing tissue repair. Uncontrolled long-lasting acute inflammation can gradually progress to chronic, causing a variety of chronic inflammatory diseases that are usually treated with anti-inflammatory drugs, but most of them are inadequate to control chronic responses and are also associated with adverse side effects. Thus, many efforts are being directed to develop alternative and more selective anti-inflammatory therapies from natural products. One main field of interest is the obtaining of bioactive peptides exhibiting anti-inflammatory activity from sustainable protein sources like edible insects or agroindustry and fishing by-products. This work highlighted the structure–activity relationship of anti-inflammatory peptides. Small peptides with molecular weight under 1 kDa and amino acid chain length between 2 to 20 residues are generally the most active because of the higher probability to be absorbed in the intestine and penetrate into cells when compared with the larger size peptides. The presence of hydrophobic (Val, Ile, Pro) and positively charged (His, Arg, Lys) amino acids is another common occurrence for anti-inflammatory peptides. Interestingly, a high percentage (77%) of these bioactive peptides can be found in alternative sustainable protein sources such as *Tenebrio molitor* or sunflower, apart from its original protein source. However, not all of these peptides with anti-inflammatory potential *in vitro* achieve good scores by the *in silico* bioactivity predictors studied. Therefore, it is essential to implement current bioinformatics tools, in order to complement *in vitro* experiments with prior prediction of potential bioactive peptides.

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1. Introduction

Inflammation is a physiological immune response triggered by noxious stimuli, and acts by restoring tissue homeostasis during infection or injury. This defense mechanism involves changes in vascular permeability, recruitment and accumulation of immune cells and release control of pro-inflammatory mediators at sites of immune reaction.[1] Acute inflammation is the first line of defense, occurring immediately after inflammatory response induction, that temporarily (from minutes to a couple of days) triggers cellular and molecular events and interactions to prevent progression of damage or infection efficiently.[1,2] Nonetheless, acute inflammatory response must be silenced over time to prevent loss of the immune function and further tissue deterioration, otherwise excessive and unregulated production of inflammatory mediators can lead to the development of chronic inflammation, a persistent inflammatory response.[2] Furthermore, abnormal activation of inflammation-related enzymes, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), lipoxygenase (LOX) and phospholipase A_2 (PLA_2), play an important part in the development of inflammatory disorders.[3]

Chronic inflammation is associated with increased risk of chronic diseases and disorders such as asthma, inflammatory bowel disease (IBD), cancer, cardiovascular disease, obesity and type-2 diabetes.[4–7] Indeed, chronic inflammatory diseases dominate present-day morbidity and mortality worldwide with more than 50% of all deaths.[8] Although the etiologies of chronic inflammatory diseases differ, the pathways that lead to pathological abnormalities are common.[2] As a result, these inflammatory pathways can be explored as prospective targets for developing therapeutic treatments.

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Regardless of initial stimuli's nature and location, the inflammatory cascade always involves the same steps: (1) noxious stimuli are recognized by cell surface receptors known as pattern-recognition receptors (PRRs); (2) inflammatory signaling pathways are triggered; (3) inflammatory mediators and signaling molecules are produced and released; and (4) blood vessels are dilated allowing inflammatory cells to accumulate in the inflamed tissue.[2] Pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs), are found in both immune and non-immune cells. PRRs can recognize conserved motifs of molecules expressed by bacterial structures called pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) which is the major component of Gram-negative bacteria cell walls, or endogenous signals activated during tissue and cell injury named alarmins or danger-associated molecular patterns (DAMPS).[9–12] Inflammatory mediators comprise cytokines of the interleukin-family (ILs) such as IL-6, IL-1β and IL-10, interferons (IFNs) like interferon-γ (INF-γ), tumor necrosis factors (TNFs) like tumor necrosis factor-α (TNF-α) and chemokines such IL-8, and they mediate inflammation through interaction with diverse cellular components or receptors such as IL-1 receptor (IL-1R), IL-6 receptor (IL-6R), and the TNF receptor (TNFR) among others.[2] Once recognition of stimuli occurs, receptor activation triggers common signaling pathways including the nuclear factor kappa-B (NF-κB) and mitogen-activated protein kinase (MAPK). Regarding the NF-κB pathway, inactive protein complex NF-κB bounds to IκB protein; when PRRs recognize noxious stimuli, IκB protein degradation is induced. This releases NF-κB subunits p50 and p65 which translocate to the nucleus regulating genes involved in the inflammatory response.[13] The NF-κB pathway regulate the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α), anti-inflammatory cytokines (IL-10), expression of iNOS, which produces nitric oxide (NO), and COX-2, which is a key enzyme in the biosynthesis of inflammatory mediators such as prostaglandins and leukotrienes.[14,15] Besides, it regulates the expression of adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1).[16] In the case of MAPK pathway, it consists of a family of serine/threonine protein kinases, divided in three subfamilies: extracellular signal-regulated kinases (ERKs); C-Jun N-terminal kinases (JNKs); and p38 MAPKs; which upregulate or downregulate inflammation-related genes through protein phosphorylation.[17] Particularly, the MAPK pathway stimulates enzyme phospholipase A2 (PLA_2) activity together with translocation of NF-κB subunits to nucleus.[18]

While a great number of commercially anti-inflammatory drugs exist, these pharmacological therapies are often related with adverse side effects due to prolonged consumption.[19,20] Non-steroidal anti-inflammatory drugs (NSAIDs), like ibuprofen, naproxen or aspirin, are some of the most frequently conventional drugs prescribed to combat chronic Inflammation.[21] NSAIDs acts by inhibiting the activity of cyclooxygenase enzymes involved in the synthesis of pro-inflammatory mediators and they are associated with severe toxicity and hypertension and are contraindicated to older patients with cardiovascular, renal or hepatic complications.[22] Therefore, natural compounds exhibiting anti-inflammatory activity, which can be included in functional foods or within nutraceutical formulations, are a potentially better alternative to synthetic drugs for the prevention and treatment of inflammatory diseases. For instance, the variety of immunomodulatory properties attributed to bioactive peptides has focused the attention on them for the treatment of inflammatory diseases. These peptides are small fragments of proteins that usually contain 2–20 amino acid residues per molecule and they achieve their biological activity once they are released from their parental protein. The release of bioactive peptides is accomplished by enzymatic or chemical hydrolysis, *in vivo* or *in vitro* simulated gastro-intestinal digestion or bacterial fermentation of the parental proteins.[23] Although there are many studies that have observed the anti-inflammatory activity of single purified or synthesized peptides, others have evaluated the activity of whole protein hydrolysates composed of a mixture of diverse bioactive peptides.[24–27] Once crossed the gastrointestinal barrier and survive enzyme degradation, active peptides are absorbed in the human body and perform a wide range of functions, including modulating physiological systems and inflammatory response due to the modulation of MAPK and NF-κB inflammatory signaling pathways through the downregulation of pro-inflammatory mediators and upregulation of anti-inflammatory mediators expression.[28,29] The biological and functional properties of peptides are normally determined by their amino acid sequence, relative proportion of specific amino acids and the type of residues present at C- and N-terminals, as well as by their hydrophobicity, molecular weight/length and net charge.[30] However, limited information about the relationship between structure and anti-inflammatory activity of bioactive peptides is available, which makes it difficult to understand the specific molecular mechanisms involved in their action.

Animal proteins from eggs, milk (casein and whey) and meat have been widely used for extracting bioactive peptides, but the production of these conventional animal-based protein sources is not feasible to meet the growing global protein demands in a sustainable manner.[31,32] Plant protein has also been commonly used as a source of bioactive peptides, being legumes such as soybean among the most used crops. Nonetheless, the use of soybeans may be inconvenient because of the amount of antinutritional components that can cause negative effects. Moreover, soybean cultivation is typically associated with environmentally unfriendly and unsustainable practices.[33–35]

The difficulties with conventional protein sources from animals and plants have prompted a search for other alternative or unconventional protein sources, such as those derived from edible insects and by-products from the food or agriculture industry. Edible insects can be produced with less negative impact on the environment than existing livestock.[36] From the wide array of edible insects, yellow mealworms known as *Tenebrio Molitor* have been reported to provide a source of

high-quality protein (53% protein in a dry basis) containing high amounts of essential amino acids.[37–40] A recent study show that insect-derived protein can be rapidly digested and effectively absorbed, with no different from a high-quality dairy protein source.[36] Alternative plant-based proteins are gaining increasing interest and a wide range of flour or defatted flours from different sources (especially legumes, cereals and oilseeds) are already in the market.[41] By-products of the extraction of sunflower (*Helianthus annuus* L.) oil are considered an interesting raw material for biopeptides production due to their high content of protein. For instance, defatted sunflower meal (principal by-product) contains about 28–42% protein (dry basis).[42,43] Other interesting crops, due to their favorable composition and availability, are Lupine family such as *Lupinus albus* or *Lupinus angustifolius* which have a high protein content (about 35%–40% in a dry basis) containing a high proportion of essential amino acids.[44] Nutritional characteristics along with low water requirements and high productivity make chickpeas (*Cicer arietinum*) a great alternative to conventional protein sources. Chickpeas are an inexpensive source rich in protein (approximately 20% dry mass) with excellent balance of essential amino acid composition, high bioavailability, and low level of antinutritional factors.[26,45] It is important to mention that pea protein, that is usually used as feed with a low added value, also contains a high content of protein (about 18% protein in a dry basis) and an adequate content of essential amino acids.[46,47] Olive (*Olea europaea*) is another interesting source of protein (17% protein in seeds), since olive oil consumption has experienced a continuous increase, and hence waste and by-products derived from the olive production (*e.g.* olive seed flour) have also increased. Recently, it has been reported a novel strategy for the revalorization of olive residues based on the extraction of bioactive peptides.[48] In addition, the recovery of protein-rich fish by-products resulting from the processing of sardine (*Sardina pilchardus*) (15.5–19.1% protein in a wet basis) is also gaining importance as well as the valorization of low commercial value and traditionally discarded fish species as blue whiting (*Micromesistius poutassou*) and horse mackerel (*Trachurus trachurus*), which contain between 14%–19% of protein (wet basis).[49–52]

One of the preferred approaches to identify bioactive peptides is the empirical approach which begins with the hydrolysis of the selected protein source, testing different types of protease and operating conditions such as enzyme/substrate ratio, time, pH or temperature.[53] Afterwards, a bioactivity screening of the protein hydrolysate is carried out. However, these hydrolysates are a complex mixture of peptides with different sizes and compositions, as well as other undesirable components such free amino acids, enzymes and nonreacted native proteins. Therefore, fractionation methods are required to separate active peptides from other non-active hydrolysate components, using common techniques such as ultrafiltration.[54,55] Subsequently, the potential active peptides are identified from the most active fraction.[56] Generally, a further purification process is required, consisting of sequential chromatographic treatments including gel-filtration chromatography and high-performance liquid chromatography (HPLC),[57–59] which are combined with mass spectrometry (MS), particularly tandem MS (MS/MS) or by a combination of liquid chromatography and mass spectrometry (LC-MS).[25,60] Finally, the bioactivity of purified peptides is validated *in vitro* and/or *in vivo* through the quantification of inflammatory mediators such as prostaglandin E2 (PGE2), TNF-α, IL-6, IL-1β, IL-10, IL-8, VCAM-1 or NO in cell lines or by measuring the inhibition of enzymes involved in inflammation such as COX-2, LOX and iNOS.[61–64] Nonetheless, current techniques for separation, purification, identification, and quantification of biopeptides from their natural matrices are limited by high technicality and high cost, which restricts their therapeutic competitiveness and industrial application.[53] Processes for peptide preparation and identification represent an analytical challenge because they implie high yield and purity for the quantification of its potential bioactivity and the validation of their structure–activity relationship.

Recently, computational methods for predicting bioactivities of peptides have emerged to reduce the experimental effort, and *in silico* tools have become an inevitable approach prior to *in vitro* and/or *in vivo* investigation.[65] Bioinformatics-driven techniques have recently been suggested to solve the major drawbacks of the empirical methodology (reagent and time investments) to identify biopeptides, although there is still room for improvement. This approach is helped by the development of profiles of protein fragments with potential biological activity, based on a template sequence from the transcriptome of the organism. Once the bioactive sequence or sequences have been identified, its bioactivity could be predicted by machine learning web-based tools or prediction servers and for further validation, they are chemically synthesized and tested individually for their bioactivity either *in vitro* or *in vivo*.[66,67] In comparison to classical approaches, in this case the trial-and-error approach of the initial stage can be avoided, and the obtaining of biologically active peptide becomes less laborious and time-consuming.

This work reviews the current literature on anti-inflammatory peptides and protein hydrolysates from natural sources, such as animals and plants, and synthetic sources, including their production and mechanism of action. Particularly, this work systematically studies the role of peptides sequence, structure and physicochemical properties on their anti-inflammatory or immunomodulatory activity. In addition, the potential release of the most bioactive sequences from sustainable protein sources was assayed. Moreover, this study evaluates the correlation between predicted anti-inflammatory activity by two available bioinformatics tools.

2. Methods

2.1. Literature search

A systematic search was carried out from the published data in literature to identify protein hydrolysates and peptide

sequences with anti-inflammatory bioactivity. The databases used were Scopus and Pubmed and the search was limited to publications from 2015 to 2022. The publications were analyzed using the following terms: "Peptides" and "Hydrolysate" in combinations with "anti-inflammatory" and "inflammation". The selected literature met the following criteria: research works that conducted mainly *in vitro*, but also *in vivo* experimental studies evaluating the anti-inflammatory effects of peptides or protein hydrolysates. Studies were excluded based on the following criteria: thesis, dissertations, reviews and, dissertations, reports, and repeated articles. Moreover, references included in the chosen studies and in review articles were reviewed to look for additional studies of interest.

Among all the peptide sequences identified in the selected literature for exhibiting anti-inflammatory potential *in vitro*, a classification was made according to the inhibition and/or production values of inflammatory markers reported for each peptide. From this classification, a threshold for each inflammatory marker was chosen. The selection threshold is determined by the value at which the activity reported is significantly higher than the rest of the activities reported for that specific inflammatory marker. The threshold selected for inhibition values of NO was 70% (30% production), 52% for iNOS, 65% for COX-2, 50% for 5-LOX, 65% for TNF-α, 75% for IL-1β, 75% for IL-6, and 800 pg mL^{-1} for the production of the anti-inflammatory cytokine IL-10. Peptide sequences showing values below these thresholds were not selected for further study. The threshold selected for production values of TNF-α was 80 pg mL^{-1}, 99 pg mL^{-1} for IL-1β, 131 pg mL^{-1} for IL-6, 50 pg mL^{-1} for IL-8 and PGE2 and 14 μM for nitrite. Peptide sequences whose values exceed this threshold were excluded from the selection. Finally, peptides that meet the selection threshold for more than one inflammatory marker (*e.g.*, they reached the inhibition threshold for COX-2 and also for TNF-α, presenting high values in both cases) were selected for further analysis.

2.2. Structural analysis of biopeptides

For the structure analysis, the PepCalc peptide property calculator (**https://pepcalc.com/**; Innovagen AB) has been used to predict properties such as isoelectric point, net charge and estimated solubility of the peptide sequences. The hydrophobicity, polarity and composition of peptide sequences has also been studied.

2.3. Alternative protein sources of anti-inflammatory peptides

BLASTp has been used to search for alternative and sustainable protein sources containing the sequences identified in the literature with the highest bioactive potential.[68] In the search, the default parameters were considered in order to represent an array of vegetal and animal sources as well as possibilities of by-products valorisation: standard databases (nonredundat protein sequences) and blastp algorithm (protein–protein BLAST). In the protein database, the chosen alternative protein sources were *Tenebrio molitor* a representative specie of insects; *Helianthus annuus*, *Cicer arietinum*, *Lupinus albus*, *Olea europaea subsp. Europaea* and *Pisum sativum* species representing plants; *Sardina pilchardus*, *Micromesistius poutassou* and *Trachurus trachurus* representative species of fish by-products.

2.4. Anti-inflammatory activity predictors

To predict the potential theoretical anti-inflammatory activity of the peptides found in literature, two predictors were used and compared. PreAIP (Predictor of Anti-Inflammatory Peptides) that was proposed by Khatun *et al.* incorporating manifold features like primary sequence, physicochemical properties, evolutionary features and structural information by combining *k*-spaced amino acid pairs, amino acids index and *k*-spaced amino acid pairs acquired from position-specific scoring matrix through a random forest (machine learning algorithm) classifier; and AIPpred (Anti-inflammatory Peptide predictor) developed by Manavalan *et al.* that uses 4 different machine learning methods (ERT, RF, k-NN and SVM) and sequence encoding features like amino acid composition, dipeptide composition, amino acid index and physiochemical properties.[69,70]

3. Results and discussion

After searching the databases, 114 articles were selected and utilized to prepare the current review. A total of 129 peptides and 46 protein hydrolysates were found from 52 different protein sources.

3.1. Peptides exhibiting anti-inflammatory activity *in vitro*

The majority of experimental studies on bioactive peptides with anti-inflammatory activity have used the murine macrophage cell line RAW 264.7, although many anti-inflammatory peptides have been identified through human leukemia monocytic cell line THP-1 and human colon adenocarcinoma cell line Caco-2 as observed in the studies compiled in Tables 1–3. Moreover, some studies have tested the inflammatory response in cell coculture which provides the opportunity to evaluate cell–cell interactions on inflammation microenvironment.[71,72] Cell cultured systems offer competitive, rapid and reproducible assays to analyze and validate the effects of peptides on different inflammatory markers such as TNF-α, IL-1β or NO.[73,74] To induce cell inflammation, LPS is one of the most common stimuli used in literature in a wide range of concentrations from 0.002 μg mL^{-1} to 1000 μg mL^{-1}, but TNF-α is also frequently used as it also stimulate the inflammatory cascade (see Table S1 in ESI†).

In addition to cell assays, there are other types of enzyme-type assays to study the anti-inflammatory potential. Inflammatory enzymes are also very attractive therapeutic targets since their expression and elevation is associated with most forms of inflammation. Several studies make use of commercial inhibition assays for COX-2, LOX and PLA_2.[27,75] For instance, the potential anti-inflammatory capacities of lupin protein hydrolysate were studied by determining its *in vitro*

Table 1 *In vitro* studies of animal-derived anti-inflammatory peptides

Protein type	Protein source	Peptide obtention	Peptide sequence[a]	Regulatory mechanism	Cell type	Ref.
Egg and dairy	Egg	Enzymatic hydrolysis *in silico* (pepsin and trypsin)	FL	Reduce IL-8 secretion, TNF-α, IL-8, IL-6 and IL-1β expressions and increased IL-10 expression	Caco-2	82
			MK			
			LL			
			CR	Reduce IL-8 secretion, TNF-α, IL-8, IL-6 and IL-1β expressions, phosphorylation of pJNK and p-p-38 and increased IL-10 expression		
			HC			
		GID (pepsin and pancreatin)	MLGATSL (ML-7)	Inhibit expressions of TNF-α, IL-8, IL-6, IL-1β, IL-12, JNK, IκB, and p38 and increase IL-10 expression	Caco-2	84
			DEDTQAMPFR (DR-10)			
			DEDTQAMPF (DF-9)			
			GW	Reduce the expression of TNF-α, ICAM-1 and VCAM1	HUVECs	83
	Milk	Enzymatic hydrolysis (trypsin)	KVLPVPQK (K-8-K)	Reduce levels of NF-κB in the nucleus and TNF-α, IL-6, and IL-1β gene expression	Caco-2	86
		Enzymatic hydrolysis (alcalase)	DQWL	Inhibit gene expression of IL-1β, COX-2, and TNF-α	RAW 264.7	85
Marine	Baijiao sea bass (*Lateolabrax maculatus*)	Chemical extraction	DAPAPPSQLEHIRAA	Inhibit NO production	RAW 264.7	89
			AADGPMKGILGY			
	Sea anemone (*Heteractis crispa*)	Chemical extraction	RGICSEPKVVGPCKAGLRRFYYDSETGECKPFIYGGCKGNKNNFETLHACRGICRA (HCRG1)	Reduce TNF-α and IL-6 secretion and IL-1β precursor (proIL-1β) expression levels	J774A.1/RAW 264.7 coculture	90
			RGICLEPKVVGPCKARIRRFYYDSETGKCTPFIYGGCGGNGNNFETLHACRGICRA (HCRG2)			
	Clam worm (*Marphysa sanguinea*)	Chemical extraction	NCWPFQGVPLGFQAPP	Inhibit IL-1β, TNF-α, NO, iNOS, and COX-2 gene expression	RAW 264.7	73
	Sea snake venom gland (*Hydrophis cyanocinctus*)	Chemical synthesis	DEQHLETELHTHLTSVLTANGFQ (Hydrostatin-SN1)	Inhibit TNF-α, IL-6, and IL-1β	RAW 264.7	91
	Mollusk Abalone (*Haliotis discus hannai*)	GID (pepsin, trypsin, α-chymotrypsin)	PFNEGTFAS	Inhibit NO production, reduce gene transcription levels of IL-1β, IL-6, and TNF-α, and suppress phosphorylation of p-p38 and p-JNK MAPKs	RAW 264.7	94
	Oyster (*Ostreidae*)	Enzymatic hydrolysis (Protamex, Neutrase) + GID (pepsin, trypsin, α-chymotrypsin, carboxypeptidase A)	YA	Inhibit NO production	RAW 264.7	183
	Bivalve visceral mass (*Meretrix meretrix*)	Enzymatic hydrolysis (trypsin, pepsin, alcalase and papain)	HKGQCC	Reduce iNOS activity, inhibit production of TNF-α and IL-1β, and prevent activation of COX-2	RAW 264.7	74
		Enzymatic hydrolysis (trypsin, pepsin, alcalase, papain) + GID (pepsin, trypsin, α-chymotrypsin)	GQCC (MMV2)			
	Sturgeon muscle (*Acipenseridae*)	Enzymatic hydrolysis (alcalase)	HLDDALRGQE	Reduce NO, IL-6, and IL-1β production and inhibit phosphorylation levels of MAPKs	RAW 264.7	63
	Herring milt (*Clupea harengus*)	Enzymatic hydrolysis (alcalase)	IVPAS	Inhibit iNOS pathway decreasing NO production	RAW 264.7	96
			FDKPVSPLL			
	Peanut worm (*Sipunculus nudus Linn.*)	Enzymatic hydrolysis (alcalase)	TVNLAYY	Inhibit NO production and reduce gene expression of iNOS, IL-6, TNF-α, and COX-2	RAW 264.7	97
			LSPLLAAH			
	Sea cucumber (*Actinopyga lecanora*)	Enzymatic hydrolysis (bromelain)	LREMLSTMCTARGA	Inhibit NO production	RAW 264.7	99
			VAPAWGPWPKG			
			AVGPAGPRG			
	Salmon skin (*Salmo salar*)	Enzymatic hydrolysis (Flavourzyme)	QA	Reduce NO, IL-6, IL-1β, and TNF-α secretion	RAW 264.7	100
			APD			
			KA			
			WG			
	Salmon bones (*Salmo salar*)	Enzymatic hydrolysis (papain)	SNKGGGRPN	Inhibit NO production and iNOS, IL-6, TNF-α and COX-2 mRNA levels	RAW 264.7	101
			TVTVYSLLR			
			PGVATAPTH			
			SLPEANSLRHR			
			LLGLGLPPA			
			TLGTGLCPV			
			LKPKGGSVP			
			FGLLVNPGA			
			NGRACSYKLWD			
	Salmon pectoral fin (*Salmo salar*)	Enzymatic hydrolysis (pepsin)	PAY	Inhibit NO, PGE2, IL-6, TNF-α and IL-1β production	RAW 264.7	60
	Sturgeon muscle (*Acipenseridae*)	Enzymatic hydrolysis (pepsin)	KIWHHTF	Reduce NO, IL-6, and IL-1β production and inhibit phosphorylation of MAPKs	RAW 264.7	63
			VHYAGTVDY			

Table 1 *(Contd.)*

Protein type	Protein source	Peptide obtention	Peptide sequence[a]	Regulatory mechanism	Cell type	Ref.
Terrestrial	Spider venom gland (*Pardosa astrigera*)	Chemical synthesis	AMMAESRKDNCIPKHHECTSRPKDCCKQNLMQFKCSCMTIIDKNNKETERCKCDNSIFQKVAKTSVNIGKAVVTK (Lycotoxin-Pa4a)	Reduce NO production *via* downregulation of the iNOS gene and COX-2, TNF-α and IL-1β expression	RAW 264.7	66
	Cecropia moth (*Hyalophora cecropia*)	Chemical synthesis	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK (Cecropin A)	Reduce NO production, mTNF-α and mIL-1β cytokine release, and COX-2 expression	RAW 264.7	107
	European red frog skin (*Rana temporaria*)	Chemical synthesis	FVQWFSKFLGRIL (Temporin-1TL)	Inhibit TNF-α and NO production, and TNF-α and iNOS mRNA expression	RAW 264.7	134
	Brazilian scorpion venom (*Tityus obscurus*)	Chemical synthesis	KIASVLGGILSPILSFF (ToAP4)	Reduced TNF-α and IL-1β gene expression and protein levels	J774, BMDMs and BMDCs	102
			KIASILGGILGPIMGIF (ToAP3)			
	Frog skin (*Hylarana guentheri*)	Chemical synthesis	GLFSKKGGKGGKSWIKGVFKGIKGIGKEVGGDVIRTGIEIAACKIKGEC (Esculentin-1GN)	Inhibit NO production, NO, IL-1β, IL6, and TNF-α gene expression and enhanced IL-10 expression	RAW 264.7	104
	Centipede (*Scolopendra subspinipes mutilans*)	Chemical synthesis	KKASKSVIKIFYKCM (Scolopendrasin IX)	Inhibit TNF-α, IL-6, and enhanced IL-10 production	Mouse neutrophil	184
	Chicken (*Gallus gallus domesticus*)	Chemical synthesis	FRASGQITITVKPRFRRIKRLFRGFR (CATH-2)	Inhibit IL-1β transcription and NO production	HD11	185
	Human (*Homo sapiens*)	Chemical synthesis	SIFGKIFKRIIRVAWK (Hs02)	Inhibit TNF-α production	C57BL/6/THP-1 coculture	71
	Hard tick (*Amblyomma variegatum*)	Chemical synthesis	HLHMHGNGATQVFKPRLVLKCPNAAQLIQPGKLQRQLLLQ (Amregulin)	Inhibit TNF-α, IL-8 and IFN-γ production	Rat splenocytes	103
	Dynastid Beetle (*Allomyrina dichotoma*)	Chemical synthesis	AFWCLIRRTVAA (Allomyrinasin)	Reduce NO and PGE2 production and COX-2 expression	RAW 264.7	67
	Chinese scorpion (*Mesobuthus martensii*)	Chemical extraction	HYGH	Reduce NO, TNF-α, IL-6, and IL-1β production and inhibit IκBα degradation, p65 nuclear translocation, NF-κB activation and phosphorylation of ERK, JNK, and p38 MAPKs	RAW 264.7	64
	Horsefly salivary glands (*Tabanus yao*)	Chemical extraction	RGQANILAGKNIKIRSGAAAGVGKTPQKANVEVLALGIW (Cecropin-TY1)	Inhibit NO, IL-1β, IL6, and TNF-α production	RAW 264.7	106
	Black fly salivary glands (*Simulium bannaense*)	Chemical extraction	GKLTKDKLKRGAKKALNVASKV (SibaCec)	Inhibit NO, TNF-α, IL-1β, and IL-6 transcription and production along with inhibiting MAPKs and NF-κB pathways	RAW 264.7	105
	Red deer velvet antler (*Cervus elaphus Linnaeus*)	GID (pepsin and pancreatin)	LAN	Inhibit NO production	RAW 264.7	59
			VH			
			IA			
			AL			
	Locust (*Schistocerca gregaria*)	GID (α-amylase, pepsin, pancreatin, and bilioA)	FDPFPK	Inhibit LOX and COX activity	Comercial assay	62
	Gastropod visceral mass (*Harpa ventricosa*)	Enzymatic hydrolysis (trypsin, alcalase and pepsin)	AKGTWK	Inhibit NO, TNF-α and IL-1β production	THP-1	58
	Hen spent muscle (*Gallus gallus domesticus*)	Enzymatic hydrolysis (protease M and Protex 50FP)	SFMNVKHWPW	Inhibit IL-6 production	U937	108
			AFMNVKHWPW			
	Chicken Feather Meal (*Gallus gallus domesticus*)	Enzymatic hydrolysis (Flavourzyme)	SNPSVAGVR	Reduce gene transcription levels of iNOS, TNF-α, COX-2 and IL-6	RAW 264.7	110
	Chicken sternal cartilage (*Gallus gallus domesticus*)	Enzymatic hydrolysis (papain)	VAIQAVLSLYASGR	Inhibit IL-1β, TNF-α, and PGE2 production	C518	109

[a] Amino acid sequence has been written from the first residue at N-terminus to the last residue at C-terminus (considering C-terminus the end of the peptide amino acid chain). Caco-2: human colon adenocarcinoma cell line. HUVECs: human umbilical vein endothelial cell line. RAW 264.7: murine macrophage cell line. J774A.1: mouse reticulum cell sarcoma cell line. BMDM: murine bone marrow-derived macrophages cell line. BMDC: murine done marrow-derived dendritic cell line. HD11: chicken macrophage cell line. THP-1: human leukemia monocytic cell line. U937: human monocytic cell line. C518: rat C518 knee joint degenerative cartilage cells.

Table 2 *In vitro* studies of plant-derived anti-inflammatory peptides

Protein type	Protein source	Peptide obtention	Peptide sequence[a]	Regulatory mechanism	Cell type	Ref.
Gramineae	Rice (*Oryza sativa* L.)	Enzymatic hydrolysis (trypsin)	IGVAMDYSASSKR	Inhibit gene expression of iNOS, IL-1β, IL-6, and TNFα	RAW 264.7	111
			DNIQGITKPAIR			
			IAFKTNPNSMVSHIAGK			
			QRDFLLAGNKRNPQAY	Inhibit nuclear translocation of p65, NO and TNF-α production and reduced TNF-α, iNOS, IL-6 and IL-1β transcription	RAW 264.7	112
	Corn zein (*Zea mays*)	Enzymatic hydrolysis (alcalase, neutral protease, thermolysin) + GID (pancreatin)	PPYLSP	Reduce gene expression of TNF-α and VCAM-1	EA.hy926	113
			IIGGAL			
			FLPPVTSMG			
Leguminosae	Black soybean (*Glycine max*)	Extraction	RGD	Inhibit NO, TNF-α, IL-1β, and IL-6 production	RAW 264.7	120
	Soybean (*Glycine max*)	Chemical synthesis	FLV	Inhibit TNF-α and IL-6 release	RAW 264.7/ 3T3-L1 coculture	72
		Enzymatic hydrolysis (alcalase and Neutrase)	YGGGGE	Reduce NO production and gene expression, TNF-α, IL-1β and IL-6 production and promote IL-10 gene expression	IEC-6	61
			SEGGFLE			
		Enzymatic hydrolysis (trypsin)	SLVNNDDRDS (S-10-S)	Reduce levels of NF-κB in the nucleus and TNF-α, IL-6, and IL-1β gene expression	Caco-2	86
	Fermented sorghum Baijiu vinasse (*Sorghum bicolor*)	Enzymatic hydrolysis (corolase PP)	KLPDHPKLPK (VPH-1)	Inhibit NO, TNF-α, IL-6 and IL-1β production	RAW 264.7	124
			VDVPVKVPYS (VPH-2)			
	Lupin seeds (*Lupinus angustifolius L.*)	Enzymatic hydrolysis (alcalase)	GPETAFLR	Reduce IL-1β, IL-6, and TNF-α production and increase IL-10 production and gene expression	THP-1	122
					ARPE-19	121
		GID (pepsin and pancreatin)	IQDKEGIPPDQQR (IQD)	Inhibit TNF-α, IL-6, IL-1β production	RAW 264.7	186
	Foxtail Millet (*Setaria italica*)	GID (pepsin and pancreatin)	QNWDFCEAWEPCF	Inhibit NO, IL-6, and TNF-α production	RAW 264.7	125
			EDDQMDPMAK			
Other plants	Hempseed (*Cannabis sativa*)	Enzymatic hydrolysis (pepsin)	IGFLIIWV	Reduce NO production levels and modulate IFN-γ, TNF-α, IL-6 and IL-10 levels	HepG2	126
			WVSPLAGRT			
	Lychee seeds (*Litchi chinensis Sonn.*)	Enzymatic hydrolysis (alcalase, Flavourzyme, Neutrase)	KVRTKLLPP	Inhibit NO production by down-regulation of iNOS and IL-6	RAW 264.7	127
			RPLVTHK			
			MKLCWQKSIHGS			
	Walnut (*Juglans regia*)	Enzymatic hydrolysis (Viscozyme L and pancreatin)	GVYY	Inhibit gene expression of COX2 and iNOS, and production of NO and PGE2	BV-2	128
			APTLW			
			LPF			

[a] Amino acid sequence has been written from the first residue at N-terminus to the last residue at C-terminus (considering C-terminus the end of the peptide amino acid chain). EA.hy926: human umbilical vein cell line. 3T3-L1: murine preadipocyte cell line. IEC-6: rat intestinal epithelial cell line. ARPE-19: human retinal pigment epithelial cell line. HepG2: human hepatocellular carcinoma cell line. BV-2: murine microglial cell line.

inhibition of enzymes involved in the inflammatory process including PLA_2 and COX-2.[76] By studying the inhibition of COX-1 and COX-2 together with LOX activities, anti-inflammatory peptides have been identified in the digest of millet grains as well as digested protein fractions from chia seed.[77,78] Furthermore, the inhibitory capacity against COX-2 and LOX of biopeptides obtained from edible insects have been measured.[62]

Table 3 *In vitro* studies of modified or designed *de novo* synthetic anti-inflammatory peptides

Protein source	Parental protein	Peptide sequence[a]	Regulatory mechanism	Cell type	Ref.
AMP	Tick defensin OsDef1	KGIRGYKGGYKGAFKQTKY (Os–C)	Inhibit NO and TNF-α production	RAW 264.7	130
		KGIRGYKGGYCKGAFKQTCKCY (Os)			
	Rana temporaria temporin-1TL (TL)	FVQWWSKWLGRIL (TL-1)	Inhibit TNF-α and NO production, and TNF-α and iNOS mRNA expression	RAW 264.7	134
		FVRWWSKWLRRIL (TL-2)			
		FVRWWSRWLRRIL (TL-3)			
		FVKWWSKWLKKIL (TL-4)			
	Pseudin-2 (Ps)	GLNALKKVFQGIHEAIKKINNHVQ (Ps-K18)	Inhibit NF-κB gene expression and TNF-α, IL-6 and IL-1α production	RAW 264.7	133
	Chensinin-1	SAVWRHWRRFWLRKHRKH (MC1-1)	Inhibit TNF-α and IL-6 production	RAW 264.7	132
	Chemokine CXCL14	YKRWKKNWAKYWKIFRK (CXCL14-C17-a2)	Inhibit NO, TNF-α and IL-6 production	RAW 264.7	131
		YKRWKKRWAKYWKKFRK (CXCL14-C17-a3)			
	Chicken cathelicidin-2 (CATH-2)	QITITVKPRFRRIKRLFR	Inhibit IL-1β transcription and NO production	HD11	185
		QITITVKPRFRRIKRLFRGFR			
Hormone	Glucagon-like peptide-1 (GLP-1)	HAEGTFTSDVSSYLEGQAAKEFI (Liraglutide)	Reduce NO, PGE2, and IL-6 production, IL-6, COX-2, and TNF-α gene expression	RAW 264.7	136
	Parathyroid hormone–related protein (PTHrP)	ETNKV	Reduce IL-6 and PGE2 production	HOb-OA	135
		ETNKVETYKEQPLKTPGKKKKGKP GKRREQEKK	Reduce IL-6, PGE2, TNF-α production and NF-κB activation		
	Protease-activated receptor-1 (PAR-1)	NPNDKYEPFWEDEEKNESGL	Reduce IL-1β production	THP-1	187
	Protease-activated receptor-3 (PAR-3)	GAPPNSFEEFPFSALEGWTGATIT			
Hybrid	Bovine lactoferrin (LfcinB)-human cathelicidin (LL-37) hybrid	RLWKKILKVIRKPRWQWRR (Lf-KR)	Inhibited NO and TNF-α expression and production	RAW 264.7	139
	Cathelicidin-2 (CATH-2)-thymopentin (TP5) hybrid	RWGRFLRKIRRFRRKDVY (CTP)	Reduce TNF-α, IL-1β, and IL-6 production	RAW 264.7	138
De novo designed	Non-existent	KKIRVRLSA (SET-M33D)	Reduce TNF-α, IL6, COX-2, iNOS and NF-κB gene expression	RAW 264.7	143
			Inhibit TNF-α, IL-6, and IL-1β, IL-8 and COX-2, iNOS and NF-κB activation	RAW 264.7	142
		GAKYAKIIYNYLKKIANALW (GW-A2)	Reduce NO, iNOS, COX-2, TNF-α and IL-6 expression levels, phosphorylation of MAPK and NF-κB activation	RAW 264.7	141
		LKWLKKLLKKL (WALK11.3)	Inhibit NO, COX-2, IL-1β, IL-6, INF-β, and TNF-α expression	RAW 264.7	140
	Spider venom glands (*Agelena koreana*)	FKGLAKLLKIGLKALAKVIQ (Ak-N′)	Reduce TNF-α, IL-6, and IL-1β gene expression	THP-1	188
		NKGLAKLLKIGLKALESVIQ (Ak-N′m)			

[a] Amino acid sequence has been written from the first residue at N-terminus to the last residue at C-terminus (considering C-terminus the end of the peptide amino acid chain). HOb-OA: human osteoblasts–osteoarthritis cell line.

3.1.1. Anti-inflammatory peptides derived from animals. Table 1 shows the peptides generated from animal proteins that showed anti-inflammatory activity *in vitro.*

3.1.1.1. Egg and dairy peptides. Egg protein is considered a high-quality protein source given the wide variety of proteins it contains. There are more than 100 different proteins in the egg white alone, among which it is worth highlighting Ovotransferrin that accounts for 12% of egg white composition and is considered a potential drug for the treatment of inflammation-related diseases such as inflammatory bowel disease (IBD) and cardiovascular diseases (CVDs).[79,80] Indeed, egg proteins are well-recognized as an excellent source of bioactive peptides.[81] From hydrolysates produced by combining pepsin and trypsin treatment on egg-ovotransferrin five anti-inflammatory dipeptides were identified, with amino acid sequences CR, HC, FL, MK and LL.[82] Moreover, from the treatment of egg-ovotransferrin with pepsin, in this case combined with pancreatin simulating a gastrointestinal digestion (GID), another dipeptide with sequence GW was found to perform effects against inflammation after digestion of its parental peptide GWNI.[83] Furthermore, simulated GID of egg-whites with pepsin and pancreatin produced peptides MLGATSL (ML-7), DEDTQAMPFR (DR-10) and DEDTQAMPF (DF-9) that showed the same anti-inflammatory mechanism as dipeptides CR, HC, FL, MK and LL inhibiting the NF-κB pathway by reducing pro-inflammatory cytokines TNF-α, IL-8, IL-6 and IL-1β

expression and increasing anti-inflammatory cytokine IL-10 expression.[84] In addition, ML-7, DR-10 and DF-9 possessed the higher IL-8 inhibitory activity between other peptides obtained in the study, reducing IL-8 production a 34.6%, 35.2% and 35.9%, respectively. Besides, among the five peptides produced by pepsin/trypsin treatment CR and HC have the greatest potential to reduce the production of the pro-inflammatory chemokine IL-8. Peptides CR, HC, ML-7, DR-10 and DF-9 also inhibited the MAPK signaling pathway by directly suppressing the phosphorylation of two MAPK, c-Jun N-terminal kinase and p-38 in TNF-α-induced Caco-2 cells. In contrast, the bioactivity of egg-derived dipeptide GW was measured differently from the previous peptides, and GW exerted a reduction of the expression of TNF-α, ICAM-1 and VCAM1 significantly, especially in the case of the last one since it was reported a 64.3% reduction compared to TNF-α treated endothelial cell line HUVECs.[83]

Similarly to egg-derived peptides, anti-inflammatory peptides from milk were among the first-studied biopeptides. In this case, the reviewed studies carried out the hydrolysis with a single enzyme instead of combining pepsin with different enzymes like is done for egg proteins. For example, properties against inflammation of peptides derived from whey bovine protein treated with alcalase have been evaluated in RAW 264.7 cells and peptide DQWL showed strong inhibitory ability on IL-1β and COX-2 with a reduction of 49.5% and 62.1%, respectively. Additionally, DQWL treatment suppressed nuclear translocation of the p65 component of NF-κB and blocked IκB kinase phosphorylation, IκB degradation and p38 activation.[85] Another study on milk-derived peptides identified a peptide with sequence KVLPVPEK (K-8-K) exerting anti-inflammatory activity through inhibition of the NF-κB pathway.[86]

3.1.1.2. Marine animal peptides. Marine-organisms derived peptides have attracted the attention of the pharmaceutical and nutraceutical industries in the hopes of using them to treat or prevent chronic diseases due to their active anti-inflammatory properties and high commercial values.[87] The first marine-derived peptide approved as a drug by the US Food and Drug Administration (FDA) in 2004 for specific indication of chronic pain was Ziconotide (Prialt®), originally isolated from a marine animal cone snail (*Conus magus*).[88] Besides, anti-inflammatory peptides naturally present in other marine organisms such as Baijiao sea bass have been identified. This sea bass is one of the most economically important cultured fish species in China and its derived peptides DAPAPPSQLEHIRAA and AADGPMKGILGY demonstrated potent anti-inflammatory capacity in murine macrophages cells suppressing NO production by 20%.[89] However, peptides named HCRG1 and HCRG2 naturally found in sea anemone *Heteractis crispa* possessed anti-inflammatory activity by reducing TNF-α, IL-6 and IL-1β precursor secretions in LPS-activated murine macrophages but they show no effect on NO production.[90] Furthermore, peptide Hydrostatin-SN1 present in sea snake (*Hydrophis cyanocinctus*) venom gland reported a reduction in inflammation by inhibiting essential pro-inflammatory cytokines along with phosphorylation of ERK1/2 and nuclear translocation of NF-κB in various cell lines: RAW 264.7, HEK293 (human embryonic kidney cells) and HT29 (human colorectal adenocarcinoma cells).[91,92] In addition, Hydrostatin-TL1, also identified from (*Hydrophis cyanocinctus*) venom gland, has significant anti-inflammatory activity *in vitro* and also *in vivo*.[93]

Apart from chemical extraction of peptides naturally present in marine-animal proteins, anti-inflammatory peptides have also been obtained by enzymatic hydrolysis of marine proteins. Anti-inflamamtory peptides with sequences PFNEGTFAS, YA and GQCC were obtained by gastrointestinal digestion with a combination of trypsin, pepsin and α-chymotrypsin of proteins from mollusk abalone (*Haliotis discus hannai*) intestine, oyster (*Ostreidae*) and bivalve (*Meretrix meretrix*) visceral mass, respectively.[74,94,95] It should be noted that tetrapeptide GQCC, produced from the digestion of its parental peptide HKGQCC obtained from hydrolysis with a combination of four enzymes (trypsin, pepsin, alcalase and papain), outperformed its parental peptide in inflammatory potential, suppressing NO production by 58% against 45% supressed with HKGQCC. These three peptides have the same regulatory action on iNOS activation by inhibiting the production of NO, showing a good IC_{50} value of 54.07 μg ml^{-1} in the case of PFNEGTFAS. Besides, they have other effects such as the regulation of the NF-κB pathway (except in the case of the dipeptide YA).

Furthermore, recent findings on fishing by-products treated with Alcalase led to the identification of two novel peptides IVPAS and FDKPVSPLL from herring milt hydrolysate and peptide HLDDALRGQE from sturgeon muscle protein hydrolysate, that showed anti-inflammatory activity by decreasing NO production in murine macrophage cells.[63,96] Another study reported the hydrolysis with Alcalase of peanut worms protein, where two identified peptides TVNLAYY and LSPLLAAH exhibited high NO-inhibitory activity along with pro-inflammatory cytokines regulation, such as IL-6 and TNF-α, and COX-2 suppression.[97] Moreover, anti-inflammatory peptides KIWHHTF and VHYAGTVDY from pepsin–sturgeon muscle hydrolysate also suppressed NO production by reducing nitrite levels and down-regulate the MAPK pathway.[98]

Another enzymatic treatment of marine proteins using bromelain has also reported good results with respect to the production of peptides with anti-inflammatory activity. For example, bioactive peptides with sequences LREMLSTMCTARGA, AVGPAGPRG and VAPAWGPWPKG from sea cucumber (*Actinopyga lecanora*) bromelain-hydrolysate, obtained using bromelain, inhibited NO production in murine macrophages showing an IC_{50} value of 572.096 mg mL^{-1} and 674.435 mg mL^{-1}, respectively.[99]

Alternatively, enzymes such as Flavourzyme and papain have also been used to produce anti-inflammatory peptides from salmon by-products. Atlantic salmon provides large quantities of low-value protein rich co-products, such as salmon skin and bones that can be upgraded from by-products to high-value functional ingredients such as biopeptides. As an example of this, hydrolysis with Flavourzyme of salmon

skin produced dipeptides QA, KA and WG and tripeptide APD, which significantly reduced production of IL-6, IL-1β and TNF-α in LPS-stimulated murine macrophages. Peptide QA had the highest overall anti-inflammatory impact of all of them having an IC_{50} value against NO production of 849.3 μM.[100] Moreover, salmon bone hydrolysis with papain, in comparison with Alcalase, Flavourzyme and Neutrase hydrolysates, produced a range of potential anti-inflammatory peptides such as peptides SNKGGGRPN and TVTVYSLLR, which were found to have a marked NO-inhibitory activity with an IC_{50} value of 2.56 μg mL^{-1} along with IL-6, TNF-α and COX-2 regulatory activity.[101] In addition, a tripeptide from salmon pectoral fin by-product, PAY, produced by a different enzyme treatment using pepsin instead of Flavourzyme or papain, also exhibited anti-inflammatory action *via* inhibiting production of NO by 63.80% plus reducing production of inflammatory markers such as PGE2 among others and activation of COX-2.[60]

3.1.1.3. Terrestrial animal peptides. Peptides exerting anti-inflammatory activity have also been found in terrestrial animal sources either after processing the protein source by enzymatic hydrolysis or by extracting or synthesizing the peptides found naturally in its protein composition.

Regarding peptides naturally present in these animals, RNA sequencing has proven to be an interesting tool to assist the identification of novel anti-inflammatory peptides. As an example, after sequencing the RNA of spider *Pardosa astrigera* venom gland and of Dynastid beetle (*Allomyrina dichotoma*) potential anti-inflammatory peptides named Lycotoxin-Pa4a (peptide toxin) and Allomyrinasin, from the two sources respectively, were selected based on their physicochemical properties and structural characteristics. Both peptides showed the ability to decrease NO production *via* downregulation of iNOS gene and suppressed the expression of COX-2 apart from regulating the production of other inflammatory mediators such as IL-1β in LPS-stimulated murine macrophages.[66,67]

Another powerful tool towards the identification of anti-inflammatory peptides with desired properties is cDNA display, which enables selection of peptide libraries that encompass thousands of sequences. Thanks to this technique, peptides ToAP3 and ToAP4 from Brazilian scorpion (*Tityus obscurus*) venom, peptide Esculentin-1GN from the skin of the frog *Hylarana guentheri* and peptide Amregulin from hard tick *Amblyomma variegatum* were identified as potentially anti-inflammatory peptides.[102–104] These four peptides act by inhibiting the production of different proinflammatory cytokines such as TNF-α, and in the case of peptides ToAP3 and ToAP4 they are capable of increasing the production of anti-inflammatory cytokine IL-10, and exerted their properties in various cell types: J774, murine bone marrow-derived macrophages (BMDMs) and dendritic cells (BMDC). In addition, peptide Esculentin-1GN is the only one that shows the ability to regulate NO production and iNOS transcription.

In addition to the *in silico* tools for peptide identification discussed above, anti-inflammatory peptide discovery has also been made by chemical extraction, as in the case of tetrapeptide HYGH purified from the crude protein extract of the Chinese scorpion (*Mesobuthus martensii*). Peptide HYGH showed different mechanisms of action by regulation of NF-κB together with MAPK inflammatory signaling pathways along with suppressing NO production by 76.8%.[64] Anti-inflamamtory peptide GKLTKDKLKRGAKKALNVASKV (Sibasec) has also been extracted from the salivary glands of black fly (*Simulium bannaense*) after determining its amino acid composition by cDNA sequencing.[105] Since flies attract attention due to their good nutritional value (17.5–67 g per 100 g protein), more peptides with anti-inflammatory potential such as Cecropin-TY1 were discovered from salivary glands of horsefly.[106] Cecropin TY1 and Sibasec share the same mechanism of action inhibiting the production of NO, TNF-α, IL-1β, and IL-6 and the activation of iNOS by 58.8% and 51.9%, respectively. Furthermore, synthesis of known antimicrobial peptides (AMPs) has also been investigated to test whether they also have effect against inflammation. A proof of this is peptide Cecropin A, an antibacterial 37-residue peptide isolated from the cecropia moth that proved to be a potential agent for prevention and/or treatment of inflammatory disorders, since its mechanism of action is very complete. Cecropin A was able to reduce NO production by total suppression of nitrite production along with reducing TNF-α and IL-1β release, COX-2 expression and phosphorylation of ERK, JNK, p38 inhibiting MAPK pathway.[107]

Apart from identifying already existing anti-inflammatory peptides in the protein sequence of terrestrial animal sources, several studies have identified peptides with this bioactivity after *in vitro* simulated gastrointestinal digestion with pepsin/pancreatin mixture, either in combination with other enzymes such as α-amylase or alone. Results of these studies reported that edible insect locust *Schistocerca gregaria* derived peptide FDPFPK has the ability to inhibit COX-2 and LOX activity giving a IC_{50} values of 7.40 and 2.85 mg mL^{-1}, respectively, and that red deer Cervus elaphus Linnaeus velvet antler derived peptides LAN, VH, IA and AL had also a strong inhibitoty activity against NO, showing greater effect in the case of the three dipeptides compared to the longer peptide LAN.[59,62]

There are also terrestrial animal-protein hydrolysates produced by with a single enzyme where peptides with good effects against inflammation have been identified. For example, visceral mass protein of gastropod *Harpa ventricose* was hydrolysed by trypsin, Alcalase and pepsin individually. The most active hydrolysate was the tryptic one where an hexapeptide AKGTWK was found to suppresed NO production up to 61.6% along with inhibition of TNF-α and IL-1β production.[58] Moreover, anti-inflammatory peptides were generated from chicken by-products using hydrolysis with individual commercial enzymes, *i.e.*, peptides SFMNVKHWPW and AFMNVKHWPW identified from spent hen muscle protein and treated with Protex 50FP reported a good IC_{50} value of 100 μg mL^{-1} for IL-6 production.[108] Another examples is chicken-collagen peptides such as VAIQAVLSLYASGR from papain hydrolysate that significantly inhibited the secretion of inflammatory cytokines IL-1β, TNF-α, and PGE2.[109] Furthermore, peptide SNPSVAGVR obtained after hydrolysis with Flavourzyme of chicken feather meal that showed inhibitory activity against

NO production with an IC_{50} value of 55.2 mM and reduced gene transcription levels of COX-2 and IL-6 by 83 and 96%, respectively.[110]

3.1.2. Anti-inflammatory peptides derived from plants. Plant proteins have also been studied as a source for bioactive peptides with anti-inflammatory activity as shown in Table 2.

3.1.2.1. Gramineae peptides. Recent studies identified peptides from trypsin hydrolysates of rice protein with good anti-inflammatory activity in murine macrophages, such as peptide QRDFLLAGNKRNPQAY that was capable of reducing NO production and iNOS transcrition along with TNF-α, IL-6 and IL-1β transcription, or peptides IGVAMDYSASSKR, DNIQGITKPAIR and IAFKTNPNSMVSHIAGK that also showed inhibition of IL-1β, IL-6, and TNF-α gene expression but do not intervene in the regulation of NO production.[111,112]

Apart from enzymatic hydrolysis with trypsin, peptides PPYLSP, IIGGAL and FLPPVTSMG with strong anti-inflammatory activity have also been identified by hydrolyzing zein, a by-product of corn starch, with an enzymatic mixture of Alcalase, neutral protease and thermolysin in combination with a subsequent simulated gastrointestinal digestion with gastric fluid and pancreatin. These three peptides exhibit their effect by decreasing TNF-α expression and inhibiting ICAM-1 by 36.5–28.6% along with VCAM-1 by 54–38.9%; and their action is believed to be due to their successful transport across Caco-2 cell monolayers without digestion by peptidases.[113]

3.1.2.2. Leguminosae plant peptides. Several anti-inflammatory peptides found naturally in soybean (*Glycine max*) protein composition have been identified. Particularly, Lunasin, which is a 43-residue peptide from soy seeds, and lunasin-like peptides have been identified as an effective agent against inflammation acting through different mechanisms in several studies. For example, Lunasin isolated from defatted soybean flour inhibited inflammation through suppression of NF-κB pathways and was also found to inhibit COX-2/PGE2 and iNOS/NO pathways in LPS-induced murine macrophages.[114,115] Furthermore, intake of Lunasin from diet or as a supplement could prevent obesity by inhibiting inflammatory cytokine production and could prevent asthma due to its effect against allergic airway inflammation in two murine models.[116–119] Smaller soybean-derived peptides has also shown anti-inflamatory activity, like tripeptide FLV which potentially prevented obesity-related adipose inflammation by inactivation of inflammatory signaling molecules of the MAPK pathway or tripeptide RGD that was extracted from black soybean by high hydrostatic pressure (HHP) and germination and effectively inhibited expression of NO, TNF-α, IL-1β and IL-6.[72,120] Interestingly, peptide sequence RGD is present in Lunasin, thus it is believed to contribute to its anti-inflammatory effects.

Other novel peptides with anti-inflammatory activity have been obtained by enzymatic hydrolysis of soybean. Specifically, peptides YGGGGE and SEGGFLE obtained by hydrolysis with a mixture of Alcalase/Neutrase reported protective activity against intestinal inflammation, as well as peptide SLVNNDDRDS, named S-10-S, obtained by trypsin hydrolysis, that exerted its effect through inhibition of the NF-κB pathway and downregulation of the gene expression of IL-1β and TNF-α.[61,86]

The enzymes Corolase PP and Alcalase have also been used successfully to hydrolyze leguminosae plant proteins and identify anti-inflammatory peptides such as GPETAFLR an octapeptide derived from lupine protein hydrolyzed with alcalase that exerted protection against inflammatory damage in retinal pigment epithelium cells, as well as modulated the inflammatory response and plasticity in human primary monocytes.[121,122] Moreover, GPETAFLR inhibited neuroinflammation by preventing inflammation in BV-2 microglial cells and potentiating neuroprotection in mouse brain.[123] Alternatively, hydrolysis with Corolase PP produced anti-inflammatory peptides VDVPVKVPYS and KLPDHPKLPK with NO inhibitory activity (89% and 84%, respectively) from Baijiu vinasse, which is a by-product of sorghum fermentation.[124]

It also can be highlighted the potential of *in vitro* gastrointestinal digested foxtail millet protein with pepsin and pancreatin as a source of anti-inflammatory peptides like QNWDFCEAWEPCF and EDDQMDPMAK, which stood out for their effect inhibiting NO, IL-6, and TNF-α production between seven novel peptides identified.[125]

3.1.2.3. Other plant sources. Enzymatic hydrolysis with pepsin of hempseed (*Cannabis sativa*) produced two different anti-inflammatory peptides, IGFLIIWV and WVSPLAGRT, which showed their effect by reducing expression of IFN-γ and TNF-α along with attenuating NO production and increasing IL-10 expression levels. Interestingly, only peptide IGFLIIWV showed the ability to reduced IL-6 levels up to 15.1%.[126] Moreover, treatment with a combination of enzymes other than pepsin, specifically Viscozyme and pancreatin, generated neuroprotective short peptides GVYY, APTLW and LPF from walnut, as well as Neutrase treatment, gave better results regarding the inhibition of NO production by anti-inflammatory peptides from Lychee seeds such as RPLVTHK, in comparison with Alcalase and Flavourzyme hydrolysates.[127,128]

3.1.3. Anti-inflammatory peptides from modified natural peptides or designed *de novo*. Unlike the anti-inflammatory peptides listed in Tables 1 and 2, Table 3 shows peptides that do not exist naturally in organisms. Despite the fact that the majority of them are synthesized chemically, the structure of these peptides correspond to modifications or hybridizations of natural molecules belonging to the microbial world (AMPs) or endogenous in mammalian organisms (like hormones). Besides, some of these peptides are *de novo* designs based on existing proteins or enzymes.

Mutations of several residues or amino acid sequence modifications of known antimicrobial peptides have been made in order to test the anti-inflammatory potential of these new synthetic peptides since AMPs are key components of the immune system as one of the most important defense mechanisms against bacterial infections in many types of organisms. A defensin-like AMP, named OsDef2, was found in the hemolymph of the tick *Ornithodoros savignyi* and used as template for the synthesis of peptide Os and its analogue Os-C. Both

peptides derived from the C-terminal region of OsDef2 and succesfully inhibited LPS/IFN-γ-induced production of NO and TNF-α by 36% and 25%, respectively.[129,130] It seems that C-terminal region of antimicrobial peptides is a good template for the creation of anti-inflammatory peptides since, in addition to peptides Os and Os–C, peptides YKRWKKRWAKYWKKFRK and YKRWKKNWAKYWKIFRK were engineered by introducing changes in C-terminal amino acids of antimicrobial chemokine CXCL14, like the addition of lysine, arginine or tryptophan, and reported almost 100% inhibition of NO production along with TNF-α and IL-6 production decrease.[131] Substitutions of other amino acid residues have also been made resulting in several anti-inflammatory peptides such as peptide MC1-1, which was created by glycine and histidine mutations in AMP chensinin-1 derived from Chinese frog (*Rana chensinensi*) skin secretions or peptide Ps-K18 produced by substituting lysine for leucine at position 18 of AMP pseudin-2 (Ps) from paradoxical frog (*Pseudis paradoxa*) skin.[132,133] Another AMP that has been modified to enhance anti-inflammatory activity is temporin-1TL (TL), in this case 4 analogous peptides TL-1, TL-2, TL-3 and TL-4 were synthesized by substituting tryptophan, arginine and lysine from the original template sequence. It should be noted that synthetic TL analogs improved anti-inflammatory activity compared with TL thanks to the mutations performed.[134] Moreover, anti-inflammatory peptides derived from modifications of hormones involved in inflammatatory response, like parathyroid hormone (PTH) and Glucagon-like peptide-1 (GLP-1), have also been reviewed.[135] Peptide ETNKV and ETNKVETYKEQPLKTPGKKKKGKPGKRREQEKK are derived from N-terminus (amino acids 107–111) and C-terminus (107–139) of parotid hormone-related protein or PTH-rP (composition extracted from UniProt database for human PTH-rP) and both favored osteoblastic function, although the C-terminal domain was more efficient than N-terminal domain which is in agreement with the previous data for peptides Os, Os–C and CXCL14-derivivatives. Another hormone-derived peptide is Liraglutide, a glucagon-like peptide 1 receptor agonist that also exerted anti-inflammatory and anti-degradative actions in osteoarthritis being suggested as a potencial therapeutic candidate for Osteoarthritis (OA) treatment along with ETNKVETYKEQPLKTPGKKKKGKPGKRREQEKK and its shorter fragment.[135,136] Besides, Liraglutide is already commercially available for treatment of type II diabetes under the name Victoza® with a longer half-life compared to endogenous GLP-1.[137]

Apart from modifying natural peptides, hybridization is also suggested as an effective approach to enhance anti-inflammatory activity. An hybrid peptide named CTP and created by combining the active center of AMP cathelicidin-2 (CATH-2) with thymopentin (TP5), which is the active site of the naturally occurring hormone thymopoietin and showed enhanced anti-inflammatory activity when amine modification was made at C-terminus.[138] These results that are in agreement with the previously suggested hypothesis that C-terminus is a region involved in the anti-inflammatory effect. Nevertheless, the hybridization of a peptide derived from the N-terminus of bovine lactoferrin, LfcinB6, with another peptide KR-12-a4 composed of amino acids from the central part of human cathelicin (LL-37) resulted in an anti-inflammatory peptide named Lf-KR, which exerts inhibitory effects against NO and TNF-α production.[139] Therefore, it can be argued that the composition of other regions in natural peptides, apart from the C-terminal end, also have an influence on the activity against inflammation.

Finally, in addition to hybridizing or modifying known peptide sequences, designing *de novo* peptides that meet structural and physicochemical requirements is also a successful strategy for creating anti-inflammatory peptides. As an example, model peptide isomers with simple amino acid composition containing tryptophan and combinations of leucine/lysine residues were designed for the development of therapies against inflammation and one of these peptides, named WALK11.3, reported potent anti-inflammatory activity by inhibiting NO, COX-2 and cytokines TNF-α, IL-6 and IL-1β production.[140] Through the design based on structural determinants such as charge or hydrophobicity peptide GW-A2 was created and reported both antimicrobial activity and anti-inflammatory activity, attracting attention for its complete mechanism of action regulating MAPK and NF-κB pathways along with pro-inflammatory cytokines, COX-2 and iNOS expression levels.[141]

Regarding the bioactivity of these modified or designed synthetic peptides compared with the previously mentioned natural peptides, it could be assumed that "non-natural" peptide sequences present a slight improvement in terms of *in vitro* activity against inflammation, particularly for the suppression of proinflammatory cytokines TNF-α, IL-6 and IL-1β production and iNOS enzyme inhibition. Supporting this theory it is worth mentioning that synthetic peptide SET-M33D (KKIRVRLSA) was reported to have a very strong potential for protection against in inflammation murine macrophage cells by inhibiting the enzymes iNOS (by 83.3%) and COX-2 (by 84%) as well as significantly reducing cytokines TNF-α (by 95.7%), IL-1β (by 77.6%) among other inflammatory mediators regulation.[142,143] This bioactivity improvement may be due to these sequences being specifically designed or mutated to fulfil the purpose of fighting inflammation and they are "upgraded" versions of natural sequences. However, no reported measures have been found for inflammatory mediators such as LOX, IFN-γ, VCAM-1 or IL-10 and their effects *in vivo* have not been evaluated in many studies. Therefore, the spectrum of action against inflammation for natural peptides is largest since more mechanisms of action have been studied. Nevertheless, in view of the variability of methods and types of measurements, doses, *etc.*, it is very difficult to establish comparisons between the studies.

3.1.4. Selection of anti-inflammatory peptides. Table 4 shows the peptide sequences, among all peptides reviewed in Tables 1–3, that meet the first criteria of selection: they present the highest bioactivity with respect to the threshold set

Table 4 Number of peptides within the selection threshold for different measurements of bioactivity

Mechanism of inhibition	Selection criteria	Threshold	Number of peptides
NO production	Peptide sequences that report values below the threshold	30%	19
Nitrite production	Peptide sequences that report values below the threshold	14 μM	16
PGE2 production	Peptide sequences that report values below the threshold	50 pg mL^{-1}	2
TNF-α production	Peptide sequences that report values below the threshold	80 pg mL^{-1}	11
IL-1β production	Peptide sequences that report values below the threshold	99 pg mL^{-1}	15
IL-6 production	Peptide sequences that report values below the threshold	131 pg mL^{-1}	14
IL-8 production	Peptide sequences that report values below the threshold	50 pg mL^{-1}	2
IL-10 production	Peptide sequences that report values above the threshold	800 pg mL^{-1}	1
TNF-α inhibition	Peptide sequences that report values above the threshold	65%	2
IL-1β inhibition	Peptide sequences that report values above the threshold	75%	3
IL-6 inhibition	Peptide sequences that report values above the threshold	75%	3
iNOS inhibition	Peptide sequences that report values above the threshold	52%	5
COX-2 inhibition	Peptide sequences that report values above the threshold	65%	3
5-LOX inhibition	Peptide sequences that report values above the threshold	50%	1

in Methods subsection 2.1 (see Table S1 in ESI† for inhibition values); but only 28 of them meet the second requirement: they report values within the threshold set for more than one inflammatory marker. Therefore, the 28 peptides that meet both requirements have been chosen among the rest, along with 3 exceptions, 3 peptides that report bioactivity values within the threshold for a single inflammatory marker but are representative of such biomarker because there are few values reported in the literature. In summary, a total of 31 peptides were selected for further analysis.

3.2. Peptides exhibiting anti-inflammatory activity *in vivo*

Based on the promising results on cell-based investigations, several bioactive peptides have been studied in animal models, which allow to evaluate the effects of these compounds in more complex physiological conditions, un-like *in vitro* studies. Table 5 summarizes the *in vivo* studies conducted on peptides with anti-inflammatory activity.

One of the animal models in which the anti-inflammatory response to peptide treatment has been studied the most is

Table 5 *In vivo* studies of anti-inflammatory peptides

Protein type	Protein source	Peptide obtention	Peptide sequence[a]	Regulatory mechanism	Cell type	Ref.
Plant	Corn silk (*Zea mays* L.)	Enzymatic hydrolysis (trypsin)	TMKLLLVTL (FK2)	Inhibit IL-1β, IKKβ activity, IκB phosphorylation and NF-κB activation	BALB/c mice	144
Animal	Beetle (*Allomyrina dichotoma*)	Chemical synthesis	AFWCLIRRTVAA (Allomyrinasin)	Inhibit IL-6 and TNF-α production	BALB/c mice and mouse skin infection model	67
Synthetic	Synthetic hybrid	Chemical synthesis	RWGRFLRKIRRFRRKDVY (CTP)	Reduce TNF-α, IL-1β, and IL-6 secretion levels	C57/BL6 mice	138
Animal	Snake venom gland (*Hydrophis cyanocinctus*)	Chemical synthesis	DEQHLETELHTHLTSVLTANGFQ (Hydrostatin-SN1)	Inhibit TNF-α, IL-6, and IL-1β production	C57BL/6 mice	91
Synthetic	AMP (Chensinin-1)	Chemical synthesis	SAVWRHWRRFWLRKHRKH (MC1-1)	Inhibit TNF-α and IL-6 production	Kunming mice	132
Animal	Earthworm coelomic fluid (*Eisenia foetida*)	Chemical synthesis	AMADQ	Inhibit TNF-α and COX-2 production, degradation of IκB and MAPK signaling pathway	Mice	57
Plant	Jiuzao (*Baijiu vinasse*)	Chemical synthesis	AYI	Inhibit TNF-α, IL-1β, IL-6, and NO expressions	Rats	145
Animal	Snake venom (*Heloderma suspectum*)	Chemical synthesis	HGEGFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS (Exendin-4)	Reduce IL-1β, IL-6, TNFα, and IFNγ production	Wistar rats	146

[a] Amino acid sequence has been written from the first residue at N-terminus to the last residue at C-terminus (considering C-terminus the end of the peptide amino acid chain).

BALB/c mice. Particularly, protection against inflammation of peptide TMKLLLVTL (FK2) obtained from hydrolysis with trypsin of corn silk and peptide was investigated in BALB/c mice model Besides, *in vitro* anti-inflammatory potential of peptide Allomyrinasin from Beetle *Allomyrina dichotoma* was verified in BALB/c mice together with a mouse skin infection *in vivo* study using *Staphylococcus pseudintermedius* bacteria.[67,144]

Another widely used strain in biomedical research to study the anti-inflammatory activity of peptides is mice strain C57BL/6, which has been used to show that apart from having *in vitro* activity, peptides CTP (cathelicidin-2 and thymopentin hybrid) and Hydrostatin-SN1 from sea snake venom gland also exerted anti-inflamatory activity *in vivo*.[91,138] However, these are not the only animal models used to investigate anti-inflammatory effects *in vivo*. For example, another powerful anti-inflammatory peptide named MC1-1 with *in vitro* effects, was tested in a different animal model of the two named above, Kunming mice.[132]

Aside from mouse, rat models have also been used to test anti-inflammatory activity. Treatment with Exendin-4, a GLP-1 receptor agonist purified from snake *Heloderma suspectum* venom, significantly attenuated inflammation in a LPS-induced rat model of inflammation and the potential mechanism of action of tripeptide AYI, isolated from a byproduct of baijiu distillation, was also investigated using a rat model.[145,146]

Interestingly, peptides CTP, Hydrostatin-SN1, AYI and Exedin-4, obtained from completely different protein sources, seem to share a mechanism of action consisting of reducing proinflammatory cytokines TNF-α, IL-1β, and IL-6 levels, although it can not be assumed that this is their only mechanism of action since they may have effects on other inflammatory mediators, but no trials have been carried out in this regard. In the same way, peptides Allomyrinasin and MC1-1 share *in vivo* effect against the cytokines IL-6 and TNF-α, but in this case the protein source is similar, even though MC1-1 is a modification of Chensinin-1 peptide found in Chinese brown frog and Allomyrinasin peptide is found naturally in a beetle, both are derived directly or indirectly from animal sources. On the contrary, peptide FK2 derived from a plant protein source acts in a different way, inhibiting compounds and mediators involved in NF-κB pathway.

Despite the fact that several studies investigated the effects of anti-inflammatory peptides *in vivo*, more research on animal models is needed before applications to human health.

3.3. Anti-inflammatory activity of protein hydrolysates

Protein hydrolysates present a variable distribution of peptides with different amino acid composition, structure and chain length. Therefore, the studies employing crude protein hydrolysates lack to identify the contribution of single peptide species (or the combination of some of them) to the anti-inflammatory response. Therefore, further work on the fractionation of protein hydrolysates for concentration of the most active peptides fraction is required.

3.3.1. Animal protein hydrolysates. Table 6 shows the reported studies on anti-inflammatory activity of animal protein hydrolysates obtained by enzymatic hydrolysis. Identification of peptide sequences within the hydrolyzed fractions that reported higher bioactivity has been carried out in several studies, but bioactivity has only been tested on hydrolysates or fractions, not on the purified peptides.

To produce hydrolysates with bioactivity against inflammation, Alcalase has proved to be among the most efficient proteases for these protein sources. This protease has produced hydrolysates with significant anti-inflammatory activity from egg and dairy products as well as from terrestrial and marine animals (Table 6). An experiment on sandfish (*Arctoscopus japonicus*) meat and roe reported that products from hydrolysis with Alcalase and Copulline MG separately showed higher NO inhibitory activity *in vitro* than the ones produced by Flavourzyme, Neutrase and Protamex.[147] Moreover tuna cooking juice the hydrolyzate with alcalase exhibited a more powerful inhibitory effect on inflammatory mediators such as TNF-α and IFN-y in murine macrophage RAW 264.7 calls than the hydrolysates produced with Orientase and Flavourzyme.[25] These results follow the line of those reported for hydrolysate of Skipjack tuna (*Katsuwonus pelamis*) dark muscle where Alcalase produced a hydrolyzate with better anti-inflammatory activity in terms of inhibiting TNF-α, IL-6, IL-1β and NO (IC_{50} > 45.44 mg mL^{-1}) compared to Flavourzyme hydrolyzate *in vitro*.[148] Nonetheless, a hydrolyzate of Skipjack tuna has also been produced with pepsin in combination with an animal protease of unspecified origin and showed good anti-inflammatory activity against TNF-α, IL-6, IL-1β and NO production *in vivo*.[149] Therefore, two assumptions can be drawn from these data. First one is that the mechanism of action of a hydrolysate seems to be more influenced by the origin of the protein source than by the enzyme used, since for Alcalase hydrolysates from different fish species the anti-inflammatory effect reported is not the same, and in order to add extra support for this hypothesis, the same substrate, SKipjack tuna, treated with different enzymes acts by the same mechanism both *in vitro* and *in vivo*, reducing the expression of TNF-α, IL-6, IL-1β and NO. Second one is that particularly compared to Flavourzyme, Alcalase leads to better results in terms of production of hydrolysates with anti-inflammatory potential, but this refers to the use of these enzymes individually and whether the combination of both enzymes could give even better results has not been mentioned. This last question has been explored for edible insect Silkworm pupae and the results provided showed that treatment with Alcalase individually produced lower NO production values, presenting greater NO inhibition activity than treatment with Flavourzyme/Alcalase in combination.[150] However, Flavourzyme hydrolysis of sea cucumber (*Stichopus Japonicus*) resulted in a pool of peptides reporting anti-inflammatory effect.[99] Besides, thanks to Alcalase broad selectivity and specificity permit the use of a wide variety of protein substrates such as milk β-Lactoglobulin, egg ovomucin and livetins or bovine bone-gelatin apart from those already mentioned above.[151–154]

Table 6 Studies of animal protein hydrolysates exhibiting anti-inflammatory activity

Protein source	Enzyme treatment	Regulatory mechanism	Study type	Ref.
Tuna cooking juice (*Thunnini*)	Alcalase	Suppress TNF-α, IFN-γ and IL-2 expression	*In vitro* (RAW 264.7)	25
Skipjack tuna dark muscle (*Katsuwonus pelamis*)	Alcalase	Reduce TNF-α, IL-6 and IL-1β secretion and inhibit NO production	*In vitro* (RAW 264.7)	148
Milk	Alcalase	Suppress TNF-α and IL-1β gene expression	*In vitro* (RAW 264.7)	151
Bovine bone-gelatin (*Bos Taurus*)	Alcalase	Reduce IL-6, NO and TNF-α release and in serum, suppress TNF-α, IL-6 and IL-1β production along with decreasing COX-2 activation	*In vitro* (RAW264.7) *In vivo* (C57BL/6 mice)	152
Egg	Alcalase	Inhibited TNF-α activation	*In vitro* (HDFs)	153
	Alcalase and pepsin	Inhibit NO, PGE2, TNF-α, IL-1β and IL-6 production and iNOS and COX-2 expression	*In vitro* (RAW 264.7)	154
Sandfish (*Arctoscopus japonicus*)	Alcalase and collupulin MG	Inhibit NO production	*In vitro* (RAW 264.7)	147
Milk	Bacterial food-grade enzyme (unknown)	ReducedIL-1α, IL-1β, IL-8 and TGF-β expression and increase IL-17 expression	*In vitro* (Caco-2) *Ex vivo* (Porcine colonic tissues)	24
Sea cucumber (*Actinopyga lecanora*)	Bromelain	Inhibit NO production	*In vitro* (RAW 264.7)	99
Chicken (*Gallus gallus domesticus*)	Corolase PP, Protamex	Reduce IL-1β, IFN-γ, TNF-α, IL-1α, IL-2, IL-6, IL-10 and MCP-1 levels in plasma	*In vivo* (C57BL/6 mice)	159
Sea cucumber (*Stichopus Japonicus*)	Flavourzyme	Suppress IL-6, TNF-α and IL-1β mRNA expression, phosphorylation of JNK, ERK and p38 and inhibit degradation of IκBα and nuclear transposition of NF-κB p65	*In vitro* (RAW 264.7)	189
Milk	Flavourzyme	Reduce NO production and synthesis of TNF-α and IL-1β	*In vitro* (RAW 264.7)	190
Silkworm pupae (*Bombyx mori*)	Flavourzyme and alcalase	Inhibit NO production	*In vitro* (RAW 264.7)	150
Bee pollen (*Apis mellifera*)	Neutrase	Suppress COX-2, NO, iNOS, IL-6 and TNF-α production	*In vitro* (RAW 264.7)	191
Milk	Neutrase and Protamex	Inhibit production of NO and reduced IL-1α, IL-6, and TNF-α production	*In vitro* (RAW 264.7)	192
Salmon bones (*Salmo salar*)	Papain	Inhibit NO production and reduced iNOS, IL-6, TNF-α and COX-2 mRNA levels	*In vitro* (RAW 264.7)	101
Crocodile hemoglobin (*Crocodylus siamensis*)	Pepsin	Reduce NO, IL-6, IL-1β, and PGE2 production	*In vitro* (RAW 264.7)	156
Milkfish (*Chanos chanos*)	Pepsin	Reduce LOX activity and NO production	*In vitro* (assay)	155
Mussel (*Mytilus edulis*)	Pepsin	Inhibit translocation of NF-κB through the prevention of IκB phosphorylation and degradation, NO and PGE2 production, iNOS and COX-2 protein and gene expressions and reduce IL-1β, IL-6 and TNF-α secretions and also inhibit the MAPK signaling pathway	*In vitro* (RAW 264.7)	193
Sturgeon muscle (*Acipenser schrenckii*)	Pepsin	Reduce NO, IL-6, TNF-α and IL-1β production and suppress the expression of MAPK, IκB, and NF-κB p65	*In vitro* (RAW 264.7)	98
Skipjack tuna (*Katsuwonus pelamis*)	Pepsin and animal protease (unknown)	Reduce IL-6 and TNF-α expression	*In vivo* (BALB/c mice)	149
Oyster soft tissue (*Crassostrea talienwhanensis*)	Pepsin, trypsin and Maxipro	Suppress TNF-α, I L-1β, IL-6 and i-NOS expression	*In vitro* (RAW 264.7)	158
Chum salmon (*Oncorhynchus keta*)	Pepsin and trypsin	Reduce NO, IL-6 and TNF-α secretions, as well as TNF-α, IL-6, iNOS and COX-2 mRNA expression	*In vitro* (RAW 264.7) *In vivo* (C57BL/6 mice)	157
Cricket (*Gryllodes sigillatus*)	Alcalase, pepsin and pancreatin (GID)	Inhibit expression of NF-κB	*In vitro* (RAW 264.7)	150
Sardine (*Sardina pilchardus*)	Brewer's spent yeast (BSY) proteases, pepsin and trypsin (GID)	Inhibit IL-8 and ICAM-1 secretion	*In vitro* (EA.hy926/Caco-2 co-culture)	160
Hen spent muscle (*Gallus gallus domesticus*)	Protease M, Protex 50FP	Inhibit IL-6 production	*In vitro* (U937)	108
Egg	Trypsin	Inhibit NO and iNOS production and reduce the phosphorylation levels of JNK and ERK	*In vitro* (RAW 264.7)	194
Sturgeon cartilage (*Acipenser schrenckii*)	Trypsin and papain	Inhibit NO production and reduced IL-6 levels	*In vitro* (RAW 264.7)	174
Milk	Virgibacillus halodenitrificans SK1-3-7 proteinase	Suppress IL-1β, IL-6, IL-8, TNF-α and COX-2 production	*In vitro* (THP-1)	195

Pepsin, used individually and not in combination with pancreatin and other enzymes to mimic human gastrointestinal digestion as occurs in many anti-inflammatory peptides studies, also leads to protein hydrolysates with anti-inflammatory activity. Interestingly, the reviewed hydrolysates exhibiting *in vitro* anti-inflammatory capacity that are produced with pepsin are from diverse marine animal sources such as milkfish (*Chanos chanos*) or crocodile hemoglobin where two potential peptides SAFNPHEKQ (SQ9) and IIHNEKVQAHGKKVL (IL15) were also identified.[155,156] Pepsin has also been used in combination with trypsin, reporting good results against inflammation for hydrolysate of Chum salmon (*Oncorhynchus keta*) myofibrillar protein (Mf) *in vitro* and *in vivo*.[157] However, for Oyster (*Crassostrea talienwhanensis*) soft tissue, pepsin hydrolysate did not give as good results against inflammation as trypsin hydrolysate *in vitro*.[158] Regarding the mechanism of action, since both pepsin hydrolysates act by different mechanisms, the first reducing LOX activity and NO production and the second reducing IL-6, IL-1β, NO and PGE2 production, we can continue with the previous assumption where we reasoned that the mechanism of action of a hydrolyzate can not be attributed directly to the enzyme used for its production.

Furthermore, different enzymes such as Corolase PP or Protamex have also produced hydrolysates with strong anti-inflammatory effect *in vivo*, even showing greater pro-inflammatory cytokine-lowering potential individually with respect to the mixture of papain/bromelain or single Alcalase in chicken protein hydrolysates, although these last two treatments also reported anti-inflammatory activities.[159] Besides, simulated gastrointestinal digestion with combinations of pepsin/pancreatin or pepsin/trypsin has also reported good results in different types of protein sources after previous hydrolysis with other proteases, as is the case of Sardine (*Sardina pilchardus*) which decreases IL-8, ICAM-1 and NO levels in EA.hy926/Caco-2 cell co-culture or Cricket (*Gryllodes sigillatus*) where three novel anti-inflammatory peptides YKPRP, PHGAP and VGPPQ were identified from the bioactive hydrolysate that reported *in vitro* inhibition of NF-κB expression.[150,160] Interestingly, apart from studies of bioactivity of hydrolysates *in vitro* and *in vivo*, the anti-inflammatory potential of hydrolyzed milk-casein with a bacterial food-grade enzyme (unknown) has been tested also in *ex vivo* models of the gastrointestinal tract, using porcine colonic tissue.[24]

3.3.2. Plant and algae protein hydrolysates. Table 7 shows the reported studies on anti-inflammatory activity of plant and algae protein hydrolysates obtained by enzymatic hydrolysis.

As observed for animal protein hydrolysates, Alcalase remains one of the most used enzymes in terms of searching for anti-inflammatory activity *in vitro* for plant protein hydrolysates. Particularly, Alcalase showed potential in the production of hydrolysates with NO inhibitory properties from legume proteins such as pigeon pea (*Cajanus cajan*), lentil (*Lens culinaris*) or chickpea (*Cicer arietinum*).[161] Nevertheless, it can not be assumed that this enzyme is responsible for this mechanism of action since for other legumes hydrolysates such as lupin (*Lupinus angustifolius* L.) or pinto bean (*Phaseolus vulgaris* L.) activity has been measured through other inflammatory markers and the effect showed is different, decreasing TNF-α, IL-1β, and IL-6 levels by 70, 40, and 45% (respectively) in the first case and inhibiting IL-6 secretion by 28% in the second case.[162,163] Furthermore, anti-inflammatory peptides in lupin protein hydrolysate were able to cross Caco-2 monolayers successfully without any modifications in their bioactivity.[162] Thus, it can be deduced that they are potentially resistant to the gastrointestinal tract and may reach the bloodstream to exert their beneficial effect. Despite this, further investigation is required to confirm these properties *in vivo* and apply this information to a tissue and organ level like as has been done in an innovative study which determined the protective anti-inflammatory effects of Alcalase-derived peptide extracts of white sorghum against the damage induced on human skin by the exposure to ultraviolet-B irradiation (UVB).[164]

In addition to the use of Alcalase, the use of trypsin has also resulted in hydrolysates of plant protein from gramineae family (known as grasses) displaying anti-inflammatory abilities by NF-κB signaling pathway regulation. Trypsin hydrolysates from rice and corn silk protein inhibited inflammatory response *in vitro* and *in vivo*, respectively.[144,165] Moreover, a recent study reported the potential used of peptides derived from trypsin hydrolysis of microalgae (*Synechococcus* sp.) to develop natural anti-inflammation food-grade ingredients, drugs, and cosmetic products.[166] The authors recovered some active fractions, reporting an IC_{50} value of 34.51 μg mL^{-1} for NO inhibitory activity but due to their interest for biotechnological applications more studies on anti-inflammatory peptides from microalgae should be done.

Apart from the use of enzymes individually, pepsin and pancreatin have been used in combination to simulate the physiological process of digestion providing hydrolysates from different plant sources with anti-inflammatory potential. To this regard, peptides fractions generated from *in vitro* gastrointestinal digestion of chia seeds showed high inhibitory activity against 5-LOX, COX-1-2 and iNOS pro-inflammatory enzymes, and its bioactivity was also validated *in vivo* murine models.[77] Similarly, gastrointestinal digested peptide fractions generated from heat treated millet grains had high COX-1 and COX-2 inhibitory activity, IC_{50} value of 0.08 and 0.12 mg mL^{-1}, along with LOX inhibitory activity, IC_{50} value of 0.14 mg mL^{-1} *in vitro*.[78] Additionally, *in vitro* digestion of chia seeds in another study resulted in peptide fractions exerting anti-inflammatory effect through the regulation of proinflammatory cytokines such as IL-1β, IL-6, TNF-α and IL-10.[167] Therefore, bioactive peptides found in chia seeds hydrolysate may have different mechanisms of action against inflammation, but in order to study their individual effect these sequences, such as peptide TGPSPTAGPPAPGGGTH, should be isolated and purified. In contrast, the study of the anti-inflammatory activity of the Amaranthaceae family of plants, such as quinoa or amaranth, has resulted in hydrolysates with an inhibitory effect on NO production in murine macrophages *in vitro*, but their activity against other proinflammatory markers has not been investigated.[168,169]

Table 7 Studies of plant and algae protein hydrolysates exhibiting anti-inflammatory activity

Protein source	Enzyme treatment	Regulatory mechanism	Study type	Ref.
White sorghum grain (*Sorghum bicolor*)	Alcalase	Reduce IL-1β, IFN-γ and TNF-α levels	*In vitro* (Human skin cultures)	164
Lupin (*Lupinus angustifolius* L.)	Alcalase	Reduce TNF-α, IL-1β, and IL-6 levels and promote expression of IL-10	*In vitro* (Caco-2/THP-1 coculture)	162
Pigeon pea (*Cajanus cajan*) Lentil (*Lens culinaris*) Chickpea (*Cicer arietinum*)	Alcalase and bromelain	Inhibit NO production	*In vitro* (RAW 264.7)	161
Pinto bean (*Phaseolus vulgaris* L.)	Alcalase and savinase	Inhibit IL-6 secretion	*In vitro* (CD-18Co)	163
Lychee seeds (*Litchi chinensis Sonn.*)	Neutrase	Inhibit NO production and reduce iNOS and IL-6 expression	*In vitro* (RAW 264.7)	127
Corn silk (*Zea mays* L.)	Trypsin	Inhibit IL-1β, NF-κB activities, IKKβ activities, IκB phosphorylation and NF-κB activation	*In vivo* (BALB/c mice)	144
Rice (*Oryza sativa*)	Trypsin	Inhibit NO and TNF-α release, TNF-α, iNOS, IL-6, IL-1β transcription and repress NF-κB pathway by impeding the nuclear translocation of p65	*In vitro* (RAW 264.7)	165
Microalgae (*Synechococcus* sp. *VDW*)	Trypsin	Reduce iNOS, TNF-α, COX-2, and IL-6 gene expression	*In vitro* (RAW 264.7)	166
Pea (*Pisum Sativum*)	Virgibacillus halodenitrificans SK1-3-7 proteinase	Suppress IL-1β, IL-6, IL-8, TNF-α and COX-2 production	*In vitro* (THP-1)	194
Quinoa (*Chenopodium quinoa Willd.*)	Papain, pepsin and pancreatin	Inhibit NO production	*In vitro* (RAW 264.7)	169
Rice bran (*Oryza sativa*)	Alcalase, pepsin and pancreatin (GID)	Suppress iNOS, IL-6 and TNF-α mRNA levels and inhibited NO production	*In vitro* (RAW 264.7)	112
Millet grain (*Panicum miliaceum* L.)	α-Amylase, pepsin and pancreatin (GID)	Inhibit COX-1, COX-2 and LOX activity	*In vitro* (assay)	78
Chia seeds (*Salvia hispanica* L.)	Pepsin and pancreatin (GID)	Reduce NO, IL-1β, IL-6, and TNF-α levels and increase levels of IL-10	*In vivo* (BALB/c mice) *In vitro* (peritoneal murine macrophages)	167
Common bean (*Phaseolus vulgaris* L.)	Pepsin and pancreatin (GID)	Reduce TNF-α, IL-1β and PGE-2 production	*In vitro* (THP-1)	195
Soybean (*Glycine max*)	Pepsin and pancreatin (GID)	Inhibit NO and PGE2 production	*In vitro* (RAW 264.7, Caco-2, HT-29 and HCT-116)	182
Chickpea (*Cicer arietinum*)	Pepsin and pancreatin (GID)	Inhibit NF-κB expression	*In vitro* (RAW 264.7)	196
Chia seed (*Salvia hispanica* L.)	Pepsin and pancreatin (GID)	Inhibit 5-LOX, COX-1-2, and iNOS production	*In vitro* (assay)	77
Amaranth flour (*Amaranthus hypochondriacus*)	Pepsin and pancreatin (GID)	Inhibit NO production	*In vitro* (RAW 264.7)	168

Regarding the bioactivity of these protein hydrolysates, compared with single peptides from natural and "non-natural" origin, they may display better anti-inflammatory effect than single peptides giving understanding that possible synergies in the peptide pool could result in increased values of bioactivity. For example, hydrolysate fractions from locust *Schistocerca gregaria* protein preparation showed higher inhibitory potential for COX-2 and LOX, IC_{50} values between 0.13–0.26 μg mL^{-1}, than the individual peptide FDPFPK identified in the hydrolysate fraction of the same insect, which reported IC_{50} values of 7.40 and 2.85 mg mL^{-1} for COX-2 and LOX inhibition, respectively.[62] On the contrary, there are also cases in which the purified peptides exhibit greater bioactivity than the hydrolyzed proteins such as peptides SNKGGGRPN and TVTVYSLLR from salmon bone hydrolysis which were found to have a marked NO-inhibitory activity compared to Skipjack tuna dark muscle hydrolysate, reporting salmon peptides an IC_{50} value of 2.56 μg mL^{-1} for this inflamamtory marker, while tuna hydrolysate reported an IC_{50} value around 45.44 mg mL^{-1}.[101,148]

3.4. Structure–activity relationship of anti-inflammatory peptides

We aimed to study the structure–activity relationship of the selected peptides exhibiting potential anti-inflammatory activity (Table 8).

It has been reported that the molecular weight and the length of the peptide chain (PCL) play an important role in their bioactivity. Out of the 31 peptides analyzed, 6 of them are di- or tri-peptides and 19 have a length between 4 and 20 amino acids, showing that bioactivity seems to be greater in smaller peptides with short amino acid sequences. This pre-

Table 8 Structure analysis of anti-inflammatory peptides studied *in vitro*

Peptide sequence	M_w (Da)	pI	Net charge (pH 7)	Water solubility	PCL	Hydrophobicity ratio (%)	Positively charged AA (%)	Polar AA (%)	Aromatic AA (%)	P (%)	G (%)	Q (%)
PAY	349.38	3.85	0.00	Poor	3	100%	0%	0%	33%	33%	0%	0%
VDVPVKVPYS	1102.28	6.55	0	Good	10	70%	10%	30%	10%	20%	0%	0%
VHYAGTVDY	1024.08	4.87	−0.9	Poor	9	56%	11%	33%	22%	0%	11%	0%
GAKYAKIIYNYLKKIANALW	2341.79	10.33	4.00	Poor	20	55%	20%	30%	20%	0%	5%	0%
QA	217.22	3.34	0	Good	2	50%	0%	50%	0%	0%	0%	50%
KA	217.27	9.91	1	Good	2	50%	50%	50%	0%	0%	0%	0%
WG	261.28	3.50	0	Poor	2	50%	0%	0%	50%	0%	50%	0%
SNPSVAGVR	885.97	10.57	1	Good	9	44%	11%	44%	0%	11%	11%	0%
SIFGKIFKRIIRVAWK	1962.43	12.18	5	Good	16	44%	31%	38%	19%	0%	6%	0%
YKRWKKNWAKYWKIFRK	2429.91	11.30	8	Good	17	41%	47%	53%	35%	0%	0%	0%
RGQANILAGKNIKIRSGAAAGVGKTPQKANVEVLALGIW	3971.61	11.57	5.00	Good	39	41%	15%	36%	3%	3%	15%	5%
EDDQMDPMAK	1179.28	3.32	−3	Good	10	40%	10%	60%	0%	10%	0%	10%
YKRWKKRWAKYWKKFRK	2487.01	11.63	10	Good	17	35%	59%	59%	35%	0%	0%	0%
FDPFPK	749.85	6.39	0	Good	6	33%	17%	33%	33%	33%	0%	0%
KKIRVRLSA	1070.33	12.16	4	Good	9	33%	44%	56%	0%	0%	0%	0%
QNWDFCEAWEPCF	1674.81	0.65	−3.1	Poor	13	31%	0%	54%	31%	8%	0%	8%
KLPDHPKLPK	1172.42	10.40	2.1	Good	10	30%	40%	50%	0%	30%	0%	0%
KIWHHTF	968.11	9.91	1.2	Poor	7	29%	43%	57%	29%	0%	0%	0%
HAEGTFTSDVSSYLEGQAAKEFI	2487.63	4.04	−2.9	Good	23	26%	9%	52%	13%	0%	9%	4%
HYGH	512.52	7.70	0.2	Poor	4	25%	50%	50%	25%	0%	25%	0%
GPETAFLR	889.99	6.86	0	Good	8	25%	13%	38%	13%	13%	13%	0%
QITITVKPRFRRIKRLFRGFR	2689.26	12.81	8	Good	21	24%	38%	52%	14%	5%	5%	5%
GKLTKDKLKRGAKKALNVASKV	2353.85	11.38	7.00	Good	22	23%	36%	55%	0%	0%	9%	0%
KGIRGYKGGYCKGAFKQTCKCY	2459.92	9.99	5.80	Good	22	23%	27%	50%	18%	0%	23%	5%
YGGGGE	538.51	1.00	−1	Good	6	17%	0%	17%	17%	0%	67%	0%
HLDDALRGQE	1153.20	4.16	−1.9	Good	10	10%	20%	60%	0%	0%	10%	10%
LKWLKKLLKKL	1410.87	11.28	5.00	Good	11	9%	45%	45%	9%	0%	0%	0%
DEQHLETELHTHLTSVLTANGFQ	2620.78	4.49	−3.7	Good	23	9%	13%	65%	4%	0%	4%	9%
HC	258.30	7.06	0	Poor	2	0%	50%	100%	0%	0%	0%	0%
CR	277.35	9.21	0.9	Good	2	0%	50%	100%	0%	0%	0%	0%
SEGGFLE	737.75	0.85	−2	Good	7	0%	0%	43%	14%	0%	29%	0%

M_w: molecular weight. pI: isoelectric point. PCL: peptide chain length. P: presence of proline residue. G: presence of glycine residue. Q: presence of glutamine residue.

vious argument is also reflected in the molecular weight data where about 42% of the most bioactive peptides reviewed have a molecular weight below 1000 Da and the remaining does not exceed 3 kDa, except for one of them that almost reaches 4 kDa. Although bioactive peptides are generally short sequences of up to 20 residues, longer peptides have also been reported to exert anti-inflammatory activities like lunasin from defatted soybean flour or the peptides HCRG1 and HCRG2 from sea anemone (*Heteractis crispa*), both containing 56 amino acid residues.[90,115] Results in literature support that both small (2–3 amino acids) and large (up to 50 amino acids) peptides can be absorbed intact through the intestine and produce biologic effects at the tissue level but the potency of the enterally administered peptides decreases as the chain length increases.[170]

Several investigations point out the role of positively charged residues on the anti-inflammatory response of bioactive peptides.[59,156,171] The positively charged region of the peptide may act as a chemokine, so the peptides modulate the immune response through their union with respective chemokine receptors.[172] In our case, the presence of specific amino acids attached to the N-terminal end was studied, being the most abundant arginine (R) and lysine (K), two basic amino acids with net positive charge at physiological pH. This feature has been reported for human lactoferricin or tuna juice hydrolysates, both presenting Arg residues at terminal position, and reported to block inflammation by binding to the popolysaccharides excreted by bacteria and macrophages.[25,173] Almost half of the biopeptides analyzed in this work contain between 25–50% of positive amino acids in their sequence (*i.e.* lysine, arginine and histidine). Moreover, the majority of the peptides present a net positive charge at physiological pH. Another characteristic shared by anti-inflammatory peptides is the presence of polar amino acids at the C-terminus.[30,135,138] According to our analysis on the peptides reviewed, 83% of the sequences have within their composition 25–75% of polar amino acids. Besides, 23% peptides reviewed have a polar residue at the C-terminus, such as lysine or histidine, indicating that this property may affect their anti-inflammatory response.

Furthermore, many studies have highlighted the importance of the presence of hydrophobic amino acids in biopeptides, especially at their N-terminus, in relation to their regulation of the inflammatory response.[30,59,113,174] The presence of hydrophobic amino acids residues (*i.e.* Val, Leu, Ile, *etc.*) may improve the interaction between peptides and cell membrane, promoting their anti-inflammatory effect. This feature agrees with the peptide sequences shown in Table 8, where 19 out of the 31 peptides analyzed contain between 25 and 100% hydrophobic amino acids. The most abundant hydrophobic amino acid was alanine, followed by isoleucine. This was the case for the tripeptide PAY (100% hydrophobicity) from salmon and the peptide VDVPVKVPYS (70% hydrophobicity) from fermented sorghum, both displaying inhibitory activity against NO production.[60,124] Otherwise, no obvious relationship has been found between the solubility of the peptides studied and their potential bioactivity. Despite the fact that short peptides (4–5 amino acid residues) are generally soluble in water, some of the anti-inflammatory dipeptides reviewed show poor solubility due to the presence of hydrophobic amino acids in their sequence.[175–177]

Other feature linked to anti-inflammatory response is the presence of aromatic amino acids in the peptide sequence.[85,178] Our analysis indicates that 55% of the sequences are composed of between 10% and 50% aromatic amino acids such as tyrosine, tryptophan or phenilalanine.[100] The anti-inflammatory response of peptides containing aromatic amino acids may be explained by their less susceptibility to intestinal peptidases and proteases. For instance, the ovotransferrin-derived peptide IRW presenting tryptophan at C-terminus, exhibited limited degradation by peptidases and proteases hydrolysis.[179] Recent studies report that peptides rich in proline, glycine or glutamine have a great potential against inflammation response.[30] For instance, milk-derived tripeptides IPP and VPP, both recognized as potent anti-inflammatory agents, are rich in proline residues.[180] To this regard, the presence of proline has been related to less amenable gastro-intestinal digestion and more likely absorption upon oral consumption, a factor that can be beneficial for future therapeutic applications.[181] This hypothesis has been confirmed by the high content of glycine in anti-inflammatory peptides from sturgeon cartilage, walnut or germinated soybean.[128,174,182] Since the presence of proline, glycine, and glutamine could enhance peptide's anti-inflammatory potencial, the amount of these three residues was studied in the reviewed peptides. The sorghum peptide with the sequence KLPDHPKLPK stands out from the rest of the studied peptides due to the proline composition of its sequence, represented by 30%. The rest of biopeptides do not have a significant amount of proline in their sequences. Soybean peptide YGGGGE its composed by a 67% of glycine. In contrast, the rest of the peptides do not show high contents of this amino acid. Regarding glutamine, it has not been seen that its presence is relevant in the peptides analyzed in this study. From these data a clear relationship between the presence of proline, glycine or glutamine in the peptide sequence and anti-inflammatory activity cannot be determined.

3.5. Potential alternative sustainable protein sources of anti-inflammatory peptides

The presence of the selected peptide sequences with anti-inflammatory potential within the proteome of alternative sustainable protein sources has been investigated using the BLASTp tool and depicted in Table 9. Particularly, we have chosen for this study several plant, insect and fish sustainable sources. Of the total peptide sequences analyzed, 77% of them are found in yellow mealworm (*Tenebrio molitor*) edible insect, in sunflower (*Helianthus annuus*) and in lupin (*Lupinus albus*) oilseed crops. A significant number of bioactive peptide sequences, about 74%, are also found in chickpea (*Cicer arietinum*) and olive (*Olea europaea*) protein. In the pea protein, similarities are also found with 8 of the 31 sequences analyzed

Table 9 Alternative protein sources for anti-inflammatory peptides

Protein type	Protein source	Sequence	New sources (BLASTp)	Hits
Synthetic	Designed	KKIRVRLSA (SET-M33D)	Lupinus albus	21
			Cicer arietinum	13
			Olea europaea subsp. Europaea	12
			Helianthus annuus	80
			Tenebrio molitor	7
	Designed	LKWLKKLLKKL (WALK11.3)	*Cicer arietinum*	8
			Lupinus albus	26
			Olea europaea subsp. Europaea	24
			Helianthus annuus	35
			Tenebrio molitor	11
	Designed	GAKYAKIIYNYLKKIANALW (GW-A2)	Helianthus annuus	28
			Olea europaea subsp. Europaea	10
			Lupinus albus	19
			Cicer arietinum	7
			Tenebrio molitor	29
	Glucagon-like peptide-1 (GLP-1)	HAEGTFTSDVSSYLEGQAAKEFI (Liraglutide)	*Cicer arietinum*	13
			Pisum sativum	4
			Lupinus albus	25
			Olea europaea subsp. Europaea	24
			Helianthus annuus	29
			Tenebrio molitor	10
	Chemokine CXCL14	YKRWKKRWAKYWKKFRK (CXCL14-C17-a3)	Helianthus annuus	65
			Olea europaea subsp. Europaea	12
			Cicer arietinum	9
			Lupinus albus	26
			Tenebrio molitor	8
	Chemokine CXCL14	YKRWKKNWAKYWKIFRK (CXCL14-C17-a2)	Lupinus albus	21
			Cicer arietinum	16
			Helianthus annuus	52
			Olea europaea subsp. Europaea	11
			Tenebrio molitor	20
	Tick defensin OsDef1	KGIRGYKGGYCKGAFKQTCKCY (Os)	*Cicer arietinum*	26
			Lupinus albus	28
			Pisum sativum	24
			Helianthus annuus	19
			Tenebrio molitor	17
	Chicken cathelicidin-2 (CATH-2)	QITITVKPRFRRIKRLFRGFR	Tenebrio molitor	11
			Olea europaea subsp. Europaea	21
			Helianthus annuus	48
			Lupinus albus	12
			Cicer arietinum	13
			Pisum sativum	3
Plant	Lupin seeds (*Lupinus angustifolius* L.)	GPETAFLR	*Cicer arietinum*	14
			Pisum sativum	1
			Lupinus albus	17
			Helianthus annuus	33
			Olea europaea subsp. Europaea	21
			Tenebrio molitor	22
	Soybean (*Glycine max*)	YGGGGE	Tenebrio molitor	20
			Helianthus annuus	36
			Olea europaea subsp. Europaea	23
			Cicer arietinum	6
			Lupinus albus	15
	Soybean (*Glycine max*)	SEGGFLE	Helianthus annuus	31
			Olea europaea subsp. Europaea	35
			Lupinus albus	15
			Cicer arietinum	13
			Tenebrio molitor	6
	Foxtail Millet (*Setaria italica*)	EDDQMDPMAK	Tenebrio molitor	13
			Olea europaea subsp. Europaea	28
			Helianthus annuus	48
			Cicer arietinum	6
			Lupinus albus	8
			Pisum sativum	5
	Foxtail Millet (*Setaria italica*)	QNWDFCEAWEPCF	Helianthus annuus	50
			Olea europaea subsp. Europaea	26
			Cicer arietinum	9
			Lupinus albus	14
			Tenebrio molitor	10
	Fermented sorghum (*Baijiu vinasse*)	KLPDHPKLPK (VPH-1)	*Cicer arietinum*	9
			Pisum sativum	2
			Lupinus albus	22
			Helianthus annuus	45
			Olea europaea subsp. Europaea	22
			Tenebrio molitor	8
	Fermented sorghum, Baijiu vinasse	VDVPVKVPYS	Lupinus albus	28
			Cicer arietinum	5
			Olea europaea subsp. Europaea	13
			Helianthus annuus	37
			Tenebrio molitor	27

Table 9 *(Contd.)*

Protein type	Protein source	Sequence	New sources (BLASTp)	Hits
Animal	Sturgeon muscle (*Acipenseridae*)	VHYAGTVDY	Tenebrio molitor	14
			Helianthus annuus	40
			Lupinus albus	14
			Cicer arietinum	15
			Olea europaea subsp. Europaea	23
	Sturgeon muscle (*Acipenseridae*)	KIWHHTF	Tenebrio molitor	10
			Lupinus albus	37
			Pisum sativum	15
			Olea europaea subsp. Europaea	29
			Helianthus annuus	39
			Cicer arietinum	16
	Sturgeon muscle (*Acipenseridae*)	HLDDALRGQE	Tenebrio molitor	21
			Lupinus albus	24
			Helianthus annuus	31
			Olea europaea subsp. Europaea	14
			Cicer arietinum	5
	Human (*Homo sapiens*)	SIFGKIFKRIIRVAWK (Hs02)	Tenebrio molitor	27
			Lupinus albus	18
			Cicer arietinum	16
			Helianthus annuus	26
			Olea europaea subsp. Europaea	20
			Pisum sativum	1
	Salmon skin hydrolysates (*Salmo salar*)	QA	No significant similarity found	
	Salmon skin hydrolysates (*Salmo salar*)	KA	No significant similarity found	
	Chinese scorpion venom (*Mesobuthus martensii*)	HYGH	Tenebrio molitor	11
			Lupinus albus	28
			Helianthus annuus	33
			Cicer arietinum	19
			Olea europaea subsp. Europaea	24
	Snake venom gland (*Hydrophis cyanocinctus*)	DEQHLETELHTHLTSVLTANGFQ (H-SN1)	Helianthus annuus	29
			Olea europaea subsp. Europaea	29
			Lupinus albus	10
			Cicer arietinum	4
			Tenebrio molitor	11
	Black fly salivary glands (*Simulium bannaense*)	GKLTKDKLKRGAKKALNVASKV (SibaCec)	Helianthus annuus	14
			Olea europaea subsp. Europaea	29
			Cicer arietinum	13
			Lupinus albus	18
			Tenebrio molitor	14
	Horsefly salivary glands (*Tabanus yao*)	RGQANILAGKNIKIRSGAAAGVGKTPQKANVEVLALGIW (Cecropin-TY1)	No significant similarity found	
	Chicken Feather Meal (*Gallus gallus domesticus*)	SNPSVAGVR	Helianthus annuus	20
			Olea europaea subsp. Europaea	34
			Lupinus albus	21
			Cicer arietinum	5
			Tenebrio molitor	10
	Salmon pectoral fin (*Salmo salar*)	PAY	No significant similarity found	
	Salmon skin hydrolysates (*Salmo salar*)	WG	No significant similarity found	
	Locusts (*Schistocerca gregaria*)	FDPFPK	Lupinus albus	17
			Cicer arietinum	14
			Olea europaea subsp. Europaea	25
			Helianthus annuus	38
			Tenebrio molitor	9
	Egg	HC	No significant similarity found	
	Egg	CR	No significant similarity found	

(26%). In contrast, in fish protein sources, sardine, blue whiting and horse mackerel, no alignments or similarities with the peptide sequences tested were found, which can be attributed to the lack of proteins sequenced for these species.

It is interesting that there are sequences very similar to synthetic peptides such as KKIRVRLSA (SET-M33D) and YKRWKKRWAKYWKKFRK inside the proteome of natural protein sources such as sunflower or the synthetic peptide GAKYAKIIYNYLKKIANALW (GW-A2) within the yellow mealworm proteome. In addition, the peptides VDVPVKVPYS and GPETAFLR, derived from sorghum and lupin, respectively, are also found in the protein of the insect yellow mealworm. Our study also found similarities between the synthetic peptide KGIRGYKGGYCKGAFKQTCKCY and the protein of legumes such as chickpea or pea, the peptide KIWHHTF from sturgeon muscle and lupin or pea protein or the peptide SNPSVAGVR isolated from chicken feather and the protein from olive. According to this *in silico* analysis, *Tenebrio molitor*, *Helianthus annuus* and *Lupinus albus* could be good sustainable sources of peptides with anti-inflammatory activity. These data confirm that peptide sequences that have been reported with great anti-inflammatory activity are present within the proteome of other organisms apart from its original source. This is an opportunity to obtain such bioactive sequences from alternative available and sustainable protein sources.

3.6. Prediction of anti-inflamamtory activity

The *in vitro* anti-inflammatory activity of the peptide sequences selected in this work has been compared to the anti-inflammatory effect obtained from two computer predictors, PreAIP and AIDpred (Table 10). The peptide sequences were ranked according to the score obtained from each predictor.

This study found seven coincidences among the ten highest peptide sequences scored for both predictors. The synthetic

Table 10 Predicted anti-inflammatory activity of peptides previously studied *in vitro*

PreAIP		AIPpred	
Peptide sequence	Score	Peptide sequence	Prob
LKWLKKLLKKL	0.667[a]	LKWLKKLLKKL	0.6488
SIFGKIFKRIIRVAWK	0.639[a]	GKLTKDKLKRGAKKALNVASKV	0.6419
KGIRGYKGGYCKGAFKQTCKCY	0.634[a]	HAEGTFTSDVSSYLEGQAAKEFI	0.6372
YKRWKKRWAKYWKKFRK	0.634[a]	RGQANILAGKNIKIRSGAAAGVGKTPQKANVEVLALGIW	0.6209
GAKYAKIIYNYLKKIANALW	0.631[a]	KGIRGYKGGYCKGAFKQTCKCY	0.6163
YKRWKKNWAKYWKIFRK	0.625[a]	QITITVKPRFRRIKRLFRGFR	0.6
QITITVKPRFRRIKRLFRGFR	0.609[a]	KKIRVRLSA	0.5953
GKLTKDKLKRGAKKALNVASKV	0.57[a]	SEGGFLE	0.5791
QNWDFCEAWEPCF	0.548[a]	SIFGKIFKRIIRVAWK	0.5535
KKIRVRLSA	0.515[a]	GAKYAKIIYNYLKKIANALW	0.5302
HAEGTFTSDVSSYLEGQAAKEFI	0.501[a]	QA	0.5116
DEQHLETELHTHLTSVLTANGFQ	0.498[a]	GPETAFLR	0.4907
GPETAFLR	0.481[a]	HC	0.4884
KIWHHTF	0.476[a]	CR	0.4884
RGQANILAGKNIKIRSGAAAGVGKTPQKANVEVLALGIW	0.474[a]	WG	0.4884
HLDDALRGQE	0.466[b]	SNPSVAGVR	0.486
VHYAGTVDY	0.449[b]	DEQHLETELHTHLTSVLTANGFQ	0.4837
KLPDHPKLPK	0.424[b]	YKRWKKNWAKYWKIFRK	0.4791
SNPSVAGVR	0.42[b]	EDDQMDPMAK	0.4698
EDDQMDPMAK	0.383[c]	KA	0.4465
YGGGGE	0.374[c]	YKRWKKRWAKYWKKFRK	0.4442
SEGGFLE	0.364[c]	VHYAGTVDY	0.4326
FDPFPK	0.353[c]	PAY	0.4326
VDVPVKVPYS	0.34[d]	KIWHHTF	0.4256
HC	0.291[d]	FDPFPK	0.4233
HYGH	0.284[d]	QNWDFCEAWEPCF	0.4209
QA	0.283[d]	KLPDHPKLPK	0.4116
CR	0.265[d]	VDVPVKVPYS	0.3791
PAY	0.261[d]	HYGH	0.3791
WG	0.258[d]	HLDDALRGQE	0.3605
KA	0.258[d]	YGGGGE	0.3209[e]

[a] High confidence. [b] Medium confidence. [c] Low confidence. [d] Negative AIP. [e] Non-AIP.

peptide LKWLKKLLKKL scored the greatest anti-inflammatory potential according to both predictors. Peptide SIFGKIFKRIIRVAWK derived from the human myosin protein also has a high score in the ranking, ranking second position in PreAIP and ninth in AIPpred. Foxtail millet-derived QNWDFCEAWEPCF peptide has a high score in the PreAIP predictor but does not appear among the 10 best in the AIPpred predictor. Same happens with Cecropin-TY1 RGQANILAGKNIKIRSGAAAGVGKTPQKANVEVLALGIW, which has a high score in AIPpred but is not among the best PreAIP peptides. Among the worst rated are the dipeptides KA, WG and CR and the tripeptide PAY. This is because the predictors used are not compatible with sequences shorter than 5 amino acids, therefore, they are considered non-anti-inflammatory. Interestingly, peptide YGGGGE from soybean that was considered as a strong anti-inflammatory peptide since it inhibited a 56.78–75.76% NO production together with reducing TNF-α, IL-1β and IL-6 levels receives a low score in both predictors, not being considered an anti-inflammatory peptide by AIPpred but being considered a low-confidence anti-inflammatory peptide by PreAIP. However, SEGGFLE peptide also derived from soybean it is considered an anti-inflammatory peptide by AIPpred although for PreAIP it is a low confidence anti-inflammatory peptide.

The correlation coefficient of the scores obtained for each peptide by both predictors is 0.2337, which indicates the lack of relationship between the two selected anti-inflammatory predictors indicating that *in vitro* assays are still necessary. In addition, both predictors have some limitations, such as the sequence length they allow to analyse. In the case of PreAIP, the score is calculated based on a fixed sequence length of 25 residues, which can cause loss of information. As a consequence, PreAIP does not allow the analysis of small peptides while AIPpred requires a minimum chain length of 5 residues and a maximum of 25 residues. This is a limitation taking into account that a proportion of the biological active peptides reported previously have sequences of 2 to 3 amino acids. Therefore, a bioinformatic tool capable of predicting the bioactivity of short sequences is necessary since the selected predictors can not estimate the bioactivity of dipeptides or tripeptides.

Furthermore, there are differences between the scores obtained with the two predictors which may be due to the method implemented to measure the potential bioactivity and to the fact that the cut-off value of each predictor is different. There is still room to improve prediction performance and machine learning algorithms of the actual anti-inflammatory prediction tools in terms of precision and efficiency to support

medical research. Nevertheless, these bioinformatic tools can be very useful in qualitative analysis of bioactive sequences previous to *in vitro* investigations, serving as a guide to provide valuable leads for proteome-wide prediction and classification of prospective anti-inflammatory candidates for experimental validations.

4. Conclusions

Over the last few decades, there has been an increasing effort on the identification of biopeptides exerting anti-inflammatory properties. The bioactivity of anti-inflammatory peptides have been validated mostly by *in vitro* (cell culture) or *in vivo* studies using animal models, but rarely confirmed by human trials. Consequently, more clinical research is required to further understand their gastrointestinal stability, bioavailability, food matrix interactions and safety concerns before using them as potential agents in the prevention and treatment of chronic inflammatory diseases. Furthermore, the identification of these peptide sequences, mostly produced by enzymatic hydrolysis, has served to design and produce novel synthetic anti-inflammatory peptides.

Considering that the anti-inflammatory peptides reviewed act through different mechanisms with no apparent relation to enzyme treatment or protein source, identifying specific physicochemical or structural features for their bioactivity its very challenging. However, there are indeed some common characteristics that seem to play an important role in their response to inflammation such as presence of hydrophobic (Val, Ile, Pro) and positively charged (His, Arg, Lys) amino acids. Nevertheless, these residues are not found in all anti-inflammatory peptides or molecular weight, where most of the bioactive peptides identified so far have 2–40 residues with a molecular weight less than 3 kDa. Other investigations confirm the positive effect on inflammatory response linked to the presence of aromatic residues or proline in terminal position.

Conventional animal and plant protein sources are undoubtedly the most used in bioactive peptides production, as evidenced by the significant body of research on the subject. However, identification of anti-inflammatory peptides in alternative protein sources, such as *Tenebrio molitor*, lupine (*Lupinus albus*) or sunflower (*Helianthus annuus*), has been possible thanks to BLASTp analysis. These findings pave the way to obtain biopeptides from more sustainable and nutritious sources than conventional meat or dairy proteins.

Bioinformatic approaches are drawing increasing attention as a tool to complement experimental trials and identify potential anti-inflammatory peptides prior to their synthesis. In our case, *in silico* bioactivity predicted by PreAIP and AIPpred computer tools showed little relation to *in vitro* experimental anti-inflammatory activity reported in literature. These predictors could be useful in hypothesis-based experimental design by classifying potential anti-inflammatory candidates, but the performance of the mentioned anti-inflammatory predictors still needs to be implemented in order to provide more accurate and reliable bioactivity prediction for the growth of new biopharmaceuticals in near future.

Author contributions

Julia Rivera-Jiménez: Methodology, formal analysis, writing – original draft. Carmen Berraquero-García: Methodology. Raúl Pérez-Gálvez: Conceptualization, methodology, supervision, writing – review & editing. Pedro J. García-Moreno: Conceptualization, methodology, supervision, writing – review & editing. F. Javier Espejo-Carpio: Conceptualization, methodology, supervision, writing – review & editing. Antonio Guadix: Conceptualization, writing – review & editing. Emilia M. Guadix: Conceptualization, supervision, writing – review & editing, funding acquisition.

Conflicts of interest

The authors declare no conflict of interest.

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