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REVIEW ARTICLE

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Utilizing proteomics to identify and optimize microalgae strains for high-quality dietary protein: a review

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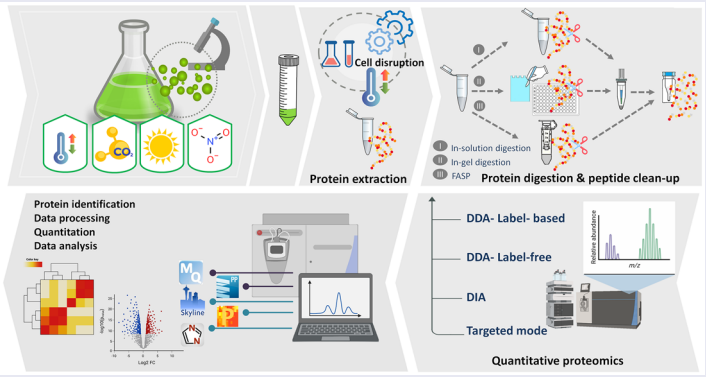
ABSTRACT

Algae-derived protein has immense potential to provide high-quality protein foods for the expanding human population. To meet its potential, a broad range of scientific tools are required to identify optimal algal strains from the hundreds of thousands available and identify ideal growing conditions for strains that produce high-quality protein with functional benefits. A research pipeline that includes proteomics can provide a deeper interpretation of microalgal composition and biochemistry in the pursuit of these goals. To date, proteomic investigations have largely focused on pathways that involve lipid production in selected microalgae species. Herein, we report the current state of microalgal proteome measurement and discuss promising approaches for the development of protein-containing food products derived from algae.

GRAPHICAL ABSTRACT



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Background/introduction

Microalgae are a diverse group of microorganisms that contain essential nutrients suitable for the human diet. These include proteins, dietary fiber, polyunsaturated fatty acids, antioxidants, and bioactive compounds [1]. Some microalgae species devote a large proportion of their biomass to protein (e.g., 66%), which make them a potentially ideal complementary protein source for human nutrition [2–4]. In addition, microalgae growth and production have significant environmental advantages compared to animal and plant-based production practices. The rapid growth rate of microalgae ensures their high biomass production, concomitant with increased protein yield [5]. Furthermore, microalgae do not need fertile land to produce protein in comparison to plant-based and animal-based products, as non-arable land can be used for their production [6].

Although microalgae have been commercially cultured for more than 50 years [7], some varieties, such as *Nostoc*, *Arthrospira* (*Spirulina*), and *Aphanizomenon*, have been consumed by people for thousands of years [8]. Proteins derived from algae are of particular interest to meet the growing demand for dietary protein,

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which underpins an expectation of substantial market growth for future commercial microalgae production. Currently, microalgae-based alternative protein products are produced by several biotechnology companies, which focus on only a few varieties, such as: *Chlorella vulgaris*, *Arthrospira* sp., *Nannochloropsis* sp., and *Dunaliella* sp. Yet, there remain hundreds of thousands of microalgae species [9], wherein little is known about their protein content, nutritional quality, and production characteristics.

Crude protein content varies markedly for different microalgae strains, ranging from 6 to 66% [3,4] and can be enhanced by modifying growing conditions. In addition, genetic engineering can be used to develop microalgae strains with specific traits that are favorable for industrial scale production, such as: rapid growth, tolerance to abiotic stresses [10,11], and/or specific nutritional attributes, such as higher levels of specific essential amino acids (EAAs). To this end, an in-depth understanding of the molecular mechanisms that underpin microalgae biochemistry is required, which can be accomplished by applying omic approaches, such as: genomics, transcriptomics, proteomics, and metabolomics. Critically, proteomics offers key information that can inform nutritional quality not offered by other omic measurements.

The definition of proteomics now extends beyond the identification of all proteins in a particular cell or cell compartment; it includes the analysis of post-genomic events in cells, including: characterization of protein isoforms, post-translational modifications (PTMs) of proteins, protein–protein interactions, protein structures, and protein complexes [12]. Proteomics can aid genomic studies as it enables us to achieve a comprehensive understanding of metabolic pathways, and, in turn, the behavior of microalgae in response to its surrounding environmental conditions. Conversely, genomics is able to complement proteomics through information acquired by complete genome sequencing [13]. However, genomic data are only available for a limited number of the thousands of known microalgae species and functional understanding of these genes is also under-represented [10]. To address this, there is an important need to utilize omic technologies to broaden our understanding of a diverse range of microalgae species/strains, especially those that show promise for applications in food production.

Proteomics can provide valuable information regarding the optimal growth conditions for algal protein productivity, protein quality for human nutrition, and food health and safety. Researchers aim to track the protein complement across growth conditions to identify optimal parameters. In terms of quality and safety, proteomics also enables the identification of peptides that are liberated during passage through the human digestive tract. These measurements provide key data to support assessments regarding functional benefits or health concerns, e.g., allergens.

With a critical mass of information regarding algal proteomics and market interest in complementary protein sources, this review seeks to: (a) evaluate a range of methods that can be used to study the algal proteome; (b) summarize current knowledge regarding the algal proteome; and, (c) identify important research approaches utilizing proteomic methods that will help identify algal species that are best suited to the production of high-quality protein ingredients for the human diet.

Protein extraction methods

A majority of microalgae species and strains have a rigid cell wall that limits the accessibility of protein molecules, thus species-specific extraction methods are often required. A key consideration is the variation in cell wall structure, which can be comprised of different polysaccharides, such as: cellulose, hemicellulose, pectin, and xylan; monosaccharides like mannose and uronic acid (acidic sugar); and polymers, e.g., sporopollenin and algaenan [14–16]. These rigid cell walls act as a physical barrier that can prevent digestive enzymes from accessing cell contents, thus inhibiting digestibility and nutrient availability. Accordingly, effective cell disruption methods are essential for food applications and enable the assessment of protein complement and the discovery of the putative peptides when these foods are digested as part of the diet. In addition, more intensive extraction methods featuring detergents or organic solvents are often used to provide further insight into the mechanisms by which algal protein yield and quality can be optimized.

Food-grade protein extraction methods

Until now most microalgal proteomic studies report the use of extraction techniques that are not food safe. For food applications, protein extraction methods must meet safety regulations, which limits the use of a broad range of chemicals that are highly effective in disrupting the tough cell wall of many microalgae species/strains, thereby necessitating tailored approaches. For instance, species such as *Haematococcus pluvialis* has a very rigid cell wall and would require more intensive cell disruption than a species like *Porphyridium cruentum* which has a very weak cell wall [17].

In defining the protein extraction method, consideration must also be given to protein solubility and energy inputs to achieve a cost-effective process [18]. A variety of disruption methods have been used to break the microalgae cell wall so that there is greater area for interaction between the soluble proteins and solvent. These disruption methods include: physical, mechanical, chemical, and biological procedures. For example, the combinations of multiple methods have been used for *Chlorella pyrenoidosa* and *Nannochloropsis gaditana*, including enzyme digestion, homogenization, and ultrasonication [17,19].

It has been reported that mechanical procedures, such as bead milling and high-pressure homogenization (HPH), can recover microalgal protein more efficiently than physical methods such as thermal treatment, sonication, and pulsed electric field (PEF) [17,20,21]. Of these methods, HPH has been characterized as the most effective method for algal disruption compared to physical and chemical processes, while also representing reasonable energy costs [14,21]. Despite the high efficiency of such a disruption procedure to recover soluble proteins, the shear force should be chosen carefully as excessive force can degrade some proteins and negatively affect the functionality of others [22]. Enzymes can serve as a viable alternative to mechanical techniques to weaken the cell walls in cases where the biomolecule is sensitive to the pressure and speed forces [23]. Regardless of the effectiveness of choosing a cell disruption method and extraction method, industrial scale-up of algal protein production will require the energy cost of the technique per unit of extracted protein to be carefully assessed as highlighted by Safi et al. [17]. Despite the low energy requirement of enzymatic hydrolysis, the considerable enzyme costs could pose limitations on the large-scale production of proteins within industrial settings. Using chemical procedures such as salting-out is considered as another safe method, as salts such as ammonium sulfate are subsequently removed by ultrafiltration.

The use of different methods to extract protein from microalgae has been reviewed recently [18,24]; however, the main comparisons were made based on total protein extracted, using estimates of total nitrogen content as a proxy protein measurement (Kjeldahl or Dumas methods) [25]. Yet, measures of protein quality – including: EAA content, amino acid and protein digestibility and bioavailability and/or peptides functionality – are often overlooked. Only a small number of studies have reported measures of protein quality [14,17,20,26], highlighting a substantial knowledge gap that needs addressing and readily addressable using high quality proteome measurements.

The bottom-up proteomic approach is hitherto under-represented in algal research, particularly with regard to food-grade protein extraction. To understand fatty acids biosynthesis in *Chlorella vulgaris*, proteins were extracted using Milli-Q water and digested for a bottom-up proteome measurement [27]. Other studies have measured the algal peptidome from food-grade extraction methods to analyze the bio-active peptides in microalgae species. In this regard, a peptidomics study of *Auxenochlorella pyrenoidosa* used a food-grade protein extraction, achieved by using HPH on microalgae-water slurry [28]. In another peptidomics study on *Arthrospira maxima*, the combination of freeze–thaw cycles, ultrasonication and homogenization was used to extract algal proteins [29]. While limited in their abundance, these studies show the utility of bottom-up proteome measurement when applied to food safe protein extractions.

Deep eutectic solvents (DESs) are another efficient and green method to extract proteins for the food industry. DES are generally composed of a quaternary ammonium salt as a hydrogen bond acceptor (such a choline chloride) and a hydrogen bond donor (such as glycerol) with a melting point lower than its constitutive components [30]. Although this method is widely used for plant seed protein extraction [31], it has not been considered for microalgal protein extraction. As such, DES may represent an interesting opportunity for microalgae food-grade protein extraction.

The application of proteome measurement to the study of edible extracted proteins from microalgae can support the identification of optimal processing methods that not only increase protein quantity but also identify proteins/peptides with nutritional and functional benefits for human health and/or identify potential allergens [32,33]. Consequently, while currently limited in its application, proteomics has great potential to offer a valuable tool – along with other established screening tests – to assess the quality and safety of algal protein.

Non-food-grade protein extraction methods

Non-food-grade protein extraction methods use a broad range of extraction buffers and solvents, such as: osmolytes (e.g., glycerol), detergents (e.g., sodium dodecyl sulfate, SDS), reducing agents (e.g., 2-mercaptoethanol; dithiothreitol, DTT), and denaturing reagents (e.g., urea). The selection of extraction buffer is crucial in proteomics as it effects the physicochemical and functional properties of proteins for

subsequent measurement [32,34]. In order to obtain the most effective protein extraction, some mechanical procedures, such as ultrasonication, are used in conjunction with extraction buffers [35]. The composition of several different buffers has been used to extract protein from microalgae. The two most used methods involve either precipitation and resolubilization in lysis buffer after removing unwanted components; or solubilization in lysis buffer before precipitation and subsequent resolubilization in lysis buffer or solubilized in buffered detergent following by filtering to remove small molecules. Supplementary Table S1 presents a summary of extraction methods used in microalgal proteomic research along with other information related to the respective proteomic approach. This information has been collated from published results since 2017 in order to cover contemporary efforts to measure microalgal proteomes.

Protein digestion and peptide clean-up

Bottom-up proteomics relies on protein identification through the analysis of peptides resulting from enzyme digests. The protein mixture in the sample is initially digested using proteolytic enzymes to obtain peptides to enable their analysis using mass spectrometry. Trypsin is the most commonly reported protease for protein digestion in mass spectrometry-based proteomics. However, trypsin is limited in that it does not cover the entire proteome as its access to cleavage sites can be constrained by tightly folded proteins, or hindered by PTMs, e.g., acetylated K or presence of glycans [36,37]. As such, alternative proteases may enable a more comprehensive assessment of the microalgal proteome. Such alternative proteolytic enzymes may include: Lys-C, Glu-C, Lys-N, Asp-N, and chymotrypsin; and nonspecific proteases, e.g., proteinase K, and elastase [38,39].

The choice of the proteolytic enzyme in algal proteomics relies on the abundance of the amino acids targeted by each enzyme. Arginine has been reported as a highly abundant amino acid in the protein sequences of microalgae [40]; and consequently, may affect the mass spectrometric identification of tryptic peptides. The high content of acidic amino acids, i.e., aspartic and glutamic acid in cyanobacteria [40], such as *Spirulina*, may suggest considering the use of Asp-N or Glu-C along with trypsin for protein digestion. Most algal proteomic studies have performed protein digestion using trypsin, nevertheless, a few studies have used the trypsin/Lys-C combination in *Dunaliella sp* [41], and sequential-digestion with Lys-C and trypsin in *Chlamydomonas reinhardtii* [42,43].

In microalgal proteomic studies, the most commonly used protein digestion method is performed in-solution using a single microtube after reduction and alkylation of proteins Supplementary (Table S1). After liberating peptides using this approach, peptide clean-up or peptide desalting is required. Peptide clean-up is accomplished by removing salts and buffers using different types of reversed phase resins, such as: C18, C8 or styrene-divinylbenzene resin (SDB), in stop-and-go-extraction tip (StageTip) or micro spin column formats [44]. Other standard approaches to sample preparation such as filter-aided sample preparation (FASP) have been less favored to date but are popular among the wider proteomics community.

Several novel protein digestion methods have been developed to process protein samples more rapidly and efficiently than the conventional processing methods. Suspension trapping (S-Trap) – the filter-based method- and phase-enhanced sample-preparation (SP3) – the paramagnetic bead-based approach are two examples of these recent processing methods [45,46]. The processing time required for centrifugation cycles in S-Trap, also available in 96-well filter plate, is reduced to less than 15 min [46].

Despite the increased adoption of in-solution and filter-based digestion methods in proteomic studies, most studies on algae continue to use in-gel digestion methods (Supplementary Table S1). Since 2017, 10% of studies that reported algal proteomics used two-dimensional gel electrophoresis (2-DE), which is considered a deprecated methodology due to its limited sensitivity to identify less abundant proteins, difficulties in the identification of non-water-soluble proteins such as membrane proteins, and poor representation of proteins of high or low molecular mass or isoelectric point [13,47]. More advanced methods of protein digestion such as S-trap and SP3 have been recently utilized in proteomic studies of *Phaeodactylum tricornutum* and *Symbiodinium tridacnidorum* [35,48]. Of relevance, a recent study described the evaluation of three methods of digestion – including on-filter digestion using FASP, SP3, and in-solution – using STAGE tips for sample clean-up in *Symbiodinium tridacnidorum* microalgae, wherein SP3 was highlighted as the best method with the highest robustness and digestion efficiency [35].

While the main reported protein processing methods are covered in this – and the previous – section, the paucity of knowledge in this space necessitates careful consideration of the efficiency of alternative and novel sample preparation methods for microalgal proteomic research.

Mass spectrometry-based analysis of microalgal proteins

Quantitative proteomics – label-free and isotopic labeling

Quantitative proteomics can be divided into two major workflows: label-free and label-based approaches. Label-free proteomics is based on: peptide sample preparation, sample separation by liquid chromatography (LC) and analysis by MS/MS, and finally data analysis including: peptide identification, quantification, and statistical analysis. In label-based techniques, peptides are commonly labeled using reagents with isobaric chemical structures that when fragmented in the mass spectrometer release reporter ions along with sequence information [49]. By extension, proteins can then be inferred with measures of relative abundance across the original samples. In addition to chemical tagging, metabolic labeling has also proven popular in the proteomics community, wherein isotopically labeled amino acids are used as a nutrient for cell and animal culture. The peptides resulting from tryptic digestion then demonstrate specific differences in their masses, which can be used for quantification, while fragmentation of these peptides provides sequence information [50,51]. Whereas chemical labeling approaches involve using chemical reactions to *in vitro* label specific chemical groups within amino acids.

Although label-free methods have been the most popular approaches in microalgal proteomics, iTRAQ has been the prevalent label-based method across a range of species, including: *Nostoc sp*., *Chlamydomonas reinhardtii*, *Chlamydomonas nivalis*, *Scenedesmus obliquus*, *Chlorella vulgaris*, and *Dunaliella salina* (Supplementary Table S1) [52–56]. Through iTRAQ labeling, the proteomic response of *Dunaliella salina* to salt stress uncovered the key role of photosynthesis and ATP synthesis for the modulation of early salinity-responsive pathways [56]. In another iTRAQ-based study of two species of *Chlamydomonas*, the molecular mechanisms of salt stress in triggering the fatty acid accumulation were revealed [53]. Therein, a decrease in the abundance of enzymes involved in the TCA cycle in salt-stressed *C. nivalis* was demonstrated to result in fatty acid biosynthesis. Moreover, the iTRAQ-based method attracted interest to investigate the molecular pathways responsible for fatty acid biosynthesis with the aim of biofuel production [53,57,58]. As an example of an iTRAQ-based method for the purpose of biofuel production is a study on the mechanism of lipid accumulation under low and high nitrogen in *Scenedesmus acuminatus*. The results of this study revealed that fatty acid synthesis and branched-chain amino acid metabolism were increased when the nitrogen supply is low for *S. acuminatus*. Some algal proteomic studies that used label-free shotgun proteomics using data-dependent acquisition (DDA) mode are discussed in section "Quantitative proteomics based on acquisition modes".

Quantitative proteomics based on acquisition modes

Depending on the MS instrumentation used, three different acquisition modes can be considered for proteome measurement: DDA, data-independent acquisition (DIA), and targeted proteomic modes, e.g., multiple reaction monitoring (MRM) [59] or parallel reaction monitoring (PRM) (Figure 1) [60]. For nearly the last three decades, liquid chromatography–tandem mass spectrometry (LC–MS/MS) in DDA mode, now widely known as shotgun proteomics, has been used for proteomic studies [61]. In DDA mode, the most intense precursor ions from a MS scan event are isolated and fragmented in a series of subsequent MS/MS scan events.

Targeted methods such as MRM and PRM are capable of reproducible and precise quantification of hundreds of proteins. However, the identities of these must be known in advance. Consequently, it is better suited to the validation phase of proteomics experiments rather than the discovery phase [62].

Advances in MS instrumentation and bioinformatics have resulted in the development of the DIA-MS approach. DIA can quantify thousands of proteins in complex samples, similar to DDA [59]. The first widely adopted DIA method was the sequential window acquisition of all theoretical fragment ion spectra (SWATH-MS) [63]. A wide range of product ions are scanned and generated by MSMS using broad isolation windows. As DIA repeatedly scans every peptide in a sample, a complex set of MS/MS data is generated that makes data interpretation difficult if there is no reference spectral library. Therefore, to quantify DIA data, DIA spectra are compared with spectral libraries, which are annotated peptide-spectrum matches from previous DDA experiments (or predicted synthetic spectra). The most widely used approach for spectral library generation is characterizing the samples of interest for SWATH-MS in initial experiments using a DDA approach [64]. However, using *in silico* spectral predictions resulting in DIA library-free approaches are progressing rapidly to perform library-free DIA data analysis [65–67].

DDA has been used in almost all published microalgal proteomics studies, while the DIA approach has been rarely reported [55,68–71]. In this regard, label-free

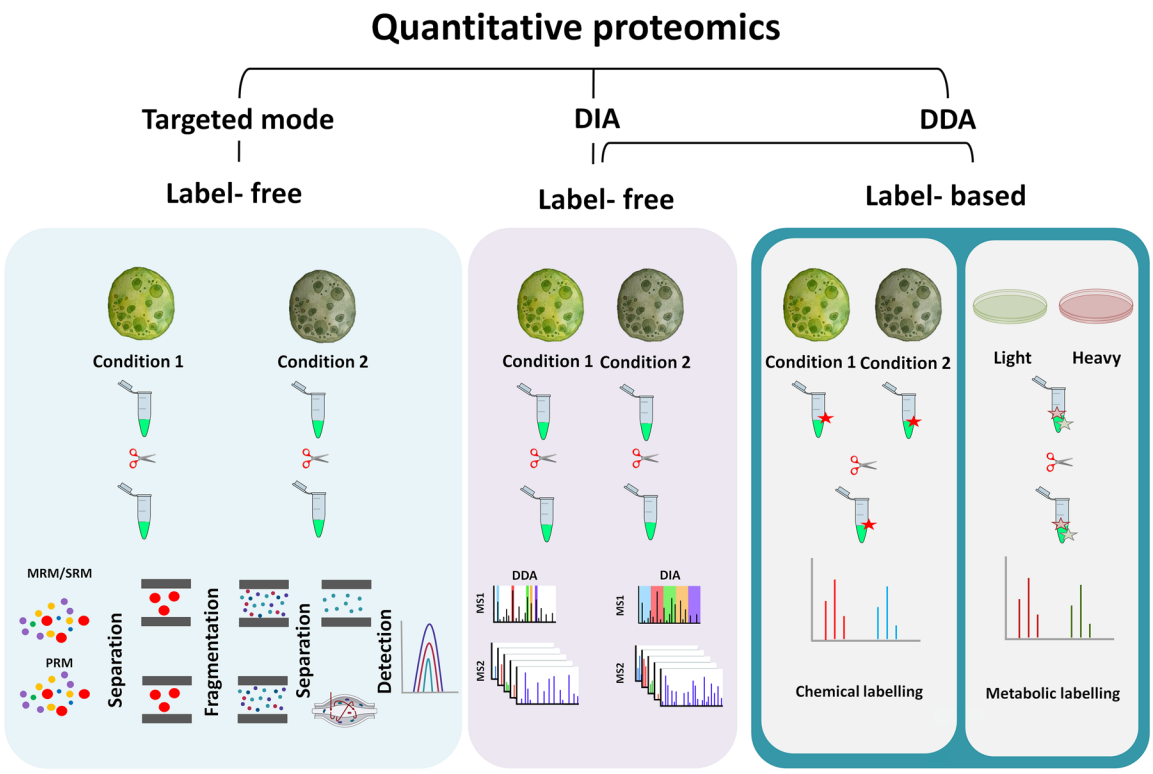

Figure 1. Workflow of three acquisition modes of quantitative proteomics. Quantitative proteomics is categorized by three acquisition modes: data-dependent acquisition (DDA), data-independent acquisition (DIA), and targeted proteomic modes (MRM and PRM). DDA mode can be categorized into two major groups of label-free and label-based approaches. In label-free methods, which are also used for DIA and targeted methods, the protein and peptide samples are prepared separately and then subjected to individual LC–MS/MS analysis. In label-based methods such as metabolic labeling and chemical labeling, depending on the method of labeling, the isotope labels are incorporated into the samples and several samples can be combined and analyzed in a single experiment.

shotgun proteomics has been mostly used to study the proteomic response of algae species to environmental perturbation, such as light stress, nutrient deficiency, etc. An example of this approach concerns the study of microalgae response to light wherein different CO_2 concentrations led to proteome reprogramming in *Nannochloropsis oceanica* [72]. In another example, the DDA method was used to reveal the high abundance of ammonia and urea transporters, as well as phosphokinases and phosphate transporters, in *Ostreococcus tauri* in response to low-nitrogen and low-phosphorus environments [73]. The membrane proteome of *Ettlia oleoabundans* was also studied using this method, showing the proteome response to nitrogen deprivation [74]. Photosynthesis-related proteins, including Photosystem II Subunit S (PSBS) and Maintenance of Photosystem II under High Light1 (MPH1), were identified as responsive to nitrogen deprivation, suggesting a similar photoprotective mechanism to prolonged nitrogen deprivation in microalgae to that found in higher photosynthetic organisms. A range of microalgae studies were performed in recent years using the DIA approach, highlighting the increasing application of this technique in microalgae research. In one recent study, changes in the proteome of *Chlorella sorokiniana* following cadmium exposure were investigated [68]. The suppression of photosynthesis and oxidative phosphorylation as well as the activation of photorespiration were uncovered in response to the presence of this heavy metal. This DIA study along with other algal proteomic studies that used this approach used DDA to generate DIA spectral libraries [55,68–70]. A DIA library-free approach has been only reported in one recent study regarding the nutrition, allergenicity, and physiochemical qualities of food-grade protein extracts in *Nannochloropsis oculata* [75].

Post-translational modifications

PTMs are chemical modifications of a protein that involves the addition of chemical moieties to the protein or elicits a structural change which is important in cell and biological processes as they play key roles in modulating protein function, activity, stability, and/or localization [76].

Among the wide range of PTMs, protein phosphorylation, acetylation, and ubiquitination are the most frequent PTMs. Phosphorylation plays a key role in the regulation of significant cellular processes, including: signal transduction pathways, replication, transcription, and response to environmental stresses. Acetylation also regulates various biological processes including protein–protein interaction, cell metabolism, and nuclear transport [77]. Ubiquitination serves as a critical regulator of substrate degradation, thereby contributing to the maintenance of cellular homeostasis and the essential functions required for sustaining life activities [78]. Protein methylation and glycosylation are also categorized among the top 10 major PTMs that shape biological processes [77].

Several studies on microalgae PTMs have reported phosphoproteomic alterations in response to changing environmental conditions such as nutrient stress [79–83]. Only a limited number of studies have investigated glycosylation pathways in *Chlamydomonas reinhardtii* [83–85], algal protein acetylation [86], and methylation [87].

It has been demonstrated that phosphorylation is an effective method of enhancing the functional properties of food proteins [88] and glycosylation can increase the solubility and global stability of proteins [89]. Apart from PTMs that are catalyzed by enzymes, numerous nonenzymatic PTMs (nePTMs) occur, some of which result from environmental or process factors rather than biological processes, such as: glycation, carbamylation, and carbonylation. NePTM formation is prevalent in food processing and storage [90,91]. Therefore, NePTMs are best studied and understood using proteomics.

Data analysis

Database creation

Microalgal proteomics studies have mostly focused on *C. reinhardtii* as the premier reference organism given its considerable genetic characterization [92]. Given the extensive number of different microalgae species, the whole genome sequence of only 105 microalgae species is publicly available which limits broad genomic and proteomic research. However, the growing availability of genomic sequences for non-model organisms is rapidly accelerating with the advancement of high-throughput sequencing technologies [93].

Sequence databases are an essential component of MS-based proteomic analysis in terms of protein identification. Genome availability of the species of interest influences the efficiency of protein identification. Incomplete genome databases may lead to missing protein identifications and hinder the ability to uncover novel proteins. Using a database search also offers the opportunity to identify a higher number of peptides compared to solely employing *de novo* methods, since the preexisting knowledge reduces the level of evidence required for a successful identification [94]. Using sequence repositories such as UniProtKB (https://www.uniprot.org), and NCBI (https://www.ncbi.nlm.nih.gov) provide access the proteomic resources for the target microalgae. In addition, algae-based databases such as Phycocosm (https://mycocosm.jgi.doe.gov/Algae/Algae.info.html), contain genomic information for more than one hundred algae species. However, when a sequence database of a non-model species is absent, cross-species identification, *de novo* genomic and transcriptomic analysis, and *de novo* protein sequencing can be used to assist with protein identification [95] (Figure 2). As a first step to generate a database for a non-model microalgae, retrieving sequences of closely related organisms from sequence repositories such as proteomic, genomic, and transcriptomic resources could be considered [97]. In the absence of genomic data, transcriptomic data represent a useful tool for MS-based protein identifications. The use of next generation sequencing technologies in RNA-seq for transcriptome characterization can provide a high-quality sequence database relatively cheaply and rapidly, so this approach has become very important in MS-based protein identification in non-model species [98]. In a membrane proteomic study of the non-model *E. oleoabundans*, a protein database was generated using translated transcriptome (RNA seq) of this microalga [74]. In the absence of genomic and transcriptomic databases, protein identification in non-model organisms relies on cross-species identification or cross-species protein sequence similarity [99]. Proteins can be identified by identification of conserved peptides of the proteins in model species, or another related species, but assembling such peptides into proteins can be problematic [100].

Peptide and protein identification in non-model algae strains can also be achieved by *de novo* sequencing of high-quality MS/MS spectra using *de novo* software packages such as PEAKS, but it is still not as efficient as peptide to spectrum matching using a genome sequence [101]. Overall, with a non-accessible genome sequence, using the above approaches allows proteomic analysis of any given species of interest. However, the results of protein identification in this case might not be as complete as those in model species. Using complementary databases such as Alga-PrAS

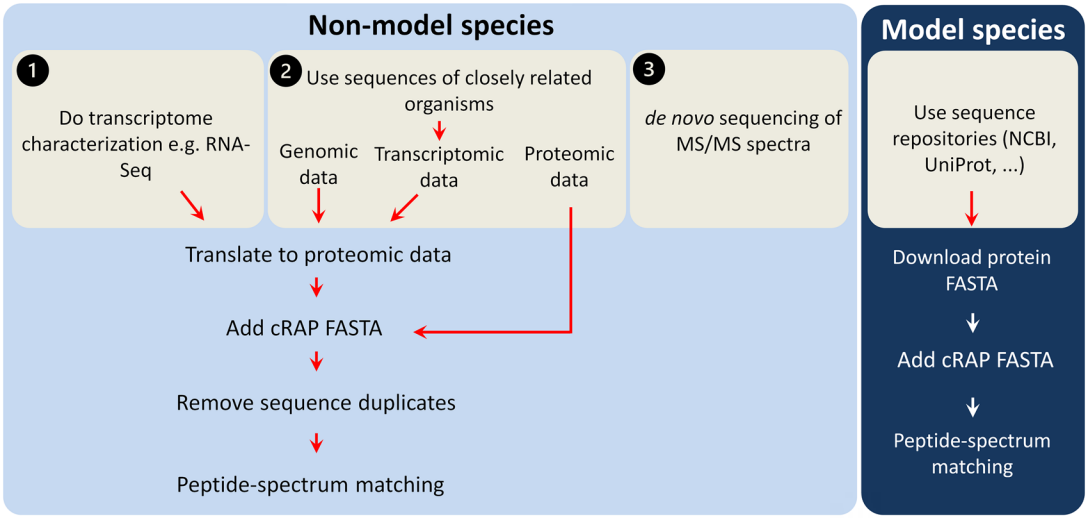

Figure 2. Workflow of database creation for model and non-model microalgae species. Common Repository of Adventitious Proteins (cRAP) in FASTA format is added to the microalgae of interest protein FASTA to include common protein contaminants. Sequence duplicates are removed using software packages, e.g., the open source tool SeqKit [96].

(Algal Protein Annotation Suite) allow the comparative analysis of physicochemical and structural properties and PTMs in algal proteomes. This database is accessible through http://alga-pras.riken.jp.

Identification of putative allergen proteins

Allergenic proteins can be found in a broad range of protein containing foods, which can cause immune responses in susceptible individuals. These allergic reactions cause biological disorders linked to gastrointestinal and respiratory tracts or skin [102]. For the most prevalent allergenic foods, such as: eggs, milk, fish, peanuts, soybeans, and wheat, extensive research effort has been undertaken to identify and quantify allergen proteins and discover marker peptides using proteomics [103]. However, little is known regarding the potential allergenicity of proteins from microalgae [104].

The combination of mass spectrometric analysis of algae to characterize peptides and use of *in silico* identification databases provides a novel/promising approach to better understand the potential allergenicity of microalgal proteins. In this regard, databases containing information for allergens are valuable tools that are publicly available at WHO/International Union of Immunological Societies (IUIS) (https://iuis.org), Allergome (https://www.allergome.org), UniProtKB (https://www.uniprot.org), Comprehensive Protein Allergen Resource (COMPARE) (https://comparedatabase.org), and Food Allergy Research and Resource Program (FARRP) (https://farrp.unl.edu). There are several programs (and accompanying web servers) such as AllerCatPro [105], AllergenPro [106], and Allermatch [107] for helping researchers to extract the allergenicity information from various databases and predict the allergenicity of proteins of interests.

The information acquired through *in silico* analysis helps to identify conserved homologous proteins that may be cross-reactive with known allergens. Cross reactivity can be predicted based on their primary sequence homology, structure, and presence of B- or T-cell epitopes in the sequence [104]. However, identification of IgE antibodies to proteins is not necessarily connected to clinical allergy. Hence, many other factors can trigger clinical cross-reactivity including food protein characteristics such as the stability of the allergen protein against pH, protease digestion or heat, immune response such as IgE antibody affinity, and host (patient) factors such as illness [108].

There is a lack of information for microalgae allergens in the WHO/IUIS and other allergen databases as research into identifying allergenic proteins in microalgae, along with experimental data like serum IgE binding tests, is currently insufficient. Only a few studies have investigated the allergic reactions after microalgae intake [109,110]. Furthermore, these studies are limited to a small number of commercially available species such as *Chlorella* and *Spirulina*. A case report showed that a 11-year-old boy who consumed *Chlorella* tablets (2 g/day) for 3 months developed acute tubulointerstitial nephritis [111]. *Spirulina* intake was also shown to cause systemic allergic symptoms such as shortness of breath, urticaria in two patients who also report mild oral reactions to some fresh fruit and raw vegetables [110]. The identification of microalgae

putative allergens has been reported recently for *Chlorella* and *Spirulina* [33]. Using shotgun proteomics and bioinformatic analysis of sequence-based homology between microalgae and known allergenic proteins, Bianco et al. [33] identified several putative allergens. Thioredoxins, superoxide dismutase, and C-phycocyanin beta-subunit were identified in *Spirulina* and calmodulin and troponin C were found in *Chlorella*; however, immunochemical tests are still required to validate the allergenicity of those proteins. Several putative inhalation-related, contact and food allergenic proteins were also identified using *in silico* methods in *Nannochloropsis oculata* [75]. MS identification of putative allergenic proteins in other microalgae specie should be considered along with human clinical studies and quantitative measurement of immunoglobulin E (IgE) upon ingestion of the microalgae of interest.

Media composition and environmental conditions can affect metabolic pathways

Proteomic methods have been used to understand how the algal proteome is modified by a range of environmental factors. These include CO_2 concentration, macronutrients, and light, which are reported to result in differing proteome response, amino acid, and total protein content of the biomass [112–114]. Much of the previous proteomic focus has been on understanding conditions that favor lipid production, particularly for biofuel generation [53,79,114,115].

Carbon dioxide levels have a significant impact on protein content and algae biomass [114,116]. The increase in growth rate and extracellular protein composition of *Chlamydomonas reinhardtii* were reported in response to increased concentration of CO_2 in algal culture [117]. Wei et al. showed that exposing *Nannochloropsis oceanica* culture to high level of CO_2, i.e., 50 000 ppm, resulted in higher protein content but no change in carbohydrates and lipid content. Using proteomics as well as transcriptomics revealed that at low CO_2 conditions, there was lower abundance of protein synthesis-related gene products at the transcript- and protein levels, which supports the observation of lower protein content. In addition, low CO_2 (100 ppm) decreased the abundance of several EAAs such as valine and isoleucine whereas non-EAAs, such as glycine, alanine, and serine, increased [114]. Although high levels of CO_2 can increase the biomass and protein content, carbon concentrating mechanism (CCM) activity declines in non-tolerant algae species as a result of exposure to high CO_2 [116]. Due to CCM shutdown, the biomass and the final protein content is adversely affected. The transcript knockdown carbonic anhydrase 2 (CA2) – one of the key components of CCM – in *Nannochloropsis oceanica* showed a greater photosynthesis rate and biomass production in high CO_2 when compared to the wild-type strain [118].

Nitrogen and phosphorus limitation act as effective pressure factors allowing the accumulation of lipids, such as triacylglycerol, in microalgae [119–122]. Thus, most omics studies have been conducted from the biofuel production perspective to analyze the response of microalgae to nitrogen and phosphorus deprivation. Cai et al. demonstrated that the low C/N ratio (12:1) was beneficial to the synthesis of glutamate in heterotrophic cultivation of *Chlorella vulgaris*, with the biomass productivity of 0.90 g/L/day, protein content of 61.6% with EAAs at 41.8% [123].

The accumulation of lipids in nitrogen and phosphorus deprived cells does not favor protein and amino acid quantity. Proteins as intracellular nitrogen storage molecules are targeted to degradation in nitrogen-deprived cells and lipids are accumulated in the cells as energy storage molecules [120,124]. The decrease in abundance of tRNA synthetases, translation initiation and elongation factors in proteomic studies of algae species may confirm the reduced protein biosynthesis of nitrogen-depleted cells [120,121]. However, less efficient carbon fixation and energy supply constraints in phosphorus-deprived cells cause a reduction in protein content [122]. Ribosomal proteins and those with functional domains that are expected to be altered in reduced growth conditions decreased in abundance in P-deprived *Scenedesmus* sp. cells [125]. Chen et al. demonstrated that the abundance of those proteins involved in photosynthesis, chlorophyll and protein biosynthesis decreased in *Thalassiosira pseudonana* grown in nitrogen and phosphorus-deficient cultures and shed light on the metabolic pathways and associated cellular functions in responses to stress at the proteome level.

Sunlight plays a crucial role in microalgal growth with the amount of light directly influencing biomass productivity. In raceway ponds, geographic location is especially important in defining the amount of light supplied and subsequent biomass productivity of the microalgae [126]. The intensity and wavelength of light also affect protein quantity and EAA content of microalgae [127,128]. In *Chlorella ellipsoidea* protein content was raised when exposed to blue light from LEDs compared to white, green, and red LEDs [128]. Protein quantity in *Dunaliella salina* was slightly higher in both blue and red than white light with no change in EAA content [127]. In complementary work, a label-based proteomic study of *Nannochloropsis oceanica* showed

higher carotenoid metabolism and ROS scavengers in response to red light when compared with blue light [129].

Microalgae exhibit flexibility in their metabolic modes, employing photoautotrophy, heterotrophy, and mixotrophy based on factors such as light availability and carbon supply [130]. Proteome and transcriptome measurements of *Chlorella vulgaris* were performed during the autotrophy-to-mixotrophy-to-heterotrophy transition [131]. The increased growth rate that was observed during mixotrophy was suggested to be linked to enhanced stress tolerance via inositol and increased resistance to oxidative stress through thioredoxin modulation [131].

In addition to studying the impact of growth medium on either protein quantity/quality or biosynthesis of a particular peptide/protein, the molecular mechanisms of microalgae tolerance to environmental factors can be explored through proteomics. The importance of the microalgae tolerance to environmental factors, such as temperature, is more valuable when cultivation is carried out in outdoor environments. Using iTRAQ-based quantitative proteome measurement, Li et al. [132] demonstrated the response of *Spirulina platensis* to low temperature. This study confirmed the suppression of protein synthetic machinery in *Spirulina* exposed to low temperature of 15 °C.

High salinity is another environmental stressor that may affect lipid accumulation in a similar way to that seen following nitrogen deprivation [53,133]; however, protein content and proteome responses to salt stress vary depending on the algae species and their habitat. For example, total protein content increased slightly in *Tetraselmis chuii* in 40 ppt salt compared to control conditions. At the same time, it decreased significantly with an increased salt concentration in *Chlorella vulgaris* [134]. The green microalgae *Dunaliella salina* is a model for investigating the molecular adaptation mechanisms in salt stress as it is the most tolerant photosynthetic unicellular eukaryotic organism to salinity [135]. *Dunaliella salina*'s enormous ability to tolerate salinity and being a good source of beta-carotene make this microalgae a valuable organism for both research and industrial purposes. Although several proteomic studies investigated the salt tolerance mechanisms in *Dunaliella salina* [56,81,136], there is still little known regarding the potential of this species to produce protein for human food applications.

Peptidomics: bioactive peptides in microalgae

Peptidomics is a sub-field of proteomics that qualitatively and quantitatively analyze the peptides in biological samples including bioactive peptides in food matrices [137]. After protein extraction from microalgae strains, peptides may be intrinsic or generated either by using one or multiple gastrointestinal enzymes, such as trypsin, pepsin, papain, etc., or food-grade enzymes such as alcalase and flavourzyme or microbial enzymes through *in situ* microbial fermentation [28,29,138,139]. The subsequent quantification and identification of peptides are performed using different methods of mass spectrometric techniques, as described previously, and using relevant bioactive databases such as BIOPEP or PepBank databases [140,141]. Native bioactive peptides can be extracted by utilizing molecular weight cutoff (MWCO) filters [142,143]; however, peptidomic studies in microalgae have focused on using gastrointestinal enzymes for peptide enrichment rather than investigating the native peptides in algal protein extracts.

Potential bioactive peptides were identified in a study on *Tetradesmus obliquus*, four of bioactive peptides were synthesized and assessed *in vitro*, demonstrating a promising rate of antioxidant and angiotensin-converting enzyme (ACE) inhibitory activities [139]. Using food-grade extracts from *Auxenochlorella pyrenoidosa*, three novel antioxidative peptides were identified [28]. Although these initial findings highlight the potential functional health effects for algae derived bioactive peptides, further research is needed to establish whether algae-derived peptides are bioavailable and exert biological activity following transit of the gastrointestinal tract. In a study by Sommella et al. [144], the peptides originated from *in vitro* digestibility method were analyzed in *Spirulina*. In this study, phycocyanin-derived peptides were identified in microalgal protein digests.

Future directions

In 2019, the global cultivation of microalgae was estimated at 56,456 tonnes across 10 countries, which is less than 0.2% of the total global cultivation of seaweed according to the FAO (Food and Agriculture Organization). Although there is currently substantial growth in the production and sales of microalgae-based food products [145] there remains huge potential, especially when compared to other related markets, such as algal production of lipids, carotenoids, and pharmaceutical proteins, which has received extensive research interest for more than two decades [146]. Multi-omics techniques have become increasingly prevalent for studying microalgal lipid synthesis, and genetic and metabolic engineering approaches have been utilized to enhance lipid production in microalgae for strain selection and enhancement. These omic studies, particularly MS-based proteomics, are essential for identifying microalgal

species best suited for producing high-quality protein ingredients for human dietary purposes.

Although 2-DE is a near-deprecated technique, there remain microalgae researchers that continue to use this technique in their investigations. Given the rapid advancement in proteome science, the use of novel sample preparation and protein digestion methods should be considered before MS analysis of microalgae peptides. In addition, the explosion of genome sequencing using high-throughput technologies is already resulting in reproducible, rapid, and comprehensive protein identification. Additionally, proteogenomic approaches are aiding the detection of novel protein sequences through advanced sequencing technologies [147].

Sample preparation throughput has long been a challenge in the field of proteome science, but pressure cycling technology (PCT) has recently emerged to substantially reduce sample preparation time. One example of PCT, PCT-SWATH, has been successfully used with tissue biopsy samples where it reduced sample preparation time to <6 h for 16 samples [148–150]. However, the use of PCT-SWATH with plant and microalgae samples still requires evaluation and optimization. Analytical throughput can also be enhanced by high-flow chromatography applied to short-gradient proteomics, e.g., 5 min [151,152]. Using a 5 min LC gradient and SWATH-MS method enabled Messner et al. [151] to precisely quantify the proteome of 180 human plasma samples per day. The ultra-high throughput proteomic method could be revolutionary for the advancement of the microalgal industry as it has the potential to enable screening of a wide range of microalgae species and in the genetic diversity in support of strain selection and optimization of growing conditions. Ultimately, these advancements can significantly increase biomass production, leading to the potential production of substantial amounts of dietary protein, EAAs, and bioactive peptides.

Conclusions

Microalgae has great potential as a source of dietary protein. Understanding the nutritional properties of algal proteins from a broad range of species, their functional health benefits and safety are needed to broaden their application and use. Current algal proteome investigations – specifically from a nutrition point of view – are limited in scope and many technical challenges remain. Shifting from outdated proteomic methods to modern techniques as well as developing genomic resources and optimized strains are key initiatives that are needed to accelerate the potential use of microalgae as a source of high-quality dietary protein for the population at large.

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