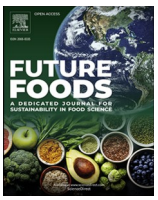
Future Foods 9 (2024) 100368

Contents lists available at ScienceDirect

Future Foods

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



Alkaline solubilization of microalgal protein and its impact on the functional properties of protein extract

Jun Wei Ng [a], Tong Mei Teh [a], Chee Fan Tan [b], Xuezhi Bi [b], Zhi En Low [a], Md. Mahabubur Rahman Talukder [a,*]

[a] Singapore Institute of Food and Biotechnology Innovation (SIFBI), Agency for Science, Technology and Research (A*STAR), 138669, Singapore
[b] Bioprocessing Technology Institute (BTI), Agency for Science, Technology and Research (A*STAR), 138668, Singapore

ARTICLE INFO

Keywords:
Chlorella vulgaris
Protein extract
Alkaline solubilization
Microalgae protein
Functional properties

ABSTRACT

Microalgal alternative proteins are increasingly recognized as a sustainable and promising source for future food production. Despite the widespread use of alkaline solubilization in protein extraction, its influence on the functional properties of microalgal protein extracts has received limited attention. This study marks the pioneering effort to assess the impact of alkaline (NaOH) solubilization on both the extraction process and the functional properties of *Chlorella vulgaris* protein extract (CVPE) obtained through isoelectric precipitation. With the gradual escalation of alkaline concentration from 0 to 0.7 M, there was a notable improvement in protein extraction efficiency, increasing significantly from 6.22 % to 56.23 %. However, elevated NaOH concentrations resulted in protein degradation, as evidenced in SDS-PAGE studies. Alkaline solubilization exhibited a positive impact on various functional properties of CVPE. It enhanced solubility, water absorption capacity, foaming capacity and stability, as well as emulsifying activity and stability. However, there was a slight decrease in oil absorption capacity with increasing NaOH concentration. Notably, the maximum solubility, oil adsorption capacity, foaming capacity, and emulsion activity of CVPE were 2.3-, 1.8-, 2-, and 1.3-fold higher, respectively, than those of commercial soy protein isolate (SPI). This highlights the effectiveness of alkaline solubilization in modifying *C. vulgaris* protein and emphasizes the high potential of CVPE as a functional food ingredient.

1. Introduction

In response to the growing global demand for sustainable and nutritious protein sources, microalgae have emerged as a promising alternative to address the challenges of conventional protein production as well as to create a greater food security and resilience worldwide. Microalgae have been substantiated as a valuable source of various biological components, encompassing carbohydrates, lipids, vitamins, pigments, and polyphenols, with proteins being particularly noteworthy (Spolaore et al., 2006; Mata et al., 2010). Specifically, the protein content of microalgae constitutes a substantial proportion when compared to lipids and carbohydrates (Finkel et al., 2016). Notably, certain species of microalgae exhibit protein quantities and qualities comparable to those found in conventional protein sources such as milk, beef, soybeans, and eggs (Gouveia et al., 2008).

Among the microalgae species, *Chlorella vulgaris* has attracted attention due to its rapid growth, substantial protein content, and excellent adaptability to diverse environmental conditions (Tomaselli, 2008). Notably, *C. vulgaris* can accumulate a total protein content ranging from 42 % to 58 % of its dry weight, depending on the growth conditions (Safi et al., 2014a). This makes *C. vulgaris* a promising sustainable source of protein. However, the rigid cell wall of *C. vulgaris* poses a challenge, leading to low intracellular component availability and hindering efficient protein extraction (Morris et al., 2008; Shelef and Soeder, 1980). Additionally, the in vitro protein digestibility of crude biomass from *C. vulgaris* remains limited, ranging from 12 % to 41 % (Becker, 2007; Kose and Oncel, 2015; Shelef and Soeder, 1980).

To overcome the aforementioned limitations, it is essential to implement effective cell wall disruption and protein solubilization techniques for extracting *C. vulgaris* protein with an industrially relevant yield. A diverse range of cell disruption methods, including mechanical actions such as bead milling and high-pressure cell disruption, ultrasound and sonication, as well as enzymatic and chemical (alkaline) treatments, have been proposed for microalgae protein extraction.

* Corresponding author.
E-mail address: rahman_talukder@sifbi.a-star.edu.sg (Md.M.R. Talukder).

https://doi.org/10.1016/j.fufo.2024.100368
Received 22 November 2023; Received in revised form 22 April 2024; Accepted 8 May 2024
Available online 9 May 2024

However, the choice of extraction method significantly influences the quality and functionality of the extracted protein, necessitating the development of improved extraction methods (Yu et al., 2007; Safi et al., 2014a). For example, the selection of NaOH concentration or pH during alkaline treatment can have a substantial impact on the protein structure and functional properties (Hou et al., 2017; Mir et al., 2019a). In a recent study, it was observed that *C. vulgaris* protein, extracted using a high-pressure cell disrupter and alkaline treatment, exhibited distinct emulsifying capacities at lower pH (pH 7) compared to higher pH (pH 12) (Ursu et al., 2014). Additionally, Garcia et al. (2018) found that protein extracted from microalgae through bead milling demonstrated superior foaming and emulsification properties when compared to commercial whey protein isolate. Furthermore, Mahali and Sibi (2019) investigated the protein from *A. platensis* microalgae extracted using ultrasound, revealing enhanced solubility and improved foaming capacity compared to protein extracted through alkaline methods. Hence, it is crucial to conduct comprehensive research on the functional properties of microalgae protein extracted through various methods, with a specific emphasis on the impacts of alkali concentration. This research is vital since the successful integration of these proteins into food products greatly relies on understanding and optimizing their functionality. While studies have reported the functional properties of microalgae protein extracts at specific pH levels (Schwenzfeier et al., 2013; Waghmare et al., 2016; Garcia et al., 2018; Chen et al., 2019), the effects of different alkaline concentrations on microalgae functional properties remain unexplored. Moreover, many previous papers have focused solely on individual functionalities, such as emulsion, foaming, or solubility, leaving other properties unexplored.

The objective of this study was to investigate the impact of varying NaOH concentrations on distinct functional properties of *C. vulgaris* protein. The protein extraction process involved a two-step approach, consisting of dry milling followed by alkaline (NaOH) solubilization. Alkaline solubilization was chosen because it is widely applied for commercial extraction of various plant proteins like soy and pea proteins, owing to its effectiveness in protein solubilization, cost-efficiency and versatility. Hence, this study focused on the influence of NaOH concentration on solubilization yield and its impact on the various functional properties, including solubility, water and oil absorption capacity, foaming capacity, foam stability, emulsifying capacity, and emulsion stability, were evaluated for the freeze-dried protein extract (CVPE) obtained through solubilization with different NaOH concentrations. Commercial soy protein isolate was selected as a benchmark for comparing functional properties with CVPE due to its extensively documented functional properties and wide-ranging applications (Singh et al., 2008). This comparison could provide valuable insights into the potential of *C. vulgaris* protein for practical food product development. The findings offer insights into *C. vulgaris* protein solubilization and the functional characteristics of the protein extract, potentially addressing the global demand for sustainable protein sources and their adaptable use in various food applications.

2. Materials and methods

2.1. Materials

The dried microalgae *Chlorella vulgaris* biomass was purchased from GO SUPERFOODS LTD (UK) and used without any treatment. Commercial soy protein isolate (SPI) was obtained from Bulk Powder company (Australia) via our collaborator Ridded Institute, New Zealand. The protein content in *C. vulgaris* biomass and SPI was about 54 and 84 %, respectively as measured by the Dumas method. Bradford reagent, bovine serum albumin (BSA), soybean oil and sodium dodecyl sulphate (SDS) were from Sigma-Aldrich. All other chemicals and solvents utilized in the study were of analytical grade.

2.2. Preparation of *C. vulgaris* protein extract

Microalgae protein was extracted from dried *C. vulgaris* biomass by a 2-step process. Firstly, the bead milling of dried biomass was performed to facilitate the disruption of *C. vulgaris* cell wall. The dried *C. vulgaris* biomass was loaded into a zirconium oxide grinding jar containing 0.5 mm diameter zirconium beads, and milled with Retsch Mixer Mill MM 400 (Retsch, Germany) at a frequency of 26 Hz for 6 × 10 min with 5-minute break interval.

Secondly, the bead milled sample was then subjected to alkaline (NaOH) solubilization followed by isoelectric precipitation and freeze drying. In brief, the sample was suspended in sodium hydroxide (NaOH) solution of five different concentrations (0.03–0.7 M), at a solid-liquid ratio of 1:20. The suspension was then stirred magnetically at 37 °C for 1 hour. The suspension was centrifuged at 8000 rpm for 20 min, and the supernatant was collected. The supernatant was adjusted to pH 4.0 with concentrated HCl for isoelectric precipitation of solubilized protein. The protein precipitate was collected by centrifugation (4000 rpm, 10 min), and subsequently adjusted to pH 7 for neutralization before freeze drying. Freeze drying was conducted using freeze dryer (Labconco, United States) set to dry at −56 °C and 0.021 mbar. The freeze-dried protein was stored for subsequent analysis. The solubilized protein in the liquid sample was measured using Bradford assay while the protein content of the freeze-dried *C. vulgaris* protein extract (CVPE) and commercial SPI were determined with Dumas method. The protein solubilization yield was calculated using following formula:

$$\text{Protein solubilization yield (\%)} = \frac{\text{Protein in supernatant}}{\text{Total protein in original biomass (54\%)}} \times 100$$

2.3. Determination of protein content in liquid sample

The solubilized protein in the liquid sample was determined by Bradford assay (Bradford, 1976) as used by other researchers for microalgae protein (Chia et al.,2019). In brief, 50 μL of sample was mixed with 1.5 mL of Bradford reagent and incubated for 10 min at room temperature. Subsequently, 250 μL of mixture was added to a 96 micro-well plate and absorbance at 595 nm was measured. Protein was quantified using calibration curve prepared using BSA as standard.

2.4. Determination of protein content in dry sample

The total protein content of the freeze-dried CVPE and commercial SPI were measured using Dumas combustion method in DUMATHERM instrument (Gerhardt Analytical Systems, Germany). A nitrogen-to-protein conversion factor of 6.25 was used (Chen et al., 2019). The protein content in freeze dried sample was about 50–67 %.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The electrophoretic profile of the CVPE in supernatant was determined following the method of Laemmli (1970) using 12 % precast polyacrylamide gel (Bio-Rad, United States). Samples were mixed with 1 % SDS, 100 mM Tris -HCl to solubilize the protein, followed by heating at 95 °C for 10 min. The samples were then mixed with 2X Laemmli sample buffer containing 50 mM dithiothreitol (Bio-Rad, United States). Sample, containing 30 μg protein, was loaded on the gel, and the electrophoresis was carried out at a constant voltage of 140 V using a Mini-PROTEAN Tetra Cell (Bio-Rad, United States) for 30 min. Gel was stained with Coomassie Brilliant Blue R-250 and destained using a mixture of methanol, acetic acid and deionized water in the proportion of 50/10/40 (v/v/v), respectively. The molecular weight of the proteins was measured using a prestained protein ladder (10–250 kDa).

2.6. Determination of functional properties

2.6.1. Solubility

Solubility of CVPE was assessed following the protocol outlined by Ma et al. (2018). Briefly, the extract was suspended in deionized water (pH 6.83) to a final concentration of 100 mg/mL. The mixture was vortexed, and then shaken (250 rpm) for 1 hour at 22–24 °C. It was then centrifuged for 10 min at 14,500 rpm. The protein content in the upper layer was quantified using Bradford assay as described in Section 2.3. Protein solubility was calculated according to the following equation:

$$\text{Protein solubility } (\%) = \frac{\text{Supernatant protein content}}{\text{Total protein content}} \times 100$$

2.6.1. Water absorption capacity (WAC)

WAC of CVPE was measured based on the procedure specified by American Association of Cereal Chemists Technical Committee (1981). Briefly, deionized water was added incrementally to 0.25 g of sample (W1), and the mixture was vortexed vigorously to produce a homogenous paste. The sample mix was kept for 80 min at room temperature, followed by centrifugation at 10,000 rpm for 1 hour. The free water droplet was removed, and the slurry weight (W2) was recorded. WAC of CVPE was determined by the following equation:

$$\text{WAC } (\%) = \frac{W2 - W1}{W1} \times 100$$

2.6.2. Oil absorption capacity (OAC)

OAC was assessed according to the method reported by Du et al. (2018). In brief, 0.5 g of the protein extract (W) were added with 5 mL of soybean oil (W1) and the mixture was vortexed for 2 min and then shaken at 200 rpm for 30 min. After the centrifugation at 10,000 rpm for 1 h, the weight of free oil (W2) was recorded. OAC was determined according to the equation below:

$$\text{OAC } (\%) = \frac{W1 - W2}{W} \times 100$$

2.6.3. Foaming capacity (FC) and foam stability (FS)

The foaming properties of the proteins were assessed using the procedure reported by Timilsena et al. (2016). Briefly, 10 mL of 0.5 % (W/V) sample was homogenized (IKA, Germany) for 1 min at 10,000 rpm. The sample volume before (V1) and after (V2) homogenization were determined. The homogenized sample was settled at 22–24 °C for 10 min and the sample volume (V3) was measured. FC and FS were expressed as follows (Yuliana et al., 2014):

$$\text{FC } (\%) = \frac{V2 - V1}{V1} \times 100$$

$$\text{FS } (\%) = \frac{V3 - V1}{V2 - V1} \times 100$$

2.6.4. Emulsifying activity index (EAI) and emulsion stability index (ESI)

EAI and ESI of the protein extract were measured with a modified method by Pearce and Kinsella (1978). In brief, 6 mL of the protein solution (1 mg/mL) was mixed with 2 mL of soybean oil. The mixture was homogenized for 2 min at 10,000 rpm (IKA, Germany) to form an emulsion. A total of 50 μL of the emulsion was added into 5 mL of 0.1 % SDS solution at 0 min and 10 min, respectively. The absorbance of the mixture at 0 min and 10 min were assessed at 500 nm using a spectrophotometer (Shimadzu Co., Japan). EAI and ESI were determined as follows:

$$\text{EAI } (m^2/g) = \frac{2.303 \times A_0 \times 2 \times d}{L \times 10000 \times \varphi \times C}$$

$$\text{ESI } (\text{min}) = \frac{A_0 - \Delta t}{A_0 - A_{10}}$$

where A_0 and A_{10} refer to the absorbance of solution at 0 and 10 min, d refers to the dilution factor ($d = 100$), L refers to the pathlength of cuvette ($L = 1$ cm), φ refers to the oil volume fraction ($\varphi = 0.25$), C refers to the initial protein concentration ($C = 0.001$ g/mL), and Δt refers to the sampling interval ($\Delta t = 10$ min10 minutes).

2.7. Statistics

Each experiment was conducted in triplicate under identical conditions, and the data were reported as mean ± standard deviation. Error bars on charts represent 0–8 % of the mean value of the data. SPSS software (IBM, USA) was used to perform one-way analysis of variance (ANOVA) with Turkey's multiple comparison tests to determine significant difference between the data collected at 95 % confidence level (p-value < 0.05).

3. Results and discussion

3.1. Effect of NaOH concentration on Chlorella vulgaris protein solubilization

The solubilization yield of protein from *C. vulgaris* broken biomass is shown in Fig. 1. The yield was found to be significantly improved (2-fold) when NaOH concentration increased from 0.03 M to 0.1 M, after which it increased in a slower pace up to 0.7 M at which about 56.0 % of *C. vulgaris* protein was solubilized. These findings align with Safi et al.'s (2014b) observations, revealing a substantial enhancement in protein yield (33.0 %) from *C. vulgaris* through alkaline treatment in comparison to water-based extraction (10.0 %). Additionally, Juul et al. (2023) and Stack et al. (2018) illustrated that employing alkaline extraction with a high pH value (pH 12), rather than low pH extraction, could result in a twofold increase in protein extraction yield for *Saccharina latissima* seaweed and *Phaeodactylum tricornutum* microalgae, respectively.

The data presented in Fig. 1 indicates a notably low protein solubilization yield at both neutral (pH 6.83) and alkaline (pH 9 with 0.0001 M NaOH) conditions, approximately 6.0 %. This suggests that bead milling alone, employed for physical cell disruption, was ineffective in liberating *C. vulgaris* protein from the cell biomass into the aqueous phase. Consequently, a higher NaOH concentration (a stronger pH) became necessary to lyse the cells and solubilize the protein. However, it is noteworthy that elevated NaOH concentrations tend to induce protein aggregation due to denaturation. The increase in protein solubilization yield therefore slowed at higher NaOH concentration (> 0.1 M). The elevated NaOH concentration might induce protein hydrolysis, resulting in the formation of peptides, thereby potentially altering the protein's functionality. Consequently, the proteins solubilized with varying NaOH concentrations were recovered and subjected to analysis, including examination of their molecular weight (size) and functional properties.

3.2. Effect of NaOH concentration on molecular weight (protein size) of C. vulgaris protein extract

The *ris* protein extract (CVPE) after solubilization with different NaOH concentrations was determined by SDS-PAGE analysis. It can be seen from Fig. 2 (SDS-PAGE) that *C. vulgaris* protein composed of diverse protein and the molecular weight of the protein extract (Lane 2, 0 M NaOH) was mainly distributed over the range of 10–18 kDA, 23–27 kDa and 40–60 kDa. Notably, the protein bands with higher molecular weight (> 15 kDa) exhibited a gradual diminishing trend in response to increasing NaOH concentration. This alteration in the protein profile implies that the proteins of *C. vulgaris* underwent conformational changes and initiated degradation, particularly pronounced at higher NaOH concentrations (> 0.1 M) (Lane 4–8). The observed protein degradation/hydrolysis is likely attributable to a robust interaction with NaOH, which has the potential to unfold the protein by disrupting

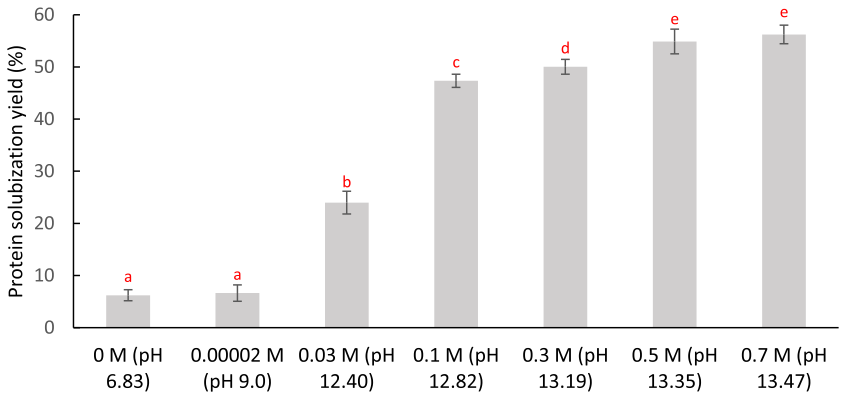


Fig. 1. Effect of NaOH concentration on solubilization of protein from bead milled *C. vulgaris* biomass. Different letters indicate the significant difference between two mean values (p-value < 0.05).

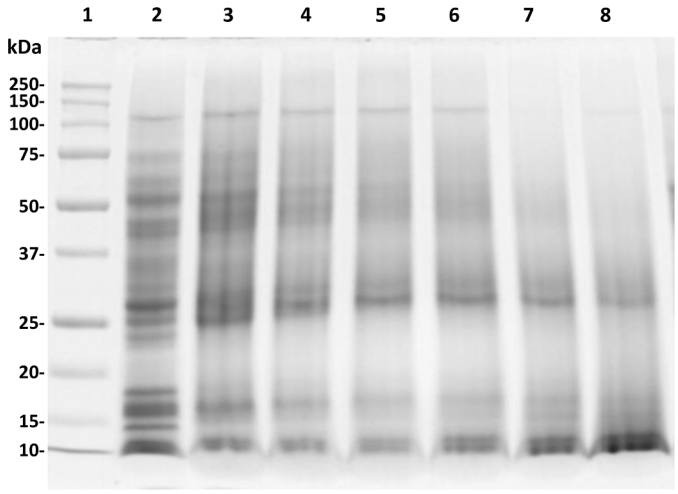


Fig. 2. SDS-PAGE of *C. vulgaris* protein extract (CVPE) obtained by different concentration of NaOH, Lanes: (1) molecular weight marker, with sizes in kDa indicated on the left, (2) 0 M, (3) 0.1 M, (4) 0.2 M, (5) 0.3 M, (6) 0.4 M, (7) 0.6 M, (8) 1.0 M.

hydrogen bonds and hydrophobic interactions, as suggested by Zhao et al. (2014), ultimately breaking peptide bonds that maintain the amino acids' linkage in the protein structure. This finding emphasizes the importance of fine-tuning NaOH concentration to uphold the integrity, functionalities, and structural properties of *C. vulgaris* proteins.

3.3. Effect of NaOH concentration on functional properties of *C. vulgaris* protein extract

3.3.1. Protein solubility

The solubility of proteins serves as a crucial parameter influencing additional functionalities such as emulsifying, foaming, or gelling properties (Kiosseoglou et al., 2021). The impact of varying NaOH concentration during protein extraction on solubility of CVPE in water was illustrated in Fig. 3. The protein extracted with 0.5 M NaOH showed maximum solubility of 64.49 %, about 2.3-fold higher than that of SPI (28.76 %). The protein extracted using 0.5 M NaOH showed a maximum solubility of 64.49 %, surpassing that of SPI by about 2.3-fold (28.76 %). Although there is no data on microalgae protein, similar observation was also reported in the previous study by Ayim et al. (2018) in which the solubility of tea protein residue increased with increasing NaOH concentration up to 0.12 M and dropped at higher concentration (0.15 M). The influence of extraction pH (NaOH concentration) on protein extract solubility is reported to be case-dependent. While extreme pH treatment (pH 12) has been reported to enhance the solubility of pea protein isolate (Jiang et al., 2017), it can adversely impact the solubility of soy protein extract (Jiang et al., 2009).

Broadly, an elevation in extraction pH or NaOH concentration tends to modify the protein configuration, leading to a disturbance in the equilibrium between protein-water and protein-protein interactions, consequently influencing solubility (Cui et al., 2020). Although additional research is needed to fully understand the influence of NaOH concentration on the solubility of *C. vulgaris* protein extract, the SDS-page results (Fig. 2) suggests that the increase in NaOH

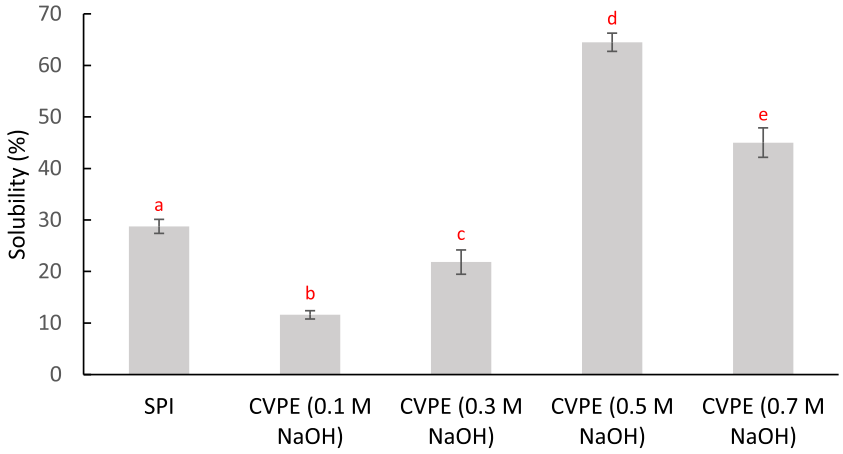


Fig. 3. Solubility of soy protein isolate (SPI) and *C. vulgaris* protein extract (CVPE) extracted by different NaOH concentrations. The detailed conditions of protein extraction were described in Materials and Methods section. Different letters indicate the significant difference between two mean values (p-value < 0.05).

concentration during extraction resulted protein degradation to smaller molecular weight. These smaller fragments may exhibit a more hydrophilic nature, potentially contributing to increased solubility. Existing literature suggests that a decrease in protein molecular size is associated with enhanced solubility in water (Hamada, 1997; Hou et al., 2017). Moreover, the high solubility of the protein extract (64.49 %) in water implies that the interaction of NaOH during extraction may lead to the formation of more globular proteins, typically soluble at neutral pH. It is also plausible that NaOH could facilitate the removal of insoluble polysaccharides linked to *C. vulgaris* protein through hydrolysis during extraction, thereby increasing solubility. However, it is important to note that the protein may undergo denaturation at high NaOH concentrations, such as 0.7 M, potentially reducing solubility. Our findings thus suggest that our finding thus suggests that NaOH concentration used for protein extraction plays a crucial role in influencing the solubility of CVPE. The high solubility of the extracts could be advantageous for their use in beverage preparation.

3.3.2. *Water absorption capacity (WAC)*

WAC of protein is defined as its ability to retain water in the presence of excess water when exposed to external forces such as heating, centrifugation and pressing (Zayas, 1997). This property is crucial in various food applications such as confectionery products, baked goods, and viscous foods like soups as they require water imbibition without dissolution of proteins, ultimately achieving the desired viscosity (Seena and Sridhar, 2005). The WAC of CVPE obtained with different NaOH concentration is depicted in Fig. 4. It can be observed that the WAC increased with the rise in NaOH concentrations used for extraction, reaching 297.0 % at 0.5–0.7 M. This suggests that CVPE is well-suited for applications in viscous foods or baked items, as its WAC falls within the specified range of 149–472 % (Aletor et al., 2002). While no prior studies have explored the impact of different NaOH concentrations in alkaline solubilization on WAC, the maximum WAC value of CVPE (297.0 %) is slightly lower than that of SPI (367.43 %). Additionally, it is comparable to the WAC of pea protein isolate (240–260 %) reported by Stone et al. (2015). Furthermore, CVPE demonstrates a higher WAC than mung bean protein (262.0 %) extracted by Du et al. (2018). Additionally, CVPE exhibits similar WAC when compared to other microalgae protein such as *Chlorella pyrenoidosa* protein (287.0 %) and *Arthospira platensis* protein (281.0 %) (Chen et al., 2019).

The rise in WAC of CVPE can be attributed to the conformational changes due to strong alkaline condition. This leads to the exposure of hydrophilic groups in the protein and the formation of smaller protein/peptide fragments, as supported by SDS -PAGE results and documented by other studies (Zhao et al., 2014; Yue et al., 2019). These changes increase the surface area, creating more sites for water binding, consequently elevating the WAC. This finding aligns with a previous study indicating that protein concentrates extracted through alkaline treatment exhibit higher WAC compared to those recovered using ultrafiltration (Boye et al., 2010). Furthermore, the improved solubility of CVPE (Fig. 3) may also positively contribute to the WAC, a correlation supported by previous research findings (Mir et al., 2019b; Wang et al., 2020).

3.3.3. *Oil absorption capacity (OAC)*

OAC is a critical parameter in formulated food, as it assesses the capability of non-polar side chains of proteins to absorb, retain, and interact with lipids in emulsions and other food systems (Zayas, 1997). This attribute holds particular significance from an industrial standpoint as it mirrors emulsifying capacity, a highly desirable trait in products like mayonnaise. As depicted in Fig. 5, the OAC of CVPE showed a declining trend with increasing NaOH concentrations. However, all CVPE samples obtained with various NaOH concentrations exhibited higher OAC compared to the commercial SPI (143.05 %). Specifically, the OAC of CVPE with 0.1 M demonstrated approximately a 1.8-fold increase compared to that of SPI. Other studies have reported the OAC of SPI to be in the range of 89–154 % (Kinsella, 1979; Tang et al., 2021). These results suggest that CVPE possesses a larger surface area and more hydrophobic proteins compared to SPI. The decrease in OAC in CVPE is likely attributed to protein degradation, as observed in SDS-PAGE (Fig. 2), and a reduction in surface hydrophobicity. The enhanced OAC of CVPE further stress the potential application in various process foods, including sausages, burgers, and baked goods where a high OAC is essential.

3.3.4. *Foaming capacity (FC) and foam stability (FS)*

Protein functionality, specifically FC and FS, plays a pivotal role in the production of diverse food items, particularly in aerated products like meringues and leavened bakery goods (Campbell and Mougeot, 1999; Wong and Kitts, 2003). The impact of NaOH concentration on CVPE (your protein of interest) is illustrated in Fig. 6A and 6B. It was observed that both FC and FS of CVPE increased proportionally with the rise in NaOH concentration during extraction, reaching an optimum at 0.5 M NaOH. However, at a higher NaOH concentration of 0.7 M, a decline in both FC and FS of CVPE was noted. The CVPE showed the highest FC and FS when extracted with 0.5 M NaOH with the value of 77.0 and 87.0 %, respectively.

Comparatively, CVPE exhibited approximately twice the FC of SPI (38.33 %), highlighting its superior foaming capacity. Conversely, SPI demonstrated a higher FS at 100.0 % when compared to CVPE. While no

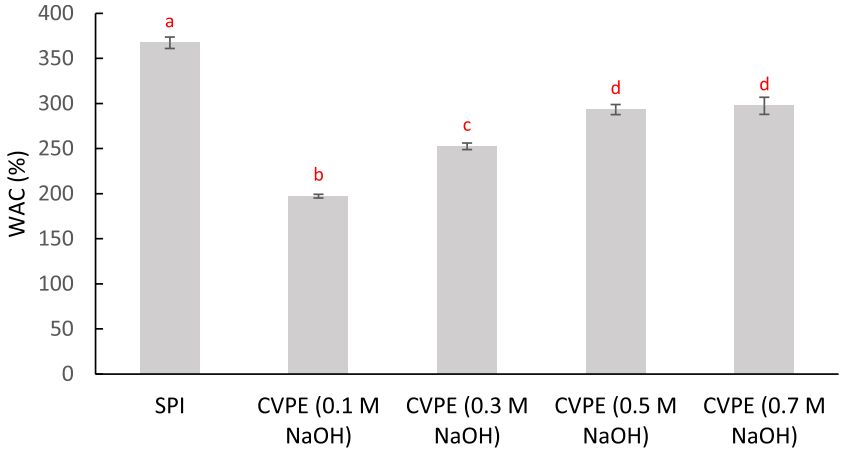


Fig. 4. Water absorption capacity (WAC) of soy protein isolate (SPI) and *C. vulgaris* protein extract (CVPE) extracted by different NaOH concentrations. The detailed conditions of protein extraction were described in Materials and Methods section. Different letters indicate the significant difference between two mean values (p-value < 0.05).

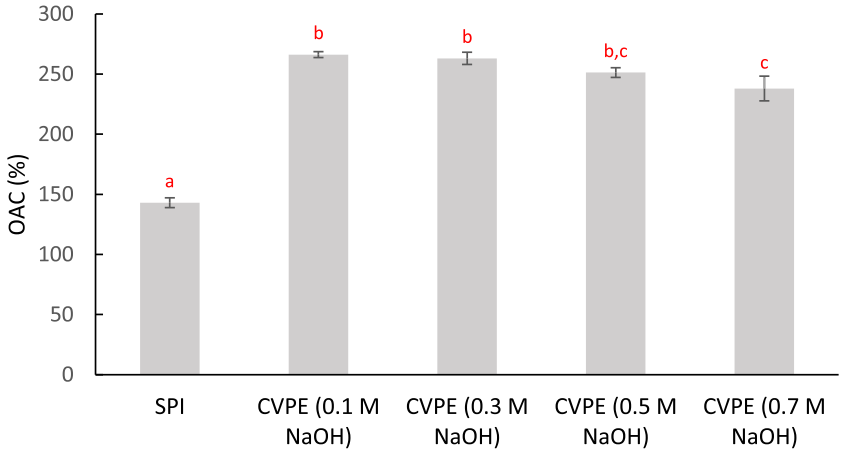


Fig. 5. Oil absorption capacity (OAC) of soy protein isolate (SPI) and *C. vulgaris* protein extract (CVPE) extracted by different NaOH concentrations. The detailed conditions of protein extraction were described in Materials and Methods section. Different letters indicate the significant difference between two mean values (p-value < 0.05).

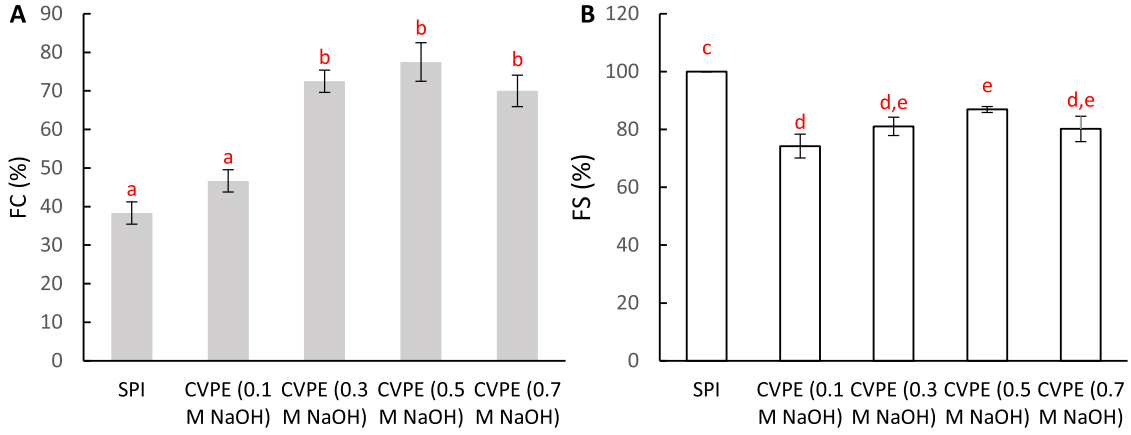


Fig. 6. (A) Foaming capacity (FC), and (B) foam stability (FS) of soy protein isolate (SPI) and *C. vulgaris* protein extract (CVPE) extracted by different NaOH concentrations. The detailed conditions of protein extraction were described in Materials and Methods section. Different letters indicate the significant difference between two mean values (p-value < 0.05).

prior research has explored on the impact of NaOH concentration on microalgal protein foaming properties, a similar trend was noted in plant proteins, specifically those derived from rice and tea (Hou et al., 2017; Ayim et al., 2018). Furthermore, the maximum FC and FS of CVPE were relatively higher than that of the protein from other microalgae such as *Chlorella pyrenoidosa* protein (FC: 36.88 %; FS: 50.0 %) and *Nannochloropsis oceanica* protein (FC: 31.50 %; FS:7.80 %) (Chen et al., 2019). To gain a comprehensive understanding of the factors influencing the foaming properties of CVPE with respect to NaOH concentration, further research is imperative to unravel the underlying mechanisms.

In general, FC and FS increases with the solubility of protein (Agboola et al., 2005; Kiosseoglou et al., 2021). It is evident from Fig. 4 that the solubility of CVPE increased with the rise in NaOH concentration. The result thus further supports the hypothesis that FC and FS are correlated with protein solubility. Additionally, the foaming capacity (FC) of CVPE surpasses that of mung bean protein (26.0 %), chickpea protein (30.4–44.3 %), and soybean protein (36.0 %) (Kaur and Singh, 2007; Aydemir and Yemenicioğlu, 2013; Du et al., 2018). This indicates that CVPE exhibits a commendable surfactant property, a crucial characteristic in foam formation. It can serve as an effective foaming agent, enhancing the palatability of food products such as bread and ice cream.

3.3.5. *Emulsifying activity index (EAI) and emulsion stability index (ESI)*

EAI and ESI were evaluated to understand the emulsifying properties of the CVPE extracted with different NaOH concentrations. Broadly, Emulsion Activity Index (EAI) gauges a protein's capacity to establish emulsions. On the other hand, Emulsion Stability Index (ESI) evaluates a protein's proficiency in sustaining a stable emulsion over a specified time frame (Boye et al., 2010; Suresh Kumar et al., 2014).

Fig. 7A and B represents the EAI and ESI of CVPE extracted at varying NaOH concentrations. It can be noted that EAI and ESI of CVPE increased as the NaOH concentrations for extraction was raised. The CPP extracted with 0.1 M NaOH showed the lowest EAI value of 65.77 m^2/g, while maximum EAI 107.0 m^2/g was observed at 0.5 M NaOH. ESI of CVPE also showed the similar trend with the increase in NaOH concentration, exhibiting maximum stability of 18.3 min when extracted at 0.7 M NaOH. It's noteworthy that the maximum EAI and ESI of CVPE were relatively higher than those of the protein from other microalgae, namely *Chlorella pyrenoidosa* (EAI: 63.58 m^2/g; ESI: 17.50 min) and *Nannochloropsis oceanica* (EAI: 59.50 m^2/g; ESI: 15.80 min) reported in previous research (Chen et al., 2019). CVPE demonstrated a comparable EAI pattern to the rice residue protein isolates (RRPI) reported by Hou et al. (2017). EAI and ESI of commercial SPI were also assessed, yielding values of 79.06 m^2/g and 14.54 min, respectively. These values were notably (approximately 1.3-fold) lower than those (106.95 m^2/g and

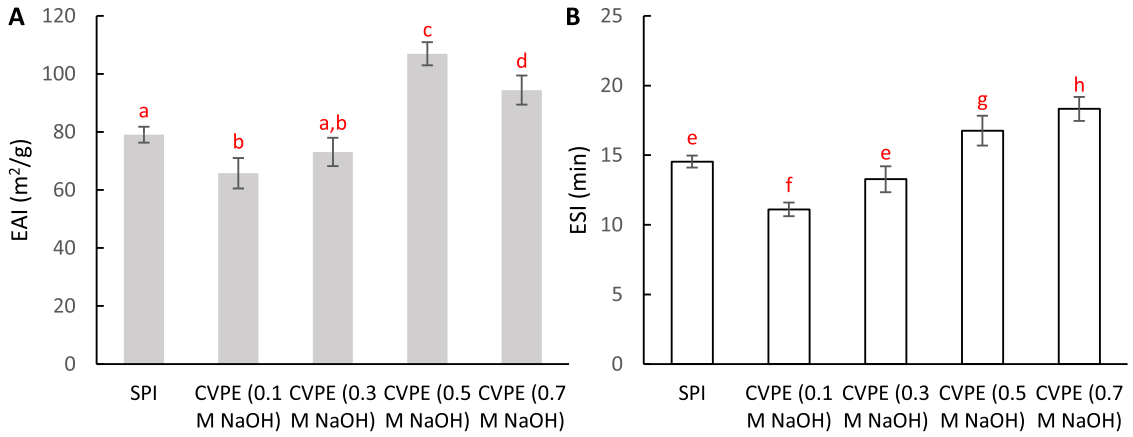


Fig. 7. (A) Emulsifying activity index (EAI), and (B) emulsion stability index (ESI) of soy protein isolate (SPI) and *C. vulgaris* protein extract (CVPE) extracted by different NaOH concentrations. The detailed conditions of protein extraction were described in Materials and Methods section. Different letters indicate the significant difference between two mean values (p-value < 0.05).

16.76 min) of CVPE obtained with 0.5 M NaOH extraction. These results highlight the potential of CVPE for application in emulsion-based food products.

The emulsifying properties of hydrolysates are predominantly influenced by the amphiphilicity, reflecting the balance between hydrophilic and hydrophobic properties of the protein. The enhanced emulsifying properties observed in CVPE extracted with higher NaOH concentration signify its improved ability to break the surface/interfacial tension (Schröder et al., 2017). Furthermore, the increased emulsifying stability suggests that NaOH treatment enhance the interaction of hydrophobic and hydrophilic residues with oil droplets and water, respectively. In general, the enhanced interaction improves emulsifying stability through the reduction of steric effect (Lam and Nickerson, 2013; Adjonu et al., 2014).

4. Conclusion

This study has illuminated the significant impact of alkaline (NaOH) solubilization of protein from bead milled *C. vulgaris* biomass on functional properties of the protein extract. The optimization of alkali concentration emerges as a crucial factor, not only in maximizing protein solubilization but also in enhancing the functional attributes of the protein extract. The protein extract obtained through solubilization with 0.7 M NaOH exhibited maximum WAC and ESI, whereas 0.5 M NaOH proved to be the optimal concentration for maximizing FC, FS, EAI, and solubility. The highest OAC was achieved at a solubilization pH of 0.1 M NaOH. The alkaline solubilization made *C. vulgaris* protein extract (CVPE) superior to soy protein isolate (SPI) in terms of solubility, OAC, FC, emulsion activity and emulsion stability. While the WAC and FS of CVPE showed improvement with higher NaOH concentration, they remained slightly lower than those of SPI. The high solubility (64.49 %) indicates that the predominant form of CVPE is globular protein. Given its noteworthy solubility, OAC, FC, and EAI, CVPE holds significant potential for use as a moisture modifier, texture enhancer, foaming agent, and emulsifying agent in diverse food products. Future research endeavours should focus on elucidating the mechanisms enhancing the functionality of CVPE and examining structural changes. Additionally, exploring potential health benefits and sustainability considerations, along with assessing the feasibility of industrial-scale production, could broaden the applicability of CVPE within the food industry.

CRediT authorship contribution statement

Jun Wei Ng: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Tong Mei Teh:** Methodology, Investigation. **Chee Fan Tan:** Methodology, Investigation. **Xuezhi Bi:** Methodology. **Zhi En Low:** Investigation. **Md. Mahabubur Rahman Talukder:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This study is part of Singapore-New Zealand Bilateral Research Programme on Future Foods which was funded by Agency for Science, Technology and Research (A*STAR) (Project number SGNZ2020-A20D3b0072).

Ethical Statement – Studies in humans and animals

I testify on behalf of all co-authors that our manuscript submitted to the Special Issue "The potential of algal protein as a sustainable functional food ingredient" of Future Foods:

This manuscript does not contain any studies involving human participants, including sensory evaluation and customer surveys, performed by any of the authors.

This manuscript does not contain any studies involving animals performed by any of the authors.

References

Adjonu, R., Doran, G., Torley, P., Agboola, S., 2014. Whey protein peptides as components of nanoemulsions: a review of emulsifying and biological functionalities. J. Food Eng. 122, 15–27. https://doi.org/10.1016/j.jfoodeng.2013.08.034.

Agboola, S., Ng, D., Mills, D., 2005. Characterisation and functional properties of Australian rice protein isolates. J. Cereal Sci. 41, 283–290. https://doi.org/10.1016/j.jcs.2004.10.007.

Aletor, O., Oshodi, A.A., Ipinmoroti, K., 2002. Chemical composition of common leafy vegetables and functional properties of their leaf protein concentrates. Food Chem. 78, 63–68. https://doi.org/10.1016/s0308-8146(01)00376-4.

American Association of Cereal Chemists Technical Committee, 1981. Method for water hydration capacity of plant protein material. Cereal Foods World 26, 291–293.

Aydemir, L.Y., Yemenicioğlu, A., 2013. Potential of Turkish Kabuli type chickpea and green and red lentil cultivars as source of soy and animal origin functional protein alternatives. LWT - Food Sci. Technol. 50, 686–694. https://doi.org/10.1016/j.lwt.2012.07.023.

Ayim, I., Ma, H., Alenyorege, E.A., Ali, Z., Zhou, C., Donkor, P.O., 2018. Effect of alkali concentration on functionality, lysinoalanine formation, and structural characteristics of tea residue proteins. J. Food Process Eng. 41 https://doi.org/10.1111/jfpe.12877.

Becker, E.W., 2007. Micro-algae as a source of protein. Biotechnol. Adv. 25, 207–210. https://doi.org/10.1016/j.biotechadv.2006.11.002.

Boye, J.I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., Rajamohamed, S.H., 2010. Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. Food Res. Int. 43, 537–546. https://doi.org/10.1016/j.foodres.2009.07.021.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1006/abio.1976.9999.

Campbell, G., Mougeot, E., 1999. Creation and characterisation of aerated food products. Trends Food Sci. Technol. 10, 283–296. https://doi.org/10.1016/s0924-2244(00)00008-x.

Chen, Y., Chen, Jianchu, Chang, C., Chen, Juan, Cao, F., Zhao, J., Zheng, Y., Zhu, J., 2019. Physicochemical and functional properties of proteins extracted from three microalgal species. Food Hydrocoll. 96, 510–517. https://doi.org/10.1016/j.foodhyd.2019.05.025.

Chia, S.R., Chew, K.W., Zaid, H.F.M., Chu, D.T., Tao, Y., Show, P.L., 2019. Microalgal protein extraction from Chlorella vulgaris FSP-E using triphasic partitioning technique with sonication. Front. Bioeng. Biotechnol. 7, 396. https://doi.org/10.3389/fbioe.2019.00396.

Cui, L., Bandillo, N., Wang, Y., Ohm, J.B., Chen, B., Rao, J., 2020. Functionality and structure of yellow pea protein isolate as affected by cultivars and extraction pH. Food Hydrocoll. 108, 106008 https://doi.org/10.1016/j.foodhyd.2020.106008.

Du, M., Xie, J., Gong, B., Xu, X., Tang, W., Li, X., Li, C., Xie, M., 2018. Extraction, physicochemical characteristics and functional properties of Mung bean protein. Food Hydrocoll. 76, 131–140. https://doi.org/10.1016/j.foodhyd.2017.01.003.

Finkel, Z.V., Follows, M.J., Liefer, J.D., Brown, C.M., Benner, I., Irwin, A.J., 2016. Phylogenetic diversity in the macromolecular composition of microalgae. PLoS One 11, e0155977. https://doi.org/10.1371/journal.pone.0155977.

Garcia, E.S., van Leeuwen, J.J.A., Safi, C., Sijtsma, L., van den Broek, L.A.M., Eppink, M.H.M., Wijffels, R.H., van den Berg, C., 2018. Techno-functional properties of crude extracts from the green microalga Tetraselmis suecica. J. Agric. Food Chem. 66, 7831–7838. https://doi.org/10.1021/acs.jafc.8b01884.

Gouveia, L., Batista, A.P., Raymundo, A., Bandarra, N., Sousa, I., 2008. Microalgae in novel food products. In: Papadopoulos, K.N. (Ed.), Food Chemistry Research Developments. Nova Science Publishers, New York, pp. 75–112.

Hamada, J.S., 1997. Characterization of protein fractions of rice bran to devise effective methods of protein solubilization. Cereal. Chem. 74, 662–668. https://doi.org/10.1094/cchem.1997.74.5.662.

Hou, F., Ding, W., Qu, W., Oladejo, A.O., Xiong, F., Zhang, W., He, R., Ma, H., 2017. Alkali solution extraction of rice residue protein isolates: influence of alkali concentration on protein functional, structural properties and lysinoalanine formation. Food Chem. 218, 207–215. https://doi.org/10.1016/j.foodchem.2016.09.064.

Jiang, J., Chen, J., Xiong, Y.L., 2009. Structural and emulsifying properties of soy protein isolate subjected to acid and alkaline pH-shifting processes. J. Agric. Food Chem. 57, 7576–7583. https://doi.org/10.1021/jf901585n.

Jiang, S., Ding, J., Andrade, J., Rababah, T.M., Almajwal, A., Abulmeaty, M.M., Feng, H., 2017. Modifying the physicochemical properties of pea protein by pH-shifting and ultrasound combined treatments. Ultrason. Sonochemistry 38, 835–842. https://doi.org/10.1016/j.ultsonch.2017.03.046.

Juul, L., Haue, S.K., Bruhn, A., Boderskov, T., Kastrup Dalsgaard, T., 2023. Alkaline pH increases protein extraction yield and solubility of the extracted protein from sugar kelp (Saccharina latissima). Food Bioproducts Process. 140, 144–150. https://doi.org/10.1016/j.fbp.2023.05.008.

Kaur, M., Singh, N., 2007. Characterization of protein isolates from different Indian chickpea (Cicer arietinum L.) cultivars. Food Chem. 102, 366–374. https://doi.org/10.1016/j.foodchem.2006.05.029.

Kinsella, J.E., 1979. Functional properties of soy proteins. J. Am. Oil Chem. Soc. 56, 242–258. https://doi.org/10.1007/bf02671468.

Kiosseoglou, V., Paraskevopoulou, A., Poojary, M.M., 2021. Functional and physicochemical properties of pulse proteins. In: Tiwari, B.K., Gowen, A., McKenna, B. (Eds.), Pulse Foods: Processing, Quality and Nutraceutical Applications. Elsevier, Amsterdam, pp. 113–146.

Kose, A., Oncel, S.S., 2015. Properties of microalgal enzymatic protein hydrolysates: biochemical composition, protein distribution and FTIR characteristics. Biotechnol. Rep. 6, 137–143. https://doi.org/10.1016/j.btre.2015.02.005.

Lam, R.S.H., Nickerson, M.T., 2013. Food proteins: a review on their emulsifying properties using a structure–function approach. Food Chem. 141, 975–984. https://doi.org/10.1016/j.foodchem.2013.04.038.

Ma, M., Ren, Y., Xie, W., Zhou, D., Tang, S., Kuang, M., Wang, Y., Du, S., 2018. Physicochemical and functional properties of protein isolate obtained from cottonseed meal. Food Chem. 240, 856–862. https://doi.org/10.1016/j.foodchem.2017.08.030.

Mahali, M., Sibi, G., 2019. Extraction methods and functional properties of protein from Arthospira platensis for Bioavailability of algal proteins. Int. J. Pharm. Chem. 5, 20. https://doi.org/10.11648/j.ijpc.20190502.12.

Mata, T.M., Martins, A.A., Caetano, N.S., 2010. Microalgae for biodiesel production and other applications: a review. Renew. Sustain. Energy Rev. 14, 217–232. https://doi.org/10.1016/j.rser.2009.07.020.

Mir, N.A., Riar, C.S., Singh, S., 2019a. Effect of pH and holding time on the characteristics of protein isolates from Chenopodium seeds and study of their amino acid profile and scoring. Food Chem. 272, 165–173. https://doi.org/10.1016/j.foodchem.2018.08.048.

Mir, N.A., Riar, C.S., Singh, S., 2019b. Structural modification of quinoa seed protein isolates (QPIs) by variable time sonification for improving its physicochemical and functional characteristics. Ultrason. Sonochemistry 58, 104700. https://doi.org/10.1016/j.ultsonch.2019.104700.

Morris, H.J., Almarales, A., Carrillo, O., Bermúdez, R.C., 2008. Utilisation of Chlorella vulgaris cell biomass for the production of enzymatic protein hydrolysates. Bioresour. Technol. 99, 7723–7729. https://doi.org/10.1016/j.biortech.2008.01.080.

Pearce, K.N., Kinsella, J.E., 1978. Emulsifying properties of proteins: evaluation of a turbidimetric technique. J. Agric. Food Chem. 26, 716–723. https://doi.org/10.1021/jf60217a041.

Safi, C., Zebib, B., Merah, O., Pontalier, P.Y., Vaca-Garcia, C., 2014a. Morphology, composition, production, processing and applications of Chlorella vulgaris: a review. Renew. Sustain. Energy Rev. 35, 265–278. https://doi.org/10.1016/j.rser.2014.04.007.

Safi, C., Ursu, A.V., Laroche, C., Zebib, B., Merah, O., Pontalier, P.Y., Vaca-Garcia, C., 2014b. Aqueous extraction of proteins from microalgae: effect of different cell disruption methods. Algal. Res. 3, 61–65. https://doi.org/10.1016/j.algal.2013.12.004.

Schröder, A., Berton-Carabin, C., Venema, P., Cornacchia, L., 2017. Interfacial properties of whey protein and whey protein hydrolysates and their influence on O/W emulsion stability. Food Hydrocoll. 73, 129–140. https://doi.org/10.1016/j.foodhyd.2017.06.001.

Schwenzfeier, A., Lech, F., Wierenga, P.A., Eppink, M.H.M., Gruppen, H., 2013. Foam properties of algae soluble protein isolate: effect of pH and ionic strength. Food Hydrocoll. 33, 111–117. https://doi.org/10.1016/j.foodhyd.2013.03.002.

Seena, S., Sridhar, K.R., 2005. Physicochemical, functional and cooking properties of under explored legumes, Canavalia of the southwest coast of India. Food Res. Int. 38, 803–814. https://doi.org/10.1016/j.foodres.2005.02.007.

Shelef, G., Soeder, C.J., Balaban, M., 1980. Algae Biomass: Production and Use. Elsevier-North-Holland Biomedical Press, Amsterdam.

Singh, P., Kumar, R., Sabapathy, S.N., Bawa, A.S., 2008. Functional and edible uses of soy protein products. Compr. Rev. Food Sci. Food Saf. 7, 14–28. https://doi.org/10.1111/j.1541-4337.2007.00025.x.

Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., 2006. Commercial applications of microalgae. J. Biosci. Bioeng. 101, 87–96. https://doi.org/10.1263/jbb.101.87.

Stack, J., Gouic, A.V.L., Tobin, P.R., Guihéneuf, F., Stengel, D.B., FitzGerald, R.J., 2018. Protein extraction and bioactive hydrolysate generation from two microalgae, Porphyridium purpureum and Phaeodactylum tricornutum. J. Food Bioactives 1. https://doi.org/10.31665/jfb.2018.1134.

Stone, A.K., Karalash, A., Tyler, R.T., Warkentin, T.D., Nickerson, M.T., 2015. Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. Food Res. Int. 76, 31–38. https://doi.org/10.1016/j.foodres.2014.11.017.

Suresh Kumar, K., Ganesan, K., Selvaraj, K., Subba Rao, P.V., 2014. Studies on the functional properties of protein concentrate of Kappaphycus alvarezii (Doty) Doty – An edible seaweed. Food Chem. 153, 353–360. https://doi.org/10.1016/j.foodchem.2013.12.058.

Tang, X., Shen, Y., Zhang, Y., Schilling, M.W., Li, Y., 2021. Parallel comparison of functional and physicochemical properties of common pulse proteins. LWT 146, 111594. https://doi.org/10.1016/j.lwt.2021.111594.

Timilsena, Y.P., Adhikari, R., Barrow, C.J., Adhikari, B., 2016. Physicochemical and functional properties of protein isolate produced from Australian chia seeds. Food Chem. 212, 648–656. https://doi.org/10.1016/j.foodchem.2016.06.017.

Tomaselli, L., 2008. The microalgae cell. In: Richmond, A. (Ed.), Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwell Science, Oxford, pp. 3–19.

Ursu, A.V., Marcati, A., Sayd, T., Sante-Lhoutellier, V., Djelveh, G., Michaud, P., 2014. Extraction, fractionation and functional properties of proteins from the microalgae Chlorella vulgaris. Bioresour. Technol. 157, 134–139. https://doi.org/10.1016/j.biortech.2014.01.071.

Waghmare, A.G., Salve, M.K., LeBlanc, J.G., Arya, S.S., 2016. Concentration and characterization of microalgae proteins from Chlorella pyrenoidosa. Bioresour. Bioprocess. 3, 1–11. https://doi.org/10.1186/s40643-016-0094-8.

Wang, F., Zhang, Y., Xu, L., Ma, H., 2020. An efficient ultrasound-assisted extraction method of pea protein and its effect on protein functional properties and biological activities. LWT 127, 109348. https://doi.org/10.1016/j.lwt.2020.109348.

Wong, P.Y.Y., Kitts, D.D., 2003. A comparison of the buttermilk solids functional properties to nonfat dried milk, soy protein isolate, dried egg white, and egg yolk powders. J. Dairy Sci. 86, 746–754. https://doi.org/10.3168/jds.s0022-0302(03)73655-8.

Yu, J., Ahmedna, M., Goktepe, I., 2007. Peanut protein concentrate: production and functional properties as affected by processing. Food Chem. 103, 121–129. https://doi.org/10.1016/j.foodchem.2006.08.012.

Yue, L., Meng, Z., Yi, Z., Gao, Q., Mao, A., Li, J., 2019. Effects of different denaturants on properties and performance of soy protein-based adhesive. Polymers 11, 1262. https://doi.org/10.3390/polym11081262.

Yuliana, M., Truong, C.T., Huynh, L.H., Ho, Q.P., Ju, Y.H., 2014. Isolation and characterization of protein isolated from defatted cashew nut shell: influence of pH and NaCl on solubility and functional properties. LWT - Food Sci. Technol. 55, 621–626. https://doi.org/10.1016/j.lwt.2013.10.022.

Zayas, J.F., 1997. Water holding capacity of proteins. In: Zayas, J.F. (Ed.), Functionality of Proteins in Food. Springer Berlin Heidelberg, Berlin, pp. 76–133.

Zhao, Y., Tu, Y., Li, J., Xu, M., Yang, Y., Nie, X., Yao, Y., Du, H., 2014. Effects of alkaline concentration, temperature, and additives on the strength of alkaline-induced egg white gel. Poult. Sci. 93, 2628–2635. https://doi.org/10.3382/ps.2013-03596.