Comparative Study of Pre-Extraction Treatments and Buffer Modifications for Macroalgal Lectin Extraction

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Abstract

Getting an efficient extraction approach is a crucial step in bioactive protein research, particularly lectin. This research aimed to examine the efficiency of cryogenic-grinding (CG) and freeze-dried-grinding (FG) pre-extraction treatments, and also the incorporation of phenylmethylsulphonyl fluoride (PMSF), Tween 80, polyvinylpolypyrrolidone (PVPP), 70% Ethanol (EtOH), or combination of the chemicals in the 20 mM phosphate buffered saline pH 7 (PBS) for extracting lectin from *Ulva lactuca*, *Sargassum polycystum*, and *Hydropuntia edulis*. The lectin content of the extracts was determined using the hemagglutination activity (HA) assay. The phenolic content was measured to determine its impact on the lectins' HA. Lectin extraction efficiency was determined by analyzing the extracts' minimum agglutination concentration (MAC) and total hemagglutination activity (THA). CG pre-extraction treatment produced slightly higher THA than FG, making it slightly more efficient. The EtOH treatment efficiently extracted lectin from *U. lactuca* and *H. edulis* by substantially reducing the polyphenol (PPs) content, lowering the MAC, and increasing the THA. The EtOH treatment significantly decreased the PPs and HA of the *S. polycystum*, suggesting that the HA is most likely produced by the PPs rather than the lectin content. Tween 80 raised the THA of *U. lactuca* by 17-fold with native rabbit erythrocyte compared to the control but did not affect the THA of *H. edulis* and *S. polycystum*. Several different effects of chemicals incorporated in the extraction buffers suggested that the optimum macroalgal lectin extraction strategy is species-dependent.

Keywords: Buffer Modification, Hemagglutinin, Lectin extraction, Seaweeds, Bioactive protein

Introduction

Lectins are carbohydrate-binding proteins with a wide range of biological and industrial applications, ranging from biotechnological research tools (Chettri et al., 2021; Divya et al., 2021; Mesquita et al., 2021; Notova & Imberty, 2023; Schnider et al., 2023; Silva et al., 2020) to various biological activities (Catanzaro et al., 2020; He et al., 2020; Hwang et al., 2020). Lectin may be extracted from all classes of organisms. However, as compared to other sources, macroalgal lectins have several unique characteristics, including low molecular weight, selective binding to glycoproteins, and divalent cation independent (Alves et al., 2020; Hung & Trinh, 2022, 2021; Hwang et al., 2020).

The first macroalgal lectin was reported by Boyd et al., (1966). Since then, macroalgal lectins have been quite intensively studied. However, only a few macroalgal lectins have been successfully isolated and characterized, particularly from 34 Rhodophyta, 19 Chlorophyta, and only one Phaeophyta, demonstrating the difficulties in extracting, isolating, and characterizing lectin from macroalgal sources.

Extraction is always a crucial first step in biological protein research since it could affect both the quantity and quality of the targeted protein. Extraction of bioactive protein, on the other hand, has always been a tedious and time-consuming research effort. Furthermore, algal bioactive protein extraction techniques have received far less attention than protein from other sources (Bleakley & Hayes, 2017; Cermeño et al., 2020). Due to the presence of anionic polysaccharide cell walls such as alginate in brown seaweed and carrageenan in red seaweed, extracting protein from algae can be challenging (Geada et al.,

2021; Joubert & Fleurence, 2008; Pliego-Cortés et al., 2020). After the algal cell wall is broken down, the polysaccharide content is released into the extraction medium as hydrocolloid compounds which may hinder the protein target's isolation process (Joubert & Fleurence, 2008). Moreover, phenolic compounds that bind to proteins shortly after the cells are lysed may alter the secondary and tertiary structures of the protein target, interfering with the effectiveness of protein extraction (Mendez & Kwon, 2021; Xu et al., 2019).

To improve the efficiency of protein extraction, several cell disruption approaches, as well as the employment of certain chemical reagents were utilized (Bleakley & Hayes, 2017; Tremblay & Beaulieu, 2021). Microwave-assisted extraction and ultrasound-assisted extraction are two modern extraction methods for macroalgal protein (Maliki et al., 2022). However, there are drawbacks to these procedures for extracting bioactive proteins, such as equipment expenses, energy costs, and the possibility of heat damage, which may affect the protein structure and bioactivities (Cermeño et al., 2020; Quitério et al., 2022; Usman et al., 2022). Thus, grinding the materials in liquid nitrogen (cryogenic-grinding), followed by extraction using a cold isotonic solution buffer, is still a common approach for extracting bioactive proteins such as macroalgal lectin (Djabayan-Djibeyan et al., 2018; Maliki et al., 2022). According to Rogers and Hori (1993), algal lectin can be extracted more easily if the material is frozen in liquid nitrogen or freeze-dried before homogenization. Additionally, selected chemical reagents, such as surfactant to increase membrane-bound protein extraction, protease inhibitor to prevent protein degradation during extraction, and polyvinyl polypyrrolidone (PVPP) to reduce phenol effects on the protein target, are included in the extraction buffers to increase extraction yield (Djabayan-Djibeyan et al., 2010; Leong et al., 2020; Liao et al., 2003; Marques et al., 2018; Nijole et al., 2012; Rogers & Hori, 1993; Rogers & Loveless, 1985; Sampaio et al., 1998b; Tavanandi & Raghavarao, 2020; Vidoviæ et al., 2020).

Since it is crucial to retain the bioactivities while obtaining an acceptable quantity of the targeted protein, achieving an effective extraction approach is a critical step in bioactive protein research, notably in the field of lectin research. However, available information on the efficiency of different cell disruption techniques and the incorporation of selected chemical reagents in macroalgal lectin extraction is very limited. Thus, the purpose of this study was to examine the efficiency of two cold cell disruption methods, as well as the addition of four chemical reagents to the phosphate-buffered saline (PBS) extraction solution for macroalgal lectin extraction. We compared cryogenic grinding using liquid nitrogen and freeze-dried grinding of the algal sample as pre-extraction treatments. The chemical reagents utilized were surfactant Tween 80, protease inhibitor phenylmethylsulphonyl fluoride (PMSF), PVPP, and 70% Ethanol (EtOH). The hemagglutination activity (HA) assay was employed to determine the presence of lectin in the sample. The effectiveness of the lectin extraction was determined by comparing the minimum agglutination concentration (MAC) and total hemagglutination activity (THA) of the extracts.

Materials and Methods

Material

We used *Ulva lactuca*, *Sargassum polycycstum*, and *Hydropuntia edulis* species as representatives of the green, brown, and red macroalgal group, respectively. The three algae were prevalent along the shore of Pantai Porok, a marine research station of the Biology Faculty of Gadjah Mada University. *Ulva lactuca*, *S. polycystum*, and *H. edulis* were collected from the intertidal zone along Pantai Porok, Yogyakarta, Indonesia (S8°8'2.71" E110°33'28.73") on October 24, 2021. Upon collection, the algae were cleaned from impurities and then stored in a cold storage (-20 °C) until further analysis. Macroalgal species were identified based on their molecular and morphological characteristics. Blood from rabbits was obtained from the Bioassay Laboratory, Research Centre for Marine and Fisheries Product Processing and Biotechnology, Jakarta, Indonesia. Unless otherwise specified, all chemicals employed in the study were Merck or Sigma analytical grade.

Experimental Design

Cryogenic grinding using liquid nitrogen (CG) and freeze-dried grinding (FG) of the algal thallus was examined as pre-extraction treatments (Figure 1). Based on the result of the pre-extraction treatment trial, CG was slightly more efficient than FG, CG was then chosen as the pre-extraction (cell disruption) treatment for the buffer modification treatment. The extraction buffer used six combination mixtures of PBS and protease inhibitor PMSF or surfactant Tween 80 or PVPP or EtOH on the extraction efficiency of macroalgal lectins (Figure 2). We incorporated each reagent at concentrations used in prior publications, as follows: 1 mM of PMSF (Han et al., 2010; Shim et al., 2012), 2 g/L of Tween 80 (Rogers & Hori, 1993), 2% of PVPP (Nijole et al., 2012), and 70% of EtOH (Sampaio et al., 1998a). The effectiveness of the lectin extraction was determined by comparing the MAC and THA of the extracts. The total phenol content was measured to find out the effect of the chemicals on

the phenol content of the lectin extracts as well as their effect on the HA.

Pre-Extraction

Each algal thallus was cut into small pieces. For the CG treatment, 25 g of algae was immediately frozen-dried using liquid nitrogen with a ratio of 1:4 (w: v) for at least 60 s, then ground into powder using a mortar and pestle. For the FG treatment, the frozen algal thallus (25 g) was put in a freeze-dryer (Martin Christ 2-8 LD Plus) for 48 h. The freeze-dried algal thallus was then ground into powder using a mortar and a pestle. The algal powders were then extracted for their protein-containing lectin.

Lectin Extraction

The basic extraction buffer used for the pre-extraction treatments study was 20 mM phosphate buffered, 0.85% NaCl, pH 7.0 (PBS). The modified PBS buffers used for the buffer modification treatment (Figure 2) were as follows: **Op1** (PBS); **Op2** (PBS + 1 mM PMSF); **Op3** (PBS + 2 g/L Tween 80); **Op4** (PBS + 1 mM PMSF + 2 g/L Tween 80); **Op5** (pre-treated with 70% cold ethanol (1:2, w:v) stirred at 4 °C for 30 mins, then filtered through Whatman No. 4 paper under vacuum, and extraction using PBS), **Op6** (PBS + 2% PVPP). So that, each treatment for the green algae *U. lactuca* were coded as Gr-Op1, Gr-Op2, Gr-Op3, Gr-Op4, Gr-Op5, and Gr-Op6; for the brown algae *S. polycystum* was coded as Br-Op1, Br-Op2, Br-Op3, Br-Op4, Br-Op5, and Br-Op6; and for red algae *H. edulis* was then coded as Re-Op1, Re-Op2, Re-Op3, Re-Op4, Re-Op5 and Re-Op6.

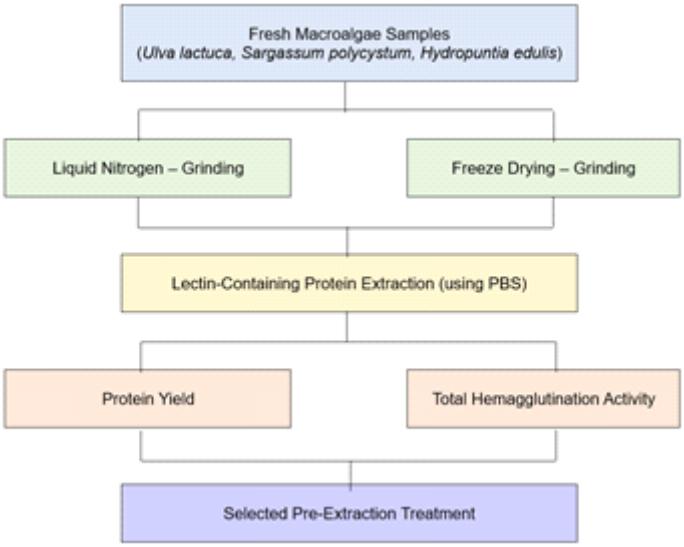

Figure 1. The flow chart of different pre-extraction treatments for macroalgal lectin-containing protein extraction.

Extraction for the algal protein containing lectin was carried out according to (Praseptiangga et al., 2012) with modifications. The algal powder was then extracted for lectin content using the PBS buffers at a ratio of 1:2 (w:v). The homogenate was then agitated at 300 rpm (IKA RT 10) for 18 h at 4 °C. After that, each algal homogenate was then centrifuged at 12,000 × *g* at 4 °C for 30 min (Beckman Coulter, JA-14 rotor). The recovered supernatant was then collected and precipitated using 75% saturation of ammonium sulfate at 4 °C overnight, and then centrifugated at 12,000 × *g* at 4 °C for 30 min (Beckman Coulter, JA-14 rotor). The precipitate was then dissolved in the least amount of buffer before being dialyzed for 10 h against the buffer using 3.5 kDa MWCO SnakeSkin dialysis tubing (Thermo Scientific), with buffer replacement for every 2 h. The internal fraction was then spun at 12,000 × *g* at 4 °C for 30 min (Beckman Coulter, JA-14 rotor), and the supernatant obtained was referred to as algal lectin-containing extract (LE), was then tested for protein content, hemagglutination activity, and total phenol content. Each treatment was carried out in triplicate.

Protein Content Determination

The protein concentration of the LEs was measured using the BCA protein assay kit (Pierce™-Thermo Scientific) according to the manufacturer's instructions, with bovine serum albumin (BSA) serving as the reference. The protein yields were calculated as follows:

$$\text{Protein Yield } (\mu g\ g^{-1}) = \frac{C \times V}{W}$$

Where *C* is the protein concentration of algal extract (µg/mL), *V* is the final volume of the algal extract (mL) and *W* is the weight of the fresh algal material before extraction (25 g). Each test was carried out in triplicate.

Preparation of 2% Native and Trypsin-Treated Rabbit Red Blood Cells

The 2% red blood cells were prepared according to Praseptiangga et al. (2012). The rabbit red blood cells were washed thrice using 50 volumes of 0.85% saline before being centrifugated at 500 × *g* for 10 min each time (Beckman Coulter). Following washing, the erythrocyte was suspended in 0.85% saline solution to make a 2% (v/v) native rabbit erythrocytes (RBC) suspension. The trypsin-treated rabbit red blood cells (TRBC) were made by adding a tenth volume of 0.5% (w/v) trypsin to a 2% native erythrocytes suspension and incubating it for 60 min at 37 °C. The erythrocytes were then washed thrice with 50 volumes of 0.85% saline and spun at 500 × *g* or 10 min each time (Beckman Coulter), and finally, the 2% (v/v) TRBC were prepared in 0.85% saline.

Hemagglutination Activity Assay

The hemagglutination activity assay was carried out in a 96-well V-bottom microplate with both RBC and TRBC according to Praseptiangga et al. (2012). Each LE sample was serially two-fold diluted in saline before being mixed with 25 ìL of 2% RBC or TRBC. The plate was gently shaken and then kept at room temperature for 1 h. Positive hemagglutination activity was indicated by the agglutination of more than half of erythrocytes. Each assay was carried out in triplicate. The minimum concentration of the macroalgal salting out fraction (SOF) to produce hemagglutination (MAC) was calculated by dividing the protein content with the hemagglutination titer of the sample. The hemagglutination titer is the reciprocal of the sample's highest two-fold dilution that still shows positive hemagglutination. The total hemagglutination activity is calculated as follows:

$$\text{Total Hemagglutination Activity (THA)} = \frac{\text{HA x V}}{\text{Av}}$$

Where *HA* is the hemagglutination titer of the algal extract, *V* is the final volume of the algal extract (µL) and *Av* is the volume of algal extract used in the HA assay (25 µL).

Total Phenolic Content

The phenolic content of the LEs was determined using a slightly modified version of Dewi et al. (2023). The test was performed in a 96-well microplate format. Approximately 25 µL of LEs were mixed with 125 µL of 10% Folin-Ciocalteau solution and 100 µL of 7.5% Na_2CO_3 solution. The mixture was then incubated at room temperature (24±2 °C) for 1 h before the absorbance reading at 765 nm using a microplate spectrophotometer (Multiskan GO, Thermo Fisher Scientific). The absorbance reading was then interpolated into the gallic acid standard curve (concentration of 0-50 µL/mL). Finally, the total phenolic content (TPC) of the LEs' is expressed as µg gallic acid equivalent (GAE)/g of fresh algal material.

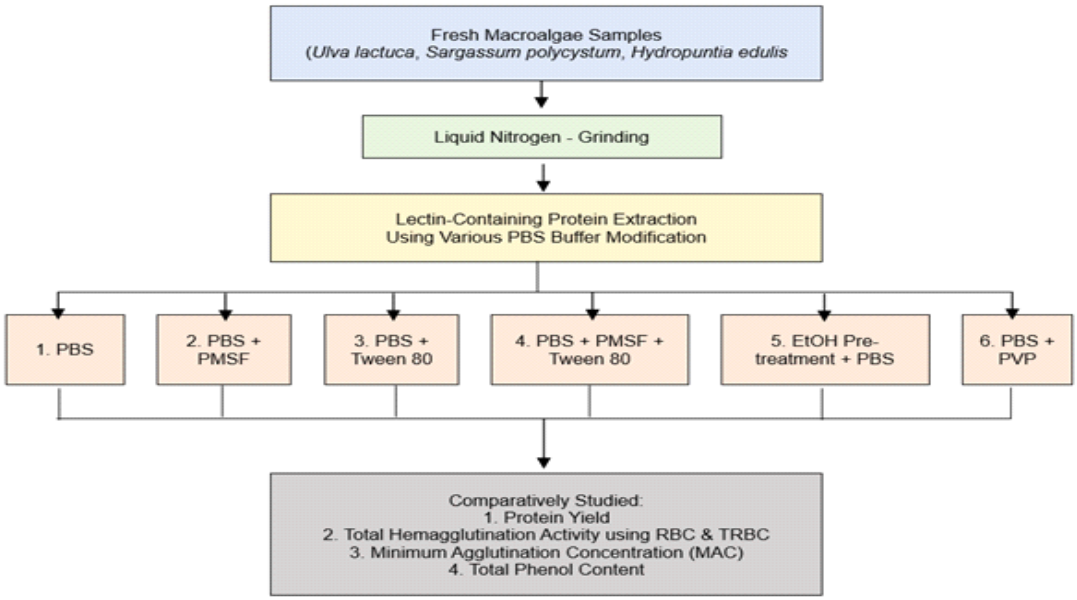

Figure 2. The flow chart of various PBS buffer modifications for macroalgal lectin-containing protein extraction.

Each test was carried out in triplicate. The TPC is determined as follows:

$$\text{TPC (µg GAE/g of fresh algal sample)} = \frac{P \times V}{W}$$

Where *P* is the phenolic concentration of the LE (µg GAE/mL), *V* is the final volume of the LE (mL), and *W* is the weight of the fresh algal material before extraction (25 g).

Statistical Analysis

All measurements were carried out in triplicate and were presented as the mean ± standard deviation. The results were analyzed using one-way analysis of variance (ANOVA), followed by post hoc Tukey's test using the SPSS software (version 23.0, SPSS Inc., Chicago, IL, USA). A *p* Â 0.05 was considered a significant difference.

Result and Discussion

Pre-extraction Treatment

The study of bioactive protein (BP) begins with the pre-extraction step, which is the raw material initial processing to reduce the loss of the protein target in the sample, such as freeze drying and grinding. As the macroalgal cell wall comprises a very complex matrix, sample material should be carefully prepared before BP extraction to achieve optimum extraction efficiency. In that regard, we studied two cold cell wall disruption methods as sample pre-extraction treatments, namely cryogenic grinding of algal tissue with the aid of liquid nitrogen and grinding of freeze-dried algal tissue (Figure 1). We used manual grinding to disrupt the algal polysaccharide cell wall to release and isolate the BP from other components of biomass. Due to the tensile strength of the polysaccharides in the cell wall, particularly cellulose, this technique is the quickest, cheapest, and most efficient way to access plant proteins (Echave et al., 2021). Considering temperature exerts a strong impact on the integrity of the BP, particularly lectin, low temperatures have to be employed during the pre-extraction processes.

Ulva lactuca, *S. polycystum*, and *H. edulis* were the samples in this study, representing the green, brown, and red algae, respectively. According to Gordalina et al. (2021), protein content of macroalgae varies by species. Brown algae has the lowest protein content among algal groups, which is in accordance with our findings (Figure 3). The cryogenic pre-extraction treatment extracted more protein from *S. polycystum* and *H. edulis*, but produced less protein from *U. lactuca* than the freeze-dried treatment. Differences in the algal cell wall composition have been suggested to have a significant effect on protein extraction (Gordalina et al., 2021). The cell wall of red algae is primarily formed of rigid structural glycans, up to 12% of which are cellulose, embedded in a variety of more flexible polymers; brown algal cell walls are composed of fucose-containing polysaccharides that interlock with â-glucan scaffold contained in an alginate matrix and cross-linking polyphenols; while green algal cell walls consist of a fibrillar polysaccharide incorporated in a matrix that can disintegrate into mucilage (Domozych, 2019; Herburger et al., 2022). When pulverized, the freeze-dried *U. lactuca* generated finer powder than the cryogenic treatment, resulting in more extracted protein. In contrast, the cryogenic treatment makes the cell structure of *S. polycystum* and *H. edulis* more brittle and easier to break, making it simpler to mill, whereas the hard and chewy freeze-dried treatment makes it tougher to mill, resulting in less extractable protein. These findings suggested that while any pre-treatment may be used to extract macroalgal protein, the optimum extraction strategy for each species should be adjusted.

Interestingly, although the protein yield of the cryogenic treatment was significantly different from the freeze-dried for all of the algae studied, there was no significant difference in MAC and total THA between the treatments. This means that both treatments may be used to extract macroalgal lectin with no significant difference. Despite the fact that the results were not statistically different, cryogenic pre-treatment of LEs resulted higher THA than the freeze-dried treatment (Figure 4). Thus, regarding the lectin yield, the cryogenic cold disruption treatment was slightly more efficient than the freeze-dried treatment.

The study showed that TRBC was more sensitive to detecting the HA of the samples than the RBC. Furthermore, LE of *H. edulis* needed a much lower concentration to cause hemagglutination than the other two species studied (Table 1). Despite producing significantly less protein yield than *U. lactuca*, *H. edulis* produced significantly higher THA than *U. lactuca* and *S. polycycstum* (Figure 4). This suggests that, among the three algae investigated, red algae *H. edulis* has a significantly greater lectin content than green algae *U. lactuca* and brown algae *S. polycystum*.

Based on the pre-extraction study result, cryogenic grinding was then chosen as the cell disruption treatment for the buffer-modified extraction study (Figure 2). Six modified PBS buffers were studied. PMSF was incorporated at 1 mM as previously used by (Han et al., 2010; Shim et al., 2012), PVPP was

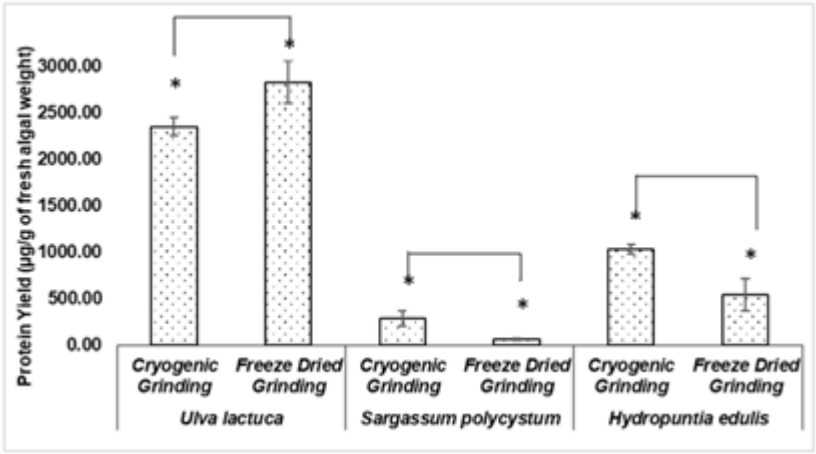

Figure 3. Protein yield of *Ulva lactuca*, *Sargassum polycystum*, and *Hydropuntia edulis* extracts with cryogenic-grinding and freeze-dried grinding pre-extraction treatment. The yield was expressed as µg per g of fresh weight of algal samples. Each bar represented the averages ±SD of three replicates. Each * indicates the treatment within each species was significantly different tested with one-way ANOVA ($p < 0.05$).

Table 1. Minimum agglutination concentration (µg/mL) of *Ulva lactuca*, *Sargassum polycystum*, and *Hydropuntia edulis* lectin-containing extract produced by cryogenic-grinding and freeze-dried grinding pre-extraction treatment. Each data represented the averages ± SD of three replicates

Macroalgae	Extraction Pre-Treatment	Type of Red Blood Cell	Minimum Agglutination Concentration (µg/mL)
Ulva lactuca	Cryogenic Grinding	RBC	30.36 ± 8.63
		TRBC	3.79 ± 1.08
	Freeze Dried Grinding	RBC	44.25 ± 3.24
		TRBC	9.38 ± 3.37
Sargassum polycystum	Cryogenic Grinding	RBC	11.24 ± 3
		TRBC	0.7 ± 0.19
	Freeze Dried Grinding	RBC	1.99 ± 0.38
		TRBC	0.12 ± 0.02
Hydropuntia edulis	Cryogenic Grinding	RBC	0.5 ± 0.02
		TRBC	0.25 ± 0.01
	Freeze Dried Grinding	RBC	0.53 ± 0.15
		TRBC	0.26 ± 0.08

Note: The hemagglutination activity was conducted against rabbit native red blood cells (RBC) and rabbit trypsin-treated red blood cells (TRBC).

incorporated at 2% as previously used by (Nijole et al., 2012), and Tween 80 was added to the PBS at a concentration of 2 g/L as previously used by (Rogers & Hori, 1993). At those concentrations, the reagents do not lyse erythrocytes and, therefore do not need to be eliminated from the lectin extract prior to the hemagglutination assay.

Even though Gr-Op6 (PVPP addition) treatment showed the highest protein yield from *U. lactuca* (Figure 5A), Gr-Op3 (Tween 80 addition) and Gr-Op4 (PMSF and Tween 80 addition) treatments produced significantly higher lectin yield, as seen by the THA values (Figure 5D). Furthermore, since Gr-Op2 (PMSF addition) treatment produced much lower lectin content than Gr-Op3 and Gr-Op4 treatments, it is possible that Tween 80 is the chemical responsible for the increase of the *U. lactuca* lectin extraction yield. Tween 80 is a mild non-ionic detergent that lyses cells, solubilizes membrane proteins, and functions as a stabilizer against protein aggregation (Danko et al., 2022; Urner et al., 2023). The addition of Tween 80 increased the THA of *U. lactuca* by 17-fold with native rabbit erythrocytes and 16-fold with TRBC. This is because the Tween 80 treatment needed a substantially lower concentration

of protein than the control to achieve hemagglutination activity (Figure 5B). Gr-Op5 (pre-treatment of 70% EtOH) treatment significantly reduced the protein yield (Figure 5A) and the phenolic content of the *U. lactuca* protein extract. Numerous studies have revealed that the binding of polyphenols (PPs) to proteins can alter the protein's structure and function (Chen et al., 2022; Chen et al., 2023; Li et al., 2021; Mendez & Kwon, 2021). Therefore, the removal of PPs content in *U. lactuca* appears to improve the lectin hemagglutination activity function, which is superior to the control (Gr-Op1).

Brown algae were known for their high PPs content. The PPs content of *S. polycystum* was more than double that of *U. lactuca* and four times higher than that of *H. edulis*. Unlike lectin HA, which is caused by a specific interaction between the lectin and erythrocytes' glycans, PPs HA was non-specific resulting in false positive HA detection of a sample (Cortés-Giraldo et al., 2012). As shown in Figure 6,

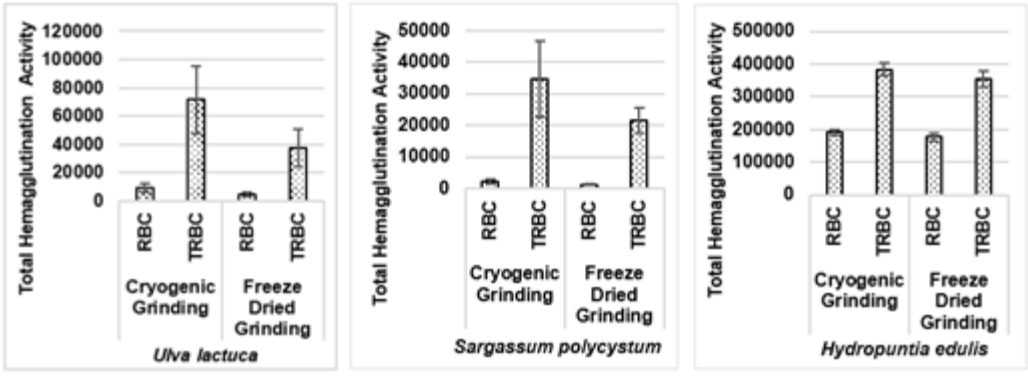

Figure 4. Total hemagglutination activity (THA) of *Ulva lactuca* (A), *Sargassum polycystum* (B), and *Hydropuntia edulis* (C) lectin-containing extract (LE) produced with cryogenic and freeze-dried pre-extraction treatment. The hemagglutination assay was conducted against native- (RBC) and trypsin-treated red blood cells (TRBC). Each data represented the averages ± SD of three replicates.

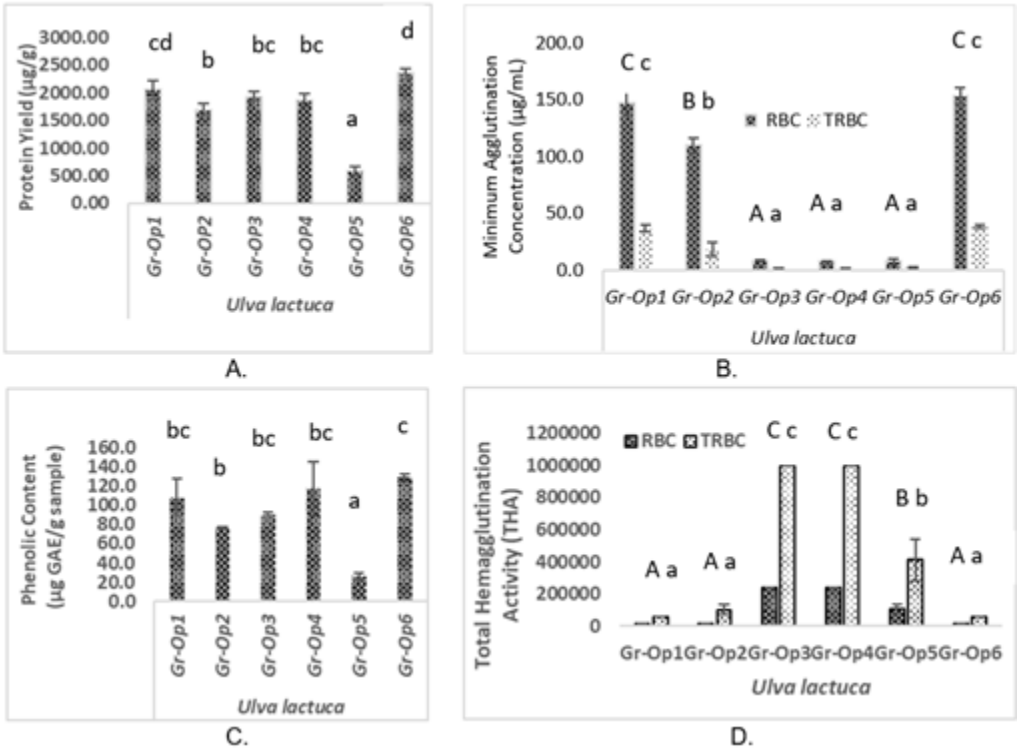

Figure 5. Protein yield (A), minimum agglutination concentration (B), total phenolic content (C), and total hemagglutination activity (D) of *Ulva lactuca* lectin-containing extract. Each data represented the averages ± SD of three replicates. Different letters indicate significant differences (Tukey post hoc, $p < 0.05$).

the EtOH pre-treatment (Br-Op5) considerably decreased the PPs content and HA of *S. polycystum*, as seen by significantly greater MAC and lower THA. On the other hand, Br-Op1 (control), Br-Op3 (Tween 80), Br-Op4 (PMSF and Tween 80), and Br-Op6 (PVPP) treatments were unable to diminish the PPs content of the brown algae resulted in a much lower MAC value and greater THA. These findings indicate that the HA observed in the *S, polycystum* protein extract is most likely induced by the PPs rather than the lectin content. According to (Cortés-Giraldo et al., 2012), the HA of PPs is two orders of magnitude lower than the HA of lectin. Polyphenols can alter the protein activity; thus, when studying materials rich in PPs, polymers that absorb phenols, particularly insoluble PVPP, are recommended to be employed. The PVPP interacts with phenolic compounds by forming stable hydrogen bonds to phenol groups, and it should be easily removed from the extract by centrifugation (Cutler & Pierpoint, 2004). However, it appears that we did not manage to eliminate the phenol-bound PVPP from the extract, as seen by the treatment's (Br-Op6) persistently high phenol levels (Figure 6C). Nonetheless, the interaction between protein and PP is

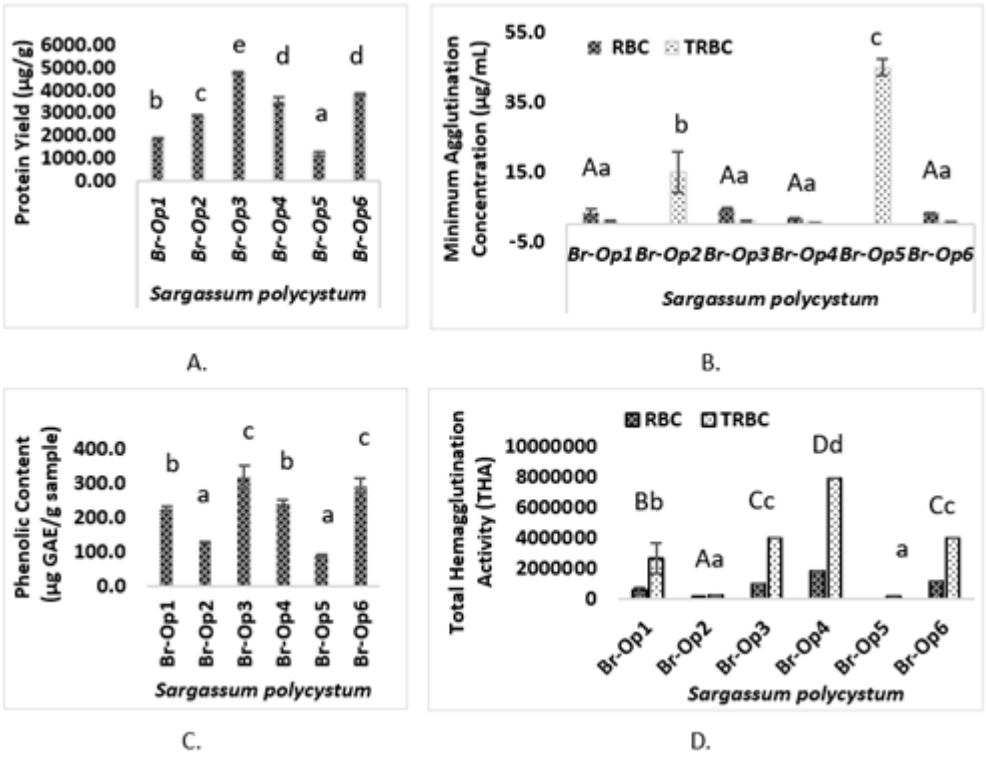

Figure 6. Protein yield (A), minimum hemagglutination activity concentration (B), total phenolic content (C) and total hemagglutination activity (D) of *Sargassum polycystum* lectin-containing extract. Each data represented the averages ± SD of three replicates. Different letters indicate significant differences (Tukey post hoc, $p < 0.05$). Note: Since the Br-Op2 and Br-Op5 treatment were inactive for hemagglutination activity to RBC, thus the minimum hemagglutination activity concentration could not be determined.

complex and still understudied, thus further research is needed, particularly in algal lectin research.

Despite the fact that the Re-Op5 (EtOH pre-treatment) of *H. edulis* produced significantly less protein yield than the other treatments, only a very small concentration of protein containing lectin (0.03 µg/ml) was required to induce hemagglutination, and thus the treatment produced the highest THA (Figure 7). This result is comparable to the EtOH treatment for *U. lactuca*. On the other hand, Tween 80 (Re-Op3) treatment on *H. edulis* showed a significantly different outcome than on *U. lactuca*. Tween 80 treatment significantly enhanced the protein yield of *H. edulis* above the control treatment (Re-Op1), but exhibited no difference in MAC and THA. The treatment, on the other hand, increased the THA of *U. lactuca* by 16-fold with TRBC, which is consistent with Rogers & Hori (1993) findings that applying 2 g/L of Tween 80 increased the HA of red algae G*riffithsia flosculosa* by 16-fold. Another intriguing finding in the *H. edulis* buffer-modified extraction study was that the employment of both PMSF and Tween 80 may

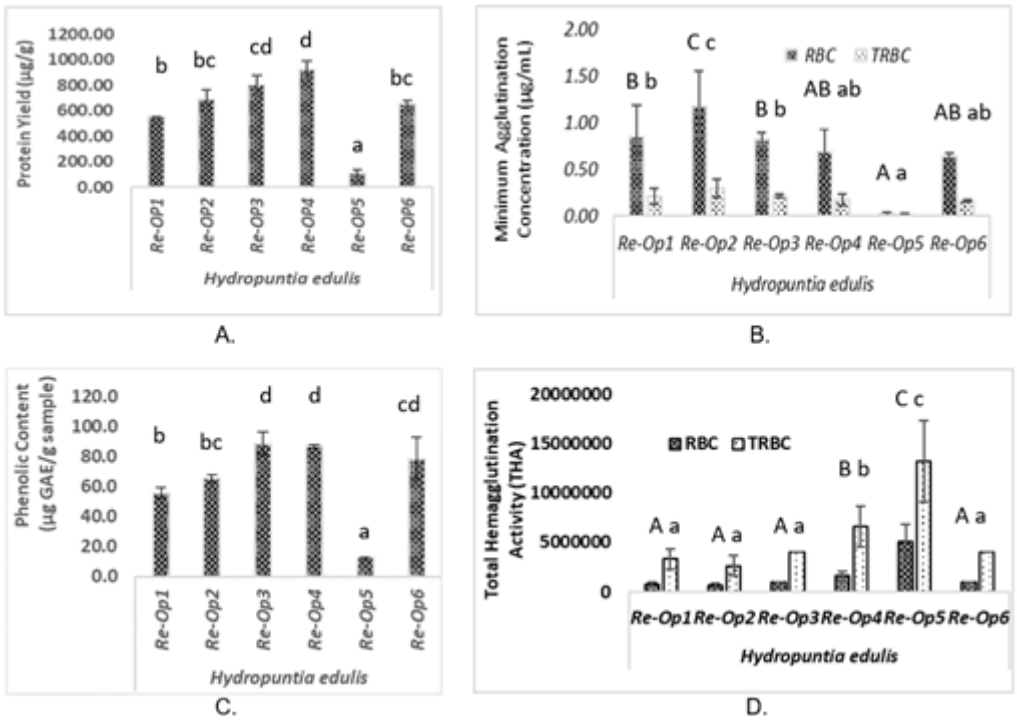

Figure 7. Protein yield (A), minimum hemagglutination activity concentration (B), total phenolic content (C), and total hemagglutination activity (D) of *Hydropuntia edulis* lectin-containing extract. Each data represented the averages ± SD of three replicates. Different letters indicate significant differences (Tukey post hoc, $p < 0.05$)

considerably enhance protein yield, lower the MAC, and increase the THA when compared to the employment of PMSF or Tween 80 only.

Several differences in the effects of different chemicals, particularly Tween 80, incorporated in the extraction buffers to different species suggested that the optimum macroalgal lectin extraction strategy should be adjusted by species. This could have been related to the enormous morphological and physiochemical variety of each algae species. Such as algal polysaccharides, which can cause problems by binding proteins or affecting bioactivity performance through modifying sample viscosity (Fido et al., 2003); and PPs, which not only may alter the secondary and tertiary structure of the protein target (Mendez & Kwon, 2021), but also can cause false detection of the lectins' HA screening (Cortés-Giraldo et al., 2012). Among the six treatments tested in this work, the EtOH pre-extraction treatment yielded the most intriguing results, as it was the most efficient in extracting macroalgal lectin from both green algae *U. lactuca* and red algae *H. edulis*. Despite severely reducing the extraction protein yield, the treatment was able to substantially lower the phenolic content, reduce the MAC required for the HA, and greatly increase the THA of both the algae.

Based on our findings, we propose cryogenic grinding as a simple yet cost-effective pre-extraction treatment for macroalgal lectin. Furthermore, since PMSF, Tween 80, PVP, and EtOH showed varied effects on *U. lactuca*, *S. polycystum*, and *H. edulis*, further experiments with other algal species will be necessary to gain a better insight into the algal lectin extraction technique efficiency. As the EtOH treatment showed an interesting lectin yield for both *U. lactuca* and *H. edulis*, it may pave the way for the utilization of the EtOH extract-by products. The EtOH extract, on the other hand, is rich in phytochemical compounds that can be employed as bioactive sources. Therefore, instead of a standalone lectin extraction process, an integrated approach for sequential extraction of phytochemicals and bioactive proteins, notably lectin, may be investigated.

Conclusion

Getting an efficient extraction approach is a crucial step in bioactive protein research, particularly lectin. Despite both cryogenic-grinding and freeze-dried grinding pre-extraction treatment extracted lectin from *U. lactuca*, *S. polycystum*, and *H. edulis* with no significant difference, the cryogenic treatment

produced slightly higher THA than the freeze-dried treatment, making it slightly more efficient. The addition of PMFS, Tween 80, PVP, and EtOH pre-treatment in the extraction buffer showed different effects on the lectin extraction efficiency of each algal species studied. It is suggested that the optimum macroalgal lectin extraction strategy should be adjusted for each species.

Human/Animal Rights, Informed Consent Statement

The study was carried out in accordance with internationally accepted guidelines and approved by The Medical and Health Research Ethics Committee Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Indonesia, with Ref. No. KE/FK/0374/EC/2022.

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Supplementary Materials

Supplementary materials is not available for this article.

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