Protein from Red Seaweeds: Extraction, Properties and Functionalities

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Abstract

Background: The global population is projected to grow more than one-third by 2050, and food demand estimated to exceed another 70%. With escalating needs for protein, animal protein production would eventually outbalance future resources. The search for a plant protein requires immediate attention to sustain natural resources and enhance food security. In this scenario, the sustainable cultivation, and attractive nutritional and functional values of red seaweeds, such as *Porphyra* and *Pyropia*, spotlight such seaweeds as reliable alternative protein sources.

Objectives: The present study aimed to systematically optimise the protein extraction protocols for red seaweeds (*Porphyra umbilicalis, Pyropia viridi*dentata and *Pyropia cinnamomea*), and investigate the physicochemical properties and functionalities of the derived seaweed protein extracts.

Methods: Four protein extraction methods were investigated, namely water, alkaline, ultrasound-assisted and enzyme-assisted extractions. Physicochemical properties of the obtained seaweed protein extracts (SPEs), including amino acid composition, molecular weight distribution, protein functional groups and thermal stability were analysed. The functional properties of the SPEs, such as solubility, emulsifying and foaming properties, were examined and compared to commercial whey and soy protein isolates. The potential antioxidant capacity of SPEs was evaluated using the DPPH and FRAP assays.

Results: The optimised conditions for each extraction protocol were as below: water extraction (biomass-water ratio of 1:40, extraction at 23 °C for 4 hr), alkaline extraction (biomass-water ratio of 1:30 at pH 12, 23 °C for 4 hr); ultrasound-assisted extraction (biomass-water ratio of 1:40 at 200 W and 20 kHz for 5 mins); enzyme-assisted extraction (biomass-water ratio of 1:40 at pH 9, 23 °C for 16 hr with Alcalase). Across all extraction protocols, SPEs derived from Alcalase-treatment exhibited the greatest protein yield of 193.20 mg protein/g seaweed and a purity of 43.01% ($p < 0.05$). Alcalase extraction efficiency doubled that of polysaccharidases, reaching up to 68% extraction yield. SPE following enzyme-assisted extraction comprised comparable proportions of essential amino acids to the commercial isolates. All SPEs had a molecular weight distribution ranging between 20 to below 10 kDa, typically lower than those from commercial isolates. From the FTIR analysis, the SPEs showed slight variances in the

absorbances of amide I, II and III bonds, referring to different protein structures. Inspecting thermal properties through Differential Scanning Calorimetry revealed higher thermal stability across all SPEs than commercial isolates. The functional properties of SPEs were consistent with their physicochemical properties. For most of the functional properties, some SPEs produced more impressive performances than commercial isolates. All SPEs demonstrated good solubility with minimal fluctuations across different pHs; in particular, SPEs obtained from enzyme-assisted extraction had over 95% solubility. All SPEs also consisted of good emulsifying properties; in particular, those obtained from enzyme-assisted extraction achieved an emulsifying capacity of 62 mg^2/g and emulsifying stability of 77.03 mins. Moreover, the SPEs illustrated good foaming properties; in particular, SPE following alkaline extraction showcased up to 280% foaming capacity, whereas the SPE after enzyme-assisted extraction formed a stable foam for 82.99 mins.

Conclusions: Based on the results of this study, red seaweed proteins, especially ones extracted enzymatically, could be a promising protein source for developing functional foods and have significant potential to be incorporated in the formulation of various food products.

Keywords: seaweed protein, protein extraction, enzymatic extraction, amino acid profiling, physicochemical properties, functional properties

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List of abbreviations

2,2’-Diphenyl-1-picrylhydrazyl	DPPH
2,4,6-Tripyridyl-s-Triazine	TPTZ
Amino acid	AA
Amino acid score	AAS
Analysis of variance	ANOVA
Angiotensin-converting enzyme	ACE
Association of Official Analytical Chemists	AOAC
Attenuated total reflection	ATR
Bovine serum albumin	BSA
Circular dichroism	CD
Coomassie Brilliant Blue	CBB
Degrees of freedom	DOF
Diode array detector	DAD
Differential scanning calorimetry	DSC
Dry weight	DW
Emulsion activity	EA
Emulsion stability	ES
Essential amino acid	EAA
Essential amino acid index	EAAI
Essential amino acid ratio	EAA:AA
Essential amino acid-non-essential amino acid ratio	EAA:NAA
Ferric ion reducing antioxidant power	FRAP
Foaming capacity	FC
Foaming stability	FS
Food & Agriculture Organisation	FAO
Fourier-transform	FT
Gas chromatography	GC
Gastrointestinal	GI
High performance liquid chromatography	HPLC
Hydrochloric acid	HCl
Infrared	IR
Ion-exchange	IE

Mass spectrometry	MS
Membrane filtration	MF
Microwave-assisted extraction	MAE
Molecular weight	MW
New Zealand	NZ
Nitrogen-to-protein conversion factor	NPCF
Oil holding capacity	OHC
Oxygen radical absorbance capacity	ORAC
Particle size	PS
Polyacrylamide gel electrophoresis	PAGE
Polysaturated fatty acid	PUFA
Pulse electric field	PEF
Reversed-phase	RP
Research and development	R&D
Seaweed protein extract	SPE
Seaweed protein isolate	SPI
Size-exclusion	SE
Sodium dodecyl sulphate	SDS
Sodium hydroxide	NaOH
Soy protein isolate	SPI
United Kingdom	UK
United Nations university	UNU
Water holding capacity	WHC
World health organisation	WHO
Whey protein isolate	WPI
Sodium dodecyl sulphate	SDS

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Chapter one

Introduction

1 Introduction

1.1 Overview

1.1.1 Background

Marine macroalgae, widely recognised as seaweeds, are ocean "vegetations" that play a crucial role in marine ecology (Bleakley & Hayes, 2017; Suryanarayana & Banerjee, 2011). Wild seaweeds have been an established part of the traditional Chinese, Japanese and Korean diets, whereas cultivated seaweeds are exploited to extract agar, alginates and carrageenans (McHugh, 2003). In New Zealand, though seaweed cultivation has not been fully commercialised, indigenous *Māori* locals have been consuming certain red seaweeds for a long time. Furthermore, the "green and clean" environment in New Zealand constitutes substantial potential to produce high-quality seaweeds for human consumption, commercial and industrial applications (Gibbons, 2014).

Seaweeds exist in three phyla, namely, *Phaeophyta* (brown seaweed), *Chlorophyta* (green seaweed) and *Rhodophyta* (red seaweed). In particular, protein content in red seaweeds could reach 47%, greater than in brown and green seaweeds, and some conventional protein sources, like cereals, eggs and pulses (Appell *et al.*, 2018; Fleurence, 1999). As the rapid growth of the global population continues, the strain on food security and the demand for proteins escalates (Aiking, 2014). However, detrimental environmental consequences during animal protein production require the immediate attention of alternative proteins (Steinfeld *et al.*, 2006). Despite the production of plant proteins would be less straining on the environment, plant sources require agricultural land, and their proteins are often deficient in essential amino acids (Garcia-Vaquero *et al.*, 2017). In this manner, extraction of red seaweed proteins would be a promising approach, as it is one of the few plant proteins that provide complete proteins with all essential amino acids (Rawiwan *et al.*, 2022).

The present thesis focuses on optimising red seaweed protein recovery from *Porphyra umbilicalis*, *Pyropia viridi*dentata and *Pyropia cinnamomea*, to ensure ameliorated protein extraction efficiency and sufficient protein yield. The physicochemical properties and functionalities of different protein extracts were further investigated and compared to commercial soy (SPI) and whey protein isolates (WPI). Ultimately, this thesis examined the potential of red seaweed proteins as an alternative non-animal protein source.

1.1.1 Research significance

Current literature mainly reported the extraction and application of polysaccharides from red seaweed. Though red seaweed protein extractions are coming into the spotlight, a perceptive understanding of red seaweed protein is absent. Up-to-date, very little literature has comprehensively compared seaweed proteins derived from various extraction protocols. Examining different protein extractions would be necessary to determine the most desirable treatment. Nevertheless, different extraction conditions would influence the nature of resulting proteins, consequently affecting protein properties, which would require more insight.

The physicochemical and functional properties of seaweed proteins have not been well-established in the literature. The literature lacks an insightful differentiation of properties between various seaweed proteins derived from differing extraction protocols. Typically, seaweed protein extracts derived from one type of extractions are subsequently forwarded to property analysis. Moreover, a systematic properties comparison between seaweed proteins and commercial protein isolates, conducted for the first time in this study, enables further development of a well-rounded protein extract.

The current study is the first to optimise and compare seaweed protein extractions through water, alkaline, ultrasound-assisted and enzyme-assisted protocols. Nonetheless, there is no literature regarding the direct comparison of properties between various seaweed protein extracts and commercially available protein isolates. Finally, findings from this study extend current scientific knowledge revolving around red seaweeds and their proteins, joining the search for a sustainable alternative protein.

1.1.2 Objectives

The overall objective of this study is to systematically optimise the protein extraction protocols for red seaweeds (*Porphyra umbilicalis*, *Pyropia viridi*dentata and *Pyropia cinnamomea*), and investigate the physicochemical properties and functionalities of the derived seaweed protein extracts. This was achieved through completing the following specific objectives:

- Optimisation of water, alkaline, ultrasound-assisted and enzyme-assisted extractions of seaweed protein through single-factor experiments and orthogonal design.
- Investigation of the physicochemical properties of seaweed protein extracts, including amino acid composition, molecular weight distribution, protein functional groups and thermal properties, compared to commercial soy and whey protein isolates.
- Investigation of the functional properties of seaweed protein extracts, including solubility, emulsifying and foaming properties, compared to commercial soy and whey protein isolates.
- Examination of antioxidant capacity of different seaweed protein extracts.

1.1.3 Scope

An extensive comprehension of seaweeds, seaweed protein, protein extraction, protein properties and antioxidant capacity would be necessary prior to practical experiments. Relevant literature would be investigated to develop appropriate procedures for examining seaweed proteins. As outlined in Figure 1, various seaweed protein extraction procedures would be trialled and optimised, thus acquiring quality proteins to analyse their physicochemical properties, functionalities and antioxidant capacity. Results were explored by employing statistical manipulation, such as *t*-tests and one-way analysis of variance. Applicable findings with regards to seaweed proteins would be corroborated accordingly. Finally, the most significant findings would be emphasised at the end of this thesis, along with potential future works and possible applications arising from this study.

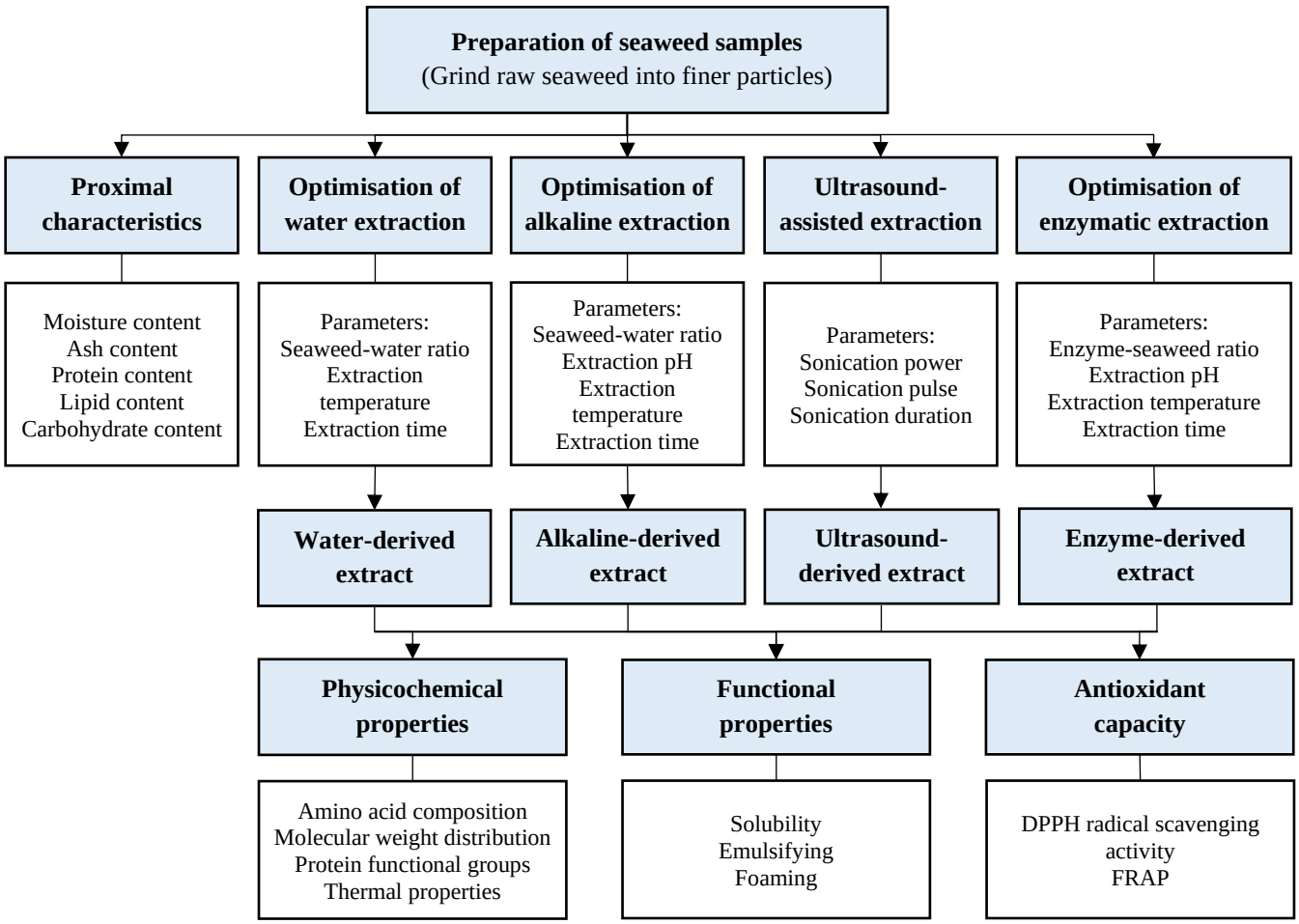


Figure 1. The experimental overview of the present study.

With the above in mind, the structure of this thesis is outlined below:

- Chapter 1 is the *Introduction*. It describes the study and outlines the thesis. It also contains a literature review regarding the general background of seaweed, seaweed protein, current procedures and techniques employed in protein extraction, protein properties and bioactivity.
- Chapter 2 is *Materials and Methods*. It lists materials used and details methods conducted, including optimisations of seaweed protein extractions and the relevant procedures for analysing protein properties, functionalities and antioxidant capacity.
- Chapter 3 is *Results and Discussion*. It determines the final extraction procedure for obtaining various seaweed proteins, interprets differences in the physicochemical and functional properties of the proteins extracted, and examines their antioxidant capacity.
- Chapter 4 is the *Conclusion and Future Work*. It discusses noteworthy outcomes and limitations of this study, concludes the research findings, and proposes potential future works.

1.2 Literature review

1.2.1 Seaweed

1.2.1.1 Global background of seaweed

Approximately 70% of Earth's surface is covered with water, and a vast diversity of aquatic organisms inhabit within. Marine macroalgae, also known as seaweeds, are ocean "vegetation" constituting 25,000 to 30,000 species (Santos *et al.*, 2015). They play a crucial role in marine ecology, stabilising ocean environments and are the fundamental base blocks for the aquatic food chain (Bleakley & Hayes, 2017). Nevertheless, seaweeds are marine photosynthesisers, acting as vital oxygen producers for the entire Earth (Suryanarayana & Banerjee, 2011).

Seaweeds have occupied various ecological niches for 600 to 900 million years (Levine, 2016). For instance, seaweed predominantly anchors to supporting material beneath the waters or is free-floating and loose-lying on water surfaces (Baweja *et al.*, 2016). Seaweeds also have a long history as a food material. Human consumption of seaweeds can be traced back to 13,000 BC, whereby Ancient Chilean civilisations utilised seaweeds for nutritional and health purposes (Baweja *et al.*, 2016). Seaweeds were recognised as the "heaven vegetable" in 600 BC in China, being delicacies offered to honourable guests and the King (Porterfield, 1922). Furthermore, seaweeds are frequently incorporated into East Asian diets. In China, Japan and Korea, seaweeds are cooked in soups, mixed into salads, crushed and served as garnishes, or wrapped around the rice to form *sushi* (McHugh, 2003). Though seaweeds are a part of the traditional human diet, they were not commercially cultivated or researched for their functional properties until the 1940s (Baweja *et al.*, 2016).

Currently, seaweeds are grown worldwide for food and non-food purposes. The Food and Agriculture Organisation (FAO, 2020) reported that the global production of seaweed has tripled within the last two decades, from 10.6 million tonnes in 2000 to 32.4 million tonnes in 2018. Chile, China, and Indonesia are leading countries producing seaweeds. Chile harvested the wildest seaweeds in 2015, totalling up to 345,704 tonnes, and critical species are Chilean kelp (*Lessonia nigrescen*), *huiro pal* (*Lessonia trabeculata*) and *Gracilaria* spp. (FAO, 2018). On the other hand, China constitutes 60% of the total global seaweed production in 2018, primarily cultivating species such as Japanese kelp (*Saccharina japonica*), Japanese *wakame* (*Undaria* spp.) and Japanese *nori* (*Porphyra* spp.) for human consumption (FAO, 2018). In contrast, Indonesia supplies seaweed-extracted agar and carrageenan for commercial and

industrial utilisation, cultivating species such as *Eucheuma* spp., *Kappaphycus* spp., *Gracilaria* spp (FAO, 2018). Finally, European countries research optimised processing techniques for natural seaweed stocks, such as extracting seaweed biopolymers for their proclaimed health and functional benefits (Tiwari & Troy, 2015).

New Zealand (NZ), in particular, is rich in marine resources, but it does not contribute an outstanding amount to global seaweed production. Despite NZ having temperate and nutritious ocean waters, NZ coastal lines are not yet fully exploited for seaweed cultivation. In NZ, the seaweeds that local Māori commonly consumes are *Pryopia* spp. and *Gigartina* spp. (White & White, 2020). Nonetheless, *Pterocladia lucida*, *Pterocladiella capillacea* and *Gigartina* spp. are predominantly hand-harvested for agar production for commercial and industrial means (White & White, 2020). As the growth and cultivation of seaweeds continue to be of global interest, NZ's "green and clean" environment has immense potential to produce high-quality seaweeds for human consumption and commercial and industrial applications (Gibbons, 2014).

1.2.1.2 Growth and cultivation of seaweed

Seaweeds flourish in ecosystems where the land meets the ocean. Seaweeds could sprout in waters worldwide under good growth conditions, such as adequate sunlight, oxygen, carbon dioxide, water and nutrients (Radulovich *et al.*, 2015). However, not all seaweed presence is of commercial value. Out of the 25,000 to 30,000 species of seaweeds, approximately 220 have commercial value (FAO, 2018). The life cycle and growth rate of seaweeds are complex and are dependent on their genetic makeup and environmental factors (Hurd *et al.*, 2014a). Different seaweeds would constitute distinct physicochemical profiles, depending on their species, the developmental stage, the season they are growing in, and the environment they occupy (Stiger-Pouvreau *et al.*, 2016).

Collecting and harvesting wild seaweeds have been a conventional practice for many coastal populations. In the 20th century, seaweed cultivation became widely established, particularly in China and Japan (Baweja *et al.*, 2016). Despite seaweeds being found in waters worldwide, seaweed species of commercial value have farming boundaries and are limited to growing in shallow coastal waters (Radulovich *et al.*, 2015). Cultivating seaweeds in such waters allow sufficient sunlight to pass through the water for optimal seaweed photosynthesis (Radulovich

et al., 2015). Moreover, growing and harvesting seaweeds in shallow coastal waters are more accessible than in deep waters.

Nowadays, commercial seaweed cultivation occurs across approximately 50 countries (FAO, 2018). In those countries, seaweeds could be cultivated vegetatively or reproductively. Generally, vegetative cultivation of seaweeds would be considered for hydrocolloid production, whereas seaweeds for human consumption undergo reproductive cultivation (Radulovich *et al.*, 2015). Once seaweed cultivation commends, seaweeds undergo significant development and drastic physicochemical changes. For instance, brown seaweed, *Sargassum horneri*, could grow from under 10 cm to 5 m within five months (Murakami *et al.*, 2011). Seaweed growth completes around early spring, which could be an appropriate season for harvesting mature seaweeds for human consumption (Murakami *et al.*, 2011).

Harvesting seaweeds could be performed by hand or machine (Radulovich *et al.*, 2015). After harvest, seaweeds are cleaned, prepared for storage and subsequent post-harvest handling procedures. Post-harvest handling procedures would be decided depending on the final seaweed product. For example, seaweeds forwarded to raw industrial processing would undergo fewer post-harvest handling procedures than seaweeds required for food purposes and biochemical extraction (Radulovich *et al.*, 2015).

Extracted seaweed hydrocolloids, including agar, alginates and carrageenan, have important economic value, as they are widely utilised in the cosmetic, food and pharmaceutical industries (Mišurcová, 2012). Nevertheless, seaweeds are increasingly recognised for their attractive nutritional value in food research and development (R&D). Seaweed biochemical compounds possess many biological benefits, such as being anti-cancer, anti-diabetic, anti-inflaming, anti-hypertensive, anti-microbial, antioxidising and anti-viral (Admassu *et al.*, 2018a; Hirayama *et al.*, 2016; Jung *et al.*, 2019; Senthilkumar & Jayanthi, 2016; Vásquez *et al.*, 2019).

A considerable advantage in seaweed cultivation is that they do not compete with territorial crops for arable land and potable water, becoming an additional crop source for worldwide food production. Furthermore, seaweed cultivation would be sustainable and further improves the global environment. This is because half the global carbon fixation is performed by algae, with seaweeds playing a crucial role in reducing eutrophication and greenhouse gases (Chung *et al.*,

2011). As a result, seaweed cultivation would simultaneously be an effective tool for carbon sequestration and combating climate change (Buschmann *et al.*, 2017).

Several factors hinder the expansion of seaweed cultivation, such as harvesting access and rights, seasonality and geographical location of seaweeds (Bleakley & Hayes, 2017). Finally, the driving force to accelerate seaweed cultivation would be the demand for seaweed products and services. Though seaweeds have growing R&D interests, national and public investments could be necessary for expanding seaweed cultivation (Radulovich *et al.*, 2015).

1.2.1.3 Types of seaweed

Unlike terrestrial and higher plants, seaweeds do not possess roots, stems or leaves (Baweja *et al.*, 2016). Due to different vegetative divisions, seaweeds could be categorised into several groups based on their thalli construction, such as filamentous, cylindrical, siphonous and siphonocladous, flattened, tubular and spherical (Baweja *et al.*, 2016; Hurd *et al.*, 2014c). Depending on the seaweed's taxonomy and stage of growth, they could be unicellular and multi-cellular (Hurd *et al.*, 2014c).

As ocean ornamentals, seaweeds are vibrantly coloured and thus are classified into three main taxonomic groups based on their natural pigmentation (Baweja *et al.*, 2016; Bleakley & Hayes, 2017). As listed in Table 1, the three main phyla of seaweeds are *Phaeophyta* (brown seaweed), *Chlorophyta* (green seaweed) and *Rhodophyta* (red seaweed), each comprising a distinctive profile of cell wall and storage polysaccharides, and are cultivated for different purposes.

Table 1. Major structural and storage carbohydrates in brown, green and red seaweeds.

Type of seaweed	Main colour pigment	Structural polysaccharide	Storage polysaccharide
Brown (*Phaeophyta*)	Fucoxanthin	Alginic acids Fucoidans Sargassans	Laminarins
Green (*Chlorophyta*)	Chlorophyll *a* and *b* *β*-carotene Xanthophylls	Cellulose Ulvans Sulphated galactans Xylans Mannans	Starch
Red (*Rhodophyta*)	Phycobilins	Agars Carrageenans Xylans	Floridean

Information acquired from Charoensiddhi *et al.* (2017).

Fucoxanthin is responsible for the olive-brown colour in brown seaweeds, *Phaeophyta* (Kadam *et al.*, 2013). Brown seaweeds are unique as they are all multicellular algae, constituting approximately 2000 marine species (Baweja *et al.*, 2016). Brown seaweeds inhabit waters throughout the globe, but are the most affected by climate changes compared to green and red seaweeds (Baweja *et al.*, 2016). As mentioned in Table 1, the main structural polysaccharides in brown seaweeds are alginic acids, fucoidans and sargassans, with laminarins being the main storage polysaccharide (Charoensiddhi *et al.*, 2017). Nevertheless, brown seaweed species such as *Ascophyllum, Durvillaea, Ecklonia, Laminaria, Lessonia, Macrocystis* and *Sargassum* provide essential sources of industrial alginate, whereas *S. japonica, Undaria pinnatifida* and *Sargassum fusiforme* are brown seaweeds cultivated for human consumption in Asia (FAO, 2018).

Green seaweeds constitute approximately 600 marine species, and are further divided into two groups: a larger group of unicellular algae known as *Chlorophyta*, and a smaller group of multicellular *Embryophyta* that are only found in freshwaters (Baweja et al., 2016). Chlorophyll *a* and *b, β*-carotene and xanthophylls produce the green-yellow colour in green seaweeds, Chlorophyta (Kadam *et al.*, 2013). Green seaweeds have high nutrient tolerance and occupy shallow coastal water (Baweja *et al.*, 2016). As listed in Table 1, the cell wall of green seaweeds is composed of cellulose, ulvans and sulphated galactans, xylans and mannans, whereas the main storage polysaccharide is starch (Charoensiddhi *et al.*, 2017). Furthermore, *Enteromorpha clathrate, Monostroma nitidum* and *Caulerpa* spp. are commonly cultivated and are substantial food resources in Asia (FAO, 2018).

Phycobilins are the red colour pigment in red seaweeds, *Rhodophyta* (Kadam *et al.*, 2013). Red seaweeds are mostly multicellular algae, constituting approximately 7000 marine species (Baweja *et al.*, 2016). Though red seaweeds inhabit waters throughout the globe, they tend to occupy temperate and tropical waters (Baweja *et al.*, 2016). As indicated in Table 1, the main structural polysaccharides in red seaweeds are agars, carrageenans and xylans, with floridean starch being the main storage protein (Charoensiddhi *et al.*, 2017). Nonetheless, *Porphyra* spp., *Eucheuma* spp., *Kappaphycus alvarezii* and *Gracilaria* spp. are commonly cultivated (FAO, 2018). *Porphyra* and *Gracilaria* are grown for human consumption, whereas *Eucheuma* and *Kappaphycus* are cultivated for subsequent carrageenan extraction (FAO, 2018).

1.2.1.4 Chemical composition of seaweed

The general nutritional composition of dried seaweeds reported by the U.S. Department of Agriculture (2020) is recorded in Table 2. Consuming 100 g of dried seaweed would provide 298 kcal of energy and many other beneficial nutrients. Seaweeds typically have high carbohydrate content (dietary fibres and sugars), followed by protein and lipids. They also include various minerals and trace elements, such as potassium, sodium, magnesium, calcium, phosphorus, iron, selenium, zinc, and copper. Seaweeds contain small amounts of vitamin A, B, C, E and K. Depending on the species, growth location and harvest season of seaweed, seaweed nutritional composition would be subjected to change (Mišurcová, 2012).

Table 2. General nutritional composition of dried seaweeds.

Nutrient	Unit	Value per 100 g dried seaweed
Proximate		
Energy	kcal	298
Water	g	6.68
Protein	g	31.84
Carbohydrates (By difference)	g	52.39
Dietary fibre (Total)	g	5.60
Sugar (Total)	g	3.04
Lipid (Total)	g	4.01
Minerals		
Calcium, Ca	mg	372
Iron, Fe	mg	24.85
Magnesium, Mg	mg	482
Phosphorus, P	mg	85
Potassium, K	mg	1244
Sodium, Na	mg	575
Zinc, Zn	mg	3.90
Copper, Cu	mg	3.36
Selenium, Se	µg	7.30
Vitamins		
Vitamin C, ascorbic acid (Total)	mg	5
Thiamine	mg	1.20
Riboflavin	mg	1.95
Niacin	mg	6.51
Vitamin B6	mg	0.33
Folate (Total)	mg	0.34
Vitamin A, RAE	µg	14
Vitamin E, α-tocopherol	mg	5
Vitamin K, phylloquinone	µg	25
Lipids		
Fatty acids, saturated (Total)	g	1.36
Fatty acids, monounsaturated (Total)	g	0.35
Fatty acids, polyunsaturated (Total)	g	1.09

Information acquired from U.S. Department of Agriculture (2020).

Protein

Proteins are complex and vital macromolecules essential for the growth and function of unicellular and multicellular organisms. Depending on the type and species of seaweeds, protein content could fluctuate between 3% to 47% seaweed dry weight (DW) (Biancarosa *et al.*, 2017; Fleurence, 1999; Marsham *et al.*, 2007). In NZ, many wild-collected and commercially cultivated seaweeds constitute proteins at approximately 9% to 32% of seaweed DW (Smith *et al.*, 2010). Additionally, peptides in seaweed proteins possess various health benefitting properties (Conde *et al.*, 2013). Seaweed proteins will be thoroughly discussed in *Section 1.2.2 – Protein.*

Carbohydrate

Carbohydrates are built from various monosaccharides, disaccharides and polysaccharides, and these are essential energy providers to the human body. The total polysaccharide content in seaweeds ranges from 4% to 76% of seaweed DW, and such polysaccharides are necessary for structural and storage purposes in seaweed (Holdt & Kraan, 2011). In NZ, many wild-collected and commercially cultivated seaweeds also constitute carbohydrates at approximately 43% to 70% of seaweed DW (Smith *et al.*, 2010). As listed in Table 2, polysaccharides are the most abundant in seaweeds. However, the taxonomic grouping of seaweeds dictates their polysaccharides' abundance and structural composition (Stiger-Pouvreau *et al.*, 2016). For instance, Table 1 lists the distinctive polysaccharide profile of brown, green and red seaweeds. Nonetheless, such polysaccharides possess particular economic and industrial importance.

Despite seaweeds having substantial hydrocolloid applications in the industry, they also carry numerous benefits for human health. The bioactivity of sulphated fucoidan from brown seaweeds has been well-established, including anti-cancer, anti-coagulant, anti-hypergamic, anti-inflammatory, antioxidant and anti-tumour activities (Chen *et al.*, 2017; Kim *et al.*, 2010; Palanisamy *et al.*, 2017; Pozharitskaya *et al.*, 2020; Senthilkumar *et al.*, 2013). Ulvans, polysaccharides from green seaweeds such as *Ulva reticulata* and *Ulva armoricana*, have great antimicrobial effects and could act as an immunostimulant (Berri *et al.*, 2017; Tran *et al.*, 2018). Agars in red seaweed, *Gelidium amansii*, display considerable antioxidant capacity (Xu *et al.*, 2018), whereas low molecular weight (MW) carrageenans in several red seaweed show antiviral activities (Kalitnik *et al.*, 2013). Recent literature reported many bioactive seaweed carbohydrates, which would enhance medicinal and pharmaceutical interest in such carbohydrates outside of their hydrocolloidal properties.

Dietary fibre

Dietary fibres are recognised for promoting beneficial physiological effects, including laxation, blood cholesterol and blood glucose attenuation, and improving overall intestinal health (Baweja *et al.*, 2016). Most carbohydrates in seaweeds exist as dietary fibres (Holdt & Kraan, 2011). The total dietary fibre in seaweeds ranges from 36% to 62% of seaweed DW, proving seaweeds as rich sources of dietary fibre (Dawczynski *et al.*, 2007). Seaweed dietary fibres include water-soluble polysaccharides, such as agar, alginic acid, furonan and laminaran; whereas fat-soluble polysaccharides constitute cellulose, mannans and xylans (Holdt & Kraan, 2011). Extracted seaweed dietary fibre could be exploited as potential prebiotics for augmenting intestinal health (Baweja *et al.*, 2016).

Lipids

Similarly to carbohydrates, lipids also offer energy to the human body. Lipids and fatty acids are present in minuscule amounts in seaweeds, ranging from 1% to 5% seaweed DW (Mišurcová, 2012). In NZ, many wild-collected and commercially cultivated seaweeds also constitute approximately 1.5% to 5% of seaweed DW (Smith *et al.*, 2010). As suggested in Table 2, unsaturated fatty acids generally exist in slightly larger amounts than saturated fatty acids. Examples of polyunsaturated fatty acids (PUFAs) found in seaweeds are long-chain linolenic acids (ω-3 fatty acids) and long-chain linoleic acids (ω-6 fatty acids) (Dawczynski *et al.*, 2007). The health benefits of long-chain PUFAs have been extensively researched and are well-established. Both ω-3 and ω-6 fatty acids are reported to reduce the risk for cardiovascular diseases and improve cardiometabolic health (Maki *et al.*, 2018; Shahidi & Ambigaipalan, 2018).

Eicosapentaenoic acid is the predominant long-chain PUFA in seaweeds (C20:5, ω-3), with concentrations up to 50% of total fatty acid content in green seaweed, *H. fusiforme* (Dawczynski *et al.*, 2007). Furthermore, multiple brown and red seaweeds have the ideal nutritional ratio of high ω-3 and low ω-6 fatty acid content, which are at higher levels when compared to green seaweed (Dawczynski *et al.*, 2007). Seaweed species with good ω-3 and ω-6 fatty acid ratios are brown *Sargassum* and red *Palmaria*, which are 0.1 and 0.3, respectively (Holdt & Kraan, 2011). Consuming foods with the ideal ω-3 and ω-6 fatty acid ratio could suppress risks of autoimmune disease, cancer, cardiovascular disease and inflammatory disease (Simopoulos, 2002). Extracting seaweed oils with the ideal fatty acid ratio could be explored to act as dietary supplements like fish oil.

Minerals

Minerals are necessary to the human diet due to the human body's inability to produce such substances (Mišurcová, 2012). Due to the unique structural polysaccharides on seaweed cell surfaces, seaweeds have an enhanced ability to uptake inorganic substances from the environment, such as minerals (Peng *et al.*, 2015). Ash and thereby mineral content could reach up to 50% of seaweed DW in some species (Holdt & Kraan, 2011). In NZ, many wild-collected and commercially cultivated seaweeds constitute minerals at approximately 19% to 43% of seaweed DW (Smith *et al.*, 2010). As listed in Table 2, seaweeds contain large amounts of essential minerals such as potassium, sodium, magnesium and calcium in descending order. Important minerals, such as calcium, copper and iron, often accumulate in higher amounts in seaweed when compared to many territorial vegetables (MacArtain *et al.*, 2007). Minerals are crucial structural materials for building tissue in the body and participate in various vital reactions as cofactors for many metalloenzymes (Mišurcová, 2012; Peng *et al.*, 2015).

Seaweeds are susceptible to acquiring undesirable metals due to their ability to uptake substances from the environment (Bleakley & Hayes, 2017). Though not listed in Table 2, heavy metals absorbed by seaweeds include arsenic, cadmium, lead and mercury (Holdt & Kraan, 2011). Such metals in seaweeds are generally below food safety limits, but their excessive intake could be detrimental to human health, increasing risks for acute and chronic diseases (Jaishankar *et al.*, 2014). Spain-grown *Hiziki* (*Hizikia fusiforme*), a brown seaweed commonly incorporated in Japanese diets, exert high inorganic arsenic and cadmium levels that exceeded the French legislation limit (Besada *et al.*, 2009). Lead in various Mediterranean seaweeds exceeded the European limit for feed and food supplements, whereas mercury was close to the limit (Squadrone *et al.*, 2018). However, heavy metal sorption in seaweeds could act as good biomonitors for environmental pollution and bio-asorbants to remove such substances (Deniz & Karabulut, 2017; Hurd *et al.*, 2014b; Mišurcová, 2012). High mineral content in seaweeds could be extracted for pharmaceuticals and nutraceuticals, but regulating environments that cultivate human-consumed seaweeds would be crucial for adhering to country-specific food regulations.

Vitamins

Vitamins are essential nutrients in the human diet. Though the human body can produce vitamins, the biosynthesis of vitamins is restricted in the body and must be acquired through the diet (Mišurcová, 2012). Due to mainly being exposed to direct sunlight in aqueous

environments, seaweeds accumulate various water-soluble and fat-soluble vitamins (MacArtain *et al.*, 2007). As suggested in Table 2, water-soluble vitamins are niacin, vitamin C, riboflavin, thiamine, folate and vitamin B6 in descending order, whereas fat-soluble vitamins are vitamin E, vitamin K and vitamin A in descending order. Likewise to minerals, vitamins also participate in vital metabolic reactions in the body, and these are only required in small quantities. Seaweeds are one of the few vegetables containing cobalamin, the water-soluble vitamin B12, needed for blood formation and neurological function (Heer *et al.*, 2015). Vitamin B12 has been discovered in brown *Ascophyllum* and *Laminaria*, green *Ulva* and red *Porphyra* and *Palmaria* (Baweja *et al.*, 2016).

Some natural coloured pigments in seaweeds could be converted into vitamins, and "function" as vitamins because they exhibit similar biochemical activities (Holdt & Kraan, 2011). Carotenoids are impressive antioxidants responsible for red, orange and yellow hues in seaweeds. Brown seaweed consists of fucoxanthin, β-carotene and violaxanthin. Fucoxanthin exhibits anti-obesity potential across various edible species, namely *Hizikia*, *Undaria*, *Laminaria* and *Sargassum* (Maeda *et al.*, 2005; Wijesinghe & Jeon, 2011). Moreover, fucoxanthin could exist up to 5 mg/g in the NZ-grown brown seaweed, *U. pinnatifida* (Fung *et al.*, 2013). Red seaweeds include α-, β-carotene and their respective derivatives, and green seaweeds have similar carotenoid compositions to higher plants (Mišurcová, 2012). Compared to trace levels of vitamins, extracted seaweed pigments could have more economical and nutritional value due to their abundance in seaweed cells.

1.2.2 Proteins

1.2.2.1 Background of general protein

Proteins are fundamental for the growth, health and optimal performance of humans, animals and plants. A protein begins as an "energetic" linear polypeptide. A sequence of amino acids (AA) is covalently connected via peptide bonds, also known as the primary structure (Appell *et al.*, 2018). Peptide bonds occur along the polypeptide backbone, forming covalent interactions between the amino group of one AA and the carboxyl group of the next. Then, neighbouring AAs within the chain interact via hydrogen bonds; the linear chain twists into coil-like α-helices, or folds plane-like into β-pleated sheets, becoming the secondary structure (Ustunol, 2015). Afterwards, simple α-helices and β-pleated sheets are stabilised by side-chain interactions, transforming into a unique and compact three-dimensional complex; this is recognised as the tertiary structure. The tertiary structure is further stabilised by molecular interactions, namely hydrophobic, Van der Waal, electrostatic and disulphide bonds; such interactions reduce the net free energy of the overall structure (Appell *et al.*, 2018; Ustunol, 2015). Finally, various intermolecular and intramolecular interactions and the aggregation of multiple tertiary structures could assemble into a quaternary protein structure (Ustunol, 2015). In simple words, proteins are built from explicit information contained within the linear sequence of AAs, and such information ultimately transforms a linear structure into a folded, functional and stable protein.

Varying proteins exhibit unique structures and functions derived from the sequence of AAs. The total MW of AAs within a protein is the overall MW of that protein. Protein MW could range from simple egg ovalbumins with an MW of 45 kDa, to complex egg ovomucins with an MW up to 7,600 kDa (Appell *et al.*, 2018; Huntington & Stein, 2001). Much of the protein's roles are related to its structure. For instance, fibrous proteins like collagen have structural roles, whereas globular proteins like β-lactoglobulin have functional roles (Appell *et al.*, 2018). Nevertheless, proteins progressing to their final state allows them to reach a lower overall energy state, which would be necessary for being stable and performing their physiological functions (Ustunol, 2015).

AAs are the initial building blocks of protein. The functionalities of AAs are determined by their structure and the chemical characteristics of their side chains. All AAs consist of carboxyl and amino terminals, each with unique sidechains (Appell *et al.*, 2018). As listed in Table 3,

AAs are recognised as positively charged and basic, negatively charged and acidic, uncharged and polar, aliphatic and non-polar, and aromatic and non-polar (Ustunol, 2015). Under biological conditions, non-polar AAs are buried within the protein centre away from water molecules due to their hydrophobicity, whereas polar AAs are on the exterior interacting with hydrophilic molecules. This phenomenon is described as hydrophobic collapse, as hydrophobicity of non-polar proteins and their burial within proteins are the influential forces driving protein folding, thus allowing the protein to reach a lower overall energy state (Ustunol, 2015; Uversky & Fink, 2002). Other forces between AAs that contribute to protein folding are disulphide bridges, ionic bonds, hydrogen bonds, and van der Waal interactions (Appell *et al.*, 2018).

Table 3. Characteristics of twenty common amino acids found in food proteins.

Amino acid	Abbreviation	Group[1]	Essentiality[2]	pKa[1,2]	Molecular weight (Da)[1]
Alanine	Ala, A	Aliphatic and nonpolar	Non-essential	-	89.1
Arginine	Arg, R	Positively charged and basic	Non-essential	12.48	174.2
Asparagine	Asn, N	Uncharged and polar	Non-essential	-	133.1
Aspartic acid	Asp, D	Negatively charged and acidic	Non-essential	3.9	132.1
Cysteine	Cys, C	Uncharged and polar	Non-essential	~8.35	121.1
Glutamic acid	Glu, E	Negatively charged and acidic	Non-essential	4.07	146.1
Glutamine	Gln, Q	Uncharged and polar	Non-essential	-	147.1
Glycine	Gly, G	Aliphatic and nonpolar	Non-essential	-	75.1
Histidine	His, H	Positively charged and basic	Essential	6.04	155.2
Isoleucine	Ile, I	Aliphatic and nonpolar	Essential	-	131.2
Leucine	Leu, L	Aliphatic and nonpolar	Essential	-	131.3
Lysine	Lys, K	Positively charged and basic	Essential	10.79	146.2
Methionine	Met, M	Aliphatic and nonpolar	Essential	-	149.2
Phenylalanine	Phe, F	Aromatic and nonpolar	Essential	-	165.2
Proline	Pro, P	Aliphatic and nonpolar	Non-essential	-	115.1
Serine	Ser, S	Uncharged and polar	Non-essential	~16	105.1
Threonine	Thr, T	Uncharged and polar	Essential	~16	119.1
Tryptophan	Trp, W	Aromatic and nonpolar	Essential	-	204.2
Tyrosine	Tyr, T	Aromatic and nonpolar	Non-essential	9.7	181.2
Valine	Val, V	Aliphatic and nonpolar	Essential	-	117.1

[1] Ustunol (2015); [2] Appell *et al.* (2018).

Of the common twenty AAs in Table 3, nine are recognised as essential AAs (EAAs): histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The human body is incapable of synthesising EAAs; hence they must be acquired from foods. The nutritional quality of protein would be dependent upon its assimilation of EAAs, which tends to be higher in animal proteins when compared to plant proteins (Appell *et al.*, 2018).

Nonetheless, the EAA contents of plant proteins are often below FAO/WHO/UNU nutrient requirements (Gorissen *et al.*, 2018).

Common animal-sourced foods with complete proteins (all EAAs) are ground beef, bovine milk, and whole egg (Appell *et al.*, 2018). Plant-based foods with complete proteins are less common; some examples are hemp (House *et al.*, 2010), quinoa (Wu *et al.*, 2020), seaweed (Naseri *et al.*, 2020b), and soy (Hughes *et al.*, 2011). Animal and plant proteins could be directly consumed from their source foods, or explicitly extracted as food products and additives. Forwarding proteins to various processing conditions could transform protein structure and function, possibly altering protein quality. An noteworthy consideration is that consuming one particular type of food would not supply all necessary proteins and EAAs. Nutrient guidelines and requirements should be satisfied from the intake of multiple types of food.

1.2.2.2 Animal-derived proteins

Animal-derived foods have been a crucial part of the human diet for generations. As mentioned previously, proteins derived from animal sources are more nutritionally complete than plant-based proteins. Widely distributed animal proteins with great nutritional value could be consumed from eggs, meats and milk, which are described in Table 4. Animal proteins could be extracted and further processed into pharmaceuticals and nutraceuticals as they consist of many bioactive properties (Madadlou & Abbaspourrad, 2018; Phelan *et al.*, 2009; Ryder *et al.*, 2016; Samaraweera *et al.*, 2011; Wang *et al.*, 2017). Moreover, some of such proteins have excellent functional properties, namely emulsifying, encapsulating, flavour-binding, foaming, gelling, solubilising, thickening and water-binding (Appell *et al.*, 2018).

Table 4. Common animal-derived proteins.

Food	Type	Protein	Approximate content in protein type
Eggs[1]	Egg white	Ovalbumin	54%
		Ovotransferrin	12%
		Ovomucoid	11%
	Egg yolk	Lipovitellin apoproteins	40%
		Livetins	9%
		Phosvitin	13%
Meats[2]	Myofibrillar	Actin	13%
(livestock, aquaculture)		Myosin	26%
	Stromal	Collagen	7%
Milks[3]	Casein	α_{s1}-Casein	44%
		α_{s2}-Casein	19%
		β-Casein	25%
		γ-Casein	5%
		κ-Casein	1%
	Whey	Immunoglobulin	9%
		α-Lactalbumin	25%
		β-Lactoglobulin	50%
		Serum albumin	6%

[1] Mine (2015); [2] Kang and Singh (2015); [3] Farkye and Shah (2015).

Eggs

Eggs are a staple food in the human diet. According to FAO (2021), more than 2.3 trillion eggs were produced worldwide in 2019. Complete proteins with all EAAs are found within the egg, proving eggs to be an excellent source of nutrients (Appell *et al.*, 2018). The relative distributions of main proteins in eggs are shown in Table 4. Both egg white and egg yolk are packed with proteins with the potential to be processed into functional foods and functional ingredients. For example, peptides extracted from egg white ovotransferrin exhibit anti-inflammatory properties (Wang *et al.*, 2017). Egg yolk phosvitins are highly phosphorylated proteins that effectively bind to iron, inhibiting oxidation (Samaraweera *et al.*, 2011). Furthermore, egg proteins are also widely employed as food ingredients. Egg white proteins produce amazing bakery foods due to their excellent foaming and gelling capacities, whereas egg yolk proteins are the emulsifying agent in many mayonnaise. Finally, innovative egg protein food packaging is currently explored in the literature (Corradini *et al.*, 2013; Huang *et al.*, 2020; Mecitoğlu *et al.*, 2006). However, egg allergy is prevalent in children; ovalbumin, ovotransferrin, ovomucoid and livetins are the main allergens in eggs (Mine, 2015; Savage *et al.*, 2007).

Meats

Meat has been consumed for most of human existence. FAO (2021) have totalled more than 381 million tonnes of meats produced in 2019 worldwide, namely from cattle, chicken, duck, pig and sheep. In contrast, global fisheries and aquaculture production reached almost 179 million tonnes in 2018 (FAO, 2020). Myofibrillar and stromal proteins are essential proteins in meat muscles, making up 25% and 60% of total meat muscle proteins (Kang & Singh, 2015). The relative distributions of main myofibrillar and stromal proteins are shown in Table 4. Muscle proteins, directly absorbed from consuming meats, are vital for skeletal and connective tissue development in the body (Alexandrov *et al.*, 2018; Durosier-Izart *et al.*, 2017). Nonetheless, meats have interesting bioactive peptide contents (Ryder *et al.*, 2016). For instance, bioactive peptides from bovine, chicken and pig meats exert good antioxidant properties (Ohata *et al.*, 2016; Ryder *et al.*, 2016; Sun *et al.*, 2012). Collagen is a well-pronounced ingredient in the cosmetics industry, currently marketed as an ingredient for skin repair with anti-ageing capacities (Subhan *et al.*, 2021). Collagen from fish waste and by-products would be further treated for the cosmetics industry or processed downstream into gelatine (Jayathilakan *et al.*, 2012). Finally, gelatine acts as a crucial gelling agent and stabiliser in ice creams, jellies and yoghurts.

Milks

Milk is known to accommodate an excellent variety of nutrients. FAO (2021) reported that the global production of fresh cow milk reached almost 750 megatons in 2019. As shown in Table 4the protein fraction of bovine milk could be classified into caseins and whey, which constitute 78% and 17%, respectively, of the milk's total weight (Appell *et al.*, 2018). The relative distributions of the main casein and whey proteins are listed in shown in Table 4.

Milk proteins have well-researched nutritional and multifunctional properties. Caseins are phosphoproteins that suspend in milk as colloidal particles (Appell *et al.*, 2018). Casein-derived bioactive exert antibacterial, antioxidant, cytomodulatory, and immunomodulatory effects; some are available as commercial functional foods and ingredients in the current market (Phelan *et al.*, 2009). Additionally, whey is a serum protein that remains soluble after casein precipitates (Appell *et al.*, 2018). Whey-derived bioactive showcase anti-hypertension, antioxidant, anti-obesity, anti-diabetes, and hypocholesterolemic impacts (Madadlou & Abbaspourrad, 2018). Application-wise, milk protein concentrates exert pronounced foaming, gelling and water-binding properties; the incorporation of milk proteins stabilises baked goods,

and frozen and liquid foods (Agarwal *et al.*, 2015). Caseins and whey proteins are successful carriers for pharmaceuticals due to having great encapsulating capacities (Madadlou & Abbaspourrad, 2018; Semo *et al.*, 2007).

Future prospects

The demand for animal products has escalated alongside the rapidly growing population, urbanisation and salaries in developing countries. As developing countries progress, their traditional household staples, such as affordable cereal, are transforming into more expensive diets consisting of dairy products, eggs, fish and meat in developed countries (Boland *et al.*, 2013). Such economic improvements in society are applaudable, yet the future supply of animal products would be of concern as the world continues to populate (Aiking, 2014). The global population is projected to grow more than one-third by 2050, with food demand estimated to exceed another 70% (Bruinsma, 2009). The need for animal proteins could eventually outbalance future resources; hence sustaining food security for future generations is in the spotlight (Aiking, 2014).

The extensive consumption of animal proteins concerns animal welfare and human health. In addition, livestock production contributes to approximately one-fifth of current greenhouse gas emissions (Steinfeld *et al.*, 2006). Carbon dioxide, methane and nitrous oxide are three greenhouse gases emitted by livestock, which are influential contributors to climate change and its consequences (Steinfeld *et al.*, 2006). Nevertheless, intensive production systems are often required to level the increasing demand for animal products from livestock. Such systems could exhaust natural resources, namely land, water, and even more resources for cultivating feed crops (Aiking & de Boer, 2020). Finally, the production of animal proteins through intensive systems depresses the efficiency of global nitrogen use, resulting in severe environmental impacts (Lassaletta *et al.*, 2014). Ultimately, a reduction in animal protein production plus its consumption and alternative plant-based proteins should be recommended to sustain natural resources and enhance food security (Aiking, 2014).

1.2.2.3 Plant-derived proteins

Plants supply another appreciable source of proteins to the human diet. Plant proteins are major structural components in the cytoplasm of plant cells, but the contents of these proteins are typically below 3% of the fruit's fresh weight (Lee, 2018). Common plant proteins are listed in

Table 5. Though plant proteins might not exhibit high nutritional values like animal proteins, plant proteins do exert functional properties, such as emulsifying, dough-forming, flavour-binding, foaming, gelling, tenderising and texturising properties (Appell *et al.*, 2018). Bioactive proteins are also found in plants, which could be extracted for the development of functional foods and ingredients (Friedman & Brandon, 2001; Hardwick & Glatz, 1989; House *et al.*, 2010; Jukanti *et al.*, 2012; Khazaei *et al.*, 2019; Kumar *et al.*, 2017; Liang *et al.*, 2018; Ma *et al.*, 2011; Real Hernandez & Gonzalez de Mejia, 2019; Serena *et al.*, 2015; Udenigwe, 2016; Zheng *et al.*, 2015). With the nutritional and functional properties of plant proteins becoming more established, plant proteins have been in the recent spotlight as potential substitutions for animal proteins in a more economical and eco-friendly way (Kumar *et al.*, 2021).

Table 5. Common plant-derived proteins.

Food	**Plant**	**Approximate protein content**
Cereals	Maise	9% to 10%[1]
	Rice	6% to 9%[1]
	Wheat	8% to 14%[1]
Pulse	Chickpea	20% to 24%[2]
	Lentil	10% to 32%[3]
	Soybean	29% to 36%[4]
Others	Hemp	21% to 53%[5]
	Marine algae	9% to 47%[6]

[1] Appell *et al.* (2018); [2] Kaur and Singh (2007); [3] Khazaei *et al.* (2019); [4] Zarkadas *et al.* (2007); [5] House *et al.* (2010); [6] Fleurence (1999).

Cereals

Cereals are key components of a balanced diet. Maise (corn), rice and wheat are the three predominant sources of cereals throughout the globe. As suggested in Table 5, the protein content of such cereals is 9% to 10%, 6% to 9% and 8% to 14%, respectively (Appell *et al.*, 2018). Cereal proteins generally lack all EAAs. For example, rice and wheat proteins are deficient in lysine and threonine, whereas lysine and tryptophan are limiting proteins in maise (Appell *et al.*, 2018).

Maise proteins from maise gluten meals reside approximately 62% to 71% protein, and exhibit high thermal stability and excellent antioxidant capacity (Hardwick & Glatz, 1989; Zheng *et al.*, 2015). Nevertheless, rice proteins from rice bran constitute 12% to 20% protein with a digestibility larger than 90% (Wang *et al.*, 1999). Rice bran proteins display anti-diabetic and antioxidant capacities (Liang *et al.*, 2018; Udenigwe, 2016). Finally, wheat proteins are the

most suited for dough-formation, as their gluten has unique rheological properties to retain gas bubbles within the dough matrix. Despite gluten being widely incorporated into baked goods, it is a typical food allergen correlated with hypoglycaemia and Type 1 diabetes (Serena *et al.*, 2015).

Pulse

Pulse is the edible seeds from legume plants and are essential foods for maintaining healthy diets. As listed in Table 5, the protein content of chickpea, lentil and soybean is approximately 20% to 24%, 10% to 21%, and 29% to 36%, respectively (Kaur & Singh, 2007; Khazaei *et al.*, 2019; Zarkadas *et al.*, 2007). Consuming legumes would supply almost all EAAs. Additionally, lysine would be abundant in legumes, which compliments diets rich in cereals that often have lysine as a limiting EAA (Kiosseoglou & Paraskevopoulou, 2011).

Chickpeas and soybeans have relatively good digestibility, enhancing the intake of their bioactive peptides (Jukanti *et al.*, 2012; Real Hernandez & Gonzalez de Mejia, 2019). Lentil-derived peptides exhibit anti-fungal, anti-hypertensive and anti-oxidant properties and have excellent encapsulating capacities (Khazaei *et al.*, 2019). Regarding the food industry, both thermally treated chickpea and lentil flours are stable and could potentially be value-adding ingredients to various foods (Ma *et al.*, 2011). The health benefits of soybean have been well-established, such as being anti-carcinogenic, lowering cholesterol, protecting against obesity and diabetes, and many other profiting physiological impacts (Friedman & Brandon, 2001). Nonetheless, soybean has been well developed into meat analogues currently sold in the market, providing meat-like textural sensations to consumers (Kumar *et al.*, 2017).

Other plant-based protein sources

The increasing demand for high-quality proteins has triggered exploration into new, non-conventional protein sources, such as hemp and marine algae. Both hemp and marine algae are of very few plant sources that constitute complete proteins with all EAAs, emphasising their potential to act as excellent animal protein alternatives (Dawczynski *et al.*, 2007; House *et al.*, 2010). Moreover, both processed hemp and marine algae proteins have up to 90% digestibility, improving the bioavailability of their proteins in the body (House *et al.*, 2010; Mæhre *et al.*, 2016).

Cannabis sativa, also known as hemp, is a traditionally cultivated crop that serves as a source of fibre, food and medicinal products. Current hemp cultivation focuses on producing seed oil and fibre, and could be value-adding ingredients in flour, milk, and snacks (House *et al.*, 2010). As indicated in Table 5, the crude protein content in hemp could reach up to 53% (House *et al.*, 2010). However, hemp protein isolates have poor functional properties attributed to aggregation from covalent bonds between different proteins (Tang *et al.*, 2006).

Macroalgae, generally known as seaweeds, are vegetation for the ocean. Unlike hemp, seaweed's human consumption and exploitation in the hydrocolloid industry are well-established. As listed in Table 5, the protein content in seaweeds could reach up to 47%, with red seaweeds having more significant protein content than brown and green types (Fleurence, 1999). Furthermore, bioactive peptides extracted from multiple seaweed proteins display various activities, namely anti-cancer, anti-hypertensive, anti-inflammatory, anti-microbial, antioxidant and anti-viral capacities (Pliego-Cortés *et al.*, 2020).

Future prospects

Increasing awareness has been shone upon the production of proteins through eco-friendly means. Due to the negative environmental implications of producing animal proteins, substituting them with plant proteins would be more attractive in the food industry. The production of animal proteins is expensive, devours unsustainable amounts of natural resources, correlates to climate change, and concerns animal welfare and human health (Aiking, 2014; Kumar *et al.*, 2021). In contrast, plant proteins might be laborious, yet their sustainable production holds a vast long-term advantage. As animal protein alternatives receive little attention, the urgency of resolving protein issues with plant sources has been highlighted in recent reviews (Aiking & de Boer, 2020; Fasolin *et al.*, 2019; van der Weele *et al.*, 2019).

The development of sustainable protein production requires a balance between animal and plant proteins, as achieving this would improve the current status of exhausting natural resources and food systems (Fasolin *et al.*, 2019). Optimising this protein transition would require an enhanced understanding of plant proteins and their subsequent processing techniques. Maximising the utilisation of plant proteins would be employing appropriate extraction procedures based on the plant's structural matrix.

Despite further technological advances, changes in human social behaviour would also be necessary. Concentrates from extruding mushroom, soy and wheat proteins have evolved into meat analogues for vegetarians, and such products have been steadily expanding in the market (Kumar *et al.*, 2017). Moreover, animal protein consumption seems to decline for those with high-income levels, hinting that economic growth could ameliorate the environmental and health impacts of consuming animal proteins (Andreoli *et al.*, 2021). Regardless of so, most consumers resist the intake of fewer animal proteins, as the dietary pattern of high meat intake is still maintained (Macdiarmid *et al.*, 2016). Ultimately, the transition to plant proteins and similar alternatives could be defined as a technological and social-institutional conundrum (Graça *et al.*, 2019).

1.2.2.4 Seaweed-derived proteins

Seaweed protein content typically ranges between 3% to 15% DW in brown seaweeds, 9% to 26% DW in green seaweeds, and as high as 47% DW in red seaweeds (Fleurence, 1999). Recent findings in Table 6 also report seaweed content within the pre-established range. It is noteworthy that seaweed protein differs depending on the harvest season, growth location and species (Mišurcová, 2012). For instance, Portugal-grown brown seaweeds, *A. nodosum* and *U. pinnatifida*, had significantly higher protein content when harvested around autumn than spring (Vieira *et al.*, 2018). Kenya-grown green *Ulva lactuca* consisted of 10.1% DW protein, whereas the same species harvested from the United Kingdom (UK) comprised of 29.0% DW (Harrysson *et al.*, 2018; Marsham *et al.*, 2007). Finally, *Porphyra* sp. and *P. palmata* are red seaweeds, one with up to 44% DW protein, and that of the latter ranged from 9.84% to 18.86% DW (Beaulieu *et al.*, 2016; Marsham *et al.*, 2007).

Table 6. Recent reports of protein content from different seaweeds worldwide.

Species	Harvest location	Harvest date	Proximal protein content	Reference
Brown seaweed				
Alaria esculenta	Norway	October 2014	10.1 ± 0.1%[1]	Biancarosa *et al.* (2017)
Ascophyllum nodosum	Portugal	April to July 2016 October to November 2016	6.9 g/100 g to 9.4 g/100 g[2]	Vieira *et al.* (2018)
Ascophyllum nodosum	Norway	October 2014	3.0 ± 0.0%[1]	Biancarosa *et al.* (2017)
Chordaria flagelliformis	Norway	October 2014	6.3 ± 0.3%[1]	Biancarosa *et al.* (2017)
Durvillaea antarctica	NZ	April 2004 August 2004	7.3 ± 0.3% [1]	Smith *et al.* (2010)
Ecklonia radiata	NZ	April 2004 August 2004	9.6 ± 0.2% [1]	Smith *et al.* (2010)
Fucus serratus	Portugal	April to July 2016 October to November 2016	11.7 g/100 g to 11.8 g/100 g[2]	Vieira *et al.* (2018)
Fucus serratus	Norway	October 2014	3.9 ± 0.1%[1]	Biancarosa *et al.* (2017)
Fucus serratus	UK	-	17.4 ± 0.2%[1]	Marsham *et al.* (2007)
Fucus spiralis	Norway	October 2014	4.0 ± 0.0%[1]	Biancarosa *et al.* (2017)
Fucus vesiculosus	Norway	October 2014	3.7 ± 0.0%[1]	Biancarosa *et al.* (2017)
Halidrys siliquosa	Norway	October 2014	4.6 ± 0.0%[1]	Biancarosa *et al.* (2017)
Hizikia fusiforme	China, Japan, Korea	-	10.9 ± 1.0%[1]	Dawczynski *et al.* (2007)
Himanthalia elongata	Norway	October 2014	5.0 ± 0.1%[1]	Biancarosa *et al.* (2017)
Hormosira banksii	NZ	April 2004 August 2004	6.1 ± 0.3% [1]	Smith *et al.* (2010)
Laminaria digitata	Norway	October 2014	6.6 ± 0.1%[1]	Biancarosa *et al.* (2017)
Laminaria digitata	UK	-	15.9 ± 0.4%[1]	Marsham *et al.* (2007)
Laminaria sp.	China, Japan, Korea	-	6.3 ± 3.8%[1]	Dawczynski *et al.* (2007)
Macrocystis pyrifera	Chile	January 2007	13.2 ± 0.0%[1]	Ortiz *et al.* (2009)
Pelvetia canaliculata	Norway	October 2014	4.1 ± 0.0%[1]	Biancarosa *et al.* (2017)
Saccharina latissima	Norway	October 2014	8.4 ± 0.0%[1]	Biancarosa *et al.* (2017)
Saccorhiza polyschides	Portugal	April to July 2016 October to November 2016	11.8 g/ 100 g to 12.4 g/ 100 g[2]	Vieira *et al.* (2018)
Sargassum muticum	Portugal	April 2012	24.4 mg/g to 29.6 mg/g[3]	Rodrigues *et al.* (2015)
Sargassum oligocystum	Kenya	-	5.64 ± 0.19%[1]	Muraguri *et al.* (2016)

Undaria pinnatifida	Portugal	April to July 2016 October to November 2016	16.5 g/100 g to 19.5 g/100 g[2]	Vieira *et al.* (2018)
Undaria pinnatifida	NZ	April to September 2004	19.7 ± 2.3% [1]	Smith *et al.* (2010)
Undaria pinnatifida	China, Japan, Korea	-	18.9 ± 9.8% [1]	Dawczynski *et al.* (2007)
Green seaweed				
Codium fragile	Chile	January 2007	10.8 ± 0.0%[1]	Ortiz *et al.* (2009)
Codium tomentosum	Portugal	April 2012	13.1 mg/g to 24.1 mg/g[3]	Rodrigues *et al.* (2015)
Cladophora rupestris	Norway	October 2014	12.0 ± 0.0%[1]	Biancarosa *et al.* (2017)
Cladophora rupestris	UK	-	29.8 ± 0.6%[1]	Marsham *et al.* (2007)
Ulva intestinalis	Norway	October 2014	11.2 ± 0.7%[1]	Biancarosa *et al.* (2017)
Ulva lactuca	Norway	October 2014	15.0 ± 0.3%[1]	Biancarosa *et al.* (2017)
Ulva lactuca	Tunisia	July 2007	8.46 ± 0.01%[1]	Yaich *et al.* (2011)
Ulva lactuca	UK	-	29.0 ± 0.1%[1]	Marsham *et al.* (2007)
Ulva fasciata	Kenya	-	10.1 ± 0.9%[1]	Muraguri *et al.* (2016)
Ulva stenophylla	NZ	April 2004 August 2004	20.4 ± 4.9% [1]	Smith *et al.* (2010)
Ulva spp.	Portugal	April to July 2016 October to November 2016	20.5 g/100 g to 23.3 g/100 g[2]	Vieira *et al.* (2018)
Red seaweed				
Ceramium sp.	UK	-	31.2 ± 0.5%[1]	Marsham *et al.* (2007)
Corallina officinalis	UK	-	6.9 ± 0.1%[1]	Marsham *et al.* (2007)
Chondrus crispus	Portugal	April to July 2016 October to November 2016	19.1 g/100 g to 19.5 g/100 g[2]	Vieira *et al.* (2018)
Chondrus crispus	Norway	October 2014	11.0 ± 0.0%[1]	Biancarosa *et al.* (2017)
Dumontia contorta	UK	-	31.7 ± 0.4%[1]	Marsham *et al.* (2007)
Eucheuma denticulatum	Kenya	-	5.06 ± 0.36%[1]	Muraguri *et al.* (2016)
Furcellaria lumbricalis	Norway	October 2014	7.5 ± 0.3%[1]	Biancarosa *et al.* (2017)
Gracilaria chilensis	Chile	January 2007	8.48 ± 0.41%[1]	Ortiz *et al.* (2009)
Gracilaria sp.	Portugal	April to July 2016 October to November 2016	24.4 g/100 g to 24.7 g/100 g[2]	Vieira *et al.* (2018)
Hypnea musciformis	Kenya	-	6.88 ± 1.22%[1]	Muraguri *et al.* (2016)
Laurencia intermedia	Kenya	-	10.1 ± 0.9%[1]	Muraguri *et al.* (2016)

Mastocarpus stellatus	Norway	October 2014	9.6 ± 0.2%[1]	Biancarosa *et al.* (2017)
Mastocarpus stellatus	UK	-	25.4 ± 0.2%[1]	Marsham *et al.* (2007)
Osmundea pinnatifida	UK	-	27.3 ± 0.1%[1]	Marsham *et al.* (2007)
Osmundea pinnatifida	Portugal	April to July 2016 October to November 2016	22.8 g/ 100 g to 24.3 g/100 g[2]	Vieira *et al.* (2018)
Osmundea pinnatifida	Portugal	April 2012	10.3 mg/g to 19.4 mg/g[3]	Rodrigues *et al.* (2015)
Palmaria palmata	Norway	May 2015	19.3% to 29.6%[1]	Bjarnadóttir *et al.* (2018)
Palmaria palmata	Norway	October 2014	10.6 ± 0.1%[1]	Biancarosa *et al.* (2017)
Palmaria palmata	Canada	June 2013	9.8 ± 0.7% to 18.9 ± 0.1%[1]	Beaulieu *et al.* (2016)
Polysiphonia sp.	UK	-	31.8 ± 0.2%[1]	Marsham *et al.* (2007)
Porphyra sp.	UK	-	44.0 ± 1.2%[1]	Marsham *et al.* (2007)
Porphyra dioica	Norway	October 2014	20.6 ± 0.3%[1]	Biancarosa *et al.* (2017)
Porphyra purpurea	Norway	October 2014	13.5 ± 0.1%[1]	Biancarosa *et al.* (2017)
Porphyra umbilicalis	Norway	October 2014	15.1 ± 0.2%[1]	Biancarosa *et al.* (2017)
Porphyra spp.	Portugal	April to July 2016 October to November 2016	27.4 g/100 g to 28.2 g/100 g[2]	Vieira *et al.* (2018)
Porphyra spp.	NZ	May to October 2004	32.7 ± 4.1% [1]	Smith *et al.* (2010)
Porphyra sp.	China	-	25.6 ± 3.6% [1]	Dawczynski *et al.* (2007)
Porphyra sp.	Japan, Korea	-	27.0 ± 7.3% [1]	Dawczynski *et al.* (2007)
Porphyra sp.	UK	-	44.0 ± 1.2%[1]	Marsham *et al.* (2007)
Pyropia acanthophora	India	July, 2013	14.1 ± 1.1 g/100g to 18.3 ± 0.90 g/100 g[2]	Kavale *et al.* (2017)
Pyropia yezoensis	Korean	January to April, 2016	36.2 ± 0.5% to 39.2 ± 0.9%[1]	Jung *et al.* (2016)

[1] % seaweed DW; [2] g protein/100 g seaweed DW; [3] mg protein/g lyophilised seaweed extract.

Aside from being directly consumed, seaweeds are essential resources for the industry. The nutritional value of seaweeds is in the spotlight, as seaweeds possess complete proteins, including all EAAs (Sutton *et al.*, 2018). Generally, red seaweeds contain higher quality proteins when compared to brown and green seaweeds (Fleurence, 1999). Red seaweed species such as *Porphyra* spp. and *Undaria* spp. have AA scores of 91 and 100, respectively (Pangestuti

& Kim, 2015). Nevertheless, the EAA content in *P. palmata* accounts for approximately 50% of its total AAs (Naseri *et al.*, 2020b).

Bioactive properties have been identified in seaweed proteins, which are valuable findings that enhance the value of seaweed and its related products (Beaulieu *et al.*, 2015; Beaulieu *et al.*, 2016; Cian *et al.*, 2015; Cian *et al.*, 2012; Harnedy & FitzGerald, 2013b). Nonetheless, seaweed proteins could exist in many forms. They could be enzymes performing various physiochemical functions, peptides that could be further developed into functional ingredients, cell-wall associating proteins with bioactive properties, and others with substantial commercial value in the industry.

Seaweed enzymes

Enzymes are bio-catalysts that participate in and enable the onset of various biochemical reactions. Though seaweed enzymes are not the primary focus in the literature, seaweed proteases and carboxylases display beneficial properties. Undariase from green *Codium fragile*, and Ulvease from green *Ulva pertusa*, are fibrinolytic proteases that could ameliorate thrombosis conditions (Choi *et al.*, 2014; Kang *et al.*, 2016). The ribulose-biphosphate carboxylase in red *K. alvarezii* and *Solieria chordalis* perform carbon dioxide fixation and oxygenation, which could act as biomarkers for pH-induced abiotic stress (Bondu *et al.*, 2015; Tee *et al.*, 2015).

Seaweed peptides

Peptides are encrypted food protein fragments constituting two to twenty AAs (Admassu *et al.*, 2018a). Bioactive peptides are one of the predominant focuses in the literature regarding seaweeds, as they have the potential to be developed into various functional products. Most bioactive peptides are antioxidants, and more biopeptides have been analysed in brown and red seaweeds than in green seaweeds. For instance, Beaulieu *et al.* (2015) discovered antibacterial properties in extracted peptides in brown *Saccharina longicruris*. Anti-hypertensive potentials have been witnessed in brown *Sargassum Maclurei* (Zheng *et al.*, 2020). Anti-cancer, anticoagulant, anti-diabetes, antihypertension, antimicrobial, and antioxidant peptides have been detailed in edible brown *U. pinnatifida* (Nadeeshani *et al.*, 2021). Furthermore, Beaulieu *et al.* (2016) identified potent antioxidant and angiotensin-converting enzyme (ACE)-inhibitory activities in peptides from red seaweed, *P. palmata*. Anti-diabetic and cardioprotective

biopeptides have been observed in red *P. palmata* (Harnedy & FitzGerald, 2013b; Harnedy *et al.*, 2015). Anti-platelet, antioxidant and ACE- inhibitory peptides that displayed increased activity after digestion have been documented in *Pyropia columbina* (Cian *et al.*, 2015). Cian *et al.* (2015) also mentioned the possible bio-accessibility and retainment of bioactivity after these biopeptides' gastrointestinal (GI) digestion. Finally, Cian *et al.* (2015) and Sun *et al.* (2019) found that biopeptides from the green seaweed, *Ulva* spp., showcased ACE- inhibitory and anti-inflammatory capacities.

Seaweed cell wall proteins

Cell wall proteins mediate many physiological functions in the cell, such as adhesion, intercellular communication and recognition (Stiger-Pouvreau *et al.*, 2016). Glycoproteins are found bound to carbohydrates, often embedded in the seaweed cell wall. Several seaweed glycoproteins have been identified with bioactive properties. For example, Senthilkumar and Jayanthi (2016) purified anti-cancer glycoproteins from green *Codium decorticatum*. Potent anti-viral activity against influenza has also been portrayed by lectins from green *Halimeda renschii* (Mu *et al.*, 2017). Anti-Alzheimers and anti-inflammatory potentials are displayed in the green *U. pinnatifida* glycoproteins (Rafiquzzaman *et al.*, 2015). Nevertheless, Hirayama *et al.* (2016) located anti-viral lectins in the red *K. alvarezii*, which could potentially ameliorate human immunodeficiency virus. Properties such as anti-cancer and anti-depressant have been observed in lectins, which are proteins bound to polysaccharides in the red *Solieria filiformis* (Abreu *et al.*, 2018; Chaves *et al.*, 2018).

Other seaweed proteins

Despite the seaweeds listed above, other health-benefiting proteins are currently being uncovered in seaweeds. For instance, phycobiliproteins are water-soluble proteins that harvest light for photosynthesis in red seaweeds (Pliego-Cortés *et al.*, 2020). Such light-harvesting pigments are commercially employed as they are helpful fluorescent markers in fluorescent applications, natural dyes in the food industry, and health-benefiting pharmaceuticals (Sekar & Chandramohan, 2008). Lee *et al.* (2017) also identified phycobiliproteins from the red *P. palmata* could exhibit anti-inflammatory effects. Another example would be mycosporine-like AAs, which are small, secondary metabolites found in many seaweeds (Pliego-Cortés *et al.*, 2020). Such secondary metabolites portray potent antioxidant capacity and are effective

cosmetic ingredients, protecting against ultraviolet radiation and preventing skin ageing (Chrapusta *et al.*, 2017; Pliego-Cortés *et al.*, 2019).

Future prospects

As the consequences of consuming and producing animal protein gain more awareness, it is evident that seaweeds have pronounced potential to become an alternative plant protein source. The protein profile of seaweeds is distinctive, as it would be one of the very few non-animal sources that constitute complete proteins with all EAAs. Many seaweeds, especially red seaweeds, exert significant amounts of quality protein and are present in quantities comparable to traditional protein-rich foods; such as some meats, cereals, and pulses (Pangestuti & Kim, 2015). Additionally, recent literature emphasises numerous health-beneficial properties possessed by various seaweeds. However, global seaweed cultivation and farming expansion would be necessary for a more comprehensive exploitation of seaweed proteins. Optimal growth conditions of seaweeds should be applied for the maximal production of quality seaweed proteins. Understanding the extraction of seaweed proteins would be next, as both conventional and novel techniques have advantages and disadvantages. Ultimately, the major challenge in seaweed protein extraction would be to degrade the complex seaweed cell walls and polysaccharides before the release of proteins.

1.2.3 Extraction and isolation of plant proteins

Protein extraction is the fundamental first step to maximise their subsequent quantification and application. As seaweed proteins are generally bound to cell wall materials, the rigid nature of seaweed cell walls is the primary obstacle that hinders cellular protein extraction. Hence, degradation of cell wall integrity and disintegration of structural polysaccharides are important for releasing seaweed proteins. Conventional extraction with various aqueous systems, novel physical extraction with current technologies and enzymatic extraction are potential procedures for protein extraction. Parameters of different extraction procedures should be optimised as they would influence the quality of extracted proteins.

1.2.3.1 Sample preparation for protein extraction

The initial harvest is essential for producing good quality samples. Harvesting seaweeds could occur at their appropriate maturity stages and should be collected according to their best physical attributes, such as size, texture, and firmness. Seaweed harvest should be conducted before warm weather; minimum protein content has been observed in summer, which could be triggered by the destruction of phycobiliproteins (Pangestuti & Kim, 2015). Post-harvest handling would be crucial in preserving the best sample quality for subsequent extraction procedures. Seaweeds are commonly washed to discard epiphytes, impurities and salt residues; then air-dried, oven-dried or sun-dried until constant weight to remove moisture. Adequate storage afterwards would maximise the retainment of seaweed quality, reducing chances of oxidation and other undesirable contamination; for instance, storage utilising airtight polyethylene bags and containers in the dark would be appropriate (Vásquez *et al.*, 2019; Vieira *et al.*, 2018). Blending, grinding or milling the dried seaweed into finer particles would be necessary for enhancing protein extraction efficiency, increasing the contact surface area with the extractant (Dumay *et al.*, 2013).

1.2.3.2 Conventional extraction of protein

Conventional extraction of proteins exploits various liquid systems, whereby the extraction system depends on the protein behaviour under different pHs. Acidic and basic solvents, buffers, and distilled water have been well-established for simple protein extraction across a range of plants and fruits; examples are red pepper seeds (Firatligil-Durmus & Evranuz, 2010), sunflower (Pickardt *et al.*, 2015), tomato (Mechmeche *et al.*, 2017) and various leaves (Zhang

et al., 2014). Such aqueous solutions solubilise multiple bonds in the cell wall, thus improving protein extraction yield (Kumar *et al.*, 2021).

For seaweeds, alkaline extraction is evidently more effective for protein extraction than acidic extraction. For instance, hydrochloric acid (0.1 M to 0.4 M HCl) extraction of protein from brown *A. nodosum* produced approximately 7% to 16% of total protein, which was significantly lower than 50% to 56% protein extracted with sodium hydroxide (0.1 M to 0.4 M NaOH) (Kadam *et al.*, 2017). Fleurence *et al.* (1995a) discovered that the water extraction of seaweed protein, followed by 0.1 M NaOH, yielded 26.8% and 36.1% of total protein in green *Ulva rigida* and *Ulva rotundata*, respectively. Nevertheless, Kadam *et al.* (2017) recovered 59% of total protein when combining acidic and alkaline extractions (0.4 M HCl first, followed by 0.4 M NaOH). Harrysson *et al.* (2018) witnessed similar results, whereby alkaline protein solubilisation followed by isoelectric point precipitation produced the highest protein concentration in red *Porphyra umbilicalis* (71% DW of total AA), green *U. lactuca* (51.2% DW), and brown *S. latissimi* (40.7% DW).

Despite the simplicity and the good protein yields from liquid systems, such conventional extraction techniques are often time-consuming and energy-intensive, and their manual operations make reproducibility a challenge (Kadam *et al.*, 2013; Kumar *et al.*, 2021). Since conventional techniques revolve around a range of pH, pressures and temperatures, they could also denature biomolecules, including proteins (Kadam *et al.*, 2013). Furthermore, some acids, bases and organic solvents are not food grade, and their prolonged usage would be detrimental to the environment. Hence, such limitations in conventional techniques urge the food industry to seek new technologies to overcome these challenges.

1.2.3.3 Novel extraction of protein

Physical extraction of proteins has several emerging technologies that have enhanced protein yield and protein extraction rate. Novel technologies such as microwave-assisted extraction, pulse-electric field, ultrasonic-assisted extraction and membrane filtration are highlighted with many advantages compared to conventional techniques mentioned above. Novel technologies revolve around cell disruption, which allows the subsequent release and extraction of desired cell contents. Nonetheless, novel technologies for protein extraction have augmented energy

and time efficiencies, cost and environmental friendliness, which are all key factors for popularising new technologies in the food industry (Kumar *et al.*, 2021).

Microwave-assisted extraction

Microwave-assisted extraction (MAE) centres on utilising its non-ionising electromagnetic energy. During this technique, microwave energy ranging from 300 MHz to 300 GHz is transferred to the sample's solution, resulting in water molecule vibrations that induce hydrogen bond breakage and dissolved ion migration (Kadam *et al.*, 2013). Such phenomenon augments pressure within the sample matrix and disrupts cell content, thus allowing solvent penetration and subsequently the extraction of intracellular compounds from the cell matrix (Kadam *et al.*, 2013).

MAE has been utilised for protein extractions in many plant-based materials. For instance, apple (Casazza *et al.*, 2020), cranberry (Spadoni Andreani & Karboune, 2020), soymilk (Varghese & Pare, 2019), and various brans (Görgüç *et al.*, 2019; Phongthai *et al.*, 2016; Wang *et al.*, 2013). On the contrary, MAE has been employed for extracting carbohydrates and polyphenols from seaweeds. For example, Singh *et al.* (2017) extracted cellulose from brown *Gelidiella aceroso* with 30 minutes of microwave irradiation at 360 W. Yuan *et al.* (2018) extracted polyphenols from brown seaweeds with 15 minutes of microwave irradiation at 2.45 GHz. Although MAE is time-efficient, improves extraction rate, yields and minimises solvent use, it would be unsuitable for extracting heat-sensitive compounds (Kadam *et al.*, 2013). Increased temperatures from MAE could denature heat-labile compounds and hinder its more comprehensive exploitation for the extraction of seaweed proteins. Therefore, a method involving MAE for seaweed protein extraction should balance its advantage in cell wall disruption and its disadvantage, such as heat generation (Kumar *et al.*, 2021).

Pulse-electric field

Pulse-electric field (PEF) emphasises the utilisation of high electrical current. PEF sends rapid pulses of high electric potential energy ranging from 10 to 80 kV/cm through samples from two electrodes in a chamber (Kumar *et al.*, 2021). This phenomenon leads to cellular perforation and triggers electroporation of the cell wall, thus disintegrating the cell, increasing its permeability and consequently freeing intracellular cell content for extraction (Kumar *et al.*, 2021).

PEF is a well-established technique for food preservation, as it is effective for inactivating unwanted enzymes and microbes. The improved yields of high-value intracellular compounds have been achieved with PEF, for example, quality lipids and pigments from microalgae have been effectively extracted through PEF (Luengo *et al.*, 2015; Zbinden *et al.*, 2013). Postma *et al.* (2018) obtained a protein yield of 15.1% when undertaking 7.5 kV/cm using 0.05 ms pulses for extracting proteins from a fresh green seaweed, *U. lactuca*. Furthermore, Prabhu *et al.* (2019) reported that PEF pre-treatment resulted in a 4-fold increase in proteins extracted from green *Ulva ohnoi*. Though PEF is considered eco-friendly, non-thermal and solvent-free, PEF has not been well-established for seaweed protein extraction compared to other emerging physical extraction procedures (Kumar *et al.*, 2021). Protein recovery results could be promising when PEF is undertaken at low temperatures with extended pulses and augmented electric field strengths (Kumar *et al.*, 2021).

Ultrasound-assisted extraction

Ultrasound-assisted extraction focuses on the utilisation of high-frequency sound waves. During ultrasound treatment, sound waves above 20 kHz are passed through samples, thus triggering mechanical vibrations that generate minuscule vapour bubbles that could violently collapse under strong ultrasonic fields (Kadam *et al.*, 2013). The implosion of microbubbles is known as cavitation; this phenomenon produces immense shear forces responsible for cell breakdown, and subsequently cellular content release (Kadam *et al.*, 2013).

Ultrasound-assisted extraction has been widely applied to plants and seeds for the extraction of desired proteins, such as proteins from brans (Kumar *et al.*, 2021), cauliflower (Xu *et al.*, 2017), sesame (Görgüç *et al.*, 2019), soy (Preece *et al.*, 2017a), and watermelon seeds (Wen *et al.*, 2019). Ultrasound treatment has been utilised for extracting seaweed alginate and carrageenan (Youssouf *et al.*, 2017), phenolic compounds (Kadam *et al.*, 2015), and pigments (Kumar *et al.*, 2020) and proteins (Kazir *et al.*, 2019). Ultrasound-assisted extraction at 400 W for 60 mins following water extraction have effectively yielded protein from brown *Sargassum muticum* (24.4 mg protein/g lyophilised seaweed), green *Codium tomentosum* (19 mg/g), and red *Osmundea pinnatifida* (10.3 mg/g) (Rodrigues *et al.*, 2015).

Ultrasound-assisted extraction could also be utilised simultaneously or in addition to other extraction techniques to maximise protein yield. For instance, ultrasound treatment has been applied to microalgae *Chlorella pyrenoidosa*, along with ethanol soaking, enzymatic digestion

and homogenisation, and achieved a protein extraction rate up to 72.4% (Zhang *et al.*, 2018). Kadam *et al.* (2017) employed a probe ultrasound equipment with 750 W capacity and 20 kHz frequency for pre-treating protein extraction in brown *A. nodosum*; this pre-treatment recovered up to 540% more protein compared to using acid hydrolysis, along with a reduction in processing time and improved liquefaction of seaweed powder. Nonetheless, the combination of alkaline and ultrasound-assisted extractions could successfully extract 57% of total *A. nodosum* protein (Kadam *et al.*, 2017). Finally, many findings have demonstrated that ultrasound treatment is simple, cost- and time-efficient, improves extraction yield and rate, has scale-up potential and is non-selective (Kumar *et al.*, 2021). Unlike MAE, ultrasound-assisted extraction yields heat-sensitive compounds with minimal damage (Kadam *et al.*, 2013). However, wave attenuation would be a challenge during ultrasound treatment as sound wave amplitude reduces with increasing distance (Kadam *et al.*, 2013).

Membrane filtration

To further isolate the protein extract, membrane filtration (MF) could be performed following MAE, PEF and ultrasound-assisted extraction. MF revolves around the molecular sizes of compounds, as they are well known for attributing their biological activities (Pangestuti & Kim, 2015). A semi-permeable membrane separates liquid fractions with compounds of various sizes (Pliego-Cortés *et al.*, 2020). Such phenomenon allows the fractionation of desired compounds, as only desired compounds of specific molecular sizes permeate through the membrane. Common types of MF are microfiltration, ultrafiltration, nanofiltration and reverse osmosis.

MF is often utilised for purifying various juices from impurities; some examples are apple, blackcurrant, cranberry and orange juices (Bhattacharjee *et al.*, 2017). In contrast, MF tends to be employed for purifying and isolating seaweed biochemical compounds. Purification of brown *A. nodosum* extracts containing laminarin was conducted using ultrafiltration with molecular cut-off membranes (Rioux *et al.*, 2010). The selectivity of MF would be ideal for extracting specific compounds with particular molecular sizes. This highlights that MF could further purify protein extracts after cell disruption, thus ensuring that the final extract is free of undesired organic compounds. For instance, Bondu *et al.* (2015) isolated antioxidant and anti-hypertensive natural bioactive peptides from seaweeds using 10 kDa cut-off membranes. Similarly, Mao *et al.* (2017) utilised 10, 5 and 3 kDa cut-off membranes to acquire anti-proliferation peptides from red *Pyropia haitanensis* protein hydrolysates. Finally, MF can filtrate a large sample quantity without damaging sensitive compounds (Pangestuti & Kim,

2015). Other advantages include being user-friendly and having moderate energy consumption (Pangestuti & Kim, 2015). However, when compared to other novel technologies, MF could have lower yields, more time-consuming, and higher inaccuracy when obtaining non-spherical compounds (Shi, 2016). Additionally, upscaling MF could be challenging as large membranes might be difficult to acquire, and the subjection of MF to liquid samples would lower its application towards solid samples.

1.2.3.4 Enzymatic extraction of protein

As the plant cell wall is primarily constructed from rigid polysaccharides that reduce the accessibility of cellular content, the exploitation of enzymes for sabotaging cell wall integrity would be promising in plant protein extraction. Enzyme-assisted extraction of plant proteins employs specific polysaccharidases to degrade complex cell wall polysaccharides, such as cellulase, hemicellulase and pectinase. Polysaccharidases are an attractive alternative to aqueous and novel physical methods for plant protein extraction (Wijesinghe & Jeon, 2012). To date, enzymatic extraction of proteins has been performed in a range of plant and fruit sources; some recent examples are cranberry (Spadoni Andreani & Karboune, 2020), potato (Waglay *et al.*, 2019), rapeseed, soybean and microalgae (Sari *et al.*, 2013), different brans (Görgüç *et al.*, 2019; Perović *et al.*, 2020; Yilmaz-Turan *et al.*, 2020), and various leaves (Akyüz & Ersus, 2021; Benhammouche *et al.*, 2021; Vergara-Barberán *et al.*, 2015). Furthermore, seed proteins acquired by enzymatic extraction showed improved physicochemical and functional properties than conventional aqueous extraction, including better emulsifying and foaming, solubility, thermal stability and water holding capacity (Jiang *et al.*, 2021).

Unlike terrestrial plants, seaweed cell walls constitute sulphated and branched polysaccharides often integrated with ions and proteins (Wijesinghe & Jeon, 2012). This demonstrates that seaweed cell walls' heterogeneous structure and composition could further impair seaweed protein extraction efficiency compared to terrestrial plants. Differing cell wall polysaccharides between seaweed phyla also leads to varying protein extraction efficiencies. For example, brown seaweeds contain higher levels of uronic acid when compared to green and red seaweeds, resulting in reduced enzymatic hydrolysis of brown seaweed cell walls (Dumay *et al.*, 2013; Jung *et al.*, 2013).

To begin, seaweeds could be initially hydrated in water or other aqueous solutions (Naseri *et al.*, 2020b). Enzymes are then incorporated into the seaweed solution, and the system is continuously stirred and incubated in a controlled manner. During incubation, enzymatic hydrolysis of the seaweed cell wall leads to the breakdown of its structural polysaccharides. A set of optimised operating parameters are refined for enzymatic hydrolysis, namely the incubation temperature, time and pH; such parameters are developed according to the optimal conditions for enzyme function. Once the structural polysaccharides are disintegrated, releasing cellular protein becomes more convenient.

Enzyme selection would be the major decision before commencing enzyme-assisted extraction. The purposes of utilising enzymes are to degrade or hydrolyse large polysaccharides and proteins or act on desired biochemicals to modify their structure (Charoensiddhi *et al.*, 2017). For seaweeds, enzyme selection should be performed with regards to their phyla-specific cell wall polysaccharides listed in Table 1, with the ability to target α-1,3, α-1,4, β-1,3, and β-1,4 bonds within the cell wall (Deniaud *et al.*, 2003; Sharma & Horn, 2016; Wu, 2012). In this case, individual enzymes or enzyme combinations could be explored for protein extraction. For example, Fleurence *et al.* (1995b) demonstrated that cell wall polysaccharide-specific enzyme combinations yielded the highest protein in red seaweeds, *Chondrus crispus* and *Gracilaria verrucosa*; but this did not apply to red *P. palmata.* Such phenomenon emphasises the complex nature of seaweed cell walls, and that it would be crucial to conduct prior trials to finalise enzyme selection.

Currently, commercially prepared enzymes are commonly exploited to extract seaweed proteins. As mentioned in Table 7, enzymes with good seaweed protein extraction yields include cellulase (Harnedy & FitzGerald, 2013a; Mæhre *et al.*, 2016), protease (Hardouin *et al.*, 2016; Naseri *et al.*, 2020b), xylanase (Bjarnadóttir *et al.*, 2018; Mæhre *et al.*, 2016). Other enzymes utilised in seaweed protein extraction also include agarase (Fleurence *et al.*, 1995b), Pectinex (Vásquez *et al.*, 2019), Viscozyme (Naseri *et al.*, 2020b). Moreover, comparing proteins obtained through enzyme-assisted extraction to a control, where seaweed proteins undergo extraction without enzyme, would enable an understanding of enzyme-assisted extraction efficiencies (Hardouin *et al.*, 2016; Harnedy & FitzGerald, 2013a; Mæhre *et al.*, 2016; Naseri *et al.*, 2020b).

Operating parameters require careful consideration. Enzyme suppliers provide recommended guidelines for their products, such as optimised incubation temperatures and pHs, yet those might not be reproducible across samples with sensitive biochemicals (Dumay *et al.*, 2013). Refined conditions for enzymatic incubation could also be experimentally confirmed; combinations with different temperatures and pHs could be tested for desired enzymes to ensure their best performance. As listed in Table 7, incubation temperature of 50° and pH 4.5 are typically the working conditions for the enzymes employed for seaweed protein extraction. Though temperature and pH are fundamental parameters for influencing enzyme activity and protein stability, optimisation of variables such as enzyme-to-substrate concentration, incubation time and seaweed particle size are also crucial for acquiring desired protein extraction yields (Dumay *et al.*, 2013).

Table 7. Optimal enzymatic extraction of proteins from seaweeds.

Species	**Harvest location**	**Harvest date**	**Protein content before treatment**	**Optimal conditions for enzymatic extraction**	**Protein recovered/yielded after treatment**	**Reference**
Brown seaweed						
Macrocystis pyrifera	Chile	January and March 2012	9.9 ± 0.2%[1]	Enzymes: cellulase pH: 4.5 Temp: 50 °C Incubation time: 12 hr Enzyme: substrate ratio: 1:10 enzyme/seaweed	Protein yield: 74.6 ± 21.3%[1]	Vásquez *et al.* (2019)
Sargassum muticum	Portugal	-	-	Enzymes: Alcalase pH: 8 Temp: 50 °C Incubation time: 24 hr Enzyme:substrate ratio: 1:20 enzyme/seaweed	Nitrogen content: 29.6 ± 0.01 mg/g[3]	Rodrigues *et al.* (2015)
Green seaweed						
Ulva armoricana	France	June 2012	24.4 ± 0.1%[1]	Enzymes: mixture of neutral and alkaline endo-proteases	Protein yield: 88.4 ± 0.2%[1]	Hardouin *et al.* (2016)

				pH: 6.2 at the start of incubation to 5.9 at the end of incubation Temp: 50 °C Incubation time: 3 hr Enzyme:substrate ratio: 6% (weight of enzyme/seaweed dry weight)		
Codium tomentosum	Portugal	April 2012	-	Enzymes: Flavourzyme pH: 7 Temp: 50 °C Incubation time: 24 hr Enzyme:substrate ratio: 1:20 enzyme/seaweed	Nitrogen content: 24.1 ± 0.4 mg/g[3]	Rodrigues *et al.* (2015)
Red seaweed						
Chondracanthus chamissoi	Chile	January and March 2012	17.60 ± 0.10%[1]	Enzymes: cellulase pH: 4.5 Temp: 50 °C Incubation time: 12 hr Enzyme:substrate ratio: 1:10 enzyme/seaweed	Protein yield: 36.1 ± 3.4%[1]	Vásquez *et al.* (2019)
Osmundea pinnatifida	Portugal	-	-	Enzymes: Flavourzyme pH: 7 Temp: 50 °C Incubation time: 24 hr Enzyme:substrate ratio: 1:20 enzyme/seaweed	Nitrogen content: 19.4 ± 0.14 mg/g[3]	Rodrigues *et al.* (2015)
Palmaria palmata	Iceland	April 2017	-	Enzymes: Celluclast/Shearzyme combination with 0.2% Alcalase pH: 7 Temp: 60 °C Incubation time: 14 hr Enzyme:substrate 0.2% (g enz/g algal biomass) Followed by N-acetyl-L-cysteine extraction.	Protein recovery: 85% to 90%[1]	Naseri *et al.* (2020b)
Palmaria palmata	Norway	May 2015	19.3% to 29.6%[1]	Enzymes: xylanase pH: 6.5 Temp: 60 °C	Protein yield: 33.4% to 54.9%[1]	Bjarnadóttir *et al.* (2018)

				Incubation time: Overnight Enzyme:substrate ratio: 37 units/300 g seaweed		
Palmaria palmata	Iceland	-	-	Enzymes: cellulase, xylanase pH: 5 Temp: 40 °C Incubation time: 24hr Enzyme:substrate ratio: 50 units of xylanase and cellulase/g seaweed Followed by NaOH extraction	Protein yield: 69.8% to 75.6%[1]	Mæhre *et al.* (2016)
Palmaria palmata	Ireland	December 2009	4.16 ± 0.17 g/ 100 g[2]	Enzymes: Celluclast, Shearzyme pH: 5 Temp: 40 °C Incubation time: 24hr Substrate/enzyme: 48 × 10^3 units/100 g seaweed Followed by NaOH-NAC extraction.	Protein yield: 11.57 ± 0.08 g/ 100 g[2]	Harnedy and FitzGerald (2013a)

[1] % dry weight; [2] g protein/100g dry weight seaweed; [3] mg nitrogen/g lyophilised seaweed extract.

Naseri *et al.* (2020b) evaluated protein extraction efficiencies of four commercially prepared enzymes, Alcalase (protease), Celluclast (cellulase), Shearzyme (xylanase) and Viscozyme (mixture of polysaccharidases), at different concentrations, pHs and temperatures, in red *P. palmata*. Enzyme combinations displayed improved protein extraction efficiency for red *P. palmata* compared to using individual enzymes. Alcalase, being a protease, demonstrated the highest protein extraction efficiency of up to 80% compared to other enzymes. As mentioned in Table 7, when Alcalase was utilised in conjunction with carbohydrases, Celluclast and Shearzyme, followed by N-acetyl-L-cysteine (NAC) extraction, achieved protein recovery reached 90% and 85% DW, for each enzyme combination, respectively. Alcalase is an enzyme with protease activity, whereby it could hydrolyse peptide bonds that link AAs together, which would augment protein extraction efficiency. Surprisingly for Alcalase, pH had little impact on its protein extraction efficiency, whereas improvements in protein extraction were observed at 45 °C and 60 °C when compared to 30 °C. Nevertheless, the utilisation of Alcalase alone produced protein extracts with the highest EAA/AA ratio of 0.44, whereas other enzymes ranged between 0.2 to 0.24. All EAAs displayed improved yields after enzyme-assisted

extraction. Lastly, Naseri *et al.* (2020b) illustrated that Alcalase, or enzymes with similar protease activity, has promising potential for extracting proteins from red seaweed, particularly when utilised in conjunction with other enzymes.

Vásquez *et al.* (2019) explored the optimisation of enzyme-assisted extraction for proteins using three commercially prepared carbohydrases, α-amylase, Cellic CTec3 (cellulase), and Pectinex ultra (pectinase), in brown *Macrocystis pyrifera* and red *Chondracanthus chamissoi*. All three carbohydrases were selected due to their potential ability to hydrolyse α-1,3, α-1,4 and β-1,4 bonds in polysaccharides within the seaweed cell wall (Sharma & Horn, 2016; Wu, 2012). α-Amylase displayed the highest hydrolytic activity for alginates, whereas cellulase displayed the highest hydrolytic activity for agar and carrageenan. In contrast to Naseri *et al.* (2020b)'s findings, Vásquez *et al.* (2019) reported that the enzyme combination of α-amylase and cellulase had no significant differences in hydrolytic activity when compared to individual enzymatic preparations. Vásquez *et al.* (2019) also suggested that this could be due to the pH incompatibilities between α-amylase (pH 7.5) and cellulase (pH 4.5), or that the efficiency of using two cabohydrases was not as drastic as using carbohydrase in conjunction with protease. The final procedure utilised Cellic CTec3, an enzyme with cellulase, hemicellulase and β-glucosidase activities. Through enzyme treatment, 4.7-fold more proteins were extracted from *M. pyrifera* than through non-enzyme treatment procedures, and 1.5-fold more proteins were extracted from *C. chamissoi*. As listed in Table 7, the protein yield utilising cellulase in optimised conditions reached 74.6% DW for *M. pyrifera*, and 36.1% DW for *C. chamissoi*. The different extraction yields from brown and red seaweeds emphasised their unique cell wall structure and structural polysaccharides. Hence optimisation of protein extraction in seaweeds could be highly dependent upon their phyla. Finally, Vásquez *et al.* (2019) proposed using cellulase for enzyme treatment of protein for seaweeds with similar cellulose-rich cell walls, and that enzyme treatment could be coupled to the recovery of polysaccharides to improve the feasibility and economic value.

Bjarnadóttir *et al.* (2018) examined protein extracted from red *P. palmata* with endo-1,4-β-xylanase and Umamizyme (endo- and exo-proteases). Unlike the findings reported by Naseri *et al.* (2020b), though the combination of xylanase and protease increased protein extraction yield (up to 35% DW) when compared to the control (up to 25% DW), xylanase alone exhibited a higher extraction yield (up to 54.9% DW). However, protease-digested samples contained increased protein content in the extracted supernatant compared to control and xylanase

extractions; thus indicating that protein cleavage by protease into smaller proteins, peptides and amino acids, all with lower molecular weights, occurred. Though the utilisation of protease would be undesirable if high molecular weight proteins were preferred, both Bjarnadóttir *et al.* (2018) and Naseri *et al.* (2020b) agreed that protease could effectively isolate small peptides and AAs with respect to bioactivity. Compared to findings from Naseri *et al.* (2020b), Bjarnadóttir *et al.* (2018) recorded that all AAs portrayed improved yields after enzyme-assisted extraction, except methionine, threonine and tryptophan. As suggested in Table 7, xylanase was confirmed by Bjarnadóttir *et al.* (2018) to be a feasible option for extracting quality proteins from red seaweed, yielding 33.4% to 54.9% DW protein (depending on nitrogen-to-protein conversion factor). At last, Bjarnadóttir *et al.* (2018) did not explore the effect of different enzyme concentrations on protein extraction. Thus, it was advised that an optimised enzyme-seaweed ratio should be sought as the incorporation of enzymes at high levels would not be feasible at industrial scaled protein extractions.

Mæhre *et al.* (2016) analysed the effect of enzymatic pre-treatment on the extractability and bioaccessibility of proteins in red *P. palmata*. Due to the large proportion of cellulose and xylan in the red seaweed cell wall, a combination of cellulase and xylanase was selected for the protein extraction. As expected, protein yield improved after enzyme-assisted extraction. As indicated in Table 7, enzymatic pre-treatment followed by alkaline post-treatment using 0.1 M NaOH extracted up to 75.6% DW protein. Furthermore, total AA content increased a significant 3-fold after enzymatic treatment with xylanase and cellulase, but no significant differences were observed between varying enzyme concentrations. Compared to findings from Naseri *et al.* (2020b), all EAA except lysine illustrated improved yield after enzyme treatment. Nevertheless, through a stimulated *in vitro* GI model, the total AA available for intestinal absorption increased by 3.2-fold for samples with enzymatic pre-treatment, suggesting that seaweed proteins' bio-accessibility could be enhanced through enzyme-assisted extraction.

Hardouin *et al.* (2016) assessed the influence of six commercial enzymatic preparations on the protein extraction efficiency in green *U. armoricana.* Commercial enzymatic preparations explored were neutral endo-protease, a mix of neutral and alkaline endo-proteases, a multiple-mix of carbohydrases, a mix of endo-1,4-β-xylanase/endo-1,3(4)-β-glucanase, cellulase and an exo-β- 1,3(4)-glucanase. A commercial mixture of neutral and alkaline endoproteases conveyed the greatest extraction efficiency when compared to other types of proteases and carbohydrases, which would be coherent with findings from Bjarnadóttir *et al.* (2018) and

Naseri *et al.* (2020b). Hardouin *et al.* (2016) suggested that proteases could destabilise seaweed cell walls, further releasing and solubilising more compounds. As indicated in Table 7, enzyme treatment with neutral and alkaline endo-proteases yielded 88.4% DW protein, which provided an extraction gain of 100% compared to the control without enzymes. Nevertheless, the AA composition from proteins solubilised by enzyme-assisted extraction differed from proteins from water incubation, such as lower alanine and glycine, and increased glutamic acid and aspartic acid from enzyme-assisted extraction. These changes in AA composition might be associated with the specific proteins present in green seaweeds, as such AA compositional changes were not observed in red seaweeds (Bjarnadóttir *et al.*, 2018; Naseri *et al.*, 2020b).

Rodrigues *et al.* (2015) examined the impact of enzyme-assisted and ultrasound-assisted extractions on the biological properties of brown *S. muticum*, green *C. tomentosum* and red *O. pinnatifida*. Four commercially prepared enzymes were explored: Alcalase, Celluclast, Flavourzyme (protease and peptidase), and Viscozyme; enzyme treatment was conducted at optimal conditions. As listed in Table 7, enzyme treatment by Alcalase had the greatest yield for *S. muticum* (29.6 ± 0.01 mg nitrogen/g protein/g lyophilised seaweed extract), whereas enzyme treatment extraction by Flavourzyme had the most outstanding extraction yields for green *C. tomentosum* (24.1 ± 0.4 mg/g) and red *O. pinnatifida* (19.4 ± 0.14 mg/g). Likewise to Naseri *et al.* (2020b), a protease displayed the highest extraction yield for red seaweed, though it was Flavourzyme and not Alcalase for Rodrigues *et al.* (2015). Regarding antioxidant capacity, *S. muticum* extracts after enzyme treatment displayed no activity, *C. tomentosum* after protease treatment showcased improved activity, and *O. pinnatifida* after enzyme treatment portrayed higher activity than ultrasound treatment and hot water extraction. Moreover, *C. tomentosum* and *O. pinnatifida* holds potential α-glucosidase inhibition activity that could help prevent and manage blood glucose level in type 2 diabetic patients.

Harnedy and FitzGerald (2013a) investigated the various protein extraction procedures in *P. palmata*, including osmotic shock, high shear force, alkaline treatment and enzyme-assisted extraction with commercial polysaccharidases. Compared to aqueous and physical extraction procedures, enzymatic pre-treatment with Celluclast (cellulase) and Shearzyme (xylanase) was the most promising procedure for cell disruption. As mentioned in Table 7, enzyme-assisted extraction with Celluclast and Shearzyme, followed by NaOH-NAC extraction, resulted in a total protein recovery of 11.57 g protein/100 g DW seaweed. Harnedy and FitzGerald (2013a) mentioned that NAC being a reducing agent and alkaline extraction at pH 12.68 could disturb

tertiary protein structure. Nevertheless, NAC usage could be associated with high levels of cysteine after the extraction of seaweed proteins (Naseri *et al.*, 2020b). Finally, a high ratio of enzyme-substrate, 48 × 10^3 units/100 g DW seaweed, was required to achieve the protein recovery of 11.57 g protein/100 g DW seaweed. However, Harnedy and FitzGerald (2013a) mentioned that enzyme-assisted extraction with a high enzyme-substrate ratio might not be feasible at an industrial level.

Literature findings portray that the enzyme-assisted extraction of seaweed proteins is a credible approach. Enzyme treatment significantly improves protein extraction yield and provides an impressive EAA/AA ratio. The addition of enzymes for sabotaging cell walls from degrading polysaccharides eases protein release. Additionally, incorporating alkaline extraction after enzyme-assisted extraction could be promising procedure to enhance protein extraction further. Compared to other novel extraction techniques, the utilisation of enzymes would minimise residual solvents and toxic chemicals, which could minimise the negative environmental impacts (Wijesinghe & Jeon, 2011). Nevertheless, enzyme-assisted extraction yields quality proteins with excellent digestibility, high thermal stability, and low viscosity, thus ameliorating their biological and functional properties (Kumar *et al.*, 2021). However, the disadvantages of enzyme treatment should not be disregarded; these include being expensive and slow, energy-intensive and inconsistent in yields (Kumar *et al.*, 2021). Furthermore, seaweed protein integrity would be disturbed due to the release of proteases from cytosolic vacuoles (Bjarnadóttir *et al.*, 2018; Bleakley & Hayes, 2017; Naseri *et al.*, 2020b). In some cases, the requirement of high enzyme-substrate ratios would economically hinder the upscale potential of enzyme-assisted extraction (Harnedy & FitzGerald, 2013a). This challenge could be conquered through the incorporation of inexpensive food-grade enzymes (Wijesinghe & Jeon, 2012), optimising enzyme-assisted extraction in conjunction with food-friendly aqueous and physical extraction techniques (Zhang *et al.*, 2018), and enzyme treatment with immobilised enzymes that are insoluble and could be readily recycled in the food industry (Sheldon *et al.*, 2021).

Precipitation of protein

Likewise to MF, protein precipitation after extraction would be necessary to segregate desired proteins from unwanted contaminants. Generally, protein precipitation incorporates the addition of acids, salts and organic solvents in large quantities to protein extracts. Doing so disturbs hydrophilic and hydrophobic residues of the protein surface, modifying protein

solubility and resulting in protein aggregates that could be obtained through centrifugation and filtration (Scopes, 1982). Desirable protein precipitation techniques are rapid, non-destructive, scalable and inexpensive (Burgess, 2009). Precipitation effectively enhances protein concentration and protein purity, enabling the obtainment of protein isolates. However, the achievement of protein isolates would be accompanied by sample loss, which would be catastrophic if micrograms of protein were derived (Feist & Hummon, 2015).

Common types of protein precipitation involve the utilisation of acetone or ethanol, protein's isoelectric points and ammonium sulphate followed by dialysis. For acetone precipitation, Crowell *et al.* (2013) elucidated that optimal protein recovery generally requires the overnight incubation of samples in 75% to 80% acetone at 4 °C to -20 °C; whereas Vásquez *et al.* (2019) utilised four volumes of ice-cold acetone to precipitate proteins for 2 hr. For ethanol precipitation, Hardouin *et al.* (2016) indicated that recovery of seaweed polysaccharides generally requires the overnight incubation of samples in ethanol at 4 °C. During isoelectric point precipitation, Naseri *et al.* (2020b) suggested that isoelectric point precipitation of seaweed proteins required lowering the pH of samples to approximately 3 using < 5 M HCl. During ammonium sulphate precipitation with dialysis, both Akyüz and Ersus (2021) and Garcia-Vaquero *et al.* (2017) elucidated that protein precipitation required 80% to 85% ammonium sulphate, and samples were dialysed at 4 °C overnight.

1.2.4 Characterisation of proteins

The characterisation of isolated protein extracts could be conducted through various analytical techniques. There are three predominant types of protein quantification methods. The Kjeldahl method is well-recognised as the official procedure for determining protein content in different foods; a nitrogen-to-protein conversion factor (NPCF) is nessecary to estimate protein content. Spectrophotometric techniques, such as Lowry and Bradford methods, are widely established for measuring protein content; a standard curve is the comparative estimate of protein content for both Lowry and Bradford methods. Further understanding of protein extracts could require AA identification and quantification. Such procedures would demand more advanced instrumentation, such as liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry.

1.2.4.1 Kjeldahl method

Introduced by Johan Kjeldahl, the Kjeldahl method has been widely accepted for measuring food protein for almost 140 years (Kjeldahl, 1883). The Kjeldahl method is recognised as the official food protein measurement protocol by AOAC, and most food protein determination procedures are based on this method (AOAC, 2005). In this method, total nitrogen content in foods acts as the basis for determining food protein. The Kjeldahl method involves three critical stages that could be completed by automated instruments: digestion, distillation and titration. Firstly, organic nitrogen in the food sample is converted to ammonia in the extreme boiling of sulphuric acid in the presence of a catalyst. Secondly, the digested sample with ammonia is neutralised by NaOH distillation to ammonia and ammonia is captured into boric acid as ammonium ions. Thirdly, the ammonia in the distilled sample is quantified by HCl titration. Finally, the calculation of ammonia in the distillate estimates crude protein content by multiplying the total amount of nitrogen by a NPCF. Though highly destructive and time-consuming, the Kjeldahl method has been extensively employed for seaweed protein measurement (Bjarnadóttir *et al.*, 2018; Dawczynski *et al.*, 2007; Kazir *et al.*, 2019; Marsham *et al.*, 2007; Muraguri *et al.*, 2016; Smith *et al.*, 2010; Vásquez *et al.*, 2019; Vieira *et al.*, 2018; Yaich *et al.*, 2011). Protein quantification through the Kjeldahl method could be calculated with the following:

$$Nitrogen\ (\%) = \frac{Conversion\ factor\ \times\ 14\ \times\ Corrected\ HCl}{Mass\ of\ sample} \times Normality\ of\ HCl\ \times\ 100\% \qquad \text{Eq. 1}$$

where conversion factor is the nitrogen-to-protein conversion factor; 14 is the molecular weight of nitrogen; corrected HCl = amount of HCl utilised during sample titration – amount of HCl utilised during blank titration; normality of HCl = HCl molar concentration (M)/1000 mL.

The official NPCF for measuring food proteins has been established to be 6.25 (Kjeldahl, 1883). This traditional NPCF hypothesises that dietary carbohydrates and fats have no nitrogen, that nearly all nitrogen in food proteins is protein-bound, and that the nitrogen content in food protein constitutes 16% (Conde et al., 2013; Maeda et al., 2005). However, the factor of 6.25 does not measure true protein content, and 6.25 has been evident for overestimating protein content in algae (Conde et al., 2013). Algae consist of higher amounts of other nitrogenous compounds, such as inorganic nitrogen (pigments, nucleic substances, choline, creatine), free AA and non-proteinaceous nitrogen (ammonia, nitrate, nitrile), which proposes a lower amount of protein-associated nitrogen (Conde et al., 2013). To ameliorate the precision of protein content calculation, adjustment of these variations should be considered, and species-specific NPCF have been explored.

Regarding seaweeds, NPCF less than 6.25, calculated from AA composition and total nitrogen content, have been identified to better represent seaweed protein content (Angell *et al.*, 2016; Biancarosa *et al.*, 2017; Lourenço *et al.*, 2002). For example, Biancarosa *et al.* (2017) showcased that the NPCF for 21 Norwegian seaweeds ranged between 3.53 to 5.13; mean brown, green, and red seaweed NPCFs were 4.17, 4.24 and 3.99, respectively. Furthermore, Lourenço *et al.* (2002) demonstrated an overall NPCF of 4.29 for 19 tropical seaweeds. Likewise to Biancarosa *et al.* (2017), mean brown (5.38) and green (5.13) seaweed NPCFs were higher than the one for red seaweed (4.59). Finally, a meta-analysis by Angell *et al.* (2016) elucidated that the traditional 6.25 factor overestimates protein content in seaweeds by 43%; hence, a universal seaweed NPCF of 5.00 was determined after accounting for the variation among 103 species across 44 studies. Unlike results from Biancarosa *et al.* (2017) and Lourenço *et al.* (2002), Angell *et al.* (2016) reported red seaweeds with the highest mean NPCF of 5.10, compared to brown and green seaweeds (4.59 and 4.49, respectively). Despite mean and median NPCFs being similar, Angell *et al.* (2016) favoured the usage of medians over means as median were less susceptible to outliers and skewed data during the determination of seaweed NPCFs. As NPCFs vary between literature and different foods, a standard protocol

for calculating NPCFs should be investigated, and the collection of NPCFs could be shared through an online library could be established for public use.

1.2.4.2 Bradford method

The Bradford method is one of two essential spectrophotometric analyses for protein content. Bradford (1976) recommended utilising a Coomassie Brilliant Blue (CBB) G-250 dye to estimate protein concentration, with protein content measured at an absorbance from 465 nm to 595 nm. The CBB dye could be directly added to the food sample, and it strongly binds to the proteins present (Kruger, 2009). Moreover, through the comparison with a BSA standard curve, total protein could be estimated (Conde *et al.*, 2013). Compared to the Lowry method, the Bradford method is simpler, faster and more sensitive; however, the Bradford method suffers from considerable variation between different proteins, and the sample's chemical interaction with the CBB dye often underestimates protein content (Kruger, 2009). Nonetheless, the high sensitivity of the Bradford method makes it more susceptible to interfering substances; hence, a sample free of impurities would be required (Mæhre *et al.*, 2018).

1.2.4.3 Lowry method

The Bradford method is the other crucial spectrophotometric analysis for protein content. Lowry *et al.* (1951) recommended utilising a Folin phenol reagent to estimate protein concentrations, with protein content measured at an absorbance of 750 nm. The Lowry method involves two main reactions: firstly, reduced copper ions under alkaline reactions bind to peptide bonds between AA, leading to the oxidation of aromatic AA (generally tryptophan, tyrosine and cysteine); secondly, the Folin phenol reagent is reduced by the copper-peptide bond complex and produces an intense blue colour depending on aromatic AA content (Waterborg, 2009). Furthermore, a standard curve with known bovine serum albumin (BSA) concentrations is often employed as a comparative estimate of total protein (Conde *et al.*, 2013). The Lowry method is a sensitive approach that occurs at room temperature (Waterborg, 2009). However, this method would be susceptible to interfering substances; hence a purified sample would be necessary (Mæhre *et al.*, 2018). Unlike the Kjeldahl method, the Lowry method is less common for determining protein content in seaweeds (Harrysson *et al.*, 2018; Kazir *et al.*, 2019; Vásquez *et al.*, 2019). Inaccurate protein content has been measured by using the Lowry method. Comparing protein measurement by the Lowry method and total AA, Harrysson *et al.*

(2018) and Mæhre *et al.* (2018) demonstrated that seaweed protein was overestimated when using the Lowry method.

1.2.4.4 Liquid chromatography

Chromatography is an analysis technique that enables the separation of a mixture. This technique physically segregates molecular compounds of interest (analytes) from the matrix (other components) within samples (Moldoveanu & David, 2013a). During chromatography, the sample is introduced into the mobile phase that progresses across the stationary phase (Moldoveanu & David, 2013a). Chromatography with gases as its mobile phase is gas chromatography, whereas chromatography with liquids as its mobile phase is liquid chromatography (LC).

High-performance LC (HPLC) is a premier type of LC utilised for identifying and quantifying chemical compounds in foods. In protein analysis, analytes are proteins in the food sample, and matrices are the components other than proteins. Prior to chromatography, necessary sample modifications occur to ensure that samples are modulated according to the chromatography conditions (Moldoveanu & David, 2013a). Firstly, protein samples (often ranging from 5 µL to 10 µL) are introduced into a flowing liquid (mobile phase) at the start of the column (Bondu *et al.*, 2015; Harrysson *et al.*, 2018; Kazir *et al.*, 2019). The mobile phase carries proteins above a column (stationary phase). Though proteins are generally polar, the slight differences in their polarity should be exploited when selecting a suitable column. Regular non-polar hydrocarbon columns for protein separation are C8 and C18, and separating proteins that are more non-polar could utilise C1 to C3 columns as non-polar proteins could adhere too strongly to C8 and C18 columns (Moldoveanu & David, 2013b). Passing proteins are retained by the column depending on their interaction strength and are subsequently released at specific retention times. Protein-stationary phase interaction occurs in an orientation-specific manner, and their retention time is dependent upon their molecular composition (Aguilar, 2004a).

Eluted proteins exit the column and enter the detection site. Proteins could be differentiated by their unique physicochemical properties, such as UV-absorption, refractive index, fluorescence, molecular mass and fragmentation (Aguilar, 2004a). For a typical chromatography analysis, a mass spectrometer (MS) is the commonly associated detection site (LC-MS). Electronic signals

produced upon protein detection in the mass spectrometer are illustrated as various peaks on a chromatogram.

Chromatographic separation for seaweed protein analysis could be based upon compound polarity, ionic charge and molecular mass. Reversed-phase HPLC (RP-HPLC) differentiates compounds based on their hydrophobic interactions with the stationary phase. RP-HPLC utilises polar mobile phases and non-polar stationary phases (Moldoveanu & David, 2013a). Advantages of RP-HPLC include significant compound differentiation, high recoveries and productivity, and exceptional reproducibility, but the operating conditions of RP-HPLC could denature proteins (Aguilar, 2004b). RP-HPLC has been utilised for analysing seaweed AA (Ortiz *et al.*, 2009; Vieira *et al.*, 2018), and bioactive peptides (Beaulieu *et al.*, 2016; Cermeño *et al.*, 2020; Cian *et al.*, 2012).

Ion-exchange chromatography (IE-HPLC) segregates proteins based on positive and negative charges. IE-HPLC utilises specific ion-exchange stationary phases, whereby compounds are retained and eluted through electrostatic interactions at different pH (Moldoveanu & David, 2013b). Advantages of IE-HPLC include short running time, high recoveries and non-denaturing to proteins; yet samples need to have a controlled pH and low ionic strength (Stanton, 2004b). IE-HPLC with Na^+-column has been employed to analyse the AA profile of seaweeds (Bjarnadóttir *et al.*, 2018; Kadam *et al.*, 2017).

Size-exclusion chromatography (SE-HPLC) isolates proteins based on their molecular sizes and weights. The advantages of SE-HPLC are being inexpensive and having non-denaturing operating conditions for proteins, but the disadvantages contain low resolution and low recovery (Stanton, 2004a). SE-HPLC has been utilised to discover molecular weights of proteins in *A. nodosum*, which were revealed to be 2 to 4 kDa (Kadam *et al.*, 2017). Bondu *et al.* (2015) and Mao *et al.* (2017) also employed SE-HPLC to distinguish groups of bioactive peptides in seaweeds.

1.2.4.5 Gas chromatography

Likewise to LC-MS, gas chromatography-MS (GC-MS) is a widespread coupled detection technique utilised for analysing the chemical composition in foods. This technique is more suitable for examining with low polarity and low boiling point, and is frequently employed to

analyse AA (Xue *et al.*, 2015). Firstly, the sample is injected into the column (stationary phase) under split or splitless modes; split injection is often employed for samples with high concentrations, and only a portion of the sample is allowed into the column (Xue *et al.*, 2015). After injection into the column, the sample is completely vapourised at the injection port and enters the volume via a carrier gas (mobile phase). Unlike liquid mobile phases in LC, GC employs a gaseous mobile phase, commonly a stable supply of pure helium. Flow rate and quality of the carrier gas are essential factors that influence the separation of compounds (Xue *et al.*, 2015). Subsequently, the column retains compounds in the passing carrier gas depending on their interaction strength and is later fed into MS for detection.

MS is controlled by computer software, and its general principle is as follows (Xue *et al.*, 2015). MS detects compounds in their ionic states. Compounds encounter an ionisation source upon leaving the GC, and through electron ionisation, molecular ions of compounds are consequently produced. Molecular ions enter the mass analyser and are fragmented according to their mass-to-change ratio. Fragmented molecular ions arrive at the electron multiplier, and through the formation of electrical signals, three-dimension information for compounds of interest is produced. Lastly, proportional abundances of molecular ion fragments are graphed, creating a mass spectrum with various peaks that directly deliver structural information of compounds.

The final chromatogram illustrates various peaks with unique retention times, which are crucial parameters for quantifying and identifying unknown compounds. Peaks have varying areas, which would be a predominant feature for quantifying the compound as the relative percentage of the total peak area (Moldoveanu & David, 2013c). However, peak areas derived from MS do not express actual compound content, as different extraction procedures and detection instruments have varying isolation properties. More accurate quantification of proteins could be achieved through isotopic labelling (Ghosh, 2016). Appropriate AA standards could also quantify proteins (Bjarnadóttir *et al.*, 2018; Bondu *et al.*, 2015; Harrysson *et al.*, 2018; Mao *et al.*, 2017).

Each peak is associated with its compound-specific retention time, another predominant feature provided by chromatograms. Retention time describes the duration for a compound to elute from the column, and it is hugely dependent upon the compound structure, nature of mobile and stationary phases, and column parameters (Moldoveanu & David, 2013c). This retention time is the main parameter for identifying unknown compounds, as retention time for specific

compounds does not differ upon consistent operating conditions. For protein identification, the addition of appropriate AA standards would reveal the identity of unknown compounds (Bjarnadóttir *et al.*, 2018; Bondu *et al.*, 2015; Harrysson *et al.*, 2018; Mao *et al.*, 2017). Another way to identify proteins is by comparing various online database search programs; examples of databases include SEQUEST, Mascot, Phenyx and X!Tandam (Kannan *et al.*, 2012). Nevertheless, contaminants, such as column bleeds, plasticisers, phthalates, various silicon compounds, carbon dioxides and water, should be disregarded when analysing the chromatogram (Xue *et al.*, 2015).

Characterisation of proteins and AA could be conducted through various techniques, namely Kjeldahl, Bradford and Lowry methods, as well as LC-MS and GC-MS. The Kjeldahl, Bradford and Lowry methods are predominantly employed to quantify protein amount, whereas LC-MS and GC-MS identify and quantify AA residues within the isolated protein extract. As mentioned above, each technique and protocol have associated advantages and disadvantages. Additionally, no characterisation technique would be able to measure absolute results. Hence, analysing protein content through several methods would be necessary for acquiring precise results.

1.2.5 Physicochemical properties of plant proteins

1.2.5.1 Amino acid composition

AAs are organic molecules composed of an acidic carboxyl group, a basic amino group and a unique functional group. As mentioned in Table 3, AAs have an MW ranging between 75 Da to 204 Da; they could be recognised as acidic, basic, charged, uncharged, non-polar, polar, aliphatic, and aromatic (Ustunol, 2015). Such classifications are determined by the nature of their functional groups. For example, the pKa of histidine's functional group is similar to neutrality, which enables it to be readily ionisable (positively charged or uncharged) at pH 7; the functional group of methionine contains sulphur, making it slightly non-polar at pH 7; the aromatic functional group in phenylalanine contributes to its hydrophobicity (non-polarity), whereas the hydroxyl group in threonine enhances its polarity (Nelson & Cox, 2013).

The seaweed AA composition constituents a major proportion of acidic AAs (aspartic acid, glutamic acid); alanine and glycine are also predominant AAs in seaweeds (Bjarnadóttir *et al.*, 2018; Cian *et al.*, 2012; Fleurence, 1999; Hardouin *et al.*, 2016; Lourenço *et al.*, 2002; Mæhre *et al.*, 2016; Naseri *et al.*, 2020b). On the other hand, the limiting AA across all seaweeds would be tryptophan, which is expected to be approximately 0.3% to 3% in seaweeds (Fleurence, 1999). Other limiting AAs would be sulphur-containing AAs, such as methionine and cysteine (Vieira *et al.*, 2018). Regardless of this, EAAs in seaweeds could reach over a significant 40% of total AAs with the appropriate protein extraction procedures (Bjarnadóttir *et al.*, 2018; Naseri *et al.*, 2020b; Vieira *et al.*, 2018). Mæhre *et al.* (2016) emphasised that EAAs in both raw and seaweed proteins following enzyme-assisted extraction are equal to or higher than the defined amount by WHO/FAO/UNU (2007).

AA composition delivers vital information for evaluating protein quality. Moreover, AA amounts could be compared to dietary requirements. Seaweed protein quality can be determined in several ways. The essential AA index (EAAI) compares the geometric mean EAA value to a high-quality reference protein (Conde *et al.*, 2013; Mišurcová *et al.*, 2014; Vieira *et al.*, 2018). The AA score (AAS) relates the actual abundance of EAA to FAO and WHO dietary requirements or to a reference protein (Mæhre *et al.*, 2016; Mišurcová *et al.*, 2014; Vieira *et al.*, 2018). The essential AA-AA ratio (EAA:AA) is the ratio between the total EAA and the sum of all AA (Naseri *et al.*, 2020b). Finally, the essential AA-non-essential AA ratio (EAA:NEAA) is the ratio between the total EAA and the sum of all non-EAA

(Dawczynski *et al.*, 2007). The analysis of protein quality through examining the AA profile would allow further insight into the dietary value of seaweed proteins.

Seaweed AA composition analysis has been frequently performed through HPLC (Bjarnadóttir *et al.*, 2018; Kadam *et al.*, 2017; Mæhre *et al.*, 2016; Ortiz *et al.*, 2009; Vieira *et al.*, 2018). Nevertheless, seaweed protein samples are hydrolysed under strongly acidic conditions prior to HPLC analysis (Bjarnadóttir *et al.*, 2018; Bleakley & Hayes, 2017; Bondu *et al.*, 2015; Harrysson *et al.*, 2018; Kadam *et al.*, 2017; Kazir *et al.*, 2019). A disadvantage of strong acidic hydrolysis would be that it destroys sensitive tryptophan and sometimes cysteine, preventing them from being quantified during HPLC (Conde *et al.*, 2013). Other AAs, such as cysteine and methionine, are also labile during this procedure (Lourenço *et al.*, 2002). The evaluation of the AA composition of seaweeds provides ways to supplement daily diets with seaweeds to ensure a rounded intake of essential nutrients.

1.2.5.2 Functional group

The uniqueness of AA arises from its functional groups, also known as AA side-chains and R-groups (Ustunol, 2015). Protein functional groups are covalently bound to the central carbon, determining the distinctive molecular properties of each AA, such as their overall ionic charges and polarities. Functional groups characterise AA and the resulting protein, giving rise to their unique physicochemical and functional properties, such as chemical reactivity and structure, denaturation, foaming and emulsifying capacities, and solubility. For instance, proteins with a large number of non-polar AAs could increase their surface hydrophobicity, which results in higher emulsifying and foaming abilities (Zayas, 1997a, 1997b). Nonetheless, compared to other AAs, the protonated aspartic acid and glutamic acid contribute more favourably to protein solubility at a high protein net charge (Trevino *et al.*, 2007).

For the molecular investigation of functional groups, protein samples could be subjected to Fourier-transform (FT) infrared (IR) spectroscopy. FTIR examines protein structure through the emission and absorption of light across the mid-infrared region. Protein bonds absorb emitted light at different wavelengths, and such information confers their molecular structure. Frequency is measured as wave numbers ranging from 4000 cm^{-1} to 400 cm^{-1} (Mutharasappan *et al.*, 2020). FTIR would also give insight into chemical bonds within functional groups, protein complexes and protein secondary structures (Mutharasappan *et al.*, 2020). FTIR is a

fast and flexible approach to understanding the molecular structure of proteins. However, samples subjected to FTIR would need prior purification, as spectroscopy results are often affected by interfering substances (Mæhre *et al.*, 2018).

1.2.5.3 Secondary structure

The secondary structure of proteins could be considered as the initial folding of proteins. The onset of the secondary structure is influential for subsequent stages of protein folding. Proteins begin as primary structures, whereby neighbouring AAs within the polypeptide chain interact via peptide bonds. Transitioning from the primary structure to the secondary structure is characterised by flexible degrees of freedom with AA, whereby the linear chain transforms into coil-like α-helices and plane-like β-pleated sheets (Ustunol, 2015). With that in consideration, exploring molecular interactions within α-helices and β-pleated sheets would enhance the understanding of protein secondary structure.

The secondary structure of proteins could be investigated in the same way as AA functional groups. FTIR provides a high-quality spectrum for accurately capturing essential interactions within proteins. Influential bands that provide protein structural information include Amide A and B bands for N-H stretch vibrations (3100 cm^{-1} and 3300 cm^{-1}, respectively); Amide I band for C=O stretch vibrations (1600 cm^{-1} to 1700 cm^{-1}); Amide II and III bands for C-N stretching and N-H bending vibrations (1480 cm^{-1} to 1575 cm^{-1}, and 1229 cm^{-1} to 1301 cm^{-1} respectively) (Carbonaro & Nucara, 2010).

Vásquez *et al.* (2019) investigated the FTIR spectrum of seaweed proteins following enzyme-assisted extraction. Protein extract characterisation was conducted in the mid-infrared mode with attenuated total reflection (ATR). N-H stretch vibrations at bands 3281 cm^{-1} and 3274 cm^{-1} were suggested to be the protein polypeptide skeleton; C=O stretch vibrations at bands 1637 cm^{-1} and 1544 cm^{-1} could correspond to peptide bonds of proteins; S=O stretch vibrations at bands 1220 cm^{-1} and 1243 cm^{-1} could propose that polysaccharides, such as fucoidan and carrageenan, co-precipitated with proteins. Vásquez *et al.* (2019) indicated that the extraction of polysaccharides could be co-conducted during enzyme-assisted extraction. Similar findings were reported by Waglay *et al.* (2019), whereby C=O stretch vibrations relating to peptide bonds for potato protein extracts were observed between 1600 cm^{-1} to 1700 cm^{-1}. Nevertheless, secondary structures of potato protein extracts analysed with FTIR demonstrated different

changes depending on the extraction procedure; this illustrates that parameter changes during protein extraction would affect the secondary structure (Waglay *et al.*, 2019).

Another technique for monitoring protein secondary structure is circular dichroism (CD). CD is a non-destructive and highly accurate spectroscopy technique based on proteins' differential absorption of left and right circularly polarised light (Mutharasappan *et al.*, 2020). CD specialises in monitoring the dynamics of protein transformations when they participate in processes such as protein folding and protein binding (Rogers *et al.*, 2019). Unlike FTIR, CD could evaluate both secondary and tertiary protein structures. The secondary structure of proteins would be examined in the far-UV CD spectrum, and tertiary structures are examined in the near-UV spectrum (Rogers *et al.*, 2019). For almond milk proteins, a signal at 208 nm is typical for α-helices, and at 216 nm for β-sheets (Devnani *et al.*, 2020). Regarding hemp proteins, the CD spectrum demonstrated that their secondary structure is dominated by β-sheets, followed by α-helices (Shen *et al.*, 2020). Nevertheless, CD illustrated that the unordered structure of secondary seed proteins became more compact and ordered after enzyme-assisted extraction (Jiang *et al.*, 2021). CD also revealed that the cavitation mechanism of ultrasound-assisted extraction leads to partial pea protein unfolding, as microbubble implosion disturbs protein bonds (Wang *et al.*, 2020a). Despite the advances, CD could be expensive, and it lacks the conveyance of site-specific protein information (Mutharasappan *et al.*, 2020).

1.2.5.4 Denaturation temperature

The onset of protein unfolding occurs when environmental conditions are undesirable for protein stability. Temperature influences the stability of protein structures. Additionally, denaturation temperature measures protein thermal stability, essential for protein functionality. Intermolecular and intramolecular interactions within proteins collapse at high temperatures, leading to irreversible conformational changes such as aggregation and precipitation (Zayas, 1997e). However, aspects of protein structure could increase their resistance to thermal denaturation. For example, β-pleated sheets have better structural stability than α-helices; hence proteins with a more significant proportion of β-pleated sheets have higher denaturation temperatures than proteins with fewer β-pleated sheets (Ustunol, 2015). Nonetheless, the heat stability of proteins would be also influenced by their polarity; proteins with greater non-polar residues have increased heat stability than proteins with fewer non-polar residues (Bigelow, 1967). Particle size has also been argued to impact protein denaturation. Smaller proteins have

lower thermodynamic parameters, resulting in ascended stability at high temperatures (Chakravarty & Varadarajan, 2000). Though protein denaturation would be accompanied by structural transformations from the native structure, the AA sequence is unaltered (Zhong & Sun, 2000).

To determine the precise onset of protein denaturation, examining protein structure at various temperatures could be performed. At the denaturation temperature of proteins, a certain amount of heat energy is required to ignite the denaturation process, often displayed as a single endotherm peak in thermograms (Das *et al.*, 2021). Spectroscopy and calorimetric techniques could be employed for further insight into protein structure at denaturation. Devnani *et al.* (2020) highlighted that almond milk protein secondary structure changes at heat treatments above 75 °C with far-UV CD; α-helical structure detected by CD increased significantly at higher temperatures, indicating a decrease in α-helix content within proteins, which would be consistent with protein denaturation. Waglay *et al.* (2019) noticed the collapse of α-helix and β-sheet regions occurred above 55 °C in potato protein extracts, supported by a drop in peak separation around 1650 cm^{-1} and 1630 cm^{-1} in the FTIR spectrum. Kaur and Singh (2007) investigated the thermal denaturation of different chickpea proteins with differential scanning calorimetry (DSC); chickpea cultivars exhibited significantly different peak denaturation temperatures. Tang *et al.* (2006) examined the thermal denaturation of hemp proteins via DSC, which happened at 95 °C shown with one endothermic peak. Investigating protein denaturation temperature would be vital for several processes, such as preventing overheating during protein extraction and limiting overheating during protein incorporation into other food systems.

1.2.5.5 Particle size distribution

Protein particles could be assemblies of small oligomers at the nanometre level or visible aggregates at the micrometre level (Ripple & Dimitrova, 2012). Particle size (PS) is a critical physical property of proteins as it impacts protein functional properties, namely gelling, emulsifying and foaming (Nicolai & Durand, 2013). Typically, protein PS is expressed as the apparent mean particle diameter, either as the volume-weighted or surface-weighted distribution (Das *et al.*, 2021; Sun *et al.*, 2020). Protein PS analysis could be conducted using laser diffraction technologies and different types of scanning microscopy (Das *et al.*, 2021; Devnani *et al.*, 2020; Preece *et al.*, 2017a; Sun *et al.*, 2020; Wang *et al.*, 2020a). However, the

detector's dynamic range could hinder the analysis of PS, and such procedures require prior purification due to poor selectivity (Ripple & Dimitrova, 2012).

An essential factor that influences protein PS is temperature. Temperature affects protein structure, which contributes to protein PS. Devnani *et al.* (2020) analysed PS with a Mastersizer and noticed that temperatures below 45 °C had no significant impact on almond milk protein PS, whereas high temperatures above 75 °C exhibited increased PS. Devnani *et al.* (2020) revealed that temperatures below 45 °C graphed one protein distribution peak at 0.1 to 1 µm. Moderate temperatures ranging from 55 °C to 75 °C graphed two protein distribution peaks, one at 0.1 to 1 µm, and another at 10 to 100 µm; thus indicating the presence of protein aggregation. Finally, high temperatures above 75 °C graphed more prominent aggregation peaks at 10 to 100 µm. Such observations are evident in that the temperature increase promotes protein aggregation, yielding larger protein PS.

pH is another vital factor that affects protein PS. As AA residues constitute various ionic charges, pH shifts could contribute to the neutralisation and ionisation of proteins, altering protein structure and PS. Larger PS from aggregation and precipitation of proteins occur at their isoelectric point, whereby negative and positive charges are balanced, with lower repulsive electrostatic and higher attractive forces (Novák & Havlíček, 2016). Generally, protein PS would be smaller at higher pH due to having enhanced solubility (Das *et al.*, 2021).

Physical extraction of proteins could also impact protein PS. The forces of cavitation during ultrasound treatment reduced the PS of proteins (Preece *et al.*, 2017a; Sun *et al.*, 2020; Wang *et al.*, 2020a). When comparing liquid extraction, MAE, ultrasound-assisted and enzyme-assisted extractions of seed protein isolates, enzyme-assisted extraction obtained the lowest protein PS; the increased PS from MAE and ultrasound treatment could be related to protein aggregation during cellular disruption (Jiang *et al.*, 2021).

1.2.5.6 Molecular weight distribution

Proteins are constructed from AAs; hence, the MW of proteins corresponds to all AAs' total weight. MW of proteins is a physical property that reflects protein structural information, ranging from under 5 kDa to thousands of kDa (Mutharasappan *et al.*, 2020). In this case, MW of various proteins within a sample could be identified via sodium dodecyl sulphate-

polyacrylamide gel electrophoresis (SDS-PAGE) (Devnani *et al.*, 2020; Phongthai *et al.*, 2016; Wang *et al.*, 2020a). MW of proteins also characterise proteins into groups; for instance, maise proteins could be classed into low MW zein proteins and high MW glutelin protein fractions (Appell *et al.*, 2018). To understand proteins at distinguished MW, SE-HPLC could be employed (Bondu *et al.*, 2015; Cian *et al.*, 2012; Kadam *et al.*, 2015; Mao *et al.*, 2017; Sun *et al.*, 2020).

MW would be related to PS, and likewise, temperature and pH are influential factors that impact molecular weight. Comparing protein MW at different parameters would provide enhanced insight into their molecular changes. For instance, aggregation of proteins at high temperatures could lead to different molecular weights. Moreover, the dissociation of proteins at acidic and alkaline pHs would result in smaller molecular weights. Kadam *et al.* (2017) reinforced that highly acidic and alkaline extractions would initiate seaweed protein hydrolysis, resulting in smaller peptides with lower MWs.

1.2.6 Functional properties of plant proteins

1.2.6.1 Solubility

The dissolution ability of proteins in an aqueous solvent could be defined as protein solubility (Tan *et al.*, 2021). This hydration property arises from the protein's intrinsic AA composition, MW and sequence; it is predominantly controlled by the polarity of AA functional groups (Appell *et al.*, 2018; Bigelow, 1967; Mutharasappan *et al.*, 2020). Extrinsic factors such as pH, temperature, ionic strength, and extraction conditions also affect protein solubility (Zayas, 1997e). Nevertheless, protein solubility is the prerequisite consideration for other protein functionalities, such as gelling, emulsifying, foaming, and water and oil holding capacities. For proteins to exhibit enhanced emulsifying stability and foaming capacity, solubility is the foremost requirement as it improves protein flexibility and hydrophobicity (Feyzi *et al.*, 2018; Zayas, 1997b). Protein solubility could be expressed as a percentage at different conditions (pH, temperature) and is calculated with the following:

$$Solubility\ (\%) = \frac{Protein\ content\ in\ supernatant}{Total\ protein\ content} \times 100\% \qquad \text{Eq. 2}$$

Protein solubility is often investigated across a pH range, as this condition relates to the subsequent formation of gels, emulsions and foams. The solubilisation of proteins is closely associated with protein isoelectric point. Proteins have no overall net charge at isoelectric point, leading to the domination of attractive intermolecular protein-protein forces, generally resulting in minimal protein solubility (Farkye & Shah, 2015; Tan *et al.*, 2021). However, at pH higher than isoelectric point, proteins have an overall negative net charge due to ionisation of hydroxyl groups, leading to greater repulsive electrostatic forces, resulting in augmented solubilisation (Das *et al.*, 2021; Phongthai *et al.*, 2016; Zayas, 1997e). Protein solubilisation could also occur at a pH lower than isoelectric point, but protein solubility is enhanced under more alkaline conditions for seaweed proteins (Das *et al.*, 2021; Harrysson *et al.*, 2018; Jiang *et al.*, 2021). PS reduction due to newly exposed ionisable AAs and carboxylic groups are observed at alkaline pHs, which would consequently decrease protein hydrophobicity and increases protein solubility (Tavano, 2013). Harrysson *et al.* (2018) reported that protein solubility was best at pH 12 (50% to 60%) for several Swedish seaweeds. Felix *et al.* (2021) observed that solubility was the greatest at alkaline pHs for red *Porphyra dioica*, namely pHs 8, 9 and 10 (40% to 43%). Garcia-Vaquero *et al.* (2017) illustrated that the lowest and highest solubilities were observed

at pH 4 (22.5%) and pH 12 (96.15%), respectively, in brown *Himanthalia elongate*. Nonetheless, alkaline conditions are often incorporated into seaweed protein extraction due to the enhanced protein solubility at alkaline pH (Fleurence *et al.*, 1995a; Harrysson *et al.*, 2018; Kadam *et al.*, 2017; Naseri *et al.*, 2020b).

As proteins undergo irreversible conformational changes at high temperatures, extensive thermal treatment would significantly reduce protein solubility (Zayas, 1997e). However, appropriate thermal treatment would accelerate protein extraction and prevent microbial contamination and growth (Tavano, 2013). Moreover, ion presence also influences protein solubility. Calcium chloride ($CaCl_2$) and sodium chloride (NaCl) are often exploited for protein precipitation; negative chloride ions bind to positive AAs and augment intermolecular repulsion, lowering protein solubility and resulting in precipitation (Tan *et al.*, 2021; Zayas, 1997e).

Protein solubility could be affected by extraction conditions, but the findings from other research could be contradicting. For instance, Jiang *et al.* (2021) reported that ultrasound treatment, shear emulsification and microwave-assisted enzymatic extraction did not significantly alter seed protein surface charges, which had little impact on protein solubility. However, ultrasound-assisted extraction improved pea and soy protein solubilities, as protein bonds are sabotaged through cavitation (Preece *et al.*, 2017a; Wang *et al.*, 2020a). Such differences in protein behaviour could be specific to types of proteins and species-specific.

1.2.6.2 Water/oil holding capacity

The ability for proteins to retain water when force is applied could be distinguished as proteins' water holding capacity (WHC). This hydration property of proteins relies on interactions between protein and water, which also influences other functional properties of food proteins, namely emulsifying and foaming, gelling, solubility, swelling, syneresis, viscosity, water binding and retention (Appell *et al.*, 2018; Zayas, 1997f). Oil holding capacity (OHC) is a binding property of proteins that exhibits a similar phenomenon; instead, oil entrapment occurs, and hence protein-oil interactions are assessed (Appell *et al.*, 2018).

Both WHC and OHC directly influence the textural characteristics of foods (Zayas, 1997f). Red *K. alvarezii* demonstrated acceptable WHC (2.22 g water/g protein concentrate) and OHC

(1.29 g oil/g protein). For protein extracts from brown *H. elongate*, WHC and OHC were determined as 10.27 g water/g protein and 8.1 g oil/g protein (Garcia-Vaquero *et al.*, 2017). Both *K. alvarezii* and *H. elongate* displayed acceptable WHC and OHC, and such seaweed proteins could be useful as texture and palatability enhancers in food formulations (Garcia-Vaquero *et al.*, 2017; Suresh Kumar *et al.*, 2014). To analyse WHC and OHC of proteins, water or oil could be added to briefly saturate protein samples (Beuchat, 1977; Phongthai *et al.*, 2016; Wang *et al.*, 2020a). Following incubation at optimised temperature and time, supernatants are removed through low-speed centrifugation. Finally, WHC and OHC of protein samples could be expressed as the amount of water or oil absorbed per gram of protein, and their calculations are as follows:

$$Water/oil\ holding\ capacity = \frac{Protein\ content\ after\ centrifugation\ and\ supernatant\ removal}{Initial\ protein\ content} \qquad \text{Eq. 3}$$

Protein WHC and OHC are both affected by protein concentration and hydrophobicity. Protein WHC could be enhanced with more polar protein groups available for water binding, and in contrast, protein OHC could be improved with more available non-polar protein groups for oil binding (Zayas, 1997d, 1997f). At pHs more acidic than protein isoelectric point, WHC reduces due to protein aggregation and fewer protein-water interactions; but OHC might not be significantly altered (Das *et al.*, 2021). Additionally, protein PS also influence WHC and OHC. For example, Wang *et al.* (2020a) showcased that decreased PS enhanced the WHC of pea proteins, and that the increased exposure of pea protein hydrophobic groups augmented the OHC. However, proteins with PS that are too small would negatively affect WHC and OHC. For instance, Phongthai *et al.* (2016) suggested that small rice bran proteins could lose the ability to capture water and oil in their structure due to lower bulk density. Moreover, protein extraction such as MAE, alkaline, ultrasound-assisted and enzyme-assisted extractions could have varying impacts on the WHC and OHC of proteins (Jiang *et al.*, 2021; Phongthai *et al.*, 2016; Wang *et al.*, 2020a). This is because different extraction procedures have varying conditions that influence protein PS. Hence an optimised extraction method would be necessary to obtain targeted proteins with profound WHC and OHC.

1.2.6.3 Gelling

The formation of three-dimensional networks through balanced protein-protein and protein-water interactions could be described as protein gelling. Gelling is a hydration property of

proteins that enables the stabilisation of emulsions and foams, and it controls the textural characteristics of foods (Appell *et al.*, 2018; Zayas, 1997c). Least gelling concentration is the minimum protein amount required to achieve self-support and no flow upon inverting the protein samples, could be performed to analyse the gelling extent of proteins (Sathe *et al.*, 1982; Wang *et al.*, 2020a).

Intrinsic factors that influence protein gelling are protein MW and AA composition. High MW proteins and more hydrophobic AA residues result in strong gel networks (Zayas, 1997c). Nevertheless, extrinsic factors that affect protein gelling include protein concentration, ionic strength, pH and temperature. A critical protein concentration is necessary for gel formation, whereas an environment with appropriate ionic strength and pH stabilises the gel (Zayas, 1997c). Regarding temperature, protein denaturation at high temperatures could induce extensive gelling (Devnani *et al.*, 2020). Proteins acquired from various extraction methods could possess different gelling properties. For instance, Wang *et al.* (2020a) suggested that ultrasound-assisted extraction increased pea protein hydrophobicity and protein-protein interaction, which resulted in reduced least gelling concentration and improved protein gel formation.

1.2.6.4 Emulsifying

A thermodynamically unstable dispersion of two or more immiscible liquids suspended by emulsifiers is known to be an emulsion (Rawiwan *et al.*, 2022). The emulsifying property of proteins is considered a surface property as proteins form the cohesive film on the exterior of emulsion particles, stabilising the overall emulsion through electrostatic repulsion and steric hindrance (Appell *et al.*, 2018). Generally, emulsions occur between oil-based and water-based solutions, such as butter, chocolate, mayonnaise and salad dressings. The amphiphilic nature of proteins, with the appropriate distribution of polar and non-polar regions, can act as emulsifying agents that “mixes” oil-based and water-based solutions. During emulsion formation, proteins reorientate to position non-polar (hydrophobic) AAs towards the oil phase, and polar (hydrophilic) AAs towards the water phase. Nevertheless, the inherent flexibility of proteins is essential for being excellent emulsifiers; emulsifiers should have little secondary structure and fast unfolding at the oil-water interphase (Appell *et al.*, 2018).

Protein extraction procedures could impact the emulsifying properties of the resulting protein. For instance, proteins from enzyme-assisted extraction exhibited better emulsifying properties, which could be associated with the increased protein disulphide bond content, strengthening the formation of viscoelastic films and resulting in stable emulsions (Jiang *et al.*, 2021). Enzyme-assisted extraction also uncovers concealed hydrophobic groups in proteins, which could augment the hydrophilic-lipophilic balance and further stabilise emulsions (Phongthai *et al.*, 2016). Nonetheless, ultrasound treatment improves protein's molecular flexibility and surface hydrophobicity, resulting in better adsorption at the oil-water interphase (Wang *et al.*, 2020a).

Generally, reduced protein PS would increase protein molecular fluidity and augment its emulsifying properties (Wang *et al.*, 2020a). However, it is essential to consider that small PS could also impair emulsifying properties of proteins as low MW peptides cannot unfold and reorientate like larger molecules (Phongthai *et al.*, 2016). Hence appropriate protein PS would be necessary to enhance emulsifying capacity and stability of proteins.

The emulsion activity (EA) and stability (ES) of proteins could be measured to further comprehend protein emulsions (Chen *et al.*, 2019; Chiu *et al.*, 2009; Jiang *et al.*, 2021; Pearce & Kinsella, 1978). Firstly, protein samples are homogenised with oil for several minutes to form an emulsion. The emulsion would be further mixed with SDS solution for absorbance measurement at 500 nm. The EA index could be estimated with the following:

$$EA\ (\frac{m^2}{g}) = \frac{2\ \times\ 2.303 \times\ A_0 \times D}{C \times \phi \times L \times 10^6} \qquad \text{Eq. 4}$$

where A_0 is absorbance of the protein sample at 500 nm; D is the dilution factor; C is weight of protein per volume; ϕ is the volumetric fraction of oil; L is the pathlength of cuvette.

The ES is an EA comparison measured some time after emulsion formation, generally 30 minutes. ES could be estimated with the following equation:

$$ES\ (\%) = \frac{EA_{0\ minutes}}{EA_{t\ minutes}} \times 100\% \qquad \text{Eq. 5}$$

where $EA_{0\ minutes}$ is absorption measured directly after homogenisation; $EA_{t\ minutes}$ is EA measured sometime after emulsion formation.

Protein concentration, pH, and temperature are influential factors that affect the emulsifying property of proteins. Suresh Kumar *et al.* (2014) suggested that the formation of stable protein emulsions would be related to protein content and the high concentration of hydrophobic AAs in red *K. alvarezii*. pH affects protein solubility and interfacial properties, indirectly impacting EC (Zayas, 1997a). Garcia-Vaquero *et al.* (2017) elucidated that across olive, peanut, rapeseed, sunflower and walnut oils, EA was the lowest at pH 4 and highest at pH 8 and pH 10 for brown *H. elongata*; such phenomenon could be linked to the hydrophilic-lipophilic balance of proteins at different pHs. Generally, emulsions prepared at pH close to isoelectric point could lead to the flocculation of protein-stabilised droplets, whereas preparations at pH away from isoelectric point repel protein molecules and thus stabilising the emulsion; a more stabilised emulsion could be observed at pH higher than isoelectric point, rather than lower pH (Barac *et al.*, 2010; Mota da Silva *et al.*, 2021; Phongthai *et al.*, 2016). Nevertheless, moderate heating could augment protein ES due to increased solubility, but excessive heating impaired EC due to protein unfolding and the subsequent protein aggregation (Zayas, 1997a).

1.2.6.5 Foaming

Foams could be understood as air-water emulsions, whereby gas is suspended in a liquid. Proteins stabilise foams by reducing the interfacial tension between gas and liquid and improving the elasticity and viscosity of the liquid phase (Zayas, 1997b). The foaming property of proteins is considered to be a surface property. Briefly, proteins diffuse to the gas-liquid interphase. Upon unfolding and re-orientating (polar groups interacting with liquid water and non-polar groups interacting with air), proteins adsorb at this interphase and construct a cohesive film to entrap gas bubbles (Zayas, 1997b).

As the foaming property of proteins is dependent on protein structure, extraction procedures that alter protein structure would influence its foaming properties. For instance, seed proteins derived from enzyme-assisted extraction expressed improved foaming properties than protein extractions via alkaline and acid means (Jiang *et al.*, 2021). Jiang *et al.* (2021) explained that this phenomenon could be associated with the smaller PS and improved hydrophobicity of proteins from enzyme-assisted extraction. Furthermore, smaller PS and improved hydrophobicity of proteins could further augment their absorption rate at the air-water interface, improving air incorporation and enhancing foaming properties (Feyzi *et al.*, 2018). For similar reasons, proteins following ultrasound treatment also display good foaming properties.

Ultrasound-assisted extraction produced partially unfolded proteins with smaller PS, and the increased exposure of hydrophobic AA groups on protein surface-enhanced foam formation (Wang *et al.*, 2020a). However, a very small PS would impair the construction of a continuous intermolecular polymer for entrapping air, thus reducing the elasticity and viscosity necessary for stable foams (Phongthai *et al.*, 2016).

Likewise to protein emulsions, the quality of protein foams could be analysed by measuring foaming capacity and stability. To analyse capacity and stability of protein foams, protein samples are homogenised and vortexed for a couple of minutes to enable aeration, and their volumetric change is immediately observed for measurement (Garcia-Vaquero *et al.*, 2017; Jiang *et al.*, 2021; Suresh Kumar *et al.*, 2014; Wang *et al.*, 2020b).

Foaming capacity (FC) describes the rapid unfolding and reorientation of proteins into a cohesive film around gas bubbles (Chen *et al.*, 2019; Jiang *et al.*, 2021). Nevertheless, FC and ES could be positively correlated (Barac *et al.*, 2010). FC could be estimated with the following:

$$FC\ (\%) = \frac{V_{0\ minutes}}{V} \times 100\% \qquad \text{Eq. 6}$$

where $V_{0\ minutes}$ is the volume of protein sample immediately after homogenisation; V is the volume of protein sample subjected to homogenisation.

Foaming stability (FS) describes the formation of continuous intermolecular polymers for entrapping gas bubbles (Chen *et al.*, 2019; Jiang *et al.*, 2021). FC is the volume of foam remaining after some time of foam formation, generally 30 minutes. FS could be estimated with the following:

$$FS\ (\%) = \frac{V_{t\ minutes}}{V_{0\ minutes}} \times 100\% \qquad \text{Eq. 7}$$

where $V_{t\ minutes}$ is the volume of protein sample some time after homogenisation;$V_{0\ minutes}$ is volume of protein sample immediately after homogenisation.

Protein concentration, pH, and temperature are influential factors that affect the foaming property of proteins. Generally, an increased protein concentration enhances FS as thicker interfacial films are formed, which results in denser and more stable foams (Zayas, 1997b). In other cases, the foaming ability of proteins could be enhanced at pH outside of isoelectric point. This phenomenon coincides with protein solubility, as augmented solubility would be observed

at pH other than isoelectric point, allowing the rapid diffusion of proteins to air-water interphase (Barac et al., 2010; Phongthai et al., 2016). Garcia-Vaquero et al. (2017) noticed that FC was lowest at pH 2 and 4 for brown *H. elongate*, whereas FC was significantly higher at pH 6, 8 and 10; this could be due to higher charges on proteins reduced protein hydrophobic interactions. Garcia-Vaquero et al. (2017) also observed low FS at pHs 2 and 4 for brown *H. elongate*, which was opposite to high FS at pHs 2 and 4 in red *K. alvarezii* observed by Suresh Kumar et al. (2014). As FS is dependent on denatured protein, the degree of denatured proteins could have varied for the two pieces of literature (Garcia-Vaquero et al., 2017). Nonetheless, moderate heating generally improves foaming properties due to diminished surface tension and the slight unfolding to uncover hidden hydrophobic AA resides; but excessive heating leads to coagulation, aggregation, and thus destabilising the foam (Zayas, 1997b). Finally, an important factor is that various plant genotypes have different protein compositions, which consequently influence the functional properties of proteins (Barac *et al.*, 2010).

1.2.7 Antioxidant capacity of plant proteins

Biomolecules could have beneficial or detrimental effects on living matter, and such a phenomenon could be recognised as the molecule's bioactivity. The various biological activities of food proteins have always been of research interest, as food provides a major source of essential nutrients and offers disease prevention properties to the human body (Pangestuti & Kim, 2015). The most common studied bioactive properties of plant protein are ACE-inhibitory activity and antioxidant capacity (Beaulieu *et al.*, 2016; Cian *et al.*, 2015; Cian *et al.*, 2012; Harnedy & FitzGerald, 2013b; Phongthai *et al.*, 2016; Wang *et al.*, 2020a).

ACE-inhibitors are well-established for reducing hypertension, which is the leading risk factor for cardiovascular diseases (Beaulieu *et al.*, 2016; Cian *et al.*, 2015; Cian *et al.*, 2012; Hayakari *et al.*, 1978). Antioxidants are well-recognised for scavenging and neutralising free radicals, protecting living matter and foods from cellular damage by various toxic, reactive oxygen species. The determination of protein antioxidant capacity could be performed through *in vitro* assays, such as 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay, ferric ion reducing antioxidant power (FRAP) assay, and oxygen radical absorbance capacity (ORAC) assay (Beaulieu *et al.*, 2016; Cian *et al.*, 2015; Cian *et al.*, 2012; Harnedy & FitzGerald, 2013b). Such assays involve adding their respective chemical reagents, and Trolox is frequently employed as a standard (antioxidant capacity expressed as Trolox equivalents) (Admassu *et al.*, 2018a). Antioxidant capacity measurements could be performed spectrometrically using automated plate readers (Harnedy & FitzGerald, 2013b). Readings could be performed at approximately 520 nm for DPPH assay, about 590 nm for FRAP assay, and excitation at 485 nm with emission at 538 nm for ORAC assay (Beaulieu *et al.*, 2016; Benzie & Strain, 1996; Brand-Williams *et al.*, 1995; Cao *et al.*, 1993; Harnedy & FitzGerald, 2013b).

1.2.8 Application of plant proteins

The demand for animal proteins is expected to escalate alongside the rapid growth of the population. Alternative protein sources are sought to enhance the sustainment of natural resources and food security. As the bioactivity and functionality of plant proteins are continuously uncovered and augmented, plant proteins serve as a promising alternative to animal proteins. Moreover, the shift in consumer trends towards plant-based foods is evident; hence there are fruitful opportunities in the market to expand and imply more plant proteins (Sutton *et al.*, 2018). Over time, the broader application of plant proteins could reduce the production of animal proteins, which is correlated to many environmental concerns (Steinfeld *et al.*, 2006).

Several plant sources are recognised as complete protein foods, such as soybean, hemp and seaweed mentioned in *Section 1.2.2.3.* Benefiting nutritional properties of plant proteins allow their incorporation into helpful functional foods, value-adding ingredients, pharmaceuticals and nutraceuticals. Furthermore, the functional properties of plant protein could promote them to be various emulsifying, foaming and gelling agents in food manufacturing and processing.

Much of the recent spotlight has been shed upon terrestrial plant proteins, yet the understanding of marine "vegetation", seaweed, is to a lesser extent. Despite seaweed polysaccharides having extensive development in the relevant industries, the exploration of seaweed proteins' properties commenced in the late twentieth century (Baweja *et al.*, 2016). The capability of seaweed proteins should not be disregarded, as they could demonstrate antibacterial, anti-cancer, anti-diabetic, anti-hypertensive, ACE-inhibiting and antioxidant properties (*Section 1.2.2.4*). Besides supplementing human nutrition, incorporating seaweed proteins into animal and aquaculture feeds could elevate the health of many living creatures (Bleakley & Hayes, 2017). Phycobiliproteins from seaweeds could act as natural colourants for food and cosmetic industries, and fluorescent markers for fluorescent application (Sekar & Chandramohan, 2008). Moreover, seaweed proteins demonstrate promising functional properties (Felix *et al.*, 2021; Suresh Kumar *et al.*, 2014). Hence, seaweed proteins hold substantial potential for their applicability in the food, pharmaceutical, and nutraceutical industries.

1.2.9 Concluding remarks

Escalating demand for proteins from the rapid growth of the population urges the search for alternative protein sources. The extraction of plant proteins is a promising approach to ease the current strains on animal protein production and the detrimental environmental impacts associated with animal protein production. The exploration of suitable non-animal proteins has spotlighted seaweeds, as they are one of the other few sources that accommodate complete proteins with all EAAs.

The extraction of seaweed proteins could be accomplished through numerous procedures and techniques as described in *Section 1.2.3*. Current novel extraction techniques, such as MF, MAE, PUF, ultrasound-assisted and enzymatic extraction, are preferred over conventional extraction procedures due to improved energy and time efficiencies, cost and environmental friendliness (Kumar *et al.*, 2021). Nevertheless, a combination of extraction techniques could be employed to enhance protein extraction further. Protein composition and extraction have been analysed and optimised for various seaweed species worldwide, except NZ seaweeds. Due to the variation between seaweed species inhabiting different geographical regions, proteins from local seaweeds could be evaluated. Moreover, the insight into seaweed proteins could possibly diversify NZ's plant protein production and deliver an additional source of premium plant protein foods.

Proteins have numerous functional properties that enable their exploitation in food, cosmetic, pharmaceutical and nutraceutical industries. As the comprehension behind seaweed proteins is still somewhat contemporary, very little literature has explored the functional properties of seaweed proteins. Hence, examining functional properties of extracted seaweed proteins would be necessary for broadening their potential industrial incorporation. For instance, understanding protein properties, such as the ones described in *Sections 1.2.5* and *1.2.6*, would augment industrial protein applications and enhance their economic value.

Ultimately, a deeper insight into the algae protein extraction, seaweed protein composition and functionalities would update current scientific literature regarding plant protein.

Chapter Two

Materials and Methods

2 Materials and methods

2.1 Sample materials

Porphyra umbilicalis, the commercial Korean (KR) seaweed sample, was purchased from Pacific Harvest NZ (Auckland, NZ). A late-season mixture of *Pyropia viridi*dentata and *Pyropia cinnamomea*, the wild seaweed sample, was sourced and harvested near Ōkiwi Bay, New Zealand (NZ). The NZ seaweed sample was kindly provided by Cawthorn Institute (Nelson, NZ). Both KR and NZ seaweed samples were in a dry and milled form upon receipt.

The raw seaweed samples were further downsized to 0.5 to 1 mm particles with a high-performance blender (Total Nutrition Centre, Vita-Mix Corporation, Olmsted Falls, OH, USA). The fine seaweed samples were stored in airtight containers, away from light exposure, and at 23 °C ± 1 (room temperature) until further experiments.

2.2 Enzymes, chemicals and reagents

Novozymes A/S (Bagsværd, Denmark) kindly provided the following enzymes: Alcalase® 2.4 L FG (protease), Celluclast® 1.5 L (cellulase), Pectinex® Ultra SP-L (polygalacturonase), and Shearzyme® 500 L (xylanase).

All chemicals and solvents used during experimental procedures were of analytical grade or higher; these include: acetic acid, acetonitrile, ammonium sulphate, boric acid, copper sulphate, ethanol, isopropanol, iron III, chloride, phenol, potassium sulphate, sodium acetate, sodium chloride and sodium hydroxide (NaOH) from ECP Labchem (Auckland, NZ); acetonitrile and hydrochloric acid (HCl) from JT Baker (Avantor, Radnor, PA, USA); acetone and titanium dioxide from May & Baker Ltd (London, UK); chloroform, hexanes, methanol and sulphuric acid from Macron Fine Chemicals (Avantor, Radnor, PA, USA); formic acid from Emsure (Merck, Kenilworth, NJ, USA); sodium citrate dihydrate and citric acid from Sigma-Aldrich (St. Louis, MO, USA); ammonia formate from Analytical Reagent (The British Drug Houses, Poole, UK).

Other reagents used include: 10x Tris/Glycine/SDS Buffer, 2x Laemmli Sample Buffer, CBB R-250 Staining Solution, CBB R-250 Destaining Solution and Precision Plus Protein Standards from Bio-Rad (Hercules, CA, USA); 2-mercaptoethanol, 2,4,6-Tripyridyl-s-Triazine (TPTZ), Amino Acid Standards Physiological (HPLC standard), Bradford Reagent, 2,2'-Diphenyl-1-picrylhydrazyl (DPPH), SDS ACS Reagent ⩾ 99% and 6-hydroxy-2,5,7,8-

tetramethylchroman-2-car-boxylic acid (Trolox) from Sigma-Aldrich; AlbumiNZ™ Bovine Albumin Low Endotoxin ⩾ 97% from MP Biomedicals NZ (Auckland, NZ).

Pure Soya Oil was purchased from Simply (Goodman Fielder, Sydney, NSW, Australia). Soy protein isolate (SPI) was provided from Myprotein (Manchester, UK). Whey protein isolate (WPI) was provided by Fonterra Co-operative Group (Auckland, New Zealand).

Deionised water and type-1 water, available on the university premise (University of Auckland, Auckland, NZ), were used when necessary.

2.3 Proximate analysis of seaweeds

2.3.1 Moisture content

The moisture content of raw seaweed samples was determined gravimetrically in a drying oven, employing the Association of Official Analytical Chemists (AOAC) 930.15 method with slight modification (AOAC, 2005; Nielsen, 2017a). Seaweed samples of 0.5 g were placed into crucibles, and dried at 105 °C overnight in a Heraeus Function Line Oven (Thermo Fisher Scientific, Waltham, MA, USA). After drying, seaweed samples were cooled and stored in a desiccator before weighing. Triplicate analyses were performed. The difference in sample mass before and after drying was recognised as the moisture content in raw seaweed samples, and was calculated by the following:

$$Moisture\ content\ (\%) = \frac{Mass\ of\ final\ sample\ (g) - mass\ of\ initial\ sample\ (g)}{Mass\ of\ initial\ sample\ (g)} \times 100\% \qquad \text{Eq. 8}$$

2.3.2 Ash content

The crude ash content of raw seaweed samples was determined gravimetrically by incineration in a muffle furnace, employing the AOAC 925.51 method with slight modification (AOAC, 2005; Ismail, 2017). Following overnight drying in an oven as described in *Section 2.3.1*, dried seaweed samples in associated crucibles were incinerated at 525 °C overnight in a KSL-1100X High Temperature Furnace (MTI Corporation, Richmond, CA, USA). After incineration, seaweed samples were cooled and stored in a desiccator before weighing. Triplicate analyses were performed. The ash content of raw seaweed samples was the mass of inorganic matter remaining in the crucible, and was calculated by the following:

$$Ash\ content\ (\%) = \frac{Mass\ of\ final\ incinerated\ sample\ (g)}{Mass\ of\ dried\ seaweed\ sample\ (g)} \times 100\% \qquad \text{Eq. 9}$$

2.3.3 Protein content

The crude protein content of raw seaweed samples was determined through Kjeldahl analysis with automated instrumentation, employing the AOAC 954.01 method with slight modification (AOAC, 2005; Nielsen, 2017b). The digestion unit was a SpeedDigestor K-425 (BUCHI

Corporation, New Castle, DE, UK), which was connected to a BUCHI Scrubber K-415; the distillation unit was a BUCHI Distillation Unit K-350.

Firstly, a known mass of raw seaweed samples, 5 g of potassium sulphate, 0.1 g of mixed titanium dioxide and copper sulphate (1:1), and 20 mL of concentrated sulphuric acid were loaded into digestion tubes and heated until the samples became transparent. After digestion, nitrogenous material from the samples was distilled into 60 mL of 2% boric acid solution (made with type-1 water). Following distillation, colourimetric titration was conducted by adding 0.1 M HCL solution dropwise to distilled samples in boric acid until the first faint grey colour was observed. The volume of HCl titrated for the desired colour change corresponds to the amount of ammonia nitrogen in the samples. Triplicate analyses were performed. With a universal seaweed nitrogen-to-protein conversion factor (NPCF) of 5.00 as proposed by Angell *et al.* (2016), protein content in raw seaweed samples was calculated with the following:

$$Protein\ (\%) = \frac{5 \times 14 \times Corrected\ HCl\ (mL)}{Weight\ of\ sample\ (g)} \times Normality\ of\ HCl \times 100\% \qquad \text{Eq. 10}$$

2.3.4 Lipid content

The crude lipid content of raw seaweed samples was determined through the Folch method (Folch *et al.*, 1957; Gosch *et al.*, 2012; Zhang *et al.*, 2017). Seaweed samples of 0.4 g in 10 mL of chloroform- methanol solution (2:1, v/v) were vortexed before heating at 60 °C for 90 mins. Following heating, samples were passed through 0.2 µm syringe filters, and were then washed with 2 mL 0.9% NaCl solution (20% of the sample's 10 mL volume). Samples were left to stand for phase separation into an upper aqueous layer and a lower chloroform layer containing lipids. After separation, the chloroform layer was collected, and evaporated under a gentle stream of nitrogen to retrieve the total lipids fraction. Triplicate analyses were performed. The lipid content of raw seaweed samples was calculated by the following:

$$Lipid\ content\ (\%) = \frac{Mass\ of\ final\ dried\ lipids\ fraction\ (g)}{Mass\ of\ dried\ seaweed\ sample\ (g)} \times 100\% \qquad \text{Eq. 11}$$

2.3.5 Carbohydrate content

The carbohydrate content of raw seaweed samples was estimated by difference (Ortiz *et al.*, 2009; Smith *et al.*, 2010; Vásquez *et al.*, 2019). The following equation was utilised:

$$Carbohydrate\ content\ (\%) = 100\% - (Moisture\ \% + Crude\ ash\ \% + Crude\ Protein\ \% + Lipid\ \%) \qquad \text{Eq. 12}$$

2.4 Extraction of seaweed proteins

2.4.1 Optimisation of water extraction

Water extraction of seaweed proteins was optimised through single-factor experiments. Parameters influencing seaweed protein extraction efficiency include biomass-water ratio, extraction temperature, and extraction duration (Wijesinghe & Jeon, 2012). In literature, a range of conditions for each parameter have been exploited for seaweed protein extraction (Hardouin *et al.*, 2016; Harnedy & FitzGerald, 2013a; Harrysson *et al.*, 2018; Kadam *et al.*, 2017; Mæhre *et al.*, 2016; Naseri *et al.*, 2020b; Vásquez *et al.*, 2019). Thus, as listed in Table 8, various levels were explored to identify the ideal level for each parameter. The optimisation of water extraction was performed with KR seaweed, and analyses of seaweed protein were performed in duplicate analyses.

Table 8. Experimental parameters for optimising water extraction of seaweed protein.

	Levels
Biomass-water ratio (g raw seaweed:mL water)	1:10, 1:20, 1:30, 1:40 1:50, 1:60, 1:70, 1:80, 1:90, 1:100
Extraction temperature (°C)	23, 40, 50, 60
Extraction duration (hr)	1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 24, 48

n = 2

2.4.1.1 Biomass-water ratio

Seaweed biomass-water ratio was the first parameter to be explored (Table 8). For instance, to achieve a biomass-water ratio of 1:10, 0.8 g of raw seaweed sample and 8 mL of deionised water were used; to achieve a ratio of 1:20, 0.8 g of seaweed and 16 mL of deionised water were used; to achieve a ratio of 1:30, 0.8 g of seaweed and 24 mL of deionised water were used; similar conditions were established for the remaining ratios listed in Table 8. The mixture of seaweed and water at various ratios was placed into centrifuge tubes, magnetically stirred at 700 rpm and at constant extraction temperature and duration. The resulting seaweed-water mixture was centrifuged at 10,000 rpm at 23 °C (room temperature), for 20 mins. Following centrifugation, the supernatant (liquid) was recovered for further tests. The ratio producing the highest protein content would be selected as the most favourable level for this particular parameter.

2.4.1.2 Extraction temperature

Extraction temperature was the second parameter to be explored (Table 8). The biomass-water ratio that yielded the most desirable amount of protein was selected. Despite the changing extraction temperatures, the remaining experimental procedure was conducted in accordance with *Section 2.4.1.1*. For instance, 1 g of raw seaweed sample was hydrated with 40 mL of deionised water (achieving a biomass-water ratio of 1:40). Hydrated samples were magnetically stirred at 700 rpm with constant extraction duration but varying extraction temperatures, namely 23 °C ± 1, 40 °C ± 1, 50 °C ± 1, and 60 °C ± 1. The temperature producing the highest protein content would be selected as the most favourable level for this particular parameter.

2.4.1.3 Extraction duration

Extraction duration was the final parameter to be explored (Table 8). Combining levels that produced the most desirable protein extraction yields from *Sections 2.4.1.1* and *2.4.1.2*, various extraction durations were explored. Despite the changing extraction durations, the remaining experimental procedure was conducted in accordance with *Section 2.4.1.1*. For instance, 1 g of raw seaweed sample was hydrated with 40 mL of deionised water (achieving a biomass-water ratio of 1:40). Hydrated samples were magnetically stirred at 700 rpm with a constant extraction temperature but varying extraction durations, namely 1, 2, 4, 6, 8, 10, 12, 14, 16, 20, 24, and 48 hr. The duration producing the highest protein yield would be selected as the most favourable level for this particular parameter.

2.4.1.4 Quantification of protein

Bradford analysis was employed to quantify the extracted seaweed protein content (Mæhre *et al.*, 2018). Firstly, a bovine serum albumin (BSA) standard with known serial concentrations (1.4, 1.2, 0.8, 0.6. 0.4, 0.3, 0.2 and 0.1 mg/mL) was prepared. Next, 5 µL of BSA standards and samples (supernatant from *Sections 2.4.1.1*, *2.4.1.2* and *2.4.1.3*) were loaded onto a 96-well microplate (Nunc MicroWell 96-Well Microplate, Thermo Fisher Scientific), followed by the addition of 250 µL Bradford reagent into each well. Incubation away from light exposure was performed for 15 mins before the microplate was loaded onto a plate reader (Enspire Multimode Plate Reader, PerkinElmer, Waltham, MA, USA). After shaking for 30 secs, the absorbance of each well was detected at 595 nm. A linear standard curve ($R^2 > 0.99$) was graphed using the serial concentrations of BSA. The seaweed protein concentration of each

sample was calculated in relation to the BSA standard curve and was expressed as mg protein/g dried seaweed. The condition producing the highest protein yield would be selected as the most favourable level for the parameter.

2.4.2 Optimisation of alkaline extraction

2.4.2.1 Orthogonal design

Alkaline solutions enables the solubilisation of seaweed protein, improving seaweed protein extraction (Harnedy & FitzGerald, 2013a; Harrysson *et al.*, 2018; Kadam *et al.*, 2017; Mæhre *et al.*, 2016). Alkaline extraction at pHs up to pH 12 displayed improved protein recovery and extraction yields (Harnedy & FitzGerald, 2013a; Harrysson *et al.*, 2018).

Combining knowledge from previous literature and own findings, this extraction was optimised through an orthogonal design with KR seaweed (Peng *et al.*, 2019; Wang *et al.*, 2021). In this case, four factor-three level orthogonal tests were conducted. The four extraction factors were extraction pH (A), seaweed biomass-water ratio (B), extraction duration (C), and extraction temperature (D). The three levels for factors A, B, C and D were selected upon preliminary experiments. Extraction pH was altered with NaOH (< 1 M). L9 (3^3) tests required for orthogonal design were conducted in accordance with the experimental conditions described in Table 9. Analyses of seaweed protein were performed in triplicate analyses.

Table 9. Orthogonal design L9 (3^3) for optimising alkaline extraction of seaweed proteins.

Sample	A (pH)	B (ratio)	C (hr)	D (°C)
1	1	1	1	1
2	1	2	2	3
3	1	3	3	2
4	2	1	2	2
5	2	2	3	1
6	2	3	1	3
7	3	1	3	3
8	3	2	1	2
9	3	3	2	1

n = 3; factors: A, extraction pH, B, seaweed biomass-water ratio, C, extraction duration, D, extraction temperature.

After seaweed protein extraction according to the conditions in Table 9, the supernatant of each sample was recovered following centrifugation and was subjected to Bradford analysis for

quantifying protein content, particle size analysis for determining particle size and uniformity, and solubility analysis.

2.4.2.2 Quantification of protein

Seaweed protein content (obtained in *Section 2.4.2.1*) was quantified with Bradford analysis, as described in *Section 2.4.1.4.*

2.4.2.3 Analysis of particle size distribution

The PS distribution of seaweed protein (obtained in *Section 2.4.2.1*) was analysed with a laser diffraction unit (Mastersizer 2000, Malvern Panalytical, Malvern, UK) in accordance with methods by Devnani *et al.* (2020) and O'Flynn *et al.* (2021) with slight modifications. Samples were first made to 5% w/v total solid content (300 g sample in 5 mL solution). Following centrifugation at 10,000 rpm in 23 °C for 20 mins, samples were added dropwise into type-1 water in the Mastersizer's sample dispersion unit (Hydro 2000SM, Malvern Panalytical) at 2000 rpm until an obscuration level of > 4%. The PS distribution of samples was measured in manual mode, with a refractive index of 1.45 for proteins (sample), and 1.33 for type-1 water (dispersant) (Johnston *et al.*, 2015). Data acquisition and analysis was executed with Mastersizer 2000 Software (Version 5.61, Malvern Panalytics).

2.4.2.4 Analysis of protein solubility

The solubility of seaweed protein (obtained in *Section 2.4.2.1*) was analysed at pHs 3, 5, 7, 9 and 12 in accordance with methods by O'Flynn *et al.* (2021), Garcia-Vaquero *et al.* (2017) and Suresh Kumar *et al.* (2014) with slight modification. Samples were first made to 5% w/v total solid content (300 g sample in 5 mL solution). Following centrifugation at 10,000 rpm at 23 °C for 20 mins, 1 mL of the supernatant was loaded into crucibles and weighed. Finally, crucibles were placed into a drying oven at 105 °C overnight. After drying, samples were cooled and stored in a desiccator before weighing. Triplicate analyses were performed. The difference in sample mass before and after drying was recognised as the insoluble solids present in the sample, and solubility was calculated by the following:

$$Solubility\ (\%) = \frac{Total\ solutes\ (g)}{Mass\ of\ SPE\ (g)} \times 100\% \qquad \text{Eq. 13}$$

2.4.2.5 Statistical analysis of data

To determine the optimal level within each factor, as well as the most influencing factor (F (2,2) 95% = 19; $p < 0.05$) during alkaline extraction of seaweed proteins, range analysis and one-way ANOVA of data from *Sections 2.4.2.2, 2.4.2.3* and *2.4.2.4* were conducted (Peng *et al.*, 2019; Shen *et al.*, 2008; Wang *et al.*, 2021; Wang *et al.*, 2020b; Xingfei *et al.*, 2020).

2.4.3 Optimisation of enzyme-assisted extraction

Enzymes could effectively sabotage rigid seaweed cell walls, hence enzyme-assisted extraction could ease the ameliorate of seaweed proteins (Bjarnadóttir *et al.*, 2018; Hardouin *et al.*, 2016; Harnedy & FitzGerald, 2013a; Mæhre *et al.*, 2016; Naseri *et al.*, 2020b; Rodrigues *et al.*, 2015; Vásquez *et al.*, 2019). As mentioned in Table 7 (*Section 1.2.3.4* in *Literature Review)*, polysaccharidases and proteases have been exploited for the extraction of seaweed protein, with the extraction timing ranging from 3 to 24 hr. The optimisation was performed with KR seaweed, and analyses seaweed protein content following enzyme-assisted extraction were performed in duplicate analyses.

2.4.3.1 Enzymatic extraction

Four different enzymes, namely Alcalase (protease), Celluclast (cellulase), Pectinex (polygalacturonase), and Shearzyme (xylanase) have been explored for seaweed protein extraction in the literature (Bjarnadóttir *et al.*, 2018; Hardouin *et al.*, 2016; Naseri *et al.*, 2020b; Vásquez *et al.*, 2019). Enzymes at 1% (% enzyme/g raw seaweed biomass), and their incubation with seaweed biomass in deionised water (1:40, w/v) were performed across various extraction durations. For comparison purposes, a control with no enzyme addition was performed for each extraction duration. Seaweed protein extraction was conducted in accordance with the experimental conditions described in Table 10.

Table 10. Experimental conditions for optimising enzymatic extraction of seaweed protein.

Enzyme	**Amount of enzyme[1]**	**pH[2]**	**Extraction temperature (°C)[3]**	**Extraction duration (hr)**
Alcalase	1%	9	23	
Celluclast	1%	5	23	1, 2, 4, 6, 8, 10, 12,
Pectinex	1%	5	23	14, 16, 20, 24, 48
Shearzyme	1%	5	23	

n = 2; [1] % enzyme/g seaweed calculated according to enzyme density provided by the manufacturer, Alcalase is 1.17 g/mL, Celluclast is 1.22 g/mL, Pectinex is 1.17 g/mL, Shearzyme is 1.09 g/mL; [2] extraction pH for each enzyme is defined by the manufacturer; [3] extraction temperature was defined by preliminary experiments, occurring at 23 °C (room temperature).

Seaweed protein extraction was performed with magnetic stirring at 700 rpm for the entire duration. After enzymatic incubation, enzymatic inactivation was trialled; protein content after inactivation and protein content without inactivation was compared to ensure minimal protein was lost during high-temperature inactivation.

Next, samples were centrifuged at 10,000 rpm at 23 °C for 20 mins. The supernatant of samples was recovered following centrifugation and vacuum filtration. Different filters were trialled for vacuum filtration to discard enzyme protein; membrane filters (Nylon Net Filters 100 µm, Millipore, Sigma) were compared with paper filters (Qualitative Filter Paper, Microscience, New Castle, DE, UK). Following this, samples were subjected to Bradford analysis (as described in *Section 2.4.1.4*) and Kjeldahl analysis (as described in *Section 2.3.3*) for quantifying protein content. Experimental conditions producing the highest seaweed protein yield would be selected as the most favourable ones for enzymatic extraction.

2.4.3.2 Enzymatic pre-treatment followed with alkaline post-treatment

Enzymatic pre-treatment followed by alkaline post-treatment of seaweed proteins could further enhance protein extractability (Mæhre *et al.*, 2016; Naseri *et al.*, 2020b). Therefore, single-factor experiments investigating the influence of various enzymes and alkaline conditions on seaweed protein extraction yield were conducted.

Firstly, enzymes such as Alcalase, Celluclast, Pectinex, and Shearzyme at 200% (% enzyme/g raw seaweed biomass), were added to a seaweed-water mixture (1:40, w/v) and were incubated for 4 hr at 23 °C, with 700 rpm mixing. After pre-treatment, supernatants and pellets were separated. Secondly, pellets were re-dissolved in alkaline water, making up to the same mass

as enzymatic pre-treatment. Pellet incubation in pH 8, 10 or 12 deionised water, and was performed with 700 rpm magnetic stirring for 4 hr at 23 °C. For comparison purposes, a control with no enzyme addition was performed for 8 hr. Finally, samples were centrifuged at 10,000 rpm at 23 °C for 20 mins after alkaline post-treatment. Seaweed protein extraction was conducted according to the experimental conditions described in Table 11.

The supernatant of samples was recovered following centrifugation and was quantified with Bradford analysis, as described in *Section 2.4.1.4*. Experimental conditions producing the highest seaweed protein yield would be selected as the most favourable ones for enzymatic pre-treatment followed by alkaline post-treatment.

Table 11. Experimental conditions for optimising enzymatic pre-treatment followed with alkaline post-treatment.

1. Enzymatic pre-treatment					**2. Alkaline post-treatment**		
Enzyme	**Amount of enzyme[1]**	**pH[2]**	**Extraction temperature (°C)[3]**	**Extraction duration (hr)**	**pH**	**Extraction temperature (°C)[3]**	**Extraction duration (hr)**
Alcalase	200%	9	23	4	8, 10, 12	23	4
Celluclast	200%	5	23	4	8, 10, 12	23	4
Pectinex	200%	5	23	4	8, 10, 12	23	4
Shearzyme	200%	5	23	4	8, 10, 12	23	4

n = 2; [1] % enzyme/g seaweed calculated according to enzyme density provided by the manufacturer, Alcalase is 1.17 g/mL, Celluclast is 1.22 g/mL, Pectinex is 1.17 g/mL, Shearzyme is 1.09 g/mL; [2] extraction pH for each enzyme is defined by the manufacturer; [3] extraction temperature was defined by preliminary experiments, occurring at 23 °C (room temperature).

2.4.4 Ultrasound-assisted extraction

Cavitation during ultrasound-assisted extraction is responsible for cell breakdown (Kadam *et al.*, 2017; Rodrigues *et al.*, 2015). Preliminary experiments (performed by a previous student in the research group) were conducted to identify appropriate sonication power, pulse, and duration for yielding the most protein content. The optimisation was performed with KR seaweed, and analyses for seaweed protein following ultrasound treatment were performed in duplicate analyses. A probe-type of ultrasound equipment was employed (Sonic Ruptor 250 Ultrasonic Homogeniser, OMNI International, Kennesaw, GA, USA).

The final ultrasound-assisted extraction protocol for KR seaweed was as follows. Firstly, raw seaweed sample of 1 g was hydrated with 40 mL of deionised water (1:40, w/v). After setting the conditions to 200 W and 20 kHz, the ultrasonic probe was placed into the seaweed-water mixture for 5 mins (sonication occurs for 1.2 sec and pauses for 0.8 sec, repeated for 5 min). The temperature increase due to ultrasonic heat generation could reach up to 70 °C ± 1. Seaweed protein content after ultrasound-assisted extraction was quantified with Bradford analysis, as described in *Section 2.4.1.4.*

2.4.5 Freeze drying of protein extracts

Supernatants from optimised seaweed extraction protocols (obtained in *Sections 2.4.1, 2.4.2, 2.4.3, and 2.4.4*) were freeze-dried (FreeZone 12 Liter Console Freeze Dry System, Labconco, Kansas City, MO, USA). Freeze-dried seaweed protein extracts (SPEs) were powdered, and stored in desiccators at 23 °C (room temperature) away from light exposure until further experiments.

2.4.6 Precipitation of protein extracts

2.4.6.1 Acetone precipitation

Acetone precipitation of seaweed protein was conducted in accordance with the method by Crowell *et al.* (2013) with slight modification. Briefly, 400 µL of acetone were added to 100 µL of SPE to achieve 80% acetone (v/v). Samples were then incubated at -20 °C overnight. After centrifugation at 10,000 rpm in 4 °C for 20 mins, the supernatant was discarded, and residual acetone was removed under a gentle stream of nitrogen gas. Finally, the remaining pellet (precipitated protein) was freeze-fried and forwarded to Kjeldahl analysis (as described in *Section 2.3.3*) to determine protein purity. Duplicate analyses were performed.

2.4.6.2 Ethanol precipitation

Ethanol precipitation of seaweed protein was performed in accordance with the method by Hardouin *et al.* (2016) with slight modification. Similar to acetone precipitation, after the addition and mixing of 400 µL of ethanol to 100 µL of SPE (achieving 80% ethanol, v/v), samples were incubated at -20 °C overnight. After centrifugation at 10,000 rpm in 4 °C for 20 mins, the supernatant was discarded, and residual ethanol was removed under a gentle stream of nitrogen gas. Finally, the remaining pellet (precipitated protein) was freeze-fried and

forwarded Kjeldahl analysis (as described in *Section 2.3.3*) to determine protein purity. Duplicate analyses were performed.

2.4.6.3 Isoelectric precipitation

Protein precipitation was performed with isoelectric point precipitation (Naseri *et al.*, 2020b; Novák & Havlíček, 2016). For the maximum recovery of proteins through isoelectric point precipitation, isoelectric point of SPE was first determined in accordance with the method by Harnsilawat *et al.* (2006) with slight modification. Briefly, an aliquot of liquid SPE extract after enzyme-assisted extraction was taken, and their pH was adjusted to 1.5 to 5.5 using < 5 M HCl and/or NaOH. Next, SPEs were precipitated at the respective acidic pH followed by centrifugation at 10,000 rpm at 23 °C for 20 min. Following centrifugation, the supernatants were collected, and their soluble proteins were measured at 280 nm with a UV-VIS spectrometer (Shimadzu UV-1280, Kyoto, Japan) spectrometer against type-1 water.

2.4.6.4 Ammonium sulphate precipitation

Protein precipitation was performed with ammonium sulphate precipitation in accordance with methods by Akyüz and Ersus (2021) and Garcia-Vaquero *et al.* (2017) with slight modification. Freeze-dried SPE was dissolved in type-1 water, with the sample's pH altered from 6.0 to 7.0 with NaOH (< 5 M NaOH). Samples were gently saturated to 80% ammonium sulphate whilst stirring at 300 rpm in 4 °C for 1 hr. Following centrifugation at 10,000 rpm in 4 °C for 20 mins, the supernatant was discarded, and the pellet containing precipitated protein was redissolved with minimal type-1 water, and subsequently dialysed (Visking Dialysis Tubing 3500 Daltons, Medicell International Ltd, London, UK) against type-1 water at 4 °C overnight. Dialysed protein isolates were retrieved, freeze-dried and forwarded Kjeldahl analysis (as described in *Section 2.3.3*) to determine protein content and purity. Duplicate analyses were performed.

2.5 Characterisation of seaweed proteins

2.5.1 Analysis of protein physicochemical properties

2.5.1.1 Analysis of protein molecular weight distribution

The molecular weight (MW) distribution of SPEs (obtained in *Sections 2.4.1, 2.4.2, 2.4.3* and *2.4.4*) was analysed with SDS-PAGE in accordance with methods by Abdollahi *et al.* (2019), Devnani *et al.* (2020) and Wang *et al.* (2020a) with slight modifications. Different concentrations of SPE were dispersed in 1 mL of 2x Laemmli Sample Buffer:type-1 water (1:1 v/v, 5% of buffer is 2-mercaptoethanol) to determine the optimal band display on the gels. Then, samples were heated for 10 mins at 100 °C. Following centrifugation at 10,000 rpm at 23 °C for 10 mins, sample supernatants and 5 µL of Precision Plus Protein Standards were loaded onto 4-15% and Any Dalton Mini-PROTEAN Precast Gels (Bio-Rad). Electrophoresis was conducted with PowerPac Basic Power Supply (Bio-Rad) at a constant voltage of 100 V. Gels were rinsed with deionised water before staining with CBB R-250 Staining Solution for 45 minutes. Next, gels were rinsed with deionised water before destaining with CBB R-250 Destaining Solution overnight. Finally, gels were photographed. Duplicate analyses were performed.

2.5.1.2 Analysis of amino acid composition

The amino acid (AA) profile of SPEs (obtained in *Sections 2.4.1, 2.4.2, 2.4.3 and 2.4.4*) was analysed and quantified through HPLC-MS. This was conducted with an Agilent 1290 Infinity LC (Agilent Technologies, Santa Clara, CA, USA), coupled with the Agilent 6460 Triple Quadrupole MS (Agilent Technologies). Triplicate analyses were performed.

Prior to commencing AA profile analysis, AAs from SPEs were retrieved through acid hydrolysis in accordance with methods by Naseri *et al.* (2020b) and Huang *et al.* (2018) with slight modification. As acid hydrolysis was conducted at different concentrations of HCl in the literature, acid hydrolysis was trailed with 1 M and 6 M HCl with 0.1% phenol (Huang *et al.*, 2018; Muramoto & Kamiya, 1990; Naseri *et al.*, 2020b); this would ensure sufficient AA breakdown was complete. Sample preparation for acid hydrolysis of SPE was as follows. Firstly, 10 mg of SPE was vortexed with 1 mL of HCl for 20 mins, and purged with nitrogen gas for 3 mins, before incubating at 110 °C for 24 hr. Following the incubation, samples were sonicated at 23 °C for 20 mins, and were then passed through 0.2 µm syringe filters.

Consecutively, 250 µL of samples were transferred into LC and were further diluted to 1 mL by adding 750 µL of 80% acetonitrile in type-1 water.

To prepare for AA analysis, a serial reference AA standard with known concentrations (2 ×, 4 ×, 8 ×, 16 ×, 32 ×, 64 ×, 128 ×, 256 ×, 512 × and 1024 × dilutions) was produced. As standards were diluted in different buffers in the literature, dilutions were trailed in citrate buffer at pH 2.2 (Yaich *et al.*, 2011), mobile phase A at pH 3 (data not yet published by another student in the research group), and acetonitrile in 20% type-1 water (Huang *et al.*, 2018); this would ensure sufficient resolution was achieved during chromatography. The diluted standards and hydrolysed samples were loaded for HPLC-MS analysis; the HPLC-MS analysis was performed in accordance with the method described by Huang *et al.* (2018) with modification. LC was performed with Agilent InfinityLab Poroshell 120 HILIC-Z (2.1 × 100 mm, 2.7 µm, Agilent Technologies) for all samples. The injection volume for all standards and samples was 1 µL, with the column maintained at 25 °C throughout the run, and the eluent mobile phase flow rate being 500 µL/min for separation.

A stock solution of 200 mM of ammonia formate in type-1 water (pH 3 with formic acid) was prepared for mobile phases A and B. Mobile phase A was diluting the stock solution to an ionic strength of 20 mM in 90% type-1 water, whereas mobile phase B was diluting the stock solution to 20mM in 90% acetonitrile; mobile phases A and B were at alterted to pH 3 with formic acid. The gradient program was as follows: 0 min, 100% B; 1 min, 100% B; 2 min, 1% A and 99 % B; 3 min, 2% A and 98% B; 7.5 min, 30% A and 70% B; 8.5 min, 100% B. A post-run of 1 min was incorporated to re-equilibrate the column. This resulted in a total cycle time of 9.5 mins per sample.

MS was operated in multiple reaction monitoring mode to produce daughter ions by an electrospray ionisation source; capillary voltage was 3.5 kV, nebuliser gas was 45 psi, drying gas was at 250 °C with a flow rate of 10 L/min, and sheath gas was at 280 °C with a flow rate of 11 L/min.

A linear standard curve ($R^2 > 0.99$) was configured based on the serial concentrations and peak areas of reference AAs. Data acquisition and analysis of chromatograms were executed with Quantitative Analysis software (Version B.07.00, Agilent Technologies). The AA

concentrations of each sample were calculated in relation to the reference AA standard curve, and were expressed as mg AA/g sample.

The protein quality of SPEs was estimated with the following equations:

$$Total\ AA\ \left(\frac{mg\ AA}{g\ SPE}\right) = Sum\ of\ all\ AAs \qquad \text{Eq. 14}$$

$$EAA{:}AA\ (\%) = \frac{Sum\ of\ EAAs\ \left(\frac{mg\ AA}{g\ SPE}\right)}{Sum\ of\ all\ AAs\ \left(\frac{mg\ AA}{g\ SPE}\right)} \times 100\% \qquad \text{Eq. 15}$$

$$EAA{:}NEAA\ (\%) = \frac{Sum\ of\ EAAs\ \left(\frac{mg\ AA}{g\ SPE}\right)}{Sum\ of\ NEAAs\ \left(\frac{mg\ AA}{g\ SPE}\right)} \times 100\% \qquad \text{Eq. 16}$$

$$AAS\ (\%) = \frac{mg\ of\ first\ limiting\ EAA\ of\ SPE}{mg\ of\ limiting\ EAA\ in\ reference\ pattern} \times 100\% \qquad \text{Eq. 17}$$

$$EAAI\ (\%) = \sqrt[n]{\frac{mg\ of\ limiting\ EAA\ of\ SPE}{mg\ of\ limiting\ EAA\ in\ reference\ pattern}} \times 100\% \qquad \text{Eq. 18}$$

where AA is amino acid; EAA is essential AA; NEAA is non-essential AA; AAS is AA score, calculated with reference to the EAA scoring pattern for children ages 3 to 10 years (Naseri *et al.*, 2020b; WHO/FAO/UNU, 2007); EAAI is EAA index, n is number of EAA entering the equation, measures the geometric mean of EAAs compared to their relative reference according to the EAA scoring pattern for children ages 3 to 10 years (Dawczynski *et al.*, 2007; WHO/FAO/UNU, 2007).

2.5.1.3 Analysis of protein functional groups and secondary structure

The protein functional groups and secondary structure of SPEs (obtained in *Sections 2.4.1, 2.4.2, 2.4.3 and 2.4.4*) were analysed with an FTIR spectrometer (VERTEX 70v FTIR Spectrometer, Bruker, Billerica, MA, USA) in accordance with methods by Vásquez *et al.* (2019) and Benslima *et al.* (2021) with slight modifications. ATR-FTIR spectra were acquired with an A225/Q Platinum ATR accessory with a single reflection diamond crystal, in the range of 4000 cm^{-1} to 400 cm^{-1} at 23 °C and 4 cm^{-1} resolution. Various scans (32 and 64 scans) were trialled to ensure sufficient data and resolution were captured in the spectrum. Data acquisition and analysis were executed with Opus Viewer (Version 7.5, Bruker).

2.5.1.4 Analysis of protein thermal properties

The thermal properties of SPEs (obtained in *Sections 2.4.1, 2.4.2, 2.4.3 and 2.4.4*) were analysed with a DSC Q1000 (TA Instruments, New Castle, DE, UK) in accordance with methods by Suresh Kumar *et al.* (2014) and Chen *et al.* (2019). A sample of 5 mg was placed into a hermetically sealed aluminium pan. Following equilibration at 23 °C for 5 mins, the sample was scanned from 20 °C to 210 °C. Various heating rates (2, 5 and 10 °C/min) were trialled to ensure sufficient data and resolution were captured in the spectrum. An empty aluminium pan was used as a reference. Onset temperature (T_0), peak denaturation temperature (T_d) and enthalpy change (ΔH) was extracted from the thermogram with Instrument Explorer (TA Instruments) and analysed with Universal Analysis 2000 (Version 4.5A, TA Instruments). Triplicate analyses were performed.

2.5.2 Analysis of protein functional properties

2.5.2.1 Analysis of protein solubility

Solubility of SPEs (obtained in *Sections 2.4.1, 2.4.2, 2.4.3 and 2.4.4*) was analysed in accordance with *Section 2.4.2.4.*

2.5.2.2 Analysis of protein emulsifying properties

The emulsifying properties of SPEs (obtained in *Sections 2.4.1, 2.4.2, 2.4.3 and 2.4.4*) were analysed at pHs 3, 5, 7, 9 and 11 in accordance with the method by Chen *et al.* (2019) and Chiu *et al.* (2009) with slight modification. All samples were made to 1% w/v total solid (100 mg SPE in 10 mL solution). Following centrifugation at 10,000 rpm at 23 °C for 20 mins, 9 mL supernatant was gently mixed with 1 mL soya oil to achieve a ratio of 9:1, and the mixture was dispersed (S 25 KD – 18 G Dispersing tool – 0020002971, IKA-werke, Staufen, Germany) with 13,000 rpm for 1 min at 23 °C. Following this, 20 uL of the emulsion from the bottom was transferred to 4.98 mL of 0.1% SDS solution at 0 and 10 mins, and their absorbances at 500 nm were subsequently determined with a UV-VIS spectrometer (Shimadzu UV-1280). Triplicate analyses were performed. Finally, EA and ES indices were the absorbance calculated by the following:

$$EA\ (\frac{m^2}{g}) = \frac{2\ \times\ 2.303 \times\ A_0 \times D}{C \times \phi \times L \times 10^6} \qquad \text{Eq. 19}$$

$$ES = \frac{A_{0\,mins} \times 10}{A_{0\,mins} - A_{10\,mins}} \times 100\% \qquad \text{Eq. 20}$$

where $A_{0\,mins}$ and $A_{10\,mins}$ were the absorbances measured at 0 and after 10 mins, respectively; D was the dilution factor (250); C was weight of protein per volume (10 mg/mL); ϕ was the volumetric fraction of oil (0.1); L was the pathlength of cuvette (10^{-2} m).

2.5.2.3 Analysis of protein foaming properties

The foaming properties of SPEs (obtained in *Sections 2.4.1, 2.4.2, 2.4.3 and 2.4.4*) were analysed at pHs 3, 5, 7, 9 and 11 in accordance with the method by Chen *et al.* (2019) with slight modification. All samples were made to 1% w/v total solid content (100 mg SPE in 10 mL solution). Following centrifugation at 10,000 rpm at 23 °C for 20 mins, 10 mL of the supernatant was dispersed (S 25 N – 18 G Dispersing tool – 0000593400, IKA-werke) at 19,000 rpm at 23 °C for 2 mins. Following this, foam volume was measured at 0 and after 30 mins. Triplicate analyses were performed. Finally, FC and FS were calculated by the following:

$$FC = \frac{V_{0\,min} - 10}{10} \times 100\% \qquad \text{Eq. 21}$$

$$FS = \frac{V_{30\,mins} - 10}{V_{0\,min} - 10} \times 100\% \qquad \text{Eq. 22}$$

where $V_{0\,min}$ and $V_{30\,mins}$ were the volumes measured at 0 and after 30 min, respectively.

2.6 Analysis of protein antioxidant capacity

Two different protocols, DPPH and FRAP, were employed to measure the antioxidant capacities of SPEs (obtained in *Sections 2.4.1, 2.4.2, 2.4.3 and 2.4.4*). Triplicate analyses were performed.

2.6.1 DPPH radical scavenging activity assay

The DPPH radical scavenging activity of SPEs was measured in accordance with the method by Brand-Williams *et al.* (1995) and Cian *et al.* (2012) with slight modification. A Trolox standard with known serial concentrations (0.8, 0.6, 0.4, 0.3, 0.2, 0.1, 0.05 and 0.025 mM), and DPPH working solution (2 mg DPPH in 50 mL methanol) were produced. Next, 10 µL of Trolox standards and samples were loaded onto a 96-well microplate. Following the addition of 190 µL DPPH working solution into each well, a one-hour incubation away from light exposure was performed. Finally, the microplate was loaded into an Enspire Multimode plate reader, and after shaking for 10 secs, the absorbance of each well was detected at 517 nm. The results were directly exported with the built-in Enspire Manager data analysis software. A linear standard curve ($R^2 > 0.99$) was configured using the serial concentrations of Trolox. The antioxidant capacity of each sample was calculated in relation to the Trolox standard curve, and was expressed as mM Trolox/g SPE.

2.6.2 FRAP assay

The FRAP of SPEs was measured in accordance eith the method by Kazir *et al.* (2019) and Benzie and Strain (1996) with slight modification. A Trolox standard with known serial concentrations (1, 0.8, 0.6, 0.4, 0.3, 0.2, 0.1 and 0.05 mM), FRAP working solution (acetate buffer at pH 3.6, TPTZ and iron (III) chloride at a ratio of 10:1:1) were produced. Next, 10 µL of Trolox standards and samples were loaded onto a 96-well microplate. Following the addition of 190 µL FRAP working solution into each well, a one-hour incubation away from light exposure was performed. Finally, the microplate was loaded into an Enspire Multimode plate reader, and after shaking for 10 secs, the absorbance of each well was detected at 593 nm for FRAP. The results were directly exported with the built-in Enspire Manager data analysis software. A linear standard curve ($R^2 > 0.99$) was configured using the serial concentrations of Trolox. The antioxidant capacity of each sample was calculated in relation to the Trolox standard curve, and was expressed as mM Trolox/g SPE.

2.7 Statistical analysis of data

Examination of seaweed protein yields during extraction (*Section 2.4*), and SPE properties (*Sections 2.5 and 2.6*) were performed in duplicates or triplicates, as specified in the method of each analysis. Their data were reported as mean ± standard deviation. Statistical analysis was performed with SPSS Statistics Version 28.0 (IBM Corp., Armonk, NY, USA). One-way ANOVA with Duncan's multiple range test was employed to identify significant differences amongst results ($p < 0.05$); *t*-tests were utilised when necessary ($p < 0.05$).

Chapter three

Results and discussion

3 Results and discussion

3.1 Proximal composition of seaweeds

Table 12 describes the proximal composition of two red seaweeds: the commercially cultivated *Porphyra* sp. from Korea (KR seaweed) and the harvested *Pyropia* spp. from wild oceans in New Zealand (NZ seaweed). Generally, carbohydrate contents of KR and NZ seaweeds contributed the most to the seaweed's proximal composition, subsequently followed by protein, ash, and lipid. Due to the superior carbohydrate content in seaweeds, seaweed carbohydrates constitute crucial economic value and serve as an extensive source of carbohydrate raw materials for the hydrocolloid industry (Mišurcová, 2012).

KR seaweed had significantly higher moisture content than NZ seaweed ($p < 0.05$), which could be influenced by their respective drying methods. KR seaweed contained significantly higher protein than NZ seaweed by 12% ($p < 0.05$), whereas NZ seaweed contained significantly higher ash and carbohydrates than KR seaweed ($p < 0.05$). An inverse relationship between proteins and carbohydrates was observed, whereby KR seaweed constitutes more protein (28.41%) yet less carbohydrate (59.11%), and NZ seaweed contained less protein (19.07%) but more carbohydrate (67.67%). Protein synthesis has an inverse relationship with carbohydrate production, as carbohydrate production would dominate under limited availability of nitrogenous nutrients for protein synthesis, which was noticed for red *Gracilaria cervicornis* and brown *Sargassum vulgare* (Marinho-Soriano *et al.*, 2006). Hence, the lower protein content in NZ seaweed could be related to the limited availability of nitrogenous nutrients during NZ seaweed growth (Kadam *et al.*, 2017). Moreover, the varying carbohydrate content between KR seaweed and NZ seaweed could be associated with variances in their cell wall constitution; thus indicating differences in their polysaccharide composition (agars, carrageenans, xylans) (Wijesinghe & Jeon, 2012).

Table 12. Proximal composition of raw KR seaweed and NZ seaweed.

	KR seaweed	**NZ seaweed**	**NZ wild seaweeds[3]**	**NZ commercial seaweeds[3]**
Moisture (%)	9.14 ± 0.04[a]	5.90 ± 0.19[b]	NA	NA
Ash (%)	10.04 ± 0.02[b]	11.81 ± 0.19[a]	19.8 – 28.43	20.62 – 42.87
Protein (%)[1]	28.41 ± 0.10[a]	19.07 ± 0.11[b]	9.60 – 32.71	9.78 – 26.36
Lipid (%)	2.44 ± 0.10[a]	1.45 ± 0.39[a]	1.24 – 3.30	0.80 – 3.13
Carbohydrate (%)[2]	59.11 ± 0.02[b]	67.67 ± 0.68[a]	45.40 – 66.90	43.99 – 69.61

KR, Korean commercial *Porphyra* sp., NZ, New Zealand wild *Pyropia* spp.; moisture content was accounted for in ash, protein, and lipid; [1] protein content measured through Kjeldahl method with the universal seaweed nitrogen-to-protein conversion factor of 5.00 (Angell *et al.*, 2016); [2] carbohydrate was estimated by difference; [3] referenced from Smith *et al.* (2010); NA, not available; different letters (a, b) indicate statistical significance among different species (One-way ANOVA with Duncan's multiple range test, n = 3, $p < 0.05$).

KR and NZ seaweeds analysed in this study are red seaweeds, typically comprising greater protein content than brown and green seaweeds, as well as some other red seaweeds (Table 6, *Section 1.2.2.4* – in *Literature review*). KR seaweed was commercially cultivated, yet it possessed varying proximal characteristics to other commercial seaweeds in Table 12. In contrast to another closely related commercial *Porphyra*, KR seaweed in this study exhibits 17% lower ash (26.62% seaweed DW), slightly higher protein content (26.36%), and 15% lower carbohydrate (43.99%) (Smith *et al.*, 2010). Furthermore, another Portugal *Porphyra* contained similar protein content (27.4 to 28.2 g protein/100 g seaweed DW) (Vieira *et al.*, 2018). In contrast, protein contents of closely related species *P. dioica* (20.6% seaweed DW), *P. purpurea* (13.5%), and *P. umbilicalis* (15.1%) from Norway were 8% to 15% less than that of KR seaweed (Biancarosa *et al.*, 2017). Finally, KR seaweed consisted of slightly higher protein content than closely related species from Japan and Korea (both 27.0% seaweed DW), and China (25.6%) (Dawczynski *et al.*, 2007), yet a UK *Porphyra* seaweed reported 16% higher protein (44% seaweed DW) than KR seaweed (Marsham *et al.*, 2007).

Protein, lipid, and carbohydrate contents of NZ seaweed were within range of some NZ wild and commercial seaweeds in Table 12. In contrast to a wild NZ red seaweed, *Porphyra* spp., NZ seaweed in this study constituted 8% lower ash (19.8% seaweed DW), 14% lower protein (32.71%), and 22% higher carbohydrate (45.4%) (Smith *et al.*, 2010). When comparing protein content to other closely related species, NZ seaweed exerts 10% lower protein content than red *Pyropia haitanensis* (29.4% seaweed DW) (Mao *et al.*, 2017), 1% to 5% higher protein content than red *P. acanthophora* (14.11 to 18.36 g protein/100 g seaweed DW) (Kavale *et al.*, 2017),

and 17% to 19% lower protein content than red *P. yezoensis* (36.15% to 38.16% seaweed DW) (Jung *et al.*, 2016).

Despite being closely related species, the proximal compositions of the seaweeds would be subjected to change depending on growth location and harvest season (Mišurcová, 2012). Variances in proximal characteristics could also arise from detection protocols and calculation procedures (Mæhre *et al.*, 2018). For instance, protein quantification in Table 12 underwent Kjeldahl analysis, whereas Kavale *et al.* (2017) measured protein with the Lowry method, and Biancarosa *et al.* (2017) measured protein with the Dumas method (estimation of total nitrogen). Nonetheless, protein measurement in Table 12 utilised a universal seaweed nitrogen-to-protein conversion factor (NPCF) of 5.00, as recommended by Angell et al. (2016). Biancarosa *et al.* (2017), Vieira *et al.* (2018), Jung *et al.* (2016), Mao *et al.* (2017), Smith *et al.* (2010), Dawczynski *et al.* (2007) and Marsham *et al.* (2007) utilise an NPCF of 6.25, which could overestimate protein content in algae (Conde *et al.*, 2013).

3.2 Extraction of seaweed proteins

3.2.1 Water extraction of seaweed proteins

Extraction of protein in water would provide a fundamental basis for seaweed protein extraction. Water extraction of red seaweed proteins was optimised through single-factor experiments. Single-factor optimisation, also known as the one-variable-at-a-time approach, involves experiments testing one changing parameter whilst keeping others constant. Classic water extraction of seaweed proteins was chosen as the control (blank) for comparison against other extraction methods (Hardouin *et al.*, 2016; Mæhre *et al.*, 2016; Naseri *et al.*, 2020b; Vásquez *et al.*, 2019). Having a control enables the differentiation of extraction efficiencies between varying methods. Nevertheless, the subsequent seaweed protein extractions could be set consistent with suitable experimental parameters selected to optimise water extraction.

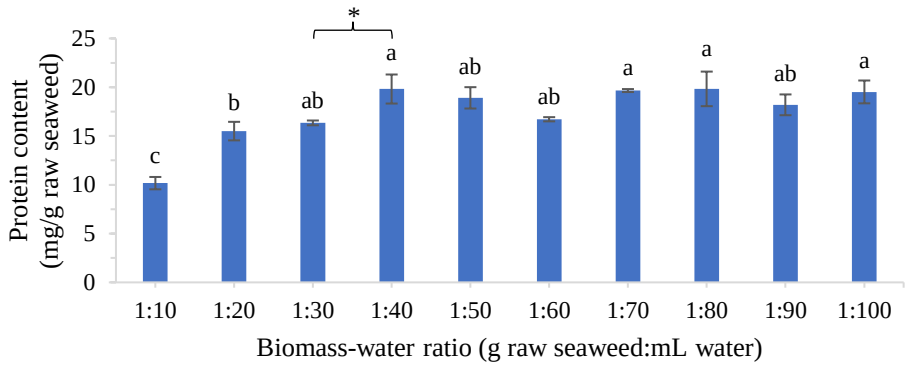
Table 13. Experimental outline for optimising water extraction of seaweed protein.

Experiment	**Biomass-water ratio (g raw seaweed:mL water)**	**Extraction temperature (°C)**	**Extraction duration (hr)**
1	1:10	50	2
2	1:20	50	2
3	1:30	50	2
4	1:40	50	2
5	1:50	50	2
6	1:60	50	2
7	1:70	50	2
8	1:80	50	2
9	1:90	50	2
10	1:100	50	2
11	1:50	23[1]	2
12	1:50	40	2
13	1:50	50	2
14	1:50	60	2
15	1:40	23	1
16	1:40	23	2
17	1:40	23	4
18	1:40	23	6
19	1:40	23	8
20	1:40	23	10
21	1:40	23	12
22	1:40	23	14
23	1:40	23	16
24	1:40	23	20
25	1:40	23	24
26	1:40	23	48

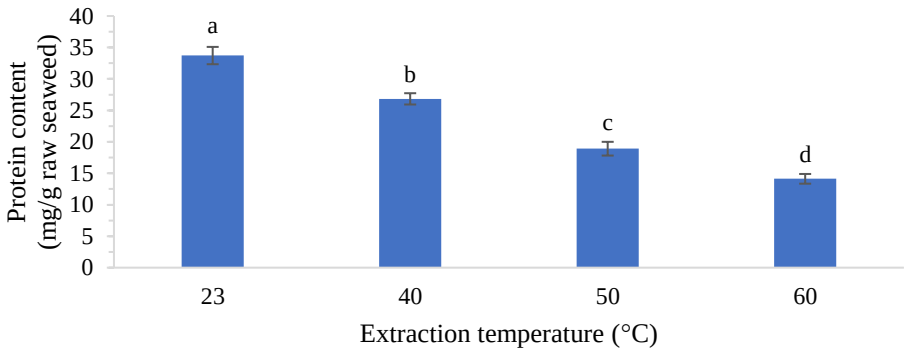
n = 2; [1] 23 °C, room temperature.

Table 13 explored various parameters and levels to identify the optimal level for each parameter: experiments 1 to 10 determined seaweed biomass-water ratio, experiments 11 to 14 determined protein extraction temperature, and experiments 15 to 26 determined protein extraction duration. All experiments occurred in deionised water. The level delivering the highest protein content would be designated as the most favourable level for that parameter (Figure 2).

A)



B)



C)

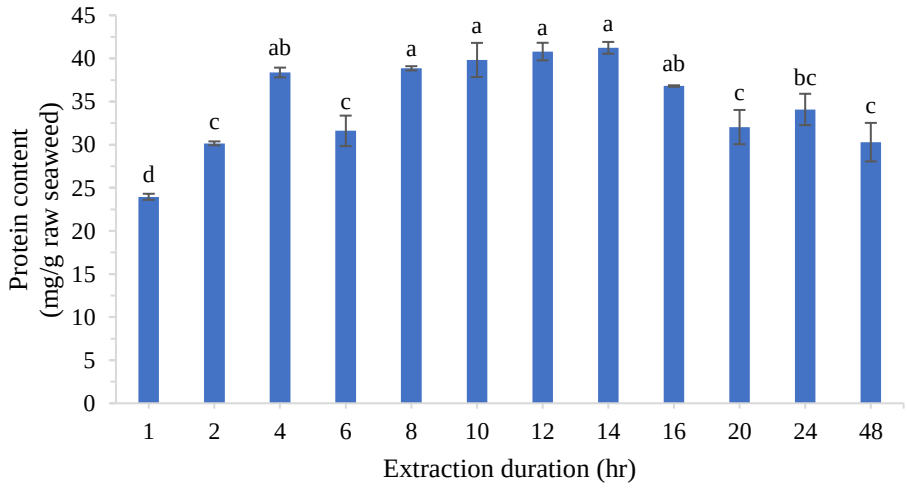


Figure 2. Seaweed protein content measured for each experimental parameter for the optimisation of water extraction, A) seaweed biomass-water ratios, B) extraction temperatures, C) extraction durations. Protein content was measured with Bradford analysis; different letters (a, b, c, d) indicate statistical significance within the same parameter (One-way ANOVA with Duncan's multiple range test, n = 2, $p < 0.05$); * confirms statistically significant difference between selected samples (*t*-test, $p < 0.05$).

3.2.1.1 Biomass-water ratio

As the seaweed biomass-water ratio changed from 1:10 to 1:100, the extracted seaweed protein content was enhanced (Figure 2A). However, protein content did not display noticeable improvement after 1:40. Preece *et al.* (2017b) proposed that after reaching a certain amount of water necessary to solubilise substrates, water would not be the limiting factor in most extractions. The direct significance between 1:30 and 1:40 would be confirmed by conducting a *t*-test (Figure 2A, $p < 0.05$), whereby the ratio of 1:40 (19.81 protein/g raw seaweed) possessed significantly more protein than 1:30 (16.34 mg/g). A sufficient biomass-water ratio would improve protein dissolution and thus augments protein yield (Preece *et al.*, 2017b). Ultimately, the most favourable seaweed biomass-water ratio would be 1:40.

3.2.1.2 Extraction temperature

As seaweed protein extraction temperature raised, seaweed protein content was reduced (Figure 2B). Extraction at 23 °C (room temperature) produced significantly higher protein content (33.71 mg protein/g raw seaweed) than extractions at 40, 50 and 60 °C, which were 26.83, 18.91, and 14.12 mg/g, respectively (Figure 2B, $p < 0.05$). Such observations were inconsistent with those by Naseri *et al.* (2020b) for red *Eucheuma denticulatum* protein, which demonstrated the weakest seaweed protein extraction efficiency at 30 °C (72.4% to 76.4%), and the greatest at 60 °C (83.8% to 84.4%). The differing observations by Naseri *et al.* (2020b) could be influenced by adding an enzyme and using an alkaline extraction solution. In most cases, as displayed in Figure 2B, heating during protein extraction induces protein denaturation, protein solubility diminishment and protein functionality modification (Devnani *et al.*, 2020; Lee, 2018). Heat-sensitive amino acids (AAs), such as cysteine, methionine, threonine, serine and tryptophan, could be damaged and lost (Dworschak & Carpenter, 1980). To maintain seaweed protein content and quality during extraction, the most favourable temperature for extraction would be 23 °C.

3.2.1.3 Extraction duration

As seaweed extraction duration increased, the extracted seaweed protein content improved (Figure 2C). Despite that, protein content did not display significant improvement after 4 hr extraction, which yielded 38.85 mg protein/g raw seaweed. As protein yield reaches its saturation concentration in water, further protein dissolution could be prohibited as the extraction system would reach equilibrium (Arakawa & Timasheff, 1985). An analogous

phenomenon was observed by Naseri *et al.* (2020a) for red *E. denticulatum* protein, whereby water extraction at 4, 6 and 8 hrs showcased no significant difference in extraction efficiency (Figure 2C, $p > 0.05$). Ultimately, the most favourable extraction duration would be 4 hr.

Through integrating results from the above single-factor experiments, optimal conditions for water extraction were utilising a seaweed biomass-water ratio of 1:40, extraction temperature at 23 °C with a duration of 4 hr. The final water extraction protocol yields approximately 23.30% red seaweed protein solids, with a protein purity of 33.57%.

3.2.2 Alkaline extraction of seaweed proteins

Extraction of protein in aqueous solutions would be a simple and effective traditional technique to understand seaweed protein in liquid systems. Alkaline extraction of seaweed proteins could improve protein yield through the solubilisation of water-insoluble hydrophobic proteins (Harnedy & FitzGerald, 2013a). Based on preliminary screening using single-factor experiments from *Section 2.4.1*, findings by Harrysson *et al.* (2018) and Harnedy and FitzGerald (2013a), alkaline extraction of red seaweed proteins was further optimised through an L9 (3^4) orthogonal design. The orthogonal design has been successfully applied to the optimisation of extracting desired compounds from various materials (Peng *et al.*, 2019; Shen *et al.*, 2008; Wang *et al.*, 2021; Wang *et al.*, 2020b; Xingfei *et al.*, 2020). The orthogonal design would also be significantly more efficient (9 experiments in Table 14), compared to 81 experiments in the case of a 4 factor 3 level comprehensive design (Peng *et al.*, 2019). Additionally, optimising protein extraction through orthogonal design would assess the cross effect of extraction factors, diminish the interference of experimental errors, and evaluate the statistical significance of each factor (for L9 (3^4), $R > 19.00$, $p < 0.05$) (Wang *et al.*, 2021).

Table 14 showcases the four extraction factors, namely extraction pH (A), seaweed biomass-water ratio (B), extraction duration (C), and extraction temperature (D). The four factors were examined to understand the effect of factors in a multidimensional system. Results from Table 14 were forwarded to the range and ANOVA analyses in Table 15 for establishing the optimal conditions of alkaline extraction. Range analysis would dictate ideal extraction conditions by combining the best level for every factor across the four extraction factors (Peng *et al.*, 2019). Moreover, ANOVA analysis was performed for each factor to obtain an F value, ensuring the best level for every factor was significantly better than other levels within the same factor (Peng *et al.*, 2019).

The ideal extraction parameters for seaweed protein extraction were determined upon findings from Table 15. For example, k values for seaweed protein content are the average protein yielded at a selected level, and R values are the maximum differences between the three k values. A greater k value reflects the higher preference of that level for a particular factor than others, whereas a greater R value reflects a factor's higher impact during protein extraction compared to others (Shen *et al.*, 2008). Hence, the most favourable extraction condition for each factor would be their maximum k value.

Table 14. Orthogonal experimental design L9 (3^3) and results for optimising alkaline extraction of seaweed proteins.

Experiment	**A (pH)[1]**	**B (Ratio)[2]**	**C (Hr)[3]**	**D (°C)[4]**	**Protein content[5] (mg protein/g seaweed)[5]**	**Particle size[6]**		**Solubility (%)[7]**
						Uniformity	**Diameter [4, 3] (volume-weighted mean, µm)**	
1	1 (8)	1 (1:30)	1 (4)	1 (23)[8]	26.96 ± 0.95	0.55	185.09	83.03 ± 1.41
2	1	2 (1:40)	2 (5)	3 (35)	23.88 ± 0.29	0.84	118.89	77.52 ± 1.76
3	1	3 (1:50)	3 (6)	2 (30)	27.73 ± 0.62	0.80	128.53	84.83 ± 1.25
4	2 (10)	1	2	2	30.52 ± 1.43	1.26	92.63	85.82 ± 2.31
5	2	2	3	1	33.85 ± 1.59	0.45	199.30	84.83 ± 0.51
6	2	3	1	3	30.07 ± 0.20	0.55	173.88	88.54 ± 1.12
7	3 (12)	1	3	3	31.46 ± 1.04	0.48	192.19	91.08 ± 1.02
8	3	2	1	2	51.66 ± 2.85	0.43	216.62	83.13 ± 0.61
9	3	3	2	1	51.66 ± 2.54	0.51	206.25	79.60 ± 0.00

[1] A, extraction pH; [2] B, seaweed biomass-water ratio; [3] C, extraction duration; [4] D, extraction temperature; [5] protein content measured through Bradford analysis; [6] particle size analysed through uniformity and volumed weighted mean diameter; [7] protein solubility analysed with 5% solid content in deionised water (w/v); [8] 23 °C, room temperature.

Table 15. Data analysis of extraction factors and levels from the orthogonal experiment optimising alkaline extraction of seaweed proteins.

	Protein content[1]				**Particle size[2]**								**Solubility[3]**			
					Uniformity				**Diameter [4, 3] (volume-weighted mean, µm)**							
	A[4]	**B[5]**	**C[6]**	**D[7]**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
k_1[8]	26.19	29.65	36.23	37.49	0.73	0.76	0.51	0.51	144.17	156.64	191.86	196.88	81.79	86.64	84.90	82.49
k_2[8]	31.48	36.46	35.35	28.47	0.75	0.57	0.87	0.62	155.27	178.27	139.26	161.65	86.40	81.83	80.98	85.71
k_3[8]	44.93	36.49	31.01	36.63	0.48	0.62	0.58	0.83	205.02	169.55	173.34	145.92	84.61	84.32	86.91	84.59
Best level[8]	A3	B3	C1	D1	A3	B2	C1	D1	A3	B2	C1	D1	A2	B1	C3	D2
R[9]	18.74	6.84	5.22	9.02	0.28	0.19	0.36	0.32	60.85	21.63	52.61	50.96	4.60	4.81	5.93	3.23
Order[10]	ADBC				CDAB				ACDB				CBAD			
SS[11]	559.79	93.25	46.82	148.78	0.14	0.06	0.22	0.16	6300.76	710.70	4272.20	4085.12	32.32	34.76	54.57	16.09
F value[12]	11.96	1.99	1.00	3.18	2.36	1.00	3.68	2.70	8.87	1.00	6.01	5.75	2.01	2.16	3.39	1.00

[1] Protein content measured through Bradford analysis; [2] particle size analysed through uniformity and volumed weighted mean diameter; [3] solubility analysed with 5% solid content in deionised water (w/v); [4] A, extraction pH; [5] B, seaweed biomass-water ratio; [6] C, extraction duration; [7] D, extraction temperature; [8] range analysis, average *k* value of each level within the factors (k_1, k_2, k_3 for levels 1, 2, 3, respectively), the largest *k* value defines the best level within each factor; [9] *R*, range between the minimum and maximum *k* values, larger *R* value implies a higher importance for the factor during extraction; [10] order, effect of factors ranked from highest to lowest, determined by the descending order of *R* values; [11] SS, sum of squares; [12] *F* value, retrieved from ANOVA Fisher's *F*-test, *F* (2,2) 95% = 19.

3.2.2.1 Protein content

An increase in seaweed protein yield was noticed as pH elevated (Table 14). For instance, seaweed protein content increased from 23.88 to 29.96 mg protein/g seaweed at pH 8, to 31.46 to 51.66 mg/g at pH 12 (Table 14). The influence of pH on protein extraction was notable in the protein extraction of brown *A. nodosum*, where the protein extraction improved from 7.97% to 16.9% at acidic pHs to 51.80 to 56.35% at alkaline pHs (Kadam *et al.*, 2017). Such findings were also concurrent with Harnedy and FitzGerald (2013a), where pH 12.68 promoted protein extraction in red *P. palmata* compared to pH 11.50. Nevertheless, alkaline conditions boosted protein extraction in microalgae, where protein extraction at pH 11 doubled the yield from pH 8.5 (Parniakov *et al.*, 2015). Alkaline conditions could enhance the solubilisation of water-insoluble hydrophobic seaweed proteins, thus assisting protein release from the rigid seaweed cell wall (Barbarino & Lourenço, 2005; Kadam *et al.*, 2017).

Different parameters were investigated in combination to understand their influence on seaweed protein extraction. In Table 15, according to range analysis, factor A (pH) holds the greatest *R* value, followed by D (extraction temperature) > B (seaweed biomass-water ratio) > C (extraction duration); thus indicating that pH had the highest impact on protein extraction, and extraction duration had the lowest impact on protein extraction. The best levels for each factor to obtain maximum protein yield (the biggest *k* value) were A3, B3, C1 and D1. Factor A displayed considerable effect from ANOVA analysis amongst the four factors (Table 15, *F* value of 11.96). Hence, optimal conditions for deriving the more seaweed protein content would be modified to A3 (pH 12), B1 (1:30), C1 (4 hr), and D1 (23 °C), as the influence of seaweed biomass-water ratio could be dismissed (*F* value of 1.99).

3.2.2.2 Particle size

Uniformity estimates the absolute median particle size (PS) deviation from the median, whereas volume-weighted mean diameter estimates the mean PS representing the volume of particles (Malvern Instruments, 2007). Both uniformity and diameter are vital to comprehending protein PS and functionality. An increased uniformity value proposes reduced heterogeneity amongst PS. For instance, experiment 8 with a uniformity of 0.41, suggests greater heterogeneity than others (Table 14). Larger PS diameter advocates for an overall bigger particle of the sample. As seen from experiment 8 in Table 14, its particles (216.62 µm) were bigger than the others. Typically, larger particles have been attributed to declined

uniformity (Hennemann *et al.*, 2021; Mirhosseini *et al.*, 2008; Zhang *et al.*, 2018). A reduction in PS improves uniformity and would be essential for achieving stable emulsions (Mirhosseini *et al.*, 2008), as well as enhancing WHC and gelation strength (Hu *et al.*, 2013).

A pronounced observation would be that seaweed protein PS increased as pH elevated. For example, the protein PS at pH 8 ranged from 118.89 to 185.09 µm, whereas protein PS at pH 12 ranged from 192.19 to 216.62 µm (Table 14). This could connote seaweed protein aggregation at high pHs. Similarly, increasing extraction pH accumulated pea protein aggregates, provoking larger PS (Gao *et al.*, 2020). Gao *et al.* (2020) unveiled that the immediate formation of disulphide bonds from free sulfhydryl groups was discovered as pH became more alkaline. Contrastingly, amaranth seed protein PS was reduced when treatment pH was increased (Das *et al.*, 2021), and the authors highlighted that alkaline pH had elevated ionic strength that reinforces hydrophobic interaction in proteins, thus inducing smaller PS. In this case, the PS increase and decrease upon alkaline pHs could be dependent on the protein source.

Based on the range analysis and ANOVA, PS did not display an apparent trend at different temperatures (Table 15). For example, in Table 15, larger diameters were identified at 30 °C compared to 35 °C, namely experiments 3 (128.53 and 185.09 µm, respectively), and 8 (192.19 and 216.62 µm, respectively); yet experiment 4 reported a smaller diameter at 30 °C compared to 35 °C (92.63 and 199.30 µm, respectively). Likewise, Devnani *et al.* (2020) emphasized that almond protein PS with heat treatments below 45 °C showcased insignificant change, whereas almond protein aggregation with larger PS was observed for heat treatments from 55 °C to 95 °C. It could be implied that extraction temperatures ranging from 23 °C to 35 °C would not lead to notable aggregation for seaweed proteins, thus maintaining their initial protein integrity.

In Table 15, the optimal conditions for deriving the ideal seaweed protein PS according to range analysis were C1 (4 hr), D1 (25 °C), A3 (pH 12), and B2 (1:40) based on uniformity; extraction duration (factor C) had the greatest influence on PS during protein extraction (*R* value order: C > D > A > B). In the contrary, A3, C1, D1, and B2 were the optimal conditions for deriving the ideal seaweed protein PS based on diameter; extraction pH (factor A) was the most important factor in obtaining the best PS during protein extraction (*R* value order: A > C > D > B). However, ANOVA results suggest that all levels were insignificant amongst the four

factors for both uniformity and diameter ($p > 0.05$). Thus, reinforcing that the four extraction parameters had no significant influence on seaweed protein PS during extraction ($p > 0.05$).

3.2.2.3 Solubility

Alkaline extraction of seaweed proteins exhibited relatively high solubility, ranging from 77.52% to 91.08% in Table 14. The solubility of seaweed proteins would be enhanced in alkaline conditions (Fleurence *et al.*, 1995a; Harrysson *et al.*, 2018; Kadam *et al.*, 2017; Naseri *et al.*, 2020b). In the current study, the solubility of KR seaweed was greater than red *P. dioica* (43.3% solubility at pH 8) (Felix *et al.*, 2021), greater than green *U. lactuca* (62.1% solubility at pH 12) and red *P. umbilicalis* (54.2% solubility at pH 12) (Harrysson *et al.*, 2018), greater than red *K. alvarezii* (58.72% in 0.5 M NaCl) (Suresh Kumar *et al.*, 2014), and similar to brown *H. elongate* (96.15% at pH 12) (Garcia-Vaquero *et al.*, 2017). At a pH higher than the protein isoelectric point, the ionisation of proteins results in an overall negative net charge, enhancing greater repulsive electrostatic forces and solubilisation (Das *et al.*, 2021; Phongthai *et al.*, 2016; Zayas, 1997e). Nonetheless, this occurrence would be more evident in alkaline conditions than acidic conditions (Das *et al.*, 2021; Harrysson *et al.*, 2018; Jiang *et al.*, 2021).

The optimal conditions for deriving the ideal seaweed protein solubility according to range analysis were C3 (6 hr), B1 (1:30), A2 (pH 8), and D2 (35 °C) (Table 15). In this case, extraction duration (factor C) had the greatest influence on solubility during protein extraction (R value order: C > B > A > D). However, all levels were insignificant from ANOVA analysis amongst the four factors for solubility ($p > 0.05$). Thus, reinforcing that the four extraction parameters had no statistically significant influence on seaweed protein solubility during extraction ($p > 0.05$).

3.2.2.4 Optimised alkaline extraction

As an L9 (3^4) orthogonal design, the F value during ANOVA analysis is to be greater than 19 to be statistically significant (3 replicates, $F(2, 2)$, $p > 0.05$). Despite so, an F value greater than 9.55 would be considered statistical significance if four replicates were conducted ($F(2, 3)$, $p > 0.05$). The largest F value within Table 15 is 11.96, conferring the influence of pH on seaweed protein content during extraction. Although three replicates were performed in the current experimental design, the minimal standard deviation for the results corroborates pH (F value of 11.96) would be a significant factor in maximising protein content during extraction.

Moreover, the second largest *F* value is 8.87 in Table 15, referring to the influence of pH on PS during extraction, reinforcing the importance of pH during protein extraction. Hence, extraction pH exerts a sizeable influence compared to other extraction parameters, and the ideal pH would be 12 (factor A, level 3).

Ultimately, through integrating the findings from *Sections 3.2.2.1, 3.2.2.2* and *3.2.2.3,* the optimal alkaline extraction conditions for seaweed protein would be A3 (pH 12), B1 (1:30), C1 (4 hr), and D1 (23 °C), The final alkaline extraction protocol yields approximately 20.81% red seaweed protein solids, with a protein purity of 37.48%.

3.2.3 Ultrasound-assisted extraction of seaweed proteins

Ultrasound-assisted extraction has been a popular method for extracting numerous food proteins (Görgüç *et al.*, 2019; Kumar *et al.*, 2021; Preece *et al.*, 2017a; Xu *et al.*, 2017). Ultrasound-assisted extraction could augment seaweed protein extraction yield as implosions of cavitation bubbles produce immense shear forces, which would sabotage cell walls and release proteins (Kadam *et al.*, 2013). The ultrasonic conditions explored by Kadam *et al.* (2017) were sonication at 750 W and 20 kHz for 10 mins as a pre-treatment to seaweed protein extraction, whereby Rodrigues *et al.* (2015) applied 400 W and 50 to 60 kHz for 60 mins for seaweed protein extraction. However, high capacities and prolonged sonication duration in ultrasound-assisted extraction illustrated sharp elevations in extraction temperature in this study, which would lead to protein denaturation and consequently diminish seaweed protein yield and quality.

Building upon preliminary screening (performed by a previous student in the research group), findings by Kadam *et al.* (2017) and Rodrigues *et al.* (2015), the conditions for ultrasound-assisted extraction of red seaweed proteins were finalised to be 200 W and 20 kHz for 5 mins (sonication occurs for 1.2 sec and pauses for 0.8 sec, repeated for 5 min), utilising a seaweed biomass-water ratio of 1:40. The final ultrasound-assisted extraction protocol yields approximately 32.38% red seaweed protein solids, with a protein purity of 32.51%.

3.2.4 Enzyme-assisted extraction of seaweed proteins

3.2.4.1 Enzymatic extraction

Due to the rigid nature of seaweed cell walls, enzymes are bio-catalysts that could degrade cell wall constituents and ease protein extraction compared to conventional chemical and mechanical processes (Wijesinghe & Jeon, 2012). Enzyme-assisted extraction of seaweed proteins has augmented protein extraction efficiencies and yields (Bjarnadóttir *et al.*, 2018; Hardouin *et al.*, 2016; Mæhre *et al.*, 2016; Naseri *et al.*, 2020b; Rodrigues *et al.*, 2015; Vásquez *et al.*, 2019). As seaweed cell wall is complex and species-dependent, careful selection of enzymes and thorough optimisation of extraction conditions would be necessary (Harnedy & FitzGerald, 2013a). Several enzymes, namely Alcalase (protease), Celluclast (cellulase), Pectinex (polygalacturonase), and Shearzyme (xylanase) were selected in this study. Alcalase targets chemical bonds within protein structures, while Celluclast, Pectinex and Shearzyme are polysaccharidases that disintegrate polysaccharides within the seaweed cell wall to assist the release of proteins.

Protein extraction by single enzymes

Figure 3 illustrates the red seaweed protein content derived with different enzymes across varied extraction durations. Compared to controls (extractions in the absence of enzymes), Celluclast, Pectinex, and Shearzyme improved protein extraction yields, whereas Alcalase displayed reduced protein extraction yields. This finding would be concurrent with results from other literature, whereby utilising polysaccharidase during seaweed protein extraction could effectively augment protein yield (Bjarnadóttir *et al.*, 2018; Harnedy & FitzGerald, 2013a; Mæhre *et al.*, 2016).

Within the same enzyme, extraction durations at 16 and 24 hr typically derived significantly more protein than other durations (Figure 3, $p < 0.05$). For instance, in Figure 3, protein extraction yields following Alcalase-treatment and Celluclast-treatment peaked at 24 hr (25.16 and 40.00 mg protein/g raw seaweed, respectively), whereas protein extraction yields following Pectinex-treatment and Shearzyme-treatment peaked at 16 hr (39.70 and 44.88 mg/g, respectively). After the peak extraction yields at those hours, protein content derived by all enzymes for more than 24 hr significantly declined ($p < 0.05$). Prolonged extraction duration could lead to excess enzymatic hydrolysis, resulting in diminished protein content (Sitthiya *et al.*, 2018).

Among different enzymes in Figure 3, Shearzyme typically derived significantly greater protein content at 16 hr (44.88 mg protein/g raw seaweed) than other enzymes ($p < 0.05$). Each enzyme specialises in the cleavage of distinctive bonds; hence the varying protein content derived by polysaccharidases could reinforce the differentiating levels of carbohydrates within the seaweed. Shearzyme extracting the most proteins could imply the presence of more xylans or xylan-bound proteins than cellulose and galactans in KR seaweed. The exploitation of enzymes could further develop into extracting desired proteins bound to particular polysaccharides, and proteins of desired molecular weights (MW) (Bjarnadóttir *et al.*, 2018; Naseri *et al.*, 2020b).

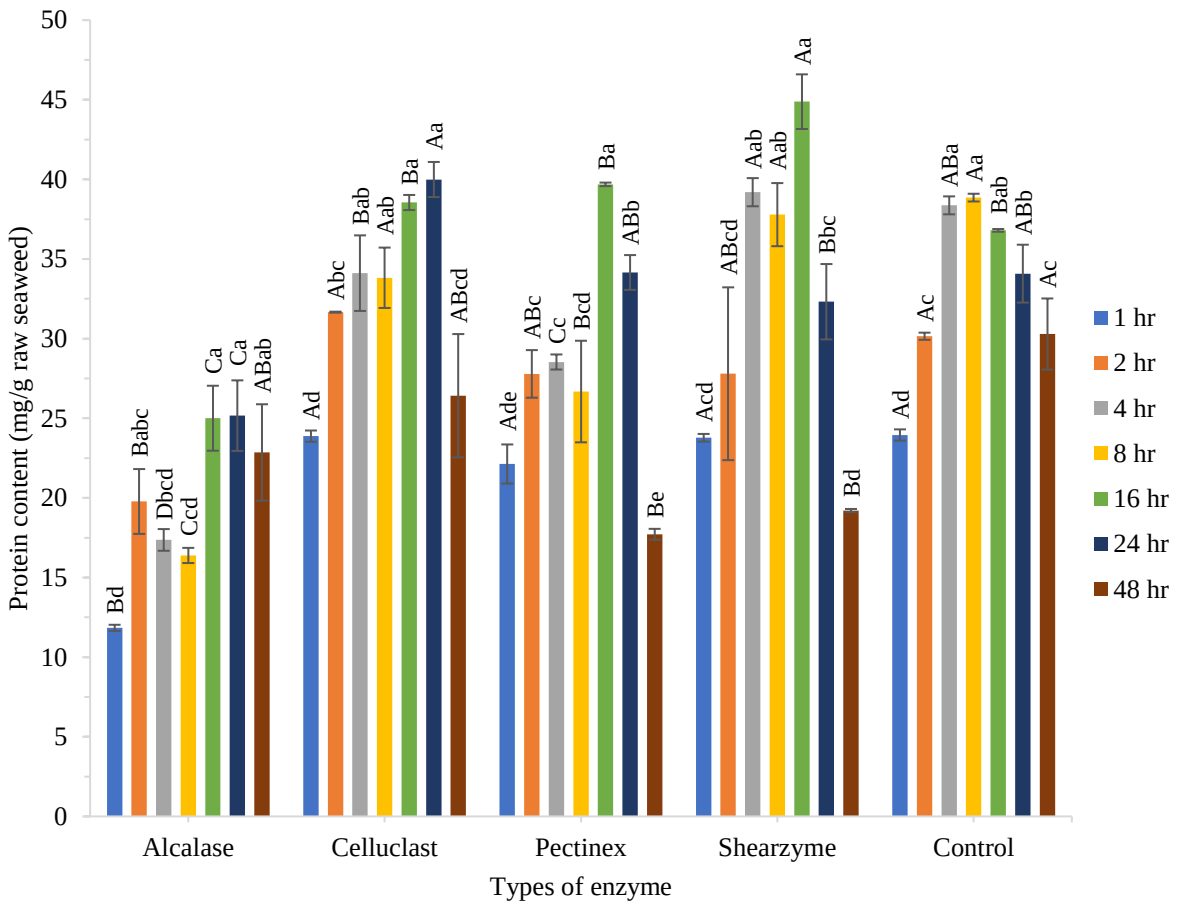


Figure 3. Seaweed protein content measured from the optimisation of enzymatic extraction using 1% single enzyme.

Protein content was measured with Bradford analysis; uppercase letters (A, B, C, D) compare different samples of the same extraction duration, while lowercase letters (a, b, c, d) compare between different extraction durations of the same sample; different letters within each case indicate statistical significance (One-way ANOVA with Duncan's multiple range test, n = 2, $p < 0.05$).

Surprisingly as shown in Figure 2, Alcalase generally extracted less seaweed protein than other enzymes and their respective controls; Alcalase derived 25.00 mg protein/g seaweed at 16 hr, yet other polysaccharidases derived 38.98 to 44.22 mg/g, and control derived 41.23 mg/g (Figure 3). This was a contrasting occurrence to that reported by Naseri *et al.* (2020b) and Hardouin *et al.* (2016). Naseri *et al.* (2020b) indicated that Alcalase doubled red *P. palmata* protein extraction efficiency (above 80%) compared to polysaccharidases (around 40%). Hardouin *et al.* (2016) also suggested that proteases (76.7% to 88.4% extraction yield) were more effective than polysaccharidases (44.6% to 45.%) during protein extraction from green *U. armoricana*. Both Harnedy and FitzGerald (2013a) and Naseri *et al.* (2020b) measured protein content with the Lowry method, whereas the current study employed the Bradford method. The specificity of different protein quantification assays could lead to variations in results. Lowry method was not employed in this study because an overestimation of seaweed protein has been witnessed when using the Lowry method (Harrysson *et al.*, 2018; Mæhre *et al.*, 2018)

As seaweed protein yield from enzyme-assisted extraction was lower than expected, some constructive trials were performed to improve extraction yield. These included modifying filters (membrane filter compared to paper filter) and adjusting enzyme inactivation protocols (temperature and duration). However, such trials implied insignificant influence on protein extraction yield (Appendix A, Table A1). Hence, enzyme-assisted extraction efficiency was likely to be substantially constrained by the rigid nature of seaweed cell wall constituents. Deniaud *et al.* (2003) elucidated that seaweed polysaccharide linkages, such as 1,4-linked xylose, could be too short for hydrolysis by polysaccharidases, or other similar structures could have disguised such linkages. Moreover, irregular distribution of polysaccharides within the seaweed cell wall could influence polysaccharide hydrolysis (Deniaud *et al.*, 2003).

Protein extraction by enzyme combinations

As Shearzyme demonstrated the most promising seaweed protein extraction ability among all selected enzymes, it was combined with other enzymes to elevate protein yield further. Exploiting enzyme "cocktails" in seaweed protein extraction have been conducted previously: Naseri *et al.* (2020b) paired Alcalase/polysaccharidases, Vásquez *et al.* (2019) paired *α*-amylase/cellulase, Bjarnadóttir *et al.* (2018) paired xylanase/Umamizyme (protease), Mæhre *et al.* (2016) paired cellulase/xylanase, and Harnedy and FitzGerald (2013a) paired

Celluclast/Shearzyme. Figure 4 illustrates the seaweed protein content derived from three enzyme combinations, namely Celluclast/Shearzyme (0.5% Celluclast and 0.5% Shearzyme), Pectinex/Shearzyme (0.5% Pectinex and 0.5% Shearzyme) and Celluclast/Pectinex/Shearzyme (0.33% Celluclast, 0.33% Pectinex and 0.33% Shearzyme).

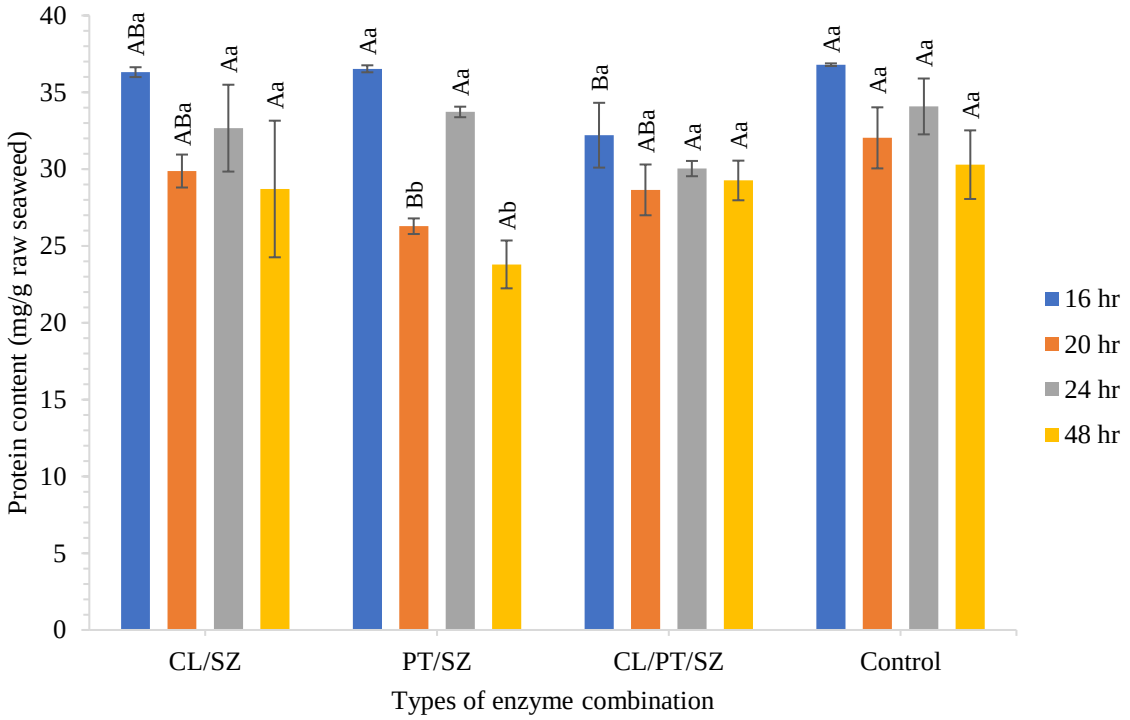


Figure 4. Seaweed protein content measured during the optimisation of enzymatic extraction with 1% enzyme combinations.

CL was Celluclast, PT was Pectinex, SZ was Shearzyme, and control was no enzyme added. Protein content was measured with Bradford analysis; uppercase letters (A, B, C, D) compare different enzymes combinations of the same extraction duration, while lowercase letters (a, b, c, d) compare between different extraction durations of the same enzyme combinations; different letters within each case indicate statistical significance (One-way ANOVA with Duncan's multiple range test, n = 2, $p < 0.05$).

Seaweed protein extracted by enzyme combinations exhibited no significant difference from their respective controls across all extraction durations (Figure 4, $p > 0.05$). Nonetheless, enzyme combinations did not ameliorate protein yield compared to the extraction with single enzymes. For example, the greatest protein amount extracted by Shearzyme was 44.88 mg protein/g of raw seaweed at 16 hr (Figure 3), yet the greatest protein amount derived from an enzyme combination of Pectinex/Shearzyme was 35.56 mg/g (Figure 4). An analogous phenomenon was noticed by Bjarnadóttir *et al.* (2018), whereby xylanase alone produced a

higher protein extraction yield in red *P. palmata* (up to 54.9% seaweed DW) compared to the combination of xylanase/Umamizyme (up to 35% DW). Furthermore, Vásquez *et al.* (2019) elucidated that polysaccharidase combination had no significant differences in activity compared to individual enzyme extractions for yielding brown *M. pyrifera* and red *C. chamissoi* proteins ($p > 0.05$). Vásquez *et al.* (2019) proposed that pH incompatibilities between mixed polysaccharidases would influence protein extraction yield. In the current study, preferred conditions for optimal enzyme activity were dissimilar between polysaccharidases, which could have disturbed protein extraction. For instance, the optimal pH for Celluclast would be 4.5 for Celluclast and Pectinex (Naseri *et al.*, 2020b; Vásquez *et al.*, 2019), compared to pH 7 for Shearzyme (Harnedy & FitzGerald, 2013a). In this study, protein extraction with enzyme combinations were conducted at pH 5 as per guidelines from the manufacturer. Therefore, the incompatibilities between polysaccharidases could be responsible for the negligible seaweed protein yield improvement during extraction with enzyme combinations.

3.2.4.2 Enzymatic pre-treatment followed with alkaline post-treatment

Both alkaline extraction and enzyme-assisted extraction revealed encouraging seaweed protein extraction ability. Thus, to further improve protein extraction, the integration of enzyme-assisted extraction with alkaline extraction into enzymatic pre-treatment followed by alkaline post-treatment was investigated. Such extraction integrations have been explored by Naseri *et al.* (2020b), Mæhre et al. (2016) and Harnedy and FitzGerald (2013a), all evincing that exploiting enzyme-assisted extraction as a pre-treatment would be an effective measure for seaweed cell wall degradation.

Table 16 portrays the relevant optimisation and results of incorporating enzyme-assisted extraction with alkaline extraction. In general, pre-treatment by polysaccharidases (Celluclast Pectinex and Shearzyme) could better facilitate extraction than protease (Alcalase). The combination of enzyme pre-treatment with Shearzyme followed by alkaline post-treatment at pH 8 demonstrated the highest seaweed protein yield of 39.88 mg protein/g raw seaweed (Table 16). Such results agree with enzyme-assisted extraction data utilising single enzyme (Figure 3). It would be interesting to note that, as the pH of the alkaline post-treatment increased, extraction yields by polysaccharidases and Alcalase conveyed the opposite trends. For polysacharidases, alkaline post-treatment at pH 8 derived more proteins than higher pHs (Table 16, $p < 0.05$). In contrast, Alcalase coupled with post-treatment at pH 10 and 12 derived

significantly more proteins than pH 8 (Table 16, $p < 0.05$). Such results could be associated with the varying enzyme working mechanisms and their targeted cleavage of different bonds, as discussed in *Section 3.2.4.1*.

Table 16. Enzymatic pre-treatment followed by alkaline post-treatment for extracting seaweed proteins.

1. Enzymatic pre-treatment		**2. Alkaline post-treatment**		
Enzyme	**Amount of enzyme (%)**	**Protein content (mg/g raw seaweed)[1]**		
		pH 8	**pH 10**	**pH 12**
Alcalase	200	2.79 ± 0.08^{Cb}	7.08 ± 0.35^{Ba}	8.70 ± 1.07^{Aa}
Celluclast	200	34.03 ± 2.87^{Aa}	28.90 ± 1.07^{Aa}	4.36 ± 0.45^{Bb}
Pectinex	200	15.97 ± 1.17^{Ba}	11.57 ± 1.73^{Ba}	1.80 ± 0.24^{Cb}
Shearzyme	200	39.88 ± 0.15^{Aa}	25.46 ± 3.52^{Ab}	6.96 ± 0.00^{Ac}

[1] Protein content measured through Bradford analysis; uppercase letters (A, B, C) compare different enzymes of the same extraction duration, lowercase letters (a, b, c) compare between different extraction durations of the same enzyme, different letters within each case indicate statistical significance (One-way ANOVA with Duncan's multiple range test, n = 2, $p < 0.05$).

Naseri *et al.* (2020b) unveiled a 90% extraction efficiency with Alcalase/Celluclast pre-treatment followed by N-acetyl-L-cysteine-assisted alkaline extraction. Mæhre *et al*. (2016) reported a 1.6-fold protein extraction yield was derived with xylanase/cellulase pre-treatment followed by 0.1 M NaOH post-treatment compared to alkaline extraction with no enzyme pre-treatment. Harnedy and FitzGerald (2013a) claimed that polysaccharidase pre-treatment in conjunction with alkaline extraction was effective for protein extraction. In contrast to findings by Naseri *et al.* (2020b), Mæhre et al. (2016) and Harnedy and FitzGerald (2013a), Table 16 conveyed that combining enzyme-assisted extraction with alkaline extraction had negligible improvement from the utilisation of single polysaccharidases in Figure 3. As mentioned previously, Shearzyme derived 44.88 mg protein/g raw seaweed at 16 hr, whereas the best enzyme-alkaline assisted extraction with Shearzyme only yielded 39.88 mg/g.

Inconsistencies between the current study and existing literature could be the nature of different seaweeds. Naseri *et al.* (2020b), Mæhre et al. (2016) and Harnedy and FitzGerald (2013a) explored protein extraction in red *P. palmata*, which are not the same species as KR seaweed. Seaweeds of different species and growth locations would have discrete characteristics due to various intrinsic and extrinsic factors (Mišurcová, 2012). Thus, varying seaweed cell wall components would sequentially influence protein extraction. Nonetheless, the protein production in seaweeds could be correlated to the concentration of nitrogenous nutrients in

seawater, which would vary during different harvesting seasons and in different oceans (Galland-Irmouli *et al.*, 1999). Therefore, additional studies would be necessary to comprehend seaweed compositional and structural characteristics better. Thus, to pursue other suitable enzymes for enzyme-alkaline-assisted protein extraction from seaweeds in this study.

It would also be essential to note that, till now, seaweed protein content has been measured with the Bradford method due to its rapid reaction and friendliness to large sample sizes. However, this method only provides a relative quantity of proteins in samples, and the varying protein types within seaweeds could substantially limit its accuracy. The Bradford reagent detects specific residues within proteins and would be unable to bind to some peptides and free AAs (Kruger, 2009). In summary, protein extraction with protease would cleave proteins into smaller peptides and free AAs, thus, diminishing the efficiency of Bradford reagent and affecting the accuracy of protein quantification.

3.2.4.3 Protein quantification of enzyme-assisted extraction

Despite being highly destructive and time-consuming, the Kjeldahl method has been well-recognised for protein quantification as it measures the total nitrogen content within food materials (AOAC, 2005; Kjeldahl, 1883). Previous enzyme-assisted extractions were revised and verified with the Kjeldahl method to decipher seaweed protein content derived from polysaccharidases (Celluclast, Pectinex and Shearzyme) and protease (Alcalase). Table 17 illustrates the amount of seaweed protein extracted by different enzymes across various extraction durations. Unlike previous results, Alcalase exhibited the greatest protein yield compared to the three polysaccharides across different extraction durations, reaching up to 193.20 mg protein/g seaweed ($p < 0.05$). Most importantly, Alcalase extraction efficiency was more than double of Celluclast, Pectinex and Shearzyme (94.50, 94.50, and 87.50 mg/g, respectively).

Table 17. Enzymatic extraction of seaweed protein measured with the Kjeldahl method.

Enzyme	**Amount of enzyme (%)**	**Protein content (mg/g seaweed)[1]**			
		Extraction duration (hr)			
		1	**6**	**16**	**24**
Alcalase	1	114.80 ± 2.80^{Ac}	145.60 ± 0.00^{Ab}	190.40 ± 5.60^{Aa}	193.20 ± 2.80^{Aa}
Celluclast	1	66.50 ± 3.50^{Bc}	84.00 ± 0.00^{Bb}	94.50 ± 3.50^{Ba}	84.00 ± 0.00^{Bb}
Pectinex	1	73.50 ± 3.50^{Bb}	87.50 ± 3.50^{Ba}	94.50 ± 3.50^{Ba}	84.00 ± 0.00^{Bab}
Shearzyme	1	77.00 ± 7.00^{Bab}	70.00 ± 0.00^{Cb}	77.00 ± 0.00^{Cab}	87.50 ± 3.50^{Ba}

[1] Protein content quantified through Kjeldahl method with the universal seaweed nitrogen-to-protein conversion factor of 5.00 (Angell *et al.*, 2016); uppercase letters (A, B, C) compare different enzymes of the same extraction duration, lowercase letters (a, b, c) compare between different extraction durations of the same enzyme, different letters within each case indicate statistical significance (One-way ANOVA with Duncan's multiple range test, n = 2, $p < 0.05$).

Discoveries from Table 17 correspond to previous findings in Figure 3, Figure 4 and Table 16. For example, protein content measured by the Kjeldahl method was typically greater than the Bradford method; Shearzyme extraction for 16 hr derived in 44.88 mg protein/g seaweed with Bradford (Figure 3), and 94.50 mg/g with Kjeldahl (Table 17). It would be essential to note that non-proteinaceous nitrogen exists in seaweeds and would be consequently detected by Kjeldahl (Conde *et al.*, 2013). Despite utilising an NPCF designated for seaweeds to counter over-estimation, interspecies variation for seaweeds requires consideration. Additionally, Alcalase displayed the best protein extraction yield (Table 17, 193.20 mg/g), opposing previous findings of Alcalase being the least effective (Figure 3, 25.00 mg/g; Table 16, 8.70 mg/g). With reference to KR seaweed protein content in Table 12, Alcalase reached 68% protein yield, whereas polysaccharidases reached 33% protein yield. In such a manner, results in Table 17 would be consistent with findings by Naseri *et al.* (2020b) and Hardouin *et al.* (2016), whereby Alcalase-treatment approximately doubled seaweed protein yield compared to no Alcalase-treatment ($p < 0.05$). Naseri *et al.* (2020b) explained that protease hydrolyses peptide bonds within the polypeptide backbone in seaweed proteins, resulting in smaller subunits that could drastically augment their extraction. Protease could also destabilise seaweed cell walls, resulting in the eased release and ameliorated solubilisation of proteins (Hardouin *et al.*, 2016).

Protease catalyses proteolysis, disintegrating proteins into smaller subunits, peptides and AAs. As mentioned earlier, the Bradford reagent recognises and binds to arginine, lysine, histidine and aromatic residues within proteins and would be insufficient for binding to free AAs or peptides under 3 kDa (Kruger, 2009). Protein extraction with Alcalase would have cleaved

some proteins into smaller peptides and AAs, leading to a heterogeneous protein profile with inadequate detection from Bradford (Xu *et al.*, 2021). Hence, results from Figure 3, Figure 4 and Table 16 could be unfitting quantifications of seaweed proteins derived from Alcalase.

On the contrary, polysaccharidases cleave bonds between polysaccharides, such as agars, carrageenans and xylans. Whole proteins would be more likely to have been extracted by Celluclast, Pectinex and Shearzyme, suggesting adequate Bradford detection. Ultimately, the Bradford method would be more sufficient for quantifying whole proteins derived from polysaccharidases, whereas the Kjeldahl method would be more suitable for quantifying peptides and AAs derived from proteases. Moreover, this amplifies the viable exploitation of enzymes with different activities to derive different bioactive compounds within seaweed. Bjarnadóttir *et al.* (2018) and Naseri *et al.* (2020b) agreed that protease could effectively isolate small peptides and AAs with respective to enhance bioavailability. Nevertheless, proteases with a low degree of hydrolysis could be pursued, as they might concurrently provide effective protein yield without damaging the native structure of proteins.

The greatest seaweed protein yield extracted by Celluclast, Pectinex and Shearzyme was 94.50, 94.50, and 87.50 mg protein/g seaweed, respectively (Table 17, $p < 0.05$). In contrast, Alcalase could reach 193.20 mg/g. It would be also worth mentioning that during seaweed protein extraction, the extraction mediums were adjusted to the enzymes' preferred conditions as recommended by the manufacturer and literature, such as pH 9 for Alcalase, and pH 5 for Celluclast, Pectinex and Shearzyme. Alcalase extraction of proteins occurred at an alkaline pH, whereas protein extraction with polysaccharidases was performed at a more acidic pH. As witnessed in the optimisation of alkaline extraction (*Section 3.2.2*) and in literature, alkaline conditions augment seaweed protein extraction (Harnedy & FitzGerald, 2013a; Harrysson *et al.*, 2018; Kadam *et al.*, 2017). Hence, extractions at pH 5 by polysaccharidases would be undesirable. Extraction of proteins with Alcalase at pH 9 could synergistically stimulate the improvement of protein extraction, as Alcalase extraction at pH 9 accommodates the advantage of Alcalase being a protease and the benefit of alkaline conditions in one extraction. As an objective of this study was to ameliorate the extraction of seaweed proteins, enzyme-assisted extraction with Alcalase successfully augmented extraction efficiency and yield. Thus, Alcalase-treatment was selected for the optimised enzyme-assisted extraction. Conclusively, the final optimal enzyme-assisted extraction conditions were with Alcalase, at extraction pH 9

for an extraction duration of 16 hr. Extraction duration of 16 hr was selected due to its insignificance compared to 24 hr ($p > 0.05$).

3.2.4.4 Protein precipitation of enzyme-assisted extraction

Protein precipitation enables the isolation and purification of proteins from the raw extract. As enzyme-assisted extraction exhibited desired seaweed protein extraction yields, seaweed protein extracts (SPEs) from enzyme-assisted extraction were precipitated to improve protein concentration and purity. For example, Crowell *et al.* (2013) elucidated that optimal protein recovery generally requires the overnight incubation of samples in 75% to 80% acetone at 4 °C to -20 °C; Hardouin *et al.* (2016) recovered seaweed polysaccharides with overnight incubation of samples in ethanol at 4 °C; both Akyüz and Ersus (2021) and Garcia-Vaquero *et al.* (2017) discovered that protein precipitation required 80% to 85% ammonium sulphate with dialysis at 4 °C overnight. Nonetheless, protein precipitations with acetone, ethanol and ammonium sulphate were utilised to identify the optimal protocol for protein precipitation. Figure 5 compares the acetone, ethanol and ammonium sulphate precipitations of proteins derived from enzyme-assisted extraction.

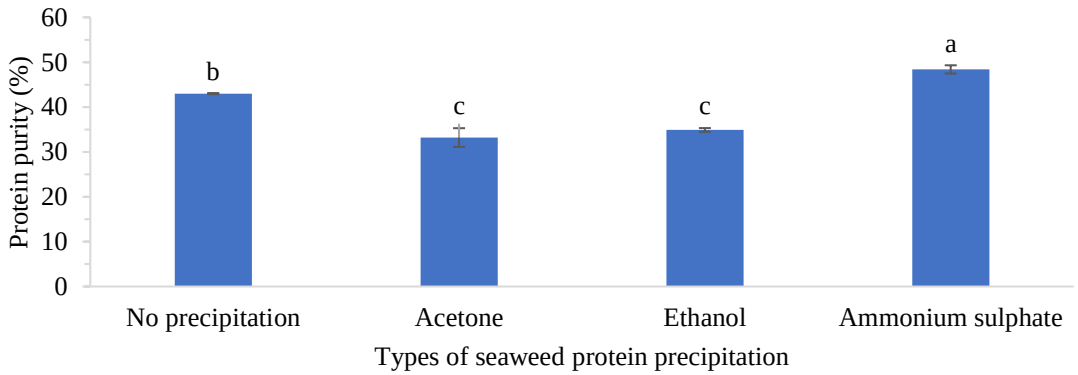


Figure 5. Precipitation of seaweed proteins from enzymatic extraction.

No precipitation was enzyme-assisted seaweed protein extract without precipitation. Protein content was measured through the Kjeldahl method using the universal seaweed nitrogen-to-protein conversion factor of 5.00 (Angell *et al.*, 2016); different letters (a, b, c, d) indicate statistical significance among different precipitation types (One-way ANOVA with Duncan's multiple range test, n = 3, $p < 0.05$).

In Figure 5, ammonium sulphate precipitation derived significantly higher protein purity (48.40%) compared to acetone and ethanol precipitations (33.21% and 34.90%, respectively), and no precipitation (43.01%) ($p < 0.05$). Moreover, precipitations by both acetone and ethanol

yielded significantly lower seaweed protein purities (33.21% and 34.90%, respectively) than SPEs from enzyme-assisted extraction without precipitation (43.01%); hence, they were deemed inappropriate for protein precipitation for this study (Figure 5, $p < 0.05$). Unlike the expected improvements in purity with acetone and ethanol precipitation, a reduction in protein purity was perceived in Figure 5. Protein precipitation with acetone or ethanol would be more effective for proteins with larger MWs than ammonium sulphate (Scopes, 1982). However, SPEs from enzyme-assisted extraction would accommodate smaller peptides and AAs, which would have smaller MWs than intact proteins. The lack of whole proteins would hinder protein precipitation with acetone and ethanol, thus diminishing protein purity.

Conversely, protein precipitation with ammonium sulphate could improve the recovery of low MW proteins and peptides (Baghalabadi *et al.*, 2021). Salt could limit interaction between water molecules and proteins and peptides, reducing protein hydration, promoting hydrophobic residue aggregation, and thus enhancing protein precipitation; this phenomenon is often recognised as "salting-out" (Scopes, 1982). Additionally, salts could elevate ionic strength during protein precipitation, impairing protein solubility, which would further stimulate "salting-out" (Duong-Ly & Gabelli, 2014). Such occurrence is portrayed in Figure 5, whereby ammonium sulphate precipitates contained significantly greater protein purity than acetone and ethanol precipitates ($p < 0.05$).

Isoelectric point protein precipitation was also conducted in accordance with Naseri *et al.* (2020b) with slight modification. The isoelectric point of SPEs following enzyme-assisted extraction was discovered to be between pH 2 to 3.5 (Appendix A, Figure A1). Naseri *et al.* (2020b) elucidated that isoelectric point precipitation of seaweed proteins required lowering the pH of samples to approximately 3 using < 5 M HCl. However, unlike acetone, ethanol and ammonium sulphate precipitations, protein aggregates at isoelectric point were negligible. A similar occurrence was noticed for red *P. palmata* seaweed proteins, whereby proteins extracted by Alcalase were difficult to precipitate at isoelectric point (Naseri *et al.*, 2020b). As new peptides by Alcalase-treatment could be different to the original seaweed protein, they would have other and more isoelectric points; the precipitation of one peptide at one pH could lead to the solubilisation of other precipitated peptides (Naseri *et al.*, 2020b).

Unfortunately, seaweed protein loss was inevitable during protein precipitation despite significantly augmented protein purity with ammonium sulphate followed by dialysis (Figure

5, $p < 0.05$). During protein precipitation, achieving 48.40% purity with ammonium sulphate precipitation sacrificed approximately 85% SPE. Though protein loss would be unavoidable for all protein precipitation techniques, an 85% loss could not be justified. A similar occurrence was witnessed by Suresh Kumar *et al.* (2014), whereby approximately 92% of red *K. alvarezii* protein was sacrificed to obtain a protein concentrate with 62.3% purity. Moreover, the purity of ammonium sulphate precipitate (48.40%) was not drastically improved from no precipitation (Figure 5, 43.01%). In addition, protein precipitation with various solvents and salts would not be food grade, consequently obstructing the scalability of this technique at an industrial level. Therefore, as the optimised enzyme-assisted extraction have fulfilled the objective of improving the extraction of seaweed proteins, protein precipitation was not considered of great importance due to exigent protein loss.

In summation, incorporating results from *Sections 3.2.4.1*, *3.2.4.2*, *3.2.4.3* and *3.2.4.4*, the optimal conditions for enzyme-assisted extraction were with Alcalase, at an extraction pH 9 for an extraction duration of 16 hr, utilising a seaweed biomass-water ratio of 1:40. The final enzyme-assisted extraction protocol yields approximately 32.29% red seaweed protein solids, with a protein purity of 43.01%.

3.2.5 Comparison of various seaweed protein extracts

Based on resulted from *Sections 2.4.1, 3.2.2, 3.2.3* and *3.2.4*, the final optimised protocols for deriving various red SPEs are described in Table 18. Enzyme-assisted extraction of seaweed proteins utilises protease-assisted extraction with Alcalase at pH 9 for 16 hr, alkaline extraction was conducted at pH 12, ultrasound-assisted extraction occurred at 200 W and 20 kHz for 5 mins (sonication occurs for 1.2 sec and pauses for 0.8 sec, repeated for 5 min), and classic water extraction happened for 4 hr in deionised water. Enzyme-assisted extraction had significantly greater protein purity than all other SPEs (43.01%, $p < 0.05$), followed by alkaline, ultrasound-assisted and water extractions.

As enzyme-assisted extraction produced the SPE with the highest purity and yielded the most protein content (Table 18), its extraction protocol was also applied to NZ seaweed to augment NZ seaweed protein extraction. Enzyme-assisted extraction of NZ seaweed yielded approximately 35.79% red seaweed protein solids, with a protein purity of 27.20%.

Table 18. Comparison between final optimised protocols for the extraction of seaweed proteins.

Types of extraction	**Extraction conditions**					**Protein purity (%)[3]**
	Biomass-water ratio (g seaweed:mL water)	**pH**	**Temperature**	**Duration**	**Other conditions**	
Water	1:40	Water[1]	23 °C[2]	4 hr	N/A	33.57 ± 0.39[c]
Alkaline	1:30	pH 12	23 °C	4 hr	N/A	37.48 ± 0.16[b]
Ultrasound -assisted	1:40	Water	23 °C	0.08 hr	200 W 20 kHz	32.51 ± 1.27[c]
Enzyme -assisted	1:40	pH 9	23 °C	16 hr	1% Alcalase	43.01 ± 0.12[a]

[1] Water, deionised water; [2] 23 °C, room temperature; [3] protein purity quantified through Kjeldahl method with the universal seaweed nitrogen-to-protein conversion factor of 5.00 (Angell *et al.*, 2016); different letters (a, b, c) indicate statistical significance among different SPEs (One-way ANOVA with Duncan's multiple range test, n = 3, $p < 0.5$).

Protein content within the Alcalase enzyme could be dismissed during enzyme-assisted extraction, as Alcalase comprised 5.98% protein and only 1% Alcalase was incorporated in

extraction (Appendix A, Table A2). Additional to constituting the greatest protein purity and content from enzyme-assisted extraction (Table 18, $p < 0.05$), SPEs following Alcalase-treatment could be exploited to obtain refined peptides and low MW proteins. Previous literature has established bioactivity within seaweed proteins. For example, Admassu *et al.* (2018b) demonstrated that red *Porphyra spp.* peptides had α-amylase inhibition activity; Beaulieu *et al.* (2016) discovered important antioxidant and ACE inhibitory potential in the peptide from red *P. palmata*. Furthermore, anti-diabetic peptides have been isolated in *P. palmata* (Harnedy *et al.*, 2015); anti-platelet, antioxidant and angiotensin-converting enzyme (ACE)-inhibition peptides that displayed increased activity after digestion have been documented in red *P. columbina* (Cian *et al.*, 2015). Nevertheless, comprehension of seaweed peptides and low MW proteins could be further analysed through AA profiling and protein MW distributions.

Although alkaline extraction produced lower protein content (37.48%) than enzyme-assisted extraction (43.01%), it required a significantly shorter duration (Table 18, 4 hr for alkaline extraction compared to 16 hr for enzyme-assisted extraction). The impact of alkaline extraction could also relate to the hydrogen-bonding nature of polysaccharides in the seaweed cell wall (Deniaud *et al.*, 2003). Extractions at high pHs destroy hydrogen bonds and covalent linkages of seaweed polysaccharides, which would strengthen the extraction of polysaccharide bound proteins. However, conducting alkaline extraction at pH 12 could be conferred to the loss of several AAs, as the availability of lysine, cysteine, serine, and threonine diminish at high pHs (Weder & Belitz, 2003). Alkaline treatment of proteins also elevates occurrences of unusual AAs, such as lysinoalanine and lanthionine (Weder & Belitz, 2003). When food proteins are subjected to high pH, β-elimination of cysteine and serine generates dehydroalanine, which interacts with lysine and cysteine to form aminoacyl cross-linked lysinoalanine (LAL) and lanthionine (LAN) (Friedman, 1999). Depending on food protein type, LAL and LAN could hinder protein quality, impairing proteins' functional and nutritional values (Weder & Belitz, 2003). Formation of LAL and LAN have been reported during the alkaline extraction of green *Ulva fenestrate* proteins at pH 8.5 and 12 (Juul *et al.*, 2021). However, reported contents of *U. fenestrata* LAL (up to 62 ng/mg protein), and LAN (up to 90 ng/mg protein) by Juul *et al.* (2021) were well-below common foods, such as oven-baked chicken thighs, boiled egg whites and milk (Weder & Belitz, 2003). Furthermore, LAL and LAN formation would be enhanced at high temperatures (Harnedy & FitzGerald, 2013a; Juul *et al.*, 2021). In the current study,

alkaline extraction was conducted at 23 °C, which would prevent the extensive formation of LAN and LAL. However, this would warrant validation through AA profiling.

Protein content derived from ultrasound-assisted and water extractions were not significantly different, 32.51% and 33.57%, respectively (Table 18, $p > 0.05$). This was unexpected as ultrasound treatment has sufficiently augmented seaweed protein yield in literature, such as 57.23% brown *A. nodosum* protein extracted (Kadam *et al.*, 2017), 86% of red *Gracilaria* sp. DW, and 70% of green *Ulva* sp. DW (Kazir *et al.*, 2019). In the present study, the lack of protein yield improvement during ultrasound-assisted extraction could be due to processing conditions and the variation amongst seaweed species. Protein aggregation in the ultrasound-assisted extraction could also hinder protein solubility, limiting protein extraction efficiency (Jiang *et al.*, 2014). Moreover, Kadam *et al.* (2017) and Kazir *et al.* (2019) combined alkaline extraction and ultrasound-assisted extraction. As mentioned in *Section 3.2.3*, Kadam *et al.* (2017) and Kazir *et al.* (2019) of literature utilised elevated ultrasonic capacities and prolonged extraction duration. Ultimately, the synergistic effect of various extraction parameters would provoke differences observed between this study and other literature.

Subsequently, to insightfully explore the differences between SPEs, their physicochemical and functional properties were analysed. The behaviour and functionality of proteins depend on their physicochemical properties. Insights into protein properties would promote successful commercialisation at an industrial scale and broaden the incorporation of red seaweed proteins as value-adding functional ingredients.

3.3 Physicochemical properties of seaweed proteins

3.3.1 Molecular weight distribution

The molecular weight (MW) of proteins reflects protein structure and provides insight into protein functionality. For seaweeds, different species of seaweeds constitute a notable variance in the type of proteins (Pangestuti & Kim, 2015). Proteins are subjected to modification when undergoing different processing protocols, as extraction conditions such as temperature and pH could alter protein structure (Kadam *et al.*, 2017; Liang *et al.*, 2020). After undergoing varying extraction protocols, this study analysed the MWs of seaweed protein extracts (SPEs) from different seaweed species. Extraction protocols were water, alkaline, ultrasound-assisted and enzyme-assisted extractions, red seaweeds were the commercially cultivated *Porphyra* sp. from Korea (KR) and harvested *Pyropia* spp. from wild oceans in New Zealand (NZ).

Figure 6 presents the MW distribution of different SPEs and commercial protein isolates. Soy protein isolate (SPI) and whey protein isolate (WPI) exhibited greater protein MW ranges, and darker protein bands compared SPEs (Figure 6). SPI and WPI illustrated higher intensities and greater widths of protein bands on the SDS-PAGE gel than SPEs, implying more protein content than SPEs, which could be further confirmed through AA profiling. Typically, SPEs contain lower MW proteins and peptides than SPI and WPI, suggesting SPEs could be exploited as a source of low MW proteins and peptides.

For SPEs, most protein bands were identified from 50 kDa to below 10 kDa. All SPEs from KR seaweed shared the bands between 15 to 20 kDa (Figure 6). Bands around 10 kDa were also noticed in all KR SPEs, but such bands were more prominent for KR SPEs from alkaline and enzyme-assisted extractions (Figure 6), suggesting some protein hydrolysis during alkaline and enzyme treatments. Kadam *et al.* (2017) conferred that alkaline extraction could initiate protein hydrolysis, yielding to a greater extent of short peptides with low MWs. Likewise, Alcalase (protease) modifies the surface hydrophobicity of proteins, consequently influencing protein structure and impacting the MW of proteins (Zhao *et al.*, 2011). Cian *et al.* (2015) observed a reduction in MW of red *P. columbina* peptides to 2.2 kDa after being treated with Alcalase. The production of small peptides from Alcalase-treatment could be developed for deriving low MW fractions of proteins (Bjarnadóttir *et al.*, 2018; Naseri *et al.*, 2020b).

KR SPE produced from ultrasound-assisted extraction contained band widths around 20, 15 and 10 kDa, which were similar to KR SPE from water extraction (Figure 6). This could be associated with their lower protein content than other SPEs; SPEs from ultrasound-assisted and water extractions showcased insignificance differences across their protein purity (Table 18, $p > 0.05$), and could be further verified with AA profiling. Compared to other SPEs, one distinguishable band around 60 kDa was noticed only for SPE from ultrasound-assisted extraction. Such occurrence could imply potential protein aggregation during ultrasound-assisted extraction, which was revealed for black bean protein isolates following ultrasound treatment (Jiang *et al.*, 2014).

When comparing KR SPE and NZ SPE derived from enzyme-assisted extraction, KR SPE portrayed enhanced separation with weaker bands, whereas NZ SPE showcased impaired separation but more prominent bands (Figure 6). For enzyme-assisted extraction, the staining of NZ SPE around 15 and 10 kDa was darker than KR SPE in Figure 6; which could emphasise that NZ SPE yielded more low MW proteins and peptides than KR SPE. Such phenomenon would arise from species differences. Nonetheless, it would be possible to ameliorate the resolution and separation of various seaweed protein samples through optimising sample preparation and gel types.

Different protein bands established by KR SPEs could be associated with specific proteins (Figure 6). Prominent staining around 20 and 15 kDa could be related to α and β units from phycoerythrin, both units have a MW of around 20 kDa (Harnedy & FitzGerald, 2013a; Munier *et al.*, 2015). Phycoerythrin is a highly pigmented and soluble phycobiliprotein in red seaweeds, commonly utilised as fluorescent markers, natural dyes and pharmaceuticals (Dumay *et al.*, 2013; Sekar & Chandramohan, 2008). Furthermore, staining between protein bands is present across all SPEs on the SDS-PAGE gel, possibly due to covalent bonding between proteins and polysaccharides (Kazir *et al.*, 2019). Moreover, low MW peptide fragments have been well established for their bioactivity (Pangestuti & Kim, 2015). Admassu *et al.* (2018b) demonstrated that < 3 kDa red *Porphyra spp.* peptides, Gly-Gly-Ser-Lys and Glu-Leu-Ser, had α-amylase inhibition activity; Beaulieu *et al.* (2016) illustrated that red *P. palmata* peptide with less than 10 kDa highlighted important antioxidant and ACE-inhibitory activities.a

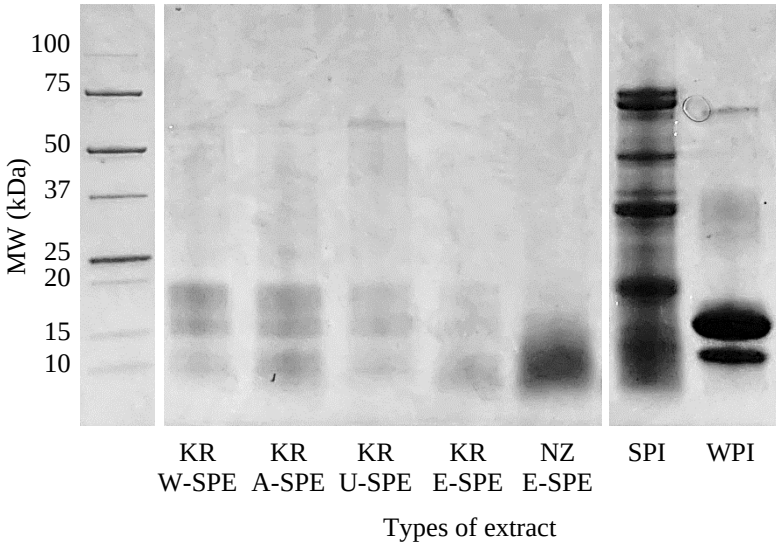


Figure 6. Molecular weight distribution of different seaweed protein extracts and commercial protein isolates.

KR, Korean commercial *Porphyra* sp.; NZ, New Zealand wild *Pyropia* spp.; SPE, seaweed protein extract; W-SPE, SPE from water extraction; A-SPE, SPE from alkaline extraction; U-SPE, SPE from ultrasound-assisted extraction; E-SPE, SPE from enzyme-assisted extraction. SPI, soy protein isolate; WPI, whey protein isolate.

MW of SPEs in Figure 6 would align with those reported in the literature. For example, Garcia-Vaquero *et al.* (2017) established five brown *H. elongate* protein bands between 71.6 to 27.1 kDa. Similarly, Harnedy and FitzGerald (2013a) conveyed that alkaline soluble and aqueous red *P. palmata* proteins have MWs from 55 to 14.8 kDA, and from 97 to 15.5 kDa, respectively. Rouxel *et al.* (2001) identified seven bands, ranging from 73.1 to 15.9 kDa in red *P. umbilicalis* protein. Protein MWs in this study were greater than the those revealed by Kadam *et al.* (2017), ranging between 3.8 to 2.6 kDa for brown *A. nodosum*.

In conclusion, MW distribution disclosed varying protein bands between SPEs and commercial protein isolates (SPI and WPI) (Figure 6). Different protein bands and MWs identified between this study and those in literature would be attributed to seaweed species, their cultivations in different geographic environments, and various seaweed protein extraction protocols (Beaulieu *et al.*, 2016; Mišurcová, 2012).

3.3.2 Amino acid composition

Amino acid (AA) composition is the primary structure of proteins, which constitutes critical information for evaluating protein quality. Table 19 quantifies the AA composition of raw seaweeds, different red SPEs and commercial protein isolates (SPI and WPI), as well as determines the quality of proteins through analysing total AA, essential AA:AA (EAA:AA), EAA:non-EAA (EAA:NAA), AA score (AAS) and EAA index (EAAI).

In Table 19, the total KR SPE AA following enzyme-assisted, alkaline, ultrasound-assisted and water extractions was 503.63, 349.42, 314.58, and 306.10 mg AA/g SPE, respectively, consistent with KR seaweed protein purity after different extraction protocols (Table 18). Other than SPE from water extraction, all KR SPEs demonstrated a greater increase in AA content than the raw KR seaweed (307.84 mg/g). In particular, KR SPE from enzyme-assisted extraction derived 163% greater AA content (503.63 mg/g) than its respective raw seaweed ($p < 0.05$); thus implying enzyme-assisted extraction would be more suitable for extracting proteins and releasing AAs from raw KR seaweed than other extraction protocols.

The total AA for NZ SPE from enzyme-assisted extraction and raw NZ seaweed was 273.31 and 233.70 mg/g, respectively, showcasing a significant 117% increase in total AA from raw NZ seaweed (Table 19, *t*-test, $p < 0.05$). For enzyme-assisted extraction, the relatively lower total AA for NZ SPE than KR SPE could be due to interspecies variation. The impressive AA extraction from enzyme treatment would be in conjunction with findings from the literature, whereby seaweed-derived protein content has ameliorated following enzyme-assisted extraction (Bjarnadóttir *et al.*, 2018; Hardouin *et al.*, 2016; Mæhre *et al.*, 2016; Naseri *et al.*, 2020b; Rodrigues *et al.*, 2015; Vásquez *et al.*, 2019).

Table 19. Amino acid composition of raw seaweeds, different seaweed protein extracts, and commercial protein isolates.

	Amino acid	Amino acid concentration (mg/g)									Scoring[3]
		Raw seaweeds[1]		SPEs[2]					Commercial protein isolate[1]		
		Raw KR seaweed	Raw NZ seaweed	KR W-SPE	KR A-SPE	KR U-SPE	KR E-SPE	NZ E-SPE	SPI	WPI	
1	Phenylalanine[4]	9.74 ± 0.42^{cd}	7.95 ± 0.37^{de}	5.91 ± 0.21^{e}	8.26 ± 0.41^{de}	6.17 ± 0.17^{e}	12.10 ± 0.16^{c}	7.75 ± 0.71^{de}	55.79 ± 1.85^{a}	41.79 ± 2.74^{b}	41[5]
2	Valine[4]	8.46 ± 0.48^{d}	4.28 ± 0.15^{efg}	3.95 ± 0.32^{fg}	5.52 ± 0.30^{e}	3.32 ± 0.07^{g}	10.24 ± 0.57^{c}	5.13 ± 0.44^{ef}	16.73 ± 0.53^{b}	22.88 ± 1.29^{a}	40
3	Proline	0.77 ± 0.06^{b}	0.77 ± 0.06^{b}	0.35 ± 0.01^{b}	0.51 ± 0.02^{b}	0.36 ± 0.02^{b}	0.91 ± 0.03^{b}	0.75 ± 0.03^{b}	9.52 ± 0.27^{a}	9.47 ± 0.87^{a}	
4	Methionine[4]	0.79 ± 0.03^{c}	0.59 ± 0.03^{cd}	0.29 ± 0.01^{e}	0.43 ± 0.05^{de}	0.31 ± 0.03^{e}	0.74 ± 0.05^{c}	0.66 ± 0.06^{c}	1.00 ± 0.03^{b}	4.63 ± 0.23^{a}	24[6]
5	Tryptophan[4]	ND	ND	ND	ND	ND	ND	ND	ND	ND	6.6
6	Leucine[4]	9.26 ± 0.05^{de}	7.68 ± 0.52^{e}	7.51 ± 0.31^{e}	11.71 ± 0.45^{d}	7.26 ± 0.52^{e}	21.49 ± 0.80^{c}	8.51 ± 0.22^{de}	38.09 ± 1.33^{b}	66.01 ± 3.66^{a}	61
7	Isoleucine[4]	9.83 ± 0.02^{d}	8.10 ± 0.58^{d}	7.94 ± 0.27^{d}	12.40 ± 0.45^{d}	7.72 ± 0.55^{d}	22.77 ± 0.69^{c}	8.98 ± 0.27^{d}	39.82 ± 1.29^{b}	73.10 ± 6.76^{a}	31
8	Alanine	8.43 ± 0.56^{bc}	5.70 ± 0.27^{cd}	15.93 ± 0.52^{a}	16.66 ± 0.86^{a}	16.34 ± 1.13^{b}	15.69 ± 0.31^{bc}	8.84 ± 0.35^{b}	2.87 ± 0.02^{d}	8.71 ± 0.78^{bc}	
9	Tyrosine	10.42 ± 0.57^{cd}	9.17 ± 0.65^{d}	4.02 ± 0.15^{f}	6.33 ± 0.38^{e}	3.49 ± 0.46^{f}	10.94 ± 0.58^{c}	4.20 ± 0.16^{f}	28.64 ± 0.98^{b}	30.98 ± 0.56^{a}	
10	Glycine	31.87 ± 1.49^{c}	22.44 ± 1.03^{d}	69.14 ± 2.41^{ab}	71.88 ± 0.18^{ab}	67.80 ± 1.86^{b}	74.91 ± 3.46^{ab}	32.66 ± 2.24^{c}	76.25 ± 4.93^{a}	35.45 ± 0.05^{c}	
11	Threonine[4]	18.41 ± 2.24^{e}	3.50 ± 0.29^{f}	31.85 ± 3.04^{d}	31.93 ± 0.72^{d}	35.47 ± 2.19^{cd}	51.24 ± 0.79^{b}	39.64 ± 0.07^{c}	49.48 ± 2.55^{b}	74.13 ± 4.34^{a}	25
12	Glutamine	20.71 ± 0.54^{e}	40.59 ± 1.25^{d}	44.29 ± 0.29^{d}	38.10 ± 2.18^{d}	56.83 ± 3.35^{c}	61.42 ± 0.71^{c}	20.58 ± 2.80^{e}	86.80 ± 6.82^{a}	72.45 ± 1.26^{b}	
13	Asparagine	0.51 ± 0.04^{c}	0.60 ± 0.07^{c}	0.76 ± 0.02^{c}	2.67 ± 0.24^{b}	0.31 ± 0.06^{c}	2.32 ± 0.02^{b}	0.66 ± 0.04^{c}	7.54 ± 0.98^{a}	3.00 ± 0.16^{b}	
14	Serine	28.85 ± 1.56^{d}	17.75 ± 0.45^{e}	21.02 ± 0.82^{e}	26.85 ± 1.78^{d}	20.24 ± 0.40^{e}	32.82 ± 1.49^{c}	19.93 ± 1.23^{e}	60.09 ± 1.68^{a}	45.38 ± 3.37^{b}	
15	Aspartic acid	64.10 ± 1.96^{de}	56.35 ± 3.58^{ef}	51.96 ± 3.66^{f}	59.44 ± 6.00^{def}	52.50 ± 1.87^{f}	87.49 ± 5.47^{c}	68.56 ± 3.59^{d}	145.32 ± 5.65^{b}	163.13 ± 2.66^{a}	
16	Histidine[4]	6.18 ± 0.49^{c}	2.40 ± 0.08^{e}	2.69 ± 0.10^{e}	4.13 ± 0.21^{d}	3.05 ± 0.17^{de}	6.13 ± 0.12^{c}	3.67 ± 0.31^{de}	23.96 ± 0.63^{a}	20.99 ± 1.76^{b}	16
17	Arginine	27.47 ± 2.40^{c}	15.00 ± 0.96^{e}	13.01 ± 0.74^{e}	20.50 ± 1.65^{d}	13.49 ± 0.65^{e}	29.16 ± 1.37^{bc}	15.04 ± 1.30^{e}	89.57 ± 2.75^{a}	32.66 ± 3.51^{b}	
18	Glutamic acid	30.27 ± 2.41^{f}	13.05 ± 0.56^{f}	12.85 ± 0.07^{d}	20.65 ± 1.52^{e}	12.66 ± 0.01^{f}	34.95 ± 2.37^{c}	13.89 ± 0.94^{f}	50.03 ± 2.93^{b}	80.60 ± 0.15^{a}	
19	Lysine[4]	22.41 ± 1.57^{c}	12.60 ± 0.75^{de}	11.46 ± 0.50^{e}	14.20 ± 0.37^{d}	11.63 ± 0.83^{e}	21.33 ± 0.40^{c}	12.28 ± 0.48^{de}	56.55 ± 1.74^{b}	90.79 ± 0.60^{a}	48
20	Cysteine	0.28 ± 0.00^{c}	0.92 ± 0.02^{c}	ND	ND	ND	ND	0.64 ± 0.02^{c}	3.54 ± 0.12^{b}	15.40 ± 1.57^{a}	
	Total AA	307.84 ± 7.88^{de}	233.70 ± 5.59^{f}	306.10 ± 13.22^{de}	349.42 ± 7.88^{d}	314.58 ± 6.31^{de}	503.63 ± 23.75^{c}	273.31 ± 4.55^{ef}	830.39 ± 7.51^{b}	891.56 ± 25.72^{a}	
	EAA:AA (%)[7]	27.64 ± 0.35^{e}	20.79 ± 0.01^{h}	23.03 ± 0.74^{g}	25.29 ± 0.35^{f}	22.84 ± 0.13^{g}	29.03 ± 0.68^{d}	31.31 ± 0.18^{c}	33.43 ± 0.66^{b}	44.23 ± 0.01^{a}	
	EAA:NEAA (%)[8]	38.20 ± 0.62^{e}	26.25 ± 0.01^{h}	29.94 ± 1.25^{g}	33.86 ± 0.62^{f}	29.61 ± 0.21^{g}	40.92 ± 1.36^{d}	45.59 ± 0.38^{c}	50.23 ± 1.48^{b}	79.30 ± 0.05^{a}	
	AAS (%)[9]	4.94	3.71	1.82	2.69	1.93	4.61	4.14	6.27	28.96	
	EAAI (%)[9]	23.88	13.21	15.16	20.96	15.37	34.68	19.96	71.28	109.81	

[1] Raw seaweeds (KR: Korean commercial *Porphyra* sp., NZ: New Zealand wild *Pyropia* spp., mg amino acid/g seaweed dry weight) and commercial protein isolates (SPI: soy protein isolate, WPI: whey protein isolate, mg amino acid/g isolate) did not undergo extraction; [2] SPE: seaweed protein extract (mg amino acid/g SPE), W-SPE: SPE from water extraction, A-SPE: SPE from alkaline extraction, U-SPE: SPE from ultrasound-assisted extraction, E-SPE: SPE from enzyme-assisted extraction; [3] amino acid (AA) requirement scoring patterns (mg AA/g protein) for children ages 3 to 10 years (WHO/FAO/UNU, 2007); [4] essential AAs (EAAs) in human nutrition; [5] phenylalanine + tyrosine; [6] methionine + cysteine; [7] EAA:AA, essential AA quantity compared to total AA quantity; [8] EAA:NEAA, EAA compared to non-EAA; [9] AAS: amino acid score, EAAI: essential AA index; AAS and EAAI calculated according to the EAA requirement scoring patterns for children ages 3 to 10 years (WHO/FAO/UNU, 2007); ND, not detected; different letters (a, b, c…) indicate statistical significance within the same row (One-way ANOVA with Duncan's multiple range test, n = 3, $p < 0.05$); statistically significant difference between selected samples were also confirmed through *t*-tests ($p < 0.05$).

The overall AA composition in Table 19 was similar to other red seaweeds (Harrysson *et al.*, 2018; Naseri *et al.*, 2020b; Pangestuti & Kim, 2015); including a predominant abundance of glycine (31.87 and 22.44 mg AA/g seaweed for raw KR and NZ seaweeds, respectively; 32.66 to 74.91 mg AA/g SPE for SPEs), aspartic acid (64.10 and 56.35 mg/g for raw KR and NZ seaweeds, respectively; 51.96 to 87.49 mg/g for SPEs), and glutamic acid (30.27 and 13.05 mg/g for raw KR and NZ seaweeds, respectively; 12.66 to 34.95 mg/g for SPEs). Aspartic acid and glutamic acid could contribute to the taste sensation of "umami", indicating SPEs, if incorporated into foods, could synergistically enhance food flavour and nutritional value (Pangestuti & Kim, 2015). Additionally, SPEs contained an impressive level of threonine. In SPEs, threonine ranges from 31.85 to 51.24 mg/g across all SPEs, reaching a 205% increase from threonine (25 mg/g) in the WHO/FAO/UNU (2007) reference scoring pattern. Threonine has limited consumption in the typical human diet; its ameliorated content in SPEs could amplify seaweed protein's feasibility in fortifying foods (Dawczynski *et al.*, 2007).

Most AAs were present across all raw seaweeds and SPEs, with exceptions being low contents of cysteine and methionine (Table 19). Cysteine and methionine are recognised as common limiting AAs in some red seaweeds (Bjarnadóttir *et al.*, 2018; Dawczynski *et al.*, 2007; Mæhre *et al.*, 2016; Pangestuti & Kim, 2015; Vieira *et al.*, 2018). Unlike other red seaweeds, histidine (6.18 and 2.4 mg AA/g seaweed for raw KR and NZ seaweeds, respectively; 2.69 to 6.13 mg AA/g SPE for SPEs) was not a limiting AAs, and was greater than the reported content in red *P. palmata* (Bjarnadóttir *et al.*, 2018; Mæhre *et al.*, 2016; Naseri *et al.*, 2020b).

Compared to the raw KR seaweed in Table 19, KR SPE derived from enzyme-assisted extraction had significantly greater EAA:AA (29.03%) and EAA:NEAA values (40.92%), while KR SPE from alkaline and ultrasound-assisted extractions portrayed significantly lower values of EAA:AA (25.29% and 22.84%, respectively), and EAA:NEAA (33.86% and 29.61%, respectively) ($p < 0.05$). EAA loss during alkaline and ultrasound-assisted extractions could be the consequence of their sensitivity and extraction conditions. For example, lysine would be susceptible to damage during alkaline extraction at pH 12 and ultrasonic heat generation during ultrasound treatment (Appell *et al.*, 2018; Weder & Belitz, 2003). Nonetheless, enhanced EAA:AA and EAA:NEAA were also observed in NZ SPE following enzyme-assisted extraction (31.31% and 45.59%, respectively), reinforcing the suitability of enzyme-assisted extraction for deriving proteins with enhanced quality from both raw KR and NZ seaweeds. The influence of varying extraction conditions on the final seaweed protein have been reported

(Harrysson *et al.*, 2018; Rodrigues *et al.*, 2015), yet a systematic comparison between extraction protocols is still missing among red seaweeds. Hence, this study delivers a direct and comprehensive insight into how various extraction protocols influence red seaweed proteins and AAs.

Despite NZ SPE following enzyme-assisted extraction having the least total AA content, it demonstrated a significantly higher EAA:AA and EAA:NEAA ratio than all KR SPEs (Table 19, $p < 0.05$). The EAA:AA of NZ SPE (31.31%) was comparable to other red seaweeds. For instance, 38.5% in the closely related *P. umbilicalis* (Harrysson *et al.*, 2018), and 36% to 47% in *P. palmata* (Bjarnadóttir *et al.*, 2018; Mæhre *et al.*, 2016; Naseri *et al.*, 2020b). Such findings could promote the commercial cultivation possibilities of NZ seaweed, as it is currently harvested from the wild.

SPEs from enzyme-assisted extraction also comprise comparable EAA:AA values to that of commercial isolates in Table 19. For instance, EAA:AA values for KR SPE and NZ SPE following enzyme-assisted extraction were 29.03% and 31.31%, respectively; whereas SPI and WPI were 33.43% and 44.23%, respectively. The good nutritional value of SPEs after enzyme-assisted extraction suggests seaweed proteins could be a promising source for developing value-adding functional foods. In addition, the raw seaweeds and SPEs generally exhibited significantly lower individual AA content and total AA content than commercial isolates in Table 19 ($p < 0.05$). However, as SPI and WPI are commercially available, their extraction and processing would be incomparable to the laboratory-scale seaweed protein extraction in the current study. Furthermore, the relative proportions of AAs (individual AA content/total AA content) would highlight that SPEs hold impressive potential for becoming marketable protein isolates.

Though similar, the proximal protein content of raw seaweeds (Table 12), the protein content of SPEs during optimisation (*Section* 3.2), and the purity of SPEs (Table 18) were not in perfect accordance with total AA content (Table 19). This could arise from utilising a universal NPCF of 5.00 for seaweeds (Angell *et al.*, 2016). As differences exist in protein structure and AA composition of raw seaweeds and their respective SPEs, a general NPCF for all could be unsuitable for accurately estimating protein content and purity. Angell *et al.* (2016) mentioned that NPCFs were calculated based on the quantification of total AAs. Hence, the total AA in Table 19 should be the most accurate measure for protein content in raw seaweeds and their

respective SPEs. Furthermore, NPCFs for different seaweed species and different extraction methods could be further explored to improve their precision in quantifying protein content.

Tryptophan was completely absent during AA profiling, due to its destruction in 6 M HCl hydrolysis (Harrysson *et al.*, 2018; Naseri *et al.*, 2020b). Dawczynski *et al.* (2007) measured tryptophan as 0.7 g/16 g nitrogen in red *Porphyra sp.*, whereas Bjarnadóttir *et al.* (2018) quantified 0.9% tryptophan in red *P. palmata*. Furthermore, cysteine and its dimer would also be sensitive to acid hydrolysis, which could have been partially destroyed, thus resulting in its underestimation and unavailability for detection in some KR SPEs (Angell *et al.*, 2016; Kazir *et al.*, 2019).

It would be worth mentioning that prior to 6 M HCl acid hydrolysis, 1 M HCl was trialled as part of method development. Total AA detected from 1 M HCl hydrolysis was almost half of 6 M HCl hydrolysis (Appendix B, Table B1: 1 M HCl hydrolysis, Table 19: 6 M HCl hydrolysis). Thus, 6 M HCl hydrolysis was determined to be the final acid hydrolysis protocol. Moreover, by comparing the AA composition for 1 M and 6 M HCl hydrolysis, the complete destruction of tryptophan and the potential partial destruction of cysteine would be the consequence of 6 M HCl hydrolysis. Ways to quantify tryptophan include alkaline hydrolysis (solely quantifies tryptophan) (Yust *et al.*, 2004), the incorporation of 0.2% tryptamine during acidic hydrolysis (prevents tryptophan destruction) (Vieira *et al.*, 2018), and lithium hydroxide dissolution of protein followed by autoclaving (Dawczynski *et al.*, 2007). Nevertheless, Several buffers were also trialled to ensure sufficient HPLC-MS resolution was established for the analysis of AA composition. This included citrate buffer at pH 2.2 (Yaich *et al.*, 2011), mobile phase A in pH 3 (data not yet published by another student in the research group), and acetonitrile in 20% type-1 water (Huang *et al.*, 2018). All three buffers demonstrated sufficient HPLC resolution (Appendix B, Figure B2). Acetonitrile in 20% type-1 water was selected as the final buffer for HPLC analysis due to its enhanced separation of AAs during HPLC-MS.

Ultimately, AA profiling of SPEs derived from enzyme-assisted extraction compared to their respective raw seaweeds and other SPEs illustrate their potential exploitation as an effective red seaweed protein extraction protocol (Table 19). The comparable nutritional values and similar EAA proportion between SPEs after enzyme-assisted extraction and commercial protein isolates (SPI and WPI) enlighten feasible commercialisation of this SPE in the food industry.

3.3.3 Functional groups and secondary structure

Fourier Transform Infrared (FTIR) analysis enables the examination of protein functional groups, allowing for the comprehension of protein secondary structure to a certain degree. Through the transmittance of wavelength and intensity of IR radiation, chemical structures of proteins could be uncovered. Figure 7 unravels the FTIR spectrums of different red SPEs and commercial protein isolates (SPI and WPI) in the range of 4000 to 400 cm^{-1}. Similar spectrums have been presented by Vásquez *et al.* (2019) for red *C. chamissoi* proteins and brown *M. pyrifera* proteins, Suresh Kumar *et al.* (2014) for red *K. alvarezii* proteins, and Chen *et al.* (2019) for microalgal proteins.

In Figure 7, the broad signal centred at approximately 3250 cm^{-1} (band 1) would be contributed by hydrogen-bonded O-H and amino-bonded N-H stretching vibrations, whereas the weak signal at approximately 2930 cm^{-1} (band 2) would arise from C-H stretching vibrations. Both bands 1 and 2 generally correspond to compounds from fatty acids, polysaccharides and polyphenols in seaweeds (Gómez-Ordóñez & Rupérez, 2011; Vásquez *et al.*, 2019). Another signal at 1030 cm^{-1} (band 7) could refer to C-O and C-C from pyranose rings in seaweed polysaccharides (Gómez-Ordóñez & Rupérez, 2011; Vásquez *et al.*, 2019). The presence of bands 1, 2 and 7 across all SPEs could indicate the existence of polysaccharides, polyphenols or fatty acids in SPEs, as they were not of 100% purity (Table 18).

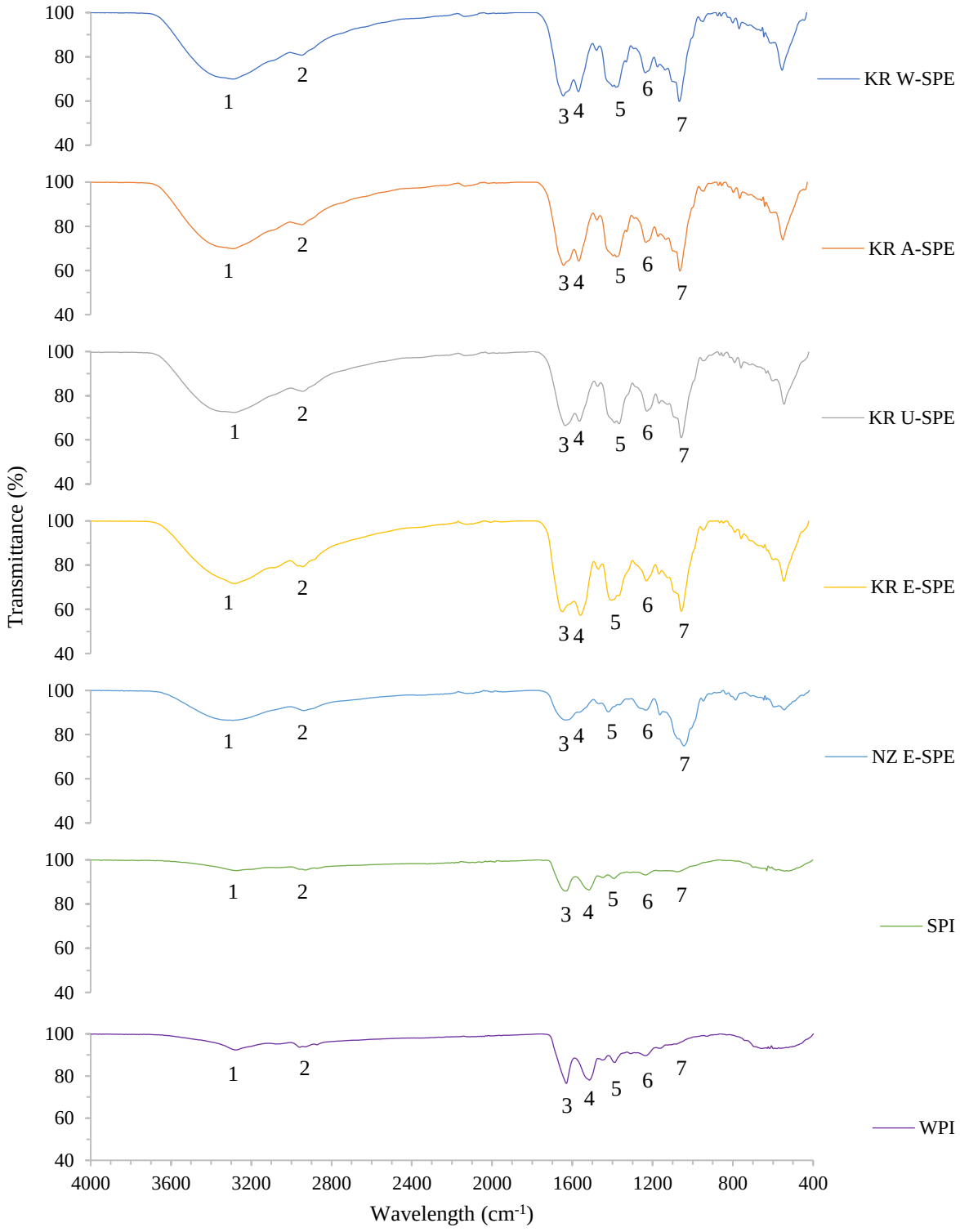


Figure 7. FTIR spectrums of different seaweed protein extracts and commercial protein isolates.

KR, Korean commercial *Porphyra* sp., NZ, New Zealand wild *Pyropia* spp.; SPE, seaweed protein extract; W-SPE, SPE from water extraction; A-SPE, SPE from alkaline extraction; U-SPE, SPE from ultrasound-assisted extraction; E-SPE, SPE from enzyme-assisted extraction; SPI, soy protein isolate; WPI, whey protein isolate; band numbers (1-7) represent characteristic transmittances.

Signals at 1630, 1530 and 1210 cm^{-1} in Figure 7 (bands 3, 4 and 5, respectively) of SPEs could be amides I, II and III in proteins (Barth, 2007). The appearance of bands 3 and 4 together could be related to C=O stretching vibrations in peptide bonds, as identified for red *C. chamissoi* and brown *M. pyrifera* proteins (Vásquez *et al.*, 2019). Band 3 could refer to amide I, characterising the protein α-helices (Barth, 2007). Additionally, band 5 could be N-H bending vibrations corresponding to the protein β-sheets, identified for microalgal proteins (Chen *et al.*, 2019). There were slight differences in bands 3, 4 and 5 between SPEs, hinting that the protein structure for SPEs would be varied. Finally, the transmittances at bands 3, 4 and 5 for NZ SPE after enzyme-assisted extraction were distinctively lower than other SPEs, corroborating lower protein content in this NZ SPE and would agree with results in purity content (Table 18) and AA profiling (Table 19).

Signals at 1350 cm^{-1} in Figure 7 (band 5) of SPEs could also be associated with tryptophan (Barth, 2007). The lack of tryptophan during AA profiling (Table 19) and its detection in Figure 7 would comply with tryptophan destruction during 6 M HCl hydrolysis. Finally, a wide transmittance at approximately 520 cm^{-1} is visible in all spectrums. As most protein functional groups would be detected above 600 cm^{-1}, this transmittance could be considered background noise or other contaminating compounds (Barth, 2007). Noise in FTIR spectrums could be minimised by utilising more advanced lasers, whereas undesired compounds could be discarded through protein purification techniques such as ultrafiltration (Barth, 2007).

The spectrums of SPEs contained more intensive transmittances than SPI and WPI spectrums in Figure 7. The transmittances in IR spectrums could be influenced by sample colour intensity (Silva *et al.*, 2006). In this case, all SPEs were vibrant shades of purple and red, yet SPI (light yellow) and WPI (white) lacked colour. The darker colours of SPEs could enable effective transmittance during FTIR, whereas fainter colours of SPI and WPI could hinder their transmittance. Despite the identifications of SPE bands in SPI and WPI, bands 5 and 7 of SPI and WPI appeared less prominent than all SPEs (Figure 7). Band 5 could be related to tryptophan, which could hint at a low proportion of tryptophan in these SPI and WPI. Diminished band 7 could be associated with the absence of polysaccharides, polyphenols or fatty acids, which would confer high protein purity and AA content in SPI and WPI, as validated during AA profiling (Table 19).

In summary, transmittances in FTIR elucidated similar functional groups and secondary structures across SPEs and commercial protein isolates (WPI and SPI) (Figure 7). Different seaweed protein extraction protocols could be responsible for the slight difference among transmittance bands responsible for secondary structures, such as α-helices and β-sheets. To further quantify α-helices and β-sheets, a more accurate and dynamic technique, such as CD, would be necessary to affirm protein transformations during processing (Rogers *et al.*, 2019).

3.3.4 Thermal properties

Differential scanning calorimetry (DSC) analysis is a convenient and non-destructive technique for understanding protein thermodynamics. As temperature influences the stability of protein structure, insight into the thermal properties of proteins would be vital to food processing strategies and heat processing designs. Table 20 unveils the thermal properties of different red SPEs and commercial protein isolates. Onset temperature (T_0), peak denaturation temperature (T_d) and enthalpy change (ΔH) would be crucial factors for the examining thermal properties of any sample. In proteins, T_0 indicates the commencement of protein instability, T_d describes the thermal stability of proteins, whereas ΔH could confer the status of ordered conformation in proteins (Chen *et al.*, 2019).

Table 20. Thermal properties of different seaweed protein extracts and commercial protein isolates.

Extracts[1]	**T_0 (°C)**[2]	**T_d (°C)**[3]	**ΔH (J/g)**[4]
KR W-SPE	145.95 ± 1.58[a]	166.29 ± 1.96[a]	-80.06 ± 2.28[g]
KR A-SPE	130.06 ± 1.94[a]	150.18 ± 2.36[b]	-178.40 ± 3.25[c]
KR U-SPE	137.99 ± 5.71[a]	161.66 ± 4.78[a]	-208.93 ± 4.11[a]
KR E-SPE	138.70 ± 10.45[a]	151.86 ± 3.61[b]	-172.50 ± 3.39[d]
NZ E-SPE	133.48 ± 6.92[a]	152.79 ± 5.41[b]	-194.97 ± 2.22[b]
SPI	106.75 ± 5.82[b]	130.93 ± 1.79[c]	-115.70 ± 0.44[f]
WPI	110.20 ± 0.29[b]	120.08 ± 0.76[c]	-139.90 ± 1.13[e]

KR, Korean commercial *Porphyra* sp., NZ, New Zealand wild *Pyropia* spp.; [1] Extracts, SPE: seaweed protein extract, W-SPE: SPE from water extraction, A-SPE: SPE from alkaline extraction, U-SPE: SPE from ultrasound-assisted extraction, E-SPE: SPE from enzyme-assisted extraction, SPI: soy protein isolate, WPI: whey protein isolate; [2] T_0, onset temperature; [3] T_d, peak denaturation temperature; [4] ΔH, enthalpy change; different letters (a, b, c…) indicate statistical significance between different samples (One-way ANOVA with Duncan's multiple range test, n = 3, $p < 0.05$).

As shown in Table 20, SPEs began to exhibit protein instability at temperatures around 130.06 °C to 145.95 °C. Sequentially, SPEs derived from water and ultrasound-assisted extractions reached significantly higher denaturation temperatures (166.29 °C and 161.66 °C, respectively) compared to other SPEs ($p < 0.05$); thus indicating greater thermal stability than other SPEs. All SPEs showed higher T_0 and T_d than SPI and WPI. Thermal stability could be controlled by the proportions of polar and non-polar residues within proteins, whereby higher stability would be evident for having a greater proportion of non-polar residues than polar residues (Kaur & Singh, 2007). With reference to AA profiling (Table 19), SPEs from water and ultrasound-assisted extractions generally had greater proportions of non-polar AA compared to other SPEs and commercial isolates. Furthermore, all SPEs reflected greater T_d

than 109.25 °C for red *K. alvarezii* proteins (Suresh Kumar *et al.*, 2014), and 73.68 °C to 100.32 °C for several microalgae proteins (Chen *et al.*, 2019).

With regards to ΔH in Table 20, it ranged between -80.06 to -208.93 J/g for SPEs. SPEs following ultrasound-assisted extraction displayed significantly greater ΔH than other SPEs ($p < 0.05$), implying a larger extent of ordered conformation in its extract. Nevertheless, SPEs from ultrasound-assisted extraction could constitute strong binding between molecules, thus requiring more energy for its protein to unfold (Chen *et al.*, 2019). SPEs showcased comparable ΔH to several microalgae proteins, observed between -14.69 J/g to -191.06 J/g (Chen *et al.*, 2019). Additionally, SPEs demonstrated greater ΔH than red *K. alvarezii* proteins, which was -5.3 J/mg (Suresh Kumar *et al.*, 2014). This might be due to sample difference and extraction variation. The presence of polysaccharides across all SPEs has been indicated in protein purity (Table 18) and FTIR (Figure 7); the potential polysaccharide-protein complexes could improve stability and thus elevate ΔH (Turgeon *et al.*, 2007).

To summarise, the thermal properties of SPEs were more impressive than commercial protein isolates (SPI and WPI) (Table 20). The T_0, T_d and ΔH were significantly greater than SPI and WPI ($p < 0.05$). Such findings reinforce that SPEs could be more stable during heat processing than commercial isolates, which could amplify the commercial and industrial possibilities of KR and NZ seaweed proteins. Nonetheless, thermogravimetric analysis of SPEs could be explored to observe their mass changes under constant heating, thus enhancing the understanding of potential structural degradation due to thermal treatments (Suresh Kumar *et al.*, 2014).

3.4 Functional properties of seaweed proteins

3.4.1 Solubility

Protein solubility corresponds to the hydrophilic residues within proteins, as protein solubilisation occurs upon the interaction between polar groups and water molecules (Rawiwan *et al.*, 2022). The solubility of proteins would be the prerequisite consideration for other protein functionalities, namely emulsifying and foaming properties. Nevertheless, pH would be a critical parameter that impacts protein solubility (Zayas, 1997e). Table 21 displays the solubility of different red SPEs and commercial protein isolates (SPI and WPI) across various pHs.

Solubilities of all SPEs were above 80% in the current study (Table 21), which were generally greater than the ones reported in the literature. For example, SPEs exhibited greater solubility than red *P. dioica* protein (< 5% to 43.3%) at similar pHs (Felix *et al.*, 2021). SPE solubilities exceeded that of green *U. lactuca* and red *P. umbilicalis*, reaching a maximum of 62.1% and 54.2% at pH 14, respectively (Harrysson *et al.*, 2018). The solubilities of SPEs across all pHs were higher than the greatest solubility of red *K. alvarezii* protein (58.72%) (Suresh Kumar *et al.*, 2014). SPE solubilities surpassed that of microalgae, whereby microalgal protein solubility progressed to 67.30% (Chen *et al.*, 2019). Contrastingly, SPEs and brown *H. elongate* protein reached similar maximum solubilities, whereby *H. elongate* protein solubility elevated from 25% at pH 2 to 96.15% at pH 12 (Garcia-Vaquero *et al.*, 2017).

Table 21. Solubility of different seaweed protein extracts and commercial protein isolates at various pHs.

Extracts[1]	**pH 3**	**pH 5**	**pH 7**	**pH 9**	**pH 11**
KR W-SPE (%)	81.09 ± 0.60^{Dd}	87.18 ± 0.37^{Bc}	89.32 ± 2.24^{BCd}	91.37 ± 0.30^{Bb}	96.03 ± 0.02^{Ab}
KR A-SPE (%)	80.21 ± 0.03^{Cd}	94.39 ± 1.49^{Ab}	93.19 ± 1.41^{Abc}	90.37 ± 1.17^{Ab}	84.83 ± 1.54^{Bd}
KR U-SPE (%)	85.22 ± 0.95^{Bc}	85.88 ± 0.51^{Bc}	84.71 ± 0.75^{Be}	85.11 ± 0.87^{Bc}	89.35 ± 0.39^{Acd}
KR E-SPE (%)	96.44 ± 0.43^{Da}	97.57 ± 0.00^{CDa}	98.67 ± 0.27^{Ca}	$>99^{Ba}$	$>99^{Aa}$
NZ E-SPE (%)	96.48 ± 0.24^{Aa}	96.32 ± 0.67^{Aab}	94.61 ± 0.15^{ABb}	91.79 ± 1.75^{Bb}	93.95 ± 1.04^{ABc}
SPI (%)	28.09 ± 0.64^{Ee}	17.70 ± 0.31^{Dd}	31.23 ± 0.67^{Cf}	39.31 ± 0.96^{Bd}	64.23 ± 0.63^{Ae}
WPI (%)	93.29 ± 0.19^{Ab}	85.80 ± 1.29^{Cc}	90.35 ± 0.40^{Bcd}	91.79 ± 1.60^{ABb}	93.57 ± 0.53^{Ac}

KR, Korean commercial *Porphyra* sp., NZ, New Zealand wild *Pyropia* spp.; [1] Extracts, SPE: seaweed protein extract, W-SPE: SPE from water extraction, A-SPE: SPE from alkaline extraction, U-SPE: SPE from ultrasound-assisted extraction, E-SPE: SPE from enzyme-assisted extraction, SPI: soy protein isolate, WPI: whey protein isolate; uppercase letters (A, B, C…) compare between different pHs of the same sample, lowercase letters (a, b, c…) compare between different samples of the same pH, different letters within each case indicate statistical significance (One-way ANOVA with Duncan's multiple range test, n = 3, $p < 0.05$).

Table 21 reinforces that the solubility of SPEs and commercial isolates would be affected by pH. For most seaweed proteins, greater solubilities have been identified at high alkaline pHs (Felix *et al.*, 2021; Garcia-Vaquero *et al.*, 2017; Harrysson *et al.*, 2018; Kadam *et al.*, 2017). Such results from literature were also apparent in this study, as most SPEs demonstrated significantly greater solubility at pH 11 compared to other pHs (Table 21, $p < 0.05$). Proteins could convey a strong net negative charge in alkaline conditions, enhancing interaction with the aqueous environment, thus improving solubility (Xu *et al.*, 2021).

For most literature, seaweed protein solubility would be low at acidic pHs. For example, solubilities were 22.5% for brown *H. elongate* protein at pH 4 (Garcia-Vaquero *et al.*, 2017), 33.72% for red *K. alvarezii* protein at pH 4 (Suresh Kumar *et al.*, 2014), and < 5% for red *P. dioica* protein under pH 5 (Felix *et al.*, 2021). Poor solubilities would be related to seaweed protein isoelectric points. As proteins have no net charge at their isoelectric point, the attractive protein-protein forces lead to protein aggregation, consequently inducing insolubility (Farkye & Shah, 2015; Tan *et al.*, 2021). The isoelectric point of seaweed proteins has been discovered to be pH 2 in both green *U. lactuca* and red *P. umbilicalis* (Harrysson *et al.*, 2018), pH 2 in brown *S. latissimi* (Vilg & Undeland, 2017), pH 3 in red *P. palmata* (Naseri *et al.*, 2020b), pH 4 in brown *H. elongate* (Garcia-Vaquero *et al.*, 2017), and pH 4 in red *K. alvarezii* (Suresh Kumar *et al.*, 2014). However, the solubilities of all SPEs at acidic pHs 3 and 5 (80.21% to 97.57%) were much greater than those listed in the literature for seaweeds, and would be comparable to that of WPI (Table 21) and peanut proteins (around 90%) (Zhao *et al.*, 2011). Moreover, small solubility fluctuations could imply that SPEs from the KR and NZ seaweeds in this study could be applied in a wide range of food systems with varying pHs (Farkye & Shah, 2015).

Unlike other SPEs in Table 21, SPE derived from alkaline extraction constituted 84.83% solubility at pH 11, significantly lower than solubilities at pHs 5, 7, and 9 (94.39%, 93.19% 90.37%, respectively; $p < 0.05$). Hindered solubility at alkaline pHs could be related to extraction protocols for SPEs, as varying processing protocols could modify seaweed protein structure, thus deriving proteins with unique properties.

Compared to other SPEs in Table 21, SPEs following enzyme-assisted extraction had the most impressive solubilities, ranging from 91.79% to above 99%. With regards to enzyme-assisted extraction, both KR and NZ SPEs portrayed the highest solubility (96.44% and 96.48%,

respectively) at pH 3 compared to other pHs ($p < 0.05$). The ameliorated solubility of such SPEs could also be related to their extraction protocol, which was conducted with Alcalase, a protease that would cleave proteins into smaller peptides. The absence of whole seaweed proteins would restrain protein aggregation despite pH reaching protein isoelectric point; this observation has been witnessed by Naseri *et al.* (2020b) and during isoelectric point protein precipitation in *Section 3.2.4.4*. As showcased in MW distribution (Figure 6), SPEs from enzyme-assisted extraction comprised a larger proportion of smaller peptides and proteins than SPEs from other extractions. Low MW could be associated with small PS, enhancing protein solubilisation (Tavano, 2013). Nevertheless, the exploitation of Alcalase could further reduce protein surface hydrophobicity, in turn elevating solubility (Zhao *et al.*, 2011). The exposure of protein surface hydrophobic residues also depends on Alcalase's degree of hydrolysis. A high enzymatic hydrolysis degree would impair surface hydrophobicity, uncover ionisable amino and carboxyl substituents, and ameliorate protein solubility (Xu *et al.*, 2021; Zhao *et al.*, 2011).

As a reference, the solubility of SPI ranged from 17.70% to 64.23%, whereas the solubility of WPI ranges from 85.80% to 93.57% (Table 21). Their lowest solubilities were observed at their isoelectric point, both around pHs 3 and 5 (Farkye & Shah, 2015; Preece *et al.*, 2017b; Torrezan *et al.*, 2007). Compared to commercial SPI and WPI, SPEs demonstrated significantly greater solubilities at different pHs (Table 21, $p < 0.05$). For example, both KR SPE and NZ SPE after enzyme-assisted extraction comprised higher solubilities at pHs 3 and 5 (96.44% and 96.48%, 97.57% and 96.32%, respectively); KR SPE from enzyme-assisted extraction showcased highest solubility at pHs 7, 9 and 11 (98.67%, >99% and >99%, respectively) ($p < 0.05$). Such observations would be consistent with the physicochemical properties of SPEs. For instance, a greater relative proportion of polar AAs would be present in SPEs compared to commercial isolates (Table 19); this would enhance SPE interaction with water molecules (Kramer *et al.*, 2012). SPEs also contain low MW proteins (Figure 6), which enables better solubilisation in aqueous conditions (Tavano, 2013).

In conclusion, solubilities of SPEs has not yet been well-explored in literature. The impressive SPE solubilities illustrate minimal fluctuations across acidic and alkaline pHs (Table 21); which would escalate their exploitation in the food industry, as SPEs could be effectively incorporated into foods and beverages of different pHs (Farkye & Shah, 2015). Finally, structures of SPEs under pH changes could be examined to further understand their solubility.

3.4.2 Emulsifying

Emulsions are recognised as a thermodynamically unstable dispersion of two or more immiscible liquids (Rawiwan *et al.*, 2022). The emulsifying properties of proteins could be analysed by studying emulsifying activity (EA) and emulsifying stability (ES). EA grants insight into the formation of an emulsion produced, whereas ES provides understanding into an emulsion's stability against change (coalescence, creaming, flocculation or sedimentation) (Ma *et al.*, 2011). Figure 8 portrays the emulsifying properties of different red SPEs and commercial protein isolates (SPI and WPI) in soybean oil across acidic and alkaline pHs (3, 5, 7, 9 and 11).

Overall, SPE EA and ES improved as pH elevated (Figure 8). Depending on the type of extraction protocol, EA and ES of SPEs were comparable to those reported in the literature. SPE EA in Figure 8A was similar to the EA of 30.83 mg^2/g to 63.58 mg^2/g for microalgae protein in soybean oil, and SPE ES in Figure 8B was greater than microalgae ES of 17 mins (Chen *et al.*, 2019). ES findings in Figure 8B were also comparable to that for red *K. alvarezii* proteins, whereby ES were 53.36, 54.33, and 75.33 mins in olive, jatropha and cedarwood oils, respectively (Suresh Kumar *et al.*, 2014).

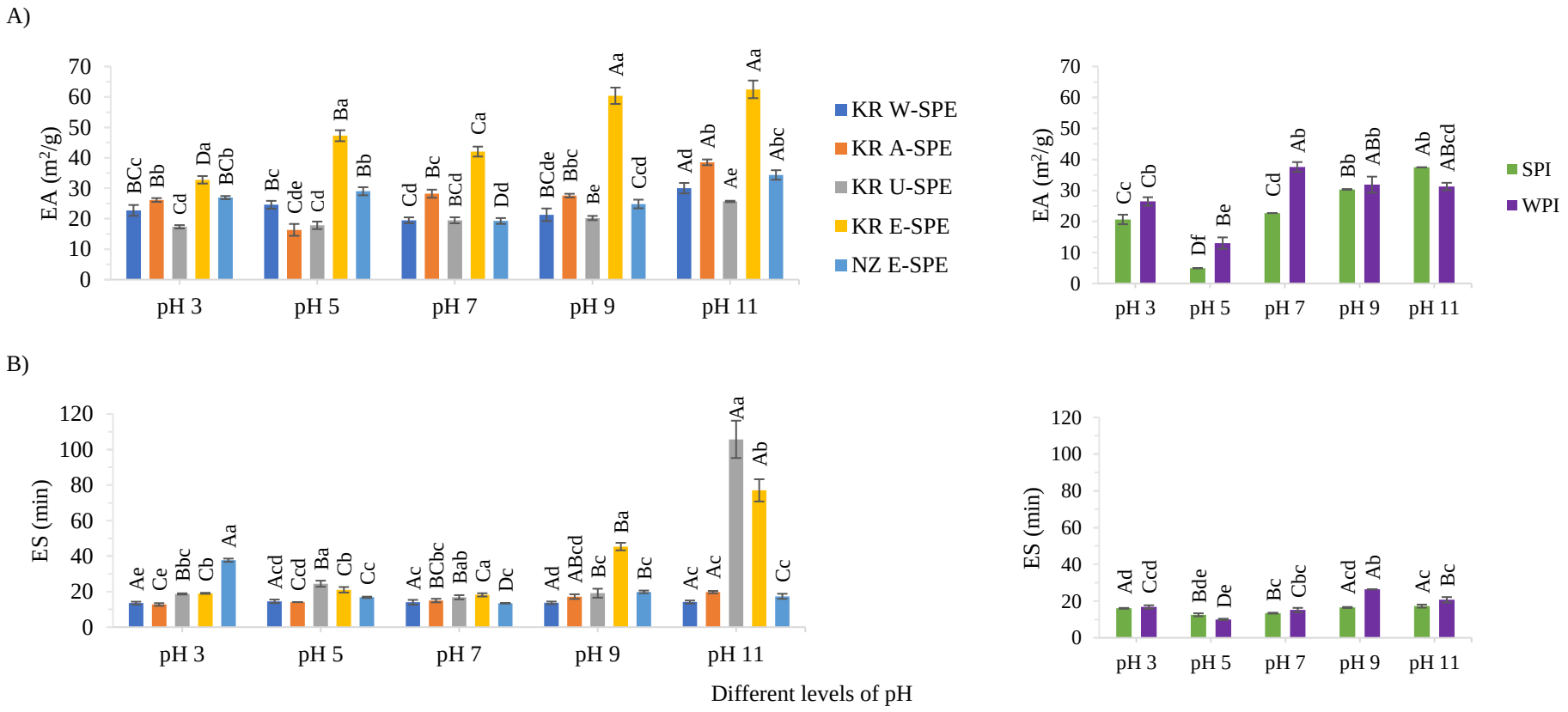


Figure 8. Emulsifying properties of different seaweed protein extracts and commercial protein isolates at various pHs with soybean oil, A) EA, emulsifying ability, B) ES, emulsifying stability.

KR, Korean commercial *Porphyra* sp.; NZ, New Zealand wild *Pyropia* spp.; SPE, seaweed protein extract; W-SPE, SPE from water extraction; A-SPE, SPE from alkaline extraction; U-SPE, SPE from ultrasound-assisted extraction; E-SPE, SPE from enzyme-assisted extraction; SPI, soy protein isolate; WPI, whey protein isolate; uppercase letters (A, B, C…) compare between different pHs of the same sample, lowercase letters (a, b, c…) compare between different samples of the same pH, different letters within each case indicate statistical significance (One-way ANOVA with Duncan's multiple range test, n = 3, $p < 0.05$).

Likewise to solubility (Table 21), the tendencies of SPE EA and ES were dependent on pH in Figure 8. As pH increased, EA in Figure 8A and ES in Figure 8B improved. Similar results were reported for red *K. alvarezii* proteins (Suresh Kumar *et al.*, 2014). The most distinguishable EA was demonstrated by KR SPE following enzyme-assisted extraction, as it was significantly greater than other SPEs across all pHs, reaching its maximum EA at pH 11 of 62 mg^2/g ($p < 0.05$). Additionally, the KR SPE from enzyme-assisted extraction exhibited higher ES than most SPEs at pH 11, reaching its maximum ES of 77.03 mins ($p < 0.05$). Tavano (2013) conveyed that EA and ES would be elevated with increased protein solubility; this was exemplified by KR SPE from enzyme-assisted extraction with a significantly greater solubility at pH 11 than most SPEs (Table 21, $p< 0.05$). Furthermore, the degree of hydrolysis by Alcalase during enzyme-assisted extraction could improve EA. Buried hydrophobic residues following Alcalase-treatment could be uncovered, thus promotes polypeptide unfolding and ameliorates interaction at the oil-water interphase (Rawiwan *et al.*, 2022; Xu *et al.*, 2021). Unlike KR SPE from enzyme-assisted extraction, the NZ SPE from enzyme-assisted extraction did not illustrate great EA and ES, possibly associated with its lower total AA content than KR SPE (Table 19).

It is interesting to observe in Figure 8B that KR SPE following ultrasound-assisted extraction showcased significantly greater ES at pH 11 compared to other SPEs, reaching 105.75 mins ($p < 0.05$). The vast improvement of ES could connote that KR SPE from ultrasound-assisted extraction had greater compatibility with soybean oil, as emulsifying properties would depend on the oil employed (Garcia-Vaquero *et al.*, 2017). Another possible reason might be that ultrasound treatment could induce polysaccharide-protein complexes, thus enhancing ES through steric repulsion and limiting droplet aggregation (Fidantsi & Doxastakis, 2001; Rawiwan *et al.*, 2022).

All SPEs consisted of comparable EA and ES to that of commercial protein isolates (SPI and WPI) in Figure 8A and B, respectively. The comparable EA between SPEs and commercial isolates could be related to the higher presence of polysaccharides in SPEs, as evident in FTIR spectrums (Figure 7, bands 1 and 7) and low purity content (Table 18), which was perceived for microalgae proteins (Chen *et al.*, 2019). Within SPI and WPI, greater EA and ES were generally observed at alkaline pHs compared to SPEs. However, KR SPE derived from enzyme-assisted extraction showcased significantly greater EA and ES at pHs 9 and 11 than SPI and WPI (Figure 8, $p < 0.05$).

To summarise, effective emulsifiers would be surface-active and could minimise surface tension (Suresh Kumar *et al.*, 2014). As KR SPE derived from enzyme-assisted extraction showcased impressive overall EA and ES in alkaline pHs, it could be explored as a potential emulsifying agent for food systems with alkaline pHs. To further exploit KR SPE from enzyme-assisted extraction as a feasible emulsifier, the surface properties of its proteins should be explored and improved.

3.4.3 Foaming

The suspension of gas in a liquid could be recognised as a foam. In this case, foaming properties depend on interfacial films that lock air bubbles in suspension and impede coalescence (Zhao *et al.*, 2011). The foaming properties of proteins could be examined by studying foaming capacity (FC) and foaming stability (FS). FC corresponds to foam volumes, whereas FS refers to the stability of foams over time (Rawiwan *et al.*, 2022). Figure 9 illustrates the foaming properties of different red SPEs and commercial protein isolates across acidic and alkaline pHs.

In Figure 9, elevated FC was witnessed for most SPEs at alkaline pHs, whereas elevated FS was observed for most SPEs at acidic pHs. Depending on the type of extract and pH, FC and FS of SPEs in Figure 9 could be greater than those reported in the literature. For most SPEs in Figure 9A, the FC was over 100% with a solid content of 1% w/v, which would be greater than the highest FC revealed in brown *H. elongate* protein of 71.53% with a solid content of 1.5% (Garcia-Vaquero *et al.*, 2017), greater than that of 53.33% in red *K. alvarezii* protein with a solid content of 2% (Suresh Kumar *et al.*, 2014), and 54.02% in microalgae protein with a solid content of 0.5% (Chen *et al.*, 2019). Furthermore, the FS of SPEs in Figure 9B varied between 13.75% to 82.99%, which was in the range of findings in *H. elongate* protein (0% to 95%) (Garcia-Vaquero *et al.*, 2017), *K. alvarezii* protein (25% to 45.33%) (Suresh Kumar *et al.*, 2014), and several microalgae proteins (5% to 50%) (Chen *et al.*, 2019).

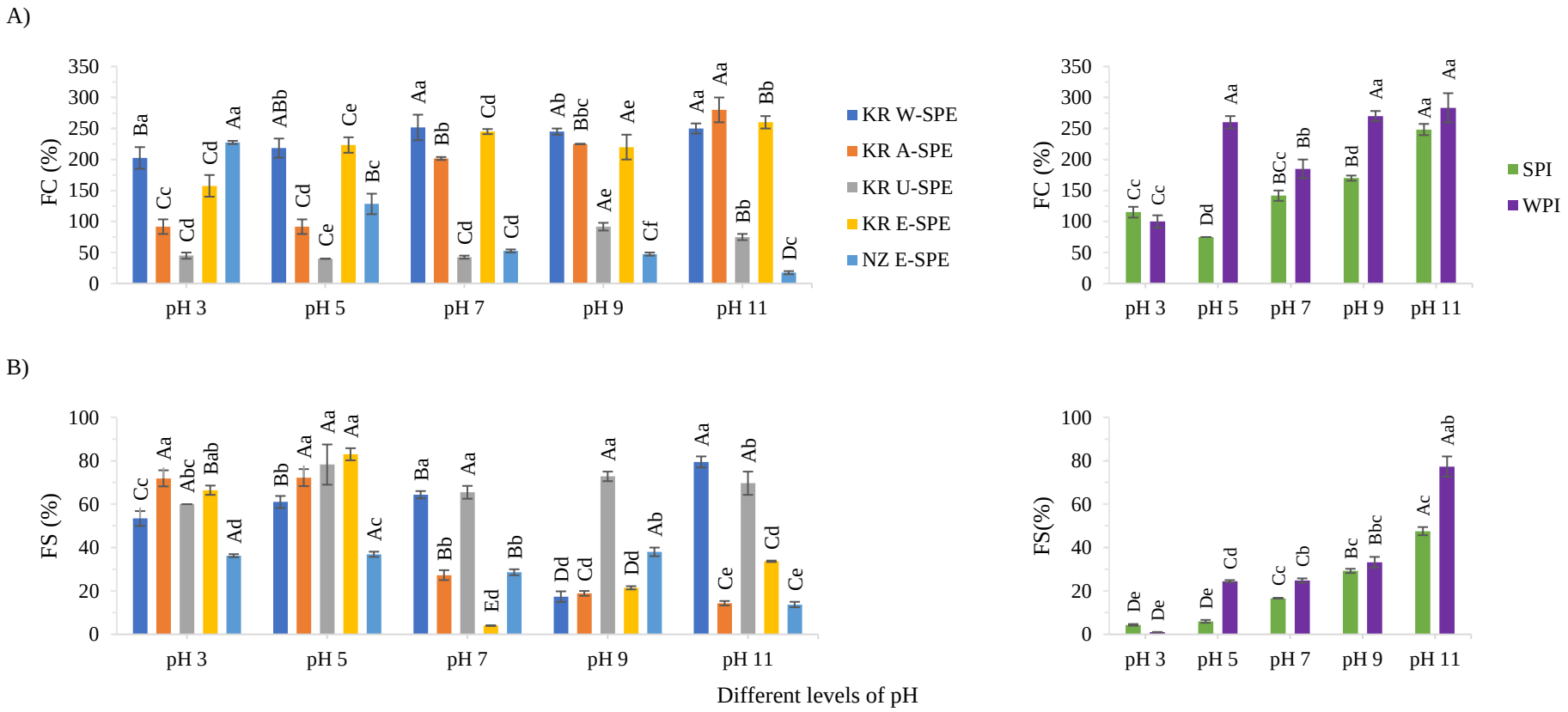


Figure 9. Foaming properties of different seaweed protein extracts and commercial protein isolates at various pHs, A) FC, foaming capacity, B) FS, foaming stability.

KR, Korean commercial *Porphyra* sp.; NZ, New Zealand wild *Pyropia* spp.; SPE, seaweed protein extract; W-SPE, SPE from water extraction; A-SPE, SPE from alkaline extraction; U-SPE, SPE from ultrasound-assisted extraction; E-SPE, SPE from enzyme-assisted extraction; SPI, soy protein isolate; WPI, whey protein isolate; uppercase letters (A, B, C…) compare between different pHs of the same sample, lowercase letters (a, b, c…) compare between different samples of the same pH, different letters within each case indicate statistical significance (One-way ANOVA with Duncan's multiple range test, n = 3, $p < 0.05$).

Likewise to solubility (Table 21) and emulsifying properties (Figure 9), the FC tendencies of SPE would be dependent upon pH in Figure 9A. For KR SPEs, FC was improved as pH elevated (Figure 9A). For instance, FC for KR SPE from alkaline extraction steadily increased from 91.67% at pH 3 to 280% at pH 11. This phenomenon was evident for brown *H. elongata* proteins, whereby significantly greater FC was present during higher pHs (71.52% at pH 10) compared to low pHs (6.98% at pH 2) (Garcia-Vaquero *et al.*, 2017).

Unlike KR SPE following enzyme-assisted extraction, with FC gradually increasing from 157.5% at pH 3 to 260% at pH 11, a contrasting FC trend was observed for NZ SPE following enzyme-assisted extraction (Figure 9A). The FC for NZ SPE from enzyme-assisted extraction exhibited a significant reduction from 227.5% at pH 3 to 17.5% at pH 11 ($p < 0.05$). This phenomenon was evident for red *K. alvarezii* protein, whereby the highest FC of 53.33% was obtained at pH 4 (Suresh Kumar *et al.*, 2014). Such opposing trends between KR SPE and NZ SPE after enzyme-assisted extraction could coincide with their solubilities at varying pHs (Zayas, 1997b). With respect to enzyme-assisted extractions, the KR SPE displayed increased solubility at more alkaline pHs, whereas the NZ SPE displayed increased solubility at more acidic pHs (Table 21). Moreover, differing FC phenomena between KR and NZ SPEs could imply that different species of red seaweeds could be exploited in food systems at various pHs.

For some SPEs, the tendencies of their FS were also dependent upon pH (Figure 9B). For example, SPEs from alkaline and enzyme-assisted extractions displayed significantly greater FS at acidic pHs than alkaline pHs ($p < 0.05$). Such observations were in accordance with FS for red *K. alvarezii* protein, whereby greater FS was maintained at acidic pHs (above 40% at pHs 2 and 4) than alkaline pHs (under 30% for pHs 6, 8 and 10) (Suresh Kumar *et al.*, 2014). This phenomenon could be associated with increased charge density and electrostatic repulsions that impede coalescence, as seen for amaranth seed proteins (Das *et al.*, 2021). In contrast, SPEs derived from water and alkaline extractions demonstrated promising FS across acidic and alkaline pHs compared to other SPEs. This could be contributed by their polysaccharide fraction (Table 18), as co-precipitation of polysaccharides with seaweed proteins during extraction was inevitable. Though polysaccharides do not directly absorb at the air-water interphase, they decrease the rate of disproportion and drainage by elevating bulk viscosity (Yang & Foegeding, 2010). Thus, the mixture of proteins and polysaccharides in SPEs could produce stable foams, as seen for microalgal proteins (Schwenzfeier *et al.*, 2013).

All SPEs included comparable FC and FS to commercial protein isolates (SPI and WPI) in Figure 9A and B, respectively. Within SPI and WPI, greater FC and ES were typically witnessed at alkaline pHs than acidic pHs. In contrast to SPI and WPI, SPEs exhibited significantly higher FS at acidic pHs than alkaline pHs (Figure 9B, $p < 0.05$). To conclude, SPEs exemplified good foaming properties at acidic pHs. Such results emphasise the feasibility of SPEs incorporation in the foaming of acidic foods. Further analysis into the interfacial properties of SPE foams would be desirable to uncover more of their functional properties.

3.5 Antioxidant capacity of seaweed proteins

Seaweed proteins have been reported to be natural antioxidants to diminish oxygen-induced cellular damage and offer disease prevention properties to the human body (Pangestuti & Kim, 2015). In particular, protein hydrolysates from several red seaweeds, such as *P. palmata* (Beaulieu *et al.*, 2016; Harnedy & FitzGerald, 2013b), *O. pinnatifida* (Rodrigues *et al.*, 2015), *P. columbina* (Cian *et al.*, 2015), *Polysiphonia howei*, *Ahnfeltiopsis concinna* and *Pterocladiella capillacea* (Kelman *et al.*, 2012) have shown strong antioxidant capacities.

Figure 10 presents the antioxidant capacity of different red SPEs assessed through DPPH scavenging potential and FRAP assays. Results from both assays are of high consistency, ranging from 10.20 to 25.67 mM Trolox/g for the DPPH assay, and from 5.48 to 25.16 mM Trolox/g for the FRAP assay (Figure 10). KR SPE following alkaline extraction exhibited the greatest FRAP (25.16 mM Trolox/g SPE), and portrayed a comparable DPPH value to KR SPE after water extraction (25.67 and 24.45 mM Trolox/g, respectively); both of which were significantly greater than other SPEs ($p < 0.05$). Similar findings were reported in the literature. For example, SPEs from red *P. palmata* showed antioxidant capacities between 1.06 to 21.56 µmol Trolox/g extract by FRAP (Harnedy & FitzGerald, 2013b); seaweed proteins derived from ultrasound treatment from red *O. pinnatifida* had a reduced DPPH value than proteins from water extraction (Rodrigues *et al.*, 2015).

The antioxidant capacity of seaweed proteins could depend on the location and quantity of hydroxyl substituents in their structure (Balboa et al., 2013). Unlike findings by Harnedy and FitzGerald (2013b), whereby seaweed protein hydrolysates after enzyme-assisted extraction illustrated the greatest antioxidant activity (FRAP), KR SPE following enzyme-assisted extraction in this study had lower antioxidant activity than KR SPEs from water and alkaline extractions. KR SPEs derived from water and alkaline extractions could contain more hydroxyl groups, as greater absorptions for O-H stretching vibrations were identified in their FTIR spectrums than other SPEs (Figure 7, band 1). Furthermore, the lowest DPPH scavenging potential and FRAP retained by NZ SPE from enzyme-assisted extraction could be from having the least total AA than other extracts (Table 19), as low protein content would impair antioxidant potential.

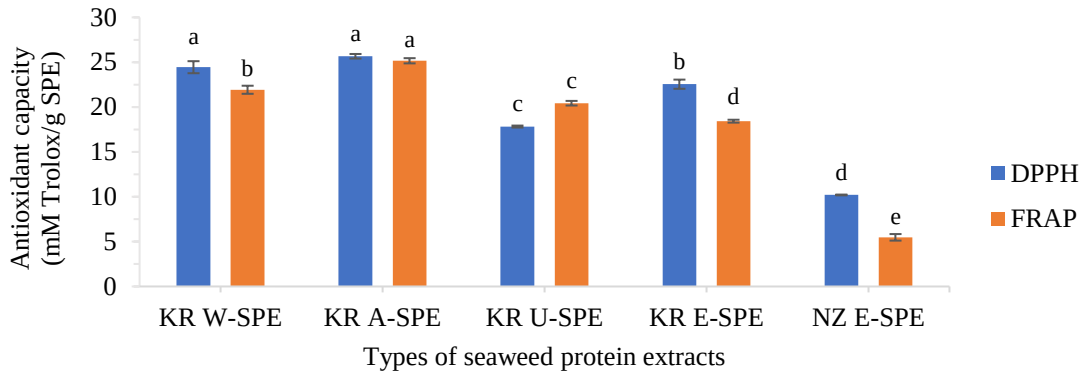


Figure 10. Antioxidant capacity of different seaweed protein extracts.

KR, Korean commercial *Porphyra* sp.; NZ, New Zealand wild *Pyropia* spp.; SPE, seaweed protein extract; W-SPE, SPE from water extraction; A-SPE, SPE from alkaline extraction; U-SPE, SPE from ultrasound-assisted extraction; E-SPE, SPE from enzyme-assisted extraction. SPI, soy protein isolate; WPI, whey protein isolate; DPPH, 2,2'-diphenyl-1-picrylhydrazyl radical scavenging assay; FRAP, ferric reducing antioxidant power assay; different letters (a, b, c…) indicate statistical significance between SPEs (One-way ANOVA with Duncan's multiple range test, n = 3, $p < 0.05$).

KR and NZ SPEs derived from enzyme-assisted extraction were expected to have high antioxidant capacity due to their low MW peptides. As presented in MW distribution (Figure 6), KR and NZ SPEs after enzyme-assisted extraction had more < 10 kDa proteins than other SPEs. Beaulieu *et al.* (2016) observed improved DPPH and FRAP values for < 10 kDa red *P. palmata* protein hydrolysates compared > 10 kDa hydrolysates. Such unexpected phenomena could result from utilising different enzymes and extraction protocols. Nevertheless, the reduced DPPH values of protein extracts following enzyme treatment have been observed in pea and peanut proteins, whereby Alcalase-treated protein hydrolysates contained significantly lower antioxidant capacity than their parent proteins ($p < 0.05$) (Xu *et al.*, 2021). Though enzyme treatment could release antioxidant peptides from some plant proteins, their antioxidant capacity depends on the protein source (Silva *et al.*, 2017; Xu *et al.*, 2021). Impaired antioxidant capacity of SPEs derived from enzyme-assisted extraction could also be associated with Alcalase hydrolysis degree during treatment. Klompong *et al.* (2007) connoted that DPPH scavenging potential was significantly lower for Alcalase-treated fish protein hydrolysates with 15% and 25% degree of hydrolysis, than 5%. Despite that, Rodrigues *et al.* (2015) and Wang *et al.* (2010) witnessed no significant improvement in DPPH values for Alcalase-treated red seaweed protein hydrolysates compared to aqueous extraction ($p > 0.05$). Therefore, further studies would be necessary to develop desired protein hydrolysate with strong antioxidant capacities from KR and NZ seaweeds in the current study

Chapter Four

General Discussion, Conclusion,

Limitations and Future Work

4 General discussion, Conclusion, Limitations, and Future Work

4.1 General discussion

Amongst red seaweed protein extraction protocols within this study, enzyme-assisted extraction with Alcalase constitutes the most desirable extraction efficiency and yields. Results within this study suggest that physicochemical properties and functionalities of seaweed protein extracts, especially those derived from enzyme treatment, hold potential in fortifying and formulating various food products. Despite the efficiency of enzyme treatment, the high costs of enzymes and processing constraints are economic challenges that restrict its potential exploitation on an industrial scale (Rawiwan *et al.*, 2022). This challenge could be conquered through incorporating inexpensive food-grade enzymes (Wijesinghe & Jeon, 2012), developing enzymatic extraction in conjunction with food-friendly aqueous and physical extractions (Zhang *et al.*, 2018), and utilising insoluble immobilised enzymes that could be readily recycled in the food industry (Sheldon *et al.*, 2021).

Further cost reduction of seaweed protein extraction could occur through "whole of plant" utilisation (Sutton *et al.*, 2018). Seaweed protein extraction could be achieved simultaneously alongside their polysaccharide retrieval, or directly derived from seaweed by-products in their other commercial processing streams. Similar procedures have been conducted for obtaining wheat gluten protein whilst isolating wheat starch, and acquiring pea protein whilst isolating pea starch (Sutton *et al.*, 2018). Maximising the exploitation of seaweed by-products would be economical and eco-friendly, as seaweed by-products could act as an additional source of inexpensive raw materials.

Several challenges hinder the widespread application and implementation of seaweed proteins. Plant protein extraction is still somewhat contemporary, especially for seaweed proteins. Understanding seaweed's cell wall integrity and structure would be necessary to enhance and ensure subsequent seaweed protein extractions. Moreover, interspecies variation exists for seaweed proteins, as protein characteristics depend on seaweed genetic makeup (Mišurcová, 2012). Another influential factor for protein variability would be the seaweed growth environment. Varying oceanic nutrients in different geographical inhabitations, differing temperature and seasonality also contribute to seaweeds' final phenotype (Murakami *et al.*, 2011). Hence, identifying a widely applicable and optimised protein extraction procedure would be challenging.

The scalability of seaweed protein extraction serves to be another obstacle, as most of the current procedures are at a laboratory scale (Pangestuti & Kim, 2015). Factors that could hinder the up-scaling of seaweed protein extraction would be food safety and processing cost. Seaweeds are known bio-absorbants of heavy metals, but such metals in seaweeds are generally below food safety limits (Holdt & Kraan, 2011). Nonetheless, seaweed protein extraction could be ameliorated through a hybrid of procedures rather than the solely employing one technique (Kumar *et al.*, 2021). Refining seaweed protein extraction into food-grade methods would widen their application opportunities in the food industry.

Commercial exploitation of seaweeds and their proteins could depend heavily upon public interest (Radulovich *et al.*, 2015). Despite the negative impacts of animal protein production, most consumers resist the intake of non-animal proteins, and the dietary pattern of high meat intake is still maintained (Macdiarmid *et al.*, 2016). Hence, the transition to non-animal protein alternatives could be a social-institutional conundrum (Graça *et al.*, 2019). Currently, the food industry is creatively developing products that mimic meat flavours and animal muscle textures.

A major advantage of cultivating seaweeds over territorial animal and plant sources is that seaweeds do not compete for arable land and potent water. Seaweeds also play a crucial role in reducing greenhouse gases, being an effective tool for carbon sequestration and combating climate change (Buschmann *et al.*, 2017; Chung *et al.*, 2011). However, mass cultivation of seaweeds offshore could alter biodiversity in the surrounding aquatic environments, threatening the local benthic ecosystems (Titlyanov & Titlyanova, 2010). Integrated seaweed mariculture with wind farms could minimise such problems, which is currently taking place in Belgium (Rawiwan *et al.*, 2022).

Seaweed protein extraction has substantial potential in New Zealand (NZ). Firstly, NZ waters are temperate and nutritious, with unoccupied coastal lines available for aquaculture cultivation. NZ's "green and clean" environment would produce quality seaweeds and seaweed products for commercial and industrial applications (Gibbons, 2014). Seaweed cultivation contains important economic and market values, and could simultaneously contribute to reducing of eutrophication and greenhouse gases (Chung *et al.*, 2011). Following the NZ government's goal to diversify protein production, extraction of seaweed proteins could be a favourable approach for manufacturing more non-animal protein foods from NZ's sustainable aquaculture systems (Sutton *et al.*, 2018).

4.2 Conclusion

With respect to the results obtained from this study, the following conclusions can be conferred:

- Korean commercial *Porphyra* sp. (KR seaweed) constituted 28.41% protein, whereas New Zealand wild *Pyropia* spp. (NZ seaweed) comprised 19.07% protein. The protein contents in both red seaweeds were comparable to other red seaweed species.
- The optimised conditions for each extraction protocol were as below: water extraction (biomass-water ratio of 1:40, extraction at 23 °C for 4 hr), alkaline extraction (biomass-water ratio of 1:30 at pH 12, 23 °C for 4 hr); ultrasound-assisted extraction (biomass-water ratio of 1:40 at 200 W and 20 kHz for 5 mins); enzyme-assisted extraction (biomass-water ratio of 1:40 at pH 9, 23 °C for 16 hr with Alcalase).
- For enzyme-assisted extraction, Alcalase-treatment (protease) significantly doubled seaweed protein efficiency compared to Celluclast (cellulase), Pectinex (polygalacturonase), and Shearzyme (xylanase) ($p < 0.05$). Alcalase yielded up to 68% protein from KR seaweed.
- Of the four extraction protocols, enzyme-assisted extraction resulted in the greatest protein purity of 43.01%.
- During AA profiling, seaweed protein extracts (SPEs) obtained from enzyme-assissted extraction exhibited significantly greater AA content than their respective raw seaweeds (163% and 117% greater for KR and NZ SPEs, respectively, $p < 0.05$), suggesting enzyme-assisted extraction would be suitable for extracting proteins from red seaweeds in this study.
- In AA profiling, SPEs derived from enzyme-assissted extraction portrayed comparable nutritional value (EAA:AA values of 29.03% and 31.31% for KR and NZ SPEs, respectively) to commercial protein isolates (SPI and WPI). In particular, NZ SPE consisted of comparable EAA:AA to other red seaweeds. This good nutritional value of red seaweed proteins would broaden their application as value-adding functional foods.
- The MW distribution of various SPEs illustrated that most extracted seaweed proteins ranged between 20 to below 10 kDa. SPEs from enzyme-assissted extraction were comprised of more low MW proteins and peptides than SPEs from other treatments.
- The FTIR spectra conveyed similar functional groups across all SPEs. The slight variance in amide I, II and III absorbances at 1630, 1530 and 1210 cm^{-1}, respectively, between SPEs, would corroborate differences in their protein secondary structures.

- The thermal properties of various SPEs were more impressive than SPI and WPI, with denaturation temperatures ranging from 150.18 °C to 166.29 °C for SPEs; thus, enhancing their thermal stability during processing.
- The SPEs from enzyme-assisted extraction showcased higher solubility than other SPEs and commercial protein isolates, reaching above 95% at ideal pHs. All SPEs had minimal solubility fluctuations at different pHs, enabling their possible incorporation into food systems at various pHs.
- KR SPE obtained from enzyme-assisted extraction demonstrated greater emulsifying properties than SPI and WPI at pH 11, with an emulsifying capacity of 62 mg^2/g and emulsifying stability of 77.03 mins.
- SPEs generally contained comparable foaming capacity to commercial isolates. In particular, KR SPEs derived from enzyme-assisted and water extractions could reach 260% and 280% foaming capacity, respectively, at pH 11. All SPEs had significantly greater foaming stability than commercial isolates at acidic pHs ($p < 0.05$), with KR SPE from enzyme-assisted extraction forming stable foams for the longest of 82.99 mins.
- Antioxidant capacity results from both DPPH and FRAP assays for all SPEs were of high consistency. SPEs following alkaline and water extractions accommodated the greatest antioxidant capacity (21.93 to 25.67 mM Trolox/g SPE), which could be associated with the greater proportion of hydroxyl groups within their SPEs. The SPEs in this study showed comparable antioxidant capacity to other red seaweeds.
- Conclusively, the physicochemical and functional properties of SPEs were comparable to commercial SPI and WPI. SPEs, especially ones extracted enzymatically, would constitute potential in the fortification and formulation of various food products.

4.3 Limitations

Although this study was conducted in a systematic way, with all pre-determined objectives well-satisfied and positive results retrieved, there are still some limitations due to the time constraints of this project and restricted access to some equipment during the global COVID-19 pandemic.

There were a couple of challenges during enzyme-assisted extraction of red seaweed proteins, including protein quantification (*Section 3.2.4.3*) and protein precipitation (*Section 3.2.4.4*). Firstly, polysaccharide co-precipitation with proteins was inevitable during seaweed protein extraction; this may limit the purity of protein extracts and further affect protein properties. Appropriate purification of protein extracts could enhance the elucidation of protein properties. Secondly, a sample-specific nitrogen-to-protein conversion factor was yet to be confirmed; thus, estimating protein through Kjeldahl would merely provide an approximate. Such challenges imply that the search for more advanced techniques would be necessary to ameliorate seaweed protein extraction and quantification.

Several limitations occurred during the AA profiling of SPEs. Firstly, 6 M HCl hydrolysis destroyed tryptophan, and partially damaged cysteine and its dimer for detection. Hence, a comparative analysis of AA composition following alkaline hydrolysis would be worth conducting if time permitted. Secondly, AA quantification could be more accurate if internal standards for each compound were included.

Finally, SPEs were harvested through freeze-drying, yet SPI and WPI were commercial products. Despite the production method of SPI and WPI may not be similar to extracts prepared in this study, SPI and WPI acted as good comparisons for the investigation into seaweed proteins.

In summary, some analytical methods in this study could be further optimised, and there is still much to be explored in the broad scope of seaweed protein. Future works will be elaborated on in the following section.

4.4 Future Work

Due to the COVID-19 pandemic and time constraints within this degree, the present study only focused on the various extractions of red seaweed proteins, and the properties and bioactivity of their subsequent protein extracts. Future work can be performed to develop and improve the outcomes of this study, as well as to further the comprehension of extraction, physicochemical and function properties, and antioxidant capacity of seaweed proteins.

Future works include and are not limited to:

- Elucidating the cell wall constituents in seaweed, thus clarifying target bonds to cleave for the further of improvement seaweed protein extraction efficiency and yield.
- Performing other conventional protein extraction techniques, thus enabling the comparison and determination of the ultimate extraction protocol for seaweed proteins.
- Purifying seaweed proteins to further comprehend their composition, through the exploration of more protein recovery techniques, thus curating a straightforward procedure that could be applied to various seaweed protein extracts.
- Analysing the proteins and their properties within seaweeds from various phyla (brown, green and red), thus aiding the selection and cultivation of seaweeds producing high-quality proteins.
- Isolating and identifying seaweed peptides, thus facilitating the understanding and refinement of their bioactivity.
- Assessing the bio-accessibility, digestibility and other health benefits of seaweed proteins, thus broadening the incorporation potential of seaweeds into pharmaceuticals and nutraceuticals.
- Conducting sensory analyses regarding the aroma and flavour of seaweed proteins alone or incorporated into appropriate foods, thus determining consumer perception of seaweed proteins.
- Optimising an applicable protocol for the simultaneous extraction of seaweed hydrocolloids and proteins, thus exploiting all parts of the seaweed to minimise industrial seaweed waste.

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Appendix

Appendix A. Trials conducted during the optimisation of seaweed protein extraction

Table A1. Comparison between different filters used and enzyme inactivation protocols conducted during enzyme-assisted extraction of seaweed proteins.

	Protein content (mg/g seaweed protein extract)
Membrane filter	40.02 ± 1.52
Paper filter	40.05 ± 0.24
Inactivation at 50°C	41.21 ± 0.20
No inctivation	40.95 ± 0.13

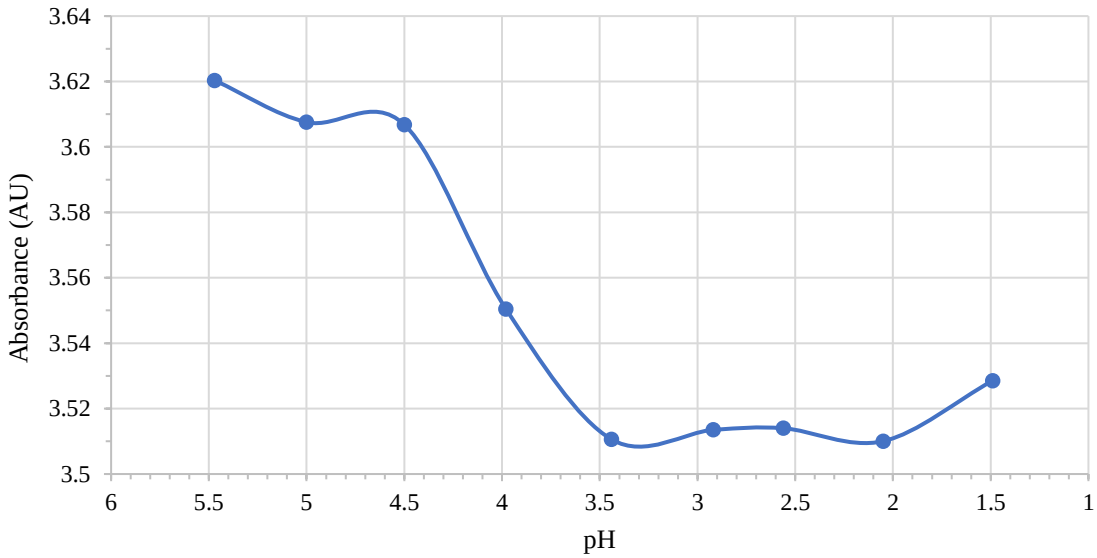

Figure A1. Isoelectric point of seaweed protein extracts following enzyme-assisted extraction measured through UV-VIS spectrometer.

Table A2. Protein content in Alcalase.

	Protein content (mg/g)
Alcalase	5.98 ± 0.17

Appendix B. Amino acid analysis

Table B1. Amino acid composition of raw seaweeds and seaweed protein extracts following 1 M HCl hydrolysis.

	Raw seaweed[1]		**SPEs[2]**				
	KR seaweed	**NZ seaweed**	**KR W-SPE**	**KR A-SPE**	**KR U-SPE**	**KR E-SPE**	**NZ E-SPE**
Phenylalanine	5.58 ± 0.23	3.57 ± 0.08	2.56 ± 0.05	4.27 ± 0.42	2.85 ± 0.08	6.91 ± 0.26	3.92 ± 0.19
Valine	0.07 ± 0.00	0.21 ± 0.02	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.07 ± 0.00	0.11 ± 0.00
Proline	2.11 ± 0.13	1.50 ± 0.05	0.94 ± 0.04	1.45 ± 0.11	1.39 ± 0.08	2.73 ± 0.21	1.49 ± 0.11
Methionine	0.07 ± 0.01	0.03 ± 0.00	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.01	0.03 ± 0.00
Tryptophan	4.61 ± 0.01	2.96 ± 0.05	2.57 ± 0.11	4.00 ± 0.37	3.61 ± 0.45	7.46 ± 0.03	2.44 ± 0.02
Leucine	1.33 ± 0.04	0.40 ± 0.02	2.22 ± 0.17	1.12 ± 0.06	0.90 ± 0.11	3.08 ± 0.19	1.55 ± 0.14
Isoleucine	6.99 ± 0.22	3.73 ± 0.10	3.02 ± 0.04	5.20 ± 0.28	3.19 ± 0.38	12.53 ± 0.23	5.45 ± 0.21
Alanine	6.73 ± 0.11	4.67 ± 0.03	18.48 ± 0.62	16.10 ± 0.73	16.50 ± 0.69	17.12 ± 1.64	9.18 ± 0.25
Tyrosine	7.25 ± 0.34	4.68 ± 0.09	3.41 ± 0.09	5.85 ± 0.47	3.79 ± 0.37	2.41 ± 0.05	5.33 ± 0.25
Glycine	51.55 ± 0.86	35.62 ± 0.14	51.73 ± 9.77	72.14 ± 7.23	94.19 ± 14.60	65.63 ± 3.69	57.38 ± 4.73
Threonine	10.19 ± 0.47	7.03 ± 0.17	9.88 ± 0.28	11.90 ± 1.04	10.42 ± 0.81	21.27 ± 1.02	9.03 ± 0.36
Glutamine	0.29 ± 0.02	0.50 ± 0.04	0.49 ± 0.01	0.15 ± 0.01	0.56 ± 0.04	0.38 ± 0.03	0.61 ± 0.05
Asparagine	10.01 ± 0.45	17.10 ± 0.07	10.33 ± 0.83	13.68 ± 1.36	18.46 ± 0.46	18.85 ± 0.09	18.71 ± 0.77
Serine	23.42 ± 1.27	22.40 ± 0.34	19.55 ± 1.66	24.03 ± 1.29	16.10 ± 0.38	38.07 ± 1.30	28.17 ± 1.01
Aspartic acid	2.46 ± 0.03	1.65 ± 0.04	1.41 ± 0.02	2.02 ± 0.08	1.52 ± 0.11	2.33 ± 0.10	1.62 ± 0.02
Histidine	11.12 ± 0.35	8.66 ± 0.04	7.63 ± 0.14	10.54 ± 0.61	7.69 ± 0.26	14.65 ± 0.37	9.29 ± 0.09
Arginine	0.19 ± 0.00	0.23 ± 0.01	0.18 ± 0.01	0.21 ± 0.01	0.16 ± 0.01	0.28 ± 0.01	0.21 ± 0.01
Glutamic acid	7.56 ± 0.01	5.26 ± 0.05	5.64 ± 0.31	6.91 ± 0.36	4.79 ± 0.06	9.27 ± 0.45	5.68 ± 0.10
Lysine	38.57 ± 0.44	27.35 ± 0.26	24.37 ± 0.99	36.49 ± 1.65	25.91 ± 1.57	52.73 ± 1.70	30.68 ± 1.08
Cysteine	39.85 ± 0.74	22.99 ± 0.60	29.13 ± 0.90	37.22 ± 2.36	29.30 ± 1.33	51.72 ± 0.21	33.14 ± 0.78
Total AA	222.01 ± 13.85	170.32 ± 1.38	189.86 ± 8.13	251.17 ± 13.85	232.28 ± 1.89	320.75 ± 5.66	219.29 ± 2.51
EAA:AA[3]	34.02 ± 0.33	27.46 ± 0.18	27.51 ± 1.69	27.59 ± 0.33	23.61 ± 1.32	30.82 ± 1.42	27.46 ± 0.25
EAA:NEAA[4]	51.60 ± 0.63	37.85 ± 0.33	38.03 ± 3.22	38.10 ± 0.63	30.94 ± 2.27	44.60 ± 2.96	37.86 ± 0.47
AAS[5]	26.34	16.01	17.81	24.13	18.73	35.80	20.88
EAAI[5]	9.63	6.89	6.17	8.63	6.75	10.63	7.11

[1]: Raw seaweeds did not undergo further extraction (mg amino acid/g seaweed dry weight); [2] SPE: seaweed protein extract (mg amino acid/g SPE), W-SPE: SPE from water extraction, A-SPE: SPE from alkaline extraction, U-SPE: SPE from ultrasound-assisted extraction, E-SPE: SPE from enzyme-assisted extraction, SPI: soy protein isolate, WPI: whey protein isolate; [3] EAA:AA, essential amino acid quantity compared to total AA quantity; [4] EAA:NEAA, EAA compared to non-EAA; [5] AAS: AA score, EAAI: essential AA index; AAS and EAAI calculated according to the EAA requirement scoring patterns for children ages 3 to 10 years (WHO/FAO/UNU, 2007).

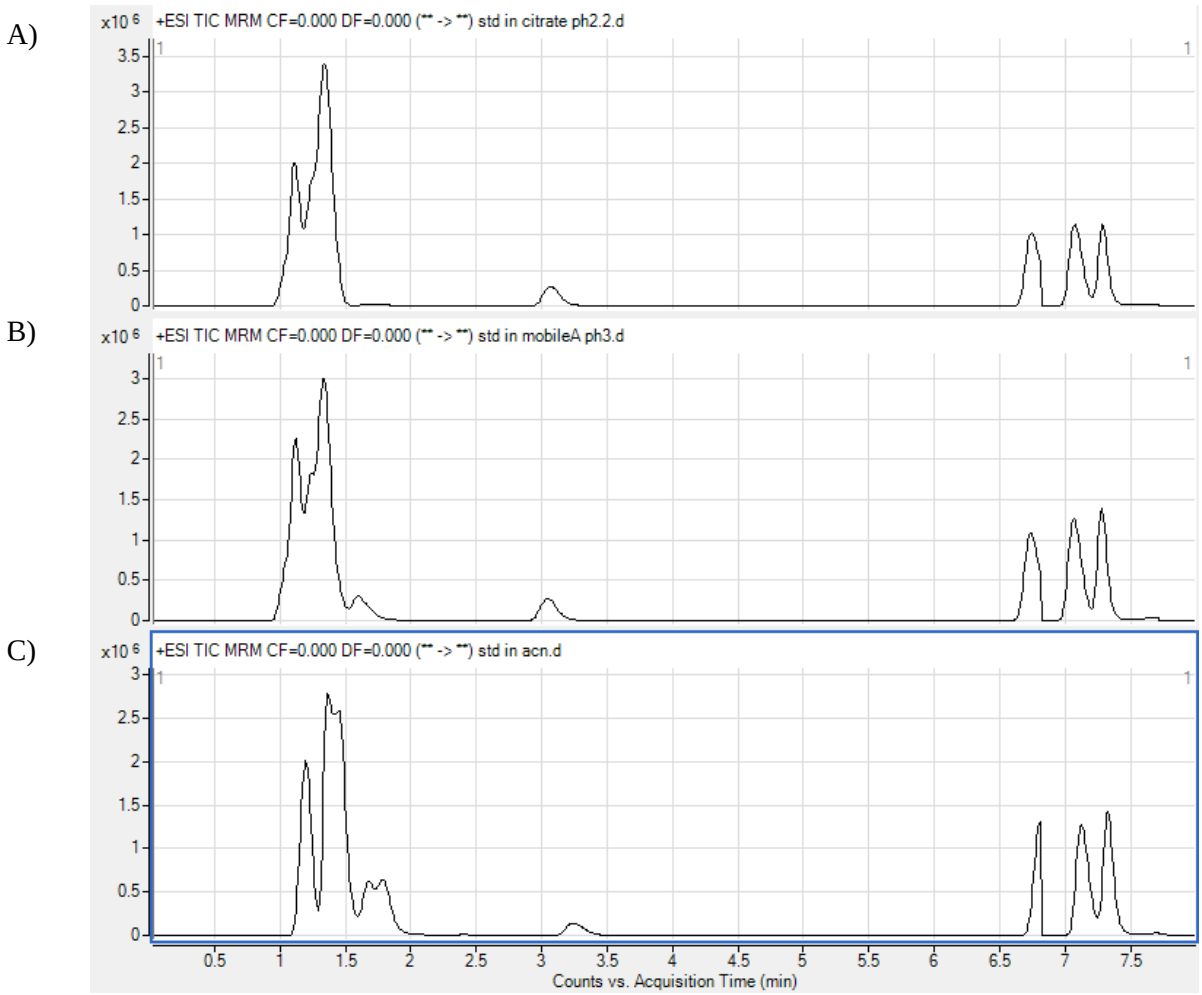

Figure B2. HPLC-MS spectra of amino acid standard in A) citrate buffer at pH 2.2, B) mobile phase A at pH 3, and C) Acetonitrile in 20% type-1 water.

Table B2. Linear curves of amino acid standards utilised to calculate the amino acid content of raw seaweeds, their seaweed protein extracts and commercial isolates.

	Slope	**y-Intercept**	**R^2 value**
Phenylalanine	-8554.9986	58810.7655	0.9997
Valine	-29976.4876	247298.4534	0.9983
Proline	-354803.2388	2205634.1802	0.9978
Methionine	-28691.7419	150212.1303	0.9995
Tryptophan	305611.1486	137462.4602	0.9952
Leucine	-7984.1295	168247.5136	0.9952
Isoleucine	-5013.5363	157704.8597	0.9990
Alanine	-19535.6204	111595.5670	0.9998
Tyrosine	1151.4118	7509.2215	0.9929
Glycine	5.5417	176.7320	0.9965
Threonine	2552.1250	3801.3705	0.9949
Glutamine	157.6957	56.3664	0.9999
Asparagine	228.0454	126.2316	0.9971
Serine	-3151.0932	29699.7527	0.9972
Aspartic acid	-13111.4339	24234.1352	0.9794
Histidine	2753.6274	288935.1989	0.9998
Arginine	3019.3018	195647.9657	0.9982
Glutamic acid	23.2878	121.0852	0.9993
Lysine	-1375.6379	27975.2147	0.9996
Cysteine	17.9222	1776.5841	0.9997

Appendix C. DPPH scavenging potential and FRAP assays

Table C1. Linear curves of DPPH scavenging potential and FRAP assays used to calculate the antioxidant capacity of SPEs.

	Slope	**y-Intercept**	**R^2 value**
DPPH	0.0006	-0.0093	0.9979
FRAP	0.0011	0.0128	0.9974

DPPH, 2,2'-diphenyl-1-picrylhydrazyl radical scavenging assay; FRAP, ferric reducing antioxidant power assay.