Recent advances in microalgae peptides: analysis and health benefits

Yuchen Li, Carmen Lammi, Giovanna Boschin, Anna Arnoldi, Gilda Aiello

Department of Pharmaceutical Sciences, University of Milan, Milan, Italy

ABSTRACT

There is now a great interest for food protein hydrolysates and food-derived peptides, since they may provide numerous health benefits. Among other foodstuffs, microalgae appear as sustainable sources of proteins and bioactive peptides to be exploited in foods and functional formulations. This review considers either protein hydrolysates or individual peptides that may be relevant in cardiovascular disease prevention, because they mimic the function of mediators involved in pathologic processes that represent relevant risk factors for its development, such as hypercholesterolemia, hypertension, diabetes, inflammation, and oxidative status. Some of these peptides are also multifunctional, i.e. offer more than one benefit. Moreover, the most efficient techniques for protein extraction and hydrolyzation are commented as well as the best methodologies for high throughput detection and quantification are discussed. Finally, current challenges and critical issues are presented.

Keywords: microalgae, bioactive peptides, LC-MS, cardiovascular prevention

INTRODUCTION

In recent years, food peptides are gaining much interest for their potential health benefits. In fact, short and medium size peptides released from food protein hydrolysis may be absorbed and modulate specific metabolic pathways by binding or inhibiting targeted receptors, with a final positive impact on metabolic diseases.t This is very relevant since the possibility of preventing or influencing the development of pathological conditions by dietary management rather than by drug use is in line with the current propensity of many persons for a healthier lifestyle.[3] In addition, the toxic or adverse effects of bioactive peptides are generally very low or not-existing.[4] Therefore, the discovery of bioactive peptides and the exploration of their mechanism of action open the possibilities of developing innovative functional foods and nutraceuticals.

Until now, the most common sources of bioactive peptides include eggs, meat, fish, soybean, wheat, milk, and its derivatives.[5] However, more and more new food matrices are under investigation. In particular, microalgae have drawn the attention of different groups due to their easy growing and high protein contents.[6] These are a group of simple organisms usually with dimensions between 3 and 20 μm that are prevalently autotrophic, i.e. capable of converting CO_2 and minerals to biomass by photosynthesis.[7]

The human consumption of microalgae dates back to very ancient times. Some edible microalgae species, such as *Nostoc commune, Arthrospira platensis* (spirulina), and *Aphanizomenon flos-aquae* have been used as food for thousands years.[8, 9] Whereas in the 1960s and 1970s, the interest in microalgae had mainly the scope of providing protein supplements to meet the increasing demand of protein linked to the exponential growth of the world population,[10] nowadays numerous studies have proven that these organisms are promising unconventional foods, since they exert a good nutritive value, being rich in nutrients, such as proteins, polyunsaturated fatty acids, polysaccharides, vitamins, pigments, and microelements [11]. Due to commercial factors, market demand, specific preparations, and European food safety regulations, the most dominant species on the European market are currently spirulina and *Chlorella vulgaris*, whereas extracts of *Dunaliella salina*, *Haematococcus pluvialis*, or *Crypthecodinium cohnii* are commercialized for their high content of β-carotene, astaxanthin, or docosahexaenoic acid, respectively.[12]

This review is focused on microalgae peptides that mimic the function of mediators involved in pathologic processes responsible of vascular damage, highlighting the role played in the prevention of cardiovascular disease. Hypertension, dyslipidemia, and diabetes are the main risk factors of coronary artery disease together with endothelial dysfunction, atherosclerosis, and obesity, which are also strictly connected to oxidative stress and inflammatory processes. This review takes into consideration the preventive action of microalgae peptides against cardiovascular disease (CVD) as well as the current trends in microalgae peptide identification, highlighting some key issues, such as the heterogeneity of microalgae hydrolysates and the stochastic nature of current methods of analysis by mass spectrometry, as well as the novel trend in technologies for bioactive peptide discovery and quantification.

MICROALGAE AS A PROMISING SOURCE OF PROTEINS AND PEPTIDES

Protein content. In order to give an overview of protein levels in microalgae, **Table 1** collects the data of the three major nutrients in some microalgae species comparing them to some common foods. Although the microalgae protein content varies in the different species, it appears to be comparable

or even sometimes superior than that of conventional foods. For example, spirulina, a blue-green microalga, has a very high content of protein (46-71% on dry weight).[10] The industrial culture of this species was started in the 1960s and 1970s and its large-scale production and commercialization have been already accomplished. As reported by FAO the global production of spirulina in 2014 has achieved 86,000 tonnes.[10]

Interestingly, microalgae show a competitive amino acid pattern as shown in **Table 2.**[13, 14] In terms of the presence of indispensable amino acids (IAA), the content in microalgae generally matches well that in conventional foods. Based on the IAA content and the digestibility of each amino acid, digestible IAA score (DIAAS), the newest protein quality measure recommended by FAO[15] in 2011 has been calculated as shown in **Table 2.** This parameter depends on the lowest value of the digestible IAA reference ratio. For example, the DIAAS of *Chlorella sp.* is related to histidine, which has the lowest digestible reference ratio among all the IAA. Compared with high quality daily foods (i.e. egg, soybean), *Chlorella sp.* presents a higher DIAAS, while the other four microalgae species are inferior to different extents. One reason for this is the low content of one single amino acid, which limits the overall protein quality. This is the case, for example, of tryptophan in spirulina and *Scenedesmus obliquus*. Another reason lies in the rigid cell wall of most microalgae species, which is a key constraint factor to the bioavailability of nutrients. Encouragingly, with the help of suitable techniques in the downstream processing of microalgae, such as ball-milling and homogenization, the cell wall can be effectively disrupted, thus increasing the digestibility of microalgae nutrients as well as improving the protein quality.[16]

As microalgae display huge potential in nutritional and pharmacological applications, it is necessary to prove their safety by severe toxicity assessment. A report by the European Commission listed 23 microalgae genus or species used for food or feed consumption.[17] Among them spirulina, *Chlorella sp., Porphyridium cruentum*, *C. cohnii* have got the GRAS (generally recognized as safe) status by the US FDA (Food and Drug Administration). Others, such as *Chlamydomonas reinhardtii*, *H. pluvialis*, *Dunaliella sp*, *Navicula sp*., *Nitzschia dissipata*, *Phaeodactylum tricornutum* have been declared to have no toxic effects.[8] The fact that, under strict supervision, no toxicity or any other adverse effects have been found in any of the microalgal food products or animal feed, supports the exploration of microalgae in nutritional development.[16]

In addition, microalgae proteins possess unique advantage in health benefits and bioactivity. Phycobiliproteins are a group of brilliantly colored and highly fluorescent protein-pigment components of the photosynthetic light-harvesting antenna complexes, generally existing in cyanobacteria, red algae and some cryptomonads.[16] Commercially used as natural colorants in food and cosmetic industries as well as fluorescent markers in biomedical research and clinical diagnostics,

phycobiliproteins have drawn much attention for their therapeutic value, since various biological properties have been reported in literature. Phycocyanin, the most important member of the phycobiliproteins family, has been mostly studied. The phycocyanin-oral treatment of numerous animal models has revealed a broad spectrum of bioactivities, including the anti-inflammatory, anti-cancer, antimicrobial, and antioxidant activities, with a protective function of multiple organs and tissues, such as heart, lung, liver, kidney, pancreas, eyes, and brain.[18, 19] Currently, the active species are supposed to be some peptides released from phycocyanin hydrolysis and not the protein itself. In particular, 5 peptides, identified in a hydrolysate obtained by treating phycocyanin with pepsin in a simulated gastric fluid, have been proven to have significant antioxidant and metal-chelating activities and have shown cytotoxic effects on human cervical adenocarcinoma and epithelial colonic cancer cell lines.[20] It is important however to underline that, although available literature is very promising, many studies are still necessary for a complete exploration of the bioactivities of peptides from phycocyanin or phycobiliproteins and other microalgae proteins.

Biological activities of microalgae-derived peptides. For this review, literature information was collected by means of ScienceDirect database using the keywords "microalgae bioactive peptides". **Figure 1** indicates that a significant increase of the number of papers published annually has taken place in the period from 1995 to May 2019, with an increasing frequency in the last decade. The continuously rising research output suggests the enormous potential value of microalgae-derived bioactive peptides. On the basis of our personal experience, this review is focused on the effects that are useful in cardiovascular disease (CVD) prevention, i.e. the antioxidant, anti-inflammatory, anti-dyslipidemic, antidiabetic, and antihypertensive activities.

Antioxidant activity. Oxidative stress derives from the imbalance of homeostasis between oxidant and antioxidant species in cells, with an excessive production of reaction oxygen species (ROS) and free radicals, such as peroxyl radicals ($ROO^{\cdot}$), hydrogen peroxide (H_2O_2), nitric oxide radicals ($NO^{\cdot -}$), hydroxyl radicals ($HO^{\cdot}$) and superoxide radicals ($O_2^{\cdot -}$). Robust scientific evidence has shown that unlimited accumulation of ROS and free radicals leads to damage to all important cellular macromolecules, such as proteins, DNA, and membrane lipids, which contributes to various pathologies including cardiovascular diseases, diabetes mellitus, inflammation, liver injury, and so on.[21-23]

In order to reduce the oxidation, many researches are currently dedicated to discover new potent antioxidant agents. Particularly, microalgae-derived peptides have received much attention as they give a good antioxidant performance, since they are endowed of interesting free radicals scavenging

activities.[24-28] This provides a reliable foundation for a further isolation of single bioactive peptide and investigation of their mechanism of action.

Table 3 lists the antioxidant peptides identified up-to-now in microalgae hydrolysates. The peptide VECYGPNRPQF from *C. vulgaris*, PGWNQWFL and VEVLPPAEL from *N. incerta*, as well as LNGDVW from *C. ellipsoidea* are known from some time and mentioned in several reviews as they exert potent free radicals scavenging ability.[3] However, numerous investigations on the microalgae species are ongoing and new antioxidant peptides are continuously discovered. A tetrapeptide MGRY, isolated from *Pavlova lutheri* by yeast fermentation, exhibits multiple ROS scavenging action, decreases the expression of melanogenesis-related proteins, and suppresses the melanin synthesis, when acting on the melanoma cells damaged by oxidative stress.[29] Using protease K to digest the protein extracts from spirulina, a unique peptide (PNN) has been identified that shows scavenging effects on free radicals (DPPH, superoxide anion, and hydroxyl radicals).[30] From another species named *Tetradesmus obliquus*, after alcalase digestion, twenty-five peptides have been identified by mass spectrometry and predicted as potential bioactive agents by *in silico* investigations. Two peptide sequences WPRGYFL and SDWDRF have been proven to give the best scavenging effect on ABTS and DPPH, respectively.[31] The structural features of the above-mentioned peptides are in line with the characteristics observed for other peptides identified in several food sources. Low molecular weight (＜1000 Da) and abundance of hydrophobic amino acids (His, Trp, Phe, Pro, Gly, Lys, Ile and Val) are the characteristics supposed to enhance the antioxidant capability of peptides because they may lead to a favorable hydrophobic micro-environment. The imidazole, indole, or pyrrolidine ring in His, Trp and Pro may serve as important proton and hydrogen donors to react with ROS and free radicals. The amino acid sequence is crucially important, involving the interaction of amino acids, the electrostatic and hydrogen-bonding properties, the location of amino acids and the steric properties of the residues at the C- and N-termini. Other minor factors, which influence the bioactivity, are the secondary structure, the stability, and their synergistic effects. In general, all the above characteristics contribute to the overall antioxidant activity of peptides.

Microalgae peptides with therapeutic effects on inflammation and related pathology. Inflammation occurs in various diseases, triggered by stimuli, such as pathogen-derived molecules, products of damaged cells, toxins, or allergens, which result in the release of inflammatory mediators, i.e. cytokines of the interleukin (IL) families, tumor necrosis factor alpha (TNF-α), prostaglandins (PG), nitric oxide (NO), and leukotrienes (LTs).[32] Up to now, the benefits of microalgae peptides in counteracting the inflammatory response and related diseases are well documented (**Table 4**). An undecapeptide with the sequence VECYGPNRPQF, isolated from *C. vulgaris* protein waste, was

reported to effectively inhibit the production of lipopolysaccharide (LPS)-induced nitric oxide (NO) *in vitro* with an IC_{50} value of 42.4 μM, exhibiting potential anti-inflammatory activity, since NO acts as the product of inflammatory macrophage.[33] Further study showed that this peptide could reduce the expression of LPS-induced inducible nitric oxide synthase (iNOS) mRNA, iNOS and nuclear factor kappa B (NF-κB) proteins and the production of two other inflammatory mediators: TNF-α and prostaglandins E2(PGE2), again inhibited by these peptides. Two peptides, P1 (LDAVNR) and P2 (MMLDF), have been purified from the peptic hydrolysates of spirulina proteins. *In vitro* experiments have shown that both peptides fight against the histamine-induced inflammation in endothelial cells by inhibiting the ROS production and interleukin (IL)-8 expression, two major products of the inflammatory process.[34] Since endothelial inflammation is involved in the atherosclerotic lesion formation, P1 and P2 have been also supposed to have anti-atherosclerotic activity. Further studies have supported this hypothesis. In addition, to alleviate the endothelial inflammation, they inhibit the production and expression of IL-6 and monocyte chemoattractant protein-1 (MCP-1) as well as the production of adhesion molecules, including P-selectin and E-selectin. All the above effects are beneficial to prevent atherosclerosis.[35]

A recent study has revealed the therapeutic effects of microalgae peptides on bowel inflammation. A *Schizochytrium sp.* meal hydrolysate, in which 172 peptide sequences have been detected, has been reported to significantly reduce the mucosal barrier damage and to prevent colonic inflammation in the colitis mice model by increasing the expression of cytokine IL-10 and to promote cell proliferation for damage repairing.[36] Notably, there are enough research evidences based on animal experiments that oral phycocyanin fights against the inflammatory process in different compartments, thus relieving various diseases including hepatitis, arthritis, colitis and brain injury.[19, 37-39] As phycocyanin is hydrolyzed in the digestive tract, it seems reasonable to suppose that this comprehensive anti-inflammatory effect is due to the peptides and amino acids contained in the digests. However, very little is known about the bioactivity of peptides deriving from phycocyanin, an issue that is certainly worth of more attention.

Microalgae peptides ameliorating dyslipidemia. Dyslipidemia takes place with an increasing frequency correlated to the modern unhealthy lifestyle, in particular owing to a high-calorie diet and lack of exercise. Numerous studies indicate that dyslipidemia is undoubtedly the major risk factor for the development of atherosclerosis and cardiovascular disease, with its distinctive characteristics including high levels of triglyceride (TG), total cholesterol (TC), low-density-lipoprotein cholesterol (LDL-C), and reduction of high-density-lipoprotein cholesterol (HDL-C).[40] Over the years, considerable research has been devoted to the discovering of compounds and drugs for lipid

metabolism regulation and microalgae peptides have been also taken into account as highlighted in **Table 5**.

In a recent report, in a spirulina protein hydrolysate produced by protamex, 217 peptides have been identified. Working on high-fat diet fed rats, this hydrolysate has significantly reduced the levels of serum and liver lipids, including TG, TC, and LDL-C, as well as alanine transaminase (ALT) and aspartate transaminase (AST), by decreasing the expressions of several genes related to fatty acid transport and lipid metabolism. All these effects represent multiple potential benefits on lipid metabolic disorder (LMD), i.e. they have anti-obesity, hypolipidemic, as well as hepatoprotective properties [41].

Phycocyanin, being the most abundant protein in spirulina, has shown enormous potential to release peptides against LMD in numerous studies. By treating rats with a spirulina concentrate (SPC) and phycocyanin, respectively, with dosage of 3% protein level, it has been found that phycocyanin contributes in a significant way to the observed hypocholesterolemic effects.[42] In hamsters, it decreases serum TC, TG, LDL-C, glutamate-oxaloacetate transaminase (GOT), and glutamate-pyruvate transaminase (GPT), and also enhances the protein expression of several antioxidant enzymes, exerting promising ability either against hyperlipidemia or oxidation.[43] It is useful to observe that, although these studies suggest potential dyslipidemia-regulating effects of microalgae peptides, only a few microalgae species have been investigated in this area and that no information is available on the mechanism of action. Thus, more efforts would be necessary to exploit microalgae peptides as effective dyslipidemia-regulating agents.

Antidiabetic activity. Diabetes is a chronic disease with the feature of high glucose level in the circulatory system. Diabetes mellitus and its complications, such as cardiovascular disease, nephropathy, retinopathy, amputation, and nerve damage, are one of the major causes of mortality, accounting for 14.5% of global mortality among aged 20-79 years.[44]

Several bioactive peptides have been discovered with potential antidiabetic effects and microalgae peptides have provided some encouraging results (**Table 6)**. After in vivo gastrointestinal digestion, phycocyanin shows notable preventive effects on the development diabetes through multiple action mechanisms. In particular, it promotes the normalization of glucose and lipid metabolism in the alloxan-induced diabetic mice by activating the insulin signaling pathway and glucokinase (GK) expression in pancreas and liver.[45, 46] This kind of animal model is featured by the destruction of pancreatic beta cell, which is the main cause of type 1 diabetes. Another research, instead, has revealed that digested phycocyanin has therapeutic effects on mice with type 2 diabetes, manifesting as insulin resistance and decreased insulin sensitivity. The administration of phycocyanin has

237 significantly ameliorated the glucolipid metabolism indexes, improving peripheral target tissues to
238 respond to insulin regulation.[47] In addition, the oral administration of phycocyanin has protected mice
239 with type 2 diabetes against diabetic nephropathy. Possibly, this has taken place via the antioxidant
240 effects of phycocyanin on urinary and renal oxidative stress markers favoring the normalization of
241 NAD(P)H expression after the treatment.[48]
242 The reduction of the activity of peptidyl-peptidase IV (DPP-IV, EC 3.4.14.5), a new therapeutic target
243 towards type 2 diabetes, makes it possible to investigate the antidiabetic activity of the bioactive
244 compounds *in vitro*. DPP-IV can degrade incretins including glucagon-like peptide-1 (GLP-1) and
245 glucose inhibitory polypeptide (GIP), resulting in the loss of their ability to enhance insulin
246 secretion.[49] Hence, the inhibition of DPP-IV feasibly improves the insulin activity. It is reported that
247 protein hydrolysates from *Porphyridium purpureum* and *P. tricornutum* exhibit *in vitro* an anti-
248 diabetic activity by inhibiting the effect of DPP-IV, with IC_{50} values of 2.28 and 2.68 mg/mL,
249 respectively.[50] Six tripeptides from *C. vulgaris* have been identified by in silico gastrointestinal
250 digestion as potential inhibitors of DPP-IV activities and then synthesized. Out of them two peptides,
251 i.e. VPW and IPR, showed the highest DPP-IV inhibitory activity either *in vitro* or in mice serum. In
252 addition, these two peptides have been confirmed to be resistant to gastrointestinal digestion and to
253 enter the circulation in order to exert their activity. Interestingly, these peptides have a Pro or Ala
254 residue as the penultimate N-terminal sequence and a hydrophobic amino acid as the N-terminal
255 amino acids. The structure-activity relationship of DPP-IV inhibitory peptides is based on numerous
256 studies: a hydrophobic or aromatic amino acid of N-terminus facilitates the binding of the peptides to
257 DPP-IV; whereas a Pro and Ala residue at the second N-terminal site contributes to the DPP-IV
258 inhibition. DPP-IV is in fact a post proline-cleaving enzyme that especially removes Xaa-Pro or Xaa-
259 Ala (Xaa being an amino acid residue) dipeptides from the N-terminus of polypeptides.[51]
260
261 **Antihypertensive activity.** Hypertension is another major risk factor in the development of CVD.
262 Many serious diseases, such as chronic kidney failure, stroke, coronary events, and heart failure, are
263 caused by persistent hypertension.[52] Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) is a main
264 therapeutic target for the development of antihypertensive drugs, since the inhibition of ACE leads to
265 the decrease of vasoconstrictor angiotensin II and the increase of vasodilator bradykinin, thus
266 resulting in an antihypertensive effect.[53] Interacting with the ACE structure, food-derived peptides
267 have been verified to exert huge potential of fighting against hypertension and more and more active
268 peptides are continuously discovered from various protein sources including microalgae. **Table 7** lists
269 microalgae ACE-inhibitory peptides. A recent review summarizes the major findings about
270 microalgae peptides with antihypertensive activity.[54] Fourteen sequences derived from *C. vulgaris,*

C. ellipsoidea, spirulina, and *Nannochloropsis aculata*, have been listed, among which the peptide IAPG derived from spirulina gave the most potent ACE-inhibitory effect with an IC_{50} value of 11.4 μM. Two more efficient ACE-inhibiting peptides, GPDRPKFLGPF and WYGPDRPKFL, have been identified in an alcalase hydrolysate of *T. obliquus* proteins with IC_{50} values of 5.73 and 0.82 μM, respectively.[31] In another study, the residues of *Chlorella sorokiniana* after hot water extraction have been hydrolyzed by Protease-N followed by gastrointestinal enzymatic hydrolysis. After fractionation, the most active fraction has been identified to include three ACE-inhibitory di-peptides VW, IW, LW, with IC_{50} values of 0.58, 0.50, 1.11 μM, respectively. Since dipeptides may derive from numerous different proteins, these hypotensive dipeptides have been also identified from other protein sources, such as ovalbumin and salmon.[55]

Two tripeptides TTW and VHW from *C. vulgaris* have been reported to be non-competitive ACE inhibitors *in vitro* with IC_{50} value of 0.61 and 0.91 μM, respectively, and to achieve considerable blood pressure decrease when administered to SHRs at a relatively low dosage.[56] These two peptides have been sorted out from 4334 peptides produced by virtual gastrointestinal digestion of *C. vulgaris* proteins. The screening criteria has been based on the in-silico prediction of ACE-inhibiting IC_{50} values as well as on what is known of the structure / activity relationship in case of ACE inhibitors: in fact, it is known that peptides with an aromatic or cyclic amino acid, i.e. Trp, Tyr, and Pro, as their C-terminal amino acid are very likely to be highly active. In addition, two ACE-inhibitory peptides, IQP and VEP, deriving from spirulina, have been identified with IC_{50} values of 5.77 and 27.36 μM.[53]

Multifunctional peptides from microalgae. Some microalgae peptides exhibit more than one biological activity and thus may be classified as multifunctional peptides, a new definition indicating those peptides that can favorably modulate more than one physiological process by affecting different targets.[57] The multifunctionality of some peptides is closely related to the multiple factors of some illnesses and the common factors at the basis of different diseases.

The most typical example is the undecapeptide VECYGPNRPQF, derived from *C. vulgaris*, that show multiple effects, including antihypertensive, antioxidant, anti-inflammatory, anti-atherosclerotic as well as anticancer activities. It fights against inflammation and oxidant stress in endothelial cells and inhibits the production of histamine, intracellular ROS and adhesion molecules, thus showing potential benefits against atherosclerosis.[33, 58] In addition, due to the prevention of oxidation-induced cell damage and inflammation in macrophages, this peptide effectively suppresses cell proliferation.[33] Similarly, two peptides, LDAVNR and MMLDF, released from spirulina, have been recognized as multifunctional peptides with antioxidant, anti-inflammatory, anti-atherosclerotic as well as anti-allergenic activity.[35] The spirulina heptapeptide TMEPGKP inhibits the activity of

ACE and is therefore hypotensive and in the meanwhile antioxidant thus improving the vascular dysfunction, again hindering the development of hypertension.

Microalgae protein hydrolysates also provide different health benefits. In particular, digested phycocyanin is very interesting, since it is antioxidant, anti-inflammatory, antihyperlipidemic, and hypocholesterolemic *in vivo*. However, since a hydrolysate has a very complex composition, it is impossible to attribute the observed effects to specific peptides, what greatly limits the research of multifunctional peptides in microalgae. This is a pity, considering the advantages of multifunctional peptides compared to monofunctional peptides.[57]

PEPTIDOMICS IN THE CHARACTERIZATION OF MICROALGAE

It is nowadays widely consolidated that MS-proteomic and peptidomic technologies are the gold standard for peptide analysis. Peptidomics has become an important tool for the characterization of bioactive peptides form food sources. The peptidomic strategy normally includes the protein extraction, the release of peptides by single or multiple enzymes, i.e. gastrointestinal, trypsin, pepsin or microbial enzymes, the subsequent separation and identification of peptides, and the biological activity measurement. This strategy allows the identification and quantification of bioactive peptides even in a very complex mixture, such as protein hydrolysates, using complementary and mutually compatible approaches ranging from protein extraction to peptide analysis. Some important aspects to consider in a driven-bioactive peptide discovery from microalgae are reported below.

Microalgae protein extraction: technological aspects. The protein extraction from microalgae is one of the main limiting steps for the production of bioactive peptides either in small or large scales.[10] Marine algae cell walls, generally consisting of polysaccharides, such as cellulose and xylan, and sometimes including sulfated polysaccharides, phenol compounds, glycoproteins and proteoglycans, are recalcitrant tissues that offer robustness and resilience to disruption.[3, 59] The algal cell walls with a high cellulose content (*C. vulgaris*) exhibit lower digestibility than those with a thinner, easier to digest cell wall (spirulina).[8]

Furthermore, the proteins entrapped inside the cell walls and cell organelles cannot exhibit their full functional potential, if not adequately extracted. For this reason, it is crucially important to select for the best the cell disruption method, depending on the cell wall structure, product location, size, and solubility. Several extraction methods have been proposed in literature for the extraction of soluble proteins from microalgae. These methods can be divided into physical and chemical. Physical ones involve mechanical shear (bead milling, high-pressure homogenization, ultrasonication, explosive decompression, microfluidization), electric fields, and thermal treatments (thermal shock,

microwaves).[8] Chemical and biochemical methods include, solvents, ionic liquids, pH shifts, and enzymatic hydrolysis.[60, 61]

Very recent, a green and innovative technology based on the modulation of both pressure and temperature coupled with ultrasound intensity, has been applied for improving the efficiency of the well-established ultrasound methodology. The combination of these three methods, called the manothermosonication (MTS), appears as a new very efficient extraction technique. Compared to conventional ultrasonic extraction techniques, it has allowed a protein recovery rate of 50% in 6 min with a continuous MTS process.[62] These findings demonstrate how the effect of pressure and temperature combined with ultrasound leads to a better cell disruption and increase mass transfer phenomena (increase of effective diffusivity) compared to ultrasound alone.[62]

Since the new trend is in favor of combined techniques, a new microwave-assisted multiphase partitioning technique (MWTPP) has been recently developed for the extraction and purification of proteins from *C. vulgaris.*[63] With microwave irradiation assistance, a yield enhancement by up to 2.54 times has been reported. However, although several technological advances have been applied to microalgae protein extraction, some issues need further attention. The development of protein-compatible process technologies for solvent extraction of high-value products from wet biomass and the development of scalable and cost-effective purification methods for the production of bioactive peptides are the two most important driving-factors for improving existing techniques. In this contest, to support industrial eco-sustainability, the enzyme-assisted extraction (EAE) has recently received more attention in the extraction of proteins form microalgae. The treatment with food-grade enzyme, such as cellulase, α-amylase, and pepsin, generally performed under mild conditions, makes the EAE an ecologically friendly, non-hazardous, and low energy alternative to mechanical and chemical techniques.[64] In some instances, the enzymatic disruption may result in a more efficient protein extraction compared to mechanical and chemical cell lysis.[65] For example, the *C. vulgaris* protoplasts have been generated using a combination of 4% onozuca, 2% macerozyme, and 1% pectinase enzymes[66] and gametolysin, an autolytic metalloprotease produced by *C. reinhardtii*, have been used to achieve cell permeabilization and up to 50% protein release.[67]

Multidisciplinary approaches for bioactive peptide discovery. Microalgae derived-peptides are routinely produced by the use of various commercial proteases, i.e. papain, trypsin, pepsin and α-chymotrypsin; the latter used for releasing antioxidant peptides from marine *Chlorella ellipsoidea*[68] or by single enzyme, i.e. pepsin, to liberate new antioxidative peptides, such as VECYGPNRPQF, from algae protein waste.[69] Food-grade enzyme such as Alcalase and Flavourzyme have been selected to hydrolyze the red microalga *P. purpureum* and the diatom *P. tricornutum* endowed of anti-diabetic

and antioxidant properties.[70] In addition, microalgae derived peptides can be produced by in situ microbial fermentation of the parental proteins. Bacterial proteinases such as subtilisin-A have been also used for releasing peptides from *Arthrospira maxima* biomass cultivated on sugarcane vinasse at laboratory and pilot scale.[71]

Nevertheless, microalgae protein hydrolysates are very complex and generally contain hundreds of peptides of different length and relative abundance, making a comprehensive detection a challenge. To overcome this problem, until now the commonly used approach is based on various purification steps (ultrafiltration, preparative HPLC on different phases, etc.) for obtaining more and more purified fractions, whose retained activity must be confirmed after each single stage (**Figure 2, Scheme A)**. However, the purification of the peptide fractions from the hydrolysates is an expensive and complex process. The isolation of a single peptide becomes an excessively difficult task, especially when a relatively large amount is required. Nevertheless, the use of these methodologies is widespread in the microalgae bioactive peptide discovery. The heptameric peptide, TMEPGKP endowed with an ACE inhibitory activity, has been obtained from a spirulina gastrointestinal hydrolysate after multiple purification steps, i.e. HiPrep DEAE FF ion-exchange column using fast liquid chromatography, followed by further purification on a PrimeSphere ODS C18 column permeation reversed-phase high-performance liquid chromatography (RP-HPLC).[72]

Likewise, IVVE (an ACE activity inhibitor with an IC_{50} of 315.3 μM), AFL (IC_{50} 63.8 μM), FAL (IC_{50} 26.3 μM), AEL (IC_{50} 57.1 μM), and VVPPA (IC_{50} 79.5 μM) from *C. vulgaris*; IAE (IC_{50} 34.7 μM), FAL, AEL, IAPG (IC_{50} 11.4 μM), and VAF (IC_{50} 35.8 μM) from spirulina have been obtained by ion exchange chromatography and gel filtration. Gel filtration chromatography and two step RP-HPLC has been employed to purify peptide IQP from an alcalase digest of spirulina. Ultrafiltration using a membrane with 10 kDa molecular weight cut off (MWCO) has been instead used for the purification of three peptide fractions from microalgae biomass with antioxidant, antimicrobial, anti-inflammatory, and/or anti-collagenase activities. A novel antioxidant peptide PNN has been obtained by ultrafiltration, gel filtration chromatography, and reverse-phase high-performance liquid chromatography.[71] However, the fractionation often does not lead to the desired results: the fractionated peptides may lose or reduce their activities, due to the lack of synergism.

The important issue of bioavailability, has suggested a new approach to bioactive peptide discovery based on Caco-2 monolayers that are used as a "natural sieve of bioavailable species".[57] In fact, the absorption as intact species across the intestinal epithelium is a main prerequisite in order to exert their bioactivity *in vivo*. The Caco-2 monolayer represents a well-established model of the intestinal epithelium to study trans-epithelial transport of nutrients, drugs, phytochemicals, and peptides, because of their ability of expressing morphologic characteristics of normal enterocytes, such as

exhibiting spontaneous enterocyte-like differentiation with morphological polarity and expressing brush-border peptidases when grown on trans-well polycarbonate membranes.[73] After absorption experiments, further investigations are focused only on absorbable peptides that are generally only a limited fraction of the parent hydrolysate. This model not only simplifies the high complexity of the hydrolytic mixtures, but also allows to obtain valid functional information about their ability to cross the epithelial barrier (**Figure 2, scheme B**).

This model has been applied for evaluating the absorption of several food-derived peptides, such as those deriving from lupin,[74] soy,[75] and dry-cured ham.[76] Up to now, instead, only one study reports the mechanisms of the trans-epithelial transport of two ACE-inhibitory peptides from spirulina, i.e. IQP and VEP.[77] Both peptides have been found to be transported intact, an evidence that underlines their resistance to hydrolysis by peptidases and makes them potentially effective as antihypertensive agents *in vivo*. This behavior is in agreement with experimental evidences according to which peptides containing proline residues are generally resistant to the degradation by peptidases.[78] For example, QIGLF, TNGIIR, RVPSL, RADHP, YAEER, LKP, IQW, and YPI, some antihypertensive egg-derived peptides, are resistant to gastrointestinal digestion, whereas FRADHPFL and YAEERYPIL are completely hydrolyzed.[50, 79]

New frontiers in the discovery and quantification of bioactive peptides by mass spectrometry.

The difficulty of isolating specific peptides from the hydrolysates arises from the fact that hundreds of peptides may be present in a given hydrolysate. Because some peptides may have very similar physicochemical properties (mass, hydrophobicity, charge, solubility, etc.), their separation and purification may result very difficult.[80] Recently, with the increased accuracy (high resolution) of MS analyzers and the development of various bioinformatic tools, the identification of peptides has become less challenging and numerous peptide sequences can be detected simultaneously.[81,82-84] The tendency to avoid long and expensive fractionations prior to MS characterization has led to the use of unfractionated hydrolysates, which are routinely characterized by LC-MS/MS, allowing the assessment of gross peptide composition. In details, the most common approach used for peptide sequence identification is called shotgun proteomics based on data-dependent acquisition (DDA). Basically, the peptide identification is achieved by comparing MS/MS spectra derived from peptide fragmentation with theoretical tandem mass spectra generated from in silico digestion of a protein database. This type of data acquisition has been adopted for almost all peptide sequences reported in this review.

Although the DDA approach allows exploratory analysis, it suffers of one limitation: its sensitivity is strongly sample-dependent. Moreover, the biased intrinsic nature of the DDA approach may cause

inconsistent run-to-run reproducibility in peptide identification especially when complex samples, such as food hydrolysates, are analyzed. The selection of the ions to fragment is "dependent" upon some criteria previously set-up in the analytical method and is usually sorted based on the abundance. Nevertheless, the peptide abundance in hydrolysates covers very huge orders of magnitude, which makes it difficult a proper investigation only with the shotgun approach. Most intense peptides deriving from very abundant proteins are usually very well characterized, whereas missing values are often observed for peptides deriving from less abundant proteins.

To overcome these limitations, a relatively new approach called data-independent acquisition (DIA) has been developed.[85] DIA fragments every single peptide in a sample.[86] The fragmentation is "independent" of any ion characteristics (such as the abundance). Because the entire precursor mass range is fragmented, no gaps in the data take place, and run-to-run reproducibility is extremely high. Therefore, the unbiased nature of data independent acquisition makes it the best technique for discovery proteomics. This new methodology has been recently applied as an alternative way for shotgun proteomics to identify proteins of *C. vulgaris* in order to select the best extraction protocols.[87] The ability to comprehensively identify peptides in very complex matrices over a large dynamic range and in an extremely reproducible mode opens up a world of application possibilities to bioactive peptide analysis by DIA.

Since beside structure elucidation, the interest is to get information about the abundance of such bioactive peptides, MS-quantitative approaches including absolute or relative quantification of peptides by using labeling or label-free methodologies are currently adopted. Even if labeled methods provide the most accurate quantitative values, they require complex experimental set-up and expensive isotope labels. On the contrary, label-free methodologies allow an easy, reliable, versatile, and cost-effective quantification. Peak intensity measurements or spectral counting are the most employed techniques for label free quantification allowing a precise and accurate evaluation of changes in abundance between samples.[88, 89]

Beside to relative quantification, the absolute one based on Multiple Reaction Monitoring (MRM) is actually the most frequent strategy used to quantify the total levels of a given peptide in a sample, within a wide linear range, either by using label or label-free approaches. In MRM experiments multiple predefined pairs of precursor and product ions, known as MRM transitions, are used in combination with defined retention times to detect and quantify peptides.

Label-free targeted MRM has been widely adopted to quantify food derived peptides, i.e. for quantifying the soy peptides IAVPGEVA, LPYP, and IVAPTGVA absorbed through Caco-2 monolayers and to study their metabolic degradation by endopeptidases.[75] The absolute quantification using MRM has been also applied to bioactive ACE-inhibitory tripeptides extracted from rye malt

sourdoughs, showing the highest concentrations in gluten sourdoughs fermented with *Lactobacillus reuteri,*[90] as well as during the bread-making process.[91] This methodology has also been used to determine the intact absorption of the ACE-inhibitory dipeptide VY into the blood of spontaneously hypertensive rat (SHR) after administration and to detect the maximal absorption amount.[92] Despite its widespread use, to the best of our knowledge, no microalgal peptides have been quantified by a targeted approach so far. However, even if MRM is considered as the golden standard for peptide quantification largely because of its excellent reproducibility and quantitative accuracy of proteins and peptides spanning over 5 orders of magnitude,[93] it remains limited in the total number of targeting peptides. In addition, the development of MRM assay requires the prior knowledge of peptide sequence to be quantified, a condition that rarely occurs in the field of food peptidomics.

To overcome these limitations, the new MS data-independent acquisition, coupled with peptide spectral library match is able to provide label-free quantification in a MRM-like manner, showing higher quantification accuracy and precision.[94] In details, by reference to prerecorded spectral libraries, targeted data extraction can be undertaken at both the MS (precursor ion) and MS/MS (product ion) levels providing quantitative abilities similar to MRM analysis.[85] Similar approach has been applied for quantifying proteins from green algae *C. vulgaris* extracted using different methods, i.e. direct lysis buffer method, TCA-acetone method, phenol method, and phenol/TCA-acetone method. This strategy has been also efficiently applied for the relative quantification of barley gluten in selectively bred barley lines.[95] These findings highlight the clear benefit of data-independent analysis in the ability to use non-tryptic peptide fragments identified in the discovery experiments to examine the relative levels of C-hordeins, a family of trypsin-resistant gluten proteins, without the need for an alternative proteolytic strategy.[95] Since many hydrolysates are produced by the use of different food grade enzymes, such as alcalase, protamex, flavorzyme, characterized by low cutting specificity, data-independent acquisition would be considerably beneficial in the analysis of biopeptides from microalgae. Definitively, bridging the gap between discovery and targeted proteomics, this new method is very promising in food technology and nutrition, because of its ability to allow the identification and quantification of low abundance peptides.

FINAL REMARKS

In conclusion, there is evidence that peptides from microalgae may potentially provide diverse health benefits, particularly in the area of cardiovascular disease prevention. However, there are some weaknesses to overcome for their practical application. In fact, most information comes from in vitro tests, whereas in vivo and clinical studies are very scarce. In addition, more research is needed to assess their bioavailability in vivo, their stability and the metabolic fate they undergo in human body

during digestion, transport, and absorption, as well as the final available concentrations. From the point of view of the analytical methods to assess their concentrations in the starting materials and in vivo, there are also some critical issues that should be addressed in a proper way. Most methods are in fact only qualitative, being focused on the identification of peptide sequences, whereas the problem of the quantification in different matrices is still an open question. Recent improvements in mass spectrometers and novel MS techniques are actually permitting a significant advance in this direction. The use of new performant analytical strategies, such as the new data-independent MS aimed at the simultaneous discovery and quantification of microalgae peptides, represent a powerful tool for subsequent structure-activity studies. In this contest, the newest tendency of analysis will support the further investigation of the peptide structural scaffolds, their functions and mechanisms of action in cardiovascular disease prevention supporting the discovery and development of novel bioactive peptides from microalgae.

AUTHOR INFORMATION

Corresponding Author

Gilda Aiello, Department of Pharmaceutical Sciences, University of Milan, via Mangiagalli 25, 20133 Milan, Italy. E-Mail: gilda.aiello@unimi.it, tel.: +390250319293

Ethics statement

This review compares literature data: no experiments have been performed for its preparation.

Conflict of interest

We declare no conflict of interest.

ACKNOWLEDGEMENT

This work was supported in part by financial support of a fellowship from the China Scholarship Council (to allow Y.L. to conduct the research at the University of Milan), in part by Cariplo Foundation, project “SUPER-HEMP: Sustainable Process for Enhanced Recovery of Hempseed Oil”, contract n. 2017-1005, in part by project “COMPETiTiVE: Claims of Olive oil to iMProvE The market ValuE of the product” financed by AGER.

ABBREVIATIONS:

ABTS, 2，2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid); ACE, angiotensin I-converting enzyme; ALT, alanine transaminase; AST, aspartate transaminase; CVD, cardiovascular diseases;

DDA, data-dependent acquisition; DIA, data-independent acquisition; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DPP-IV, peptidyl-peptidase IV; EAE, enzyme-assisted extraction; GIP, glucose inhibitory polypeptide; GLP-1, glucagon-like peptide-1; GK, glucokinase; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; GRAS, generally recognized as safe; HDL-C, high-density-lipoprotein cholesterol; IL, interleukin; iNOS, inducible nitric oxide synthase; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; LDL-C, low-density-lipoprotein cholesterol; LMD, lipid metabolic disorder; LTs, leukotrienes; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MRM, multiple reaction monitoring, MWCO, molecular weight cut off; MWTPP, microwave-assisted multiphase partitioning technique; MTS, manothermosonication; NAD(P)H, nicotinamide adenine dinucleotide (phosphate) hydrogen; NF-κB nuclear factor kappa B; NO, nitric oxide; PG, prostaglandins; PGE2, prostaglandins E2; ROS, reaction oxygen species; RP-HPLC, reversed-phase high-performance liquid chromatography; SPC, spirulina concentrate; TC, total cholesterol; TG, triglyceride; TNF-α, tumor necrosis factor alpha.

REFERENCES

1. Sultan, S.; Huma, N.; Butt, M. S.; Aleem, M.; Abbas, M., Therapeutic potential of dairy bioactive peptides: A contemporary perspective. *Crit. Rev. Food Sci. Nutr.* **2018,** *58* (1), 105-115.
2. de Castro, R. J. S.; Sato, H. H., Biologically active peptides: Processes for their generation, purification and identification and applications as natural additives in the food and pharmaceutical industries. *Food Res Int* **2015,** *74*, 185-198.
3. Fan, X. D.; Bai, L.; Zhu, L.; Yang, L.; Zhang, X. W., Marine Algae-Derived Bioactive Peptides for Human Nutrition and Health. *J. Agric and Food Chem* **2014,** *62* (38), 9211-9222.
4. Li-Chan, E. C. Y., Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. *Curr Opin Food Sci* **2015,** *1*, 28-37.
5. Nongonierma, A. B.; FitzGerald, R. J., Enhancing bioactive peptide release and identification using targeted enzymatic hydrolysis of milk proteins. *Anal Bioanal Chem* **2018,** *410* (15), 3407-3423.
6. Bleakley, S.; Hayes, M., Algal Proteins: Extraction, Application, and Challenges Concerning Production. *Foods* **2017,** *6* (5).
7. Odjadjare, E. C.; Mutanda, T.; Olaniran, A. O., Potential biotechnological application of microalgae: a critical review. *Crit Rev Biotechnol* **2017,** *37* (1), 37-52.
8. Soto-Sierra, L.; Stoykova, P.; Nikolov, Z. L., Extraction and fractionation of microalgae-based protein products. *Algal Res* **2018,** *36*, 175-192.
9. Villarruel-Lopez, A.; Ascencio, F.; Nuno, K., Microalgae, a Potential Natural Functional Food Source - a Review. *Pol J. Food Nutr Sci* **2017,** *67* (4), 251-263.
10. Lupatini, A. L.; Colla, L. M.; Canan, C.; Colla, E., Potential application of microalga Spirulina platensis as a protein source. *J Sci Food Agric* **2017,** *97* (3), 724-732.
11. Matos, J.; Cardoso, C.; Bandarra, N. M.; Afonso, C., Microalgae as healthy ingredients for functional food: a review. *Food Funct* **2017,** *8* (8), 2672-2685.
12. Chacon-Lee, T. L.; Gonzalez-Marino, G. E., Microalgae for "Healthy" Foods-Possibilities and

585 Challenges. *Compr Rev Food Sci* **2010,** *9* (6), 655-675.
586 13. Becker, E. W., Micro-algae as a source of protein. *Biotechnol Adv* **2007,** *25* (2), 207-210.
587 14. Sui, Y. X.; Muys, M.; Vermeir, P.; D'Adamo, S.; Vlaeminck, S. E., Light regime and growth
588 phase affect the microalgal production of protein quantity and quality with Dunaliella salina.
589 *Bioresour Technol* **2019,** *275*, 145-152.
590 15. FAO, Dietary protein quality evaluation in human nutrition. Report of an FAO Expert
591 Consultation, **2011.**
592 16. Neumann, U.; Derwenskus, F.; Gille, A.; Louis, S.; Schmid-Staiger, U.; Briviba, K.; Bischoff, S.
593 C., Bioavailability and Safety of Nutrients from the Microalgae Chlorella vulgaris, Nannochloropsis
594 oceanica and Phaeodactylum tricornutum in C57BL/6 Mice. *Nutrients* **2018,** *10* (8).
595 17. Christien, E.; Matthias, P.; Maria, B.; Lolke, S.; Mauro, V.; Claudia, P.; Emilio, R. C.,
596 Microalgae-based products for the food and feed sector: an outlook for Europe. EUR - Scientific and
597 Technical Research Reports, **2014**.
598 18. Fernandez-Rojas, B.; Hernandez-Juarez, J.; Pedraza-Chaverri, J., Nutraceutical properties of
599 phycocyanin. *J. Func Foods* **2014,** *11*, 375-392.
600 19. Gdara, N. B.; Belgacem, A.; Khemiri, I.; Mannai, S.; Bitri, L., Protective effects of phycocyanin
601 on ischemia/reperfusion liver injuries. *Biomed Pharmacother* **2018,** *102*, 196-202.
602 20. Minic, S. L.; Stanic-Vucinic, D.; Mihailovic, J.; Krstic, M.; Nikolic, M. R.; Cirkovic Velickovic,
603 T., Digestion by pepsin releases biologically active chromopeptides from C-phycocyanin, a blue-
604 colored biliprotein of microalga Spirulina. *J Proteomics* **2016,** *147*, 132-139.
605 21. Gupta, R. K.; Patel, A. K.; Shah, N.; Chaudhary, A. K.; Jha, U. K.; Yadav, U. C.; Gupta, P. K.;
606 Pakuwal, U., Oxidative stress and antioxidants in disease and cancer: a review. *Asian Pac J Cancer*
607 *Prev* **2014,** *15* (11), 4405-9.
608 22. Panth, N.; Paudel, K. R.; Parajuli, K., Reactive Oxygen Species: A Key Hallmark of
609 Cardiovascular Disease. *Adv Med* **2016,** 9152732.
610 23. Afanas'ev, I., Signaling of reactive oxygen and nitrogen species in Diabetes mellitus. *Oxid Med*
611 *Cell Longev* **2010,** *3* (6), 361-73.
612 24. Afify, A. E. M. R.; El Baroty, G. S.; El Baz, F. K.; Abd El Baky, H. H.; Murad, S. A., Antioxidant
613 and antiviral activity of proteins hydrolyzed by three enzymes. *J Genet Eng Biotechnol* **2018,** *16* (2),
614 399-408.
615 25. Lisboa, C. R.; Greque de Morais, M.; Vieira Costa, J. A., Development of Bioactive Nanopeptide
616 of Microalgal Origin. *J Nanosci Nanotechnol* **2017,** *17* (2), 1025-030.
617 26. Kang, K. H.; Qian, Z. J.; Ryu, B.; Kim, S. K., Characterization of Growth and Protein Contents
618 from Microalgae Navicula incerta with the Investigation of Antioxidant Activity of Enzymatic
619 Hydrolysates. *Food Sci Biotechnol* **2011,** *20* (1), 183-191.
620 27. Alzahrani, M. A. J.; Perera, C. O.; Hemar, Y., Production of bioactive proteins and peptides from
621 the diatom Nitzschia laevis and comparison of their invitro antioxidant activities with those from
622 Spirulina platensis and Chlorella vulgaris. *Int J Food Sci Technol.* **2018,** *53* (3), 676-682.
623 28. Barkia, I.; Al-Haj, L.; Hamid, A. A.; Zakaria, M.; Saari, N.; Zadjali, F., Indigenous marine
624 diatoms as novel sources of bioactive peptides with antihypertensive and antioxidant properties. *Int*
625 *J Food Sci Technol* **2019,** *54* (5), 1514-1522.
626 29. Oh, G. W.; Ko, S. C.; Heo, S. Y.; Nguyen, V. T.; Kim, G.; Jang, C. H.; Park, W. S.; Choi, I. W.;
627 Qian, Z. J.; Jung, W. K., A novel peptide purified from the fermented microalga Pavlova lutheri
628 attenuates oxidative stress and melanogenesis in B16F10 melanoma cells. *Process Biochem* **2015,** *50*
629 (8), 1318-1326.
630 30. Yu, J.; Hu, Y.; Xue, M.; Dun, Y.; Li, S.; Peng, N.; Liang, Y.; Zhao, S., Purification and
631 Identification of Antioxidant Peptides from Enzymatic Hydrolysate of Spirulina platensis. *J*
632 *Microbiol Biotechnol* **2016,** *26* (7), 1216-23.
633 31. Montone, C. M.; Capriotti, A. L.; Cavaliere, C.; La Barbera, G.; Piovesana, S.; Zenezini Chiozzi,
634 R.; Laganà, A., Peptidomic strategy for purification and identification of potential ACE-inhibitory
635 and antioxidant peptides in Tetradesmus obliquus microalgae. *Anal Bioanal Chem* **2018,** *410* (15),

636 3573-3586.
637 32. Dadar, M.; Shahali, Y.; Chakraborty, S.; Prasad, M.; Tahoori, F.; Tiwari, R.; Dhama, K.,
638 Antiinflammatory peptides: current knowledge and promising prospects. *Inflamm Res* **2019,** *68* (2),
639 125-145.
640 33. Sheih, I. C.; Fang, T. J.; Wu, T. K.; Lin, P. H., Anticancer and antioxidant activities of the peptide
641 fraction from algae protein waste. *J Agric Food Chem* **2010,** *58* (2), 1202-7.
642 34. Vo, T. S.; Ryu, B.; Kim, S. K., Purification of novel anti-inflammatory peptides from enzymatic
643 hydrolysate of the edible microalgal Spirulina maxima. *J. Funct Foods* **2013,** *5* (3), 1336-1346.
644 35. Vo, T. S.; Kim, S. K., Down-regulation of histamine-induced endothelial cell activation as
645 potential anti-atherosclerotic activity of peptides from Spirulina maxima. *Eur J Pharm Sci* **2013,** *50*
646 (2), 198-207.
647 36. Wang, X.; Wang, H.; Pierre, J. F.; Wang, S.; Huang, H.; Zhang, J.; Liang, S.; Zeng, Q.; Zhang,
648 C.; Huang, M.; Ruan, C.; Lin, J.; Li, H., Marine microalgae bioengineered Schizochytrium sp. meal
649 hydrolysates inhibits acute inflammation. *Sci Rep* **2018,** *8* (1), 9848.
650 37. González, R.; Rodríguez, S.; Romay, C.; Ancheta, O.; González, A.; Armesto, J.; Remirez, D.;
651 Merino, N., Anti-inflammatory activity of phycocyanin extract in acetic acid-induced colitis in rats.
652 *Pharmacol Res* **1999,** *39* (1), 55-9.
653 38. Romay, C.; Gonzalez, R.; Ledon, N.; Remirez, D.; Rimbau, V., C-phycocyanin: A biliprotein
654 with antioxidant, anti-inflammatory and neuroprotective effects. *Current Protein & Peptide Science*
655 **2003,** *4* (3), 207-216.
656 39. Zhu, C. H.; Ling, Q. J.; Cai, Z. H.; Wang, Y.; Zhang, Y. B.; Hoffmann, P. R.; Zheng, W. J.; Zhou,
657 T. H.; Huang, Z., Selenium-Containing Phycocyanin from Se-Enriched Spirulina platensis Reduces
658 Inflammation in Dextran Sulfate Sodium-Induced Colitis by Inhibiting NF-kappa B Activation. *J.*
659 *Agric Food Chem* **2016,** *64* (24), 5060-5070.
660 40. Fidele, N.; Joseph, B.; Emmanuel, T.; Theophile, D., Hypolipidemic, antioxidant and anti-
661 atherosclerogenic effect of aqueous extract leaves of Cassia. occidentalis Linn (Caesalpiniaceae) in
662 diet-induced hypercholesterolemic rats. *Bmc Complem Altern Med* **2017,** *17*.
663 41. Hua, P. P.; Yu, Z. Y.; Xiong, Y.; Liu, B.; Zhao, L. N., Regulatory Efficacy of Spirulina platensis
664 Protease Hydrolyzate on Lipid Metabolism and Gut Microbiota in High-Fat Diet-Fed Rats. *Int. J.*
665 *Mol. Sci.* **2018,** *19* (12).
666 42. Nagaoka, S.; Shimizu, K.; Kaneko, H.; Shibayama, F.; Morikawa, K.; Kanamaru, Y.; Otsuka,
667 A.; Hirahashi, T.; Kato, T., A novel protein C-phycocyanin plays a crucial role in the
668 hypocholesterolemic action of Spirulina platensis concentrate in rats. *J Nutr* **2005,** *135* (10), 2425-
669 30.
670 43. Sheu, M. J.; Hsieh, Y. Y.; Lai, C. H.; Chang, C. C.; Wu, C. H., Antihyperlipidemic and
671 Antioxidant Effects of C-phycocyanin in Golden Syrian Hamsters Fed with a Hypercholesterolemic
672 Diet. *J Tradit Complement Med* **2013,** *3* (1), 41-7.
673 44. Santos, A. D.; Cecilio, H. P. M.; Teston, E. F.; de Arruda, G. O.; Peternella, F. M. N.; Marcon,
674 S. S., Microvascular complications in type 2 diabetes and associated factors: a telephone survey of
675 self-reported morbidity. *Cien Saude Colet* **2015,** *20* (3), 761-770.
676 45. Ou, Y.; Lin, L.; Pan, Q.; Yang, X.; Cheng, X., Preventive effect of phycocyanin from Spirulina
677 platensis on alloxan-injured mice. *Environ Toxicol Pharmacol* **2012,** *34* (3), 721-6.
678 46. Ou, Y.; Ren, Z.; Wang, J.; Yang, X., Phycocyanin ameliorates alloxan-induced diabetes mellitus
679 in mice: Involved in insulin signaling pathway and GK expression. *Chem Biol Interact* **2016,** *247*,
680 49-54.
681 47. Ou, Y.; Lin, L.; Yang, X. G.; Pan, Q.; Cheng, X. D., Antidiabetic potential of phycocyanin:
682 Effects on KKAy mice. *Pharm Biol* **2013,** *51* (5), 539-544.
683 48. Zheng, J.; Inoguchi, T.; Sasaki, S.; Maeda, Y.; McCarty, M. F.; Fujii, M.; Ikeda, N.; Kobayashi,
684 K.; Sonoda, N.; Takayanagi, R., Phycocyanin and phycocyanobilin from Spirulina platensis protect
685 against diabetic nephropathy by inhibiting oxidative stress. *Am J Physiol Regul Integr Comp Physiol*
686 **2013,** *304* (2), 110-20.

687 49. Koska, J.; Sands, M.; Burciu, C.; Reaven, P., Cardiovascular effects of dipeptidyl peptidase-4
688 inhibitors in patients with type 2 diabetes. *Diab Vasc Dis Res* **2015,** *12* (3), 154-63.
689 50. Xu, Q. B.; Hong, H.; Wu, J. P.; Yan, X. H., Bioavailability of bioactive peptides derived from
690 food proteins across the intestinal epithelial membrane: A review. *Trends Food Sci Technol* **2019,**
691 *86*, 399-411.
692 51. Zhu, Q. S.; Chen, X. J.; Wu, J. J.; Zhou, Y.; Qian, Y.; Fang, M.; Xie, J. L.; Wei, D. Z., Dipeptidyl
693 peptidase IV inhibitory peptides from Chlorella vulgaris: in silico gastrointestinal hydrolysis and
694 molecular mechanism. *Eur Food Res Technol.* **2017,** *243* (10), 1739-1748.
695 52. FitzGerald, R. J.; Murray, B. A.; Walsh, D. J., Hypotensive peptides from milk proteins. *J. Nutr*
696 **2004,** *134* (4), 980S-988S.
697 53. Lu, J.; Ren, D. F.; Xue, Y. L.; Sawano, Y.; Miyakawa, T.; Tanokura, M., Isolation of an
698 antihypertensive peptide from alcalase digest of Spirulina platensis. *J Agric Food Chem* **2010,** *58*
699 (12), 7166-71.
700 54. Ejike, C.; Collins, S. A.; Balasuriya, N.; Swanson, A. K.; Mason, B.; Udenigwe, C. C., Prospects
701 of microalgae proteins in producing peptide-based functional foods for promoting cardiovascular
702 health. *Trends Food Sci Technol.* **2017,** *59*, 30-36.
703 55. Lin, Y. H.; Chen, G. W.; Yeh, C. H.; Song, H.; Tsai, J. S., Purification and Identification of
704 Angiotensin I-Converting Enzyme Inhibitory Peptides and the Antihypertensive Effect of Chlorella
705 sorokiniana Protein Hydrolysates. *Nutrients* **2018,** *10* (10).
706 56. Xie, J.; Chen, X.; Wu, J.; Zhang, Y.; Zhou, Y.; Zhang, L.; Tang, Y. J.; Wei, D., Antihypertensive
707 Effects, Molecular Docking Study, and Isothermal Titration Calorimetry Assay of Angiotensin I-
708 Converting Enzyme Inhibitory Peptides from Chlorella vulgaris. *J Agric Food Chem* **2018,** *66* (6),
709 1359-1368.
710 57. Lammi, C.; Aiello, G.; Boschin, G.; Arnoldi, A., Multifunctional peptides for the prevention of
711 cardiovascular disease: A new concept in the area of bioactive food-derived peptides. *J. Func Foods*
712 **2019,** *55*, 135-145.
713 58. Cherng, J. Y.; Liu, C. C.; Shen, C. R.; Lin, H. H.; Shih, M. F., Beneficial effects of Chlorella-11
714 peptide on blocking LPS-induced macrophage activation and alleviating thermal injury-induced
715 inflammation in rats. *Int J Immunopathol Pharmacol* **2010,** *23* (3), 811-20.
716 59. Doi, R. H.; Kosugi, A., Cellulosomes: Plant-cell-wall-degrading enzyme complexes. *Nat Rev*
717 *Microbiol.* **2004,** *2* (7), 541-551.
718 60. Günerken, E.; D'Hondt, E.; Eppink, M. H.; Garcia-Gonzalez, L.; Elst, K.; Wijffels, R. H., Cell
719 disruption for microalgae biorefineries. *Biotechnol Adv* **2015,** *33* (2), 243-60.
720 61. Phong, W. N.; Show, P. L.; Ling, T. C.; Juan, J. C.; Ng, E. P.; Chang, J. S., Mild cell disruption
721 methods for bio-functional proteins recovery from microalgae-Recent developments and future
722 perspectives. *Algal Res* **2018,** *31*, 506-516.
723 62. Vernès, L.; Abert-Vian, M.; El Maâtaoui, M.; Tao, Y.; Bornard, I.; Chemat, F., Application of
724 ultrasound for green extraction of proteins from spirulina. Mechanism, optimization, modeling, and
725 industrial prospects. *Ultrason Sonochem* **2019**.
726 63. Chew, K. W.; Chia, S. R.; Lee, S. Y.; Zhu, L. D.; Show, P. L., Enhanced microalgal protein
727 extraction and purification using sustainable microwave-assisted multiphase partitioning technique.
728 *Chem Eng J.* **2019,** *367*, 1-8.
729 64. Wijesinghe, W.; Jeon, Y. J., Enzyme-assistant extraction (EAE) of bioactive components: A
730 useful approach for recovery of industrially important metabolites from seaweeds: A review.
731 *Fitoterapia* **2012,** *83* (1), 6-12.
732 65. Al-Zuhair, S.; Ashraf, S.; Hisaindee, S.; Al Darmaki, N.; Battah, S.; Svistunenko, D.; Reeder, B.;
733 Stanway, G.; Chaudhary, A., Enzymatic pre-treatment of microalgae cells for enhanced extraction of
734 proteins. *Eng Life Sci* **2017,** *17* (2), 175-185.
735 66. Yamada, T.; Sakaguchi, K., Comparative studies on chlorella cell-walls - induction of protoplast
736 formation. *Arch Microbiol.* **1982,** *132* (1), 10-13.
737 67. Sierra, L. S.; Dixon, C. K.; Wilken, L. R., Enzymatic cell disruption of the microalgae

738 Chlamydomonas reinhardtii for lipid and protein extraction. *Algal Res* **2017,** *25*, 149-159.
739 68. Ko, S. C.; Kim, D.; Jeon, Y. J., Protective effect of a novel antioxidative peptide purified from a
740 marine Chlorella ellipsoidea protein against free radical-induced oxidative stress. *Food Chem Toxicol*
741 **2012,** *50* (7), 2294-302.
742 69. Sheih, I. C.; Wu, T. K.; Fang, T. J., Antioxidant properties of a new antioxidative peptide from
743 algae protein waste hydrolysate in different oxidation systems. *Bioresour Technol* **2009,** *100* (13),
744 3419-25.
745 70. Stack, J.; Le Gouic; A.V., T.; P.R., G.; F., S., D.B.,; FitzGerald, R. J., Protein extraction and
746 bioactive hydrolysate generation from two microalgae, Porphyridium purpureum and Phaeodactylum
747 tricornutum. *J. Food Bioact.* **2018***;*(1)153–165
748 71. Montalvo, G. E. B.; Thomaz-Soccol, V.; Vandenberghe, L. P. S.; Carvalho, J. C.; Faulds, C. B.;
749 Bertrand, E.; Prado, M. R. M.; Bonatto, S. J. R.; Soccol, C. R., Arthrospira maxima OF15 biomass
750 cultivation at laboratory and pilot scale from sugarcane vinasse for potential biological new peptides
751 production. *Bioresour Technol* **2019,** *273*, 103-113.
752 72. Heo, S. Y.; Ko, S. C.; Kim, C. S.; Oh, G. W.; Ryu, B.; Qian, Z. J.; Kim, G.; Park, W. S.; Choi, I.
753 W.; Phan, T. T.; Heo, S. J.; Kang, D. H.; Yi, M.; Jung, W. K., A heptameric peptide purified from
754 Spirulina sp. gastrointestinal hydrolysate inhibits angiotensin I-converting enzyme- and
755 angiotensin II-induced vascular dysfunction in human endothelial cells. *Int J Mol Med* **2017,** *39* (5),
756 1072-1082.
757 73. Kumar, K. K.; Karnati, S.; Reddy, M. B.; Chandramouli, R., Caco-2 cell lines in drug discovery-
758 an updated perspective. *J Basic Clin Pharm* **2010,** *1* (2), 63-9.
759 74. Lammi, C.; Aiello, G.; Vistoli, G.; Zanoni, C.; Arnoldi, A.; Sambuy, Y.; Ferruzza, S.; Ranaldi,
760 G., A multidisciplinary investigation on the bioavailability and activity of peptides from lupin protein.
761 *J. Func Foods* **2016,** *24*, 297-306.
762 75. Aiello, G.; Ferruzza, S.; Ranaldi, G.; Sambuy, Y.; Arnoldi, A.; Vistoli, G.; Lammi, C., Behavior
763 of three hypocholesterolemic peptides from soy protein in an intestinal model based on differentiated
764 Caco-2 cell. *J Func Foods* **2018,** *45*, 363-370.
765 76. Gallego, M.; Grootaert, C.; Mora, L.; Aristoy, M. C.; Van Camp, J.; Toldra, F., Transepithelial
766 transport of dry-cured ham peptides with ACE inhibitory activity through a Caco-2 cell monolayer.
767 *J Func Foods* **2016,** *21*, 388-395.
768 77. He, Y. Y.; Li, T. T.; Chen, J. X.; She, X. X.; Ren, D. F.; Lu, J., Transport of ACE Inhibitory
769 Peptides Ile-Gln-Pro and Val-Glu-Pro Derived from Spirulina platensis Across Caco-2 Monolayers.
770 *J Food Sci* **2018,** *83* (10), 2586-2592.
771 78. FitzGerald, R. J.; Meisel, H., Milk protein-derived peptide inhibitors of angiotensin-I-converting
772 enzyme. *Br J Nutr.* **2000,** *84*, S33-S37.
773 79. Liao, W.; Jahandideh, F.; Fan, H.; Son, M.; Wu, J., Egg Protein-Derived Bioactive Peptides:
774 Preparation, Efficacy, and Absorption. *Adv Food Nutr Res* **2018,** *85*, 1-58.
775 80. Piovesana, S.; Capriotti, A. L.; Cavaliere, C.; La Barbera, G.; Montone, C. M.; Zenezini Chiozzi,
776 R.; Laganà, A., Recent trends and analytical challenges in plant bioactive peptide separation,
777 identification and validation. *Anal Bioanal Chem* **2018,** *410* (15), 3425-3444.
778 81. Capriotti, A. L.; Cavaliere, C.; Piovesana, S.; Samperi, R.; Lagana, A., Recent trends in the
779 analysis of bioactive peptides in milk and dairy products. *Anal Bioanal Chem* **2016,** *408* (11), 2677-
780 2685.
781 82. Dallas, D. C.; Guerrero, A.; Parker, E. A.; Robinson, R. C.; Gan, J.; German, J. B.; Barile, D.;
782 Lebrilla, C. B., Current peptidomics: applications, purification, identification, quantification, and
783 functional analysis. *Proteomics* **2015,** *15* (5-6), 1026-38.
784 83. Panchaud, A.; Affolter, M.; Kussmann, M., Mass spectrometry for nutritional peptidomics: How
785 to analyze food bioactives and their health effects. *J Proteomics* **2012,** *75* (12), 3546-59.
786 84. Sanchez-Rivera, L.; Martinez-Maqueda, D.; Cruz-Huerta, E.; Miralles, B.; Recio, I., Peptidomics
787 for discovery, bioavailability and monitoring of dairy bioactive peptides. *Food Res Int.* **2014,** *63*, 170-
788 181.

85. Gillet, L. C.; Navarro, P.; Tate, S.; Röst, H.; Selevsek, N.; Reiter, L.; Bonner, R.; Aebersold, R., Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: a new concept for consistent and accurate proteome analysis. *Mol Cell Proteomics* **2012,** *11* (6), O111.016717.
86. Liu, Y. S.; Huttenhain, R.; Surinova, S.; Gillet, L. C. J.; Mouritsen, J.; Brunner, R.; Navarro, P.; Aebersold, R., Quantitative measurements of N-linked glycoproteins in human plasma by SWATH-MS. *Proteomics* **2013,** *13* (8), 1247-1256.
87. Gao, Y.; Lim, T. K.; Lin, Q.; Li, S. F., Evaluation of sample extraction methods for proteomics analysis of green algae Chlorella vulgaris. *Electrophoresis* **2016,** *37* (10), 1270-6.
88. Gallego, M.; Mora, L.; Toldrá, F., Perspectives in the Use of Peptidomics in Ham. *Proteomics* **2018,** *18* (18), e1700422.
89. Bantscheff, M.; Schirle, M.; Sweetman, G.; Rick, J.; Kuster, B., Quantitative mass spectrometry in proteomics: a critical review. *Anal Bioanal Chem* **2007,** *389* (4), 1017-31.
90. Hu, Y.; Stromeck, A.; Loponen, J.; Lopes-Lutz, D.; Schieber, A.; Gänzle, M. G., LC-MS/MS quantification of bioactive angiotensin I-converting enzyme inhibitory peptides in rye malt sourdoughs. *J Agric Food Chem* **2011,** *59* (22), 11983-9.
91. Zhao, C. J.; Hu, Y.; Schieber, A.; Ganzle, M., Fate of ACE-inhibitory peptides during the bread-making process: Quantification of peptides in sourdough, bread crumb, steamed bread and soda crackers. *J Cereal Sci* **2013,** *57* (3), 514-519.
92. Nakashima, E. M. N.; Kudo, A.; Iwaihara, Y.; Tanaka, M.; Matsumoto, K.; Matsui, T., Application of C-13 stable isotope labeling liquid chromatography-multiple reaction monitoring-tandem mass spectrometry method for determining intact absorption of bioactive dipeptides in rats. *Anal Biochem* **2011,** *414* (1), 109-116.
93. Picotti, P.; Bodenmiller, B.; Mueller, L. N.; Domon, B.; Aebersold, R., Full dynamic range proteome analysis of S. cerevisiae by targeted proteomics. *Cell* **2009,** *138* (4), 795-806.
94. Huang, Q.; Yang, L.; Luo, J.; Guo, L.; Wang, Z.; Yang, X.; Jin, W.; Fang, Y.; Ye, J.; Shan, B.; Zhang, Y., SWATH enables precise label-free quantification on proteome scale. *Proteomics* **2015,** *15* (7), 1215-23.
95. Colgrave, M. L.; Byrne, K.; Blundell, M.; Heidelberger, S.; Lane, C. S.; Tanner, G. J.; Howitt, C. A., Comparing Multiple Reaction Monitoring and Sequential Window Acquisition of All Theoretical Mass Spectra for the Relative Quantification of Barley Gluten in Selectively Bred Barley Lines. *Anal Chem* **2016,** *88* (18), 9127-35.
96. Kang, K. H.; Qian, Z. J.; Ryu, B.; Karadeniz, F.; Kim, D.; Kim, S. K., Antioxidant peptides from protein hydrolysate of microalgae Navicula incerta and their protective effects in HepG2/CYP2E1 cells induced by ethanol. *Phytother Res* **2012,** *26* (10), 1555-63.
97. Shih, M. F.; Chen, L. C.; Cherng, J. Y., Chlorella 11-peptide inhibits the production of macrophage-induced adhesion molecules and reduces endothelin-1 expression and endothelial permeability. *Mar Drugs* **2013,** *11* (10), 3861-74.
98. González, R.; Rodríguez, S.; Romay, C.; Ancheta, O.; González, A.; Armesto, J.; Remirez, D.; Merino, N., Anti-inflammatory activity of phycocyanin extract in acetic acid-induced colitis in rats. *Pharmacol Res* **1999,** *39* (1), 55-9.
99. Lu, J.; Sawano, Y.; Miyakawa, T.; Xue, Y. L.; Cai, M. Y.; Egashira, Y.; Ren, D. F.; Tanokura, M., One-Week Antihypertensive Effect of Ile-Gln-Pro in Spontaneously Hypertensive Rats. *J Agric Food Chem* **2011,** *59* (2), 559-563.

Captions of Figures

Figure 1. Number of papers about microalgae published from 1995 to May 2019.

Figure 2. Multidisciplinary strategy proposed for bioactive peptide discovery *vs* the classical approach. The blue (scheme A) represents the classical approach to peptide identification, whereas in orange (Scheme B) are reported the new proposed steps of analysis.

843

844

845

846 **Table 1.** General composition of different conventional food sources and microalgae (% of dry biomass)

847

Species	Protein	Carbohydrate	Lipid
Meat	43	1	34
Milk	26	38	28
Soybean	37	30	20
Wheat	13	71	1.5
Arthrospira maxima	60-71	13-16	6-7
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella sp.	20-57	12.2-32	6-15
Haematococcus pluvialis	17-27	37-40	25
Nannochloropsis sp.	35	7.8	18
Nitzchia sp.	26	9.8	13
Phaeodactylum tricornutum	30	8.4	14
Scenedesmus obliquus	50-56	10-17	12-14
Arthrospira platensis	46-63	8-14	4-9

848

Table 2. IAA profile and DIAAS of several microalgae species and conventional food source

Source	IAA content (mg/g protein)									DIAAS (%) [a]
	His	*Ile*	*Leu*	*Lys*	*SAA*	*AAA*	*Thr*	*Trp*	*Val*	
IAA reference pattern [b]	16	30	61	48	23	41	25	6.6	40	-
Egg (row)	24	66	88	53	55	100	50	17	72	74 (Leu)
Egg (cooked)	24	66	88	53	55	100	50	17	72	100 (Lys)
Soybean (cooked)	26	53	77	64	32	87	40	14	53	97 (SAA)
Chlorella sp.	20	38	88	84	36	84	48	21	55	111 (His)
Nannochloropsis sp.	21	48	78	61	20	104	55	16	65	23 (His)
Phaeodactylum tricornutum	17	49	56	56	23	107	54	16	59	75 (Leu)
Scenedesmus obliquus	21	36	73	56	21	80	51	3	60	40 (Trp)
Arthrospira platensis	22	67	98	48	34	106	62	3	71	34 (Trp)

a The calculation method of DIAAS is shown in Supplementary Information.

b Here the IAA reference pattern is especially for the population group of older children, adolescent and adult, as recommended by FAO criterion [15]

His histidine. *Ile* isoleucine. *Leu* leucine. *Lys* lysine. *SAA*=sulphur amino acids (methionine+ cystine). *AAA* = aromatic amino acids (phenylalanine+ Tyrosine). *Thr* threonine. *Trp* tryptophan. *Val* valine.

Table 3. Antioxidant peptide sequences derived from microalgae

Peptides sequence	Protein Source	Hydrolytic enzyme	In vitro/in vivo	Methodology and Potency	References
VECYGPNRPQF	*Chlorella vulgaris*	Pepsin	In vitro	• ABTS (9.8 μM) • DPPH (58.0 μM) • Hydroxyl radical (8.3 μM) • Superoxide radicals (7.5 μM)	69
PGWNQWFL VEVLPPAEL	*Navicula incerta*	papain	in situ (HepG2/CYP2E1 cell)	Protecting hepatocyte from ethanol-induced oxidative stress	96
LNGDVW	*Chlorella ellipsoidea*			Peroxyl radicals (0.02 mM) DPPH (0.92 mM) Hydroxyl radicals (1.42 mM)	68
MGRY	*Pavlova lutheri*	Fermentation by yeast Hansenula polymorpha	In vitro and in situ (B16F10 melanoma cells)	• DPPH (IC_{50} 0.285 mM) • Hydroxyl radicals (IC_{50} 0.068 mM) • Hydrogen peroxide (IC_{50} 0.988 mM) • Melanin synthesis suppression	29
PNN	*Arthrospira platensis*	Protease K	In vitro	Scavenging ability of • DPPH (81.44%) • Superoxide anion (47.84%) • Hydroxyl radicals (54.01%) • Superoxide Dismutase (SOD) (12.55%) at 100 μg/ml	30
WPRGYFL	*Tetradesmus obliquus*	Alcalase	In vitro	ABTS (EC_{50} 4.70 μmol L^{-1})	31
SDWDRF	*Tetradesmus obliquus*	Alcalase	In vitro	DPPH (EC_{50} 13.97 μmol L^{-1})	31

Table 4. Anti-inflammatory peptides derived from microalgae

Peptides sequence	Protein Source	Hydrolytic enzyme	In vitro/in vivo	Mechanism of action	References
VECYGPNRPQF	*Chlorella sp.*	Pepsin, flavourzyme, alcalase and papain	In vitro and in vivo (rats)	Inhibition of inflammatory mediators (NO, iNOS, NF-κB, TNF-α, PGE2, MDA)	58, 97
LDAVNR MMLDF	*Spirulina maxima*	Trypsin, α-chymotrypsin，pepsin	In vitro (mast cells and endothelial cells)	Inhibition of the inflammation in endothelial and potential anti-atherosclerosis activity (suppression of ROS, IL-8, IL-6, MCP-1 and adhension molecules)	34, 35
Hydrolysates including 172 peptide sequences	*Schizochytrium sp.*	Pepsin, trypsin	In vivo (mice)	Resistance of colitis (inhibition of IL-10, cell proliferation)	36
C-PC digests	*Arthrospira platensis*	Gastrointestinal digestion	In vivo (rats, mice)	Resistance of inflammation in different issues (hepatitis, arthritis, colitis, brain injury)	19, 38, 39, 98

Table 5. Microalgae peptides ameliorating dyslipidemia

Peptides sequence	Protein Source	Hydrolytic enzyme	In vitro/in vivo	Mechanism of action	References
Protein hydrolysates including 217 identified peptides	*Arthrospira platensis*	Protamex	In vivo (rats)	TG, TC, LDL-c, ALT, AST, HDL-c, regulating the expression of genes related to lipid metabolism (SREBP-1, ACC, PPARγ, AMPK, PPARα)	[41]
Digested phycocyanin	*Arthrospira platensis*	Gastrointestinal digestion	In vivo	Hypocholesterolemia	[42]
Digested phycocyanin	*Arthrospira platensis*	Gastrointestinal digestion	In vivo (hamsters)	Hypolipidemia and anti-oxidantion (serum cholesterol, TG, LDL, GOT, GPT)	[43]

Table 6. Anti-diabetic peptides derived from microalgae

Peptides	Protein Source	Hydrolytic enzyme	In vitro/in vivo	Mechanism of action and Potency	References
Digested phycocyanin	*Arthrospira platensis*	Gastrointestinal digestion	In vivo (mice with type 1 diabetes)	Activation of insulin signaling pathway and GK expression	[45, 46]
Digested phycocyanin	*Arthrospira platensis*	Gastrointestinal digestion	In vivo (mice with type 2 diabetes)	Improving the sensitivity of tissues to the insulin regulation	[47]
Digested phycocyanin	*Arthrospira platensis*	Gastrointestinal digestion	In vivo (mice with type 2 diabetes)	Alleviation of the diabetic nephropathy; decrease of the oxidant stress in urine and kidney	[48]
Protein hydrolysates	*Porphyridium purpureum*	Alcalase, Flavourzyme	In vitro	DPP-IV inhibition (IC_{50} 2.28 mg/mL)	[70]
Protein hydrolysates	*Phaeodactylum tricornutum*	Alcalase, Flavourzyme	In vitro	DPP-IV inhibition (IC_{50} 2.68 mg/mL)	[70]
VPW IPR	*Chlorella vulgaris*	Virtual digestion (pepsin, trypsin, chymotrypsin)	In vitro and in mouse serum	DPP-IV inhibition (IC_{50} of VPW 348.6 μM, IC_{50} of IPR 376.6 μM)	[51]

Table 7. ACE-inhibitor peptides derived from microalgae protein hydrolysis

Protein Source	Hydrolytic enzyme	Peptide sequence	IC_{50} (μM)	Reference
Tetradesmus obliquus	Alcalase	GPDRPKFLGPF	5.73	[31]
		WYGPDRPKFL	0.82	
Chlorella sorokiniana	Protease N, pepsin, pancreatin	WV	307.61	[55]
		VW	0.58	
		IW	0.50	
		LW	1.11	
Chlorella vulgaris	In silico digestion (pepsin, trypsin, chymotrypsin)	TTW	0.61	[56]
		VHW	0.91	
Arthrospira platensis	Alcalase	IQP	5.77	[53, 99]
		VEP	27.36	
Spirulina sp.	Pepsin, Trypsin, α-chymotrypsin	TMEPGKP	132	[72]

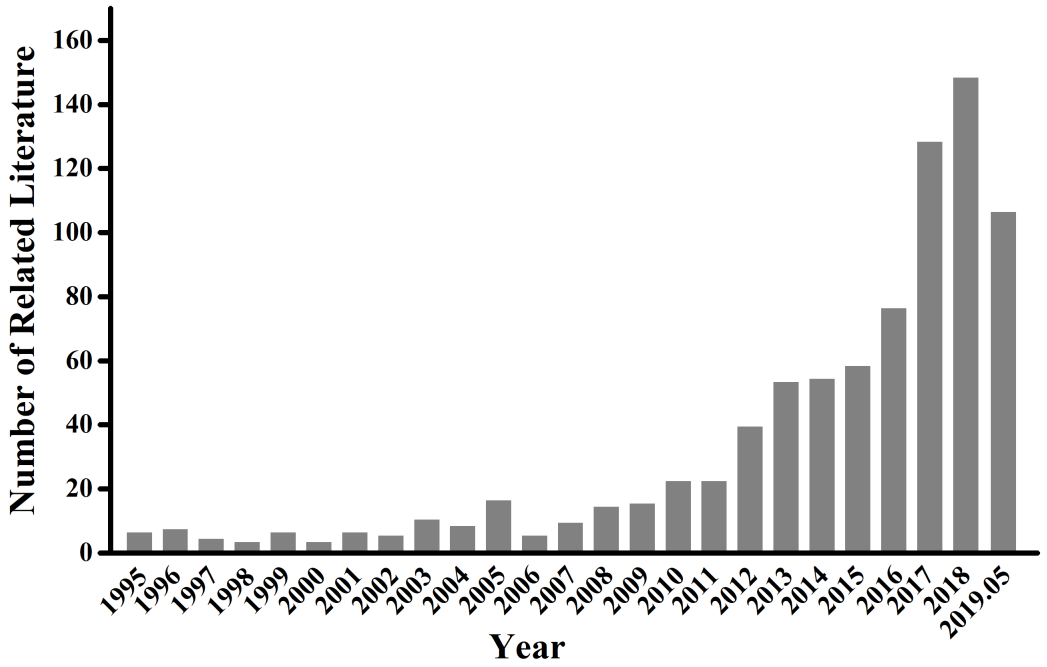

Number of Related Literature
160
140
120
100
80
60
40
20
0
1995
1996
1997
1998
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019.05
Year

Figure 1.

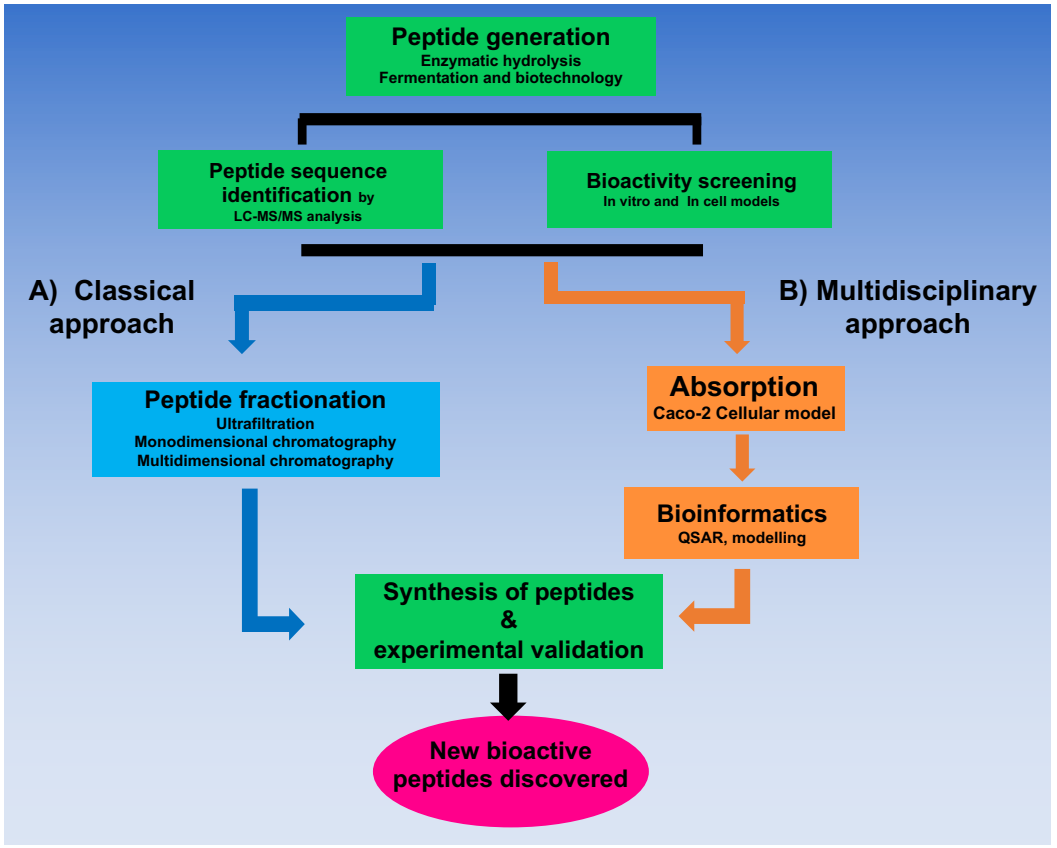
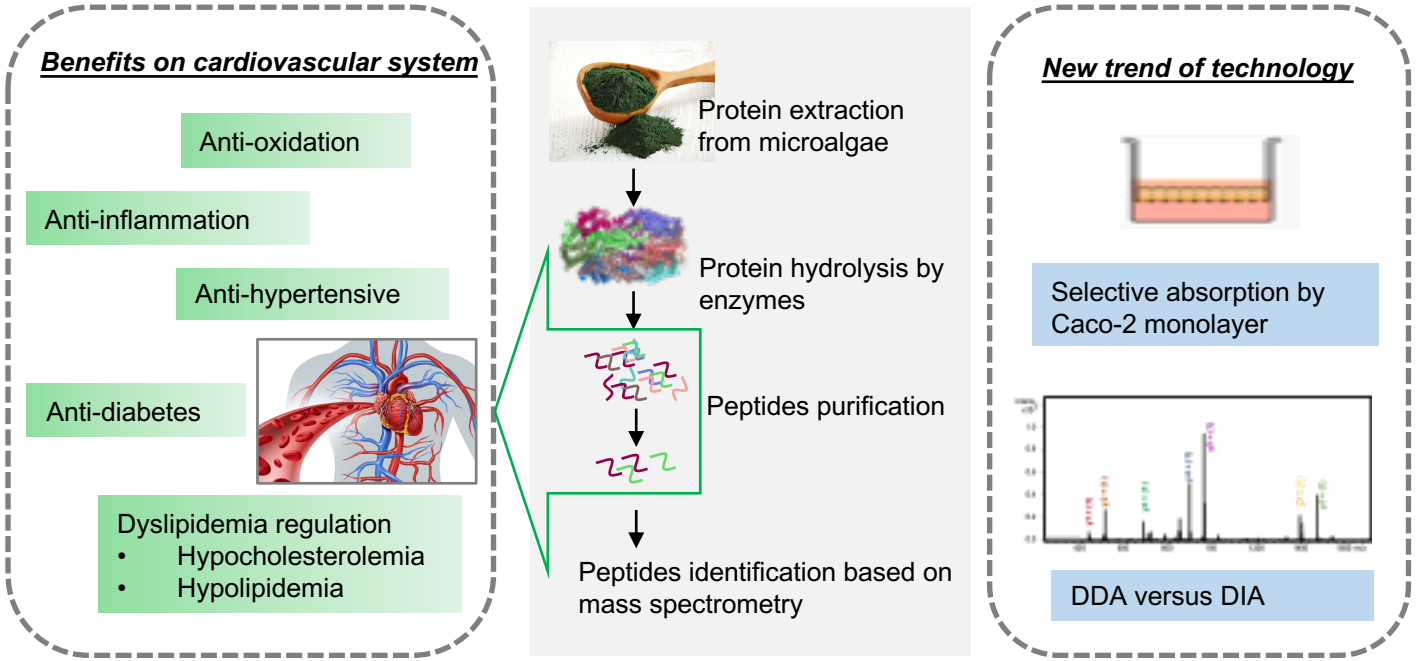

Peptide generation
Enzymatic hydrolysis
Fermentation and biotechnology
Peptide sequence identification by
LC-MS/MS analysis
Bioactivity screening
In vitro and In cell models
A) Classical approach
B) Multidisciplinary approach
Peptide fractionation
Ultrafiltration
Monodimensional chromatography
Multidimensional chromatography
Absorption
Caco-2 Cellular model
Bioinformatics
QSAR, modelling
Synthesis of peptides & experimental validation
New bioactive peptides discovered

Figure 2.

Recent advances in microalgae peptides: analysis and health benefits



TOC