

UNLOCKING THE PROTEIN POTENTIAL OF *ULVA*:A COMPARATIVE ANALYSIS OF EUROPEAN AND ASIAN SPECIES AND THEIR GASTROINTESTINAL DIGESTIBILITY

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Green seaweeds along the shores of Sokcho (South Korea), 11 October 2025—the beginning of a thesis journey and a year shaped by Ulva.

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Ghent, June 2026

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USE OF GENAI TOOLS IN THE MASTER'S DISSERTATION

- ☐ No use of GenAI tools
☒ One or more GenAI tools used

Indicate and describe, in case of using one or more GenAI tools, how you have used them:

	GenAI tool used as	Description	Used tool(s)
☐	Idea generator		
☒	Search engine	Searching literature	Gpt-5.5
☒	Writing assistant	Rewriting my text in scientific English	Gpt-5.5, Notebook LM
☒	Designer	Rewriting my text in scientific English	Gpt-5.5
☐	Practice tool		
☒	Data analysis	Help with statistics	Gpt-5.5
☒	Efficiency booster	Help with generating code and tables.	Gpt-5.5
☒	Used in another way	Help with generating code and tables.	Gpt-5.5

LIST OF ABBREVIATIONS

- **AA:** Amino Acids
- **ACE:** Angiotensin-Converting Enzyme
- **APC:** Algal Protein Concentrate
- **AS:** Ammonium Sulfate
- **ASC:** Aquaculture Stewardship Council
- **BCA:** Bicinchoninic Acid assay
- **BSA:** Bovine Serum Albumin
- **CF:** Conversion Factor
- **CLSM:** Confocal Laser Scanning Microscopy
- **CMEMS:** Copernicus Marine Environment Monitoring Service
- **CWS:** Calcofluor White Staining
- **DF:** Dietary Fibre
- **DH:** Degree of Hydrolysis
- **DHA:** Docosahexaenoic Acid
- **DMSO:** Dimethyl Sulfoxide
- **DM:** Dry Matter
- **DPP-IV:** Dipeptidyl Peptidase IV
- **DW:** Deionised Water; Dry Weight
- **EAA:** Essential Amino Acids
- **EFSA:** European Food Safety Authority
- **EPA:** Eicosapentaenoic Acid
- **FAO:** Food and Agriculture Organization of the United Nations
- **Fast Green FCF:** Fast Green For Colouring Food
- **GAE:** Gallic Acid Equivalents
- **IVPD:** *In Vitro* Protein Digestibility
- **KHOA:** Korea Hydrographic and Oceanographic Agency
- **KOPTRI:** Korea Polymer Testing & Research Institute
- **LOD:** Limit Of Detection
- **LOQ:** Limit Of Quantification
- **MWCO:** Molecular Weight Cut-Off
- **MSC:** Marine Stewardship Council
- **OECD:** Organisation for Economic Co-operation and Development
- **OPA:** o-phthaldialdehyde assay
- **PFD:** Photon Flux Density
- **PSU:** Practical Salinity Units
- **PUFA:** Polyunsaturated Fatty Acid
- **QSAR:** Quantitative Structure–Activity Relationship
- **RAAS:** Renin-Angiotensin-Aldosterone System
- **RT:** Room Temperature
- **SCFA:** Short-Chain Fatty Acids
- **SD:** Standard Deviation
- **SDS-PAGE:** Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
- **SGF:** Simulated Gastric Fluid
- **SIF:** Simulated Intestinal Fluid
- **SPM:** Sodium Phosphate Monobasic
- **Spp.:** Species
- **SSF:** Simulated Salivary Fluid
- **TEAA:** Total Essential Amino Acids
- **TPC:** Total Phenolic Content
- **UNU:** United Nations University
- **UPI:** Undigested Protein Isolate
- **US:** Ultrasound
- **WHO:** World Health Organization
- **WR:** Working Reagent

ABSTRACT

The growing demand for sustainable protein sources has intensified interest in seaweeds as alternative ingredients due to their low environmental impact and ability to generate bioactive peptides during gastrointestinal digestion. This study compared four *Ulva* species from Europe and South Korea in terms of protein digestibility and peptide release following standardized *in vitro* gastrointestinal digestion (INFOGEST 2.0), with all samples processed under identical conditions to enable interspecies comparison. Protein extracts were evaluated using biochemical assays and quantitative confocal laser scanning microscopy (CLSM). Clear interspecies differences were observed, with protein digestibility ranging from 38–83%, demonstrating strong species dependency. *Ulva australis* (75.63% ± 8.77) and *Ulva linza* (83.61% ± 4.58) showed the highest digestibility and most favourable peptide release profiles, indicating enhanced protein breakdown. In contrast, *Ulva fasciata* consistently exhibited lower digestibility (38.62% ± 10.09) and a more resistant structural organisation after digestion, as confirmed by CLSM observations. These findings highlight that protein utilization in *Ulva* is strongly influenced by species-specific structural properties. Overall, this study identifies distinct differences in digestive performance that are critical for the targeted selection of seaweed biomass for functional food and protein valorisation applications.

NEDERLANDSE SAMENVATTING

De toenemende vraag naar duurzame eiwitbronnen heeft de interesse in zeewieren als alternatieve ingrediënten versterkt, onder meer vanwege hun lage milieu-impact en hun potentieel om tijdens gastro-intestinale vertering bioactieve peptiden te genereren. In deze studie werden vier *Ulva*-soorten uit Europa en Zuid-Korea vergeleken op het vlak van eiwitverteerbaarheid en peptidenvrijstelling na gestandaardiseerde *in vitro* gastro-intestinale digestie (INFOGEST 2.0), waarbij alle stalen onder identieke omstandigheden werden verwerkt om interspecifieke vergelijking mogelijk te maken. De eiwitextracten werden geanalyseerd met biochemische assays en kwantitatieve confocale laserscanning microscopie (CLSM). Er werden duidelijke interspecifieke verschillen vastgesteld, met een eiwitverteerbaarheid tussen 38 en 83%, wat wijst op een sterke soortafhankelijkheid. *Ulva australis* (75.63% ± 8.77) en *Ulva linza* (83.61% ± 4.58) vertoonden de hoogste verteerbaarheid en de meest gunstige peptidenvrijstellingsprofielen, wat duidt op een efficiëntere eiwitafbraak. *Ulva fasciata* daarentegen vertoonde consequent een lagere verteerbaarheid (38.62% ± 10.09) en een meer resistente structurele organisatie na digestie, zoals bevestigd door CLSM-observaties. Deze resultaten tonen aan dat eiwitbenutting binnen *Ulva* sterk wordt bepaald door soortspecifieke structurele eigenschappen. Samengevat benadrukken deze bevindingen duidelijke verschillen in digestieprestaties, die van belang zijn voor de gerichte selectie van zeewierbiomassa voor toepassingen in functionele voeding en eiwitvalorisatie.

INTRODUCTION

The global community faces a huge challenge: securing a nutritious and sufficient food supply for a continuously growing world population whilst mitigating climate change and environmental degradation. The food industry is seeking new and more sustainable food sources to meet the demands of a growing global population, which is expected to reach 9.7 billion people by 2050 (United Nations, 2017).

This task is further complicated by the fact that current food production systems are a leading contributor to environmental degradation and global warming. The expansion of conventional agriculture often leads to severe ecological damage, including the desertification of arable land, which in turn increases food shortages (Xu et al., 2023). This cycle of production and degradation necessitates an urgent global transition toward more sustainable and resilient food systems. To meet this imperative, we must look beyond traditional terrestrial agriculture and explore novel resources. The ocean, which covers over two-thirds of our planet's surface and contains an "arable land" area estimated to be 15 times that of land, represents a vast and largely untapped reservoir of potential that can alleviate these pressures and redefine our approach to global food security (Xu et al., 2023).

Among the ocean's diverse biological resources, seaweed emerges as a highly promising and sustainable solution to the challenges of modern food production. The strategic cultivation of marine organisms like seaweed offers a powerful means to diversify our food supply while simultaneously mitigating environmental impact. The sustainability advantages of seaweed cultivation are profound and multifaceted, positioning it as a promising component of future food systems. Key benefits include (i) a minimal environmental footprint, as seaweed farming has a low carbon impact, does not compete with terrestrial agriculture for land or freshwater resources, and generally requires no synthetic pesticides (Gephart et al., 2021). (ii) A strong bioremediation capacity, since seaweed absorbs excess nutrients such as nitrogen and phosphorus from the water column, thereby mitigating coastal eutrophication driven by nutrient runoff and acting as an effective natural nutrient sink (Gephart et al., 2021; Neveux et al., 2018). (iii) Global abundance and accessibility, with tens of thousands of seaweed species present in marine ecosystems worldwide, many of which remain undescribed. Beyond these environmental advantages, the nutritional composition of seaweed further enhances its potential as a human food source (**cf. Literature review: section 3**), supporting its further evaluation as an alternative protein resource.

In addition to their macronutrient profile, seaweeds are often described as "superfoods" due to their rich and diverse range of bioactive compounds (Peñalver et al., 2020). Among these, proteins and protein-derived bioactive peptides are of particular interest. Bioactive peptides can be generated during human gastrointestinal digestion and may exhibit a range of health-promoting properties relevant to functional food applications (O'Brien et al., 2022). However, the quantity and nature of peptides released during digestion of seaweed protein extracts can vary depending on both species and the intrinsic composition of the extract. In turn, this composition is influenced by the extraction method used to isolate proteins from seaweed biomass. A key challenge is therefore to evaluate the potential for bioactive peptide formation following gastrointestinal digestion of protein extracts derived under comparable conditions from different seaweed species.

In this context, the present study compares four *Ulva* species (two from South Korea and two from Europe) in terms of their potential for bioactive peptide production following simulated gastrointestinal digestion of their protein extracts. The objectives of the study are to (i) compare digestibility patterns of protein extracts across species, (ii) identify factors influencing protein digestibility, including chemical composition and structural characteristics of the extracts, and (iii) evaluate the resulting digests in terms of the proportion of low-molecular-weight peptides. Ultimately, this study aims to integrate these criteria to identify the *Ulva* species with the most favourable performance in this functional context, thereby supporting a more targeted selection of seaweed biomass for functional food applications.

LITERATURE REVIEW

1. Seaweed production and cultivation context: Europe versus South Korea

1.1. Production in South Korea

Historically, wild harvesting was the main source of seaweed and still predominates in Europe and North America (Juul et al., 2024). However, production has largely shifted towards cultivation, with most of the seaweed now produced in Asia, particularly in China (67%), Indonesia (27%), South Korea (5%), and Japan (1%) (OECD, 2025). Global seaweed production exceeded 34 million tons fresh weight in 2022, more than double the 2005 level (OECD, 2025), and consisted almost exclusively of brown and red seaweeds. Approximately 85% of the produced seaweed is used for human consumption (FAO, 2018), compared to only 20% of global soy production (353 million tons in 2020) (Juul et al., 2024).

Zooming in on South Korea, seaweed cultivation technologies have been progressively developed since the 1970s. In 2017, marine farms produced 1,761,526 tons of seaweed, with production largely concentrated on *Pyropia*, *Undaria* and *Saccharina*. Among these, *Pyropia* is of greatest economic importance and accounts for roughly two thirds of the total production value. South Korea is the world's leading exporter of *Pyropia* products and exported approximately 525 million US dollars' worth of these products to 110 countries in 2018 (Hwang & Park, 2020). Considerable efforts have been made to improve productivity through selective breeding, and since 2012 the South Korean government has implemented a formal system for the certification of seaweed varieties. To date, nineteen cultivars have been officially registered, comprising thirteen *Pyropia*, five *Undaria* and one *Saccharina* variety (Hwang & Park, 2020).

Beyond increasing biomass production, the industry is increasingly focused on adding value through processing. A wide range of convenience foods and snack products has been developed, targeting consumers with an interest in nutrition and health. At the same time, sustainability has become an explicit objective of the sector. In 2019, a South Korean seaweed company became the first in the world to obtain combined ASC and MSC certification, with further certifications expected in the future (Hwang & Park, 2020).

1.2. Production in Europe

Seaweeds have long been used by coastal populations in Europe as a source of food, feed, and fertilizer, with commercial exploitation starting in the seventeenth century for potash (K_2O -equivalent potassium content) production and later shifting towards iodine and hydrocolloids such as alginic acid (Childs, 2021; Porse & Rudolph, 2017). Today, seaweeds are increasingly valued for their nutritional and biochemical properties and their potential applications in food, energy, biofuels, bioplastics, and biofiltration (Holdt & Kraan, 2011; Wei et al., 2013; Cahill et al., 2010). Despite this growing interest, the European seaweed industry remains largely dependent on wild harvesting, raising concerns about ecosystem degradation (FAO, 2014; Buschmann et al., 2014).

While Asia accounts for 99% of the global marketed seaweed, producing 34.7 million tons annually worth 14.85 billion USD, Europe's industry remains largely in the hands of startups. In 2019, only 3.8% of the 287,033 tons harvested were produced domestically, mostly from wild stocks (Jueterbock et al., 2025). Yields in Europe are still much lower than in South Korea, as cultivation practices are not yet as optimized. Nonetheless, seaweed farming is recognized as a key component of the EU's strategic guidelines for sustainable aquaculture (Jueterbock et al., 2025). More specifically, the North Sea offers high potential for seaweed farming due to its nutrient-rich waters, although large-scale cultivation is currently limited. Modelling studies suggest substantial production potential with limited ecosystem impacts, provided nutrient availability and light conditions are sufficient (Reith et al., 2005; van der Molen et al., 2018). Nutrient dynamics are critical, as seaweed farms can both cause local nutrient depletion and provide ecosystem services through nutrient recycling and bioremediation (Burson et al., 2016; Neori et al., 2003). Species identified as particularly suitable for North Sea cultivation include *Ulva lactuca*, *Saccharina latissima*, *Laminaria digitata*, and *Palmaria palmata* (Fig. 1).

2. Seaweeds: Taxonomy, ecology, and general characteristics

2.1. Classification of macroalgae

2.1.1. Phylogeny

Marine macroalgae, commonly known as seaweeds, are a diverse group of multicellular, photosynthetic eukaryotes that inhabit a wide range of aquatic environments. While often resembling terrestrial vegetation, macroalgae are fundamentally different. They lack the specialized tissue characteristics of land plants, such as true roots, internal vascular structures, seeds, or flowers (Graham & Wilcox, 2000).

The approximately 9,000 species of macroalgae are traditionally divided into three primary taxonomic clades based on deep evolutionary divergences, which for most species correspond to distinct pigmentation patterns. These groups comprise the brown algae (Phaeophyta), red algae (Rhodophyta), and green algae (Chlorophyta) (**Fig. 1**) (Khan et al., 2009). This classification is also reflected in their evolutionary relationships: red and especially green algae share a closer ancestry with terrestrial plants (**Fig. 2**), including legumes, whereas brown algae are phylogenetically more closely related to diatoms (Guiry & Guiry, 2022).

Although this pigment-based system is practical for field identification, phylogenetic analyses have demonstrated that it encompasses lineages with substantial evolutionary divergence. Specifically, brown algae are classified within the kingdom Chromista, while red and green algae belong to the kingdom Archaeplastida (Umen, 2014; Guiry & Guiry, 2022).

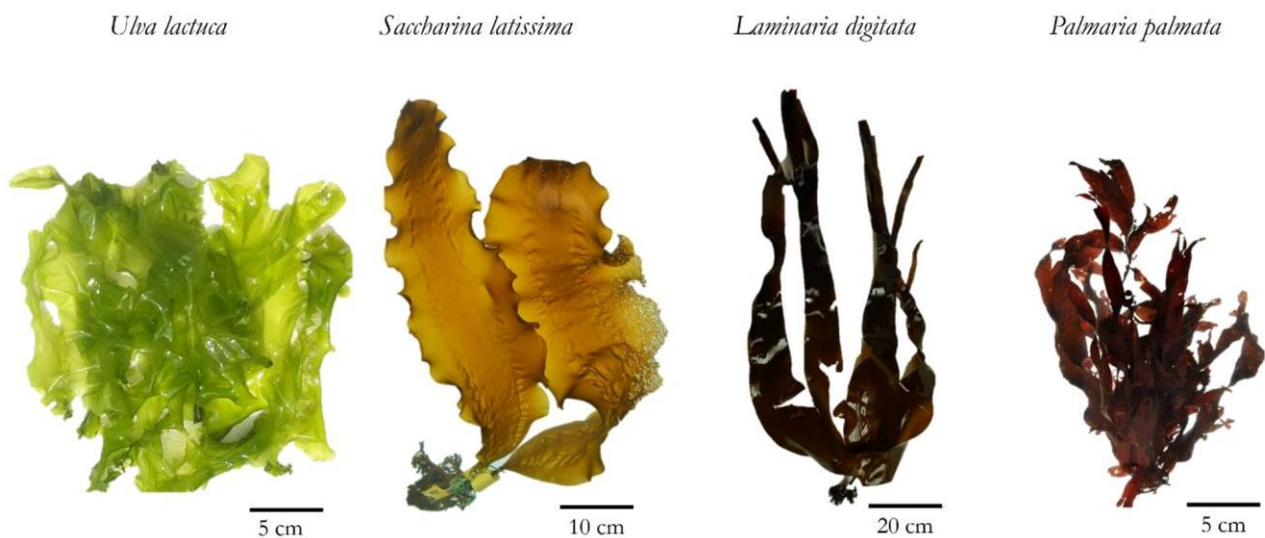


Fig. 1. Different classes of macroalgae (Lubsch, 2019).

The origin of photosynthesis in these eukaryotic lineages is a classic example of endosymbiosis. The first photosynthesizing eukaryote was formed through a primary endosymbiotic event, wherein a heterotrophic host cell engulfed a cyanobacterium. Over evolutionary time, this cyanobacterium became fully integrated into the host cell, eventually evolving into the specialized photosynthetic organelle known as a plastid (Archibald, 2009; Keeling, 2010).

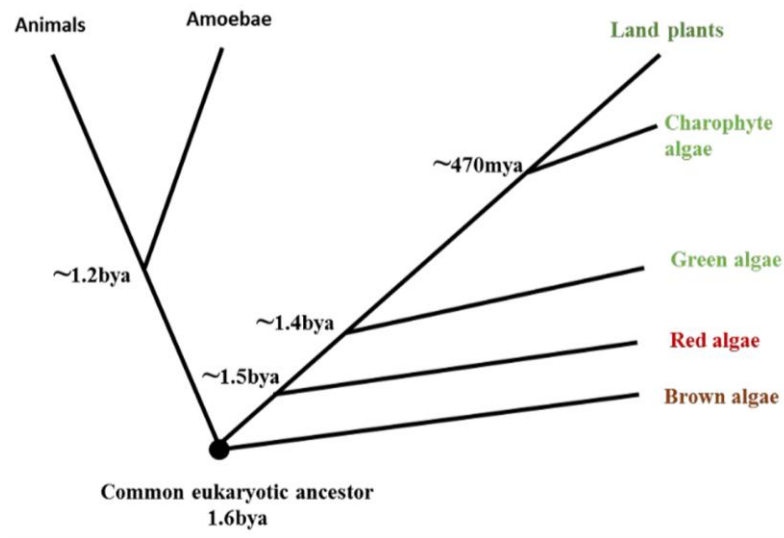


Fig. 2. A conceptual representation of the eukaryotic phylogenetic tree demonstrating the evolutionary positions of green, red, and brown seaweeds (Ghaderiardakani, 2019).

2.1.2. The three primary divisions of seaweed

Rhodophyta (Red algae)

Rhodophyta, or red algae, constitute a large and diverse division characterized by the presence of phycobilin pigments (phycoerythrin and phycocyanin), which mask the green colour of chlorophylls and give them their distinctive red, pink, or purple hues (Manzoor et al., 2024). These pigments are highly efficient at absorbing blue and green light, allowing many red algae to thrive in deeper waters where light penetration is limited. Representative species include the commercially important edible dulse (*Palmaria palmata*) (Lubsch, 2019), nori (*Porphyra sp.*), and various *Gracilaria* species used for agar production. The red alga *Delisea pulchra* serves as a key model organism for studying chemical ecology and host-pathogen interactions (Ghaderiardakani, 2019).

Phaeophyta (Brown algae)

The brown algae are characterized by the pigment fucoxanthin, which gives them their typical olive-green to dark brown colour (Manzoor et al., 2024). This group includes the largest and structurally most complex seaweeds, such as the giant kelps that form vast underwater forests. Brown algae are dominant in temperate and polar coastal ecosystems. Notable examples include sugar kelp (*Saccharina latissima*), oarweed (*Laminaria digitata*) (Lubsch, 2019), the floating mats of *Sargassum* that define the Sargasso Sea, and knotted wrack (*Ascophyllum nodosum*). The small, filamentous brown alga *Ectocarpus siliculosus* has been established as a genomic model for studying this lineage (Ghaderiardakani, 2019).

Chlorophyta (Green algae)

Chlorophyta, commonly known as green algae, contain chlorophylls *a* and *b* as their primary photosynthetic pigments, which confer a vibrant green colour similar to that of terrestrial plants, along with accessory carotenoids (Manzoor et al., 2024). This pigment composition is no coincidence; the green algal lineage is ancestrally linked to the evolution of all terrestrial plants as shown in **Figure 2** (Bennici, 2008; Leliaert et al., 2012). While most diverse in freshwater environments, marine green algae are conspicuous members of coastal ecosystems worldwide. The most extensively documented marine genus is *Ulva*, which includes species such as sea lettuce (*Ulva lactuca*), gutweed (*Ulva intestinalis*), and the model organisms *Ulva mutabilis* and *Ulva rigida* (Ghaderiardakani, 2019).

Figure 3 shows the green lineage, known as Viridiplantae, originated from an ancestral green flagellate. This lineage split into two major phyla: Streptophyta and Chlorophyta. Streptophyta include charophyte algae and the entire land plant kingdom (Embryophyta). Chlorophyta encompass a vast diversity of green algae, which are further divided into several classes. Among these, the class Ulvophyceae, which includes the genus *Ulva*, evolved from the chlorophyte

branch. This phylogenetic placement highlights that while *Ulva* and its relatives are related to land plants, they belong to a sister lineage that remained in aquatic environments and pursued a different evolutionary path of multicellular organization (Ghaderiardakani, 2019).

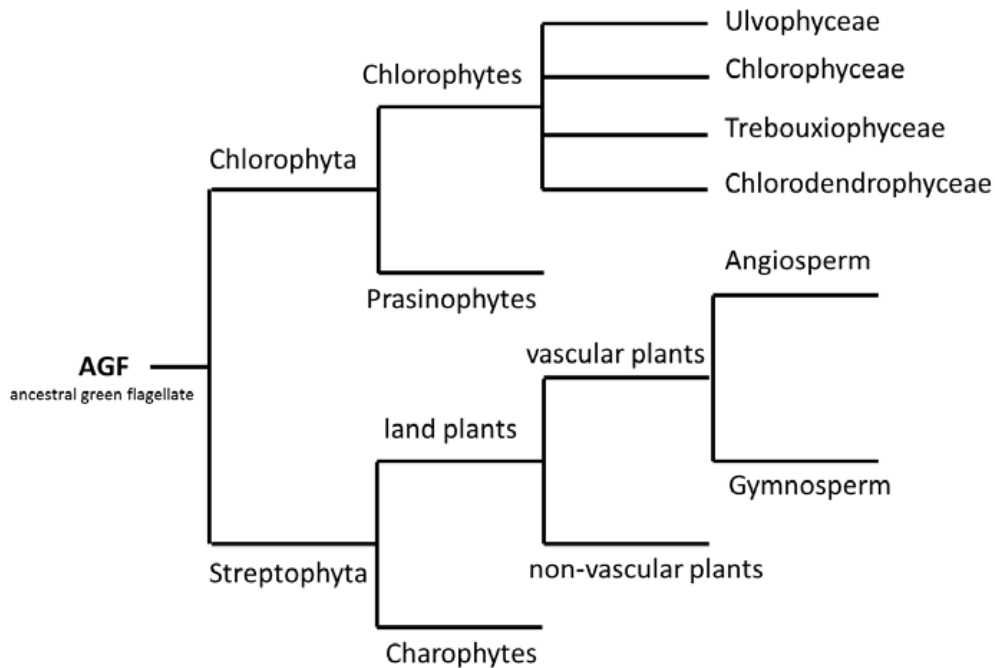


Fig. 3. Summary of the phylogenetic relationships among the main lineages of green plants (Viridiplantae), showing the divergence of Chlorophytes and Streptophytes from an ancestral green flagellate (Ghaderiardakani, 2019).

2.1.3. Taxonomic challenges: The case of the genus *Ulva*

Species-level identification within macroalgae is often complicated by high morphological plasticity. The green algal genus *Ulva* exemplifies this problem, as its simple thallus structure is strongly influenced by environmental conditions. Morphological traits traditionally used for identification, such as branching, have proven unreliable. For instance, *Ulva intestinalis* can develop branching under low-salinity conditions, making it difficult to distinguish from *Ulva compressa* (Blomster et al, 1998).

These limitations led to major taxonomic revisions based on molecular phylogenetics. Formerly separate genera, including *Enteromorpha*, were shown to be nested within *Ulva* and were therefore merged into the latter (Hayden et al, 2003). As a result, molecular markers such as *rbcl* and *tufA* are now essential for accurate species identification within the genus, highlighting the importance of combining molecular and morphological approaches in macroalgal taxonomy (Ghaderiardakani, 2019).

2.2. Growth cycle and zonation of seaweeds

As an example, *Ulva* species will be discussed as these species is also the subject of this thesis. The "isomorphic biphasic" life cycle of *Ulva* is a sophisticated evolutionary adaptation that enables rapid colonization while maintaining genetic diversity. By employing two morphologically identical but genetically distinct multicellular generations, the genus can produce vast quantities of reproductive swimmers (zoospores and gametes), allowing it to swiftly dominate bare substrates such as jetty walls and maritime vessels (Costa et al, 2024).

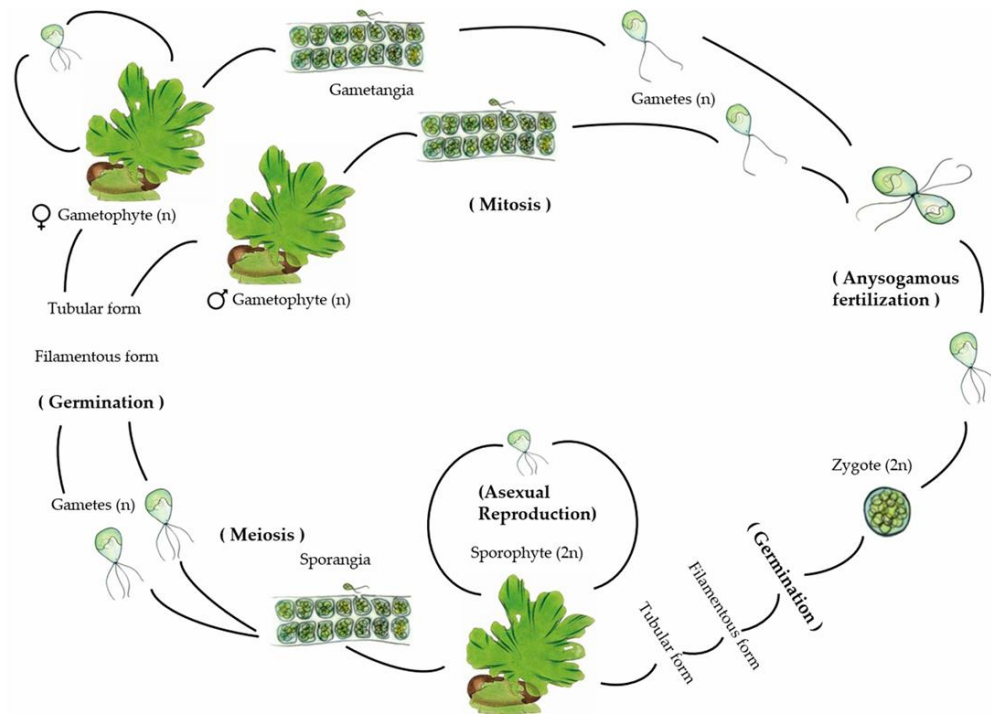


Fig. 4. Typical life cycle of *Ulva* spp. (Costa et al., 2024).

Following **Figure 4**, this haplodiplontic cycle proceeds through the following sequential stages:

1. **Diploid sporophyte (2n):** The multicellular adult generation. Upon maturation, vegetative cells transition into sporangia, producing quadriflagellate zoospores via meiosis.
2. **Haploid gametophytes (n):** Zoospores germinate into male or female gametophytic thalli. While genetically haploid, these are morphologically indistinguishable from the sporophyte.
3. **Gametogenesis:** Mature gametophytes produce biflagellate gametes mitotically, typically released from the distal margins of the thallus.
4. **Syngamy:** Anysogamous fertilization occurs as male and female gametes fuse to form a diploid zygote (2n). This zygote germinates into a multicellular adult sporophyte, completing the cycle.

2.3. *Ulva* spp.: Distribution and ecological plasticity

As a cosmopolitan genus, *Ulva* possesses an extraordinary capacity to colonize diverse aquatic systems, including rocky shores, salt marshes, and even freshwater environments (Costa et al., 2024). The global distribution of *Ulva* species is currently documented as follows:

- Asia: 56 species (18 endemic)
- Australia: 40 species (11 endemic)
- Europe: 38 species (9 endemic)
- North America: 34 species (2 endemic)
- Africa: 31 species (6 endemic)
- South America: 20 species (1 endemic)
- Antarctica: 12 species

The ecological success of *Ulva* is largely attributed to its pronounced ecological plasticity, enabling rapid physiological adjustments to variations in light intensity, temperature, and salinity (**Table 1**). This flexibility allows *Ulva* species to outcompete more specialized macroalgae in dynamic and unstable coastal environments (Costa et al., 2024).

Table 1. Environmental factors illustrating the ecological plasticity of *Ulva* species. PSU = Practical Salinity Units, PFD= Photon Flux Density.

Factor	Identified thresholds and impacts
Salinity (PSU)	Optimal spore release and growth between 20–35 PSU. Spore biomass is significantly limited at 5 PSU (Sousa et al., 2007; Han et al., 2008).
Light intensity (PFD)	Growth rate increases linearly up to 30 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$; saturation occurs beyond 70 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (Mantri et al., 2020).
Temperature	Reproduction is optimally induced between 15–20 °C. High temperature increases can double growth rates in certain species (Mantri et al., 2020; Costa et al., 2024).

A critical survival strategy involves the response to temperature shock. A rapid decrease in temperature, specifically a 4 °C shock for 10 minutes, can trigger a massive reproductive response, peaking in gamete release within two days (Carl et al., 2014; Gao et al., 2017). This mechanism suggests *Ulva* treats thermal stress as a signal for reproductive dispersal to ensure progeny survival (Costa et al., 2024).

3. Nutritional composition of seaweeds

This section opens with an overview of the principal chemical constituents of the three major seaweed clades, as summarized by Trigo (2023). These compositional data are further contextualized through comparison with conventional plant- and animal-derived protein sources, providing a benchmark to evaluate the nutritional and functional potential of seaweed biomass. Importantly, as illustrated in **Tables 1** and **2**, the chemical composition of macroalgae varies widely depending on species, developmental stage, environmental conditions, and nutrient availability (Pereira et al., 2024)

Table 2. Macronutrient composition (% dry weight) of seaweed clades compared to terrestrial protein sources of plant and animal origin (Trigo, 2023).

	Carbohydrates	Protein	Ash	Lipids	References
Brown seaweed	41-72	4-11 ^a	20-48	1	(Albers et al., 2021; Harrysson et al., 2018; Schiener et al., 2015; Stévant, Marfaing, et al., 2017)
Green seaweed	26-48	5-20 ^a	17-22	1-4	(Harrysson et al., 2018; Marsham et al., 2007; Morrissey et al., 2001; Steinhagen et al., 2021, 2022)
Red seaweed	42-75	8-35 ^a	9-32	<1-3	(Galland-Irmouli et al., 1999; Harrysson et al., 2018; Marsham et al., 2007; Mouritsen et al., 2013; Rødde et al., 2004; Vasconcelos et al., 2022)
Soybean	33	40	5	22	(USDA, 2018a)
Beef	< 0.1	65	3	33	(USDA, 2018b)

Note that all data was retrieved from seaweed cultivated in Europe, regardless of whether it was harvested from aquaculture or wild

^aTotal crude protein (N×5)

3.1. Carbohydrates and dietary fibre

Carbohydrates represent the predominant component of most seaweeds. Green seaweeds, including *Ulva spp.*, generally display the lower end of the reported carbohydrate range (**Table 2**). Although soybean shows comparable total carbohydrate levels, its carbohydrate composition differs markedly. In soybean, approximately 10% of the dry weight consists of soluble sugars, with the remaining fraction comprising structural polysaccharides classified as dietary fibres (DFs). In contrast, carbohydrates in *Ulva spp.*, as well as in brown and red seaweeds, are predominantly present as dietary fibre rather than as simple sugars (Berk, 1992; Holdt & Kraan, 2011). The type of polysaccharides

present varies substantially among seaweed groups. Brown seaweeds are rich in alginate, fucoidan, and laminarin, whereas green seaweeds contain sulphated polysaccharides such as ulvan, alongside sulphated galactans, xylans, and some starch. Red seaweeds are characterized by agar, carrageenan, xylans, and floridean starch. Cellulose is commonly found across seaweed taxa irrespective of colour (Holdt & Kraan, 2011; Prabhu et al., 2019).

These dietary fibres are defined as carbohydrate polymers that resist digestion in the upper gastrointestinal tract. Once in the colon, they can be fermented by the gut microbiota, resulting in the formation of beneficial metabolites such as short-chain fatty acids (SCFAs) (Holdt & Kraan, 2011; Makki et al., 2018). Seaweed-derived DFs have been associated with diverse bioactivities, including anti-inflammatory, antioxidant, and antiviral effects, which depend on their chemical structure and origin (Tanna & Mishra, 2019). Both soluble and insoluble fibre fractions are present. Soluble fibres can contribute to lower circulating cholesterol and glucose levels by slowing digestion and nutrient uptake, while insoluble fibres enhance faecal bulk and stimulate intestinal transit (Benjama & Masniyom, 2011). In particular, ulvan from green seaweeds is a representative soluble fibre, and consumption of seaweeds rich in soluble DFs has been linked to improved blood lipid profiles and a reduced risk of certain chronic diseases (Huang et al., 2022; Ratana-Arporn & Chirapart, 2006).

3.2. Lipids, vitamins and minerals

Although seaweeds are generally low in energy due to their limited lipid content relative to carbohydrates and proteins, their lipid composition is nutritionally significant (Narayan et al., 2008). In commonly consumed species, total lipids typically account for less than 5% of dry weight (**Table 2**). Despite these low levels, seaweeds are rich in polyunsaturated fatty acids (PUFAs), particularly omega-3 and omega-6 fatty acids (Miyashita et al., 2011). Omega-3 PUFAs support mitochondrial function (Kendel et al., 2015) and serve as precursors for multiple biochemical and physiological pathways (Holdt & Kraan, 2011). The generally favourable omega-6 to omega-3 ratio in seaweeds may help prevent chronic inflammatory conditions (Dawczynski et al., 2007; Shannon & Abu-Ghannam, 2019). Essential long-chain omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), must be obtained from the diet (Kumari et al., 2010). Notably, certain red seaweeds contain up to ~1% EPA of dry weight, whereas soybean only provides negligible amounts (Harrysson et al., 2018; Livsmedelsverket, 2023b).

Vitamins are essential organic micronutrients that must be obtained through the diet (Wells et al., 2017). Seaweeds are a valuable source of both water- and fat-soluble vitamins, with *Ulva* species providing a broad spectrum that can help prevent deficiencies (Corino et al., 2019; Kumar et al., 2008). In addition, seaweeds are among the few non-animal sources of vitamin B12 (Pereira et al., 2024), further highlighting their nutritional relevance.

Finally, seaweeds are additionally rich in minerals essential for physiological processes. Compared with terrestrial plants, they generally exhibit higher total mineral content, particularly sodium, potassium, and calcium. For instance, *Ulva intestinalis* and *U. pertusa* display favourable sodium-to-potassium ratios, supporting fluid balance (Benjama & Masniyom, 2011). Magnesium, a key enzymatic cofactor, is also abundant in *Ulva* and *Enteromorpha* species (Mabeau & Fleurence, 1993). Essential trace elements such as iron, manganese, copper, zinc, cobalt, selenium, and iodine occur in seaweeds at concentrations or forms not commonly found in land plants (Corino et al., 2019; Rupérez, 2002). However, consumption should remain within safe limits, as excessive intake may lead to acute or chronic toxicity. In contrast, other elements that may be present in seaweeds, such as lead, cadmium, mercury, and arsenic, are considered undesirable due to their potential health risks (EFSA et al., 2023; Lozano Muñoz & Díaz, 2022).

3.3. Proteins and amino acids

In terms of composition, proteins typically represent the second or third most abundant fraction in seaweeds, alternating with ash. Green and red species generally contain higher protein levels than brown seaweeds, in some cases approaching those of soybean, although both remain at least twofold lower than raw beef on a dry-weight basis (**Table 2**). Beyond quantity, the quality of seaweed proteins is of particular interest. They contain all the essential amino acids required for human nutrition, with essential amino acids accounting for nearly half of the total amino acid content (Černá, 2011; Echave et al., 2022). Amino acid profiles of *Ulva* species are remarkably similar to that of egg ovalbumin (Moustafa et al., 2015), supporting their potential as a high-quality protein source. Glutamic acid and aspartic acid are typically the dominant amino acids, contributing to the characteristic umami taste of seaweeds (Kazir et al., 2019; Milinovic et al., 2021).

In addition to their nutritional value, seaweed proteins are associated with a wide range of bioactive compounds exhibiting antioxidant, anti-inflammatory and antimicrobial activities. Bioactive peptides in particular, derived from seaweed proteins, have attracted increasing attention due to their potential role in the prevention and mitigation of chronic diseases (Pereira et al., 2024; cf. **Literature review: section 6**).

Seaweeds can contribute to meeting daily protein requirements while providing additional health benefits linked to their diverse nutrient composition (Espinosa-Ramírez et al., 2023). However, their relatively low protein density limits their use as a primary protein source. Based on an average adult body weight of 63.3 kg and a recommended protein intake of 0.75 g kg⁻¹ day⁻¹ (EFSA, 2017; WHO/FAO/UNU, 2007), the estimated dry-weight consumption required to meet daily needs would be approximately 431 g for brown seaweeds, 238 g for green seaweeds, and 148 g for red seaweeds, compared with 119 g for soybean and 73 g dry weight (≈239 g fresh weight) for beef (Trigo, 2023). As seaweed is commonly consumed either dried or fresh (Trigo, 2023), these values highlight the relatively large intake required for seaweed to serve as a primary protein source.

Furthermore, protein digestibility in seaweeds may be reduced due to their high dietary fibre content, which can limit protein accessibility in whole biomass (Trigo, 2023; Livsmedelsverket, 2023a). As current intake recommendations assume digestibility comparable to egg protein (WHO/FAO/UNU, 2007), actual consumption requirements are likely to be higher. Consequently, enhancing protein accumulation during cultivation or concentrating proteins through extraction processes is essential to improve the feasibility of seaweeds as a protein ingredient.

Given the importance of protein content within the scope of this thesis, an overview of abiotic factors influencing protein accumulation in *Ulva* species is provided in **Table 3** (Trigo, 2023).

Table 3. Summarisation of abiotic factors influencing protein content in seaweed species, more specifically *U. fenestrata* and *S. latissima* (Trigo, 2023).

Abiotic factor	Seaweed	Change leading to higher protein levels	Control group	Reference
Environmental-related	<i>U. fenestrata</i>	↓ Irradiance ($50 \mu\text{mol m}^{-2} \text{s}^{-1}$)	100 and $160 \mu\text{mol m}^{-2} \text{s}^{-1}$	(Toth et al., 2020)
	<i>S. latissima</i>	↓ Water temperature (13°C)	18°C	
		↓ Water temperature	-(correlation) ¹	(Coaten et al., 2023)
		↑ Wind speed		
		↑ Relative humidity		
		↓ Atmospheric pressure		
		↑ August–September ²	April–July	(Forbord et al., 2020)
Depth		↑ Depth (8–9 m)	1–2 m	
Media/seawater composition	<i>U. fenestrata</i>	= Nitrate (150, 500 μM)	< 5 μM	(Toth et al., 2020)
		= Phosphate (< 1 and 50 μM)	-	
		= pCO ₂ (200, 400, 2500 ppm)	-	
	<i>S. latissima</i>	↑ Nitrite	-(correlation) ¹	(Coaten et al., 2023)
		↑ Phosphate		
		↑ Ammonium		
		↓ Total phosphorus		
		↓ pH		
Tide depth		↑ Tide depth		(Coaten et al., 2023)

¹Relationship between abiotic factors and protein content was established through the correlation of data obtained from wild *S. latissima* harvested at different locations; ²This result is likely related to the other listed environmental factors.

These factors, including light intensity, nutrient availability (particularly nitrogen), temperature, and salinity, strongly influence metabolic activity and nitrogen assimilation, and therefore directly affect protein synthesis and overall biomass composition.

4. Seaweed protein extract: A next step in functional foods

Protein extracts obtained from seaweeds refer to the fraction of proteins isolated from seaweed biomass through various physical, chemical, or enzymatic extraction methods. These extracts comprise proteins and protein-derived compounds that can be applied in the formulation of functional foods, due to their nutritional value and potential health promoting properties.

4.1. Production and isolation of seaweed protein extracts

Seaweed protein extraction typically involves three main steps: cell disruption, protein solubilization, and protein concentration, as summarised by Trigo (2023) (Fig. 5).

Cell disruption can be achieved through mechanical, physical, or enzymatic approaches. Mechanical pre-treatments such as milling (Angell et al., 2017), bead milling (Steinbruch et al., 2023), and pressing (Juul et al., 2022) are commonly applied prior to extraction to reduce particle size and increase surface area. Physical methods, including freeze–thaw cycles, induce ice crystal formation that disrupts cellular structures and enhances permeability (Wijers et al., 2020). More advanced techniques, often applied during extraction in a liquid medium, include high-speed homogenization (Postma et al., 2018), high-pressure processing (O'Connor et al., 2020), pulsed electric fields (Robin et al., 2018), ultrasound (Kadam et al., 2017), autoclaving (O'Connor et al., 2020), and osmotic shock (Vilg & Undeland, 2017). Unlike mechanical pre-treatments, these methods rely on physical forces or pressure changes within a solvent system to further destabilize the cell wall matrix. In addition, enzymatic treatments targeting structural polysaccharides such as ulvan (Harrysson et al., 2019), carrageenan (Fleurence et al., 1995), and cellulose (Steinbruch et al., 2023) can be applied to weaken the cell wall and facilitate protein release.

Following disruption, protein solubilization is carried out, and its efficiency strongly depends on the degree of cell wall breakdown. Extraction under alkaline conditions is one of the most widely used strategies, as elevated pH enhances protein solubility by increasing electrostatic repulsion and unfolding protein structures (Vilg & Undeland, 2017; Juul et al., 2023, **Fig. D1**). Additional approaches include mild heating to improve diffusion (Wijers et al., 2020), the use of antioxidants to prevent phenolic–protein interactions (Juul, Møller, et al., 2021), organic solvents for hydrophobic proteins (Mendez & Kwon, 2021), reducing agents to cleave disulfide bonds (Wong & Cheung, 2001), and aqueous two-phase systems for selective partitioning (Fleurence, Le Coeur, et al., 1995). After solubilization, centrifugation is typically applied to separate soluble proteins from insoluble residues, and in some cases lipids and other interfering compounds are removed prior to extraction (Harrysson et al., 2018).

Following protein solubilization, proteins are commonly recovered using a pH-shift method (Trigo et al., 2021; Molina-Gilarranz et al., 2025). Alternatively, protein recovery can be achieved through precipitation techniques such as ammonium sulphate salting-out (Harrysson et al., 2018; Wong & Cheung, 2001) or isoelectric precipitation (Magnusson et al., 2019), occasionally preceded by freeze–thaw treatment to enhance recovery efficiency (Abdollahi et al., 2019). Protein concentrates are subsequently obtained using techniques such as freeze-drying, which removes water while preserving protein functionality (Harrysson et al., 2018).

In practice, combining multiple extraction techniques is often more effective than applying a single method. This is because seaweed cell walls are structurally complex, consisting of various polysaccharides that limit protein accessibility (*cf. Literature review: section 4.3*). For example, freeze–thawing followed by ultrasonication, which generates cavitation forces, breaks down cell structures and enhances mass transfer, while alkaline conditions improve the solubility of the released proteins. The combination of these methods therefore acts synergistically: physical disruption increases accessibility, while chemical conditions maximize solubilization. Such integrated approaches have been shown to significantly improve protein yield compared to individual treatments alone (Juul et al., 2021; Vega-Gómez et al., 2024).

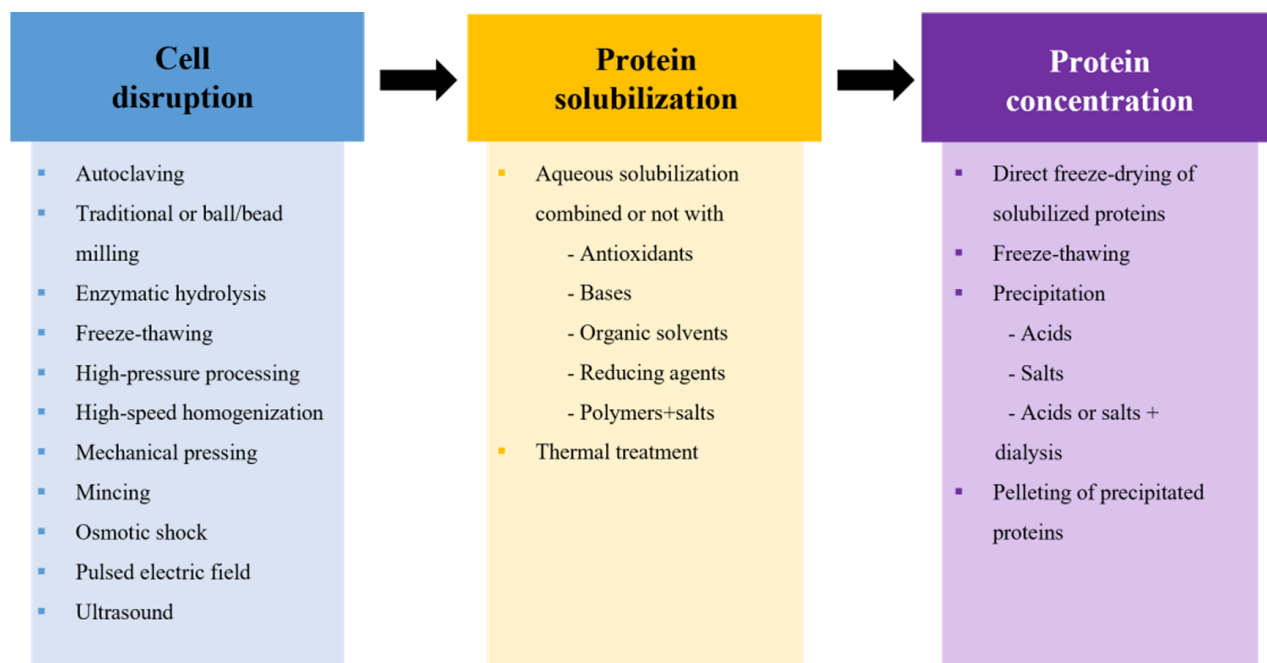


Fig. 5. Overview of the various reported methods employed at different stages of protein extraction from seaweed (Trigo, 2023).

4.2. Protein extract yields

The yield of protein extracted from seaweed is highly dependent on both the species and the extraction method applied. As summarized in **Table 4**, reported protein extraction yields for *Ulva spp.* range from 11% to 59% of dry weight, highlighting the substantial variability associated with different seaweed species and extraction protocols. However, protein functionality is not determined by extraction yield alone. Preserving protein quality is equally important, as many downstream applications require proteins to retain their native conformation and avoid denaturation in order to maintain structural integrity and biological activity. For example, the high extraction yields reported by Wong & Cheung (2001) were achieved using β -mercaptoethanol, a reducing agent unsuitable for food applications. Over time, extraction methods have progressively evolved toward more food-compatible and efficient approaches. Currently, pH-shift extraction, which combines alkaline solubilization with acid precipitation, is among the most widely applied strategies for *Ulva spp.*, with ongoing optimization based on previous research.

Table 4. Overview of reported protein extraction yields (% dry weight) from different *Ulva spp.*, including applied extraction method.

Seaweed Species	Extraction Method	Protein Extraction yield (%)	Reference
<i>U. lactuca</i>	pH-shift (using β -mercaptoethanol)	36.4 $\pm$ 0.35	(Wong & Cheung, 2001)
<i>U. ohnoi</i>	Solvent extraction	12.28 – 21.57	(Angell et al., 2016)
<i>U. lactuca</i>	pH-shift (pH 13 - pH 2)	19.6	(Harrysson et al., 2018)
<i>U. lactuca</i>	pH-shift (optimized)	29	(Harrysson et al., 2019)
<i>U. ohnoi</i>	Microwave extraction	23.9	(Magnusson et al., 2019)
<i>U. fenestrata</i>	Alkaline extraction (pH 8.5)	38	(Juul et al., 2021)
<i>U. fenestrata</i>	pH-shift	11.1 $\pm$ 3.7	(Trigo et al., 2021)
<i>Ulva spp.</i>	Ultrasonication+Alkaline extraction (pH 12)	59.06 $\pm$ 0.02	(Vega-Gómez et al., 2024)

4.3. Challenges in seaweed protein extraction

The large-scale commercialization of seaweed protein ingredients remains limited, mainly due to low extraction yields and complex, water-intensive processing methods that complicate industrial upscaling (Trigo, 2023).

Several structural and biochemical characteristics of seaweed biomass contribute to these difficulties. First, seaweed cell walls are highly rigid, consisting of dense polysaccharide networks intertwined with proteins, which restrict solvent access (Mæhre et al., 2016; Vásquez et al., 2019). Second, many seaweed polysaccharides have gel-forming properties that hinder protein diffusion and reduce solubility (Fleurence et al., 1995; Zhong et al., 2020). Third, proteins may interact with charged polysaccharides and phenolic compounds, further limiting their extractability (Molina-Gilarranz et al., 2025; Tibbetts et al., 2016).

Seaweed protein extraction is often compared with soybean processing, since soy protein represents a well-established industrial benchmark (Rajpurohit & Li, 2023). However, seaweed tissue is structurally and biochemically more complex (**Fig. 6**). In soybean, proteins are mainly stored in abundant, solvent-accessible protein bodies (Politiek et al., 2022), and a large proportion consists of salt-soluble globulins, which share similar physicochemical properties and are relatively easy to extract (Day, 2013). In contrast, seaweed proteins are dispersed throughout the cell and compartmentalized within different structures, creating multiple physical barriers to solvent penetration (Tenorio et al., 2018). Microscopic observations by Prabhu et al. (2019) confirmed the cellular organization represented in **Figure 6**, showing intracellular starch granules embedded within chloroplast and thylakoid structures in *Ulva ohnoi* cells (**Fig. D3**). Moreover, seaweed proteins are highly heterogeneous in charge, solubility, and hydrophobicity due to the functional diversity of photosynthetic tissues (Mendez & Kwon, 2021; Tenorio et al., 2018). For example, RuBisCo can account for 7–37% of total soluble protein (Iñiguez et al., 2019) in seaweeds.

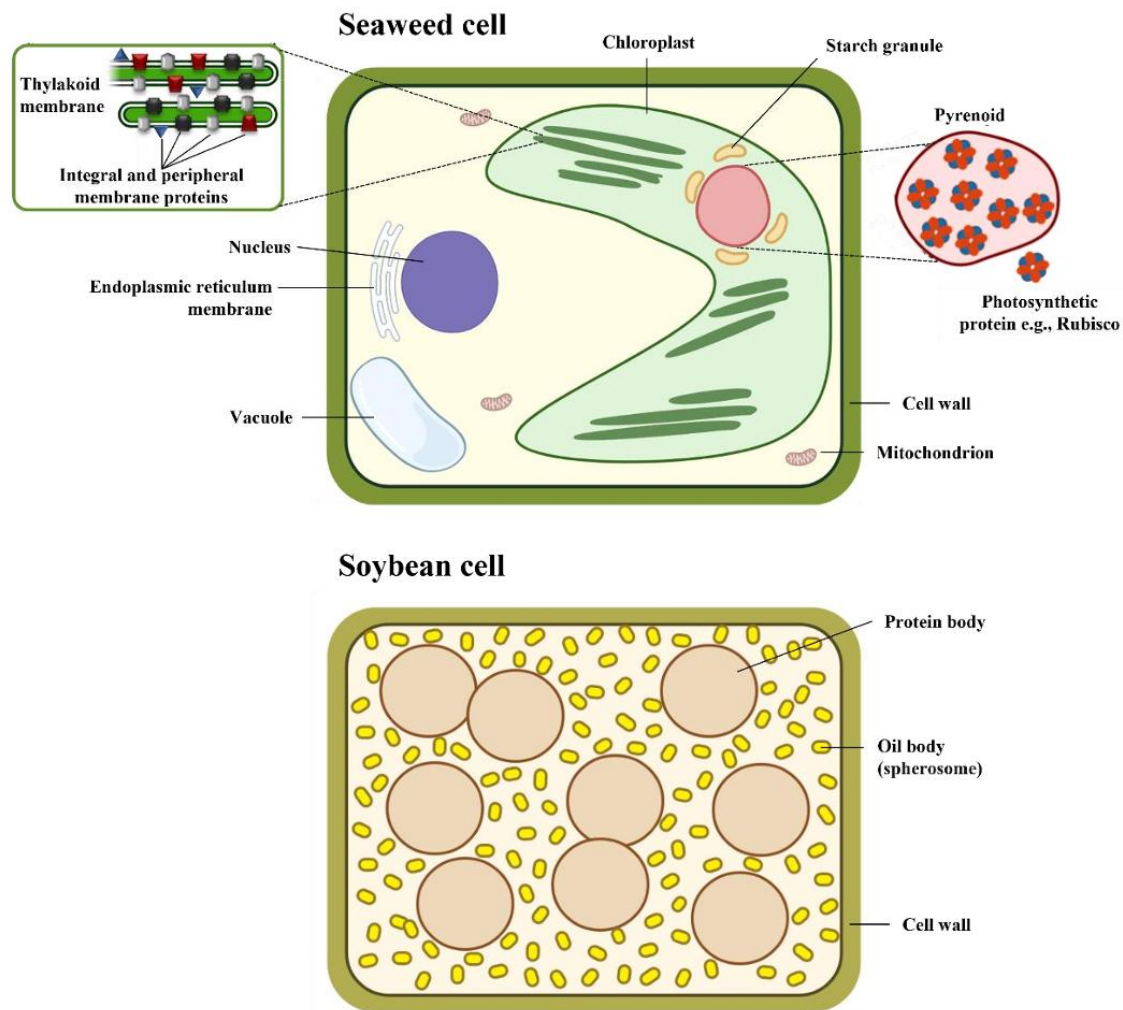


Fig. 6. Schematic drawings of a general seaweed cell and soybean cotyledon cell. Pyrenoid is defined as a carbon-concentrating structure found in the stroma of chloroplasts in algae, where it enhances the carboxylase activity of RuBisCo by concentrating CO_2 around it, thus facilitating increased CO_2 sequestration (Mishra et al., 2022). Drawings are not scaled (Trigo, 2023).

Because of these structural and compositional differences, traditional industrial approaches such as the pH-shift method, which is based on alkaline solubilization and isoelectric precipitation (Endres, 2001), are less efficient for seaweed than for legumes. Consequently, adapted pH-shift strategies and alternative extraction techniques specifically tailored to seaweed biomass have been developed over the past decades (Trigo, 2023).

5. Gastrointestinal digestion of seaweed protein extracts

5.1. Human gastrointestinal digestion

The human digestive system is a sophisticated, multi-scale physicochemical process, far exceeding the functionality of a simple conduit for food transport. It is an integrated network designed to convert heterogeneous food matrices into bioavailable molecular units through a series of highly coordinated mechanical and chemical events. This system is fundamentally controlled by an intricate neural network and hormonal feedback loops that regulate motility, secretion, and absorption (Sensoy, 2021).

Physiologically, the digestive tract is an open-ended tube approximately 8 to 9 meters in length, extending from the mouth to the anus. This transit is supported by accessory organs, including the teeth, tongue, salivary glands, liver, gallbladder, and pancreas (Fig. 7). Each digestive organ exhibits a distinct set of physicochemical conditions including pH, enzymatic composition, and transit time that are finely tuned to facilitate the efficient breakdown of specific classes

of biomolecules, such as proteins, polysaccharides, lipids, and nucleic acids. These organ-specific environments ensure maximal enzymatic activity and substrate accessibility, thereby optimizing nutrient extraction and absorption (Sensoy, 2021).

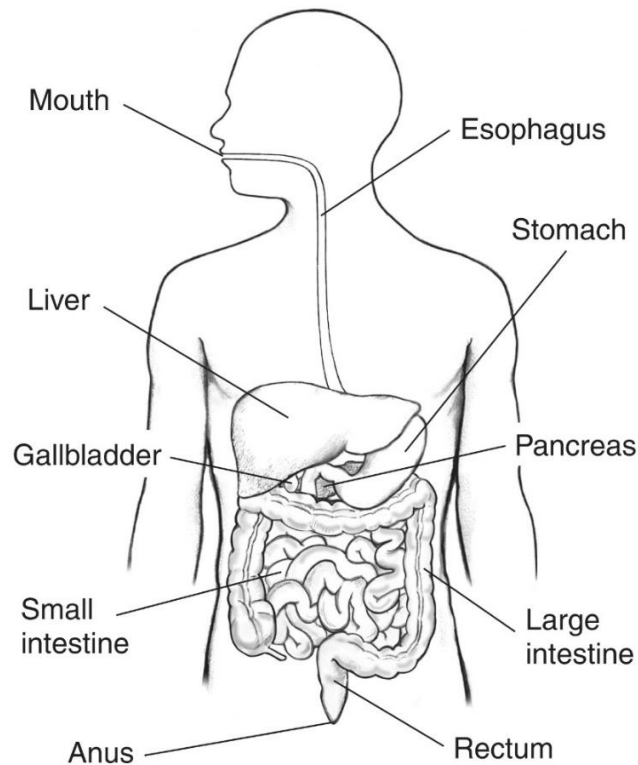


Fig. 7. The human digestive system with sections labelled (National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health, n.d.).

Table 5 provides a comprehensive overview of the physical and chemical processes occurring throughout the successive stages of human digestion. Among these, the progressive variation in pH along the digestive tract plays a pivotal role, as this directly influences enzyme activation, protein structure, and the kinetics of hydrolysis. In addition, the residence time within each compartment determines the extent to which macronutrients are exposed to digestive enzymes, thereby affecting the degree of breakdown and the profile of resulting metabolites.

Since this study concerns seaweed protein extracts and particularly the generation of protein breakdown products, the focus will be on the gastric and intestinal phases of digestion. During the oral phase, protein digestion is negligible, as salivary enzymes primarily target carbohydrates such as starch. In contrast, the gastric phase initiates substantial proteolysis under acidic conditions through the action of pepsin, while the intestinal phase further advances protein hydrolysis via pancreatic enzymes, leading to the formation of peptides and free amino acids (**Table 5**). These two phases are therefore most relevant for evaluating the digestion pattern, hydrolysis kinetics, and potential bioactive peptide release from seaweed-derived proteins.

Table 5. Overview of physical and chemical processes occurring during digestion in humans. SCFA= short-chain fatty acids. Adapted from Sensoy (2021).

Section	Physical Process	Chemical Process	Conditions (adult)	References
Mouth	Mastication Food breakdown Mixing	Enzymatic hydrolysis Starch breakdown (α -amylase) Lipid breakdown (lingual lipase)	pH: 5-7 Transit time: 5 s – 2 min Saliva flow rate: 0.042–1.83 mL/min (unstimulated), 0.77–4.15 mL/min (stimulated) Biting force: 100–400 N	(Chen, 2009; Marze, 2017a,b; Mosca and Chen, 2016; Rogers, 2011)
Esophagus	Peristalsis Bolus transport	–	Transit time: 8–10 s solid; 1-2 s liquid	(Bender et al., 2005; Buettner et al., 2001; Rogers, 2011)
Stomach	Contractions/Peristalsis Food breakdown Mixing Gastric transport Gastric sieving	Enzymatic hydrolysis Protein breakdown (pepsin) Lipid breakdown (gastric lipase) Acid hydrolysis Food softening and dissolution (gastric acid)	pH: 1-3 Transit time: 15 min - 4 h Gastric juice secretion: 1–3 L/day Contraction frequency: 3 cycles/min	(Chen et al., 2011; Miftahof, 2017; Rogers, 2011; Sullivan, 2009)
Small Intestine	Peristalsis Chyme transport Segmentation Mixing Absorption Monosaccharides Amino acids, peptides Monoacylglycerides, glycerol Phosphates, pentose sugars Minerals and vitamins	Enzymatic hydrolysis Starch breakdown Protein breakdown Lipid breakdown Nucleic acid breakdown	pH: 6–7.5 Transit time: 1–5 h Pancreatic juice secretion: ~1.5 L/day	(Rogers, 2011; Saladin, 2017; Smith and Morton, 2010; Sullivan, 2009)
Large Intestine	Peristalsis Chyme transport Segmentation Mixing Absorption Water, ions, minerals, vitamins, fats and organic molecules	Fermentation Production of SCFA	pH: 5-7 Transit time: 12–24 h Microbiota: $\sim 10^{11}$ - 10^{12}	(Rogers, 2011; Saladin, 2017; Smith and Morton, 2010; Sullivan, 2009)

5.2. *In vitro* digestion models – INFOGEST 2.0

When performing experiments related to human digestion systems, there is a need for digestion models. These are valuable tools for evaluating novel food products by predicting their behaviour in the human gastrointestinal tract. Food digestion can be studied either *in vivo* (in humans or animals) or *in vitro*. Although *in vivo* studies provide physiologically relevant data, they are costly, ethically constrained, and subject to interindividual variability. Consequently, *in vitro* digestion models are widely used due to their lower cost, faster execution, and higher reproducibility under standardized conditions (Sensoy, 2021). Furthermore, bioaccessibility can also be assessed *in vitro* using tissues, cell cultures, or artificial membranes to estimate permeability and absorption (Vega-Rojas et al., 2021; Caicedo-Lopez et al., 2019).

Most *in vitro* digestion systems are static, operating under fixed conditions in simple reactors. While practical and suitable for screening multiple samples, these models cannot reproduce mechanical forces or dynamic physiological changes occurring *in vivo*. Key digestion parameters such as enzyme-to-substrate ratios, pH variation, and transport of digestion products are therefore only partially represented. To overcome these limitations, dynamic digestion models have been developed, aiming to more closely simulate physiological conditions with improved reproducibility (Dupont et al., 2019; Liu et al., 2019).

In this thesis, the static INFOGEST 2.0 protocol (Brodkorb et al., 2019) is used to simulate human gastrointestinal conditions *in vitro*. This protocol was developed to address the lack of standardization in earlier *in vitro* digestion models, which varied widely in enzyme concentrations, pH, electrolyte composition, and digestion times, limiting comparability between studies. To overcome this, the international INFOGEST network was established, uniting experts

from over 35 countries in food science, nutrition, gastroenterology, enzymology, and engineering. This collaboration led to the INFOGEST 2.0 protocol, an internationally harmonized standard for static *in vitro* digestion.

Although dynamic digestion models are capable of simulating complex physiological processes such as gradual gastric emptying, peristaltic movements, and continuous pH changes, their technical complexity, limited throughput, and high operational costs restrict their widespread implementation. In contrast, the static INFOGEST 2.0 protocol applies fixed ratios of food to simulated digestive fluids, standardized enzyme activities, and predefined pH conditions for each digestive phase. This approach ensures reproducibility, accessibility, and interlaboratory comparability. Such standardization is particularly important for evaluating protein hydrolysis patterns, assessing nutrient bioaccessibility, and supporting the development of functional food ingredients, including seaweed derived proteins. **Figure 8** gives a summary of the protocol, in what comes each phase is briefly touched up on.

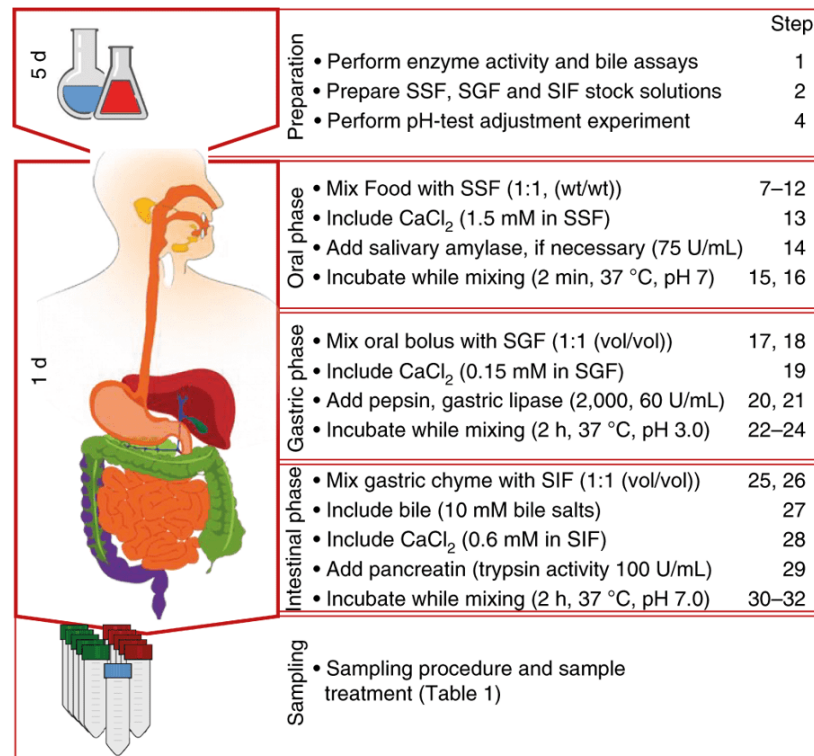


Fig. 8. Timing and flow diagram of the INFOGEST 2.0 *in vitro* digestion protocol for food. Expected time frame (left), stages and corresponding step numbers in the procedure (right) are given. SGF=simulated gastric fluid; SIF=simulated intestinal fluid; SSF=simulated salivary fluid (INFOGEST 2.0; Brodkorb et al., 2019).

The oral phase: Initiation of the digestive path

The oral phase is the first step of the INFOGEST 2.0 protocol, regardless of the food's initial physical state. This phase ensures a standardized dilution and initiates the enzymatic breakdown of starch, while mimicking the formation of a "swallowable bolus." For solid foods, simulated mastication via mincing is required to achieve the necessary surface area for subsequent enzymatic attack.

The gastric phase: Proteolysis and lipolysis

The gastric phase serves as the primary site for the initiation of protein degradation and lipid breakdown. A major refinement in the INFOGEST 2.0 protocol is the inclusion of gastric lipase, recognizing that it accounts for 10–25% of total lipid hydrolysis *in vivo*. Gastric lipase exhibits S_N3 stereospecificity, preferentially releasing short and medium-chain fatty acids, which is critical for the subsequent triggering of pancreatic lipase activity. A notable limitation of this static approach is the constant pH 3.0, which neglects the dynamic pH-gradients and gradual acid secretion found *in*

vivo. To address this, "semi-dynamic" models (Dupont et al., 2019) have been proposed to allow for gradual emptying and acid addition.

The intestinal phase: Nutrient breakdown and bioaccessibility

The intestinal phase is the critical endpoint for determining nutrient bioaccessibility. The transition to this phase involves a strategic pH shift to 7.0, which serves a dual purpose: it provides the optimal environment for pancreatic enzymes and effectively halts the activity of gastric pepsin through proteolytic autolysis (Brodkorb et al., 2019).

5.3. Gastrointestinal digestion behaviour of seaweed proteins

As already stated, protein digestibility in seaweed varies among species and is strongly influenced by their structural and biochemical characteristics. **Table 6** provides an overview of factors contributing to the moderate protein digestibility of seaweed samples (Trigo, 2023). These include the presence of complex polysaccharides, interactions between proteins and phenolic compounds, and the overall matrix structure that can limit enzyme accessibility.

Table 6. Overview of factors contributing to the moderate protein digestibility of seaweed samples. Adapted from Trigo (2023).

Factor	Underlying mechanism(s)	References
Relatively high fiber content	- Proteins are less accessible to digestive enzymes - Fibers increase chyme viscosity, slowing protein digestibility	(Mæhre et al., 2016; Marrion et al., 2003)
Presence of glycoproteins	- Glycosylation protects proteins against digestion by digestive enzymes	(Fleurence et al., 1999)
Presence of antinutrients such as phenolics, lectins, phytic acid, and protease inhibitors	- Phenol-mediated protein polymerization reduces protein accessibility - Phenolics, phytic acid, and protease inhibitors (e.g., trypsin inhibitors) can reduce digestion efficiency by inhibiting enzymes - Lectins may affect intestinal epithelial permeability (mainly shown in <i>in vivo</i> or cell models)	(Lund, 2021; Oliveira et al., 2009; Tibbetts et al., 2016; Vizcaíno et al., 2020)

5.4. Evaluating the progress of gastrointestinal protein digestion

Several methods can be used to monitor the progress of protein hydrolysis, with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and o-phthaldialdehyde (OPA) assays being among the most common.

SDS-PAGE separates denatured proteins and polypeptides based on their molecular weight, providing qualitative insight into both the digestion process and the composition of the protein sample. It is commonly used to monitor protein hydrolysis by tracking the breakdown of large proteins into smaller fragments over time. As digestion progresses, enzymes cleave the proteins into smaller peptides, resulting in bands of lower molecular weight or the disappearance of the original bands. **Figure 9** from Kazir et al (2019) provides a clear overview of the bands expected after analysis of *in vitro* digested samples.

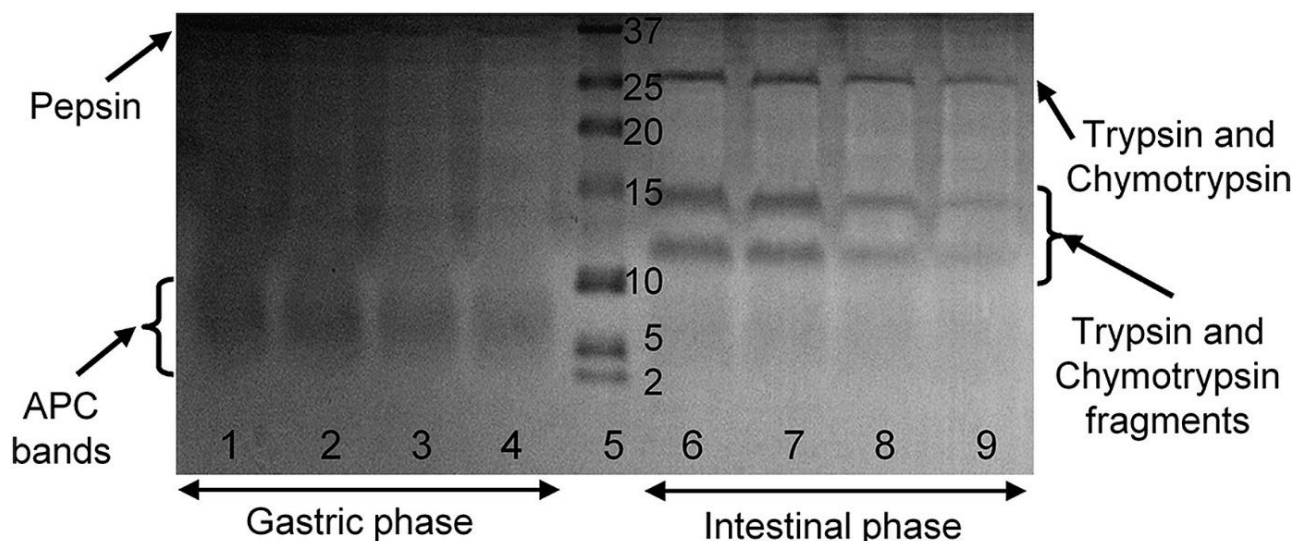


Fig. 9. SDS-PAGE analysis during simulated digestion of *Ulva* spp. APC=Algal Protein Concentrates, Lanes: (1) $t = 0$ min, (2) $t = 30$ min, (3) $t = 60$ min, (4) $t = 120$ min, (5) size marker: 2, 5, 10, 15, 20, 25 and 37 kDa, (6) $t = 125$ min, (7) $t = 135$ min, (8) $t = 180$ min, (9) $t = 240$ min (Kazir et al., 2019).

To quantitatively monitor the progression of protein digestion, the o-phthaldialdehyde (OPA) method can be used to determine the degree of hydrolysis (%DH) throughout the *in vitro* digestion process. This assay measures the concentration of free amino groups released during proteolysis, providing a numerical indication of protein breakdown. **Figure 10** illustrates the digestion progress of two seaweed species, expressed as %DH relative to casein protein that was subjected to the same protocol (Kazir et al., 2019).

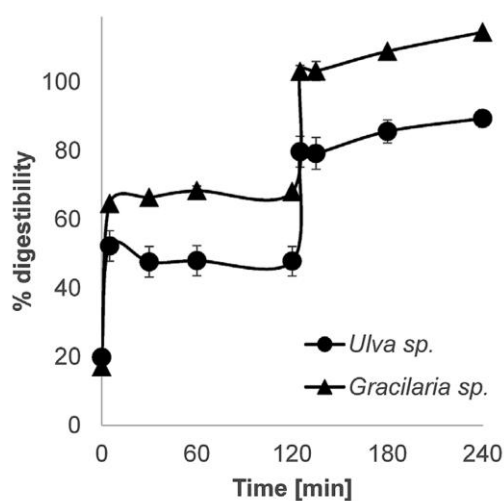


Fig. 10. Progress of proteolysis, (based on the OPA-method, relative to casein processed under the same conditions), during simulated gastro-intestinal digestion of Algal Protein Concentrates or APCs (Kazir et al., 2019).

In conclusion, both quantitative and qualitative methods are necessary to properly assess digestion progress. Numerical data indicate the extent of hydrolysis, while visual analysis confirms changes in protein size and integrity.

6. Production of bioactive peptides during digestion

During digestion, proteins are broken down by enzymatic activity into smaller fragments called peptides (**Fig. 11**). This process is mediated by proteolytic enzymes with distinct specificities. Pepsin (EC 3.4.23.1) exhibits broad specificity, preferentially cleaving C-terminal to bulky hydrophobic and aromatic residues, particularly Phe and Leu (Ahn et al., 2013). Trypsin (EC 3.4.21.4) cleaves specifically at the C-terminal side of Lys and Arg residues (Olsen et al., 2004), while chymotrypsin (EC 3.4.21.1) preferentially hydrolyses peptide bonds C-terminal to aromatic residues such as Tyr, Trp, and Phe (Yazawa & Numata, 2014).

In addition, protein structure strongly influences proteolysis efficiency. Pepsin, which is active at very low pH (≈ 1.2), is more efficient on native, folded proteins due to acid-induced denaturation that improves accessibility to cleavage sites. In contrast, pancreatic enzymes show limited activity on folded proteins, although heat treatment can further enhance their hydrolytic efficiency (Fu et al., 2021). This suggests that pepsin primarily acts on native proteins, whereas pancreatic enzymes mainly generate free amino acids and short peptides for absorption.

Notably, besides enzymatic hydrolysis, peptides can also be produced via high-pressure processing or lactic acid fermentation (Tagliazucchi et al., 2019; Echave et al., 2021).

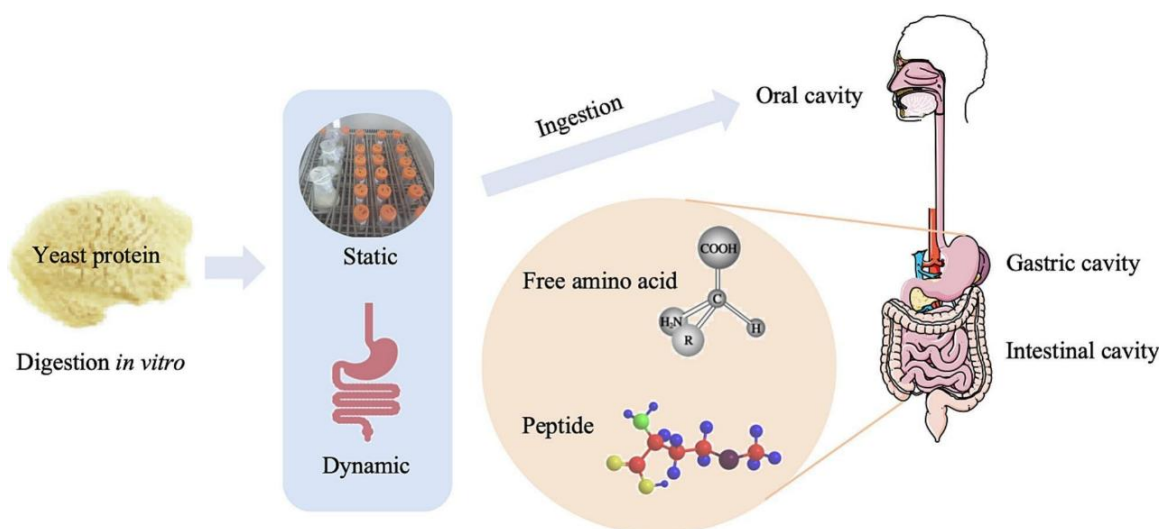


Fig. 11. Formation of peptides from yeast protein using in vitro digestion models (Qiao et al., 2025).

Following simulated gastrointestinal digestion, proteins and peptides derived from green seaweeds, particularly species of the genus *Ulva*, have demonstrated several bioactive properties, including antihypertensive, antioxidant, and anti-inflammatory effects relevant to human health (**Table 7**). Several studies indicate that peptides isolated from green seaweeds can inhibit enzymes associated with metabolic disorders, such as ACE 1 (EC 3.4.15.1) and renin (EC 3.4.23.15) (Sun et al., 2019, Pan et al., 2016). These enzymes are key components of the renin-angiotensin-aldosterone system (RAAS), which regulates blood pressure and fluid balance (Hayes, 2021; Pujiastuti et al., 2019). Moreover, regular seaweed consumption in countries such as Japan, China, and South Korea has been linked to lower incidences of cardiovascular diseases and diabetes compared to Western populations (Bermano et al., 2020; Ganesan et al., 2019).

Beyond blood pressure regulation, *Ulva*-derived peptides have also been reported to exhibit antioxidant activity (Kazir et al., 2019) and DPP-IV (EC 3.4.14.5) inhibitory activity, indicating potential anti-diabetic applications (Echave et al., 2022).

Table 7. Overview of *Ulva* spp. and reported bioactivities (IC_{50} : concentration required for 50% enzyme inhibition). Adapted from O'Brien et al. (2022).

Seaweed Species	Reported Bioactivity	Amino Acid Sequence	IC_{50}	Reference
<i>Ulva lactuca</i>	Renin inhibition	None reported	None reported	(Garcia-Vaquero et al., 2019)
<i>Ulva</i> sp.	Anti-inflammatory	None reported	None reported	(Kazir et al., 2019)
<i>Ulva lathrata</i>	ACE-1 inhibition	PAFG	35.9 μ M	(Pan et al., 2016)
<i>Ulva intestinalis</i>	ACE-1 inhibition	FGMPLDR, MELVLR	219.35 μ M, 236.85 μ M	(Sun et al., 2019)
<i>Ulva rigida</i>	ACE-1 inhibition	IP, AFL	0.020 mg/mL, 0.023 mg/mL	(Paiva et al., 2016)
<i>Ulva lactuca</i>	Renin inhibition	None reported	None reported	(Garcia-Vaquero et al., 2019)
<i>Ulva</i> sp.	Anti-inflammatory	None reported	None reported	(Cian et al., 2018)

7. Synthesis and research gaps

Marine macroalgae, particularly species within the genus *Ulva*, represent a promising and sustainable alternative protein source for food and feed. Their cultivation does not compete with terrestrial agriculture and provides additional ecosystem services (Xu et al., 2023; Gephart et al., 2021; Neveux et al., 2018). From a nutritional perspective, green seaweeds combine moderate protein levels with essential amino acids, polyunsaturated fatty acids, minerals, and vitamins (Trigo, 2023). However, the high polysaccharide content and complex cell wall architecture of *Ulva* substantially limit protein extractability and gastrointestinal digestibility when whole biomass is consumed (Trigo, 2023). These structural and biochemical constraints contribute to low extraction yields and considerable variability across studies. Reported protein contents of seaweed extracts range widely (11–59% dry weight, **Table 4**), largely reflecting differences in extraction methodologies. To date, no standardized protocol exists that simultaneously maximizes protein yield, preserves functionality, and remains compatible with downstream human digestion and peptide characterization.

Beyond methodological variability, important biological knowledge gaps remain. Most studies focus on a single species, restricting insight into interspecific differences in protein digestibility. Interregional variation is also rarely addressed, despite strong evidence that environmental conditions influence seaweed biochemical composition (**Table 1 & 3**). The effects of abiotic factors on protein extractability, amino acid composition, phenolic–protein interactions, and final digestibility therefore remain insufficiently understood.

This thesis addresses these limitations through an integrated, comparative, and mechanistic framework. By analysing four *Ulva* species, two originating from Europe and two from South Korea, it systematically evaluates both interspecific and environmental variability. Comprehensive biochemical profiling is used to link compositional traits and structural protein accessibility to *in vitro* digestion behaviour. Finally, digested fractions are characterized to relate hydrolysis patterns to the release of essential peptide fractions.

MATERIALS AND METHODS

1. Study design and sample origin

This study was conducted as a comparative laboratory-based analysis of *Ulva* seaweed species originating from Asia and Europe. The experimental work was performed in two consecutive phases using identical protocols. The first phase was carried out in South Korea and focused on Korean *Ulva* species, specifically *Ulva fasciata* and *Ulva linza*. The second phase was performed in Belgium and involved the analysis of two European *Ulva* species, specifically *Ulva australis* and *Ulva intestinalis*. All experimental procedures described below were applied consistently across both phases to ensure comparability of results.

Samples were manually harvested and subsequently transported to the laboratory under cooled conditions using ice packs and cold water in both cases, in order to minimize degradation.

1.1 Sampling

South Korean seaweed species were collected on 18 January 2025 from beaches in Busan. Upon arrival at the laboratory, samples were stored overnight at 4 °C prior to further processing. European seaweed species were collected on 28 October 2025 at Ambleteuse, France, in the North Sea area. **Figure 12** represents the four collected species, with their collection locations detailed in **Figure 13**.

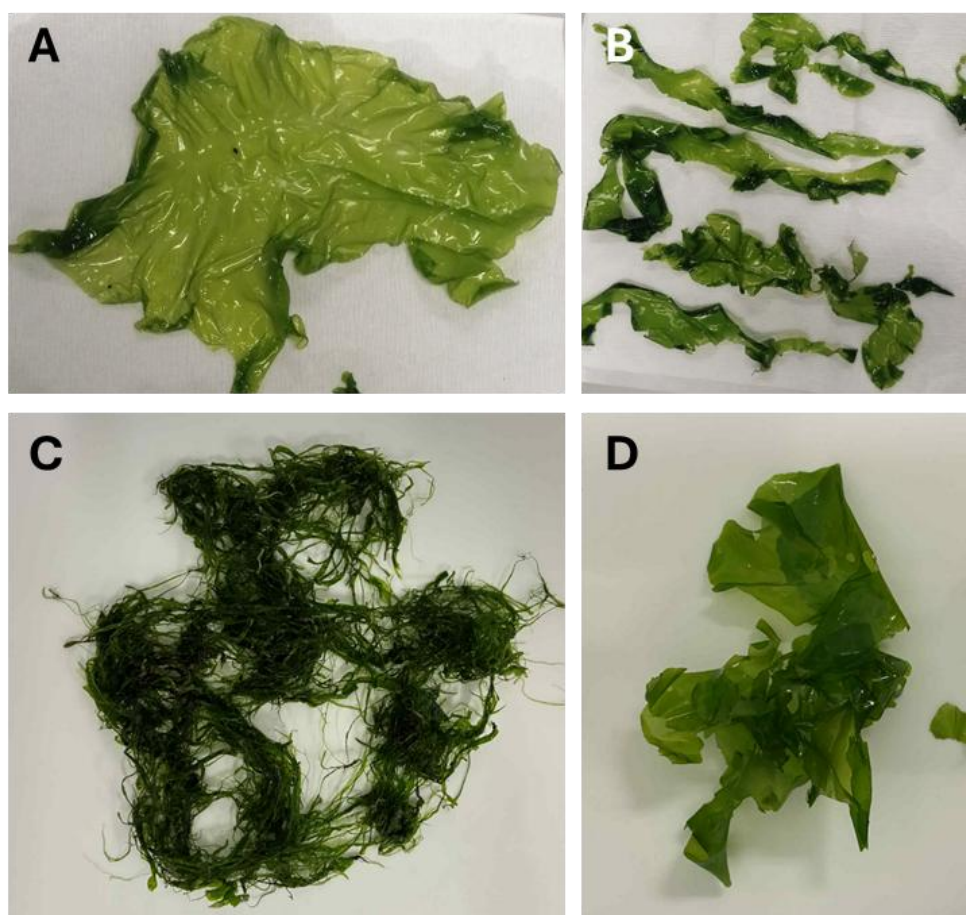


Fig. 12. Ulva seaweed samples. (A) *Ulva fasciata*, South Korea; (B) *Ulva linza*, South Korea; (C) *Ulva intestinalis*, Europe; (D) *Ulva australis*, Europe.



Fig. 13. Collection sites of *Ulva* seaweed indicated by a red star icon. (A) Gijang-gun, Busan, South Korea (35°17'59" N, 129°15'35" E). (B) Ambleteuse, France (50°48'21" N, 1°35'58" E).

1.2 Conditions

Abiotic factors at the time of harvesting were recorded for both South Korean and European *Ulva* species, as these environmental conditions may influence the results of subsequent analyses. The most relevant abiotic parameters are summarized in Table 8.

Table 8. Key abiotic factors recorded during *Ulva* biomass harvesting. South Korea: Korea Hydrographic and Oceanographic Agency (KHOA); Europe: Copernicus Marine Environment Monitoring Service (CMEMS).

Location	Water Temperature (°C)	Salinity (PSU)	Air Temperature (°C)
South Korea (18.01.2025)	10.73	33.71	4.66
Europe (28.10.2025)	13.83	34.06	16

2. Biomass preparation

Fresh *Ulva* biomass was rinsed twice with tap water and subsequently with distilled water to remove adhering sediment, salt residues, and other impurities. The cleaned samples were stored at -80 °C for 48 h and subsequently freeze-dried (South Korea: iShinBioBase, vacuum 10 mTorr for 12 h; Belgium: FreeZone 4.5 L Benchtop Freeze Dry System, LabConco, condenser temperature -50 °C, vacuum 0.200 mbar). During freeze-drying, samples were placed in plastic containers and covered with aluminium foil to prevent contamination. Moisture content for European species was determined by weighing freshly collected, washed seaweed samples and comparing them to their freeze-dried counterparts.

After freeze-drying, seaweed biomass was ground using a Kenwood grinder at stage 6 for a minimum of 15 minutes (Korean species) or using a fine grinder (IKA® A11 basic analytical mill) for 30 seconds (European species) until a homogeneous fine powder was obtained. For optimal extraction, the resulting powder was sieved sequentially through mesh sizes 40 (425 µm) and 60 (250 µm) to standardize particle size (Gilson Company Inc, USA). The fraction that passed through the sieves was collected, transferred into airtight polyethylene bags and stored at -20 °C until further analysis.

3. Seaweed biomass characterization

3.1. Taxonomic identification

The *Ulva* specimens used in this study were taxonomically identified by DNA barcoding. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA samples were subsequently stored at -20°C until further processing. DNA quality of European samples was assessed with the NanoDrop™ spectrophotometer (Version, 2000c; Thermo Scientific™) with the quality standards being based on the 260/230 and 260/280 absorbance ratios. For Korean samples, DNA quality was evaluated using a Qubit 4 Fluorometer (Thermo Scientific™) according to the standardized protocol described by Haendiges et al. (2020).

For Korean species, DNA identification and sequencing were performed by Macrogen. PCR amplification of the *tufA* gene was carried out using the degenerate primers *tufA_F* (5'-TGAAACAGAAMAWCGTCATTATGC-3') and *tufA_R* (5'-CCTTCNCGAATMGCRAAWCGC-3'). Primers were resuspended to a stock concentration of $100\text{ pmol }\mu\text{L}^{-1}$ and stored at -20°C . For European species, barcode amplification and sequencing were performed by Novogene Netherlands B.V. under comparable conditions. Sequences were searched against the standard database (nt) using BLASTn.

3.2. Determination of total protein content

Total protein content of the dried *Ulva* biomass was determined by quantifying total nitrogen, either using Kjeldahl analysis (Kjeldahl, 1883; Fig. A1) (Belgium) or by means of an elemental analyser. Nitrogen content was converted to protein using a nitrogen-to-protein conversion factor of 4.24, as commonly applied for green seaweeds (Biancarosa et al., 2017). Protein content was expressed as percentage of dry weight.

For Kjeldahl analysis, samples were digested for 2 h at 400°C in 16 mL of concentrated sulfuric acid (95–97%), using a Tecator Digestion System 20 (model 1015 Digester). A catalyst mixture consisting of 1.4% copper (II) sulfate, 94% sodium sulfate and 1.5% selenium was added to enhance digestion efficiency. After cooling, approximately 70 mL of distilled water was added prior to neutralization with 30% sodium hydroxide (NaOH). The samples were then subjected to steam distillation using a UDK 139 semi-automatic K-360 distillation unit (VELP Scientifica). The released ammonia (NH_3) was trapped in 1% boric acid (H_3BO_3) and subsequently titrated with 0.01 M hydrochloric acid (HCl).

For samples analysed in South Korea, elemental composition was determined using a FLASH SMART elemental analyser (Thermo Scientific™). In this machine, samples are combusted at high temperature in an oxygen-rich environment, converting elements such as carbon, hydrogen, nitrogen and sulfur into their respective gases. These gases are subsequently separated by gas chromatography and quantified using a thermal conductivity detector, enabling rapid and automated determination of nitrogen content.

3.3. Ash content determination

Ash content of freeze-dried seaweed powder was determined to quantify the inorganic residue remaining after complete combustion. Porcelain crucibles were dried at 100°C for at least 2 h, cooled in a desiccator, and weighed to obtain the tare weight. For each sample, 1.5–2.0 g of dried material was accurately weighed into the crucibles, and the combined weight was recorded. Crucibles containing the samples were placed in a muffle furnace (South Korea: Electric muffle furnace DMF-4.5, lab house; Belgium: Gelman, Germany) and gradually heated to 550°C for a total of 6 h until complete combustion was achieved. After cooling to room temperature, the crucibles were weighed again. Each sample was analysed in triplicate. Ash content was calculated as the percentage of residue relative to the initial dry sample mass using following equation:

$$\text{Ash (\%)} = \frac{R - T}{W - T} \times 100 \quad (1)$$

where T is the tare weight of the empty crucible (g), W is the weight of the crucible with the sample before ignition (g), and R is the weight of the crucible containing the ash after combustion (g).

4. Crude protein extraction from *Ulva* species

Protein extraction was performed using a combination of physical (freeze-thaw cycling and ultrasonication) and chemical (alkaline-assisted) treatments to disrupt seaweed cell wall structures and maximize protein yield while avoiding harsh conditions that could denature the proteins (Juul et al., 2021; Trigo, 2023). A schematic overview of the process is shown in **Figure 14**.

4.1. Freeze-thaw assisted extraction

First, freeze-dried *Ulva* powder (10 g) was suspended in 200 mL of sodium phosphate monobasic (SPM) (pH 7.4; 0.05M) working buffer (1:20 w/v) and stirred continuously at 7 °C for 24 h (Laing & Christeller, 2004; Zhang et al., 2019). The homogenate was transferred to plastic containers and frozen at -20 °C for at least six hours, then thawed at room temperature for a maximum of two hours to preserve protein integrity. This freeze-thaw cycle was repeated five times (Laing & Christeller, 2004; Zhang et al., 2019).

4.2. Ultrasonication

Following freeze-thaw treatment, samples were transferred to a 500 mL beaker and maintained in an ice bath during ultrasonication. Korean species were processed using a probe sonicator (KSS-N900DT, KoproTech Co., Ltd.) at 50% power (450 W) for 30 min, with the probe centrally positioned and operated in pulsed mode (0.1 s on / 1 s off). European species were sonicated using a probe sonicator (Vibra-Cell™ VCX-130) for 19 min with a 1 s on / 1 s off cycle.

The probe sonicator in South Korea was operated according to the following formula:

$$\text{Power (W)} = \frac{\text{Percentage Setting}}{100} \times 900 \text{ W} \quad (2)$$

The sonicated suspension was aliquoted into 50 mL Falcon tubes and centrifuged twice at 3900 × g and 4 °C for 20 min (South Korea: Centrifuge 5920 R; Belgium: Heraeus Megafuge 40 R) to separate the soluble fraction. The supernatant containing water-soluble proteins was collected and designated as S1, while the remaining pellet was retained for alkaline extraction (P1).

4.3. Extraction of alkaline-soluble proteins

The pellet obtained after separation of water-soluble proteins (P1) was resuspended in 200 mL of 125 mM NaOH per 10 g of dried seaweed powder and stirred for 1 h at 7 °C. Following a second centrifugation at 3900 × g and 4 °C for 15 min, insoluble debris was discarded, and the alkaline-soluble protein fraction was collected as S2 (Pimentel et al., 2020).

Aliquots of 1 mL from both S1 and S2 were reserved for protein quantification using the BCA assay (**cf. Materials & Methods: section 5.1**). Prior to combining the fractions, the pH of S2 was adjusted to 7.0–7.5 using 2.0 M HCl. The neutralized S2 was then slowly added to S1 under continuous stirring, and the mixture was stirred for 1 h at 7 °C. Protein extraction yield was calculated as the ratio of the extracted protein mass (mg) to the total protein content in the original dry seaweed biomass. For the European species, extract salinity prior to purification was measured using a salinity probe (WTW Multi 3630 IDS) to evaluate its potential impact on protein digestibility.

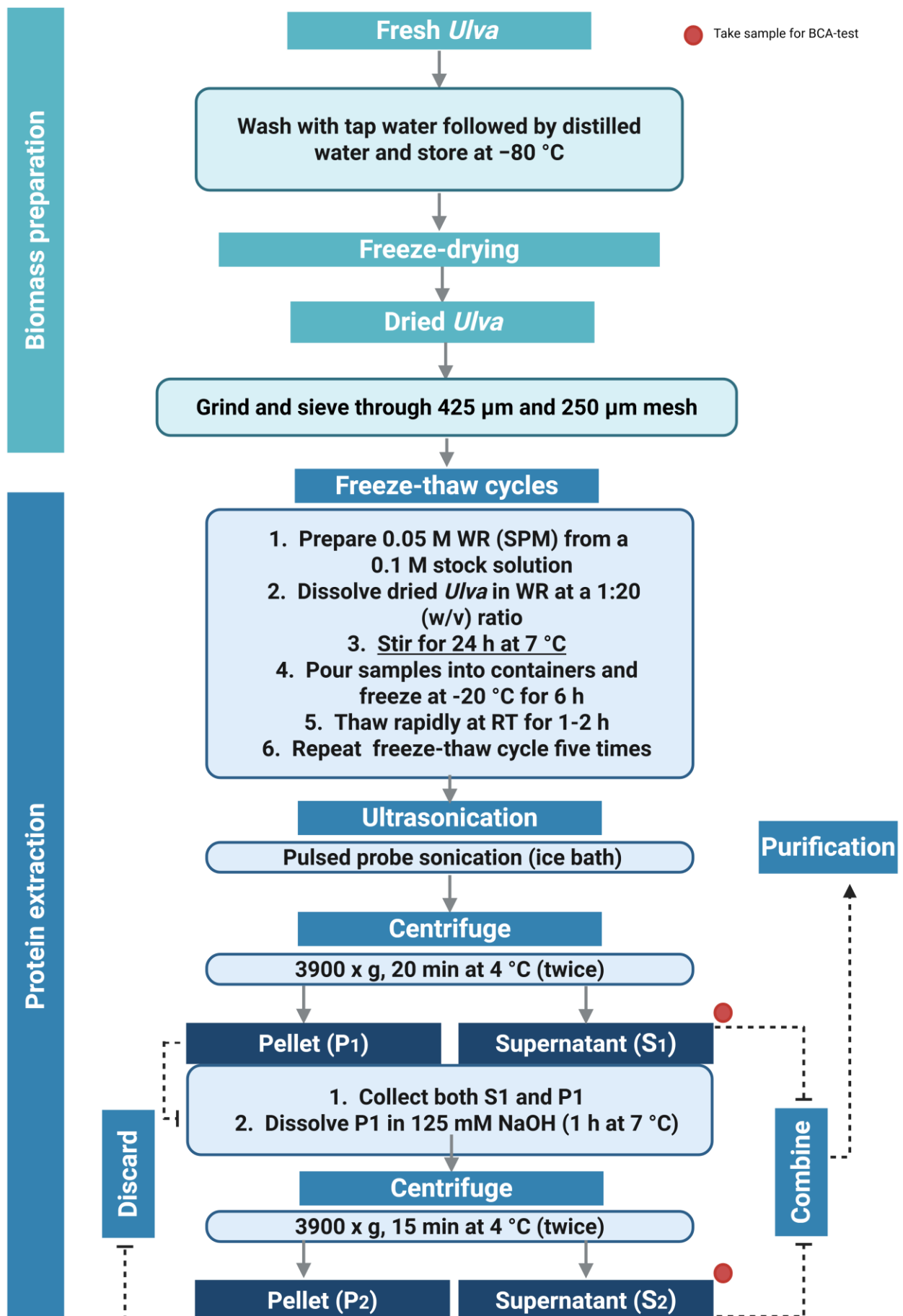


Fig. 14. Overview of biomass preparation and protein extraction workflow used in this thesis. The workflow includes two phases: biomass preparation and protein extraction. WR= working reagent, RT= room temperature. Made with Biorender.

4.4. Partial protein purification through ammonium sulfate precipitation

Partial purification of the extracted proteins was achieved by ammonium sulfate $((\text{NH}_4)_2\text{SO}_4)$ precipitation, a method that selectively concentrates and separates proteins based on their solubility. At high salt concentrations, protein solubility decreases, resulting in a phenomenon known as the “salting out” effect, allowing unwanted contaminants to remain in solution while the target proteins precipitate (Duong-Ly & Gabelli, 2014).

Solid $(\text{NH}_4)_2\text{SO}_4$ was gradually added to the crude protein extract under continuous stirring on ice until 30 % saturation was reached, following the protocol of Zhang et al. (2019) with modifications for green seaweeds. The total volume of the combined protein extract was measured, and the amount of $(\text{NH}_4)_2\text{SO}_4$ required to achieve the desired saturation at 4 °C was calculated according to EnCor Biotechnology (2026) using the following formula, taking into account the starting concentration, target molarity, saturation molarity, and the specific volume of $(\text{NH}_4)_2\text{SO}_4$.

$$G = \frac{\text{Sat} \cdot (M_2 - M_1)}{\text{SatM} - \left(\frac{\text{SpecVol}}{1000} \cdot 132.14 \cdot \text{SatM} \cdot M_2 \right)} \quad (3)$$

With:

- G = grams of $(\text{NH}_4)_2\text{SO}_4$ to add per liter of initial solution (g L^{-1})
- Sat = concentration of $(\text{NH}_4)_2\text{SO}_4$ in a saturated solution at the working temperature T (g L^{-1})
- M_2 = desired molarity of $(\text{NH}_4)_2\text{SO}_4$ (mol L^{-1})
- M_1 = initial molarity of $(\text{NH}_4)_2\text{SO}_4$ (mol L^{-1})
- SatM = molarity of a saturated $(\text{NH}_4)_2\text{SO}_4$ solution at temperature T (mol L^{-1})
- SpecVol = specific volume of $(\text{NH}_4)_2\text{SO}_4$ at temperature T (mL g^{-1})
- 132.14 = molecular weight of $(\text{NH}_4)_2\text{SO}_4$ (g mol^{-1})

The salt was added incrementally with continuous stirring, allowing approximately 30 s between successive additions to ensure complete dissolution. After the final addition, the mixture was stirred for 1 h at 7 °C to allow protein precipitation. The samples were subsequently centrifuged at 3900 x g for 15 min at 4 °C. These steps of the purification process are visualised in **Figure 15**.

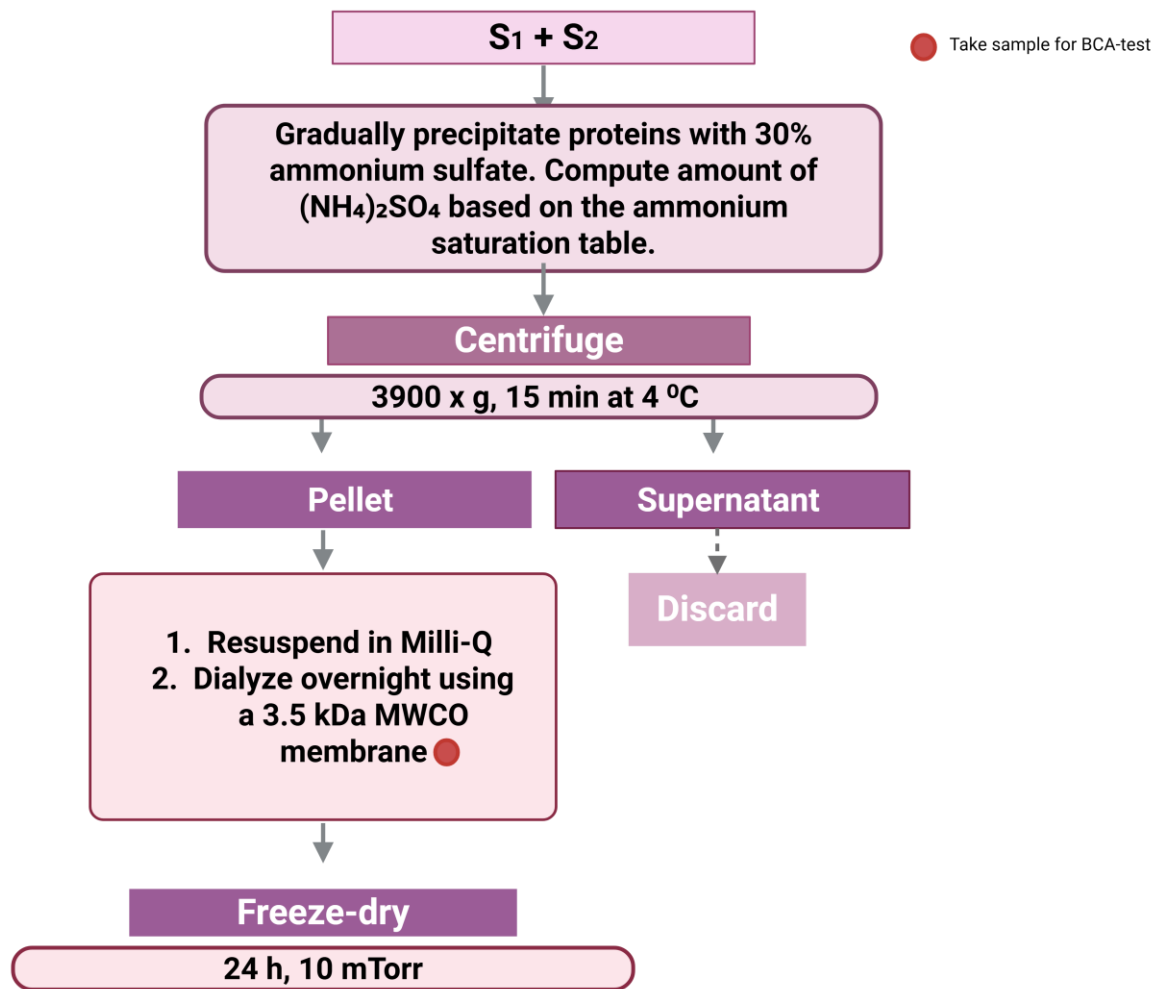


Fig. 15. Partial protein purification workflow using ammonium sulfate precipitation. MWCO= molecular weight cutoff. Made with Biorender.

The resulting pellet from each 50 mL falcon tube was resuspended in 1-2 mL Milli-Q water (pH 7.4), pooled, and stirred for 1 h at 7 °C. To remove excess salts, dialysis was performed (Fig. C3) using a 3.5 kDa molecular weight cut-off tubing (SnakeSkin™ Dialysis Tubing) against Milli-Q water for 24 h at 7 °C, secured with dialysis closures (Sigma-Aldrich). The dialysis buffer was replaced three times: first after 2–3 h, second after 12–16 h, and third after 4–6 h, using a minimum buffer volume of 100× the sample volume (Zhang et al, 2019).

After dialysis, parts of the samples were aliquoted, frozen at –80 °C, freeze-dried, and stored as protein powder at –20 °C for subsequent analysis. One mL of the dialyzed protein solution was retained for protein quantification. Protein recovery yield was calculated as the ratio of purified protein (mg) to total protein content prior to purification. In **Figure 16**, a complete overview of the protein extraction and purification process is given.

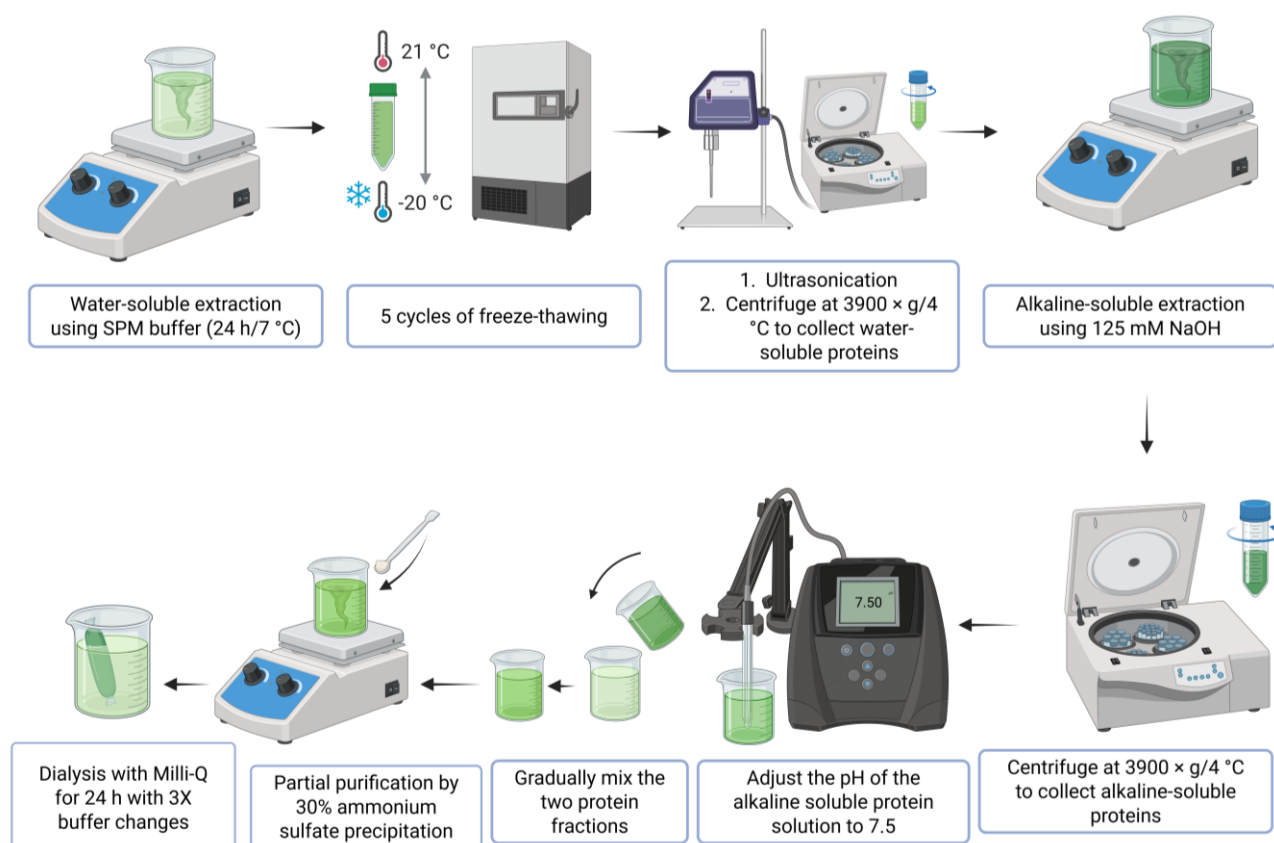


Fig. 16. Protein extraction (physical/chemical) and purification steps used in this thesis. SPM= sodium phosphate monobasic.
Made with Biorender.

5. Chemical analysis of protein extracts

Chemical characterization included (1) determination of protein content, (2) total carbohydrate content, and (3) total phenolic content, as further detailed below.

5.1. Protein quantification using the BCA assay

Protein concentration of both crude and partially purified extracts was determined using the Bicinchoninic Acid (BCA) assay (Walker, 1994) according to the kit manufacturer's instructions (Pierce™ BCA Protein Assay Kit). The assay is based on the reduction of Cu^{2+} to Cu^{+} by proteins under alkaline conditions, followed by the formation of a purple BCA-Cu^{+} complex, the intensity of which is proportional to protein concentration.

Bovine serum albumin (BSA) was used as a standard (concentration: $0.016\text{--}2\text{ mg mL}^{-1}$; LOD: 0.004 mg mL^{-1} ; LOQ: 0.011 mg mL^{-1}) and diluted in Milli-Q water. Standards and samples were prepared in triplicate. Briefly, 25 μL of each standard or sample was transferred into a 96-well microplate, followed by the addition of 200 μL of freshly prepared BCA working reagent (reagents A and B mixed at a 50:1 ratio). Plates were covered and incubated at 37 °C for 30 min (South Korea: Dry oven, Hangil science; Belgium: VWR® Incubating Mini Shaker), then cooled to room temperature for 10 minutes. Absorbance was measured at 562 nm using a UV-VIS spectrophotometer (South Korea: SpectraMax Id3; Belgium: Infinite M200, TECAN).

5.2. Total carbohydrate content

Total carbohydrate content was determined using the phenol-sulfuric acid colorimetric method, described by DuBois et al. (1956), adapted for microplate analysis according to Masuko et al. (2005). The assay is based on the dehydration of carbohydrates by concentrated sulfuric acid (H_2SO_4) to form furfural derivatives, such as hydroxymethylfurfural from

hexoses. These intermediates subsequently react with phenol to produce a coloured complex, which can be quantified spectrophotometrically (Fig. 17).

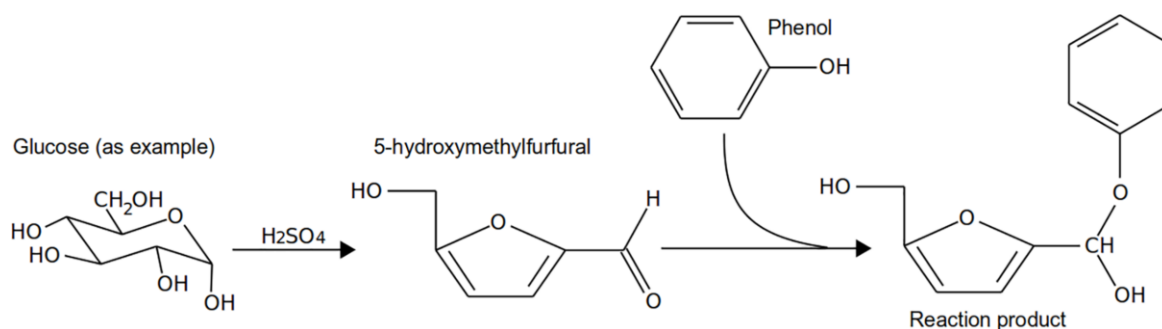


Fig. 17. Example reaction of the phenol–sulfuric acid assay with glucose (Braissant et al., 2020).

Briefly, 50 μL of each sample or standard was transferred into a 96-well microplate. Subsequently, 150 μL of concentrated H_2SO_4 was rapidly added to ensure thorough mixing, followed immediately by the addition of 30 μL of a 5% (w/v) phenol solution. The plate was then incubated for 5 min at 90 $^\circ\text{C}$ to allow complete colour development. After incubation, the microplate was cooled to room temperature, and the absorbance was measured at 490 nm using a spectrophotometer. Standards and samples were prepared in triplicate, with glucose used as the calibration standard (concentration: 0.01–2.5 mg mL^{-1} ; LOD: 0.015 mg mL^{-1} ; LOQ: 0.045 mg mL^{-1}), and total carbohydrate content expressed as glucose equivalents (mg glucose g^{-1} DW).

5.3. Total phenolic content

Total phenolic content was determined using the Folin–Ciocalteu method, adapted from Cox et al. (2010). This assay is based on the reduction of heteropolyphosphomolybdate–tungstate complexes present in the Folin–Ciocalteu reagent under alkaline conditions. During the reaction, phenolic compounds reduce molybdenum (Mo^{6+}) and tungsten (W^{6+}) ions to lower oxidation states, mainly Mo^{5+} and W^{5+} , resulting in the formation of a blue-coloured complex commonly referred to as molybdenum blue. The intensity of the blue colouration is proportional to the concentration of phenolic compounds present in the sample (Fig. 18).

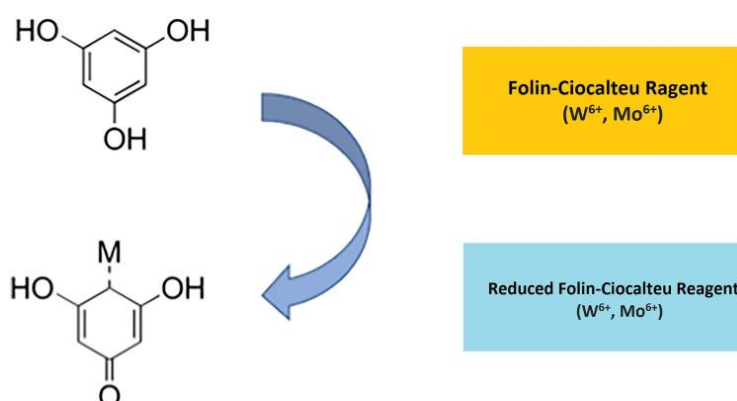


Fig. 18. Principle of the Folin–Ciocalteu method for total phenolic content determination (Shi et al., 2022).

Briefly, 10 μL of each sample extract was transferred into a microplate well and mixed with 200 μL of 2% (w/v) sodium carbonate (Na_2CO_3) solution. The mixture was incubated for 2 min at room temperature. Subsequently, 10 μL of 50% diluted Folin–Ciocalteu's reagent (Sigma-Aldrich) was added, and the reaction mixture was incubated for 30 min at room temperature in the dark. After incubation, absorbance was measured at 720 nm using a spectrophotometer. Gallic acid was used as the calibration standard (concentration: 0.02–2.5 mg mL^{-1} ; LOD: 0.023 mg mL^{-1} ; LOQ: 0.069 mg mL^{-1}),

and results were expressed as mg gallic acid equivalents per gram dry weight of extract (mg GAE g⁻¹ DW). Standards and samples were prepared in triplicate.

6. Amino Acid (AA)-profiling of protein extracts

Amino acid (AA) composition of all samples was analysed by an external specialised facility in South Korea, the Korea Polymer Testing & Research Institute (KOPTRI), regulated under ISO 17025 standards. Samples were subjected to acid hydrolysis followed by analysis using a Hitachi L-8900 automated amino acid analyser. Hydrolysis was performed using 6 M HCl containing 0.05% (w/v) 2-dimethyl sulfoxide (DMSO, C₂H₆SO), with a volume approximately 1000-fold relative to the protein content (typically 10 mL per sample). Prior to hydrolysis, samples were kept at low temperature and degassed to remove dissolved oxygen. Hydrolysis was carried out in sealed tubes at 110 °C for 22–24 hours.

After hydrolysis, samples were cooled, opened, and subjected to vacuum evaporation at 40 °C to remove excess HCl. The residues were reconstituted in 0.2 M sodium citrate (Na₃C₆H₅O₇) buffer (pH 2.2) or 0.02 M HCl. Insoluble material was removed by filtration through a membrane filter prior to analysis. Amino acid separation was performed on the L-8900 analyser using a Na⁺ form ion exchange column (60 mm × 4.6 mm) under controlled temperature conditions. Post-column derivatization with ninhydrin reagent allowed photometric detection at 570 and 440 nm. The system parameters were set as follows: flow rate 0.4 mL min⁻¹, injection volume 10 µL, column temperature 57 °C, reaction unit temperature 135 °C, and total run time 33 min. Quantification of amino acids was performed using external calibration standards. Final concentrations were calculated using following formula:

$$\text{AA (mg/g)} = \frac{\text{Concentration of test solution (mg/100 mL)} \times \text{Final volume (mL)} \times \text{Dilution factor}}{\text{Sample mass (mg)}} \quad (4)$$

7. Production of protein hydrolysates from *Ulva* species

7.1. *In vitro* simulated gastrointestinal digestion

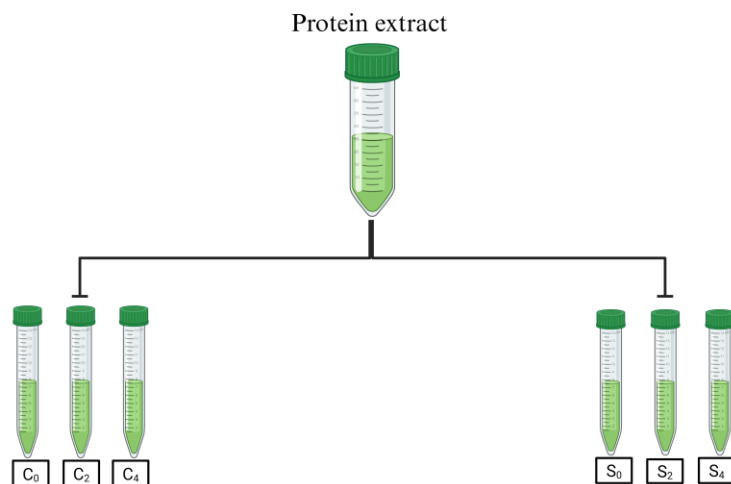
The simulated gastrointestinal digestion of *Ulva* protein extracts was performed following the standardized static INFOGEST 2.0 protocol (Brodkorb et al., 2019) with minor modifications. This protocol reproduces the physiological conditions of the human oral, gastric, and small intestinal phases, using fixed ratios of food to digestive fluids, controlled pH-values, and standardized enzyme activities. Protein samples were digested under these controlled conditions to assess hydrolysis patterns and peptide release. The model was applied as a static endpoint assay and was not intended for kinetic studies. Detailed compositions of simulated gastric and intestinal fluid (SGF and SIF) are provided in **Appendix B**, and the full digestion procedure is summarized in **Figure 19**.

The oral phase of the INFOGEST 2.0 protocol was omitted, as it mainly affects carbohydrates (**cf. Literature review: section 5.1**). Focus was placed on the gastric and intestinal phases, where proteins are hydrolysed by pepsin and pancreatin, respectively. These phases were used to assess protein digestion, hydrolysis extent, and the release of bioactive peptides from seaweed-derived proteins.

7.2. Preparation of simulated digestive fluids

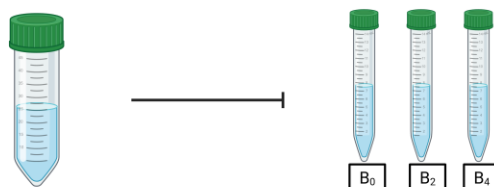
Simulated gastric fluid (SGF, pH 3) and simulated intestinal fluid (SIF, pH 7) were prepared as electrolyte stock solutions at 1.25× concentration using standardized salt compositions (**Appendix B**). Stock solutions were stored at –20 °C and prewarmed to 37 °C prior to use. Calcium chloride (CaCl₂) was added immediately before digestion to avoid precipitation. All enzyme solutions and bile solutions were freshly prepared on the day of digestion, diluted in cold solutions and kept on ice until use to minimize enzymatic activity prior to the digestion experiment.

Step 1



Step 2

Deionized water

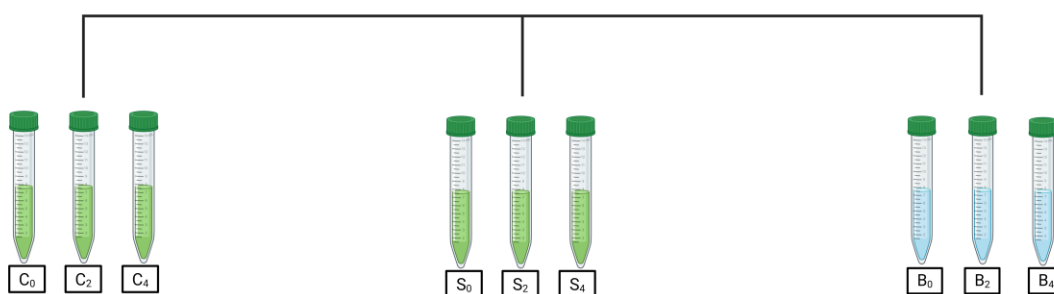


Step 3

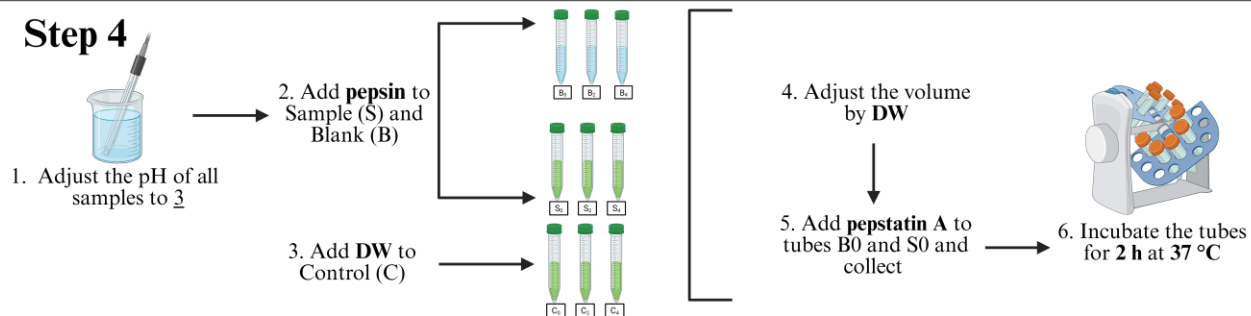
1.25X SGF solution



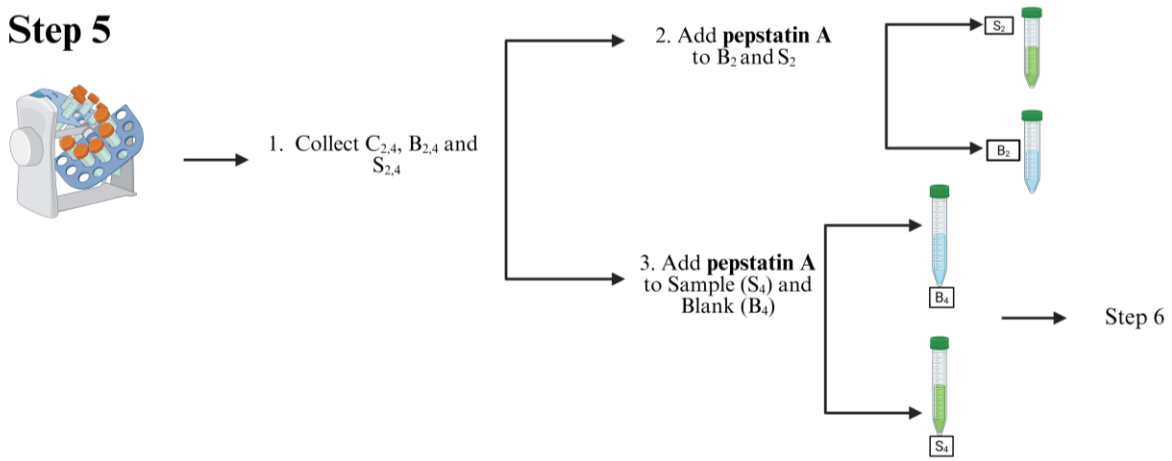
Add at a 1:1 (v/v) ratio



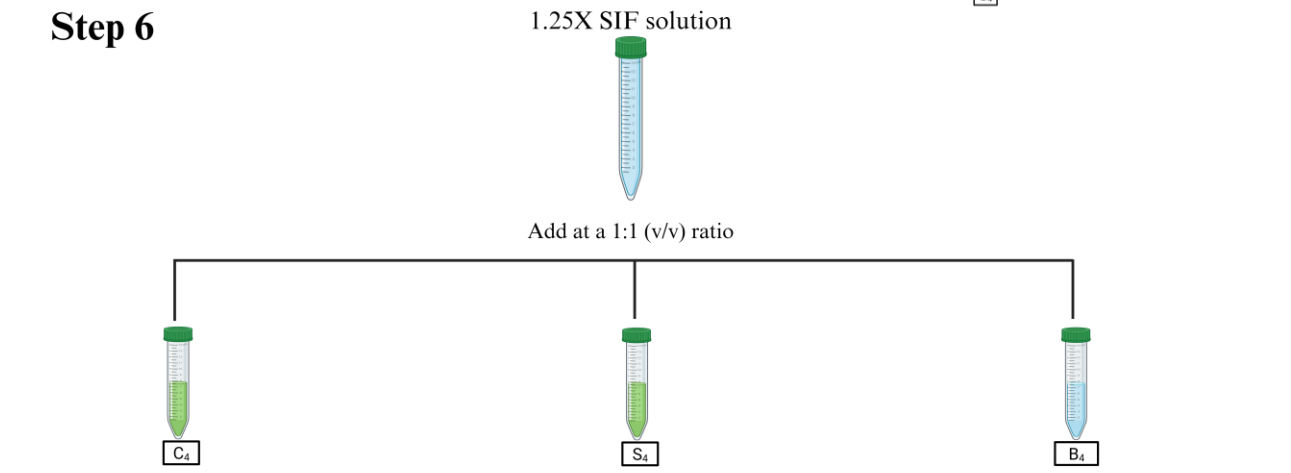
Step 4



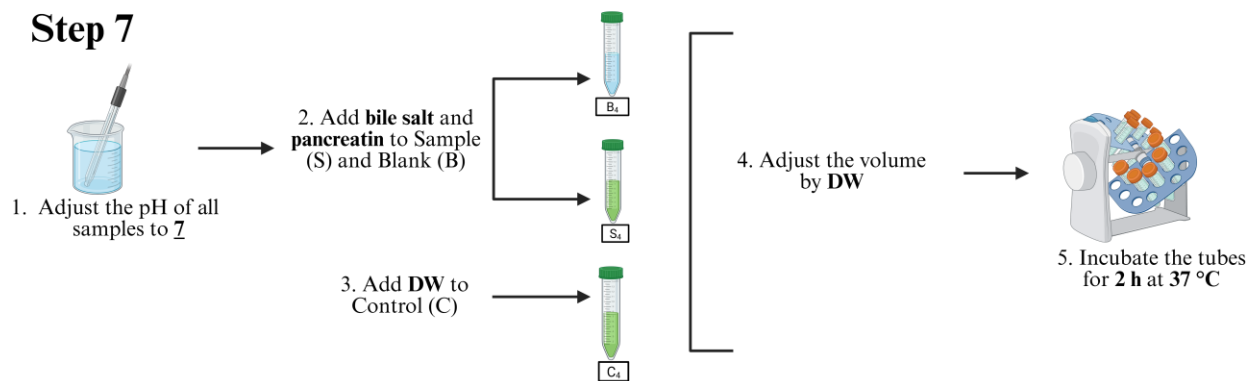
Step 5



Step 6



Step 7



Step 8

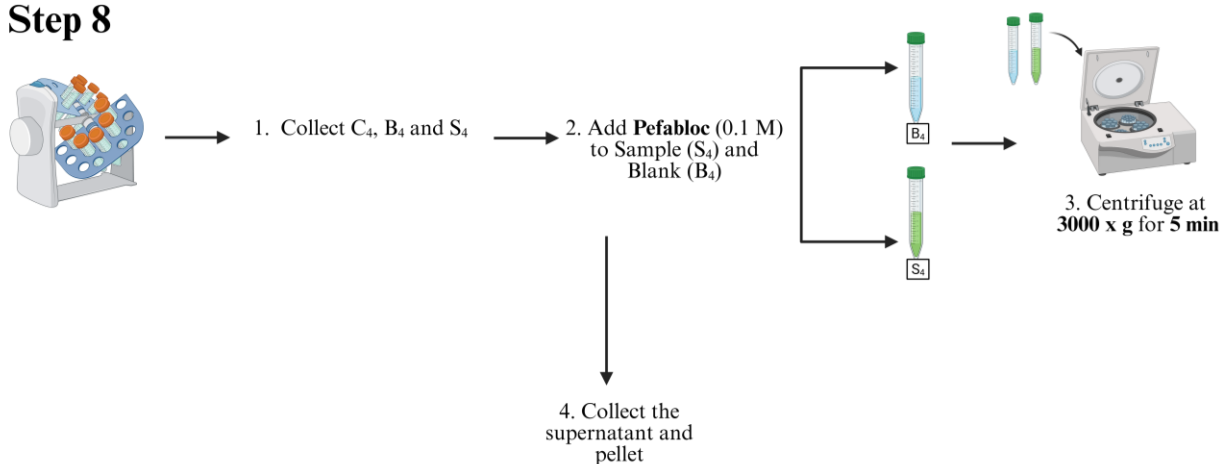


Fig. 19. Schematic overview of the in vitro simulated gastrointestinal digestion of Ulva protein extract following the INFOGEST 2.0 protocol. SGF= simulated gastric fluid, SIF= simulated intestinal fluid, DW= Deionised water. Only gastric and intestinal phases were included. C = control, B = blank, S = sample. All steps were conducted simultaneously under standardized conditions to allow assessment of protein hydrolysis and peptide release. Made with Biorender.

7.3. Digestion procedure

Protein solutions were prepared at a concentration of 10 mg mL⁻¹. For time-resolved sampling, separate digestion tubes were prepared for each sampling point to ensure homogeneity of digesta (**Fig. 19**). All samples were processed using the same protocol throughout the simulated digestion, including appropriate controls and blanks to ensure accurate assessment of protein hydrolysis. A control sample containing protein solution without enzymes was included to evaluate non-enzymatic protein degradation. In addition, a blank containing only digestive fluids and enzymes, without protein substrate, was used to account for enzyme-derived peptides and background signals.

7.3.1. Gastric phase

The gastric phase was initiated by mixing *Ulva* protein extract (10 mg mL⁻¹) with SGF at a 1:1 (v/v) ratio. The pH was adjusted to 3.0. Pepsin (100 µL, dissolved in Milli-Q water, Sigma Aldrich) was added to reach a final activity of approximately 2000 U mL⁻¹. Samples were incubated at 37 °C under continuous agitation (South Korea: RotoMini™ PLUS; Belgium: Multi RS-60) for 120 min. Aliquots were collected at predefined time points, and enzymatic activity was stopped by adding pepstatin A (Sigma Aldrich) dissolved in a 9:1 (v/v) methanol–acetic acid solution.

7.3.2. Intestinal phase

Transitioning to the intestinal phase, the complete gastric digest was mixed with SIF at a 1:1 (v/v) ratio and the pH was adjusted to 7.0. Bile extract (Sigma Aldrich) was added to a final concentration of 10 mM, and pancreatin (500 µL, dissolved in 1 mM HCl and vortexed for 10 min, Sigma Aldrich) was added to reach a final trypsin activity of approximately 100 U mL⁻¹. The mixture was incubated at 37 °C under continuous agitation for 120 min. Aliquots were collected at predefined time points, and enzymatic activity was stopped by adding Pefabloc® SC (Sigma Aldrich) to a final concentration of 0.1 M.

Upon collection at each time point, the pH of all samples, controls, and blanks was quickly increased to 12 to fully solubilize proteins prior to analysis. This step is particularly important for green seaweed proteins, which generally exhibit low isoelectric points (pI) and tend to precipitate under the acidic conditions (pH 3) of *in vitro* gastric digestion. Protein resolubilization upon alkaline pH adjustment has previously been demonstrated by Harrysson et al. (2018) (**Fig. D2**). Subsequently, samples were neutralized to pH 7–7.5 to maximize protein recovery and allow accurate assessment of digestion and hydrolysis products.

8. Evaluating the progress of *Ulva* protein digestion

8.1. OPA assay

The extent of proteolysis during *in vitro* digestion was quantified using the o-phthalaldehyde (OPA) assay. In this method, OPA (Sigma-Aldrich) reacts with primary amine groups of amino acids, peptides, and proteins under alkaline conditions in the presence of a thiol, forming a chromophore detectable spectrophotometrically (**Fig. D4**). The absorbance is proportional to the concentration of free amino groups released during protein hydrolysis (Church et al., 1985; Montgomery et al., 2021).

A calibration curve was generated using L-leucine (South Korea: DAEJUNG; Belgium: Sigma Aldrich) standards (concentration: 0.078–1.25 mg mL⁻¹; LOD: 0.035 mg mL⁻¹; LOQ: 0.106 mg mL⁻¹) following the Sigma-Aldrich OPA assay protocol. Free amino groups were quantified by interpolating sample absorbance on this curve and expressed as leucine equivalents (mg mL⁻¹). Values were normalized to protein content (**BCA assay, Materials & Methods: section 5.1**) and reported as µmol leucine equivalents per mg protein (µmol L mg⁻¹).

The degree of hydrolysis (%DH), defined as the percentage of peptide bonds cleaved during digestion, was calculated by comparing the measured amino group release with the total release obtained after complete protein hydrolysis as mentioned in following formula based on Trigo et al (2021):

$$\%DH = \frac{h_{\text{sample}} - h_{\text{digestion blank}}}{h_{\text{tot, sample}}} \times 100 \quad (5)$$

Where:

- h = Amount of primary amines ($\mu\text{mol L mg}^{-1}$)
- h_{tot} = Total primary amines after complete acid hydrolysis ($\mu\text{mol L mg}^{-1}$)

Total protein hydrolysis was performed by acid hydrolysis (**Fig. 20**) using 6 M HCl (500 μL per mg protein). Samples in screw-cap vials were flushed with nitrogen for one minute (South Korea: MD 200 Sample Concentrator; Belgium: Techne™ Sample Concentrator) to remove oxygen and prevent oxidative degradation, then incubated at 110 °C for 24 h (Gorissen et al, 2018; Hou et al, 2017). After cooling, samples were neutralized to pH 7.0 prior to OPA analysis.

Aliquots (10 μL) of samples, standards, and blanks were pipetted into a 96-well microplate, followed by the addition of 200 μL of OPA reagent to each well. The plate was immediately covered, protected from light, and incubated at room temperature for 2 min. Absorbance was measured at 340 nm using a UV-VIS spectrophotometer.

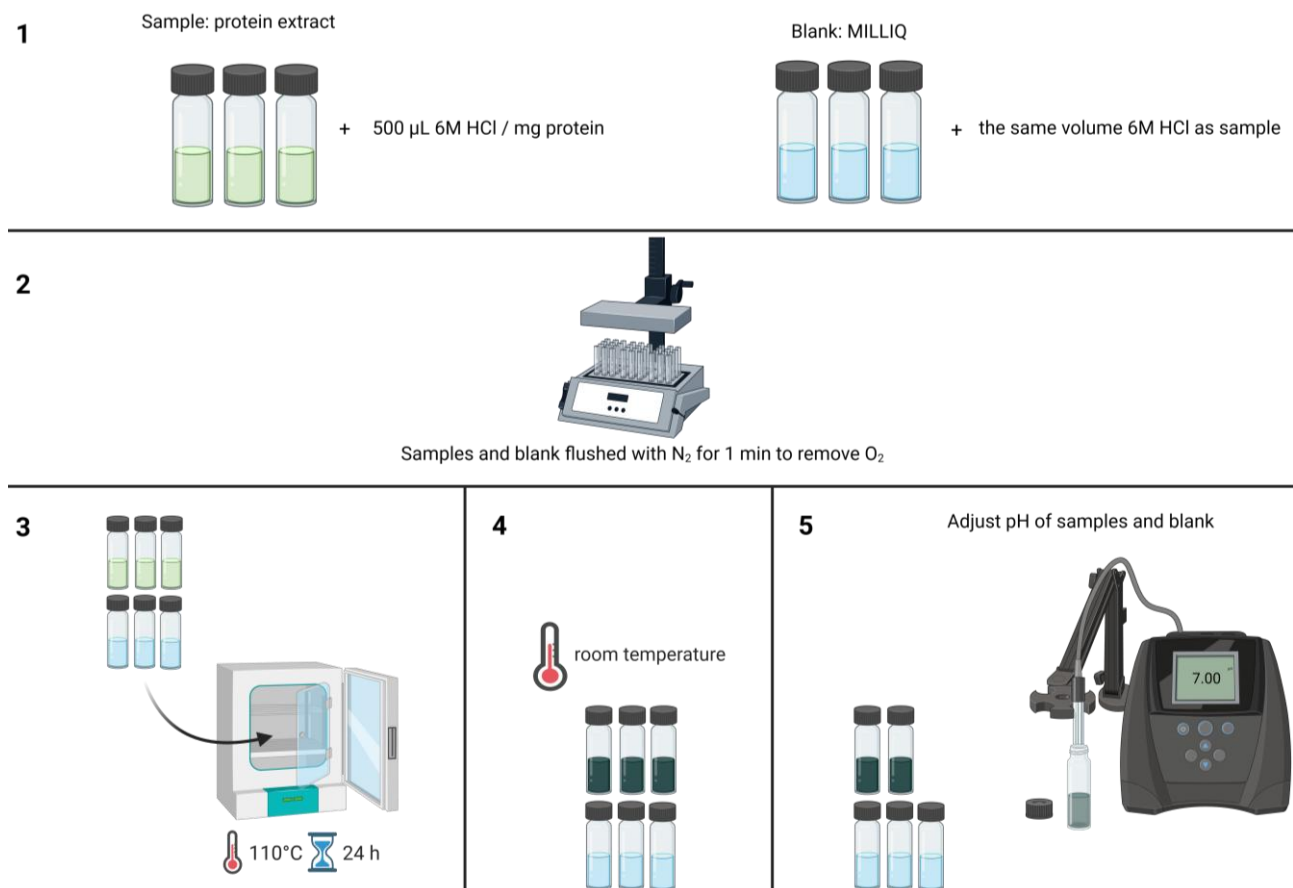


Fig. 20. Acid hydrolysis procedure for complete protein hydrolysis. Made with Biorender.

8.2. SDS-PAGE analysis

Protein molecular weight profiles before and after digestion were analysed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE separates proteins primarily based on polypeptide chain length and molecular weight.

South Korea

Protein extracts (diluted to 2 mg mL⁻¹), and undiluted digested samples were prepared for clear band visualization. Sample preparation was performed according to the manufacturer's protocol for PROTEAN® TGX precast gels (Bio-Rad). Samples were mixed with 4× Laemmli sample buffer in a 3:1 ratio (45 µL sample: 15 µL buffer) and supplemented with β-mercaptoethanol at a 1:10 ratio relative to the Laemmli buffer. Mixtures were vortexed thoroughly and heated at 95 °C for 10 min in a thermoshaker (Thermo Scientific™) to ensure complete protein denaturation. Next, 40 µL of each sample was loaded onto Mini-PROTEAN® TGX precast gels (Bio-Rad) along with 5 µL of prestained protein marker (EcoBand Protein Marker, 5–250 kDa). Electrophoresis was performed at 200 V in 1× Tris/Glycine/SDS running buffer until the dye front reached the bottom of the gel.

After electrophoresis, gels were removed from the cassette, rinsed with distilled water, and fixed for 30 min on a rocking platform (DAIHAN® Platform Rocker, 12 rpm) in 50% (v/v) methanol and 10% (v/v) acetic acid. Gels were then stained for 30 min with 0.05% (w/v) Coomassie Brilliant Blue R-250 in the same solution. Destaining was performed in 40% (v/v) methanol and 10% (v/v) acetic acid with three 30 min washes. Protein bands were visualized using a ChemiDoc XRS+ imaging system (Bio-Rad).

Belgium

SDS-PAGE was performed similarly to the procedure applied in South Korea, with some modifications. The sample buffer was prepared by mixing 3.55 mL deionized water with 1.25 mL 0.5 M Tris-HCl (pH 6.8), followed by 2.5 mL glycerol, 2.0 mL 10% (w/v) SDS, and 0.2 mL 0.5% (w/v) bromophenol blue. β-mercaptoethanol (50 µL per 950 µL buffer) was added immediately prior to use. Protein extracts (2 mg mL⁻¹) and undiluted digested samples were prepared by mixing 10 µL of sample with 20 µL of sample buffer and were heated at 95 °C for 5 min (Biometra TSI ThermoShaker, Analytik Jena). Subsequently, 10 µL of each sample was loaded onto precast 10% Mini-PROTEAN® TGX Stain-Free™ Protein Gels (Bio-Rad) along with 5 µL of Precision Plus Protein™ Unstained Standard (15–250 kDa). Electrophoresis was performed at 200 V for 60 min in running buffer containing 3.03 g L⁻¹ Tris, 14.4 g L⁻¹ glycine, and 1.0 g L⁻¹ SDS. Gels were briefly rinsed with deionized water to remove excess SDS.

Protein bands were visualized using Bio-Rad Stain-Free technology, which generates fluorescent bands by UV activation of a trihalo compound reacting with tryptophan residues. Gels were imaged directly using the GelDoc GO Gel Imaging system (Bio-Rad) with automatic exposure settings, and bands were analysed using ImageJ (v1.53k).

8.3. Molecular weight distribution of peptides in digested protein hydrolysates

Following simulated gastrointestinal digestion, protein hydrolysates were fractionated by molecular weight using Amicon® Ultra centrifugal filter units (**Fig. 21A**) with nominal molecular weight cut-offs (MWCO) of 10, 5, and 3 kDa (**Fig. 21B**). Filtration units were preconditioned by centrifugation with Milli-Q water prior to use.

Approximately 10 mL of digested sample was first loaded onto the 10 kDa filter and centrifuged at 3900 x g for 10–20 min until the retentate reached the membrane level. The retained fraction (>10 kDa) was collected, and the permeate was transferred to a 5 kDa filter to isolate the 5–10 kDa fraction. The permeate from the 5 kDa unit was further processed through a 3 kDa filter to obtain the 3–5 kDa fraction, while the final permeate represented proteins <3 kDa. The volume of each fraction was recorded after centrifugation. Protein content was quantified using the BCA assay and expressed as the percentage contribution of each fraction (>10 kDa, 5–10 kDa, 3–5 kDa, <3 kDa) relative to the total protein content prior to ultrafiltration.

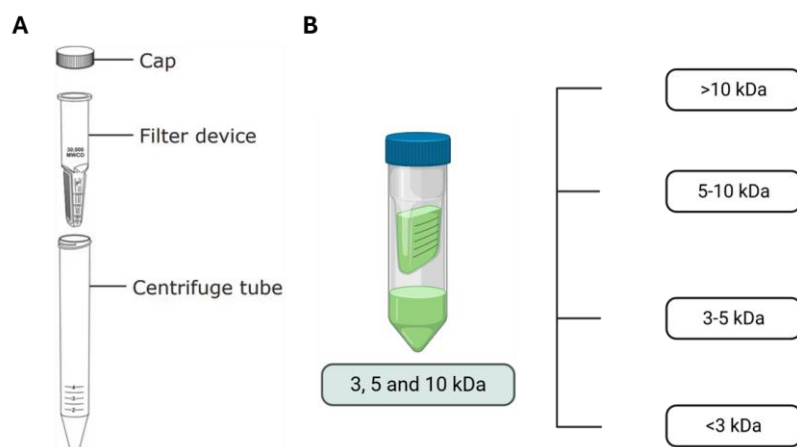


Fig. 21. Ultrafiltration and fraction collection. (A) Amicon® Ultra centrifugal filter unit from Merck Millipore. (B) Ultrafiltration workflow and molecular weight fractions (>10 kDa, 5–10 kDa, 3–5 kDa, <3 kDa). Made with Biorender.

9. Confocal Laser Scanning Microscopy (CLSM) of protein extracts

CLSM was used to visualize proteins and polysaccharides in *Ulva* protein extracts before and after digestion. This fluorescence technique provides high-resolution optical sections by eliminating out-of-focus light, making it suitable for thick biological samples. Dual staining was performed using Calcofluor White (Sigma-Aldrich) to visualize polysaccharides and Fast Green FCF (DAEJUNG) to stain proteins. Comparison of samples before digestion (intact structure) and after digestion (non-soluble residues) allows assessment of the distribution and resistance of proteins and polysaccharides to enzymatic breakdown, as well as their potential interactions during digestion. Non-soluble fractions obtained after *in vitro* digestion were collected upon centrifugation ($3,000 \times g$, 3–5 min) to pellet insoluble residues. The supernatant was retained as the protein hydrolysate after digestion, and the pellet was gently resuspended in Milli-Q water to a final concentration of $0.05\text{--}0.5 \text{ mg mL}^{-1}$. Samples prior to digestion were diluted to the same concentration for comparison.

Dual fluorescent staining was performed to visualize polysaccharides and proteins, following the protocol of Molina-Gilarranz et al. (2025). A $2\times$ staining solution was freshly prepared containing 0.02% (w/v) Calcofluor White (CWS) and 0.2% (w/v) Fast Green FCF. Equal volumes of sample and staining solution were mixed to obtain final concentrations of 0.01% (w/v) CWS and 0.1% (w/v) Fast Green FCF. Samples were incubated for 15 min at room temperature in the dark, followed by centrifugation and a washing step with Milli-Q water to remove excess stain.

For imaging, 5 μL of stained suspension was mounted on glass slides, covered with a coverslip, and kept in the dark until analysis. Imaging was performed on a APEXVIEW APX100 Benchtop Fluorescence Microscope (Evident) with a $40\times$ objective. CWS was excited at 405 nm (detected at 420–500 nm) and Fast Green FCF at 635–647 nm (detected at 650–720 nm; Table 9). Sequential scanning minimized spectral overlap. Image analysis was performed using ImageJ (v1.53k).

Table 9. Overview of the two stains used to visualize the samples

Parameter	Calcofluor White (CWS)	Fast Green FCF
Target	Polysaccharides	Proteins
Final concentration	0.01% (w/v)	0.1% (w/v)
Excitation (laser)	405 nm (Violet / UV)	635–647 nm (Red)
Emission detection	420–500 nm (Blue)	650–720 nm (Red/ far-Red)
Binding mechanism	Binds β -1,3 and β -1,4 glucans	Binds proteins

RESULTS

1. Taxonomic identification of *Ulva* species

Table 10 shows taxonomic identification of the species and their associated E-value. The E-value is extremely low and is reported as zero by BLAST, indicating a very strong sequence match.

Table 10. BLASTn-based taxonomic identification of tufA sequences, including query name, length (bp), subject accession (AC), species, E-value, and percentage identity (Pct).

Name	Length (bp)	AC	Species	E-Value	Pct.(%)
Ulva_A_tufA_F	1338	KT882614.1	<i>Ulva fasciata</i>	0.0	99
Ulva_C_tufA_F	1440	KX058323.1	<i>Ulva linza</i>	0.0	98
Ulva_X_tufA_F	774	HQ610321.1	<i>Ulva intestinalis</i>	0.0	99
Ulva_X'_tufA_F	774	HQ610383.1	<i>Ulva australis</i>	0.0	99

2. Biochemical composition of *Ulva* biomass

2.1. Total protein content

Figure 22 shows that the total protein content, calculated from total nitrogen content, differs significantly among the four *Ulva* species, with *Ulva australis* exhibiting the highest value (19.70%).

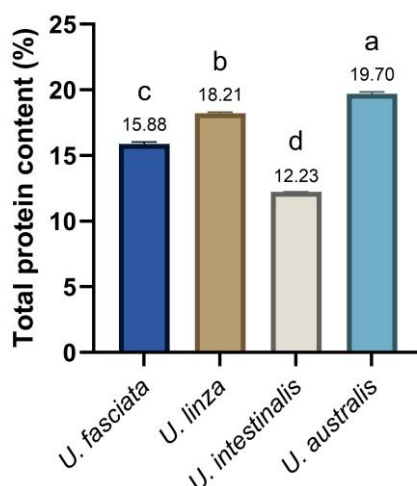


Fig. 22. Bar plot showing the total protein content of four *Ulva* species on a dry weight basis. Protein content was calculated from total nitrogen using a conversion factor of 4.24 for green seaweeds. Error bars represent the standard deviation of two replicates. Different letters indicate statistically significant differences between species (ANOVA, $p < 0.05$).

2.2. Ash content

Ash content across all species is shown in **Figure 23** and differed significantly among the four *Ulva* species, indicating clear species-dependent variation in inorganic content. **Figure C1** shows *Ulva fasciata* seaweed powder before and after combustion treatment, which removes organic matter and leaves only the mineral fraction behind.

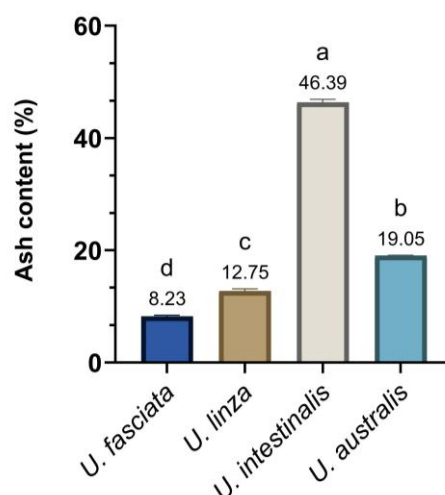


Fig. 23. Bar plot of ash content in four *Ulva* species on a dry weight basis. Error bars represent standard deviation ($n = 3$; *U. linza* $n = 2$). Different letters indicate significant differences between species (ANOVA, $p < 0.05$).

3. *Ulva* protein extraction process

3.1. Water- and alkaline-soluble extracted proteins

Following freeze–thaw treatment and ultrasonication, proteins were extracted via alkaline-soluble extraction. **Figure C2** shows both the water-soluble (S1) and alkaline-soluble (S2) protein fractions prior to purification for *Ulva fasciata* and *Ulva intestinalis*. A clear difference in colour is observed between the water-soluble fractions. The different protein fractions prior to purification are shown in **Figure 24**. These results are not directly representative of extraction efficiency, as total protein content varies between species. Notably, *Ulva australis* exhibits significantly higher levels of water-soluble protein.

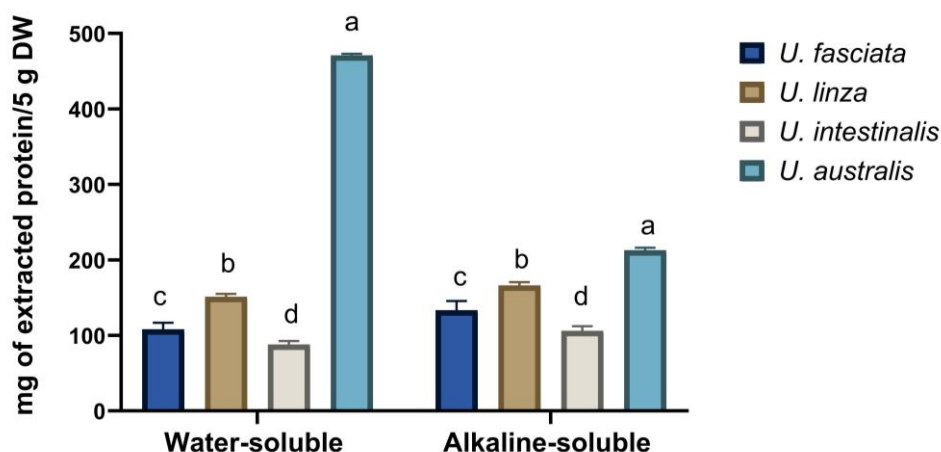


Fig. 24. Bar plot of protein fractions from four *Ulva* species, expressed as mg of extracted protein standardized to 5 g dry weight (DW). Error bars represent standard deviation ($n = 3$). Different letters (a, b, c, d) denote statistically significant differences between species within a specific category (ANOVA, $p < 0.05$).

Extraction rates, calculated based on the amount of total water- and alkaline-soluble extracted protein over the total protein content of each species, are shown in **Figure 25**. Significant differences in extraction yield were observed between species, with *Ulva australis* showing a clearly higher extraction yield compared to the other species. No significant difference was observed between *Ulva intestinalis* and *Ulva fasciata*.

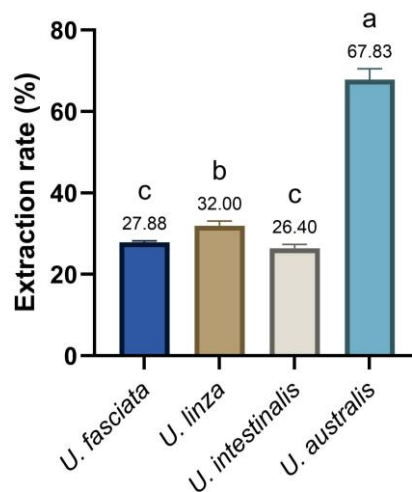


Fig. 25. Bar plot showing the extraction rate of four *Ulva* species after extraction. Error bars represent the standard deviation ($n = 3$). Different letters indicate statistically significant differences between species (ANOVA, $p < 0.05$).

3.2. Salinity

Due to the significantly higher ash content in *U. intestinalis* compared to other seaweeds (Fig. 23), the salinity of protein extracts obtained from *U. intestinalis* was measured prior to purification and compared with that of *U. australis*, which had a lower ash content. This analysis was performed to evaluate the potential effect of higher salt concentrations in *U. intestinalis* on protein digestibility. As shown in Figure 26, a significant difference in extract salinity was observed between *Ulva australis* (5.55 PSU) and *Ulva intestinalis* (7.00 PSU).

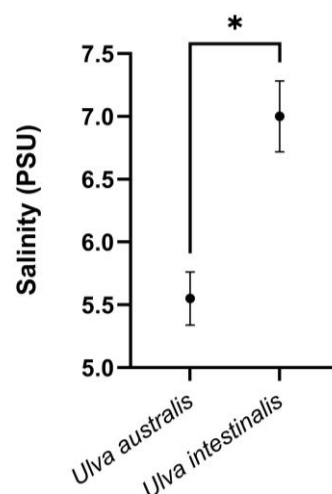


Fig. 26. Salinity of European *Ulva* extracts prior to purification. Error bars represent standard deviation ($n = 2$ biological replicates). * Indicates a significant difference between species ($p < 0.05$, Welch's t -test).

3.3. Protein purification

Purification was performed according to the scheme shown in Figure 15. As illustrated in Figure C3, the procedure consisted of ammonium sulfate precipitation followed by centrifugation and dialysis to remove excess salts. Recovery rates are presented in Figure 27, with significant differences observed between all four species.

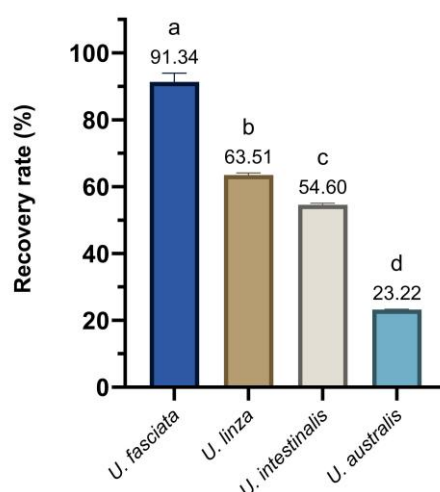


Fig. 27. Bar plot showing the recovery rate of four *Ulva* species after protein purification. Error bars represent the standard deviation ($n=3$). Different letters indicate statistically significant differences between species (ANOVA, $p < 0.05$).

4. Characterisation of *Ulva* protein extracts

4.1. Chemical composition

For all assays, concentrations were converted to total content (mg) using the same extract volume and expressed as a percentage of the dry weight of the corresponding freeze-dried extracts. Protein content of the protein extracts, determined after ammonium sulfate (AS) purification using the BCA assay, did not differ significantly among species (Fig. 28A).

Carbohydrate content differed significantly among the four *Ulva* species (Fig. 28B), with *U. fasciata* showing the highest value ($13.40\% \pm 2.30$), followed by *U. intestinalis* ($10.68\% \pm 0.99$) and *U. australis* ($8.18\% \pm 0.69$), while *U. linza* had the lowest value ($3.18\% \pm 0.86$). *U. intestinalis* did not differ significantly from *U. fasciata* or *U. australis*, indicating an intermediate level.

Phenolic content also differed significantly among species (Fig. 28C), with highest values for *U. fasciata* ($3.75\% \pm 0.25$) and lowest for *U. australis* ($2.21\% \pm 0.02$).

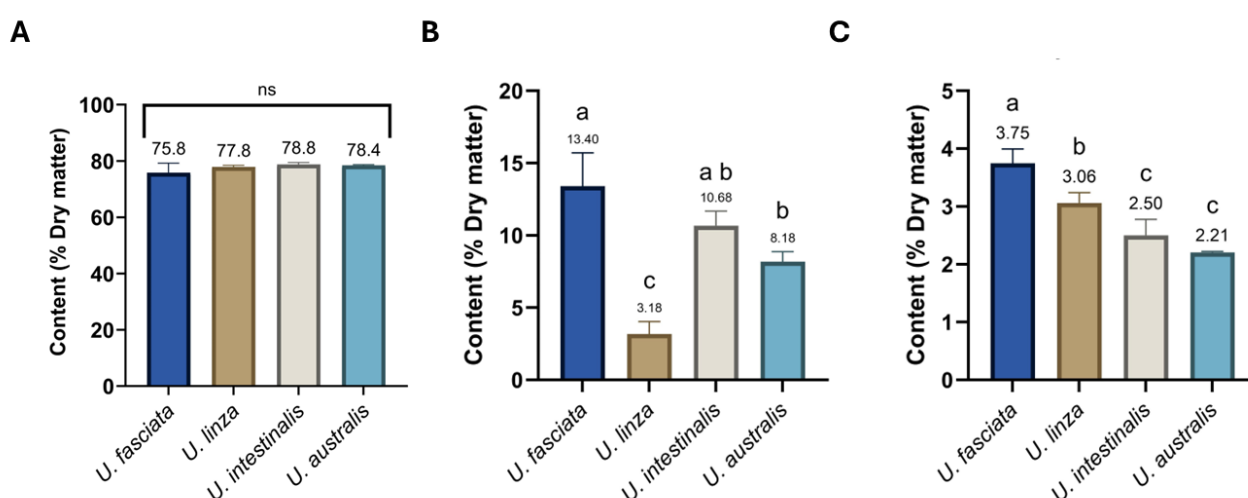


Fig. 28. Quantitative composition of protein extracts from four *Ulva* species. (A) Protein content, (B) carbohydrate content, and (C) phenolic content. Error bars represent the standard deviation of replicates ($n = 3$). Different letters indicate statistically significant differences between species (ANOVA, $p < 0.05$).

Figure 29 presents a summary of the chemical composition of all species. *U. fasciata* had the highest phenolic ($3.75\% \pm 0.25$) and carbohydrate ($13.40\% \pm 2.30$) contents, while protein levels did not differ significantly. As a result, *U. linza* showed a higher “others” fraction due to its lower carbohydrate and phenolic content.

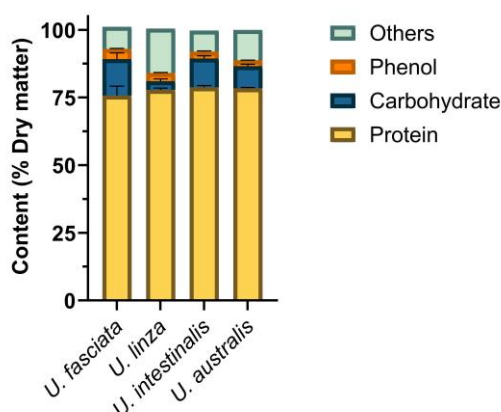


Fig. 29. Total chemical composition of four *Ulva* species. Bars represent the relative contribution of protein, carbohydrates, phenolics, and the residual “others” fraction ($100\% - \text{protein} - \text{carbohydrate} - \text{phenolic content}$).

4.2. Amino acid profile

Amino acid analysis was performed to characterize the nutritional quality of the protein extracts obtained from four *Ulva* species and to evaluate their potential as precursors of bioactive peptides during gastrointestinal digestion. 16 different amino acids were selected for this analysis. The results are summarised in **Table 11**. To facilitate comparison, amino acid abundances were expressed as relative percentages of the total amino acid content (mg) for each species. For reference, the relative amino acid composition of ovalbumin, the main protein in eggs, was included alongside the results of the four species.

Table 11. Relative composition of 16 amino acids across four species, with essential amino acids indicated by *, and ovalbumin included as a reference (Fujiwara-Arasaki et al., 1984). Abundances are expressed as percentages of the total amino acid content (mg) for each species. ND= not detected.

Amino acid composition of *Ulva* spp. (% of total AA).

	<i>U. fasciata</i>	<i>U. linza</i>	<i>U. intestinalis</i>	<i>U. australis</i>	Ovalbumin
Alanine	6.13	6.86	4.25	5.49	7.64
Arginine	4.60	5.23	5.10	3.89	13.34
Aspartic acid	8.46	5.37	8.27	9.19	7.07
Glutamic acid	16.35	15.74	11.49	11.58	11.29
Glycine	4.65	4.49	3.61	4.85	3.87
Histidine*	1.08	1.79	2.46	2.17	4.67
Isoleucine*	3.92	4.62	2.27	3.89	5.48
Leucine*	9.46	10.06	7.49	9.82	7.07
Lysine*	3.78	5.18	4.08	4.01	8.78
Methionine*	4.07	4.16	3.00	3.67	3.53
Phenylalanine*	13.19	11.76	23.00	18.18	4.67
Proline	ND	ND	ND	ND	3.19
Serine	8.27	7.69	8.12	7.32	7.76
Threonine*	6.60	6.96	6.61	6.70	3.42
Tyrosine	ND	ND	0.69	0.32	2.05
Valine*	9.43	10.08	9.57	8.91	6.15

* Essential AA

Some amino acids were not detected or were present at very low levels, possibly below the detection limit of the analytical method. Therefore, these amino acids were reported as ND rather than considered absent. From a nutritional

perspective, particular attention was given to the essential amino acids (*), as these cannot be synthesized by humans and must be obtained through the diet. Overall, the results indicate that, for most essential amino acids (except histidine and isoleucine) the relative abundances are comparable to or even exceed those reported for ovalbumin. For a clearer visualization of the results, **Figure C4** shows a bar plot of all analysed amino acids, grouped into essential (A) and non-essential (B) categories, facilitating the identification of potential outliers.

During gastrointestinal digestion, seaweed proteins are hydrolysed into peptides, where the amino acid composition of the original protein extract influences peptide sequence, hydrophobicity, charge, and potential bioactivity. Especially hydrophobic and aromatic residues are of interest, as higher proportions may indicate greater potential for bioactive peptide formation. Comparison across the four species (**Table 12**) showed clear differences, with *Ulva intestinalis* exhibiting the highest proportion of aromatic amino acids (23.69% of total AA), mainly driven by phenylalanine, while *Ulva linza* showed the highest proportion of total hydrophobic amino acids (35.79% of total AA).

Table 12. Total hydrophobic (Ala, Val, Leu, Ile, Met) and aromatic (Tyr, Phe) amino acids across four species (% total AA).

Type	<i>U. fasciata</i>	<i>U. linza</i>	<i>U. intestinalis</i>	<i>U. australis</i>
Aromatic	13.19	11.76	23.69	18.50
Hydrophobic	33.02	35.79	26.58	31.77

4.3. SDS-PAGE analysis

To assess the protein composition and the efficacy of the purification process, SDS-PAGE analysis was performed on extracts from all four *Ulva* species. To allow for a semi-quantitative visual comparison, all samples were normalized to a protein concentration of 2 mg mL⁻¹ prior to loading (**Fig. 30**).

SDS-PAGE before digestion

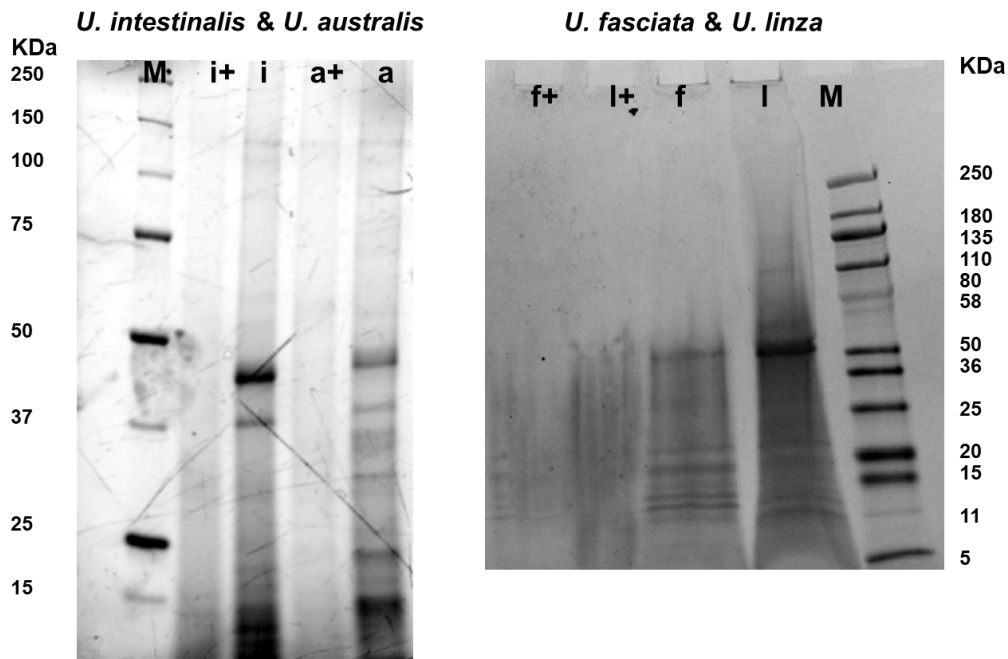


Fig. 30. SDS-PAGE profiles of protein extracts from South Korean and European *Ulva* species. Samples were standardized to 2 mg mL⁻¹ prior to loading. The left panel shows European species *U. intestinalis* (*i*) and *U. australis* (*a*), while the right panel shows South Korean species *U. fasciata* (*f*) and *U. linza* (*l*). *M* denotes the molecular weight marker (kDa). Lanes marked with '+' contain crude extracts, exhibiting characteristic smearing due to matrix impurities; lanes *i*, *a*, *f*, and *l* contain purified extracts with distinct protein bands.

The analysis reveals a significant improvement in protein resolution following the purification step. In the crude extracts (indicated by '+'), a distinct protein 'smearing' is observed across all molecular weights. This phenomenon is likely attributed to the presence of co-extracted interfering compounds, such as sulfated polysaccharides (ulvans) and polyphenols, which are known to hinder electrophoretic separation in macroalgae. In contrast, the purified fractions (lanes without '+') show well-resolved protein profiles. A dominant band at ~50 kDa is consistently observed across all *Ulva* species, suggesting a conserved protein fraction within the genus. This band is likely the RuBisCo large subunit, an enzyme accounting for up to 37% of the total soluble protein fraction in seaweeds (cf. Literature review: Section 4.3, Fig. 6). Under denaturing conditions, the intact ~550 kDa holoenzyme dissociates into eight large (51–58 kDa) and eight small (12–18 kDa) subunits, making them individually visible on the gel (Grácio et al., 2023). While the SDS-PAGE serves primarily as a visual confirmation of protein presence and purity, these results confirm that the extracts are of sufficient quality for downstream analysis.

5. In vitro gastrointestinal digestion of protein extracts

5.1. SDS-PAGE analysis

To monitor the *in vitro* simulated digestion process, SDS-PAGE analysis was used to visualize protein degradation over time. Figure 31 provides an overview for all four species, with the two upper panels representing the South Korean species and the two lower panels representing the European species.

SDS-PAGE provides a temporal overview of how the *Ulva* protein matrix responds to gastrointestinal conditions. A critical observation across all species is the high stability of the protein fractions during the initial stages of digestion. At S0 and S2, the persistence of high-molecular-weight smears indicates that the proteins remain largely intact or are only partially unfolded despite exposure to gastric conditions (pepsin; pH 3; 37°C). The most significant differences occur between the start of the intestinal phase (S2) and the end of the simulation (S4). By the final stage, the initial complex smears have effectively transitioned into a series of distinct, lower molecular weight bands. This shift confirms that the intestinal enzymes (pancreatin) are highly effective at hydrolysing the *Ulva* proteins into smaller peptide fragments. The appearance of these well-defined bands suggests that while the proteins are successfully broken down, the digestion leads to specific peptide intermediates rather than a complete degradation into free amino acids, which would be undetectable via SDS-PAGE.

A critical factor in interpreting these profiles is the visual presence of the digestive enzymes themselves. As observed in the S4 samples and the corresponding blanks, the protein bands are not exclusively derived from the *Ulva* extracts. Porcine pepsin is typically visible around 35 kDa, while components of pancreatin (primarily trypsin and chymotrypsin) appear in the 23–28 kDa range (Fig. 9). Enzymatic artefacts are particularly evident in the European samples, where they are clearly present in both species. Indeed, the importance of using blank samples is clearly demonstrated in the analysis of *Ulva linza* (Fig. C5). The comparison between the S4 sample and the B4 blank (enzymes without extract) reveals nearly identical banding patterns, indicating that enzyme autolysis and the inherent protein signal of the added enzymes contribute significantly to the total profile. Consequently, these enzymatic contributions must be accounted for when calculating the Degree of Hydrolysis (%DH) to avoid overestimating the protein breakdown of the seaweed itself.

Further evidence of enzymatic efficiency is provided by the control samples for *Ulva fasciata* (Fig. C5). While the S4 sample shows clear degradation, the C4 control (extract subjected to digestion conditions without enzymes) retains the characteristic high-molecular-weight smear similar to the crude protein extract (UPI). Although minor degradation is reported in the C4 samples, likely due to fluctuating environmental conditions such as pH shifts and temperature, the strong contrast between C4 and S4 proves that the observed proteolysis is predominantly enzyme driven.

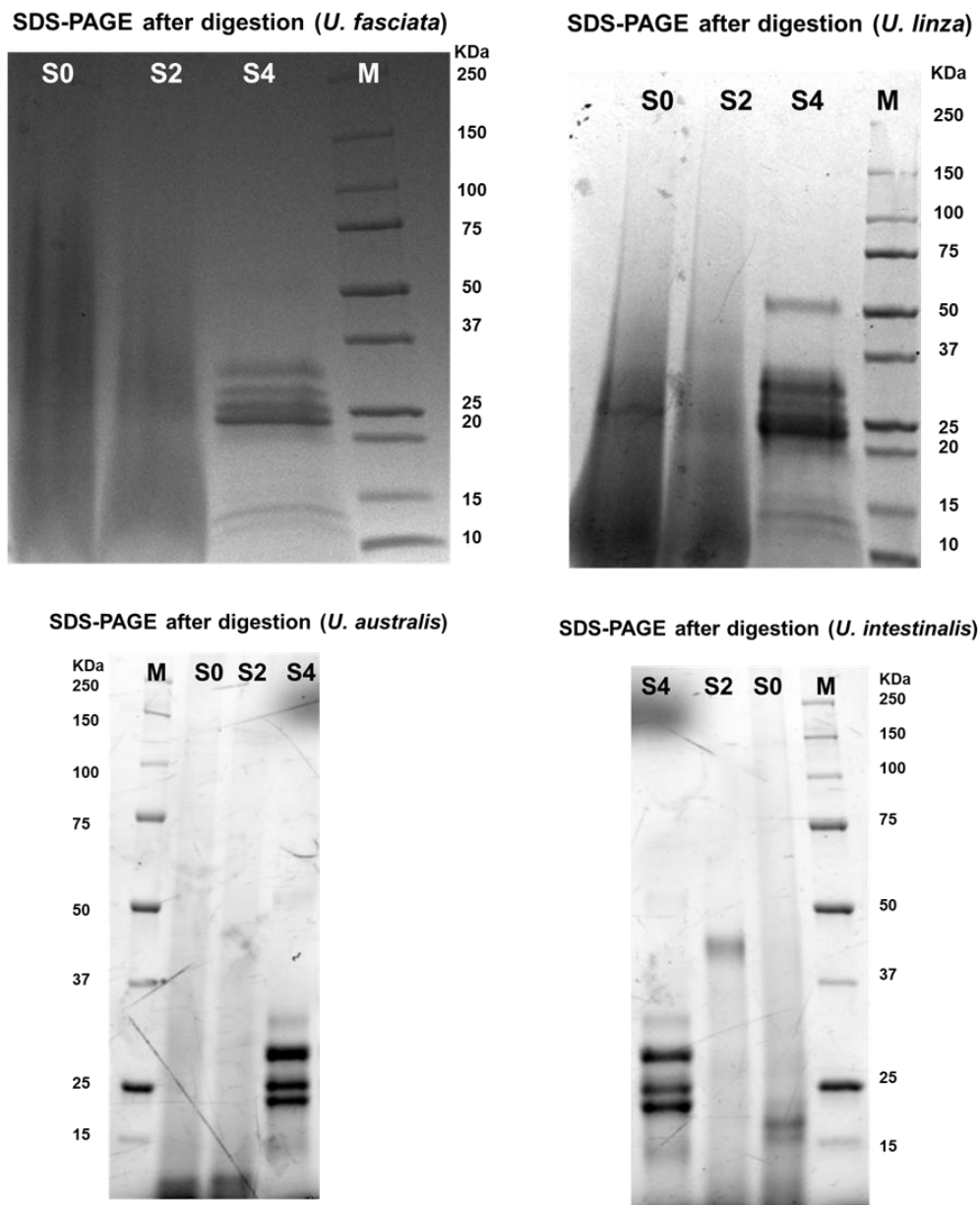


Fig. 31. Evaluation of the *in vitro* simulated digestion process over time for four *Ulva* species. SDS-PAGE analysis of undiluted samples at different digestion stages: **S0** (baseline, 0 h), **S2** (start of intestinal phase, 2 h), and **S4** (end of simulation, 4 h). **M** denotes the molecular weight markers: Precision Plus Protein™ Unstained Standard (15–250 kDa) for European species and EcoBand Protein Marker (10–250 kDa) for South Korean species. The progressive disappearance of high-molecular-weight smears and the appearance of distinct low-molecular-weight peptide bands at S4 illustrate the enzymatic breakdown of the protein matrix. The clear transition in banding patterns confirms successful proteolysis during the simulated gastrointestinal transit.

5.2. Degree of hydrolysis

Next to visual assessment, the *in vitro* digestibility of different protein extracts was also assessed quantitatively (**Fig. 32A**) by determining the degree of hydrolysis (%DH), as described in the methodology (**Formula 5**). Total hydrolysis of the samples was used as a reference, representing complete protein breakdown. **Figure C6** provides a graphical overview of the hydrolysis profiles for samples obtained in Europe and South Korea separately.

Control samples collected throughout the digestion simulation showed no noticeable increase in hydrolysis, as expected in the absence of enzymatic activity. At the starting point (S0, 0 h), a baseline level of hydrolysis was observed for all

four species, likely due to the acidic conditions of the gastric phase (pH 3, 37°C). During the gastric phase (0–2 h), no substantial increase in the %DH was detected across all species. This observation is consistent with the SDS-PAGE analysis (cf. **Results: section 5.1**), where no significant differences in molecular weight distribution were observed between the S0 and S2 lanes.

A marked increase in proteolysis was only observed upon initiation of the intestinal phase (pancreatin; pH 7; 37°C), continuing until the final time point at 4 h. Final hydrolysis values (**Fig. 32B**) indicate that *Ulva linza* ($83.61\% \pm 4.58$) and *Ulva australis* ($75.63\% \pm 8.77$) exhibited the highest %DH, showing no significant difference. They were followed by *Ulva intestinalis* ($64.26\% \pm 3.03$), while *Ulva fasciata* showed significantly lower digestibility ($38.62\% \pm 10.09$).

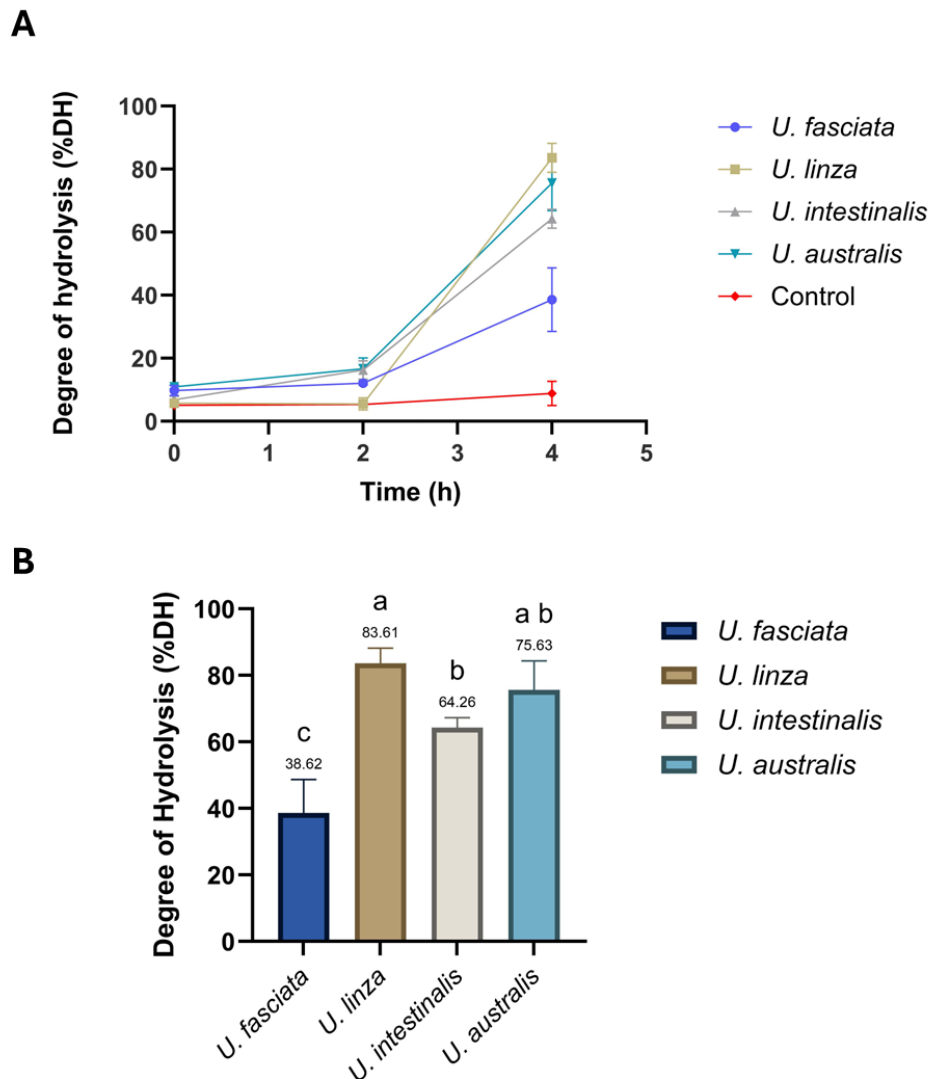


Fig. 32. Quantitative analysis of in vitro protein digestibility of four *Ulva* species. (A) Degree of hydrolysis (%DH) over time during simulated gastrointestinal digestion (gastric phase: 0–2 h; intestinal phase: 2–4 h). (B) Final %DH values at 4 h. Data are presented in triplicate. Different letters indicate significant differences between species (ANOVA, $p < 0.05$).

Correlation analyses were performed to assess relationships between chemical composition and %DH using Spearman's rank correlation ($n = 4$). Spearman's method evaluates monotonic relationships based on ranked data and does not assume normality, making it appropriate for small datasets. No significant correlations were observed at $\alpha = 0.05$. Carbohydrate content (**Fig. C7A**) showed a negative but non-significant association with %DH ($\rho = -1.000$, $p = 0.0833$), while phenolic content (**Fig. C7B**) showed no clear relationship ($\rho = -0.400$, $p = 0.7500$). **Figure C7C** presents both variables plotted against %DH for comparison.

6. Molecular weight distribution of peptides

Because small bioactive peptides generated during gastrointestinal digestion are largely responsible for the bioactivity and health-promoting effects of proteins after digestion, the peptide profiles of the four digested products were compared following the ultrafiltration process. The relative distribution (%) for each species is shown in **Figure 33**, while interspecies comparisons for each fraction are provided in **Figure C8**.

A clear trend is observed across all species (**Fig. 33, Fig. C8**), where the largest (> 10 kDa) and the smallest (< 3 kDa) fractions are most prevalent. The European species, *U. australis* and *U. intestinalis*, exhibit significantly higher percentages in the > 10 kDa fraction, indicating a larger portion of partially digested proteins or long-chain peptides remaining after 4 hours. Conversely, the < 3 kDa fraction, which represents highly digested small peptides and potential free amino acids, is most prominent in the Asian species, with *U. linza* showing the highest relative value (33.62%).

The intermediate fractions (10-5 kDa and 5-3 kDa) consistently show the lowest values across all species (**Fig. 33, Fig. C8**). This "U-shaped" distribution suggests a rapid transition from intermediate peptides to smaller fragments during the intestinal phase. It should be noted that during ultrafiltration, a total recovery of 100% is rarely achieved due to peptide adherence to the membranes; however, the relative differences remain statistically significant.

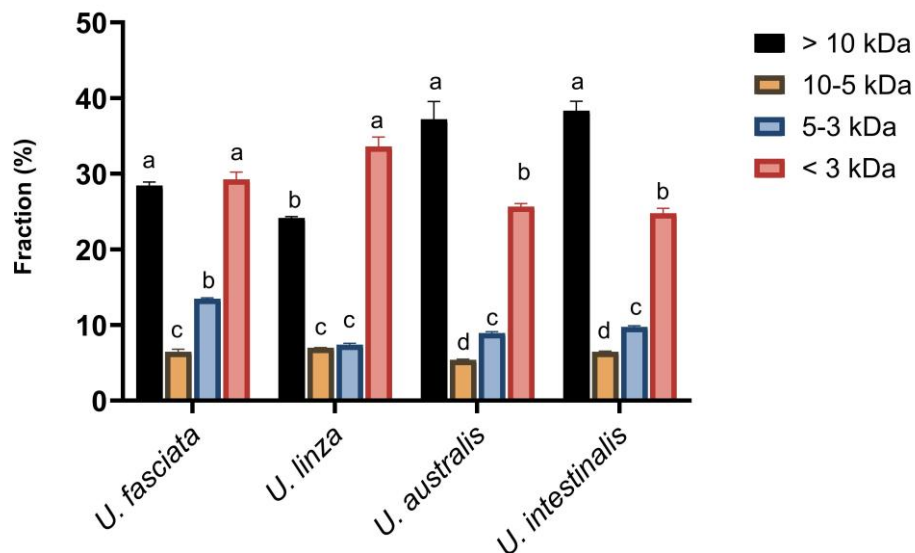


Fig. 33. Molecular weight distribution of peptides following *in vitro* digestion. Relative percentages grouped per species for each size fraction (> 10 kDa, 10–5 kDa, 5–3 kDa, and < 3 kDa) are shown. Data are presented as the mean relative fraction (%) $\pm$ standard deviation ($n=3$). Different lowercase letters (a, b, c, d) denote statistically significant differences between size categories within a specific species (ANOVA, $p < 0.05$). High prevalence of the < 3 kDa fraction in *U. linza* correlates with its high Degree of Hydrolysis.

7. Confocal Laser Scanning Microscopy (CLSM)-analysis

CLSM imaging (**Fig. 34**) revealed clear differences in the spatial organization and co-localization of proteins (red) and polysaccharides (blue) among the four *Ulva* species, both before and after *in vitro* gastrointestinal digestion.

In the undigested protein isolates (**Fig. 34, A-D**), proteins dominate the structural matrix, appearing as dense red aggregates with variable morphology. Polysaccharides are present but less prominent, confirming a protein-rich extract prior to digestion. Following digestion (**Fig. 34, E-H**), a pronounced structural transformation is observed across all samples. The red protein signal is visibly reduced, indicating extensive proteolysis. In parallel, the blue polysaccharide signal becomes more prominent, revealing specific but variable structural architectures. This is expected, as the analysed material represents the non-digestible pellet remaining after centrifugation at the end of digestion (S4; after

4 h). However, in *Ulva fasciata* (Fig. 34, B-F), a relatively strong residual protein signal is still observed after digestion. This suggests that its proteins are less accessible to digestive enzymes, potentially due to a more compact structural organization or physical entrapment within the rigid polysaccharide network.

To objectify and statistically validate these visual observations, image analysis was performed using ImageJ to quantify the fluorescence intensity ratios of red (proteins) to blue (carbohydrates) (Fig. 34, right panel). Prior to digestion, all species exhibited similar initial ratios (> 1), indicating a predominance of intact proteins relative to carbohydrates with no significant differences between the samples. Post-digestion, a clear decrease in protein-associated fluorescence was observed for all species, consistent with the CLSM images. While *U. australis*, *U. intestinalis*, and *U. linza* showed extensive hydrolysis with ratios dropping significantly to between 0.22 and 0.49, *U. fasciata* exhibited only a limited decrease. It retained a significantly higher post-digestion ratio of 0.74. Concretely, this indicates that while digestion did occur, a substantially higher proportion of the protein signal is retained relative to the carbohydrate matrix compared to the other species. This confirms the visual persistence of the protein signal and suggests that the structural matrix of *U. fasciata* may provide a higher resistance to enzymatic digestion.

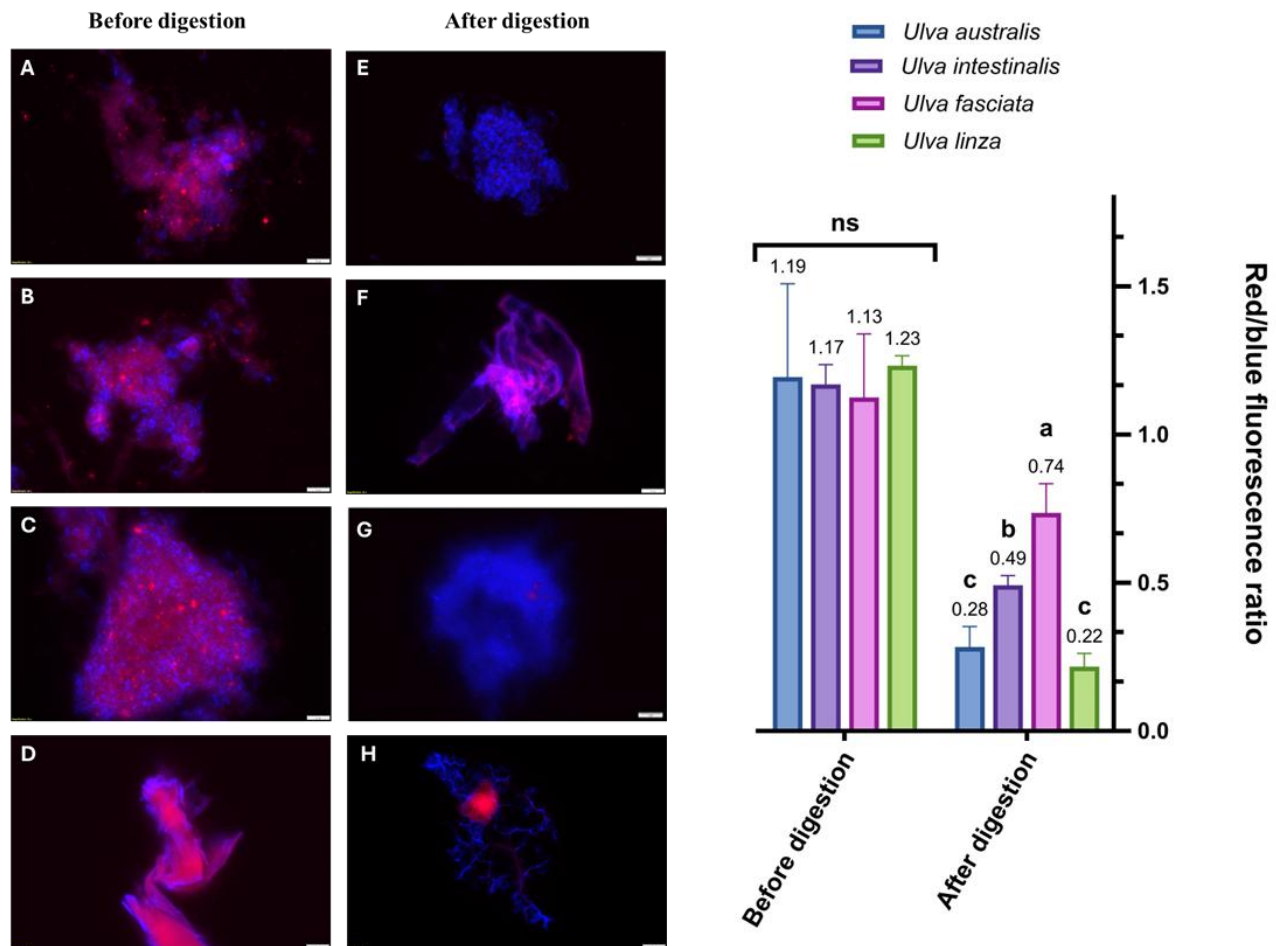


Fig. 34. CLSM visualization and fluorescence intensity analysis of protein-polysaccharide complexes in *Ulva* protein isolates before and after in vitro gastrointestinal digestion. Left panel: CLSM images of protein-polysaccharide complexes from *U. linza* (A-E), *U. fasciata* (B-F), *U. australis* (C-G), and *U. intestinalis* (D-H). Images show protein isolates prior to in vitro gastrointestinal digestion and the undigested fractions remaining after digestion. Samples were stained with Fast Green FCF (red) for proteins and Calcofluor White staining (CWS; blue) for polysaccharides. Scale bars correspond to 10 μ m. Right panel: Red/blue fluorescence intensity ratios from ImageJ analysis of CLSM images before and after in vitro digestion. Red fluorescence represents proteins and blue fluorescence represents carbohydrates. Data are presented as the mean ratio $\pm$ SD ($n=3$) calculated for different images. Different lowercase letters (a, b, c) denote statistically significant differences between species at each time point (ANOVA, $p < 0.05$).

DISCUSSION

1. Biochemical composition of *Ulva* biomass

Four different *Ulva* species originating from two distinct sampling locations, collected at different time points, were included in this study. Consequently, substantial environmental variation between sampling sites should be considered when interpreting the obtained results. In South Korea, samples were collected from shallow rocky shore environments where the seaweeds remained continuously submerged. In contrast, the European samples collected in Ambleteuse originated from rocky intertidal shores strongly influenced by tidal dynamics, resulting in the formation of tidal pools. Importantly, environmental factors (Table 1 & 3) such as light intensity, temperature, salinity, nutrient availability, and hydration status are known to strongly influence seaweed morphology and biochemical composition.

As shown in Table 2, green seaweeds generally contain average ash contents between 17–22% DW and protein contents ranging from 5–20% DW. However, an important consideration when comparing protein contents across studies is the nitrogen-to-protein conversion factor used in Kjeldahl-based protein estimations. Many studies apply the conventional conversion factor (CF) of 6.25, which may overestimate seaweed protein levels. The use of this factor is based on two assumptions: (1) proteins contain 16% nitrogen, and (2) protein represents the sole nitrogen source present in the sample, with negligible amounts of non-protein nitrogen (Biancarosa et al., 2017). In seaweeds, however, substantial quantities of non-protein nitrogen compounds such as chlorophylls, nucleic acids, free amino acids, and inorganic nitrogen are present, potentially leading to inflated protein estimations when using the standard factor. Therefore, based on the findings of Biancarosa et al. (2017), a conversion factor of 4.24 was applied in the present study for green algae. Nonetheless, differences in CF across studies (Table 13) complicate comparison, highlighting the need for standardisation.

Table 13 compares the ash and total protein contents (% DW) obtained in this study with values previously reported in literature. Considerable variation between studies is evident, which is expected given that seaweed chemical composition varies depending on species identity, morphology, geographic location, environmental conditions, weather, and seasonality (Jansen et al., 2022; El-Beltagi et al., 2022; Khairy et al., 2013).

Table 13. Ash and protein content of seaweeds in the present study compared to literature. CF= conversion factor.

Seaweed	Origin	Total ash (%)	Total protein (%)	CF	References
<i>U. fasciata</i>	South Korea	8.23 ± 0.19	15.88 ± 0.15	4.24	Present study
<i>U. linza</i>	South Korea	12.75 ± 0.37	18.21 ± 0.09	4.24	Present study
<i>U. intestinalis</i>	Belgium / Europe	46.39 ± 0.50	12.23 ± 0.01	4.24	Present study
<i>U. australis</i>	Belgium / Europe	19.05 ± 0.05	19.70 ± 0.15	4.24	Present study
Reported species					
Green seaweeds (average)	-	20-30	4-25	-	Farghali et al., 2023
Green seaweeds (average)	Egypt	3-22	27-43	-	Ismail et al., 2025
<i>U. fenestrata</i>	Sweden	-	16.5	5	Trigo et al., 2021
<i>U. fasciata</i>	India	2-12.5	24-27	-	Soufi et al., 2024
<i>U. intestinalis</i>	Morocco	61.2	10.6	6.25	El Mellouki et al., 2025
<i>U. linza</i>	Bangladesh	21	22	-	Aziz et al., 2021
<i>U. spp.</i>	-	-	8.7	5.12	Kazir et al., 2019
<i>U. spp.</i>	Spain	25	22	5.13	Vega-G. et al., 2024
<i>Caulerpa lentilifera</i>	Indonesia	63.83	12.56	6.25	Kasmiati et al., 2022

One notable outlier in the present study was *Ulva intestinalis*, which showed a substantially higher ash content (46.39% ± 0.50 DW) compared to the other species. As shown in Figure 12C, this species exhibits a tubular thallus morphology, which may facilitate the retention of sediment particles such as sand and other mineral-rich debris, potentially contributing to the elevated ash fraction. This was supported by visual observations of the dried biomass and extraction

pellets, where residual inorganic material and a distinct white salt layer were evident. In line with this, a higher salinity level was measured in the protein extracts of *U. intestinalis* (Fig. 26), which showed a significantly higher value (7.00 PSU).

However, the elevated ash content is unlikely to be solely explained by external contamination, as high mineral levels in *U. intestinalis* have also been reported in the literature. El Mellouki et al. (2025) reported ash contents of 61.2% DW, while Kasmiati et al. (2022) observed similarly high values in *Caulerpa lentilifera* (63.83% DW), attributing these levels to the naturally high mineral and sodium content of the biomass. In contrast, lower ash contents have also been reported for *U. intestinalis*, such as 19.95% DW in samples from the Gulf of Thailand (Peasura et al., 2015), indicating strong environmental and compositional variability. Taken together, the high ash content in *Ulva intestinalis* likely reflects morphology-driven sediment retention, environmental exposure, and its naturally high mineral content, with retained salts also contributing.

Next to ash and protein content, the moisture content (Table C1) of *U. intestinalis* ($77.85\% \pm 1.76$) and *U. australis* ($74.00\% \pm 3.33$) was measured and found to be consistent with previously reported values in the literature (65–80%) (Ismail et al., 2025).

2. Characterisation of *Ulva* protein extracts

2.1. Extraction efficiency

Clear differences in extraction efficiency between species were observed (Fig. 25). As extraction efficiency directly affects the composition of the resulting protein extract, this parameter was thoroughly investigated. The higher extraction yield ($67.83\% \pm 2.83$) observed for *Ulva australis* in the Belgian experiments may be attributed to species-specific differences in cell wall architecture and protein–polysaccharide interaction as well as variations in sample preparation, particularly particle size. Although all samples were passed through a 250 μm (0.25 mm) sieve (mesh no. 60), the absence of a defined lower particle size limit, combined with the use of a more efficient grinding system (IKA® A11 basic analytical mill) in Belgium, resulted in a finer powder fraction. The reduced particle size increased surface area and improved solvent accessibility, thereby enhancing extraction efficiency. Notably, although *Ulva intestinalis* was ground into the same fine powder, one would expect similarly elevated values as observed for *Ulva australis*. However, compared to the Korean species, it still showed the lowest extraction efficiency ($26.40\% \pm 0.93$; Fig. 25). This was also observed qualitatively during extraction, where phase separation, layering and sedimentation occurred in contrast to the other three species. As previously stated, *U. intestinalis* exhibits a more compact and thicker morphology, which may correspond to a more rigid or thicker cell wall structure, thereby limiting protein release. Additionally, a higher content of sediments or minerals such as salts, as reflected in the ash content, may further hinder extraction efficiency.

The substantially higher extractability of *U. australis* is clearly reflected in the distribution of the separate protein soluble fractions. As shown in Figure 24, the increased protein yield is mainly driven by the water-soluble fraction, whereas the alkaline soluble fraction contributes considerably less. This indicates that the difference in extractability is primarily due to a higher proportion of readily soluble proteins rather than an increased recovery of structurally bound proteins.

In this thesis, a combination of physical and chemical extraction methods was applied to maximise protein yield. Freeze-thawing and ultrasonication were used to enhance cell disruption (Harrysson et al., 2018; Trigo, 2023), followed by aqueous extraction and alkaline re-extraction of the remaining pellet (Angell et al., 2017), with final protein recovery through ammonium salt precipitation (Harrysson et al., 2018). Over the years, studies have increasingly combined multiple extraction methods to optimise protein extraction efficiency. However, comparatively less attention has been given to the optimisation of initial sample preparation steps, such as particle size reduction. Kazir et al. (2019) describe

manual grinding using a mortar and pestle, while Trigo et al. (2021) used a meat grinder with a 4.5 mm hole plate. Similarly, Pootthachaya et al. (2023) milled biomass to pass through a 1.0 mm screen, resulting in relatively coarse particles. In contrast, El Deeb et al. (2025) demonstrated that reducing seaweed particle size from above 355 μm to 53–25 μm significantly enhances extraction efficiency and increases the yield of bioactive compounds (Fig. D6), highlighting the critical importance of particle size in extraction processes. As mentioned above, particle size presumably also contributes to the differences in extraction efficiency observed between South Korea and Europe.

Literature (Table 4) shows that protein extraction yields are strongly method-dependent, suggesting that combining approaches may improve overall yield. The present study however, applies a single extraction protocol and still observes differences in protein extraction yield between species, indicating a potential species-specific contribution to protein extractability. To finalise this discussion, building on the findings of Harrysson et al. (2018), Vega-Gómez et al. (2024) reported protein extraction yields of $59.06\% \pm 0.02$ for *Ulva spp.* using a five-minute ultrasound (US) pretreatment followed by alkaline solubilization at pH 12. This approach did not include a grinding step and instead used blender-homogenized frozen or freeze-dried biomass resuspended in Milli-Q water, followed by additional homogenization prior to pH shift. Ultrasonication was performed using a probe system at 550 W and 50% amplitude. Increasing the ultrasound pretreatment time to 30 minutes did not significantly improve protein yield compared to 5 minutes (Table D1), while no significant changes in protein concentration were reported. Notably, this yield ($59.06\% \pm 0.02$) is already relatively close to the 67.83% obtained for *Ulva australis* in the present study.

2.2. Ammonium sulfate protein recovery

Ammonium sulphate (AS) precipitation was used as a purification step to selectively precipitate proteins, followed by dialysis to remove excess salts. Recovery rates varied strongly among species. To ensure consistency, a fixed AS saturation of 30% was applied to all samples, although differences in extraction efficiency and protein content likely require species-specific optimal AS concentrations. Consequently, applying a uniform AS concentration may have reduced recovery efficiency for certain species, as illustrated by the contrast between *Ulva fasciata* ($91.34\% \pm 2.61$) and *Ulva australis* ($23.22\% \pm 0.09$). Literature further highlights the importance of AS optimization, with reported optimal saturations ranging from 30% to 85% depending on the species and extraction method. For example, Harrysson et al. (2018) achieved protein yields of $51.2\% \pm 2.1$ in *Ulva lactuca* using pH-shift extraction combined with 80% AS saturation, whereas Chew et al. (2019) identified 30% AS as optimal within their experimental setup. Similarly, Arbita et al. (2024) reported 50% AS saturation as optimal following dialysis-based salt removal. Indeed, individual protein fraction analysis (Fig. 24) reveals that *Ulva australis* contains a markedly higher water-soluble protein fraction. This abundance of hydrophilic proteins subsequently complicates salting out, potentially leading to a lower recovery yield. Nonetheless, SDS-PAGE analysis (Fig. 30) revealed clear removal of contaminants after purification compared to the crude extracts for all species, resulting in more distinct and well-defined protein banding patterns.

2.3. Chemical composition of protein extract

Comparing the chemical composition of protein extracts is essential when evaluating protein digestibility, as digestibility can be negatively affected by non-protein components such as polysaccharides and phenolics (Fleurence et al., 1999; Tibbetts et al., 2016). Therefore, extraction methods should not only concentrate proteins but also reduce interfering compounds that may impair digestion (Trigo et al., 2021). The chemical composition of seaweed protein extracts strongly depends on the extraction protocol used (Table 14). Kazir et al. (2019), for example, evaluated five different extraction methods and reported substantial variation in protein content (20–62%), “phenol + others” content (20–60%), and carbohydrate content (5–37%). Importantly, the compositional values in Table 14 were not normalized to 100%, as also illustrated in Figure 29, indicating the presence of a residual “others” fraction likely consisting of minerals and phytochemicals such as pigments (Trigo et al., 2021).

Table 14. Comparison of the chemical composition of protein extracts from the four investigated *Ulva* species with literature. DM= dry matter. Phenol values from Kazir et al. (2019) represent the combined "phenol + others" fraction.

Seaweed	Origin	Carbohydrate (% DM)	Phenol (% DM)	Protein (% DM)	References
<i>U. fasciata</i>	South Korea	13.40 ± 2.3	3.75 ± 0.25	75.78 ± 3.45	Present study
<i>U. linza</i>	South Korea	3.18 ± 0.86	3.06 ± 0.18	77.83 ± 0.68	Present study
<i>U. intestinalis</i>	Belgium / Europe	10.68 ± 0.99	2.50 ± 0.28	78.76 ± 0.70	Present study
<i>U. australis</i>	Belgium / Europe	8.18 ± 0.689	2.21 ± 0.02	78.41 ± 0.31	Present study
Reported species					
<i>U. lactuca</i> (pH-shift)	-	-	-	51.2 ± 2.1	Harrysson et al., 2018
<i>U. spp.</i> (different methods)	-	5 - 37	20 - 60	20 - 62	Kazir et al., 2019
<i>U. fenestrata</i> (pH-shift)	-	-	1.31 ± 0.16	58.4 ± 5.7	Trigo et al., 2021
<i>U. fenestrata</i> (pH-shift)	-	-	-	62.3 ± 2.5	Juul et al., 2021
<i>U. lacinulata</i> (pH-shift)	-	29.00 ± 2.70	-	32.45 ± 1.22	Molina-G. et al., 2025

Overall, the obtained compositions were consistent with previous studies. Despite differences in extraction efficiency and protein recovery between species, no significant differences in final protein content were observed. This is likely due to compensatory effects between extraction efficiency and recovery. Notably, *Ulva fasciata* showed the highest carbohydrate and phenolic content. This is particularly relevant, as these elevated levels were subsequently associated with reduced protein digestibility in the following results, further supporting the inhibitory role of non-protein components on seaweed protein digestion.

2.4. Amino acid profiles: Interpretation and limitations

When comparing amino acid (AA) profiles with ovalbumin (Table 11), it is important to note that this represents a relative comparison based on the percentage contribution of each amino acid to the total AA-pool, rather than absolute concentrations. As such, these data do not reflect total protein abundance, since absolute AA levels in ovalbumin are considerably higher than in seaweed extracts. Nevertheless, this relative approach is useful for highlighting compositional differences and identifying proportionally enriched amino acids relevant to the nutritional evaluation of *Ulva*-derived proteins.

A similar comparative framework has been applied in previous studies, including Kazir et al. (2019) and Trigo et al. (2021), which also reported AA-profiles as relative distributions (Table D2). However, methodological constraints should be acknowledged. Due to the use of acid hydrolysis during analysis, amino acids such as tryptophan and cysteine cannot be recovered and are therefore not included in the profiles (Trigo et al., 2021). From a nutritional perspective, the proportion of essential amino acids (TEAA or EAA%) is a key indicator of protein quality (WHO/FAO/UNU, 2007). Recent work by Vega-Gómez et al. (2024) reported TEAA contents in seaweed protein extracts ranging from 37% to 41%, values that are comparable to conventional plant protein sources such as soy (27%), pea (30%), and corn (32%) (Gorissen et al., 2018). This supports the potential of *Ulva spp.* proteins for food-related applications, despite species- and method-dependent variability. Indeed, AA profiles reported in literature vary considerably depending on species and extraction methodology (Table D2). Kazir et al. (2019), for instance, reported a TEAA proportion of 25.03% in extracts derived from a mixture of *Ulva rigida* and *Ulva fasciata*, with arginine as the predominant amino acid, while Trigo et al. (2021) reported a TEAA value of 47.07%. The present study shows relatively higher and consistent TEAA proportions across all four species (51–58%), suggesting improved extraction efficiency and methodological consistency and confirming potential for food (and feed) related applications.

Notably, elevated phenylalanine levels (11.76–23.00%) were observed across all species, whereas proline was not detected. This is most likely a methodological artefact rather than a true absence. Proline, an imino acid, is typically detected at 440 nm in ninhydrin-based post-column derivatization methods, whereas α-amino acids are mainly quantified at 570 nm. Although both wavelengths were monitored during analysis, proline was likely underestimated or not detected, possibly due to its low concentration in the samples (Seracu, 1987). Similarly, the detection of tyrosine

in only two of the four species is unlikely to reflect true biological variation and is more plausibly explained by concentrations falling below the detection limit in *U. fasciata* and *U. linza*.

The amino acid composition of the protein extracts may reflect the potential bioactivity of peptides released during gastrointestinal digestion. Prakash Nirmal et al. (2023) report that hydrophobic and aromatic amino acids are frequently enriched in antioxidant, antihypertensive, and antidiabetic peptides. QSAR (Quantitative Structure–Activity Relationship) studies further underline the importance of aromatic residues (Trp, Tyr, Phe) for angiotensin-converting enzyme (ACE) inhibitory activity (Manzanares et al., 2019). In addition, Sánchez and Vázquez (2017) describe that ACE-inhibitory peptides often contain aromatic or basic residues at the N-terminus and hydrophobic or positively charged residues at the C-terminus, while hydrophobic residues at the N-terminus have been associated with dipeptidyl peptidase-IV (DPP-IV) inhibitory activity (Prakash Nirmal et al., 2023). Accordingly, a higher proportion of hydrophobic and aromatic amino acids in the present samples (Table 12) may indicate increased potential for bioactive peptide formation after digestion. However, as emphasized by Sánchez and Vázquez (2017), bioactivity is not determined by composition alone, but also depends on peptide sequence, length, and structural conformation.

3. Assessment of protein digestibility: *In vitro* gastrointestinal digestion

To standardise methodological differences, the INFOGEST 2.0 protocol was developed as a harmonised framework for *in vitro* digestion models (Brodkorb et al., 2019). However, these models remain simplified and cannot fully replicate the dynamic physiological conditions of human digestion. Gastric pH is kept constant, gastric secretions and emptying are not gradual, and enzyme activity is fixed regardless of substrate composition. In addition, the intestinal phase is represented as a single step rather than the sequential and physiologically distinct duodenal, jejunal, and ileal phases. These simplifications limit detailed kinetic interpretation of digestion processes (Brodkorb et al., 2019).

3.1. Degree of hydrolysis validation (%DH)

As shown in Figure 32A, several notable trends can be observed regarding the degree of hydrolysis (%DH) during *in vitro* digestion. First, all species already exhibited measurable %DH values at S0, prior to the actual progression of digestion. Since the OPA assay quantifies free amino groups, this suggests that peptide bond cleavage may already have occurred during protein extraction and/or sample preparation. A similar phenomenon was reported by Trigo et al. (2021) for *Ulva fenestrata*, where approximately $2.7\% \pm 0.3$ DH was already present in pH-shift protein extracts prior to digestion. However, an important distinction should be made when comparing these values to the present study. In the study by Trigo et al. (2021), %DH was measured before exposure to digestion conditions, whereas in the present study measurements at S0 were performed after samples had already been subjected to harsh gastric phase conditions (pH 3, SGF, 37°C). This likely explains the higher initial %DH values observed here.

Figure C6 further separates the Korean and European samples and includes their respective control groups, allowing a clearer comparison between controls and digested samples for each species individually. Across all species, a pronounced increase in %DH was observed over the 4 h digestion period compared to the controls, which remained relatively stable throughout the experiment. Nevertheless, a slight increase in control %DH values was still detectable, potentially due to the harsh experimental conditions themselves causing limited non-enzymatic or spontaneous hydrolysis. Another striking observation is that all species showed a strong increase in %DH starting from 2 h digestion time, corresponding to the onset of the intestinal phase. During the gastric phase, %DH remained relatively constant, with only minimal increases observed. This trend is consistent with previously reported observations by Kazir et al. (2019), who similarly reported for *Ulva* and *Gracilaria* species that major increases in %DH only occurred after 120 min (Fig. 10), while little to no hydrolysis was observed during the gastric phase. Their results likewise showed a nearly horizontal trend during gastric digestion followed by a marked increase upon transition to intestinal conditions. These

findings were not only evident numerically, but also visually in the SDS-PAGE analysis (**Fig. 31**). No significant differences in banding patterns were observed between the S0 and S2 samples, whereas the S4 samples displayed clearly defined protein bands. To further explore this trend, Fleurence (1999) compared the *in vitro* digestibility of proteins extracted from *Ulva pertusa* under strong alkaline conditions using either pepsin or pancreatin alone. Digestibility, expressed relative to casein, was substantially higher for pancreatin (66.6%) than for pepsin (17%). These findings suggest that intestinal enzymes may hydrolyse seaweed proteins more effectively than gastric enzymes alone.

Interestingly, recent work by Molina Gilarranz et al. (2025), building on Vega-Gómez et al. (2024), applied the INFOGEST 2.0 protocol to *Ulva* extracts obtained via pH-shift extraction combined with ultrasound (cf. the present methodology). Reported digestibility values ranged from 35% to 75%, with the highest value observed for extract E-US12.1 (ultrasonication with pH shift from 12 to 1). This upper range aligns with the highest hydrolysis levels in the present study.

3.2. Potential factors affecting digestibility

Linking these results to compositional data reveals a consistent pattern. *U. fasciata*, which exhibited the lowest protein digestibility ($38.62\% \pm 10.09$), also showed the least favourable biochemical profile in terms of carbohydrate and phenolic content. *U. intestinalis* displayed intermediate values for both composition and digestibility. Although this suggests an inhibitory role of non-protein components, individual contributions of carbohydrates and phenolics were not assessed. Nevertheless, Tibbetts et al. (2016) reported that phenolic compounds can reduce protein digestibility by forming hydrogen bonds with proteins and amino acids and by promoting protein oxidation, thereby decreasing their susceptibility to enzymatic hydrolysis. In their study, a highly significant inverse non-linear relationship was observed between total phenolic content (TPC) and *in vitro* protein digestibility (IVPD) (**Fig. D5**). To confirm these trends, correlations between final %DH and chemical composition (carbohydrates and phenolics) were assessed (**Fig. C7**). No significant relationships were found, although correlation with carbohydrates showed a strong negative trend ($p = -1$). In contrast to literature, no clear pattern was observed for phenolics. Given the limited sample size ($n = 4$), the Spearman correlation provides only an exploratory indication of potential associations, which should be interpreted cautiously.

Another possible factor influencing %DH is salinity, as indicated by the elevated salt levels observed when comparing *Ulva intestinalis* and *Ulva australis* (**Fig. 26**). Ionic strength is known to affect protein hydrolysis by influencing both protein structure and enzyme performance. Changes in ionic strength can modify protein solubility and aggregation behaviour, thereby altering the accessibility of enzymatic cleavage sites and overall hydrolysis efficiency (Butré et al., 2012). Therefore, ionic conditions of gastric and intestinal fluids are included as a key parameter in standardized *in vitro* digestion models (INFOGEST; Minekus et al., 2014).

Finally, CLSM imaging (**Fig. 34**), analogous to the approach of Molina-Gilarranz et al. (2025), was used to investigate the structural relationship between carbohydrates and proteins before and after digestion. The obtained images and accompanying quantitative image analysis further supported our findings, showing that *Ulva fasciata* exhibited the highest retention of intact protein post-digestion. Interactions between proteins and polysaccharides have previously been reported to exert a protective effect against proteolysis. For instance, seaweed-derived hydrocolloids like agar and carrageenan have been shown to significantly delay casein digestion (Fontes-Candia et al., 2022). Overall, these findings indicate that protein digestibility is influenced not only by carbohydrate content, but also by species-specific structural organization and the clustering of proteins and polysaccharides, which may limit enzyme accessibility and consequently reduce protein hydrolysis.

4. Formation of bioactive peptides

During simulated gastrointestinal digestion, proteins are progressively hydrolysed into peptides of varying molecular weight. To characterise this complexity, ultrafiltration is commonly used to fractionate digests into defined size ranges, enabling assessment of links between peptide size distribution and potential bioactivity (Sun et al., 2019). As shown in **Figure 33**, *U. linza* exhibited the clearest shift towards low-molecular-weight peptides after digestion, characterized by the highest relative abundance of the <3 kDa fraction together with a markedly reduced >10 kDa fraction, consistent with more extensive protein hydrolysis (**Fig. 32B**). In contrast, *U. fasciata* also showed relatively high levels of low-molecular-weight peptides, although no significant difference was observed between the >10 kDa and <3 kDa fractions. Similar patterns were observed across species within each fraction (**Fig. C8**). In this context, Camporesi et al. (2026) showed that INFOGEST digests filtered at 3 kDa best preserves epithelial integrity in Caco-2 models while still reflecting a physiologically relevant intestinal peptide pool. Their results support that gastrointestinal digestion strongly enriches peptides <3 kDa, many of which are associated with bioactivity, whereas >10 kDa peptides largely represent incompletely hydrolysed intermediates requiring further digestion before absorption.

However, static *in vitro* digestion models do not reflect the dynamic kinetics of proteolysis (Brodkorb et al., 2019). *In vivo* digestion is continuous and time-dependent, whereas static systems capture only endpoint distributions. This is particularly relevant for the intermediate 3–10 kDa fraction, which appears relatively depleted in **Figure C8**. This pattern likely reflects transient formation of intermediate peptides that are rapidly further hydrolysed into smaller fragments or retained in the >10 kDa fraction. In addition, ultrafiltration introduces a binning effect: the <3 kDa and >10 kDa fractions are open-ended, while the 3–10 kDa range is constrained, which can exaggerate apparent accumulation at the extremes and produce a U-shaped distribution driven by both enzymatic kinetics and methodological partitioning rather than a true physiological equilibrium.

Finally, it is important to note that the values shown in these graphs are relative and do not represent absolute peptide levels between species. Peptide distributions were normalised within each species to enable comparison, as total peptide recovery varied due to cumulative losses during sequential ultrafiltration. Overall recovery ranged from ~72% in *U. linza* to ~80% in *U. intestinalis*. This is consistent with reported per-step recoveries of >90% for Ultracel® membranes; assuming ≥ 0.9 recovery per filtration step, three sequential MWCO filtrations (10, 5 and 3 kDa) yield a compounded recovery of $\geq 0.9^3 \approx 0.73$, excluding additional losses from adsorption, fouling and handling.

5. Methodological considerations and limitations

This study was conducted across two different laboratories on different continents, each with their own equipment, workflows, and research focus. Although protocols were aligned as much as possible, minor methodological differences may have influenced certain outcomes. On the other hand, this multi-laboratory approach provided valuable insight in how processing conditions can affect extraction and digestion results. The comparison included four *Ulva* species originating from Europe and South Korea. Since the biomass was not cultivated under identical controlled conditions, environmental factors such as nutrient availability, salinity, light exposure, and seasonal variation may have contributed to differences in protein content, extract composition, and structural properties in addition to species-specific effects. As a result, the findings should be interpreted within the context of the selected samples and growth conditions.

Finally, the use of the standardized INFOGEST 2.0 protocol enhances comparability with existing literature by providing a controlled and reproducible approximation of gastrointestinal digestion. However, as a static *in vitro* model, it cannot fully capture the physiological complexity and dynamic nature of human *in vivo* digestion. Moreover, the focus of this study was placed on comparative digestion behaviour rather than detailed peptide kinetics, and therefore dynamic monitoring of peptide formation over time was not performed.

CONCLUSION

1. General outcomes

In this study, four different *Ulva* species originating from Europe and South Korea were compared in terms of protein digestibility, structural protein characteristics, and the composition of the resulting digested fractions. By integrating protein content, digestibility behaviour, peptide fraction distribution, and potential inhibitory components such as phenolics and carbohydrates, this thesis aimed to identify the most promising species within the context of functional food applications.

Overall, the results demonstrate clear interspecies variability, confirming that protein digestibility in *Ulva* is influenced by multiple interacting compositional and structural factors rather than protein content alone. Based on the integrated qualitative evaluation presented in **Table 15**, *Ulva australis* and *Ulva linza* showed the most favourable overall performance within the scope of this research. Both species combine high protein digestibility with a favourable compositional profile, highlighting their potential as promising candidates for future functional food and protein valorisation applications. In contrast, *Ulva fasciata* consistently showed the least favourable performance across most evaluated parameters, which was additionally supported by CLSM observations indicating a more resistant structural organisation after digestion.

Importantly, the findings also highlight that species ranking remains context-dependent and should not be interpreted as absolute. The qualitative scoring system used in **Table 15** provides an integrated overview of performance within the specific functional framework of this thesis, but the “best” species may vary depending on the intended application, desired bioactivity, extraction strategy, or nutritional target.

Table 15. Comparative qualitative evaluation of *Ulva australis*, *Ulva linza*, *Ulva fasciata*, and *Ulva intestinalis* based on protein content and functional properties of their protein extracts relevant to digestibility and peptide recovery. Arrows (↑/↓) indicate whether higher or lower values were considered favourable for each parameter. Scores were assigned semi-quantitatively based on the statistical significance of the results as “++” (highly favourable), “+” (favourable), “-” (unfavourable), and “--” (highly unfavourable). Overall performance was ranked from * to *** according to the cumulative number of favourable scores across all evaluated parameters.

Factors	<i>Ulva australis</i>	<i>Ulva linza</i>	<i>Ulva fasciata</i>	<i>Ulva intestinalis</i>
Protein content (↑)	++	+	-	--
Digestibility (↑)	++	++	-	+
Phenolic content (↓)	++	-	--	+
Carbohydrate content (↓)	+	++	--	-
Peptide fraction <3 kDa (↑)	-	++	+	-
Total score	***	***	*	**

2. Implications and future perspectives

The growing interest in seaweed research is strongly linked to its potential application in functional foods and health-oriented ingredients. In particular, *Ulva* species are promising as a sustainable source of bioactive peptides with potential metabolic health benefits. At the same time, industrial relevance is increasing, as recent studies already investigate how processing conditions and *in vitro* digestion influence the structural behaviour, rheology, and peptide release of processed *Ulva* biomass, with the broader aim of optimizing large-scale peptide production and recovery (Souto-Prieto et al, 2025).

Building further on the results of this thesis, ongoing doctoral research by Reyhaneh Ebrahimi focuses on selecting the *Ulva* species with the highest DPP-IV inhibitory activity and subsequently identifying the peptide fractions responsible for this bioactivity, relevant in the context of anti-diabetic applications (Cian et al, 2022). Since no LC-MS/MS or proteomic analyses were performed in this study, the specific peptides underlying the observed trends remain unknown. The continuation of this work therefore includes peptide identification, *in silico* peptide selection and synthesis, and subsequent permeability assessment using Caco-2 models, analogous to approaches already described in literature for other seaweed species and peptide extracts.

Another interesting perspective, especially considering the environmental variability identified as a limitation in this study, would be the cultivation of seaweeds under controlled laboratory conditions while systematically varying parameters such as temperature, light intensity, aeration, or nutrient availability. Previous cultivation studies on *Ecklonia cava* (brown algae) and *Palmaria palmata* (red algae) demonstrated that environmental conditions can strongly influence seaweed growth and biochemical composition, highlighting the possibility of modulating culture conditions toward enhanced production of specific health-promoting compounds (Semmour, Lee, et al., 2025; Semmour, Vanhercke, et al., 2025). Extending this approach toward digestibility and peptide formation could provide valuable insight in how environmental conditions influence the nutritional and functional properties of *Ulva* biomass.

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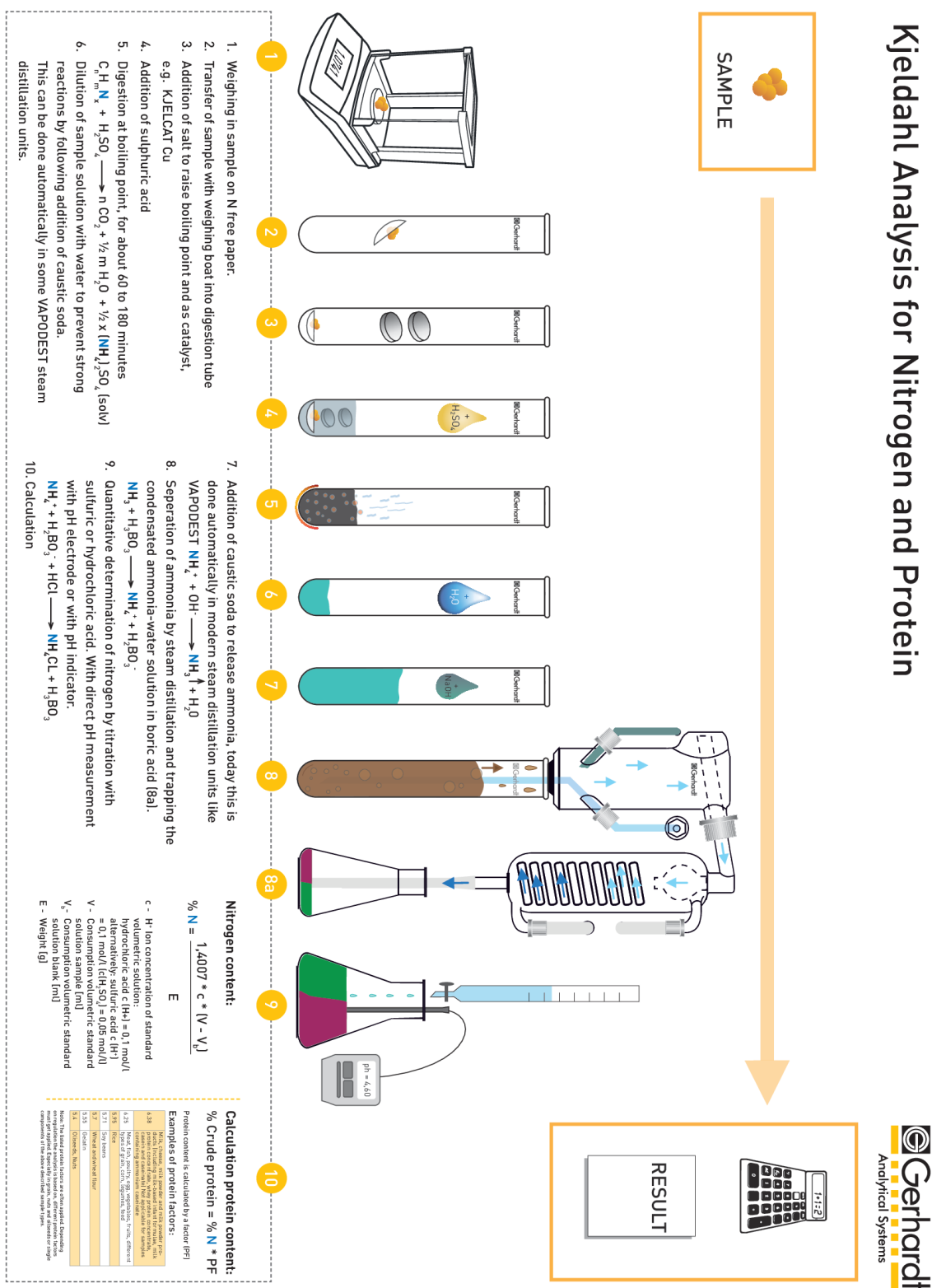
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A. KJELDAHL METHOD



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Fig. A1. Overview of the Kjeldahl procedure for the determination of total nitrogen and calculation of protein content in the European samples. The method consists of three main steps: digestion of the sample with sulfuric acid to convert organic nitrogen into ammonium, followed by distillation of ammonia, and subsequent quantification by titration. The measured nitrogen content is then converted to protein content using an appropriate conversion factor (C. Gerhardt Analytical Systems).

B. SGF AND SIF STOCK SOLUTIONS

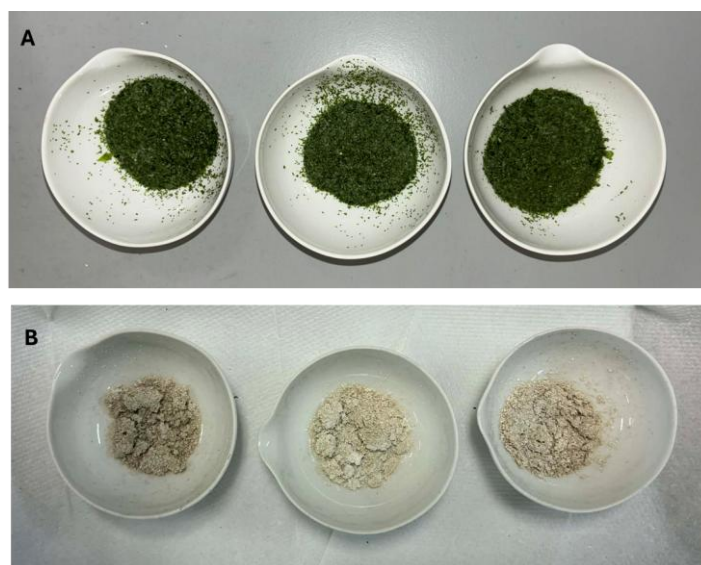
Table B. Preparation of electrolyte stock solutions for simulated digestive fluids (final working volume: 400 mL; 1.25× concentrate, subsequently diluted with water). Simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF) stock solutions were prepared as 1.25× concentrates and diluted to working concentrations using distilled water. In this study, only SGF and SIF were applied, as no salivary phase was included. Stock solutions were adjusted according to the required working volumes while maintaining identical final concentrations (INFOGEST 2.0 protocol; Brodkorb et al., 2019).

Salt solution added	Stock concentrations		SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
			Milliliters of stock added to prepare 0.4 L (1.25×)	Final salt concentration in SSF	Milliliters of stock added to prepare 0.4 L (1.25×)	Final salt concentration in SGF	Milliliters of stock added to prepare 0.4 L (1.25×)	Final salt concentration in SIF
	(g/L)	(M)	(mL)	(mM)	(mL)	(mM)	(mL)	(mM)
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH ₂ PO ₄	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
NaHCO ₃ ^a	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	–	–	11.8	47.2	9.6	38.4
MgCl ₂ (H ₂ O) ₆	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
(NH ₄) ₂ CO ₃ [*]	48	0.5	0.06	0.06	0.5	0.5	–	–
HCl		6	0.09	1.1	1.3	15.6	0.7	8.4
CaCl ₂ (H ₂ O) ₂ ^b	44.1	0.3	0.025	1.5	0.005	0.15	0.04	0.6

^aThe use of carbonate salts in the electrolyte solutions requires the use of sealed containers with limited headspace, see also CRITICAL STEP in Step 24. ^bCaCl₂(H₂O)₂ should be added immediately before use. Volumes in Table 2 are indicated for a typical experiment of 5 mL of SSF.

C. ADDITIONAL DATA

Ash content



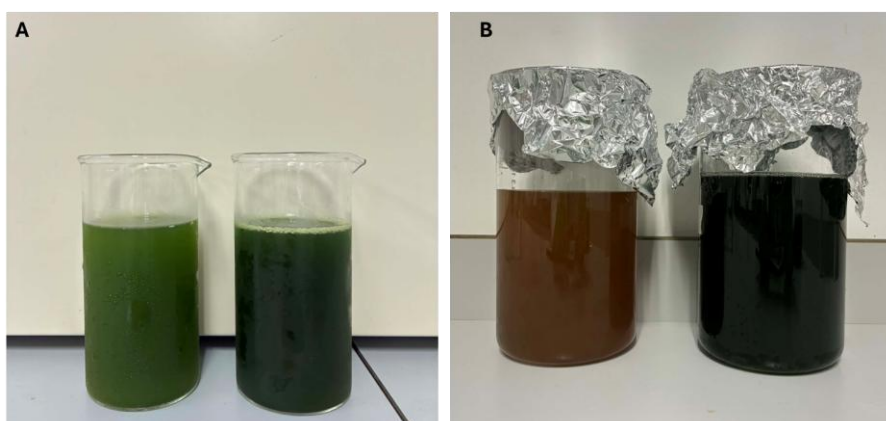
*Fig. C1. Ash content determination of *Ulva fasciata* performed in triplicate. (A) Sample prior to combustion and (B) sample after combustion.*

Moisture content

*Table C1. Moisture content (%) of the two collected European species. Standard deviation (SD) was calculated from repeated weighing. *Ulva intestinalis* is based on 18 measurements, and *Ulva australis* on 12 measurements.*

Species	Mean $\pm$ SD
<i>Ulva intestinalis</i>	77.85 $\pm$ 1.76
<i>Ulva australis</i>	74.00 $\pm$ 3.33

Extraction fractions



*Fig. C2. Protein fractions extracted prior to purification. (A) *Ulva fasciata* (South Korea) and (B) *Ulva intestinalis* (Europe). In each image, the left measuring cylinder contains the water-soluble protein fraction (S1), while the right measuring cylinder contains the alkaline-soluble fraction (S2). Clear colour differences can be observed between samples from South Korea and Belgium.*

Protein purification

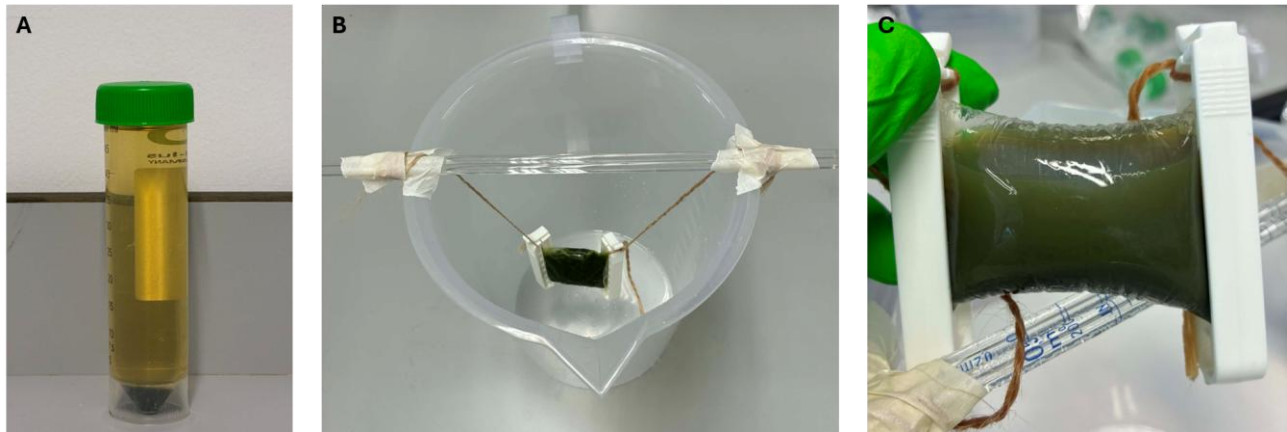


Fig. C3. Protein purification workflow. (A) Protein extract following ammonium sulfate precipitation and centrifugation: precipitated proteins form a pellet, the supernatant is discarded, and the pellet is subsequently resuspended in Milli-Q water. (B) Dialysis setup used during purification. (C) Dialysis membrane containing the purified protein extract after dialysis.

AA-profiling

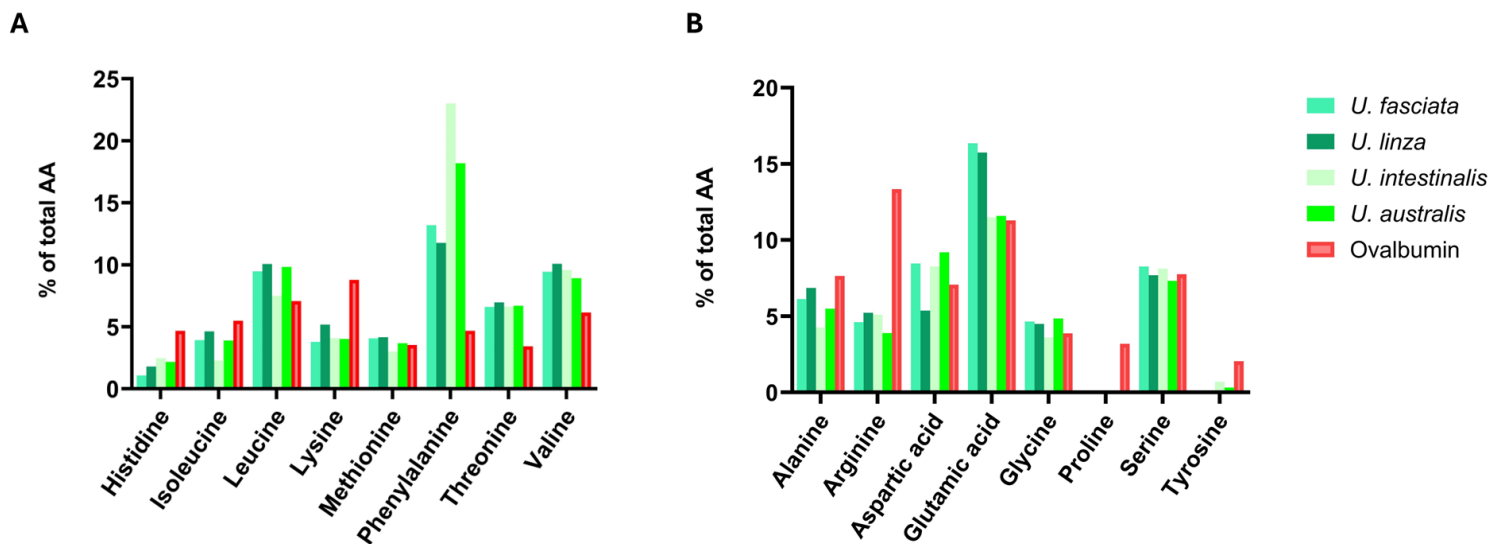
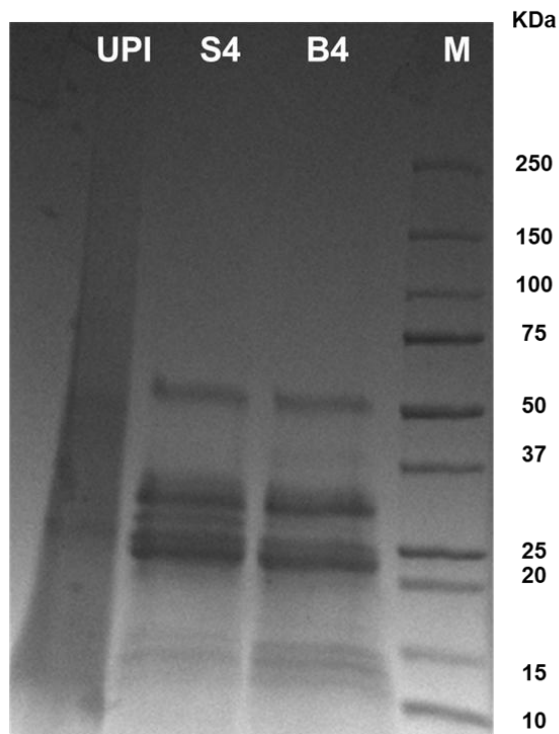


Fig. C4. Relative contribution of amino acids in protein extracts from four *Ulva* species, compared with ovalbumin. (A) Essential amino acids; (B) non-essential amino acids. Proline was not detected, while tyrosine was only present at low levels in the European species (*U. intestinalis* and *U. australis*). Phenylalanine proportions were relatively high across all species compared to ovalbumin.

SDS PAGE

SDS-PAGE after digestion (*U. linza*)



SDS-PAGE after digestion (*U. fasciata*)

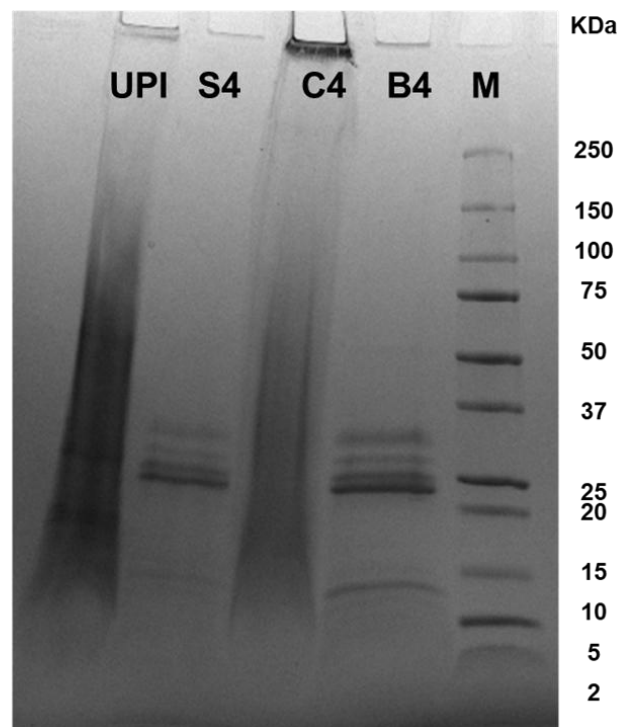
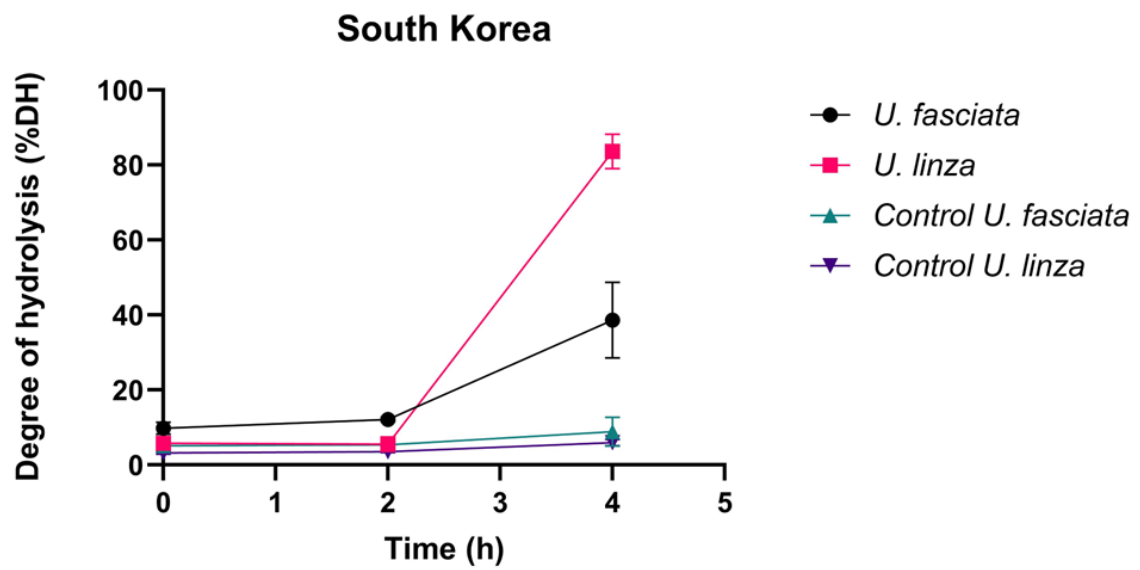


Fig. C5. SDS-PAGE profiles of Korean *Ulva* species after digestion. The left panel shows similarity between samples and the blank after 4 h of digestion for *U. linza*, indicating the presence of residual enzymes and possible autolysis of enzymes. The right panel shows *U. fasciata* samples (S4, C4, and B4); the C4 control clearly indicates that no digestion occurred, as no enzymes were added. Abbreviations: UPI = undigested protein isolate; S4 = sample after 4 h digestion; C4 = control after 4 h digestion; B4 = blank after 4 h digestion.

A



B

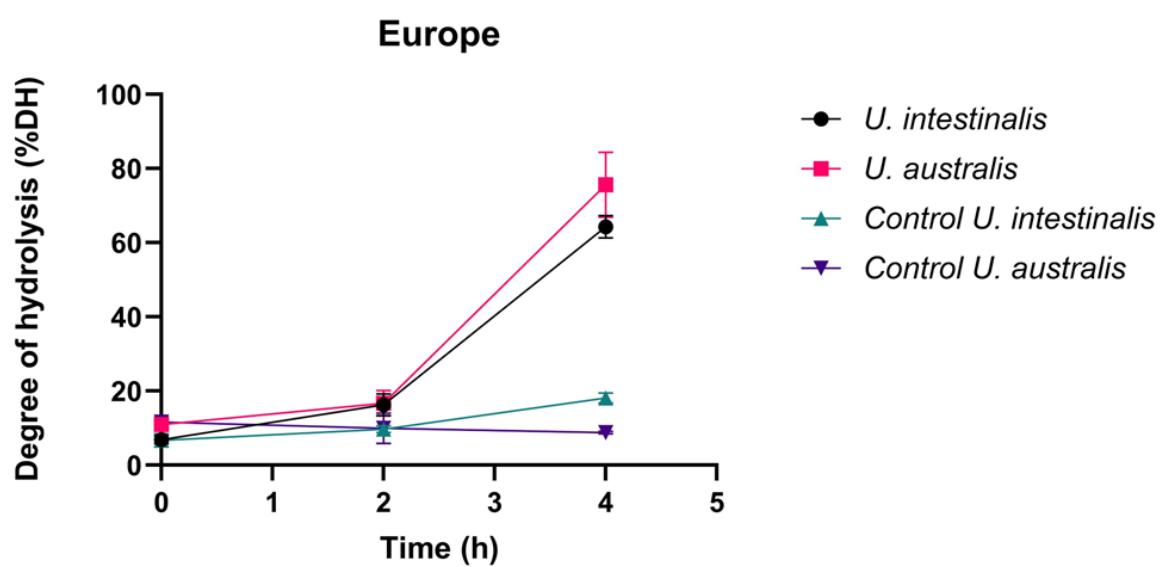


Fig. C6. Quantitative analysis of in vitro protein digestibility of four *Ulva* species. Degree of hydrolysis (%DH) over time during simulated gastrointestinal digestion (gastric phase: 0–2 h; intestinal phase: 2–4 h). (A) South Korean species and (B) European species, including their respective control groups.

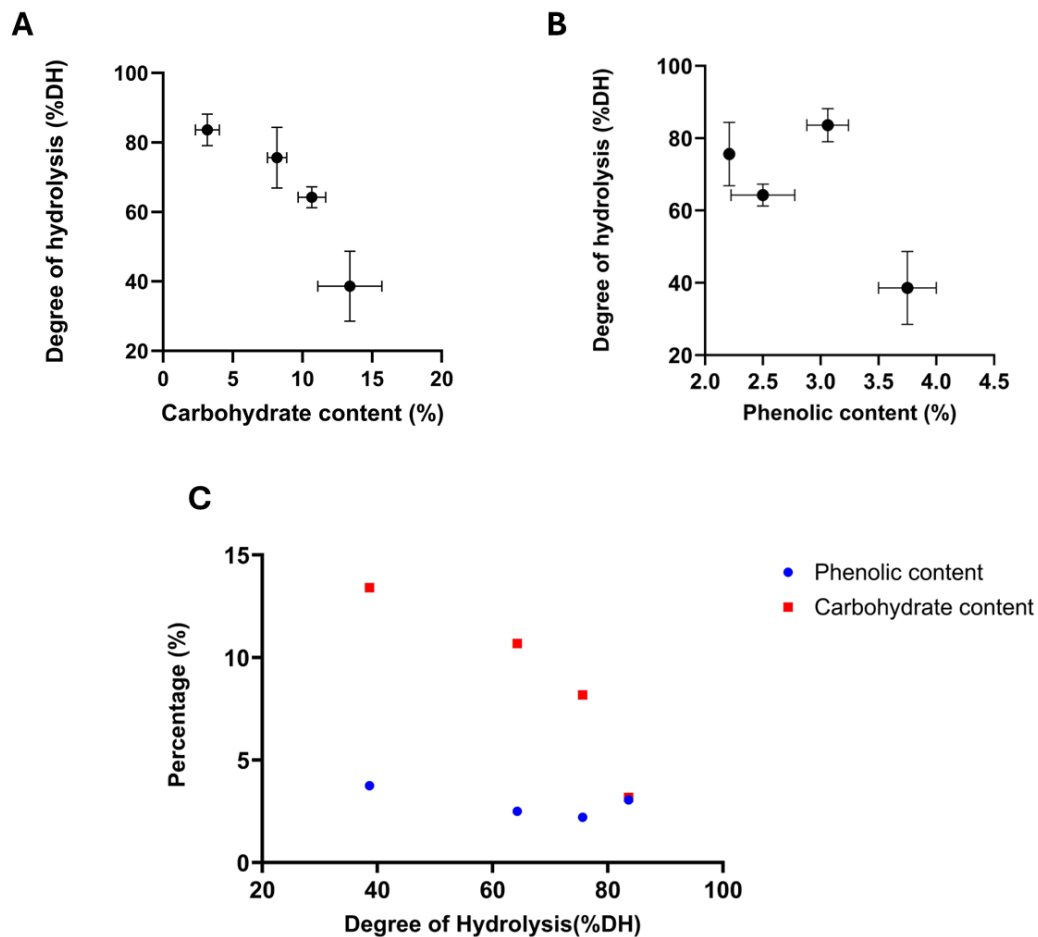


Fig. C7. Spearman correlation between chemical composition and degree of hydrolysis (%DH). (A) Carbohydrate content versus %DH; (B) phenolic content versus %DH; both include SD error bars for x and y and are ranked from high to low content. (C) Combined overview of both variables plotted against %DH. No significant correlations were observed ($\alpha = 0.05$).

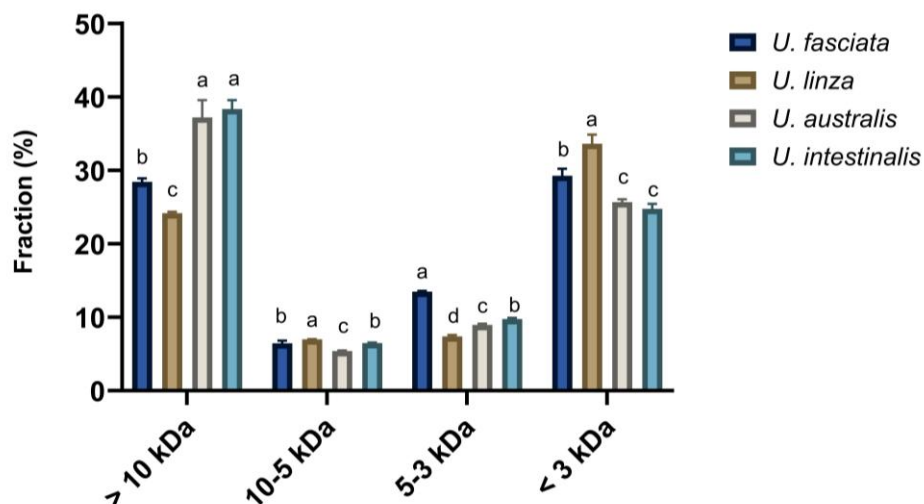


Fig. C8 Molecular weight distribution of peptides following in vitro digestion. Relative percentages of four size fractions (> 10 kDa, 10–5 kDa, 5–3 kDa, and < 3 kDa) were determined for the four *Ulva* species. Data are presented as the mean relative fraction (%) $\pm$ standard deviation ($n=3$). Different lowercase letters (a, b, c, d) denote statistically significant differences between species within a specific size category (ANOVA, $p < 0.05$). The high prevalence of the < 3 kDa fraction in *U. linza* correlates with its high Degree of Hydrolysis.

D. SUPPORTING LITERATURE

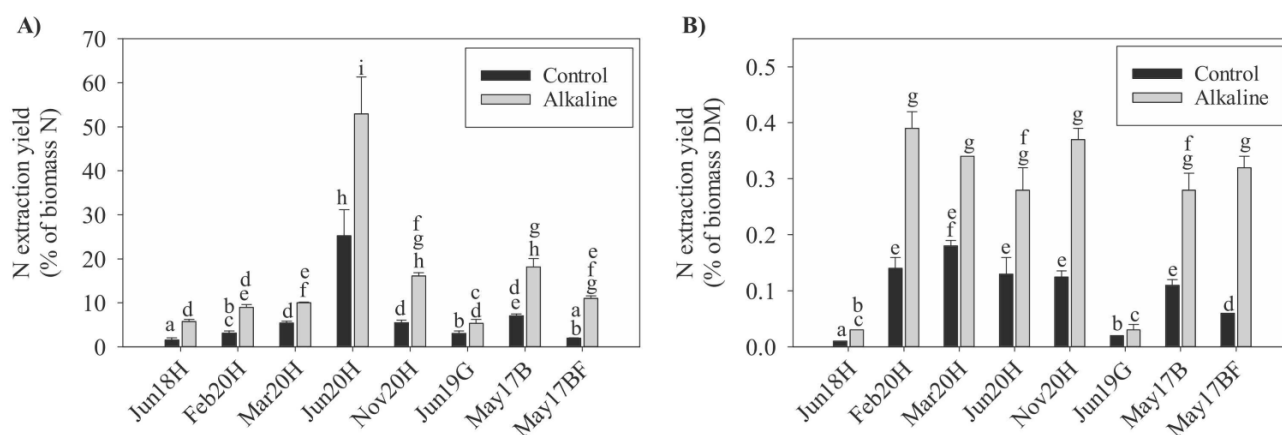


Fig. D1. Nitrogen (N) extraction yields of different *S. latissima* biomasses obtained through control and alkaline extraction methods, expressed as the percentage of N recovered in the protein extracts relative to (A) total biomass N and (B) biomass dry matter (DM). Sample names correspond to the harvest period (month and year), while the final letter indicates the cultivation site (H: Hjarnø, G: Grenå, B: Bergen, BF: fermented Bergen biomass). Data are presented as mean $\pm$ SD. Different letters above the bars indicate significant differences ($p < 0.05$) (Juul et al., 2023).

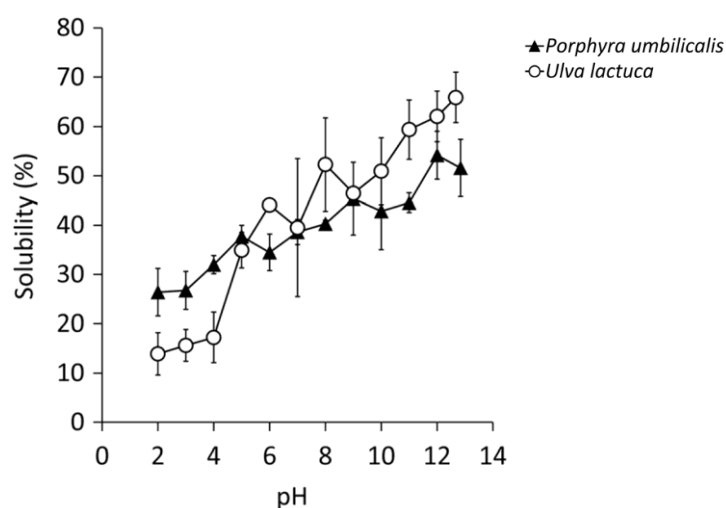


Fig. D2. Protein solubility of *Porphyra umbilicalis* and *Ulva lactuca* at different pH levels using the pH shift method. Adapted from Harrysson et al. (2018).

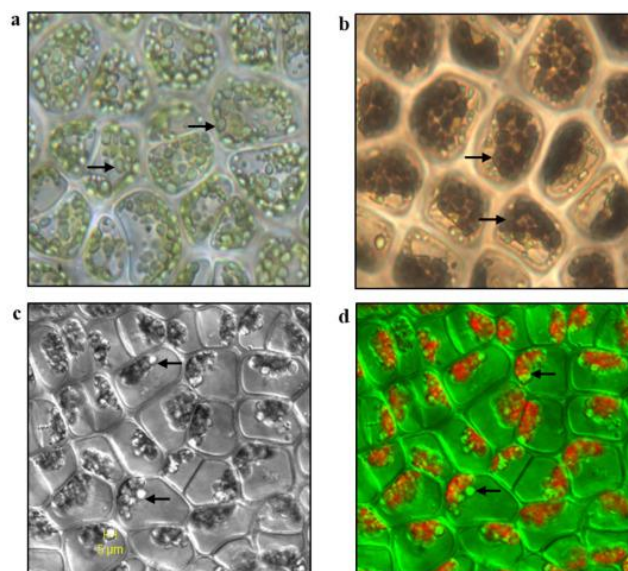


Fig. D3. Microscopic observations of *Ulva ohnoi* thallus: (a) light microscopy, (b) light microscopy after iodine staining (starch granules indicated in blue), (c) confocal microscopy without autofluorescence, and (d) confocal microscopy with autofluorescence (starch granules indicated in red). All images were observed at 40 $\times$ magnification. Adapted from Prabhu et al. (2019).

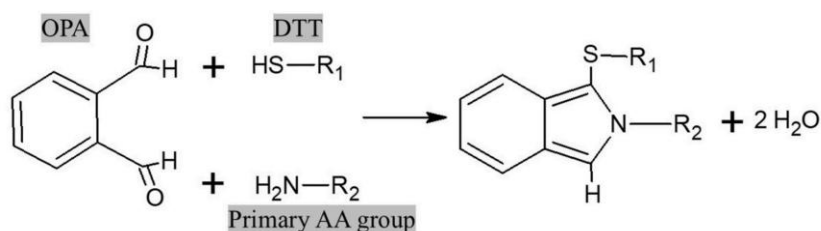


Fig. D4. Schematic of the OPA assay: OPA reacts with primary amines in the presence of dithiothreitol (DTT), forming a chromophore measured at 340 nm (Ramezani et al., 2026).

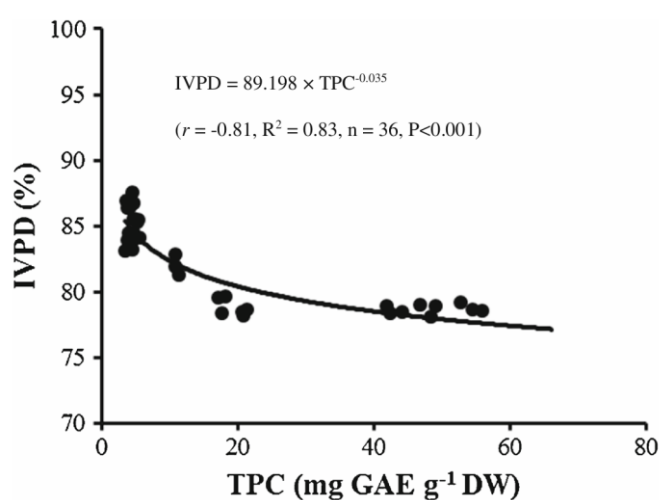


Fig. D5. Inverse non-linear relationship between total phenolic content (TPC) of seaweed biomass and in vitro protein digestibility (IVPD) in seaweeds, adapted from Tibbetts et al., 2016. TPC is expressed as mg gallic acid equivalents (GAE) g⁻¹ dry weight (DW), while IVPD represents the percentage of protein digested in vitro. A significant negative correlation between TPC and IVPD was observed ($P < 0.001$).

Table D1. Effect of ultrasound (US) pre-treatment duration (1, 5, and 30 min) on dry matter solubilization, protein concentration in the liquid fraction, and protein solubilization yield from frozen *Ulva* spp. biomass. Values are expressed as mean $\pm$ standard deviation. Different superscript letters within the same column indicate significant differences between treatments ($p \leq 0.05$). Adapted from Vega-Gómez et al. (2024).

Sample	Dry matter yield (%)	Protein concentration (%)	Protein yield (%)
E-US1	32.75 $\pm$ 0.91 ^b	7.48 $\pm$ 0.46 ^a	11.31 $\pm$ 0.91 b
E-US5	42.79 $\pm$ 2.01 ^a	10.13 $\pm$ 1.30 ^a	21.78 $\pm$ 3.22 a
E-US30	48.23 $\pm$ 2.50 ^a	9.97 $\pm$ 1.91 ^a	22.68 $\pm$ 3.38 a

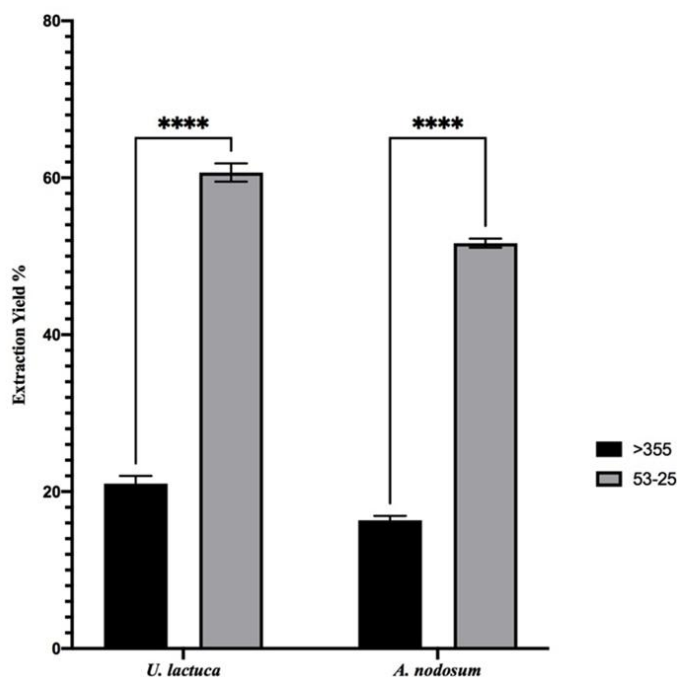


Fig. D6. Comparison of total bioactive extraction yields obtained from fine (53–25 μm) and coarse (>355 μm) particle sizes of *U. lactuca* and *A. nodosum* using optimized extraction protocols based on flavonoid, phenolic and protein recovery. Data are presented as mean $\pm$ SD ($n = 3$). Statistical analysis was performed using two-way ANOVA with multiple comparisons. Significance levels are indicated as **** ($p < 0.0001$). Adapted from El-Deeb et al. (2025).

Table D2 Comparison of the amino acid composition and relative abundance (% of total amino acids after rescaling) of protein extracts obtained from the four investigated *Ulva* species in the present study with values reported by Kazir et al. (2019) and Trigo et al. (2021). TEAA represents the total essential amino acid content.

Relative Amino acid composition of protein extracts of <i>Ulva</i> spp. (% of total AA).	<i>Ulva</i> spp. (Kazir et al., 2019)						<i>Ulva fenestrata</i> (Trigo et al., 2021)
	<i>Ulva fasciata</i>	<i>Ulva linza</i>	<i>Ulva intestinalis</i>	<i>Ulva australis</i>	<i>Ulva</i> spp.		
Alanine	6.13	6.86	4.25	5.49	8.6		6.08
Arginine	4.60	5.23	5.10	3.89	23.03		6.58
Aspartic acid	8.46	5.37	8.27	9.19	16.11		11.05
Glutamic acid	16.35	15.74	11.49	11.58	12		11.47
Glycine	4.65	4.49	3.61	4.85	2.11		4.32
Histidine*	1.08	1.79	2.46	2.17	0.64		4.8
Isoleucine*	3.92	4.62	2.27	3.89	3.6		5.22
Leucine*	9.46	10.06	7.49	9.82	4.31		8.99
Lysine*	3.78	5.18	4.08	4.01	6.34		6.31
Methionine*	4.07	4.16	3.00	3.67	0.81		1.92
Phenylalanine*	13.19	11.76	23.00	18.18	2.06		6.12
Proline	ND	ND	ND	ND	3.32		3.55
Serine	8.27	7.69	8.12	7.32	6.93		4.85
Threonine*	6.60	6.96	6.61	6.70	4.35		6.63
Tyrosine	ND	ND	0.69	0.32	2.88		5.02
Valine*	9.43	10.08	9.57	8.91	2.92		7.08
TEAA (%)**	51.54	54.61	58.48	57.35	25.03		47.07

* Essential amino acid

** TEAA = Total essential amino acid

AA-composition