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Review

Bioactive peptides in plant-derived foodstuffs

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ABSTRACT

A literature survey covering the presence of bioactive peptides in plant-derived foodstuffs is presented. Examples are given of plant peptides associated with a beneficial effect on human health. The main bioactive effects of these peptides are defined and their mechanism of action described, when known. Current understanding of the way in which these molecules are adsorbed, distributed, metabolized and finally excreted is discussed. A particular focus is given to potentially immunomodulatory peptides. The leading analytical assay methods used to evaluate their activity are outlined. Inspection of crop proteomic data revealed that at least 6000 proteins may harbour bioactive peptides. The analysis of these proteins using a Gene Ontology approach has provided a number of insights regarding their occurrence and relevance.

Biological significance

The review reports an updated survey on bioactive peptides present in food crop plants, with a particular focus on immunomodulatory peptides which might be relevant for therapeutic applications. It employs a bioinformatic approach to search for proteins of crop plants potentially harboring bioactive peptides, summarising through Gene Ontology the main classes of peptide-containing proteins in food.

1. Introduction

Much of the interest in plant-derived bioactive peptides present in foodstuffs lies in their potential pharmaceutical and/or nutraceutical benefit [1]. The advantage of naturally occurring bioactive peptides over synthetic chemicals as pharmaceuticals is particularly strong in less developed parts of the world, both from a cost and an availability point of view. Strategies designed to overcome potential problems associated with the use of therapeutic peptides have been explored by Agyei and Danquah [2], while Hou et al. [3] have proposed that food processing residues could be exploited as a source of proteins from which bioactive peptides could in principle be derived.

In 1991, Japanese legislators enacted a set of "Foods for Specified Health Use" (FOSHU) measures, which regulated the use of food for therapy [4]. Products carrying a FOSHU label are now certified with respect to their pharmaceutical activity [5]; an example is the use of "Fine Rice", rice grains as a treatment of rice-associated atopic dermatitis. The list of category 2 FOSHU products includes soybean-derived peptides which control the level of cholesterol in the blood, while further plant-derived products are represented in categories #4 (blood pressure reduction) and #10 (serum triacylglycerol level and lipid reduction). In Europe, Regulation (EC) 1924/2006 covers health claims made for food products [6]; this requires that all applications be reviewed by the European Food Safety Authority (EFSA). As part of a comprehensive review of the opioid activity of peptides derived from milk carried out by EFSA in 2009 [7], it was concluded that food peptides should not require a formal risk assessment procedure. In 2012 EFSA rejected claims made by a Finnish company that the two milk tripeptides IPP and VPP are effective in maintaining blood pressure [8], and have similarly ruled out the suggestion that the consumption of certain soy proteins actively lowers blood low density lipoprotein cholesterol content [9].

Among the benefits to human health claimed for plant peptides are antibiosis, a reduction in blood pressure, a reduction in blood cholesterol level, antithrombosis and antioxidation, the enhanced absorption of trace minerals, cytoimmunomodulation and opioid activity. After a survey on the most important features of bioactive peptides, this review will focus on peptides exhibiting immunomodulatory activity.

2. Peptides present in foodstuffs

Some of the bioactive peptides present in plant-based foodstuffs are hydrolysis products of proteins which in their native form are pharmaceutically inactive, and are sometimes referred to as "cryptides". They differ from those peptides, which are present in planta where they function either as part of the host's defence against pathogen attack or as signalling molecules [10]. The former set can be generated by microorganisms (especially as a product of fermentation), by gastro-intestinal digestive enzymes (in particular pepsin, trypsin and chymotrypsin) or by in vitro procedures involving treatment with either a proteolytic enzyme or following exposure to high temperature and/or extreme pH conditions. A number of peptides ac-

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cumulate during the aging or storage of foodstuffs, notably in cheese and yogurt [11]. Where naturally occurring bioactive peptides are identified for marketing purposes as "nutraceuticals", it is because they have been either deliberately added to the product or have become enriched during its processing. A list of commercial products containing bioactive peptides has been recently published [12], only one of which is of plant origin.

Descriptions of the conventional procedures used for identifying peptides in foodstuffs have been given by Arihara [13] and Picariello et al. [14]. A recent review focusing on the discovery and validation of bioactive peptides has been published by Sanchez-Rivera et al. [15], and several databases have been created to collate what is known about specific bioactive peptides (Table 1).

Table 2 lists known bioactive peptides present in edible plants; following the convention proposed by many authors they are restricted to those composed of between two and 20 residues and with a molecular mass of < 6 kDa [16].

An important class of immunomodulatory plant-derived peptides are the cyclotides, disulfide-rich peptides, of about 30 amino acids in size, with the unique structural features of a head-to-tail cyclized backbone and knotted arrangement of three-disulfide bonds [90]. Firstly discovered in the Rubiaceae family, up to date they have been found in Violaceae, Apocynaceae, Cucurbitaceae, Fabaceae, Solanaceae and Poaceae, including wheat, maize, and rice [91,92]. Utilising a combination of proteomics and transcriptomics an increasing number of cyclotides have been found and are estimated to be an excess of 50,000 in the Rubiaceae alone [92]. Although the immunosuppressive properties of plant cyclotides have been characterized in vitro and their scaffold offers great potentiality for drug development [93] up to now only aqueous plant extracts, in the form of tea or topic skin application, are utilised [94], thus cyclotides do not belong to the category of bioactive peptides comprising foodstuff of plant origin bioactive peptides considered in this review.

Table 1.
Online sources of information of relevance to bioactive peptides present in food (as of April 2015).

Database name	Description	URL	Institution
ACEpepDB	Collects anti-hypertensive peptides	http://www.cftri.com/pepdb/	Central Food Technological Research Institute (India)
AHTPDB	Collects antihypertensive peptides	http://crdd.osdd.net/raghava/ahtpdb	CSIR, Institute of Microbial Technology (India)
BIOPEP	Collects bioactive peptides	www.uwm.edu.pl/biochemia/index.php/en/biopep	University of Warmia and Mazury in Olsztyn (Poland)
EROP-Moscow	Collects regulatory oligopeptides	erop.inbi.ras.ru	A. N. Bach Institute of Biochemistry, Russian Academy of Sciences (Russian Federation)
PepBank	Collects peptides shorter than 20 residues	pepbank.mgh.harvard.edu	Massachusetts General Hospital, Harvard University (USA)
PeptideLocator	Identifies bioactive peptides within protein sequences	http://bioware.ucd.ie/~compass/biowareweb/Server_pages/biopred.php	University College Dublin (Ireland)
PhytAMP	Collects plant antimicrobial peptides	phytamp.pfba-lab-tun.org	Institute of Applied Biological Sciences Tunis (Tunisia)

The large majority of the peptides listed in Table 2 is associated with antihypertension activity, achieved by inhibiting the conversion of angiotensin I to angiotensin II, whereas just 10 are immunomodulatory, 9 are opioid, 30 are antioxidant and 14 hypocholesterolemic.

The list comprises 211 distinct peptides ranging from 2 to 19 residues (mean length 5.1 residues, standard deviation 3.3), involving a total of 1084 residues; the most frequent residue is proline (124 occurrences), often representing the C terminus [95], whereas cysteine is the least represented residue (15 occurrences). The most frequent C terminal residues are proline (35 peptides), tyrosine (28 peptides), arginine (23 peptides) and phenlyalanine (20 peptides). A statistical comparison of the occurrence of a specific residue at the C terminus and its overall frequency suggests a non-random pattern ($\chi^2_{(19\ \text{d.f.})} = 113.8$, $p < 0.0001$). The most frequent N terminal residues are leucine (28 peptides), valine (27 peptides), isoleucine and tyrosine (each 17 peptides). C terminal residues possessing hydrophobic or aromatic side chains have been associated with inhibition of angiotensin-converting enzyme (ACE) activity [96]. In particular, the final residue is typically either aliphatic–hydrophobic or small, while the fourth residue from the C terminus tends to be bulky and hydrophobic [97]. The non-random identity of the C terminal residues presumably reflects a preference of the peptidase(s) responsible for the release of these peptides. A proline at the C terminus can be considered a common feature to many peptides, because it renders the molecule resistant to further digestion, allowing its transmembrane transport in intact form [98].

Considering the relevance of disulfide bonds as an important factor in the structure of plant derived bioactive peptides (as in cyclotides, [90]), a statistical analysis of the peptides considered in this study was conducted to highlight the abundance pattern of cysteine (Cys) both in the whole peptides sample and in comparison with the abundance levels of the other amino acids. Considering all the peptides in Table 2, using "plant species" and "bioactive effect" as independent variables (fixed factors) and each amino acid number in each of the peptides as dependent variables, a two-way MANOVA was conducted with IBM SPSS Statistics for Windows Version 23. The result of the omnibus multivariate test within MANOVA was that, considering a confidence interval of 95% for the dependent variables, there was a significant difference in the amino acids number in the whole peptides sample according to "plant species", "bioactive effect" and the interaction of the two (plant species* bioactive effect). In this analysis, the main effects "plant species" and "bioactive effect" explained about 23% of the variability within the dependent variables, their interaction explained 20% of this variability. The results of the "between subject" tests (ANOVA) was taken into consideration only for Cys. This test showed that the content of Cys was significantly different from the other amino acids only when considering the factor "plant species", but not in connection to "bioactive effect" nor to their interaction (plant species ∗ bioactive effect). Pairwise Tuckey's HSD post hoc test conducted within the "plant species" revealed that Cys was significantly more abundant in peptides derived from potatoes in respect to all the other plant types together. All results are reported in Supplementary Table 1A.

Thus it is not possible to attribute to Cys abundance any statistical significant relevance in the immunomodulatory effect of the considered peptides.

Table 2.
Bioactive peptides of plant origin present in food products from plants and their effects. For each peptide, when available, information on origin and sequence are provided.

Plant species	Origin and original plant protein, if known (name of peptide)	Bioactive effect[a]	Experimental method[b]	Sequence information when available	Reference
Cereal grains					
Barley (*Hordeum vulgare* L.)		AH: Antihypertensive and antibacterial	In vitro	EVSLNSGYY	[17]
Buckwheat (*Fagopyrum esculentum* Moench)	Unknown, water extract from tissues	AH: antihypertensive	In vitro[a]	GPP	[18]
	Lactobacillus fermentation of sprouts	AH: antihypertensive	In vitro[a]	DVWY, FQ, VVG	[19]
	Hydrolysis with pepsin, chymotrypsin, trypsin	AH: ACE inhibition	In vitro[a], in vivo (SH rats)	AY, FY, INSQ, ITF, LF, LGI, PSY, VK, YQY	[20]
Kamut (*Triticum turanicum* Jakubz.)	Sourdough fermentation	AO: antioxidant	In vitro[b]	YEWEPTVPNFDVAKDVTDM; GVSNAAVVAGGH; DAQEFKR; HKEMQAIFDVYIMFIN	[21]
Maize (*Zea mays* L.)	α-Zein, native; hydrolysis of protein with alcalase and neutrase; gamma-zein	AH: ACE inhibition, decrease of blood pressure	In vivo (SH rats 30 mg kg^{-1})	LRP, LSP, LQP and others; LPP, VHLPP, VHLPPP	reported in [22]
	Zein hydrolysates with Alcalase	AO: chelation of radicals, scavenging	In vitro[b]	YA, LMCH	[23]
	Hydrolysis of gluten meal with Alcalase	AH: ACE inhibition	In vitro[a], in vivo (SH rats, 50 mg kg^{-1})	AY	[24]
	Hydrolysis of gluten meal with Alcalase	AO: antioxidants, scavenging	In vitro[b]	FPLEMMPF	[25]
Oat (*Avena sativa* L.)	Sourdough fermentation	AH: ACE inhibition	In vitro[a]	VPP, IPP, LQP, LLP	[26]
Rice (*Oryza sativa* L.)	Albumin (oryzatensin), tryptic digestion	IM, OP: modulation of immune system, antagonist of opioids	In vitro[c]	GYPMYPLPR	[27]
	Proteolysis (neutrase) of defatted endosperm proteins	AO: antioxidative effect, radical scavenging	In vitro[b]	FRDEHKK, KHDRGDEF	[28]
	Digestion with alcalase	AH: antihypertensive	In vitro[a], in vivo (SH rats 30 mg kg^{-1})	TQVY	[29]
	Protein hydrolysate with alcalase, trypsin	AH: ACE inhibition	In vitro[a], in vivo (SH rats 5 mg kg^{-1})	VNP, VWP	[30]
	hydrolysis of heat-stabilized defatted bran, alcalase	IM: anti-cancer, inhibition of growth of cancer cells	In vitro[d]	EQRPR	[31]
Rice (*Oryza sativa* Japonica Group)	Heating in water and acid extraction	IM, AO: antiinflammatory, anti-neuroinflammatory, immunomodulatory, radical scavenging	In vivo (mice 100 μg kg^{-1})	AEMIDLAAKMLSEGRG	[32]
Rye (*Secale cereale* L.)	Sourdough fermentation	AO: antioxidant	In vitro[b]	RLSLPAGAPVTVAVSP; LCPVHRAADL; PAEMVAAALDR; KVALMSAGSMH	[21]
	From secalin	AH: ACE inhibition		HHL, DLP, VY, PR	Reported in [33]
Spelt (*Triticum spelta* L.)	Sourdough fermentation	AO: antioxidant	In vitro[b]	GNQEKVLELVQR; PAGSAAGAAP; EALEAMFL; AAGAAAAARSAGQCGR; ITFAAYRR; HPVPPKKK	[21]
Wheat (*Triticum aestivum* L.)	Hydrolysis of gliadin with acid protease	AH: ACE inhibition, decrease of blood pressure	In vitro[a], in vivo (SH rats)	IAP	[34]
	Autolysis of bran	AH: ACE inhibition	In vivo (SH rats 10 mg ml^{-1})	VY, IY, TF, LQP, IQP, LRP	[35]
	Hydrolysis of gluten	OP: agonist of opioids (exorphins)	In vitro[c], in vivo (mice, 30–300 mg kg^{-1})	GYYPT (exorphin A5), YPISL (exorphin C)	[36,37]
	Gluten	Antagonistic effect to gliadin toxicity in celiac disease	In vitro[e]	QQPQDAVQPF (pDAV), QQPQRPQQPF (pRPQ)	[38]
	Fermentation or hydrolysis of germ proteins (Alcalase)	AO: antioxidant	In vitro[b]	VHHH	[39]
	Hydrolysis of germ proteins (Alcalase)	AH: ACE-inhibition	In vitro[a]	TF, LY, YL, AF, IY, VF, IVY, VFPS, TAPY, TVPY, TVVPG, DIGYY, DYVGN, TYLGS, GGVIPN, APGAGVY	[40]
	Sourdough fermentation	AO: antioxidant	In vitro[b]	MAPAAVAAAEAGSK; DNIPIVIR	[21]

Table 2. *(Continued)*

Plant species	Origin and original plant protein, if known (name of peptide)	Bioactive effect[a]	Experimental method[b]	Sequence information when available	Reference
	Digestion of α-gliadin (gliadinomorphin-7)	OP: Opioid (exorphin)	In vitro[c]	YPQPQPF	[41]
	α-Gliadin	IM: Immunomodulatory peptides	In vitro[e]	PPYCTIVPFGIFGTNYR	[42]
Legumes, pulses					
Bean (*Phaseolus vulgaris* L.)	Hydrolysates with pepsin, pancreatin, alcalase and others, from phaseolin	AH: ACE inhibition	In vitro[a]	PVNNPQIH	[43]
Chickpea (*Cicer arietinum* L.)	Digestion with alcalase of legumin fraction	AH: antihypertensive	In vitro[a]	MDFLI and others	[44]
	Hydrolysates with alcalase	AO: antioxidant, iron chelating peptide	In vitro[b]	NRYHE	[45]
	Enzymatic hydrolysis of albumin	AO: antioxidant	In vitro[b]	RQSHFANAQP	[46]
Mung bean (*Vigna radiata* (L.) R.Wilczek)	Digestion with alcalase of defatted flour	AH: antihypertensive	In vivo (SH rats, 600 mg kg^{-1})	KDYRL; VTPALR; KLPAGTLF	[47]
Pea (*Pisum sativum* L.)	Hydrolysis of proteins	AH: ACE inhibition, renin inhibition, decrease of blood pressure	In vitro[a]	IR, KF, EF, LR, NR, FT	[48]
Soybean (*Glycine max* (L.) Merr.)	Hydrolysate, fermentation of proteins; glycinin acid digestion	AH: inhibition of angiotensin I-converting enzyme, decrease of blood pressure	In vitro[a], in vivo (SH rats, different doses)	NWGPLV and others, HHL, WL, IFL	[49–52]
	Digestion with alcalase	AH: antihypertensive	In vitro[a]	DLP, DG	[53]
	Glycinin, enzymatic digestion with Protease P, thermolysin	Antihypertensive; smooth muscle relaxation through Ca^{2+} influx inhibition	In vitro[a]	VLIVP; HGK	[54]
	Whey proteins, enzymatic digestion	AH: antihypertensive		VAP, VKP, VTP, LAP, LN, LHP, LKP, VTY, LYQA, YEAP, YQAP	reported in [55]
	Pepsin digestion of proteins	AH: ACE inhibition	In vitro[a], in vivo (SH rats, 2 g kg^{-1})	YLAGNQ, FFL, IYLL, VMDKPQG, IA,	[56]
	Soy protein alcalase hydrolysates, SPAH	IM, LL: modulation of immune system, increase of phagocyte activity, modulation of lymphocyte proliferation, hypocholesterolemic effect	In vitro, in vivo	HCQRPR and QRPR	[57]
	Trypsin digest of β-conglycinin α' subunit (soymetide). Acid proteinase	AH, IM: stimulation of phagocytosis. ACE inhibition	In vitro[a,e], in vivo (mice)	MITLAIPVNKPGR; LAIPVNKP, LPHF	[52,58]
	Glycinin (soystatin)	LL: binds bile acid, inhibits solubility of cholesterol and adsorption	In vitro, in vivo (rats)	VAWWMY, LPYP, LPYPR	[59–61]
	β-Conglycinin; glycinin	LL: affects the transcription of the receptor of LDL	In vitro	FVVNATSN; FKTNDRPSIGN, SSPDIYNPQAGS, DTPMIGT	[62]
	β-Conglycinin (soymorphin-5)	LL, OP: decreases plasma triglycerides and glucose, opioid (anxiolytic-like)	In vivo (mice 10 mg kg^{-1})	YPFVV, also YPFV, YPFVVNA	[63,64]
	β-Conglycinin; glycinin, lipoxygenase, trypsin inhibitor	LL: inhibits synthesis of triglycerides	In vitro (HepG2 cells), in vivo (rats)	SY; VK; KA	[65]
	β-Conglycinin, simulated gastrointestinal digestion	AO: Antioxidative properties	In vitro[b]	25 peptides, including LLPHHADAD	[66]
	Globulin	LL: binds bile acid. Increases degradation of LDL	In vitro	IAVPGEVA	[67]
	Soy protein Alcalase hydrolysates	LL: inhibits solubility of micellar cholesterol	In vitro	WGAPSL	[68]
	Hydrolysate with pepsin and pancreatin	IM: immunomodulatory: decrease in the production of cytokines and inflammatory mediators	In vitro	RQRK and others	[69]
		AH: antihypertensive and antimicrobial (bacteria and fungi)	In vitro	PGTAVFK	[17]
	Digestion of protein with peptidase R (Glycinin G4 subunit)	IM: Immunostimulating	In vitro[f]	GGRKQGQHQQEE	[70]

Table 2. *(Continued)*

Plant species	Origin and original plant protein, if known (name of peptide)	Bioactive effect[a]	Experimental method[b]	Sequence information when available	Reference
Cruciferous vegetables					
Broccoli (*Brassica oleracea* L.)	Unknown, water extract from tissues	AH: ACE inhibition	In vitro[a]	YPK	[71]
Canola (*Brassica*)	Defatted meal digested with alcalase	AH: ACE inhibition	In vitro[a]	FL, VSV	[72]
Rapeseed (*Brassica napus* L.)	Enzymatic digestion	AH: antihypertensive, renin inhibition	In vitro[a], in vivo (SH rats, 12.5 mg kg^{-1})	IY, RIY, VW, VWIS, GHS, RALP, LY, TF	[73–75]
Liliaceous vegetables					
Garlic (*Allium sativum* L.)	Unknown, water extract from tissues	AH: antihypertensive	In vitro[a], in vivo (SH rats)	SY, GY, FY, NY, SF, GF, NF	[76]
Oil seeds					
Sunflower (*Helianthus annuus* L.)	Hydrolysate with pepsin and pancreatin, from helianthinin	AH: ACE inhibition	In vitro[a]	FVNPQAGS	[77]
Sesame (*Sesamum indicum* L.)	Protein hydrolysate	AH: ACE inhibition	In vitro[a], in vivo (SH rats)	LVY, LSA, LQP, LKY, IVY, VIY, MLPAY	[78]
	Trypsin hydrolysate	AO: Antioxidative, zinc chelating peptides	In vitro[b]	SM, NCS, LAN	[79]
Walnut (*Juglans regia* L.)	Hydrolysate	AH: ACE inhibition	In vitro[a]	WPERPPQIP	[80]
		AO: antioxidant	In vitro[b]	ADAF	[81]
Potatoes					
Sweet potato (*Ipomoea batatas* (L.) Lam.)	Tuber protein digested with proteases	AH: ACE inhibition	In vitro[a], in vivo (SH rats, 500 mg kg^{-1})	ITP, IIP, GQY, STYQT	[82]
	Tuber defensin digested with trypsin	AH: ACE inhibition	In vitro[a]	FK, GFR, GPCSR, IMVAEAR, CFCTKPC, MCESASSK	Reported in [83]
	THIOREDOXIN digested with pepsin	AH: ACE inhibition		EVPK, VVGAK, FTDVDFIK, MMEPMVK	Reported in [83]
	TRYPSIN INHIBITOR digested with pepsin	AH: ACE inhibition	In vitro[a]	AH, LR, RF, VRL, HDHM, KIEL, SNIP, TYCQ, GTEKC, VKAGE	[84]
Other plants					
Cocoa (*Theobroma cacao* L.)	By-product of cocoa processing hydrolysed with proteases (21 kDa seed protein)	AO: Antioxidant, delay in body paralysis due to Alzheimer disease	In vivo (*Caenorhabditis elegans*)	DNYDNSAGKWWVT	[85]
Flaxseed (*Linum usitatissimum* L.)	Hydrolysates, gastrointestinal digestion in vitro	AH: ACE inhibition	In vitro[a]	WN[IL]NA, N[IL]DTD[IL]	[86]
	Protein digested with proteases	AH: ACE inhibition	In vitro[a], in vivo (SH rats, 200 mg kg^{-1})	RW, QGR, SVR, QQQG, ASVRT, RDLPG, RGLERA, DYLRSC, ARDLPGQ, GQMRQPI, TCRGLERA	[87]
Grape (*Vitis vinifera* L.)	In red wine	AH: ACE inhibition	In vitro[a]	AWPF, SWSF, YYAPF, WVPSVY, LIPPGVPY, YYAPFDGIL	[88]
Spinach (*Spinacia oleracea* L.)	Hydrolysis from RUBISCO with pepsin and pancreatin	AH: ACE inhibition	In vitro[a], in vivo (SH rats, 20–100 mg kg^{-1})	MRW, MRWRD, LRIPVA, IAYKPAG	[89]

[a] Bioactive effects are classified into main categories: AH, antihypertensive; AO, antioxidant; IM, immunomodulatory; LL, lipid lowering; OP, opioid.

[b] Information about the assay procedure has been reported when available. For in vivo studies, the dose of oral administration has been reported. SH, spontaneous hypertensive. For in vitro studies: a—in vitro ACE inhibitory assay; b—in vitro assays for radical scavenging, inhibition of peroxidation; c—in vitro assays on affinity for opioid receptors; d—in vitro assay on cancer cell lines; e—in vitro assays on cells from donors; f—in vitro assays on spleen cells from mice.

By conducting a one-way MANOVA only for "bioactive effect" fixed factor, comprising the 5 groups reported in Table 2, this independent variable had a highly significant effect on the overall amino acids variability within the peptides considered in this study, with a significant effect size (see Supplementary Table 1B). Further single one-way ANOVAs for each amino acid were performed within the 5 groups comprising the fixed factors. Ala, Glu, Gln, Asp, Arg, Pro, Met, His, Lys, and Gly differences were highly significant, Arg and Gln had an effect size of almost 16% and 13% respectively (Supplementary Table 1B). Also in this instance Cys was not significantly different within the five "bioactive effect" groups. Post hoc Sheffé test for unequal sample size was performed only on the significantly different amino acids. When considering the immunomodulatory (IM) peptides group, Glu, Gln and Lys were significantly the most abundant amino acids, His, Glu, and Ala were the most abundant in the AO group, Gln and Pro in the OP group.

3. Bioavailability of peptides in foodstuffs

The delivery to, and the availability of peptides once in the cell are of particular importance in the context of their therapeutic efficacy. An experimentally based assessment of the health benefit of plant-derived peptides is difficult to achieve, since ingested proteins are processed by a variety of proteases and peptidases during their

passage through the gastro-intestinal tract. Additional confounding effects can be generated by interactions with other plant compounds and host metabolites. Feeding hydrolysates of a particular protein(s) is complicated by a degree of uncertainty as to the number and type of peptides present. Basing experiments on a purified peptide(s) may appear more straightforward, but these molecules too can be processed by the gastro-intestinal tract. Simulation of the human gastro-intestinal tract offers an innovative experimental approach [99,100]. Experiments have been described in which bacteria sourced from the human gut are exposed to plant proteins, with a view to exploring the extent to which the intestinal flora, either in the presence or absence of probiotic supplements, can drive the production of bioactive peptides [1,101].

The processes underlying absorption, distribution, metabolism and excretion (ADME) are still obscure, a fact which, for the moment, inhibits the replacement of synthetic chemicals as pharmaceuticals by naturally occurring peptides [102,103]. Studies about the fate of dietary antigens can shed some light on ADME aspects of dietary peptides [104]. An improved delivery of peptides may become possible by designing effective carrier compounds, although these would need to be carefully chosen to avoid potential toxicity or allergenicity problems.

3.1. Absorption

As previously discussed, bioactive peptides can be generated from food materials during digestion processes in the gastro-intestinal tract. Differently, in those cases where bioactive peptides have accumulated in foodstuffs before consumption, as a result of processing, fermentation or aging, their bioactivity can be expressed only if they remain unaltered in their passage through the gastro-intestinal tract. In principle, small peptides, particularly those carrying a proline–proline dipeptide at their C terminus, are the least prone to degradation by proteases and peptidases present in the stomach, secreted by pancreas, or contained in the brush border membrane in the small intestine [105]. Larger peptides (6 residues or more, mass > 700 Da) tend to exert their biological activity without entering the intestinal epithelium cell — this is particularly relevant for peptides affecting gastrointestinal function and for food allergens [104]. In the case of immunogenic peptides generated from the breakdown of cereal gluten, both the 33mer α2- and the 26mer γ-gliadin are highly resistant to protease degradation, thanks to their high content of glutamine and proline. Very short (2–6 residues) peptides appear to be more easily transported across the intestinal epithelium than are free amino acids [106]. According to Sarmadi and Ismail [16] and Renukuntla et al. [107], the possible entry routes available are paracellular (through tight junctions between cells, without energy consumption), transcellular (across the epithelium, by passive diffusion), endocytosis (upon recognition by receptors) and transport across the plasma membrane. Di- and tripeptides can be transported by the H^+/peptide co-transporter PepT1, which is localized in the apical membrane of duodenum and jejunum enterocytes [106,108], in a transport which depends on membrane potential and pH. PepT1 appears to be capable of transporting all possible di- and tripeptides deriving from digestion of proteins, as well as free amino acids [109], but peptides with large hydrophobic surfaces are favoured [110]. The absorption of the valine–tyrosine dipeptide occurs rapidly after ingestion [111]. Enterocytes possess high peptidase activity, and the peptides which are resistant to proteolysis cross the intestine epithelium in a transcellular manner exploiting a peptide transporter localizing to the basolateral membrane, not yet identified [106]. The M (microfold) cells overlying the Peyer's patches are capable of endocytosis, which allows for the delivery of peptides to the underlying tissue connected with the lymphatic system. Small peptides can therefore enter the blood stream via the lymphatic system in the thoracic duct [112], thereby by-passing the liver. The colon may also represent a further absorption site, since it is free of peptidase activity and the residence time is long [107]. Wheat gliadins — not just in coeliac patients — appear to increase the permeability of epithelial cells to other peptides by reorganizing the cytoskeleton and altering cell junctions [113]. The transcellular transmission of the gliadin peptides responsible for gluten intolerance can result in their degradation in the lysosomes; the paracellular route is also possible, as a result of "leaky" tight junctions; the inhibition of this entry route is currently a target for some alternative therapies [114].

One method for evaluating the absorption and transport of peptides uses monolayers of cultured Caco-2 cells, derived from a human colorectal carcinoma, as a mimic of human enterocytes from the small intestine: one recent application of this approach [115] has been used to demonstrate that a 7-residues peptide (VLPVPQK) could be transported across the cells in few minutes, but it was at the same time subjected to hydrolysis at the apical surface of cells, since the peptide VLPVPQ was recovered at the basolateral side. It was also shown that the paracellular and transcellular routes were not involved in its transport, opening the possibility that oligopeptide transporters could be involved. A detailed discussion of other in vitro and in vivo methodologies available to study intestinal absorption of peptides is reported in [116].

3.2. Distribution

Detection of peptides in plasma is technically challenging, because of their low concentrations estimated at picograms per millilitre [117]. Peptides comprised of up to 5–6 residues could be detected in the blood after feeding volunteers with peptide-enriched drinks [117]. Larger peptides can be found intact in plasma, as in the case of the immunomodulatory lunasin after consumption of soybean [118]; animal models have been used to demonstrate that this 43-residue peptide can reach several tissues and cross the blood–brain barrier [119]. Within 15 min of ingestion of an IPP tripeptide-enriched yogurt beverage, the tripeptide could be identified in picomolar amounts [120], with its concentration returning to the baseline level after two hours, demonstrating that small oligopeptides can be transported from the gastrointestinal tract to the blood without degradation. Other di- and tripeptides identified in plasma were all present at low levels, and were unrelated to the food intake, representing therefore the metabolic turnover of proteins. The concentration in the blood of the VY dipeptide also increased following the ingestion of an enriched drink, in a manner proportional to the dose [111]. It has been calculated that the half-life of the dipeptide VY was longer than the half-life of the tripeptide IPP; the half-life could be increased by the simultaneous supplementation with a meal, opening the possibility that peptidase inhibitors present in food could be a contributing factor [120]. The allergic reaction to certain food proteins mediated by IgE confirms that peptides derived from foodstuffs can find their way into the blood. Nevertheless, the presence of peptidase activity in both the endothelium and the plasma results in a half-life of these peptides measured in minutes; as a result it is argued that peptides introduced to the body are likely to have a low biological efficacy [121]. The high bioavailability of lunasin has been in fact attributed to the simultaneous presence of protease inhibitors, allowing 30% of the peptide to reach the target tissues [119].

3.3. Metabolism

Although specific data are not available, it is generally assumed that peptides are catabolized within the host cell, allowing the resulting free amino acids to be used as building blocks for protein synthesis. As previously reported, peptidases are present at the brush-border, in enterocytes, in plasma, in the endothelium and inside the target cells. Peptides can be internalized via endocytosis and digested in the lysosome [102]. A different metabolic process occurs in the case of the immunogenic gluten peptides, because glutamine residues are either de- or transamidated, whereupon they are transformed into glutamic acid, a step which is required for the binding of epitopes to HLA-DQ2 or DQ8, and for the accumulation of peptides in the lamina propria of the brush-border epithelium, followed by their recognition by antibodies [122].

3.4. Elimination

The peptides present in the blood can be detectable in urine [123], and it is known that peptide-based commercial drugs are accumulated to quite high concentrations in the liver and kidney [102]. Peptides can be filtered in the glomerular site according to their shape and size (mass below 25 kDa); once present in the filtrate they can be re-absorbed by the kidney tubules, as occurs with endogenous peptides such as the interleukins; others are hydrolysed in the tubular epithelium [124]. Small peptides are transported by PepT2 and PepT1 into renal epithelial cells, followed by their hydrolysis in the cytoplasm [106]. A proportion of peptides is excreted with the bile following their metabolism in the liver. In the case of the antihypertensive valine–tyrosine dipeptide, 50% is eliminated in this way in just over 3 h [111].

4. Major biological effects of plant-derived peptides

4.1. Hypocholesterolemic peptides

Several peptides derived from the hydrolysis of soybean proteins have been shown to suppress cholesterol and lipid levels in the blood (Table 2). The mechanistic basis of their beneficial effect varies (see Figure 1 in Howard and Udenigwe [125]): some stimulate the secretion of bile acids, some modify lipid metabolism lipids in the liver, and some affect hormones and cholesterol receptors. They are thought to lower the synthesis and release of triglycerides and cholesterol, suppress the presence of low density lipoproteins (LDLs) and very low density lipoproteins and enhance the presence of high density lipoproteins. They also interact with various constituents of the bile to stimulate the conversion of cholesterol in the blood. A quite different mode of action is represented by the induction of expression of genes encoding LDL receptors in the hepatocyte [126]. The most functionally important residues in these peptides are the hydrophobic ones (such as leucine, tryptophan and tyrosine in soystatin). Some bioactive dipeptides derived from soybean proteins are able to suppress the synthesis of triglycerides. Hydrophobic residues can establish interactions with lipids and with the hydrophobic moieties of bile compounds. On the other hand, hydrophilic peptides are able to inhibit the function of various biosynthetic enzymes. A diet enriched with soybean protein has long been known to lower the blood concentration of LDLs, a fact recognized by the US Food and Drug Administration's approval of the claims that soy-based food can help reduce the risk of coronary disease [127].

4.2. Opioid peptides

Peptides showing opioid-like activity have been termed “exorphins” thanks to their structural similarity to endorphins and enkephalins, which underlies their analogous interaction with the relevant receptors. The requirement for binding the receptors is an N terminal tyrosine, along with an aromatic amino acid in the third and fourth position away from the N terminus [128]; the presence of a proline in the second position is considered as a requirement for the correct orientation of the side chains. In addition, exorphins can have a direct influence over gut function via their interaction with smooth muscles and increase in electrolyte absorption [129]. The opioid peptides present in both wheat- and soybean-based foodstuffs (Table 2) exhibit a binding activity equivalent to that of endogenous ligands [130]; their physiological effects include behavioural alteration, loss of appetite, decrease of intestinal motility. The GM7 peptide derived from a wheat α-gliadin mimics the effects of morphine and inhibits the absorption of cysteine, thereby compromising cellular redox status, the equilibrium between the reduced and oxidized forms of glutathione and methylation capacity [131]. In consequence, it has been suggested that a wheat-free diet could reduce the risk of gut inflammation, while at the same time avoid neurological problems caused by the presence of the opioid peptides. The soybean peptide soymorphin-5 is a μ-opioid able to both suppress intestinal contractions and relieve anxiety. However, it also affects glucose and lipid metabolism, and has been shown in the mouse to exhibit anti-diabetic effects [64]. This dual behaviour provides further evidence of the connection between opioid receptors and diabetes, and more generally between the nervous system and nutrition.

4.3. Angiotensin I Converting Enzyme inhibitory peptides

Regulation of the renin–angiotensin pathway via the inhibition of ACE has an anti-hypertensive effect. ACE is a dipeptidyl carboxypeptidase which converts the decapeptide angiotensin I into the octapeptide angiotensin II. Peptide-induced inhibition of ACE activity occurs in both a competitive and a non-competitive manner [132]. ACE inhibitory peptides are characteristically short and carry hydrophobic residues (invariably including proline) along with some positively charged residues [126]; the presence of an aromatic amino acid (phenylalanine or tyrosine, but particularly tryptophan) at their C terminus promotes ACE I inhibition [133]. Some of these peptides — documented in foodstuffs prepared from flaxseed, pea and hemp [126] — directly inhibit renin activity. They are presumed to successfully resist degradation during their passage down the gut. Other plant-derived peptides have a vasoprotective effect, achieved by inducing a decrease in the intracellular concentration of Ca^{2+} in smooth muscle cells of blood vessels [134], which inhibits the function of membrane channels and interferes with certain signalling pathways. They are particularly prominent in foodstuffs prepared from pulses (Table 2) and are typically produced following hydrolysis or protease digestion. As a result, they are relevant as contaminants of fermented soybean, but they also are present in cereal dough [83]. Roy et al. [135] have claimed that the ACE inhibitory peptides present in pea-derived foodstuffs are more resistant to digestion than are those originating from milk. Autolysates of cocoa beans also contain ACE inhibitory peptides [136]. Certain vasoprotective peptides, derived from the soybean protein glycinin, act on cellular Ca^{2+} concentration [134].

4.4. Antioxidative peptides

Antioxidative peptides suppress the damage caused by reactive oxygen species, thereby restricting the peroxidation of essential fatty acids. Cysteine, lysine, histidine, methionine, tryptophan and tyrosine are all effective as radical scavengers [126]. Antioxidative peptides can be found in wheat germ, as well as in a number of other plant-based foodstuffs [16]. Beer contains the barley lipid transfer protein LTP1, a cysteine rich, 91 residue, 10 kDa peptide, which acts as an antioxidant and contributes to the beer's flavour by eliminating the reactive oxygen species responsible for the product's deterioration [137]. Other active peptides are generated by digestion or fermentation. A comprehensive study of the antioxidative peptides released by a simulated gastrointestinal digestion of soybean β-conglycinin has been given by Amigo-Benavent et al. [66]. The presence of antioxidative peptides has also been identified in sourdough products, and their origin has been traced to several cereal proteins [21]. The antioxidant activity shown by cocoa-based products has also been attributed to the presence of bioactive peptides; in animal models, some of these have been suggested to provide protection against Alzheimer's disease [85]. The antioxidant activity associated with a range of plant foodstuffs (among these notably olive oil and coffee) represents the joint effect of peptides, polyphenols and flavonoids [138]. Metal chelation can be an important component of the inhibition of free radical formation, and some peptides are effective as chelators, particularly those containing cysteine, histidine, aspartic acid and glutamic acid [139]. These have been identified in protein hydrolysates from chickpea, sesame, soybean and sunflower [139]. As demonstrated by Langelueddecke et al. [140], metals can enter the body through food, linked to peptides such as metallothionein or phytochelatin, which are synthesized by plants in response to heavy metal exposure [141]. Glutathione is present in a number of plant-derived foodstuffs; the molecule is poorly absorbed by the gut, but does have a protective effect against oxidative damages in the intestine [142,143].

4.5. Immunomodulatory peptides

Plant peptides exhibiting antimicrobial activity have been suggested as potential alternatives to conventional antibiotics for the treatment of bacterial infection [10]. Immunomodulatory peptides on the other hand contribute to the host's defence response, but do not interact directly with the pathogen. The relevance of dietary peptides in immunomodulation is strengthened by the involvement of the gastro-intestinal tract in immune response of the organism [144]. Several peptides are known to either stimulate or inhibit an immune response [2]; the best characterized of these are the degradation products of various milk proteins [145]. The inflammatory process is central to a number of diseases (including some cancers and cardiovascular disease), so anti-inflammatory peptides have a potential medicinal value [146]. Immunomodulatory peptides enhance the proliferation and maturation of immune cells, stimulate “natural killer cell” (NK cell) activity and macrophage phagocytosis, up-regulate the synthesis of antibodies, chemokines and cytokines, and inactivate inflammatory compounds. Peptides which stimulate the proliferation of human lymphocytes in peripheral blood contain glycine, along with other residues [147]. However, peptides exhibiting an inhibitory effect over lymphocyte proliferation are also considered to be immunomodulatory, being able to reduce hypersensitivity and allergic reactions, as exemplified by the phenomenon of ‘oral tolerance’ in infants [148]. The soybean-derived peptide soymetide (Table 2) is derived from β-conglycinin. It interacts with receptors on the surface of macrophages and neutrophils, eliciting an immune system signal similar to that produced by a bacterial infection. Given that cultivars of soybean differ with respect to their soymetide content [149], the elaboration of nutraceuticals needs to take into account the genetic background of the plant raw material. The soybean-derived 43 residue peptide lunasin (Table 2) has several beneficial immunomodulatory properties [150]; similar molecules have been identified in the leading small grain cereals [151]. As previously described, lunasin is present intact in the blood and liver [152]. It inhibits histone acetylation in the mammalian nucleus [152]. Its interaction with endogenous cytokines regulates gene expression in NK cells, leading to their enhanced activation. It also inhibits inflammation via the suppression of the NF-κB pathway [153] and prevents cell transformation induced by carcinogens and viral oncogenes [154]. Immunomodulatory peptides from rice and soybean stimulate the formation of reactive oxygen species as a component of the host's non-specific defence against environmental stress [155]. In oilseed rape, hydrolysates have been identified which inhibit recombinant HIV protease activity [156].

5. Leading assays used to assess immunomodulatory activity

Immunomodulatory effects of peptides and other compounds can be assessed with different methods, addressing specific cell types and functions. The wide range of assays available is justified by the non-specific action of immunomodulatory peptides, and we consider here both suppression and stimulation of immune system functions. Two recent reviews have described some in vitro assays used for this purpose, analysing their advantages and disadvantages [157,158]. Most of the assays address basic functions of immune cells, such as proliferation, phagocytosis, differentiation, cytokine production, using material from human donors or from experimental animals. We report here the methods currently used to study immunomodulatory effects of peptides (Table 3), citing those papers in which the assays have been described in detail. As highlighted by Foltz et al. [121] in vitro testing has limitations due to the lack of information about real bioavailability and ADME properties of the relevant peptides.

Most of the assays can be performed with two experimental approaches: (i) in vitro, where cell cultures are exposed to the peptides; and (ii) ex vivo, where experimental animals, e.g. mice, are fed with peptides and sacrificed to obtain cells and tissues for analysis (Table 3). Immunosuppression and anti-inflammatory activities are assessed by exposing cells or animals with known inducers of immune responses, treating with peptides and evaluating the mitigating effects in treated samples.

The induction or repression of proliferation is tested by exposing treated and untreated cells to MTT (3-(4.5-dimethylthiazoyl-2-yl)-2.5-diphenyltetrazolium bromide) or MTS (3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and PES (phenazine ethosulfate). The formation of formazan provides a measure of the number of viable cells, and a proliferation stimulation index is then computed from the ratio of the absorbance of treated vs untreated cells. Positive controls are provided by mitogen ConA and the proliferation effect is quantified on the basis of differences in the optical density between samples in the presence and in the absence of the mitogen. Alternatively, proliferation can be evidenced by incorporation of labelled nucleotides in DNA molecules.

Phagocytic capacity is usually assessed ex vivo, with collection of macrophages from the peritoneum of treated mice and challenging the cultures with a suspension of heat-killed *Candida albicans* or *Saccharomyces cerevisiae* [166]. The phagocytic activity of the macrophages is quantified on the basis of the frequency of activated macrophages containing at least one yeast cell [161].

Table 3.
A survey of assays exploited for assessing the immunomodulatory activity of peptides.

Endpoint	Target cells or molecules	Experimental setup	Analytical techniques	Interpretation of results	Reference
Proliferation of immune cells (activity of mitochondrial dehydrogenase)	–Neutrophils isolated from blood (animals) –Peritoneal macrophages harvested from mice by washing the peritoneal cavity –Peripheral blood mononuclear cells (PBMC) from blood donors –Splenic lymphocytes from mice –Macrophage cell line e.g. RAW 264.7	In vitro, ex vivo: cell culture challenged with peptides in the medium (comparison with known inducers, mitogen, e.g. ConA, LPS), reagent WST-1, MTT or MTS	–WST-1 test: absorbance reading at 450 nm with spectrophotometer –Absorbance of formazan with spectrophotomer reading –Sulforhodamine B (SRB) assay, absorbance reading at 510 nm	Stimulation Index for proliferation. Increase (immunostimulation) or decrease (immunosuppression) of proliferation; interaction with known inducers	[3,69,159–162]
Proliferation of immune cells (DNA synthesis)	–Peripheral blood mononuclear cells (PBMC) from blood donors –Splenic lymphocytes from mice	In vitro: cell culture challenged with peptides in the medium (comparison with known inducers, e.g. PHA, mitogen ConA)	–Measurement of incorporation of BrdU by detection with anti-BrdU-POD and reading of absorbance at 492 nm with spectrophotometer –Tritiated thymidine and scintillation counter	Increase (immunostimulation) or decrease (immunosuppression) of proliferation. Stimulation Index: ratio between values in treated cells and values in control non stimulated cells	[159,160,163]
Mixed lymphocyte reaction (MLR), stimulation of T lymphocytes proliferation (DNA synthesis)	Dendritic cells (DC)	Cell cultures challenged with peptides are irradiated and incubated with responder cells (heterologous PBMC)	Measurement of incorporation of tritiated thymidine by liquid scintillation spectroscopy	Peptides can change the behaviour of dendritic cells towards heterologous immune cells	[38]
Phagocytosis activity	–Ex vivo: Peritoneal macrophages harvested from mice by washing the peritoneal cavity –In vitro: Macrophage cell lines e.g. RAW 264.7	–Feeding mice with peptides before harvesting cells –Ex vivo culture of cells, incubated with peptides –Incubation with neutral red –Incubation with heat killed *Candida albicans*/Escherichia coli/*Saccharomyces cerevisiae* cells	–For neutral red, reading of absorbance in washed cells at 540 nm with spectrophotometer –Number of activated macrophages harboring at least 1 yeast cell with microscope –Fluorescence of internalized cells	Increase of phagocytic activity (immunostimulation)	[159,161,164–166]
Natural Killer (NK) cell activity	Splenic cells (splenocytes) from mice	Ex vivo: Feeding mice with peptides before harvesting cells, followed by incubation with target cells (e.g. lymphoma cell line YAC-1, erythroleukemia cell line K562) and addition of MTT	Absorbance of formazan at 550 nm with spectrophotomer reading: target cell control, blank control and effector cell control	The NK cell activity is calculated by comparing the samples with controls, as cytotoxic activity on target cells	[70,161,167,168]
Phenotypic maturation	Dendritic cells derived from monocytes (purified from PBMC)	In vitro, cultured cells challenged with peptides in the medium (comparison with known inducers)	Detection of surface maturation markers by flow cytometry after staining with antibodies		[38]
Phenotype of immune cells (e.g. population of T cells)	–Peripheral blood mononuclear cells (PBMC) from blood donors –Splenocytes from mice	In vitro, cell culture challenged with peptides in the medium (comparison with known compounds, e.g. PHA)	Flow cytometry after staining with specific antibodies against antigens of the cell surface	Changes in the population of lymphocytes: –CD4 + (T helper) –CD8 + (T cytotoxic) –CD11b + (macrophages) –Cd49b* (NK cells)	[70,161,163]
B cell population in intestine	B cells in intestine sections from mice	In vivo: mice are fed with peptides, then sacrificed and the intestine is prepared for microscopical observation	Number of IgA + and IgG + cells detected by Immunofluorescence with antibodies specific against IgA and IgG	Increase in immune surveillance by antibody-producing cells in the intestine	[164,166]

Table 3. *(Continued)*

Endpoint	Target cells or molecules	Experimental setup	Analytical techniques	Interpretation of results	Reference
Upregulation of activating cytokine production, downregulation of immunosuppressor cytokines	Cytokines: Th1: IFN-γ, IL-2 Th2: IL-4, IL-5, IL-10, GM-CSF, IL-13 Th17: IL-17 Proinflammatory: TNF-α, IL-6, IL-1β, IFN-γ Immunosuppressor: TGFβ –Macrophage activity: IL-12 –Neutrophil activation: IL-8 –Chemokines: MCP-1, MCP-3, Gro-α	–Ex vivo: mice are fed with peptides, then sacrificed and the intestine is prepared for microscopical observation or spleen is recovered –In vitro: supernatant from cultures of cells, e.g. PBMC incubated with peptides, or cultured macrophages (e.g. RAW 264.7) incubated with peptides and LPS	Analysis of cytokine content in medium by sandwich ELISA	Peptides can increase the production (immunostimulatory) or repress the induction caused by a known immunostimulant (immunosuppression)	[69,160,163,169]
Cytokine production in the intestine or spleen	Cytokines in intestine sections or spleen cells from mice: –Differentiation of B cells to plasmocytes: IL-4, IL-5, IL-10, TGFβ –Differentiation to plasma cells: IL-6 –Proinflammatory: TNF-α, IFN-γ –Macrophage activity: IL-12	Ex vivo: mice are fed with peptides, then sacrificed and the intestine is prepared for microscopical observation	Number of cells producing cytokines by Immunofluorescence test with specific antibodies against cytokines	Decrease of the cytokine-producing cells in the intestine can show antiinflammatory effect Increase shows contribution to differentiation of B cells to IgA secretory cells	[70,164,166]
Nitric oxide	–Nitrite, indicative of NO generation –iNOS (inducible nitric oxide synthase)	Ex vivo: culture of cells, peritoneal macrophages or macrophage cell lines (e.g. RAW 264.7 NO(−)), stimulated with LPS and then incubated with peptides	–Supernatant of cell culture assayed with Griess reagent in comparison with a standard curve of sodium nitrite, absorbance at 540/550 nm with spectrophotometer –Evaluation of iNOS protein by Western blotting	Increase (immunostimulation) or decrease in NO generation or in iNOS expression induced by LPS (antiinflammatory)	[69,162,164]
Induction of protein marker for inflammation	Pro-inflammatory enzymes: –COX-2 (cyclooxygenase 2) –PLA2 (phospholipase A2) –Thrombin –TG (transglutaminase)	Ex vivo: culture of cells, e.g. peritoneal macrophages, stimulated with LPS and incubated with peptides	–Measurement of enzymatic activity –Isolation of proteins, electrophoresis, Western blotting	Increase (immunostimulation) or decrease in protein induction by LPS (antiinflammatory)	[69,162,170]
Transcriptional activation of proinflammatory factors	–Nuclear translocation of p65 –NF-κB transcriptional activity –IκBα degradation (kinase IKK)	Ex vivo: culture of cells, e.g. peritoneal macrophages, stimulated with LPS and incubated with peptides	Isolation of cytoplasmic and nuclear proteins, electrophoresis, Western blotting:	Degradation of IκB, upregulation of NF-κB binding activity, nuclear translocation of p65 indicate activation of transcription of inflammatory genes	[162]

Main abbreviations: ConA, concanavalin A; IFN, interferon; IL, interleukin; LPS, bacterial lipopolysaccharides; PHA, phytohaemoagglutinin; PMA,; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium); MTT, (3-(4,5-dimethylthiazoyl-2-yl)-2.5-diphenyltetrazolium bromide); Th1, Th2: T helper cells; TNF, tumour necrosis factor.

Natural Killer (NK) cells are responsible for non-specific defence reactions and are susceptible to compounds interfering with immune functions. In the common ex vivo assay of NK cell activity [168], mouse splenocytes are incubated with sensitive cancer cells, and the viability of cancer cells is evaluated by the MTT/formazan test. The level of activity of the NK cells is calculated by comparing test samples with controls (where no splenocytes are present).

Differentiation and maturation of immune system cells can be verified in vitro, starting from mouse splenocytes, or in vivo in specific organs, such as intestine and lung. The number of macrophages, NK cells, plasma cells, T helper and T cytotoxic lymphocytes is assessed using flow cytometry after reaction with antibodies specific for surface antigens [42]. The representation of T cells, monocytes and B cells in samples of peripheral blood mononuclear cells can be obtained by the use of antibodies bound to magnetic beads. The mouse sera can also be tested by ELISA for the presence of IgE, IgA, IgM and IgG and the small intestine can also be sampled for cells secreting IgA.

Many assays on immunomodulatory activities of peptides rely on evaluation of production of cytokines and inflammatory mediators, responsible for activation of the immune response; since they are released early in the immune response, their quantification can give indications about immunostimulation or immunosuppression effects of peptides.

Peripheral blood mononuclear cells prepared from human blood or cultured macrophages can be compared with positive controls treated with phytohaemoagglutinin (PHA) or stimulated with lipopolysaccharides (LPS) to induce an inflammatory response [69].

Immunogenic peptides increase the production of cytokines in non-stimulated cells, whereas with immunosuppressing ones, cytokine production is suppressed.

The supernatants of the cultures are tested for cytokines and chemokines in an ELISA format [171]: Gro-α, monocyte chemoattractant proteins (MCP-1, MCP-3), interferon (IF*N*-γ), tumour necrosis factor alpha (TNF-α), transforming growth factor beta (TGFβ), and interleukins (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13). Cytokines measurement is seen as a promising approach to understand immunomodulation [158]. The production of prostaglandin E2, a mediator which suppresses the production of proinflammatory cytokines, is monitored by using an enzyme immunoassay based on monoclonal antibodies [69].

The production of nitric oxide, an important component of the inflammatory response, is quantified by spectrophotometric measurement of the level of nitrite generated in the presence of sulfanilamide and N-1-naphthylethylenediamine (Griess reagent [172]).

Modulation of inflammatory activity can be assessed by monitoring the activity of enzymes involved in inflammation (notably phospholipase A2, cyclooxygenase, thrombin) or determining the level of gene expression of relevant genes [162]. The inhibitory effect of peptide(s) on the activity of commercially available enzymes has also been used, as described by Millan-Linares et al. [170].

A number of relevant animal models have been used to assess the effect of peptides on specific pathologies. Apolipoprotein E (ApoE) knockout mice, which rapidly develop atherosclerosis and display an altered lipid metabolism, represent an informative model for the inflammatory response. They have been used to test the immunogenicity of several bioactive peptides [173]. NC/Nga mice provide a model for dermatitis, since they readily develop symptoms when exposed to certain allergens; the response involves the activation of macrophages, mast cells and lymphocytes [174]. Spontaneously hypertensive rats [175] have also been used to test for inflammatory effect, while animal models for colitis are useful for monitoring inflammation in the intestine.

6. An in silico scan for immunomodulatory bioactive peptides in crop species proteomes

Previous attempts to identify bioactive peptides in plant proteins have been reported, for instance focused on the degradation products of cereal endosperm storage proteins [176]. Here, an emphasis has been placed on immunomodulatory peptides. The chosen starting point was a set of 97 entries likely and/or confirmed immunomodulatory peptides represented in the BIOPEP database (Table 1), supplemented by a further 30 sequences reported in recent literature. The length range of these peptides was 2 to 43 residues and their sequences are reported in Supplementary Table 2. The PeptideMatch algorithm (http://research.bioinformatics.udel.edu/peptidematch/index.jsp) was applied to all peptides composed of at least three residues across the UniProtKB database (release 2014_07); the number of proteins harboring potential immunomodulatory peptides was in excess of 56 million. Of these, > 1.6 million were derived from higher plants (Supplementary Table 2). A small number of the peptides proved to be plant-specific, namely GYPMYPLPR (oryzatensin), MITLAIPVN and MITLAIPVNKPGR (soymetide-9 and -13), GGRKQGQHQQEE (soybean glycinin), AEMIDLAAKMLSEGRG (rice RA1), PPYCTIVPFGIFGTNYR (wheat gliadin) and SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD (soybean lunasin). Restricting the scan to crop plants and setting the minimum peptide length to five residues limited the hits to 6055 (Supplementary Table 3). The most frequently represented food crop species were *Brassica* spp., soybean, barley, rice, bean, peach, pearl millet, tomato, potato, wheat, grapevine and maize. Most of the proteins containing potential immunomodulatory peptides were of unknown function.

In order to understand the main biological features of the food plants proteins containing potential immunomodulatory peptides we applied the Gene Ontology tool QuickGO (http://www.ebi.ac.uk/QuickGO/Gannotation [177]) to the whole dataset: it resulted in 9246 biological process assignations, 9748 molecular function assignations and 3506 cellular component assignations. To provide a global survey of the most represented categories, we applied ReviGo software [178] to generate a pictorial representation of the predominant GO biological processes featured in the set of 6055 proteins (Fig. 1), notably "metabolism", "protein phosphorylation" and "regulation of transcription": this analysis simply represents the most significant terms in the whole dataset, without calculation of enrichment factors. A detailed analysis of GO terms with PANTHER overrepresentation test (pantherdb.org, [179]) was possible only for those species where a significant number of hits could be classified and recognized: rice and grapevine (Supplementary Table 3). The enrichment of GO categories in the two data sets is represented graphically in Fig. 2 (A: 239 mapped rice proteins, B: 204 mapped grapevine proteins). The GO terms with high enrichment levels correspond to metabolic processes and transport activities for ions and lipids.

This analysis can lead to useful suggestions concerning the possible relevance of specific food products of plant origin in the delivery of naturally occurring immunomodulatory peptides. The data set we proved in Supplementary Table 3 will continue to provide useful information as more yet unknown proteins are identified, annotated and assigned to GO categories.

7. Conclusion

Food represents not just a source of calories, energy and the building blocks for cellular processes, but also a source of compounds important for promoting health and sustaining a range of physiological processes, a fact which was recognized by ancient physicians in both Europe and Asia. The presence of bioactive molecules provides a partial explanation for the health benefits of some fermented foods, which have developed in diverse human cultures. So-called "functional food" contains enhanced amounts of bioactive compounds which promote health and can help in both the prevention and treatment of some diseases [103]. Naturally occurring bioactive peptides are a particularly important constituent of functional foods; they exert effects on several aspects of human physiology. Their beneficial effects on immunity, inflammation, infection, hypertension, hypercholesterolemia, diabetes, some cancers and various neurological problems are of great interest in the developed world, where these diseases are becoming ever more prominent. Plant proteins represent a major source of naturally occurring bioactive peptides. Well known examples are soymetide (from soybean) and oryzatensin (from rice). In both cases, the bioactive peptide is a degradation product of an abundant protein present in the consumed part of the plant. The present bioinformatics approach has identified large numbers of proteins in food-producing plants from which immunomodulatory peptides could be generated (Supplementary Table 3).

Delivering bioactive peptides through food is an attractive alternative to administering synthetic drugs; in some cases, the peptide binds highly selectively to its target and generally the risk of toxic metabolites is low. At present, their exploitation for medicinal purposes is inhibited by a lack of knowledge regarding their mode of action. Models for quantitative structure–activity relationships may help to over-

come this lack [97]; they have already been well developed in the context of ACE inhibitory peptides. Using such models should aid in the design and production of synthetic peptides. A further range of problems to be overcome relate to the stability, delivery and bioavailability of bioactive peptides [126], but strategies to improve delivery, enhance membrane transport and increase the level of resistance against degradation are under development [2].

In many situations, hydrolysates are easier to obtain than purified peptides. The production of bioactive plant-based peptides from residues, waste and other by-products of food processing is an attractive proposition [130], since this would represent a low-cost and otherwise valueless feedstock. At present the commercialization of "nutraceuticals" or "pharmafood" is hindered by strict regulatory requirements surrounding health claims: in particular, obtaining clinical evidence for the beneficial effect of a functional food requires a large capital investment [6,8,180], while at the same time data obtained from in vitro based tests or from the use of animal models are not considered to be adequate for this purpose [121]. The absence of reliable experimental models and measurable endpoints remains a hindrance to acquiring usable indirect data [99].

Several features aspect relating to bioactive peptides present in functional foods still need to be elucidated. The extent of their bioavailability and the mechanism of their absorption, distribution, metabolism and excretion remain processes are largely unexplored in most cases. Similarly, whether their effect is exerted directly in the gut or only after their absorption and transport through the bloodstream is unclear. The contribution of gut microflora in amplifying or suppressing their effects has scarcely been explored. Many research perspectives surrounding bioactive peptides have yet to be realized.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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