Extraction of proteins from seaweed with high functionality and the applications of seaweed in food products

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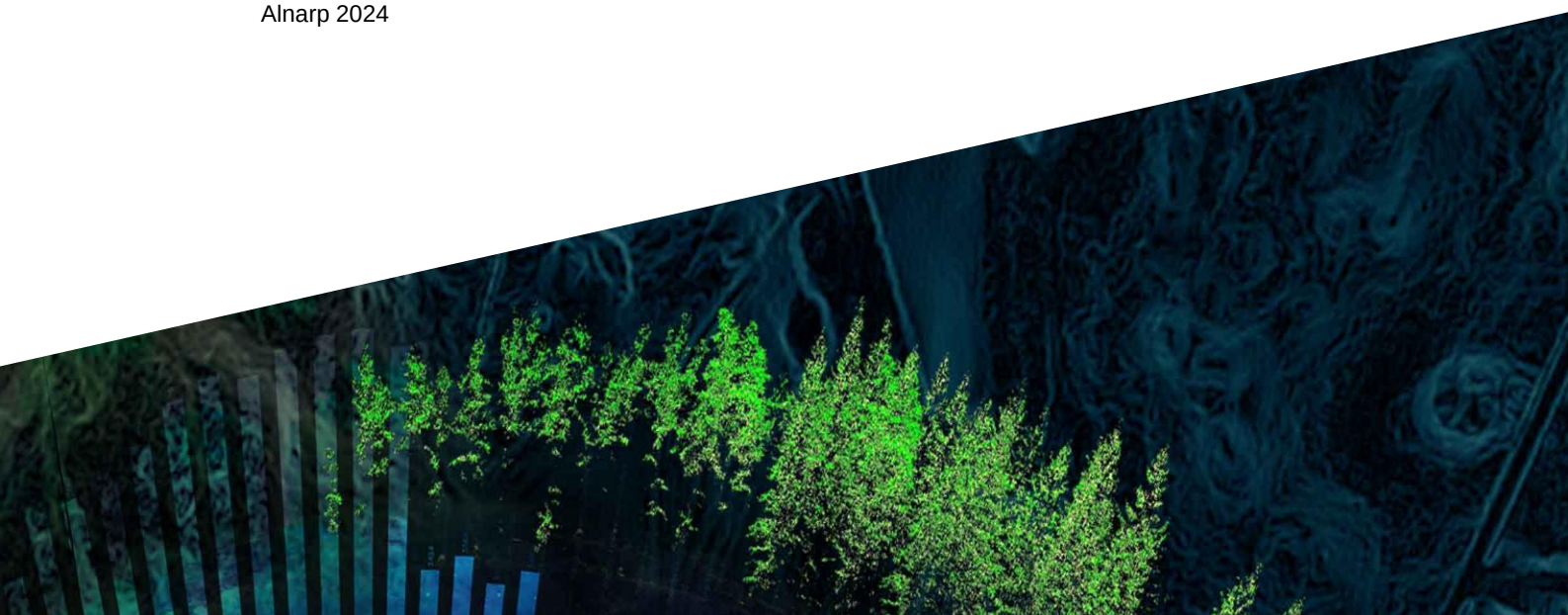

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Abstract

Demand for food production has increased due to population growth. There is a necessity to explore alternative proteins that can substitute animal-derived proteins. Seaweed, a sustainable and nutrient-rich resource, has gained attention as a potential alternative protein and essential nutrient source that has the potential to contribute to the growing global demand for sustainable food solutions. Numerous conventional methods are available for extracting protein from seaweed; however, these traditional techniques frequently result in low yields because of the complex cellular matrix. In this study, the aim was to develop a novel method that could potentially enhance protein extraction from dried seaweed biomass. For protein extraction from brown seaweed (*Saccharina latissima*), three different methods were developed. In the first method, seaweed powder was added to milli-Q water (1:50) and stirred for 1 h, in the second phase solition was stirred for 24 h, and for the third phase, solution was subjected to overnight freezing at -80 ℃. Following these steps, protein was extracted by pH method. Among the methods evaluated, the O-N freezing technique demonstrated superior efficacy in protein extraction. Protein concentrations were higher in samples extracted using the Over Night Freezing (O-N freezing) method compared to the 1 h method. The 24 h method demonstrated a higher ζ-potential and larger particle size than the other methods, contributing to enhanced solution stability. Additionally microstructural analysis revealed that emulsions prepared with the 24 h method had a more uniform droplet size distribution. SE-HPLC analysis revealed that most of the high and low molecular weight proteins were effectively extracted during the initial SDS-phosphate buffer extraction, with minimal protein recovery in subsequent steps. Additionally, seaweed was incorporated into tortilla bread, and sensory evaluations indicated positive consumer interest and acceptance, with most participants expressing willingness to recommend the seaweed-enriched bread.

Keywords: Seaweed, Protein, extraction, protein functionality, bread, consumer perception

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Abbreviations

GHG	Greenhouse gases
FAO	Food and Agriculture Organization of the United Nations
SDGs	Sustainable Development Goals
UN	United Nations
BCA assay	Bicinchoninic acid assay
HPLC	High-Performance liquid chromatography
O-N Freezing	Over Night Freezing

1. Introduction

The global population is expected to reach 8.5 billion by 2030 and approximately 10 billion by 2050 (United Nations 2019). According to estimates, the amount of food needed to feed the world's population in 2050 is expected to doubled (Aiking 2014). As a result of population expansion and increasing demand of food, the world natural resources are under a great deal of strain (Fasolin et al. 2019). In addition to the increase in demand due to population growth, other factors driving the global need for protein include socio-economic shifts such as rising incomes, increasing urbanization, and aging populations (Popkin et al. 2012).

The current global protein requirement is estimated to be 200 Mt per year (Henchion et al. 2017). About 60% of the protein consumed worldwide comes from plant-based sources; the remaining 40% comes from dairy, eggs, meat, and fish (Henchion et al. 2017). Global dairy and meat consumption trends are expected to rise by 158% and 173%, respectively, between 2010 and 2050 (Fasolin et al. 2019). Reducing the amount of animal-based proteins is considered to be a key strategy for achieving a more sustainable food supply chain in the future. Thus, the need for additional plant protein sources is necessary (Černá 2011). On a global scale, plant based protein is of immense importance and there is significant interest in its ability to meet the growing demand to fulfill future needs for animal proteins. Moreover, plant-based protein is preferred over animal-based protein from an environmental perspective as it is associated with a lower land use requirement, and it is generally accepted that plant-based foods produce lower levels of greenhouse gas (GHG) emissions (Tilman & Clark 2014). In addition to using protein as a substitute for animal proteins, the demand for plant-based proteins as emulsifiers has increased recently (Le Roux et al. 2020). The food industry is one of the many sectors that heavily relies on the use of emulsions and emulsifiers. Selecting an appropriate emulsifier is a key strategy for food manufacturers to reduce or prevent emulsion instability, which is crucial for maintaining product quality and consistency (Muna et al. 2008)

Marine plants, such as seaweed, represent a promising and novel future protein source (Gleison et al. 2023). Seaweed, also known as macro-algae, differs from micro-algae, which are typically microscopic and unicellular. Seaweeds are complex multicellular plants that grow in marine environment. They are divided into three groups: Rhodophyta or red seaweeds, Phaeophyta or brown seaweeds, and Chlorophyta or green seaweeds (Gleison et al. 2023).

Seaweeds thrive in coastal regions worldwide, flourishing in environments rich in nutrients and sunlight (Pérez-Lloréns et al. 2018). They can grow at depths ranging from 2 to 20 meters, depending on the species (J. McHugh 2016). Seaweeds attach to hard substrates, such as rocks, in areas where light and saltwater conditions support photosynthesis (Kılınç et al. 2013).

Unlike plants or animals, seaweeds are unique in their classification, lacking the specialized vascular systems found in plants as well as the ability to produce seeds or fruits. Instead, they absorb nutrients directly from the ocean through their surface (O'Connor 2017). Seaweeds can vary significantly in size: brown seaweeds, such as massive kelp, can grow up to 20 meters in length, with others reaching 2-4 meters, while smaller brown seaweed species range from 30 to 60 centimeters. In contrast, red seaweeds are typically smaller, with lengths ranging from a few centimeters to almost one meter (J. McHugh 2016).

Given their high protein concentration, favorable amino acid composition, carbohydrate content, and abundance of minerals, vitamins, and antioxidants, seaweeds are promising candidates for future applications in food production (Thiviya et al. 2022). The crude protein content and amino acid profile of seaweeds offer further evidence of the benefits of incorporating seaweed proteins as a valuable protein source in the human diet (Heidelbaugh et al. 1975). Seaweeds' significant potential and contribution to an adequate and nutritious global food supply for a population predicted to rise by 70% by 2050 have been referenced as the reasons for the rising interest in seaweed (Van den Burg et al. 2021). The production of seaweed biomass doubled globally between 2005 and 2018, according to FAO observations (Van den Burg et al. 2021).

Seaweed has been an essential component of the foods in the Asian countries such as China, Korea, and Japan. Recently, seaweed consumption has increased noticeably in western countries due to growing public knowledge of its health advantages (Lopez-Santamarina et al. 2020). Also, an increasing trend has been observed among consumers favoring plant-based products, which is largely driven by heightened environmental awareness, health considerations, and concerns regarding animal welfare (Thakur et al. 2024).

The main challenge in extracting algal proteins, particularly in macroalgal protein extraction processes, stems from the breakdown of the cell wall (Echave et al. 2021). Proteins in seaweed species are linked to non-protein compounds such as polysaccharides and polyphenols (Wijesinghe & Jeon 2012). Conventional techniques for protein extraction (aqueous, acidic, and alkaline) have demonstrated decreased profits (Bleakley & Hayes 2017). The lack of sustainable approaches to overcome the limitations of traditional methods results in higher costs and reduced efficiency (Jeon et al. 2011). In response to the reasons described, non-conventional extraction methods are currently under investigation and development to enhance extraction yield while reducing time and resource requirements (kadam et al. 2017). For instance, microwave-assisted extraction, ultrasound-assisted extraction, and

high hydrostatic pressure extraction (Echave et al. 2021) can improve the yeild extraction (Kadam et al. 2017). Although novel protein extraction methods have demonstrated increased extraction yields; nevertheless, they have not yet reached a level of development that makes them commercially viable (Vásquez et al. 2019). In this study, we focus on a specific part of the food chain by examining seaweed, exploring both the processes involved in extracting proteins from seaweed and how these proteins consumed by the public.

1.1 History of seweed

Seaweeds have been utilized for thousands of years worldwide for a wide range of food and non-food purposes. For more than 2,000 years, seaweed has been utilized as food in China, Korea, and Japan (Brijesh & Declan 2015). Seaweed from Porphyra species is used in Japan to manufacture "nori", a dried sheet of seaweed used to make sushi. Seaweeds are consumed fresh as salad in Indonesia and Malaysia. While the use of seaweeds in food has a long history in South East Asian countries, its application in the some western countries has primarily been linked to nonfood purposes. As early as 100 BC, seaweed was utilized to feed animals in Greece. Red seaweeds were utilized as medicine in Mediterranean countries. Farmers in Ireland and Scotland used seaweeds for soil mulch and other agricultural purposes (Brijesh & Declan 2015). European historical records point to the fact that local communities in coastal areas have long been consuming seaweed (O'Conner 2013). For instance, Palmaria palmata (red seaweed) has been utilized as food in Norway (Delaney et al. 2016). In coastal areas on the country's west and north coasts, P. palmata, Chondruscrispus, Mastocarpus stallatus, and Porphyra umbricallis were consumed. Seaweed was seen as a seasonal food item that might be sold locally or consumed at home. Because of this, there was no demand for edible algae outside of coastal areas (Delaney et al. 2016). The only way that seaweed was consumed by humans in France was when milk was jellied and used to produce famous black "far" (buckwheat custard). In Wales, laver (a Porphyra spp.) was either fried with oatmeal to form laverbread or boiled and eaten with cockles and bacon (O'Conner 2013). Seaweed production is still quite new in Europe; in 2018, less than 0.1% of the world's total was produced here. Nonetheless, the production and use of seaweed in Europe has the potential to benefit the three Ps of sustainability: profit, planet, and people (Van den Burg et al. 2021)

1.2 Consumer acceptance

Changes in food production systems and consumption patterns are required to facilitate the shift towards the use of alternative proteins (Poulson et al. 2020). Alternative protein sources have attracted more attention from consumers in recent years, and this trend is anticipated to continue positively (Poulson et al. 2020). However, for a variety of reasons, attitudes on alternative protein might differ significantly throughout countries (Onwezen et al. 2021). Within a country, variations in a subject's preference to consume different proteins may also be influenced by their personal attributes, such as their personality, and awareness of protein sources (Tuccillo et al. 2020). Acceptance may even vary between alternative dishes that have the same food source (Grahl et al. 2018). Significantly less research have examined the acceptability of certain food items produced from seaweed (Chapman et al. 2015). This is especially crucial to take into account since it has been found that the primary barriers to consumers accepting seaweeds as a food source are low familiarity with eating seaweeds and higher trait levels of food neophobia and food technology neophobia (avoidance of novel foods and foods produced with novel food technologies) (Losada-Lopez et al. 2021). Seaweed is a product category that most European customers are unfamiliar with in connection to the cuisine of their own country (Mouritsen et al. 2013). Previous research showed that a number of factors influence consumers' decisions on seaweed-based food items. Young et al. (2022), for instance, demonstrate that while taste, nutritional value, and overall healthiness are important factors encouraging Australians to eat seaweed, the primary obstacles were restricted availability, expensive cost, and unappealing packaging. Use of seaweeds as an extra component in other well-known items might increase consumer acceptability of seaweeds rather than presenting them as an edible food in general or in isolation (Birch et al. 2019).

1.3 A seaweed aquaculture to meet global sustainability targets

Seaweeds represent a highly productive and sustainable bioresource, offering greater efficiency than terrestrial plant biomass (Balina et al. 2017).Unlike land-based crops, seaweeds do not compete for arable land and can be cultivated without the need for fertilizers, pesticides, or insecticides, making them a more sustainable and environmentally friendly resource (Ganesan et al. 2019). Seaweed farming is considered the most environmentally friendly form of aquaculture, requiring

minimal investment to establish. It plays a crucial role in protecting coastal and aquatic ecosystems from climate change impacts such as ocean acidification and de-oxygenation (Meena et al. 2020). Seaweed farming provides three key ecosystem services. First, it offers cost-effective raw materials for producing food, feed, and energy (Buschmann et al. 2017). Second, it supports vital environmental processes such as natural oxygenation cycles, carbon sequestration, food production, waste purification, and ecosystem maintenance (Hasselström et al. 2018). Finally, it can enhance social programs by fostering natural weather interactions, recreational opportunities, and promoting the importance of aquatic biodiversity, which requires further exploration (Langton et al. 2019). Thus, seaweed farming presents a promising approach for coastal nations to contribute to climate change mitigation.

Seaweed has the potential to significantly contribute to global sustainability (United Nations 2024). Reducing resource consumption is necessary to meet the Sustainable Development Goals (SDGs) of the United Nations (UN), which include aims for the environment, biodiversity, and climate (United Nations 2024). Additionally, the expanding global population—which is predicted to reach 9.7 billion people by 2050 increases the need for healthy food, clean energy, and other resources (United Nations 2024). Discovering new methods to fulfill the UN SDGs and provide the necessary resources will be required to resolve this dilemma. Seaweed farming offers a distinct, scalable, and long-term answer to this problem, and that realizing this option's full potential is essential for a sustainable future (Duarte et al. 2022).

1.4 The impact of seaweed aquaculture on various ecosystem services

The production of various ecosystem services by seaweed aquaculture contributes directly to the advancement of several SDGs (SDGs 2, 3, 7, 13 and 14), which in turn give integrative benefits that support other SDGs (SDGs 1, 5, 9, 15 and 17) (Duarte et al. 2022).

a) Zero Hunger (SDG 2) and Good Health and Well-Being (SDG 3)

Currently, 90% of the seaweeds grown are utilized for human food, either directly or as additives (Mazarrasa et al. 2013). In addition, seaweeds are beneficial additions to human diets because they include antioxidants, fiber, healthy fatty acids, and macro- and micronutrients that help lower the risk of a number of diseases (Holdt et al. 2011).

b) Affordable and Clean Energy (SDG 7)

Through a variety of processes, including fermentation, hydrogen release, transesterification, pyrolysis, liquefaction, and gasification, seaweed biomass may

be utilized to create bio based energy, such as ethanol, butanol, biogas, biodiesel, bio-oil, or hydrogen (Duarte et al. 2022).

c) Climate Action (SDG 13)

Seaweed farming can help adapt to climate change by locally reducing ocean acidification and deoxygenation (Duarte et al. 2017).

d) Life below Water (SDG 14)

In addition to providing food, seaweeds promote biodiversity by creating intricate habitats and altering the biogeochemical and physical characteristics of the surrounding environment (Duarte et al. 2022).

e) No Poverty (SDG 1)

Seaweed aquaculture has been known as a technology for the impoverished because it requires little capital to begin up (less than US$15,000 ha in Mexico, for example) and doesn't require the purchase of large machinery or land, or post-harvest processing facilities on land (Robledo et al. 2013).

f) Gender Equality (SDG 5)

Women who work as seaweed growers in communities in Africa and Indonesia have gained status as well as power within their communities (Larson et al. 2021).

g) Industry Innovation and Infrastructure (SDG 9)

Seaweed represents a source of durable and sustainable biomolecules applicable to various sectors. These include high-value compounds and seaweed-derived biopolymers, which can be utilized in construction, packaging, and textiles, as well as in pharmaceuticals, nutraceuticals, and cosmetics (Duarte et al. 2022).

h) Life on Land (SDG 15)

The growth of land-based production systems is still the primary cause of tropical deforestation, having already converted 50 million km^2 or 46.6% of non-frozen area into farming, pasture, and agriculture (Duarte et al. 2022). Because seaweed farming doesn't require freshwater or arable land, it uses less water resources than other food production methods. Similarly, with seaweed aquaculture, no pesticides or herbicides are used. Therefore, the deterioration of natural ecosystems is limited when seaweed production is added to land-based vegetable production (Duarte et al. 2022).

i) Partnerships for the Goals (SDG 17)

Partnerships are needed to stimulate innovation through West-East and South-North collaborations across the whole production chain to create a balance between supply and demand in order to realize this potential(Robledo et al. 2013). To fully realize the benefits of the seaweed sector and, thus, promote a sustainable ocean economy, a triple-helix cooperation including academics, industry, and government is necessary (Robledo et al. 2013).

1.5 Limitations in the extraction of protein from seaweed

A significant factor influencing the separation and extraction of proteins from seaweed is the complex cellular matrix of the seaweed itself, which serves as a physical barrier (Harnedy & Fitzgerald 2013). It indicates that in order to increase the extraction yield, macroalga biomass must undergo a pretreatment step that involves many disruptive approaches before extraction. Protein recovery is increased using a combinatorial strategy that uses a coordinated pretreatment and extraction method (Harnedy & Fitzgerald 2013). Conventional processing methods, such extended heating and stirring, high water volumes, and other energy-intensive processes, can lead to decreased production efficiency, nutritional component losses, and labor-intensive processes (Echave et al. 2021). In addition, conventional protein extraction techniques are time-consuming, require substantial amounts of solvents, and exhibit limited extraction efficiency (Harnedy & Fitzgerald 2013).

The protein extraction process should ideally achieve high protein recovery while being time-efficient, economically viable, and non-destructive to the extracted protein (Jeon et al. 2011). The use of a high shear homogenization technique produced one of the highest extraction protein yields (39%) seen for *Ulva sp* (Postma et al. 2018). Mechanical pressing and acid precipitation produced a total protein yield of 5.3%, whereas alkaline extraction produced 8.95% yield for Ulva (Juul et al. 2021a). Biancarosa et al. (2017) selected the autoclave method, which combines both heat and pressure, for the extraction of protein from seaweed. The autoclave pre-treatment approach was previously employed to remove carbohydrates from seaweeds and shown to be effective in dissolving the cell wall. O' Connor et al. (2020) employed the autoclave method combined with a centrifugation step to assess whether this approach could enhance protein extraction from seaweed. Veide Vilg and Undeland. (2017) recently applied the pH-shift process, with alkaline protein solubilization followed by isoelectric precipitation, with the addition of an osmotic shock step to *S. latissima*. It yielded 16% protein in total by this method. Kadam et al. (2017) examined several extraction techniques, including sequential extraction, which involves an initial acid treatment followed by an alkaline treatment, and found that this method resulted in the highest protein yield. Specifically, this method achieved a protein extraction efficiency of 59.76% from Irish brown seaweed.

2. Aim and research hypotheses

The aim of this project was to develop effective protein extraction methods from different seaweed types (brown, red and green). An additional aim was to gain a comprehensive understanding of its protein molecular profile and functional properties, which are crucial for determining its suitability for various food applications. The social part of this study included bread baking experiments using wheat flour enriched with the seaweed to systematically assess the addition of seaweed on sensory attributes of the bread, such as taste, aroma and flavor. A tasting survey was conducted to evaluate the consumer acceptance, is also included in this study.

Based on the aims of this work, the study posits the following hypotheses:

1) Methodology developed in this study can extract high amount of proteins from seaweed with suitable structural and functional properties for food applications.

2) Incorporating seaweed into wheat flour and bread baking serves as a means to enhance human dietary protein intake, bioactive compounds and fiber, offering a viable soure of nutrition.

To address both hypotheses, this study was divided into two parts, each focusing on distinct aspects. The first part involves laboratory work consists of extracting protein and examining its functional characteristics. The second part aims to gather consumers' opinions about adding seaweed to bread and to evaluate the sensory characteristics of the bread.

3. Materials and Methods

3.1 Materials

Brown, red and green dried seaweed were provided by the Swedish seaweed producer (Nordic Seafarm AB). Seaweed protein samples were grinded to a particle size of 0.5 mm and stored at -20 ℃ until further use. For analysis, chemical compounds used were of analytical grade.

All previous methodologies employed fresh seaweed for protein extraction. In our study, we initiated the extraction process using dried seaweed. Initially, protein extraction was attempted from three different types of seaweed; however, results were only obtained from brown seaweed. Consequently, we proceeded with the extraction from brown seaweed (*Saccharina Latissima)* using three distinct methods.

3.1.1. 1 h stirring

For protein extraction, 10 grams of the ground seaweed was added to a 500 ml beaker containing 500 ml of milli-Q water. The resulting mixture was homogenized for 2 min using a stirrer. There after, ultrasonic treatment was applied for 10 min at 400W (hielscher UP400St). During the ultrasonic treatment, samples were kept in ice to prevent exposure to excessive heat, which could lead to protein denaturation and aggregation. The ultrasonic treatment performed in cycles of 5 min activation and 5 min rest to avoid sample overheating. After ultrasonication treatment, samples were stirred for 1 h at 300 rpm. The pH of the solution was then measured using a pH meter, it was 6.5. To adjust the pH to 12, 1M NaOH was gradually added. The pH adjustment process under constant pH meter monitoring lasts 1 h and 30 min, maintaining the pH 12. Following pH adjustment, centrifugation was 8000 rpm and a temperature of 8 ℃. After centrifugation, the supernatant was collected and used for proteins extraction. To obtain the proteins in the supernatant, pH was adjusted to 2 using 1M HCl. To collect the precipitated proteins, samples were centrifuged for 20 min at 4000 rpm. Extracted proteins were collected and stored at -80 ℃. Samples were later lyophilized and stored at -20 ℃ until further use. The measurements were conducted in triplicate, and the obtained data were analyzed using JMP statistical software.

24 h stirring method

This method is identical to the 1 h procedure, with the exception that the sample is allowed to stir overnight.

Over night freezing

In this case, following ultrasonication, the sample was frozen overnight at -80 ℃. Subsequently, the sample was thawed the following day, subjected to a second round of ultrasonication, and the remaining steps were performed as previously described.

3.1.2 Measuring the amount of nitrogen

Initially, 5 ± 1.0 mg of protein extracted by three different methods was placed into tin capsules (5 x 8 mm), weighed, and carefully sealed using tweezers. The total nitrogen (N) content is then analyzed using a Flash 2000 Thermo Scientific analyzer. Acetanilide/N-phenylacetamide, with 71.09% C, is used as the external

standard, and Thermo Scientific Alfalfa is used as the reference standard. Each sample were replicated three times.

3.1.3 Preparation of emulsions

Initially, 10 mg of protein was dispersed in 10 ml of milli-Q water and left to stir overnight to completely dissolve the proteins. Thereafter, the suspensions were centrifuged at 4200 rpm for 10 min to remove any insoluble components. Oil-in-water (O/W) emulsions were then made by adding 1 ml rapeseed oil into protein suspensions. These suspensions were pre-homogenized for 2 min at 12000 rpm using an Ultra Turrax IKA T18 homogenizer. Subsequently, the samples were subjected to ultrasonication for 10 minutes at 200 W and 100% amplitude using a Hielscher UP400ST ultrasonic processor. Ice-bath was used throughout the ultrasonication process to prevent overheating and protein denaturation.

3.1.4 Particle size and Zeta- Potential

The ZetaSizer Nano-ZS (Malvern Instruments Ltd., Malvern, Sweden) was utilized to determine the droplet size and zeta potential of emulsions. Dynamic light scattering (DLS) was employed to assess the droplet size at 25 ˚C. The measurements were performed using a disposable, folded capillary cell, DTS1070. Each measurement consisted of three cycles with a 120s equilibrium time. Initially, an emulsion containing 1% protein was prepared. Subsequently emulsion samples were diluted to a concentration of 0.05 mg/ml using milli-Q water. Each preparation and measurement of the fresh emulsion were replicated three times. The measurements were conducted in triplicate, and the obtained data was analysed in JMP statistical software.

3.1.5 Microstructure of emulsions

Emulsions were examined at the particle level using a DAS microscope DM LB (UCL condenser) with a camera to observe the interaction of proteins and oil in the emulsions. A single droplet of each sample was placed on a microscope glass slide, then covered with a slide cover. Samples were examined at 20× magnification and images were taken. The obtained images were analyzed using Image J software.

3.1.6 Measurement of protein concentration

The bicinchoninic acid assay (BCA) was utilized for determining the samples' total protein content. Proteins from the samples connect to copper ions during this test resulting in a complex that is purple in color which a spectrophotometer can analyse and compare to a protein standard. To conduct the experiment, five standards by varying concentrations were employed, and the proteins obtained through three distinct methods were utilized. Initially, a suspension containing 2% protein was prepared. BSA standard was made with 5 different concentrations. Both the standards and the samples were replicated three times. BCA Reagent A and BCA Reagent B were prepared in specific concentrations and then mixed together for use. For analysis, 25 µl of standard samples were used, and the 25 µl of suspension containing protein was utilized. These samples were transferred to a 96 well plate and after that, 200 µl of reagent was added to well. The plate was then covered and placed on a shaker for two hours. Following this, the sample was analyzed using a spectrophotometer at a wavelength 2.163 mm.

3.1.7 HPLC

The molecular profile of extracted proteins was studied with size exclusion high-performance liquid chromatography (SE-HPLC). For HPLC, the method described by Muneer et al. (2014) was used with slight modifications. The HPLC procedure comprised of three steps of extraction.

Step 1:

Samples were accurately weighed (16.5 ± 0.05 mg) and transferred into Eppendorf tubes. Each sample received 1.4 mL of buffer solution (SDS-phosphate) and was then vortexed for 10 seconds. The samples were subsequently shaken for 5 minutes at 2000 rpm. Following the shaking process, the samples were centrifuged for 30 minutes at 10,000 rpm. After centrifugation, the supernatant was separated and transferred into HPLC vials.

Step 2:

To the pellet obtained from Step 1, an additional 1.4 mL of buffer solution was added. The sample was sonicated for 30 seconds using a Soniprep 150 at an amplitude of 5 microns. The sample was then centrifuged for 30 seconds at 10,000 rpm, and the supernatant was separated and transferred into HPLC vials.

Step 3:

This step was repeated for the second pellet obtained, an additional 1.4 mL of buffer solution was added. Sonication duration extended to 1 minute and 30 seconds using a Soniprep 150 at an amplitude of 5 microns. The sample was then centrifuged for

30 seconds at 10,000 rpm, and the supernatant was separated and transferred into HPLC vials.

For analysis of each sample, 20 µl was injected at an isocratic flow of 0.2 ml/min (50 % acetonitrile, 0.1 % TFA; 50 % H2O, 0.1 % TFA) for 30 min. Chromatograms were obtained by UV-detection at a wavelength of 210 nm using a Waters 996 Photodiod Array Detector (Waters, USA). Chromatograms were integrated and divided into two groups, polymeric proteins (retention 7-14 min) and monomeric proteins (14-28 min).

3.1.8 Bread baking

The tortilla bread was prepared following Henriksson s ' (2020) recipe, with a slight modification.The tortilla breads were prepared using three different ratio of wheat flour to whole seaweed flour: one containing 2% seaweed, another containing 4% seaweed, and a control without seaweed flour. The preparation process began by mixing 345.5 g of wheat flour with 7 g of seaweed for 2% seaweed bread, followed by a subsequent batch of 338.5 g of wheat flour combined with 14 g of seaweed for 4% seaweed bread, and 352.5 g of wheat flour for the control. Additionally, 2.5 g of baking powder and 4 g of salt were incorporated into the flour and seaweed mixture. This initial blend was mixed for 20 seconds using a mixer. Subsequently, 19 g of rapeseed oil and 187.5 g of water were added to the mixture. The combination of all ingredients was mixed for a further 60 seconds. Following the mixing process, the dough was placed in the freezer for 15 minutes. After freezing, the dough was kneaded and divided into 70 g portions. For the baking process, each portion was cooked on one side for 50 seconds and then flipped to cook the other side for an additional 40 seconds.

3.2 Questionnaire for assessment of sensory consumer acceptance

To assess public opinion on the incorporation of seaweed into food products, an experimental study was conducted using tortilla bread. The objective of our study was to examine the sensory attributes of bread subsequent to the incorporation of seaweed. A comprehensive questionnaire was designed as described by Nicolas et al. (2010) with modifications (Appendix 1) and administered to gather participants' opinions. Participants for the sensory trial were invited via email and verbal invitation. Participants were invited from the Plant Breeding department at SLU with diverse background. This investigation sought to understand not only the

sensory characteristics of bread, such as taste, texture, and aroma, but also to explore its acceptance and preference as an ingredient in food products.

4. Results and discussion

4.1 Laboratory tests results

4.1.1 Protein extraction results

By measuring the total nitrogen content in seaweed, it was determined that the nitrogen concentration was 2.03% for *Saccharina Latissima*. The conversion factor of 6.25 was subsequently applied, resulting in the determination that the total protein content in 10 g of seaweed is equivalent to 1.27 g. Protein extraction yield varied depending on the method employed. Based on the results obtained, it was determined that the O-N freezing method yielded the highest amount of protein, approximately one-third of the total protein content present in the seaweed (0.39 g) (Figure 1). This was followed by the 24 h method, which extracted a moderate amount of protein, approximately one-fifth of the total protein content (0.26 g). The 1 h method resulted in the lowest protein extraction, yielding approximately one-sixth of the total protein content present in the seaweed (0.21g). The statistical analysis revealed that the O-N freezing method differed significantly from the other two methods in terms of protein extraction, while no significant differences were observed between the other two methods.

The main reason for the decreased protein extraction efficiency is that seaweeds' cell walls have a more complex and varied structure than terrestrial plants (Charoensiddhi et al. 2017). They are made up of branching and sulfated polysaccharide combinations linked to proteins (Wijesinghe & Jeon 2012). Within the cell wall structure, seaweed proteins are interconnected with polysaccharides through disulfide bonds (Cerme˜no et al. 2020). The high dispersion viscosity of anionic or neutral polysaccharides, along with the polyphenol-rich composition of seaweed cell walls, obstructs the release of proteins during extraction. Moreover, achieving protein purity during seaweed extraction necessitates the removal of non-protein nitrogenous compounds (Gleison et al. 2023). Furthermore, it is very difficult to keep seaweed protein at the same quality and amount throughout the year due to environmental conditions, harvesting sites, and times that most likely resulted in variation in the protein content (Pliego-Cortés et al. 2020).

The O-N freezing method demonstrates potential as an effective technique for protein extraction, yielding a protein content of 30.71% of the total protein. This

aligns with the findings of Hemlata et al. (2011), who observed that the freezing method produced a higher protein yield due to the rapid thawing and freezing processes, which led to the formation of ice crystals that disrupted cell walls. As Abdollahi et al. (2019) further noted, greater structural damage to the cell wall during freezing facilitates the release of more protein, contributing to the efficiency of the extraction process. Abdollahi et al. (2019) evaluated several pretreatment methods for extracting protein from fresh biomass of S. latissima, finding that the yields varied depending on the technique used. Protein extraction yields from freeze-thawing at -20°C (79.9%) and -80°C (65.7%) differed from those obtained through freeze-drying (90.9%) and ensiling (25.4%). Overall, freeze-thawing doubled protein production and improved protein precipitation (Abdollahi et al. 2019). Similarly, we also found that freeze-thawing extracted a higher amount of protein.

According to Wang et al. (2013), freezing time was observed to increase the protein, lipid, and solid contents in soymilk. The protein level in soymilk produced from treated soybeans reached 3.14 ± 0.02%, while soymilk made from untreated soybeans had the lowest protein content at 2.76 ± 0.01%. This indicates that freezing treatment significantly enhances extraction efficiency (Wang et al. 2013)

During protein extraction, ultrasound was employed in a two-stage process as part of the O-N freezing method to enhance efficiency by facilitating cell wall disruption. This aligns with the findings of Kadam et al. (2017), who highlighted that advanced extraction techniques, such as ultrasound-assisted extraction, are often more effective in achieving higher extraction yields due to their ability to improve cell wall permeability and release intracellular components more efficiently. There are compression when ultrasonic is applied. Bubbles occur in liquid vapor when the pressure exceeds its tensile strength. When exposed to strong ultrasonic, these bubbles burst, creating a cavitation effect (Vilkhu et al. 2008). The release of the chemical is facilitated by cavitation, which causes peeling, erosion, particle breakup, and degradation of the solid-liquid interfaces (Vilkhu et al. 2011).

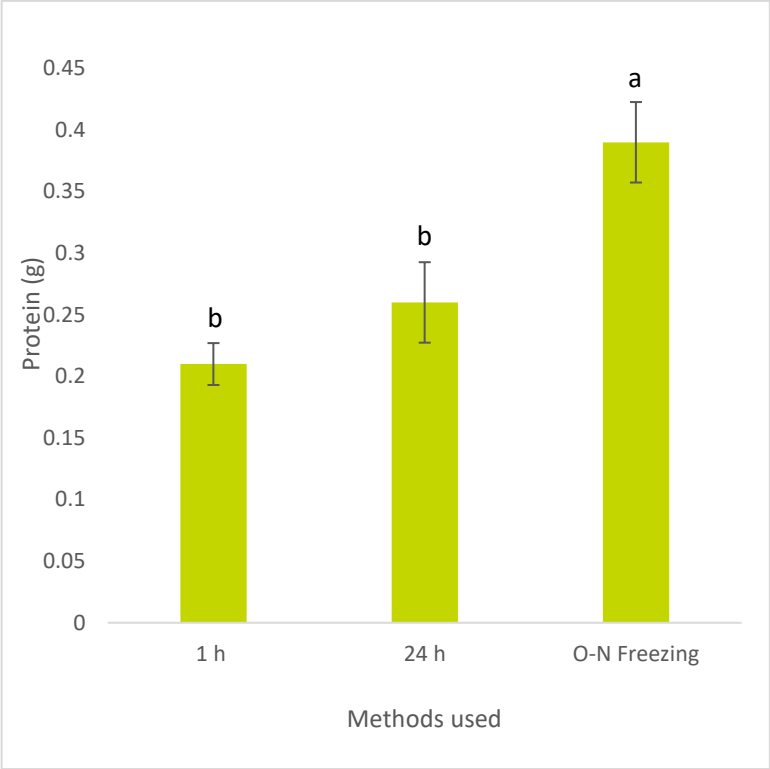


Figure 1. The quantity of protein extracted using three distinct methods with three replicates: 1 h extraction, 24 h extraction, and an O-N freezing methods. Different letters represent the difference between samples.

4.1.2 Protein concentrations in extracts

As shown in Figure 2, in the solution containing 25 µl of protein obtained via the O-N freezing method, the measured protein concentration was approximately 0.04 mg/ml, whereas in the solution prepared using the 1 h method, the protein concentration was approximately 0.01 mg/ml. Based on the obtained results, it was determined that the protein yield from the O-N freezing method was higher compared to the 1 h method.

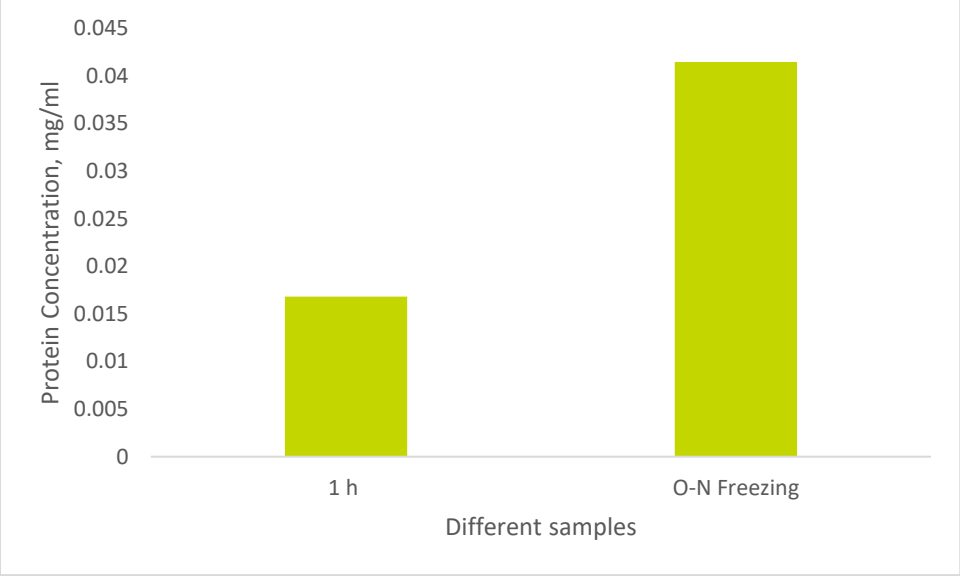


Figure 2. Protein concentration in mg/ml: Protein extracted by O-N freezing and 1 h methods.

4.1.3 Zeta test and particle size

The ζ-potential and particle size analysis were performed on the prepared protein-oil emulsions. The ζ-potential indicates the degree to which the emulsion droplets interact with one another (Wang et al. 2013). In addition, the potential stability of an emulsion system can be shown by the ζ-potential, a measurement of the surface charge density of proteins (Shanmugam & Ashokkumar 2014). Charges on the surface of the oil droplets may be provided by the protein that has been adsorbed on oil droplets in the emulsion (Dan et al. 2020).

Figure 3 presents the ζ-potential distributions for prepared emulsions. Notably, all examined samples showed negative ζ-potential values. The emulsion prepared using protein from O-N freezing method exhibited a ζ-potential that was -57.71 mV , indicating a relatively lower ζ-potential. In contrast, the 24 h method resulted in a ζ-potential of approximately -68.71 mV showed slight higher zeta potential .Consequently, the 24 h method demonstrates enhanced stability, whereas the O-N freezing method is associated with reduced stability. Li et al. (2016) demonstrated that the ζ-potential of pea protein increased with the intensification of the grinding process. the proteins adhered to the surface of the emulsion droplets tended to reach saturation, resulting in an increase in the surface charge of the oil droplets (Li et al. 2016). It contributes in enhancing the repulsion between droplets and keeps them from sticking together, improving the stability of the emulsion system (Qiaozhen et al. 2020). According to these results, it can be inferred that in the 24 h method, the

increased negative charge prevents particle aggregation, leading to a more stable emulsion.

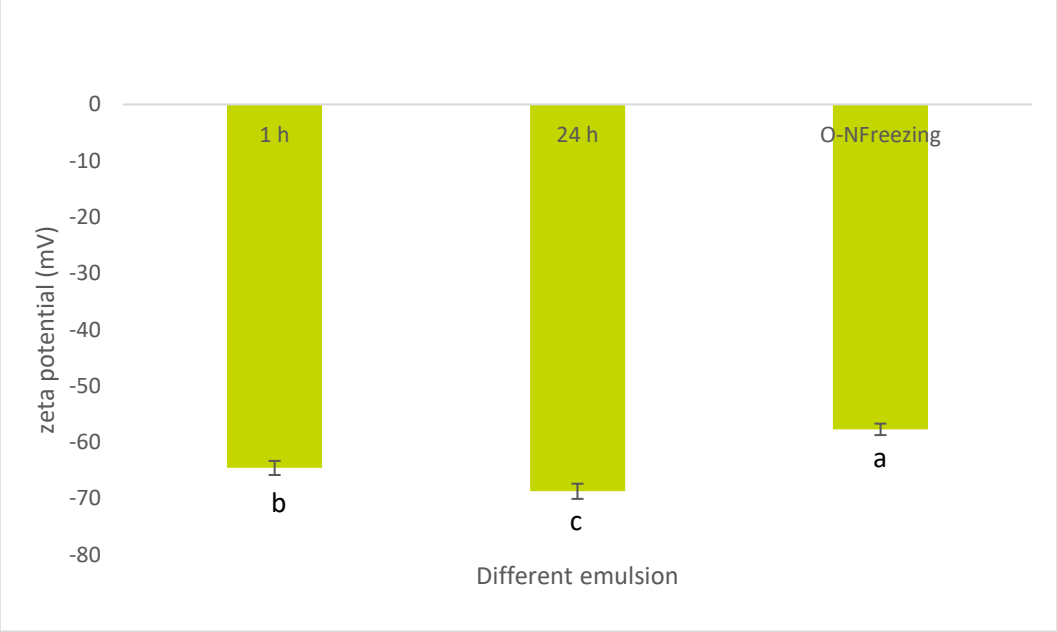


Figure 3. The zeta potential (mV) of emulsions prepared from protein extracted using three different methods: a 1 h extraction, a 24 h extraction, and an O-N freezing. Different letters represent the difference between samples

Particle size is considered to be a crucial indicator for assessing the stability of an emulsion as it may be used to see how the insoluble phase has dispersed throughout the emulsion (Zhang et al. 2022). The particle sizes are presented in Fig 4, indicating that the emulsion prepared by the 24 h method exhibited the largest particle size, approximately 1441.49 d.nm. In contrast, the emulsion prepared via the O-N freezing method, which achieved the lowest ζ-potential, had the smallest particle size, approximately 1145.98 d.nm. Wang et al. (2013) discovered that, with increased freezing time, the volume percentage of smaller soymilk particles (<10 μm) increased, while the proportion of larger particles (>50 μm) decreased. This shift in particle size distribution was found to enhance the extraction efficiency of soybean components, which is in alignment with the findings of our study.

Based on the obtained results, it was determined that the particles produced using the freezing method were smaller in size compared to those obtained through the other two methods. Additionally, the ζ-potential of these particles was reduced, leading to a corresponding decrease in the emulsion's stability. In contrast, the 24-hour method resulted in larger particle sizes and an increased ζ-potential, which contributed to a higher stability of the emulsion. This contrast highlights the

significant impact of the extraction method on both particle characteristics and emulsion stability.

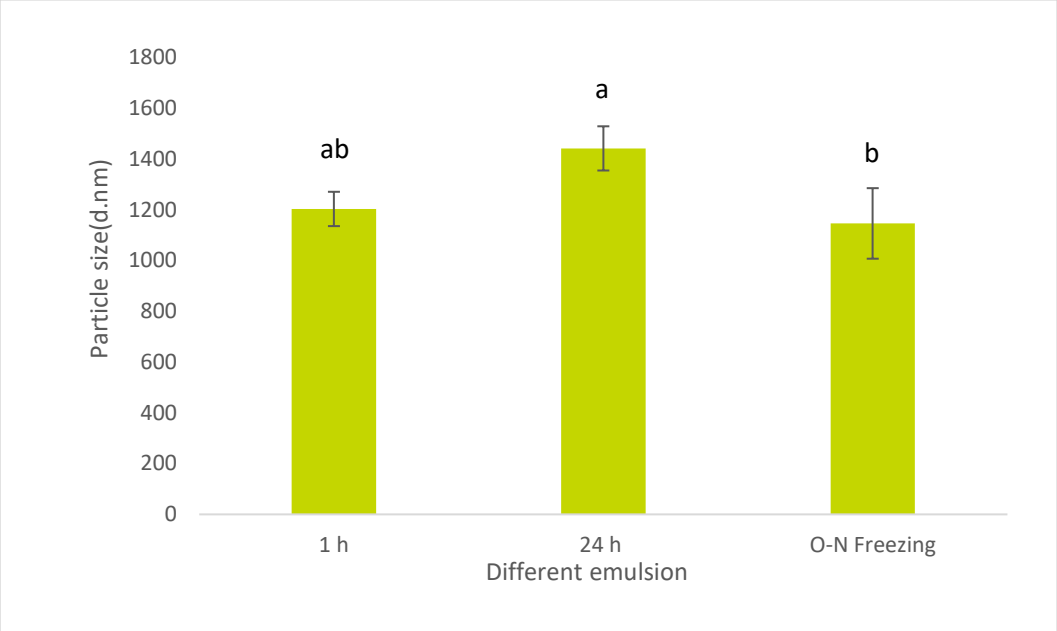
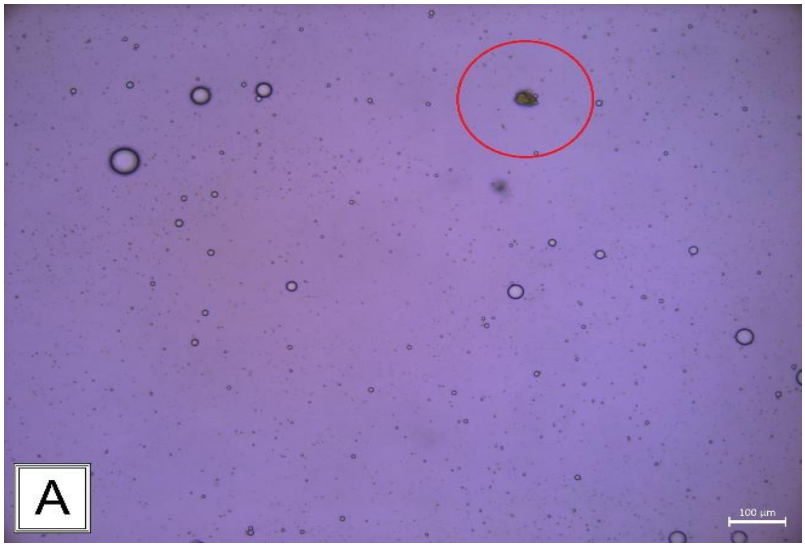


Figure 4. Particle size (d.nm) of emulsions prepared from protein extracted using three different methods with three replicerates: a 1 h extraction, a 24 h extraction, and an O-N freezing. Different letters represent the difference between samples

4.1.4 Microstructure of emulsions

The particle size and molecule aggregation of emulsion were observed by optical microscope (Figure 5). The droplet size distribution of 24 h emulsions were more uniform, compared to other methods. For 1 h insoluble residues were present.The all three types of protein emulsion exhibited a clear ring structure, and the protein was fully bonded to the oil droplet's surface.



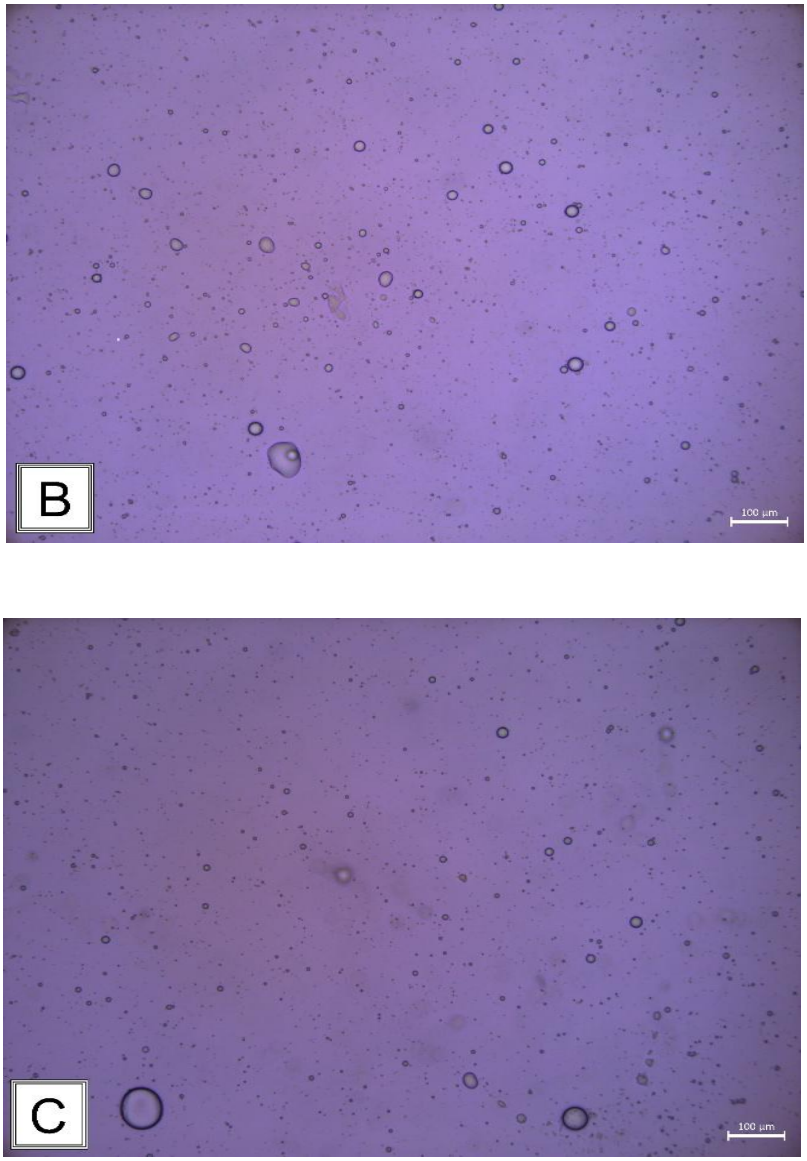


Figure 5. Microstructure of emulsions.A: 1 h - B: 24 h - C: O-N Freezing

4.1.5 HPLC results

SE-HPLC analysis was conducted to examine the molecular profile of seaweed proteins extracted using various methods. The results, shown in Figure 6, indicate only slight variations in the molecular profile. Most of the high molecular weight (HMW) and low molecular weight (LMW) proteins were successfully extracted during the initial extraction using an SDS-phosphate buffer. In subsequent extraction steps (2nd and 3rd extractions with sonication), only small amounts of

proteins were recovered. Therefore, the proteins extracted during the first step were considered representative for studying the protein profile. The substantial extraction of both HMW and LMW proteins using the SDS-phosphate buffer suggests that the proteins were less aggregated or crosslinked, making them more easily extractable with the SDS buffer. Also, large amount of LMW proteins were observed as compared to HMW proteins in all the samples (Figure 6). Furthermore, this study also shows that the variation in extraction method did not largely contribute to changes in protein profile.

Previous studies have shown relatively higher amounts of HMW proteins in seaweed proteins extracted from fresh biomass proteins compared to low molecular weight (LMW) proteins (Abdollahi et al. 2019). However, a key factor that may have contributed to the extraction of smaller amounts of HMW proteins in this study is the use of ultrasonication during the extraction process. Ultrasonication could have disrupted the crosslinks in the larger protein chains, leading to their breakdown.

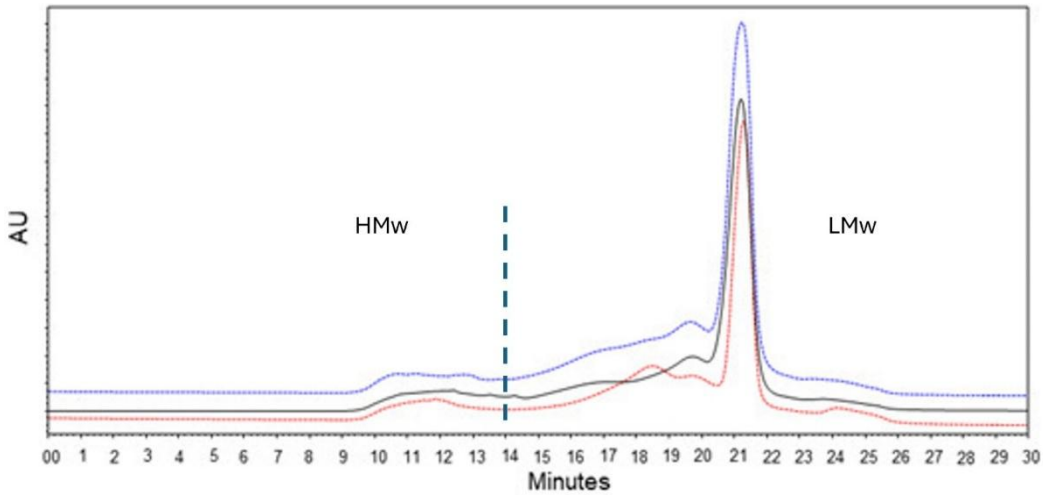


Figure 6. Molecular profile of seaweed protein extracted with 1h (red dotted line), 24h (blue dotted line) and O-N freezing (solid black line).Chromatogram area from 9-14 min represent high molecular weight proteins and from 14-26 min represent low molecular weight proteins.

4.2 Sensory evaluation results of tortilla bread

The bread tasting trial involved 22 participants (36% males and 64% females). The age distribution of the sample population was as follows: 27% of the participants were between 18 and 30 years old, 55% were between 30 and 42 years old, 9% were between 42 and 54 years old, and the remaining 9% were over 54 years old. The prepared samples acoording to Figure 7, including the control sample, bread containing 2% seaweed, and bread containing 4% seaweed, were provided to the participants for sensory evaluation. These samples were accompanied by a questionnaire to assess the sensory characteristics (appendix 1). In the

questionnaire, participants were provided with detailed explanations regarding seaweed and its associated benefits.

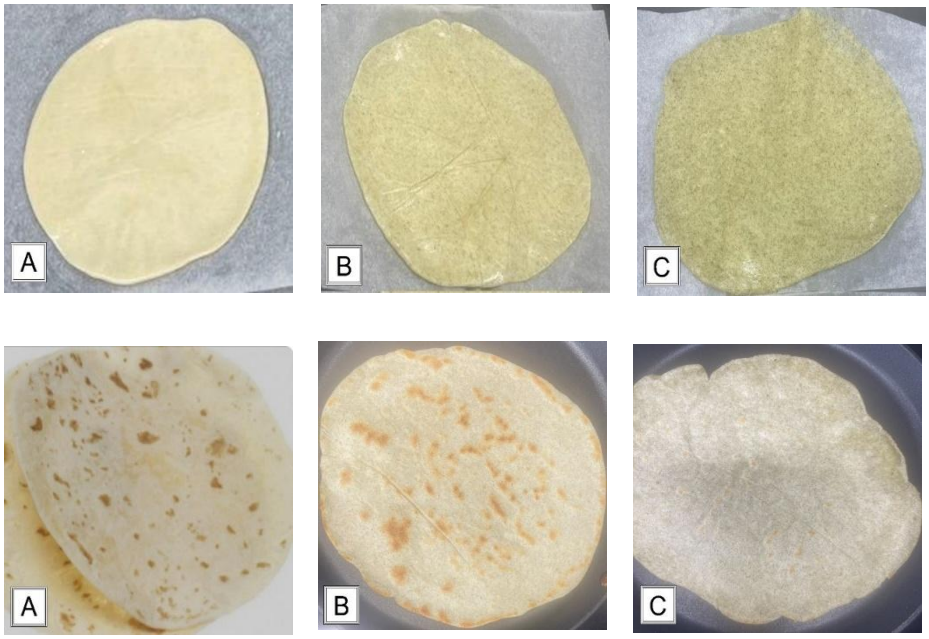
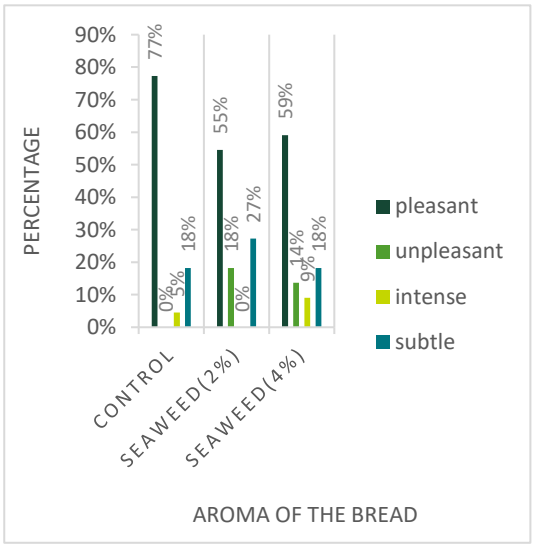
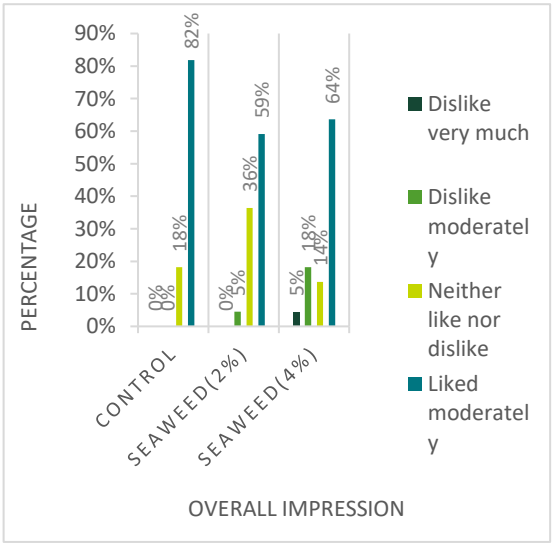


Figure 7. Pictures of the prepared tortilla bread before and after baking . A : the control bread, B:the bread containing 2% seaweed, and C the bread containing 4% seaweed





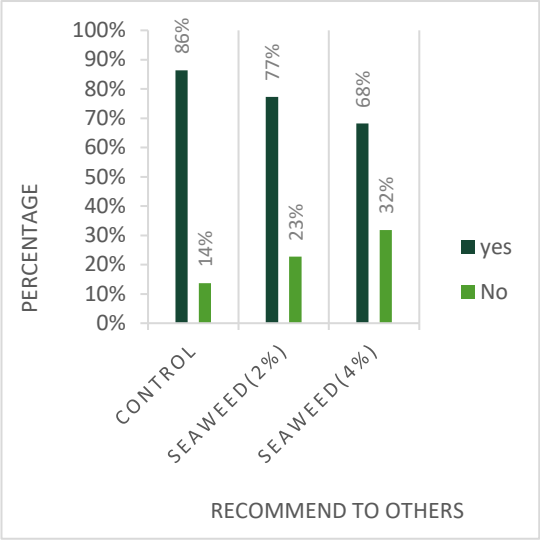


Figure 8. Sensory evaluation of prepared bread, aroma of the bread, overall impression, and recommend to others

Based on the obtained results, it was determined that overall impression according to Figure 8, showed that the majority of participants expressed a preference for bread with seaweed. In the study, 64% of participants expressed a preference for bread containing 4% seaweed, while only 5% indicated a dislike for it. Additionally, for bread containing 2% seaweed, 59% of participants preferred it, and none reported a dislike. In the overall impression assessments, the response category "Like a lot" was not included. Consequently, the absence of this category in the evaluations may have influenced the data, potentially resulting in different outcomes. According to a study conducted by Fredriksson et al. (2023), it was found that Swedish consumers had a favorable attitude toward consuming seaweed in food products which aligns with the data presented in Figure 8, where the majority of participants expressed a preference for bread containing seaweed. According to Quitral et al. (2022), seaweeds significantly influence the sensory attributes of baked and farinaceous products, particularly their taste. Based on sensory qualities, the maximum recommended percentages of seaweed for various products are as follows: 10% for noodles, 4% for bread, 5% for biscuits, and less than 10% for cookies. These findings are consistent with our data, which indicate that the inclusion of 4% seaweed resulted in the most favorable overall impression.

In this study, 68% of participants indicated that they would recommend the 4% seaweed bread to others, while 77% expressed a willingness to recommend the 2% seaweed bread. These results highlight that even higher concentrations of seaweed can sustain positive consumer acceptance. These findings align with the study

conducted by Kim et al. (2011), which emphasized participants' willingness to consume the bread themselves and recommend it to others, with the majority responding positively. Furthermore, Kim et al. (2011) found that incorporating seaweed into bread at relatively low concentrations, such as 0.5% to 1%, significantly enhanced its acceptability.

The inclusion of seaweed was not found to be unpleasant by the participants. As the majority of participants who tested the bread perceived its taste to be pleasant. Only a small proportion of individuals believed that the addition of seaweed imparted an intense flavor to the bread. Additionally, the aroma of the bread containing a concentration of 4% seaweed was more pleasant compared to that of the bread containing a concentration of 2% seaweed.

The majority of participants 67%identified saltiness as the dominant flavor in both types of seaweed-fortified bread. This is consistent with the findings of Jönsson et al. (2023), who reported that seaweed species are characterized by a high salinity, which likely influenced the taste profile of the bread. In the case of the flavor, both types were found to possess a well-balanced taste, neither excessively strong nor lacking in flavor.

Regarding the aroma's potential to evoke memories, the majority of participants reported that none of the types of bread elicited recollections of specific food items. However, a small subset of participants indicated that the bread containing 4% seaweed evoked sensory associations with grass and fish. Most participants who tested the bread perceived the bread containing 2% seaweed as having a texture between dry and moist. In contrast, the bread containing 4% seaweed was considered soft and moist.

The results from sensory tests can be significantly connected to the consumption of seaweed for positive sensory attributes, such as a pleasant taste and appealing texture, can encourage consumers to incorporate seaweed into their diets.

Previous studies have shown that consumer perceptions significantly influence their willingness to try seaweed. For instance, when consumers perceive seaweed as typically less tasty, their willingness to try it decreases (Palmieri & Forleo 2020). However, consumers who are aware of seaweed's use as an ingredient in popular dishes, such as sushi, are more likely to incorporate it into their diets (Birch et al. 2019). Familiarity with specific food products, like bread, which is commonly consumed, has also been demonstrated to increase purchase intent (Kam et al. 2012).

Research indicates that people tend to view seaweeds more positively when they are framed as part of main meals, while their perception declines when presented in sweet foods and beverages (Wendin & Undeland 2020). Additionally, Chapman et al. (2015) suggest that providing consumers with tasting opportunities can enhance acceptance of less familiar products. Nonetheless, food neophobia poses a significant barrier to the adoption of seaweeds among consumers (Embling et al.

2022). Interestingly, the negative effects of food neophobia can be somewhat mitigated by perceptions of product familiarity and taste/edibility, indicating that these attributes may help alleviate the detrimental impact of food neophobia on consumer acceptance (Embling et al. 2022).

5. Conclusions

This study concluded that protein extraction from brown seaweed using the O-N freezing method yielded the highest efficiency, recovering approximately 30.71% of the total protein content, exceeding the quantities obtained by the other two methods. The O-N freezing method also demonstrated a higher protein concentration compared to the alternatives.

The 24 h method exhibited a higher ζ-potential, larger particle size, and more uniform particle distribution, indicating enhanced stability of the seaweed-enriched emulsion. Additionally, SE-HPLC analysis showed that the initial SDS-phosphate buffer extraction effectively recovered both high and low molecular weight proteins from the seaweed.

In the sensory evaluation, participants generally liked the bread containing seaweed. Increased liking of seaweed-enriched bread is likely to drive greater consumption, which in turn could lead to higher demand for seaweed products. This increased demand can benefit local economies. Furthermore, the cultivation and harvesting of seaweed provide significant environmental benefits. Therefore, enhancing the sensory appeal of seaweed not only promotes healthier dietary habits but also contributes to broader environmental sustainability efforts.

Future research

Conduct more detailed sensory analysis involving a larger and more diverse panel of consumers to investigate how variables such as different concentrations of seaweed protein, types of seaweed used, and bread formulations influence consumer preferences in terms of taste, texture, aroma, and appearance. Additionally, research on consumer attitudes toward seaweed protein products, as well as how education and awareness campaigns might impact its acceptance as a mainstream protein source, would provide valuable insights for promoting seaweed-based foods.

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Appendix 1

Seaweed Fortified Wheat Bread Tasting Questionnaire

Seaweed is considered as superfood being rich in protein, bioactive compounds and dietary fiber. The protein derived from seaweed has the potential to replace animal protein in food preparations because of its high content of essential amino acids. Seaweed dried powder can be added as an ingredient in food sources to improve their nutritional quality. Three different types of bread were prepared for the study to evaluate the impact of seaweed on aroma, taste and flavor of the bread. The control bread was baked using only wheat flour,, second variant included 2% seaweed and third variant contained 4% seaweed.

1. What is your age?

a) 18-30
b) 30-42
c) 42-54
d) 54 or older

2. What is your gender?

a) Male
b) Female

3. Describe the aroma of the bread.

Control	**2% seaweed**	**4% seaweed**
a) Pleasant	a) Pleasant	a) Pleasant
b) Unpleasant	b) Unpleasant	b) Unpleasant
c) Intense	c) Intense	c) Intense
d) Subtle	d) Subtle	d) Subtle

4. Does the aroma evoke any memories or associations for you?

Control	**2% seaweed**	**4% seaweed**
a) Yes	a) Yes	a) Yes
b) No	b) No	b) No
If yes, please describe: _____	If yes, please describe: _____	If yes, please describe: _____

5. What flavors do you perceive when tasting the bread?

Control	**2% seaweed**	**4% seaweed**

a) Balanced	a) Balanced	a) Balanced
b) Overpowering	b) Overpowering	b) Overpowering
c) Lacking	c) Lacking	c) Lacking

6. Which tastes do you detect? (Select all that apply)

Control	**2% seaweed**	**4% seaweed**
a) Sweetness	a) Sweetness	a) Sweetness
b) Saltiness	b) Saltiness	b) Saltiness
c) Sourness	c) Sourness	c) Sourness
d) Bitterness	d) Bitterness	d) Bitterness

7. How does the bread feel in your mouth (mouthfeel)?

Control	**2% seaweed**	**4% seaweed**
a) Moist	a) Moist	a) Moist
b) Dry	b) Dry	b) Dry
c) Somewhere in between	c) Somewhere in between	c) Somewhere in between

8. Considering all the sensory attributes you have assessed, what is your overall impression of the bread?

Control	**2% seaweed**	**4% seaweed**
a) Disliked very much	a) Disliked very much	a) Disliked very much
b) Disliked moderately	b) Disliked moderately	b) Disliked moderately
c) Neither liked nor disliked	c) Neither liked nor disliked	c) Neither liked nor disliked
d) Liked moderately	d) Liked moderately	d) Liked moderately

9. Would you recommend this bread to others based on your experience?

Control	**2% seaweed**	**4% seaweed**
a) Yes	a) Yes	a) Yes
b) No	b) No	b) No

10. Any additional comments or suggestions?

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