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Crude proteins, total soluble proteins, total phenolic contents and SDS-PAGE profile of fifteen varieties of seaweed from Semporna, Sabah, Malaysia

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Abstract

It is crucial to determine several protein-related parameters at the initial stages of proteomic analysis of any biological samples. In this study, crude protein content, total soluble protein, total phenolic content and the SDS-PAGE profile of fifteen varieties of seaweed from Semporna, Sabah, Malaysia were analysed. The crude protein, total soluble protein and total phenolic content of all seaweed samples were in the range of 3.99 to 13.18 % of dry weight, 0.52 to 1.45 mg/mL in acetone dried powder samples and 8.59 to 48.98 mg PGE/g dry weight, respectively. In general, the differences (crude protein, total soluble protein and total phenolic content) among all fifteen varieties of seaweeds were significant ($p<0.05$). There was also a strong positive correlation between crude protein and total soluble protein concentration (Pearson's Correlation Coefficient (r)=0.923; p=0.01) in these fifteen varieties of seaweed. A distinctive protein pattern was observed in the SDS-PAGE gels between three different seaweed classes of green, red and brown colours. All of these results are important in sample preparations (extractions) before furthering proteomic analysis in order to identify and characterize seaweed proteomes.

Introduction

As people become increasingly aware of the relation between diet and good health, the consumption of natural food products will most likely increase. According to data from the World Health Organization (WHO), eighty percent of the world's population, primarily in developing countries, relies on plant-derived medicines for their healthcare (Gurib-Fakim, 2006). Currently, there is much consumer attention towards natural bioactive compounds as functional ingredients in the diet. Especially, bioactive compounds have been derived from marine organisms that have served as a rich source of health-promoting components (Li and Kim, 2011). Among marine organisms, edible seaweeds have been identified as an under-exploited plant resource. From previous findings, seaweed extracts can be a treasure of natural compounds with beneficial health, biological activities and beneficial properties for humans (Mohamed *et al*., 2012; Brown *et al*., 2014).

A wide-ranging diversity in the biochemical composition of various seaweeds paves the way for an exploration of a variety of compounds that consist of a wide range of physiological and biochemical characteristics, many of which are rare or absent in other taxonomic groups (Holdt and Kraan, 2011). Today, more than 15,000 primary and secondary metabolites from different metabolic pathways have been reported in various seaweeds with various different applications (Grosso *et al*., 2011). Both primary and secondary metabolites are of extreme importance since some can display remarkably positive effects on organisms, including anti-inflammatory (Matta *et al*., 2011), anti-mutagenic, anti-tumor, anti-diabetic, and anti-hypertensive properties (Yoon *et al*., 2008; Zubia *et al*., 2009: El Gamal, 2010; Pangestuti and Kim, 2011).

To date, very little attention has been placed on protein components from this marine source. However, numerous algal species, in particular the red seaweeds, have been shown to possess significant levels of protein and in some cases contain higher quantities than some conventional protein-rich foods, such as soybean, cereals, eggs, and fish (Kaliaperumal, 2003; Fleurence, 2004). The protein content of marine algae varies greatly between species and seasons. Reports have shown that in general, red seaweeds contain high levels of protein [maximum

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47% (w/w) dry weight], green seaweeds contain moderate amounts [9% to 26% (w/w) dry weight], while brown algae contain much lower protein content [3% to 15% (w/w) dry weight] (Fleurence, 2004; Harnedy and Fitz Gerald, 2011). Therefore, to assess the potential applications of proteins from various seaweeds, it is of paramount importance to identify and characterize seaweed protein extracts, which is known as proteomics studies. The proteomic analysis of plants is more challenging compare to other organisms, because plant tissues contain large amounts of interfering compounds such as phenolic compounds, carbohydrates, terpenes and pigments (Cánovas *et al*., 2004; Wang *et al*., 2008; Wu *et al*., 2014). Thus, the extraction of high-quality proteins from plant tissues is crucial for successful proteomic analyses (Wu *et al.*, 2014).

Proteins are involved in almost every biological function, so a comprehensive analysis of the proteins in the cell provides a unique global perspective on how these molecules interact and cooperate to create and maintain a working biological system. The cell responds to internal and external changes by regulating the level and activity of its proteins, so changes in the proteome, either qualitative or quantitative, provides a snapshot of the cell in action (Vercauteren *et al.*, 2007). However, up to now, the structure and biological properties of proteins extracted from seaweeds were not as widely documented as that of polysaccharides and the use of proteomics in seaweed biology and aquaculture has been limited.

The protein content of seaweeds varied depending on two main factors: the chemical composition of the species, and its morphological and structural characteristics (Barbarino and Lourenco, 2005). The chemical composition of the fifteen seaweed species and varieties was very significant (Ahmad *et al*., 2012) and there were also very distinctive differences in terms of their morphological and structural characteristics. For example *Caulerpa lentillifera* (green seaweed) is a grass-green in color, with a soft and succulent texture characterized by thallus consisting of long horizontal stolons with a few rhizoidal branches below, and many erect grapelike branches above. The erect branches are populated with many small capitates ramuli crowdedly attached to the main axis. Distinctive of this species are the spherical tips of the short ramular stalk and the base of the globose head (Mary *et al*., 2009). Meanwhile, *Kappaphycus alvarezii* [var. aring-aring] (red seaweed) has yellow-green rhizoidal filaments of branched algae and possesses a hard-leathery thallus. *Turbinaria conoides* (brown seaweed) have erect and dark brown thalli with leaves that are triangular and bell-like, turbinate in shape and toothed at the edge. Therefore, in theory, such factors may lead to differences in protein and other biochemical content (Barbarino and Lourenco, 2005).

In Malaysian waters, more than 386 taxa of marine algae (seaweeds) are found; Chlorophyta: 13 families, 102 taxa; Rhodophyta: 27 families, 182 taxa; Phaeophyta: 8 families, 85 taxa; and Cyanophyta: 8 families, 17 taxa (Phang, 2006; Phang, 2010). These seaweeds are found in various habitats ranging from rocky shores, coral reefs, sandy shores, mudflats, mangroves, to estuaries. In the area of West and East Coast of Sabah, there are about 58 taxa that have been recorded by Gilik (2004), including 25 species of Rhodophyta, 20 species of Chlorophyta and 13 species of Phaeophyta. In that study, the most abundant seaweed species were found in Semporna, Sabah, accounting for seventy-eight percent of overall seaweed species collected (39 species) compared to Tawau, Sandakan and Kudat, Sabah. In Sabah, some of the seaweed are collected and eaten either as raw or blanched in salads including the Rhodophytes *Gracilaria changii, Gracilaria tenuispitata*, *Eucheuma, Kappaphycus* species and the Chlorophytes *Caulerpa lentillifera* and *Caulerpa racemosa* (Gilik, 2004; Phang, 2006).

Considering that seaweed is part of the diet in many countries and constitute a source of beneficial nutrients, such as dietary fibre, minerals, vitamins, protein and lipids, its use as a healthy food should be investigated, as it might be important in the prevention or healing of many health problems (Bocanegra *et al*., 2009). Furthermore, to evaluate the functional properties of various seaweeds requires a clear idea about their biochemical composition, which can provide a platform for identification of the molecules responsible for various biological activities (Mendis and Kim, 2011). In order to pursue proteomics research in seaweed, this study took the first few steps necessary to determine crude protein, total soluble protein as a target compound, SDS-PAGE profile of seaweed proteins and total phenolic content as an interfering compound in fifteen different green, red and brown seaweeds that are found in Semporna, Sabah, Malaysia. The results will provide a better understanding of the sample preparation process for proteomics studies.

Materials and Methods

Sample collection and preparation

A total of 15 varieties of seaweed consisting of two green seaweeds *(Caulerpa lentillifera*

and *Caulerpa racemosa*), four brown seaweeds (*Sargassum polycystum*, *Hormophysa cuneiformis*, *Padina gymnospora* and *Turbinaria conoides*) and nine red seaweeds (*Kappaphycus alvarezii* var. aring-aring, *Kappaphycus alvarezii* var. green tambalang, *Kappaphycus striatum* var. sacol [katunai green], *Kappaphycus striatum* var. sacol [katunai brown], *Kappaphycus striatum* var. sacol [katunai yellow], *Eucheuma denticulatum* [var.yellow], *Gracilaria verrucosa*, *Laurencia* sp. [yellow] and *Laurencia* sp. [brown]) were collected from Semporna, Sabah in 2013 for analysis in the present study. The seaweed identification was guardedly confirmed based on morphological characteristics. Immediately after collection, the seaweed samples was carefully cleaned and washed with seawater to remove sand, debris, epiphytes and other extraneous matter and transported to the laboratory in an ice cooler box to maintain a low temperature (less than 15°C) and retain moisture during the journey. In the laboratory, the samples was cautiously sorted and then thoroughly cleaned by rinsing with distilled water and further dried by using tissue paper to remove excess water. The seaweed was cut into small pieces and unwanted parts removed using a razor blade. A cleaned sample was separated into two, and one portion was wrapped with aluminium foil and then immediately stored at -80°C for preservation until later use. The other portions (500 g) were thermally dried at 40±1°C until a constant weight for crude protein and total phenolic content analysis. The dried samples were homogenized to powder using a Waring blender, packed in airtight plastic bags covered with aluminium foil and stored at -20°C until further use.

Crude protein analysis

The crude protein content of the various seaweeds was determined according to the method described by AOAC (2000) with slight modifications as recommended for a Kjeltec 2300 apparatus (Foss Analytical, Denmark). Briefly, a 2 g sample was weighed into digestion tubes, then two Kjeltabs Cu 3.5 (catalyst salts) was added into each tube. About 12 mL of concentrated sulphuric acid (H2SO4) was carefully added into the tubes and then shaken gently. A digestion procedure was performed using a preheated (420°C) digestion block of InKjel 625M (Behr, Germany) for 60 minutes until a clear blue/green solution was obtained. Digested samples were cooled for 10-20 minutes. Distillation was then performed using a Kjeltec 2300 distillation unit (Foss, Denmark) and the percentage of protein was calculated by multiplying the percentage of nitrogen found by a factor of 4.86 (Diniz *et al.*, 2011). The samples and analysis were measured by six replicates and presented as mean ± standard deviation.

Acetone dried powder (AcDP) preparation

AcDP was prepared according to the method described by Wang *et al.* (2003) and Awang *et al.* (2010) with some modification to the first steps to efficiently eliminate the interfering compounds. The samples were frozen in liquid nitrogen and were ground to a fine powder using a pre-chilled mortar and pestle. This step disrupts cells and homogenizes tissues through the pressure and friction generated when a moving pestle pinches the samples against the wall of the mortar. The samples were ground directly in liquid nitrogen into a fine powder for 4 to 5 minutes. The resulting powder was weighed (± 5) g in a falcon tube and then re-suspended in 10 mL of ice-cold acetone and vortexed for 5 min. After centrifugation (10,000 × g, 5 min, 4°C), the supernatant was discarded. This step was performed five times and the resulting polyphenol-free white powder was air-dried. AcDP was stored at -80°C until protein extraction.

Total soluble protein extraction

The extraction of total soluble protein was performed according to Carpentier *et al.* (2005), Rodrigues *et al.* (2009) and Awang *et al.* (2010) with several modifications. A volume (5 mL) of extraction buffer (50 mM Tris–HCl pH 8.5, 100 mM KCl, 1% DTT, 30% w/v sucrose) was added to AcDP (0.5 g) and vortexed (30 s). Ice-cold Tris-buffered (pH 8.0) phenol (5 mL) was added to a sample mixture. The samples were then vortexed for 15 min at 4°C and centrifuged 10,000 × g for 3 min at 4°C. The phenolic phase was collected and re-extracted with an equal volume of extraction buffer. The phenolic phase after centrifugation was allowed to precipitate overnight with 5 times volume 100 mM ammonium acetate in methanol at -20°C. The supernatant was discarded after centrifugation (10,000 × g, 30 min at 4°C). The resulting pellet was washed twice in ice-cold acetone containing 0.2% DTT and between the two rinsing steps, the sample mixture was incubated for 1 hour at -20°C. The air-dried pellets were resuspended in 200 µL resolubilisation buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.2% Bio-Lyte 3-10 and 1% DTT). All chemicals at this stage are electrophoresis grade and were bought from BioRad, USA. The sample mixture was then vortexed (60 min) at an air conditioned room temperature (20°C). When necessary, samples were cooled on ice to prevent heating. The supernatant was collected after centrifugation (10,000 × g, 30 min at 20°C).

Estimation of total soluble protein concentration

The total soluble protein in the seaweed samples was determined using the Bradford assay with some modification (Bradford, 1976). Protein concentration was calculated according to the standard protein curve of bovine serum albumin (BSA). The absorbance at 595 nm was measured after 5 min in microtiter plates according to the manufacturer's instructions using a Thermo Scientific microplate reader (Multiskan Ascent V1.25 Plate Reader with Ascent Software version 2.6: Thermo Electron Corporation, Vantaa, Finland). The total soluble protein concentration was expressed as miligram per mililiter (mg/mL) of acetone dried powder samples with six replicates and presented as mean ± standard deviation.

Determination of total phenolic content

Total phenolic content (TPC) was determined by the Folin-Ciocalteu reagent in accordance with a protocol described by Singleton and Rossi (1965) with some modifications and using phloroglucinol as a standard compound. The sample extract (1 mL) was mixed with 5 mL of the Folin-Ciocalteu reagent (10% in distilled water in a test tube). After 5 min, 4 mL of 7.5% (w/v) Na_2CO_3 was added to each tube, the test tubes were cap-screwed and vortexed for 20 seconds. After incubation at room temperature for 2 hours in the dark, the absorbance of the reaction mixture was measured at 740 nm using a UV-Visible Spectrometer (Perkin Elmer) against a blank sample. TPC was expressed as milligram phloroglucinol equivalents per gram of dry weight sample (mg PGE/g dry weight). The samples were measured by six independent experiments and presented as mean ± standard deviation.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) profile

Protein-denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970) with some modifications using a Mini-PROTEAN Tetra Cell (Bio-Rad, USA) with a mini size (8.3 x 7.3 cm) and 1 mm thick gel. The amount of protein load to each well is fixed (1 µg) for all samples.

Protein visualization and imaging

The gels were visualized by silver staining as described by Berkelman and Stenstedt (1998) with minor modifications. Briefly, the gel was fixed overnight initially in a fixation solution containing 40% (v/v) ethanol and 10% (v/v) acetic acid. The gel was washed for 2 min and then sensitized for 20 min in a solution containing 30% (v/v) ethanol, 0.2% (w/v) sodium thiosulphate, 6.8% (w/v) sodium acetate and 0.125 % (v/v) glutaraldehyde, and then afterwards washed with distilled water (three times for 10 min each). The gel was stained for 20 min in 0.25% (w/v) silver nitrate with 0.015% (v/v) formaldehyde before washing with distilled water again (twice for 1 min each). The gel was developed in 2.5% (w/v) sodium carbonate containing 0.0074% (v/v) formaldehyde. The reaction was stopped with 1.5% (w/v) ethylenediaminetetraacetic acid, disodium salt. The stained gels were scanned with GS-800 calibrated densitometer using Quantity-One 1-D analysis software (Bio-Rad, USA). Electrophoresis was run in three independent experiments for three replicates of each variety of seaweed protein extract.

Statistical analysis

Data collected in this study was analysed using SPSS (Statistical Package for the Social Sciences) version 19.0. A one-way ANOVA test was used to compare differences in the means of the crude protein, soluble protein and total phenolic contents among different species and seaweed varieties. This was followed by a Tukey post-hoc analysis to further determine their differences. A significant difference was considered at the level of $p<0.05$. Meanwhile, Pearson's correlation test was used to correlate the crude protein, total soluble protein and total phenolic content values.

Results and Discussion

Crude protein content

The data regarding crude protein content in seaweeds from tropical environments are relatively limited (Fong *et al*., 2001; Lourenco *et al*., 2006) and previous results frequently show lower crude protein content in the subtropical coastal environments compared with other areas (Kaehler and Kennish, 1996; Wong and Cheung, 2000). In some Brazilian environments, Ramos *et al.* (2000) found that the percentage of crude protein (N × 6.25) in 14 seaweeds varied from 2.30 to 25.6% of dry weight. From this study, the range of crude protein content in all three different classes of seaweeds varied and ranged from 3.99% to 13.18% dry weight in red seaweeds followed by green seaweeds (8.44% to 10.22% dry weight) and brown seaweeds (4.63% to 5.70% dry weight) (Figure. 1). The red seaweeds, *Laurencia* sp. (var. yellow) contained the highest ($p<0.05$) amount of crude protein (13.18% dry weight) followed by *Laurencia* sp. (var. brown) (11.64% dry weight) and both were significantly different ($p<0.05$) from the others. Next were the green variety of *Caulerpa*

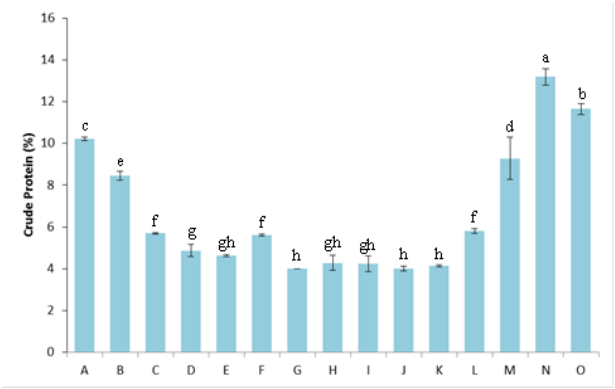

Figure 1. Protein content (% dry weight) of fifteen seaweed samples. A *Caulerpa lentillifera*, B *Caulerpa racemosa*, C *Sargassum polycystum*, D *Hormophysa cuneiformis*, E *Padina gymnospora*, F *Turbinaria conoides*, G *Kappaphycus alvarezii* (var. aring-aring), H *Kappaphycus alvarezii* (var. green tambalang), I *Kappaphycus striatum* var. sacol (katunai green), J *Kappaphycus striatum* var. sacol (katunai brown), K *Kappaphycus striatum* var. sacol (katunai yellow), L *Eucheuma denticulatum* (var.yellow), M *Gracilaria verrucosa*, N *Laurencia* sp. (var. yellow) and O *Laurencia* sp. (var. brown). Values presented as mean ± standard deviation (n=6) and different letters indicate significant differences between samples at $p<0.05$.

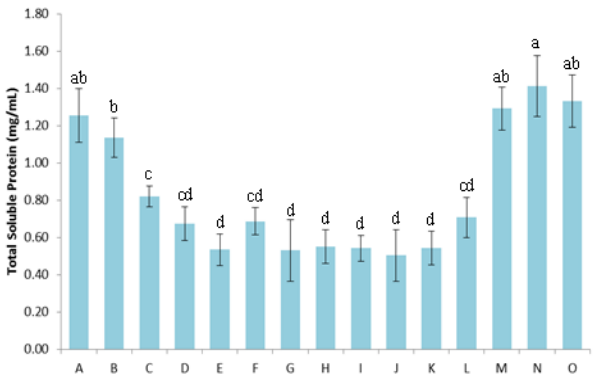

Figure 2. Total soluble protein (mg/mL) of fifteen seaweed samples. A *Caulerpa lentillifera*, B *Caulerpa racemosa*, C *Sargassum polycystum*, D *Hormophysa cuneiformis*, E *Padina gymnospora*, F *Turbinaria conoides*, G *Kappaphycus alvarezii* (var. aring-aring), H *Kappaphycus alvarezii* (var. green tambalang), I *Kappaphycus striatum* var. sacol (katunai green), J *Kappaphycus striatum* var. sacol (katunai brown), K *Kappaphycus striatum* var. sacol (katunai yellow), L *Eucheuma denticulatum* (var.yellow), M *Gracilaria verrucosa*, N *Laurencia* sp. (var. yellow) and O *Laurencia* sp. (var. brown). Values presented as mean ± standard deviation (n=6) and different letters indicate significant differences between samples at $p<0.05$

lentillifera (10.22 % dry weight), red variety of *Gracilaria verrucosa* (9.28% dry weight) and green variety of *Caulerpa racemosa* (8.44% dry weight). They were also significantly different ($p<0.05$) from each other and the rest. This was then followed by *Eucheuma denticulatum* (var. yellow) (5.80% dry weight), *Sargassum polycystum* (5.70% dry weight) and *Turbinaria conoides* (5.61% dry weight), which were not significantly different ($p>0.05$) from each other, but significantly different ($p<0.05$) from the rest. Among the brown seaweed samples, *Sargassum polycystum* and *Turbinaria conoides* contained the highest ($p<0.05$) amount of crude protein followed by *Hormophysa cuneiformis* (4.87% dry weight) and *Padina gymnospora* (4.63% dry weight). Meanwhile, the other samples that showed no significant difference ($p>0.05$) in crude protein content and also contained the lowest amount of crude protein were *Kappaphycus alvarezii* (green tambalang) (4.29% dry weight), *Kappaphycus striatum* var. sacol (katunai green) (4.24% dry weight), *Kappaphycus striatum* var. sacol (katunai yellow) (4.14% dry weight), *Kappaphycus striatum* var. sacol (katunai brown) (4.02% dry weight) and *Kappaphycus alvarezii* (aring-aring) (3.99% dry weight).

In this study, our data for crude protein content in seaweed was in accordance with the information that available in the literature (e.g. Wong and Cheung, 2001; McDermid and Stuercke, 2003; Burtin, 2003; Marinho-Soriano *et al*., 2006; Matanjun *et al*., 2009; Polat and Ozogul, 2009; Holdt and Kraan, 2011) indicating that crude protein content differs according to species, and is generally low in brown seaweeds (5.96% to 7.33% dry weight) compared with green or red seaweeds (5.14 to 16.96% dry weight). These differences may be due to species composition (Fleurence, 1999), taxonomic traits and species-specific differences of seaweeds in taking up dissolved nutrients (Martinez-Aragon *et al*., 2002). The mean crude protein content of both the red and green seaweeds (with the exception of *Eucheuma denticulatum* (var.yellow), *Kappaphycus alvarezii* (var. aring-aring and green tambalang) and *Kappaphycus striatum* var. sacol (katunai green, yellow and brown) were significantly higher ($p<0.05$) compared to the brown seaweeds. Burtin (2003) reported that the crude protein content in brown seaweeds are generally lower (ranging from 5 to 15% of dry weight) compared to red and green seaweeds (ranging from 10 to 30%). A similar range of crude protein content in seaweeds was also reported by Chakraborty and Santra (2008) and Manivannan *et al.* (2009). In general, researchers have indicated predominantly low concentrations of crude protein content in seaweed compared to the terrestrial plants. This trend may be related to the natural characteristics of marine environments; predominantly oligotrophic, with low availability of N (Oliveira *et al.*, 1997; Ovalle *et al*., 1999). As a consequence, low concentrations of protein would be

accumulated by some natural seaweed populations.

From the literature, most of protein from seaweed contain almost all of the essential amino acids. For example, in *Enteromorpha* spp., 9 of the 10 essential amino acids for vertebrates were discovered at higher quantity that in an equivalent content of as in soy beans (Aguilera-Morales *et al.*, 2005). Some very important bioactive proteins can be extracted from seaweeds, including lectins, which bind with carbohydrates and participate in many biological processes like intercellular communication. Seaweeds also have anti-bacterial, anti-viral or anti-inflammatory activities (Mohamed *et al.*, 2012). Wong and Cheung (2000) and Matanjun *et al*. (2009) also found that most seaweed proteins contained all the essential amino acids at levels close to that recommended by FAO/WHO. However, the protein content in seaweed varied according to the season and the species (Fleurence, 1999; Murata and Nakazoe, 2001).

Total soluble protein concentration

The total soluble protein concentrations of 15 varieties of seaweed samples ranged from 0.52 mg/mL to 1.45 mg/mL (Figure 2). Total soluble protein concentration of the extracts from nine different varieties of red seaweed showed variation from 0.52 to 1.45 mg/mL. *Laurencia* sp. (var. yellow) was found to have the highest ($p<0.05$) soluble protein concentration (1.45 mg/mL) followed by *Laurencia* sp. (var. brown) (1.37 mg/mL), *Gracilaria verrucosa* (1.33 mg/mL), *Eucheuma denticulatum* (var.yellow) (0.73 mg/mL), *Kappaphycus alvarezii* (green tambalang) (0.57 mg/mL), *Kappaphycus striatum* var. sacol (katunai yellow) (0.56 mg/mL), *Kappaphycus striatum* var. Sacol (Katunai green) (0.55 mg/mL), *Kappaphycus alvarezii* (aring-aring) (0.54 mg/mL) and *Kappaphycus striatum* var. sacol (katunai brown) (0.52 mg/mL). They were no significant differences between the variety of *Kappaphycus alvarezii* and *Kappaphycus striatum*. The highest ($p<0.05$) soluble protein concentration between two species of green seaweed was *Caulerpa lentillifera* (1.29 mg/mL) and *Caulerpa racemosa* (1.16 mg/mL). Meanwhile, the highest ($p<0.05$) soluble protein content among four species of brown seaweed was *Sargassum polycystum* (0.84 mg/mL), followed by *Turbinaria conoides* (0.71 mg/mL), *Hormophysa cuneiformis* (0.69 mg/mL) and *Padina gymnospora* (0.55 mg/mL), respectively. There was significant difference in soluble protein value ($p<0.05$) among these brown seaweeds, except for *Turbinaria conoides* and *Hormophysa cuneiformis*, which showed no significant difference between similar classes.

A comparison of the total soluble protein concentration among seaweeds is difficult because of methodological differences (Berges *et al*., 1993). One of the main problems with protein analysis in seaweed is the protein extraction, done with different degrees of success by various researchers (Fleurence *et al*., 1995). Differences in the cell wall composition of algae and in procedures for protein extraction can establish strong and negative effects on the final results (Fleurence, 1999). Sample preparation is one of the most critical steps in gaining high-quality resolution of proteins in proteomic analysis, yet it can be challenging (Görg *et al*., 2000). Proteins isolated from seaweed and other plant tissues are often difficult to resolve by two-dimensional gel electrophoresis (2-DE) analysis due to the abundance of secondary metabolites (Wu *et al.,* 2014). Normally, protein extraction starts with breaking of the protective cell wall and plasma membrane. As such, there is a definite release of other intracellular components that interfere with subsequent proteomic analyses, such as polyphenols, polysaccharides, proteases, lipids, and numerous secondary metabolites. Obviously, these components have to be removed from the protein sample.

Carpentier *et al.* (2005) compared several extraction protocols that deal with this problem and found that phenol extraction was the most preferable. Saravanan and Rose (2004) also proved that phenol extraction is better when dealing with recalcitrant plant tissue. To the best of our knowledge, the present work describes for the first time, a phenol extraction method for the extraction of proteins from fifteen varieties of seaweed found in Semporna, Sabah, Malaysia. The preliminary results indicated that the phenol extraction method gave significantly higher protein concentration than that obtained using other methods for *Caulerpa lentillifera* (data not shown). This higher protein concentration may be attributed to the use of phenol as efficient solvent for protein, which can minimize molecular interactions between proteins and other compounds (Wang *et al*., 2003). Recent reports have also demonstrated similar observations indicating that phenol-based methods could generate higher protein yield than that produced using TCA methods (Vincent *et al*., 2006).

Total phenolic content

The variation of phenolic content was quite large and significant differences were found ($p<0.05$) among different seaweed species, ranging from 8.59 to 48.98 mg PGE/ g dry weight (Figure 3). This study indicated that both green seaweeds and brown seaweeds (not including *Hormophysa cuneiformis*)

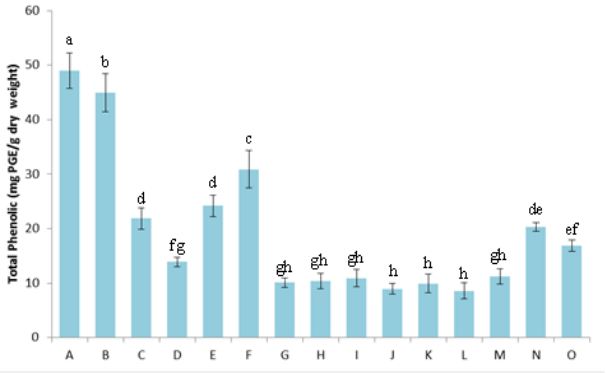

Figure 3. Total Phenolic content (mg PGE/gm dry weight) of fifteen seaweed samples. A *Caulerpa lentillifera*, B *Caulerpa racemosa*, C *Sargassum polycystum*, D *Hormophysa cuneiformis*, E *Padina gymnospora*, F *Turbinaria conoides*, G *Kappaphycus alvarezii* (var. aring-aring), H *Kappaphycus alvarezii* (var. green tambalang), I *Kappaphycus striatum* var. sacol (katunai green), J *Kappaphycus striatum* var. sacol (katunai brown), K *Kappaphycus striatum* var. sacol (katunai yellow), L *Eucheuma denticulatum* (var.yellow), M *Gracilaria verrucosa*, N *Laurencia* sp. (var. yellow) and O *Laurencia* sp. (var. brown). Values presented as mean ± standard deviation (n=6) and different letters indicate significant differences between samples at $p<0.05$.

contained higher amounts ($p<0.05$) of polyphenols than red seaweeds. Green seaweeds *Caulerpa lentillifera* (48.98 mg PGE/ g dry weight) showed the highest ($p<0.05$) total phenolic content followed by Caulerpa racemosa (44.91 mg PGE/ g dry weight) and both were significantly different ($p<0.05$) between them and from the others. Meanwhile, for brown seaweed *Turbinaria conoides* (30.90 mg PGE/ g dry weight) showed the highest ($p<0.05$) total phenolic content followed by *Padina gymnospora* (24.18 mg PGE/ g dry weight), *Sargassum polycystum* (21.84 mg PGE/ g dry weight) and *Hormophysa cuneiformis* (13.93 mg PGE/ g dry weight). Among red seaweeds, *Laurencia* sp. (var. yellow) (20.31 mg PGE/ g dry weight) was found to have the highest ($p<0.05$) total phenolic content followed by *Laurencia* sp. (var. brown) (16.82 mg PGE/ g dry weight) and both were not significantly different ($p>0.05$) between them but showed significantly different ($p<0.05$) from the others. Next, followed by *Gracilaria verrucosa* (11.27 mg PGE/ g dry weight), *Kappaphycus striatum* var. sacol (katunai green) (10.90 mg PGE/ g dry weight), *Kappaphycus alvarezii* (var. green tambalang) (10.39 mg PGE/ g dry weight), *Kappaphycus alvarezii* (var. aring-aring) (10.09 mg PGE/ g dry weight), *Kappaphycus striatum* var. sacol (katunai yellow) (9.90 mg PGE/ g dry weight), *Kappaphycus striatum* var. sacol (katunai brown) (8.94 mg PGE/ g dry weight) and *Eucheuma denticulatum* (var.yellow) (8.59 mg PGE/ g dry weight), respectively, and were not significantly different ($p>0.05$) between each other.

This study also indicated that total phenolic content was significantly different ($p<0.05$) among the seaweed species. The range of total phenolic content in all three different classes of seaweeds varied and ranged from 44.91 to 48.98 mg PGE/ g dry weight in green seaweeds followed by brown seaweeds (13.93 to 30.90 mg PGE/ g dry weight) and red seaweeds (8.59 to 20.31 mg PGE/ g dry weight). Similar findings have been reported where the total phenolic content varies with species and generally that the green seaweeds have higher free-radical scavenging properties, followed by brown seaweeds, then red seaweeds (Santoso *et al*., 2004; Duan *et al*., 2006; Chandini *et al*., 2008; Matanjun *et al*., 2008). The findings for total phenolic content of various seaweeds in this study can be used for further research on seaweed antioxidant properties. As such, the selection of a suitable extraction solvent is an important factor for obtaining phenolic compounds in seaweed. Therefore, additional study can be done to select the best solvent and subsequently the determination of their antioxidant activities. Further analysis is necessary because the antioxidant activity of extracts from seaweed is not directly correlated with total phenolic content (Lim *et al*., 2002; Chandini *et al*., 2008).

Pearson correlation analyses

Pearson correlation analyses were used to determine relationships between variables (Figure 4). Results indicated that crude protein content in the fifteen varieties of seaweed samples showed a strong positive correlation with their total soluble protein ($r = 0.923$) as compared to a moderate positive correlation with their total phenolic content ($r = 0.417$). Meanwhile, the correlation between their total soluble protein concentration and total phenolic content also showed a positive correlation, but also with moderate strength ($r = 0.431$). All correlations were significant at the 0.01 level (2-tailed). Strong positive relationships between crude protein content and total soluble protein of these fifteen varieties of seaweed ($r = 0.923$; $p=0.001$), indicated that a higher soluble protein yield may be obtained after the extraction process for those variety of seaweed with a higher crude protein content compared to seaweed with a lower amount of crude protein content. Therefore, crude protein content can be a very useful indicator for estimating the amount of seaweed sample required during a single extraction process in order to have a sufficient amount of protein for 2-DE analysis. The correlation of total phenolic content with protein

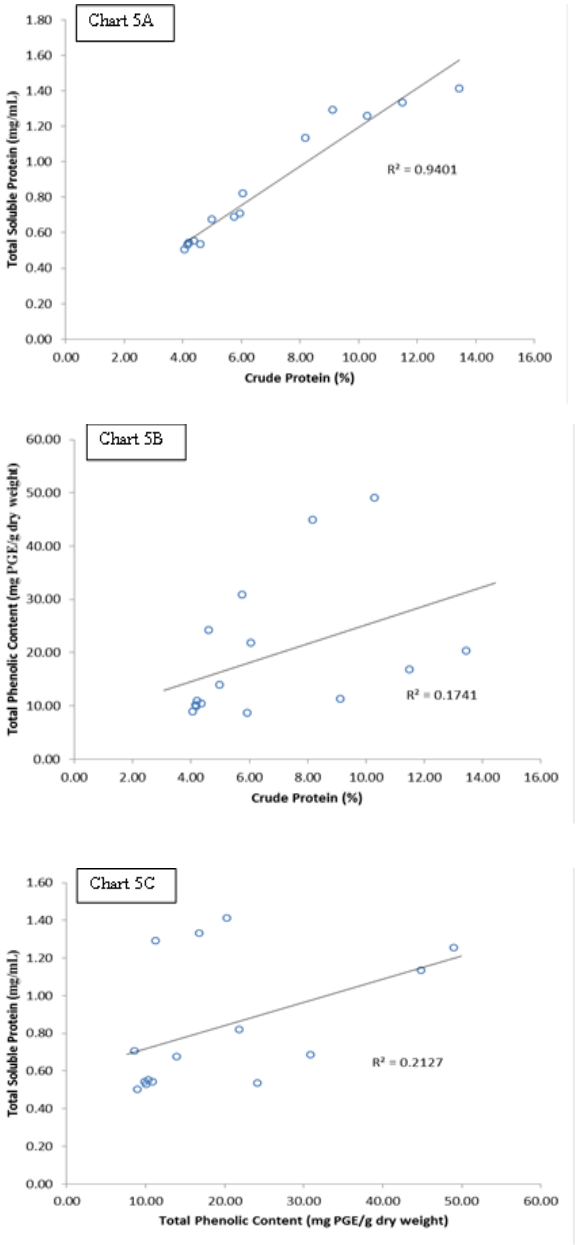

Figure 4. Relationship between crude protein and total soluble protein values of fifteen seaweed samples. A: the relationship between crude protein and total soluble protein; B: the relationship between crude protein and total phenolic content; C: the relationship between total phenolic content and total soluble protein concentration.

content and total soluble protein concentration are also illustrated in Figure 4, respectively, and both were significantly positive correlated, but with moderate strength. In line with this finding, some precautions have to be made during the extraction of protein from seaweed with high protein content as it may also have a high amount of total phenolic compound, which can interfere with subsequent protein separation and identification (Contreras-Porcia *et al*., 2008).

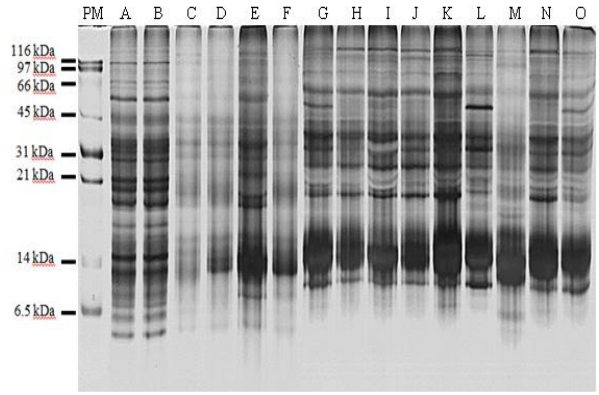

Figure 5. SDS-PAGE gels of proteins from fifteen seaweed samples. lane A; *Caulerpa lentillifera*, lane B; *Caulerpa racemosa*, lane C; *Sargassum polycystum*, lane D; *Hormophysa cuneiformis*, lane E; *Padina gymnospora*, lane F; *Turbinaria conoides*, lane G; *Kappaphycus alvarezii* (var. aring-aring), lane H; *Kappaphycus alvarezii* (var. green tambalang), lane I; *Kappaphycus striatum* var. sacol (katunai green), lane J; *Kappaphycus striatum* var. sacol (katunai brown), lane K; *Kappaphycus striatum* var. sacol (katunai yellow), lane L; *Eucheuma denticulatum* (var. yellow), lane M; *Gracilaria verrucosa*, lane N; *Laurencia* sp. (var. yellow) and lane O; *Laurencia* sp. (var. brown). In total, 1 µg of extracted protein was loaded and the 12.5 % SDS-PAGE gels were stained with silver staining. The molecular mass of protein standards are indicated on the left (PM).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) rofile

General features of SDS-PAGE analyses of seaweed protein extracts revealed that a majority showed patterns containing approximately 10 to 25 discrete bands with molecular weights of 6.5 to 116 kDa (Figure 5). Comparable patterns of protein bands were observed between lane A and lane B of green seaweeds C*aulerpa lentillifera* and *Caulerpa racemosa*, but both band arrangements were different from others. Meanwhile, brown seaweeds were shown in lane C; *Sargassum polycystum*, lane D; *Hormophysa cuneiformis*, lane E; *Padina gymnospora* and lane F; *Turbinaria conoides*. From the observation, lane C, lane D and lane F showed lower intensity, with low background compared to the lane E. Meanwhile, the SDS-PAGE profile of red seaweed were shown from lane G to lane O, from lane G; *Kappaphycus alvarezii* (var. aring-aring), lane H; *Kappaphycus alvarezii* (var. green tambalang), lane I; *Kappaphycus striatum* var. sacol (katunai green), lane J; *Kappaphycus striatum* var. sacol (katunai brown), lane K; *Kappaphycus striatum* var. sacol (katunai yellow), lane L; *Eucheuma denticulatum* (var.yellow), lane M; *Gracilaria verrucosa*, lane N; *Laurencia* sp. (var. yellow) and lane O; *Laurencia* sp. (var. brown), respectively. Most of the bands were

present, but their intensity varied among the extracts of red seaweeds.

In general, the SDS-PAGE profile of protein extracts for all the seaweed samples showed that the protein bands were resolved clearly without having too much smearing and with not too much background (Figure 5, lanes A-O), suggesting that phenol extraction method might be applicable to a wide range of seaweed species. Bands containing abundant proteins were observed in all the seaweed species groups, indicating that common proteins are shared among these plants. Band sizes with low molecular weight (< 15 kDa), however, varied, revealing the differences in types of protein among various seaweed samples. Significant differences in term of protein bands were observed in the electrophoretic patterns among three different classes of seaweed (green, red and brown seaweed).

Conclusions

The outcome of the present study reveals that protein and total phenolic content are diverse depending on the seaweed species. Strong positive relationships between crude protein content and total soluble protein of these fifteen varieties of seaweed indicated that a higher soluble protein yield may be obtained after the extraction process for those variety of seaweed with a higher crude protein content compared to seaweed with a lower amount of crude protein content. Therefore, crude protein content can be a very useful indicator for estimating the amount of seaweed sample required during a single extraction process in order to have a sufficient amount of protein for 2-DE analysis. As crude protein content increases, total phenolic content is also seem to increase. Thus, suitable method of extraction is needed that can eliminate phenolic and other non-protein interfering compounds. Further studies are necessary to identify and characterize the protein, both structurally and functionally, by proteomic methods. These investigations are now in progress.

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