

Comparison of Protein Extraction Protocols for Proteomic Analysis of Red Algae, *Eucheuma cottonii*

Lim, H. T.* and Teo, S. S.

UCSI University, Kuala Lumpur Campus (South Wing), No. 1, Jalan Menara Gading, UCSI Heights, 56000 Cheras, Kuala Lumpur, Malaysia

ABSTRACT

Eucheuma cottonii is a red seaweed of the family *Rhodophyceae*, which is mainly harvested in South East Asia. *E. cottonii* has long been explored as a major source of kappa carrageenan, which is of great economic and industrial importance. The proteomics of marine macroalgae is always a tough task due to low protein concentration and a greater possibility for the co-extraction of contaminants such as anionic polysaccharides, polyphenols and salts, which may massively deteriorate the resolving power of SDS-PAGE and current proteomic tools. The main objective of this study is to compare three different phenol based protein extraction protocols: (i) Phenol/lysis buffer extraction, (ii) TRI reagent/chloroform extraction, and (iii) Phenol/SDS buffer extraction in terms of total protein yields and resolving patterns of single dimension SDS-PAGE. Among the three phenol based extraction protocols, the phenol/lysis buffer produced slightly higher protein yields (0.027 ± 0.000 mg/g), followed by the phenol/SDS buffer (0.024 ± 0.002 mg/g) and the TRI reagent/chloroform (0.018 ± 0.001 mg/g). In SDS-PAGE, all the three extraction protocols showed clear protein profiles, with several intense protein bands observed from 27 kDa to 158 kDa. The phenol/SDS buffer extraction protocol was recommended for proteome study of *E. cottonii* as it is the cheapest and relatively easy in preparation as compared to the other two protocols in this study. This study represents an initial attempt to study the proteome of *E. cottonii*. Further proteomic works, such as two-dimensional gel electrophoresis, mass spectrometry and protein crystallography, can be carried out in the near future. A successful establishment of *E. cottonii* proteome is important to facilitate the exploration of *E. cottonii*

and other carrageenan rich seaweed species due to its agricultural significance.

Keywords: *Eucheuma cottonii*, 1DE, Macroalgae proteomics, Phenol based protein extraction

ARTICLE INFO

Article history:

Received: 25 March 2014

Accepted: 4 December 2014

E-mail addresses:

hooting58@gmail.com (Lim, H. T.),

teoss@ucsiuniversity.edu.my (Teo, S. S.)

* Corresponding author

INTRODUCTION

Eucheuma cottonii is an eye-catching element of the marine flora in Southeast Asia. It is abundantly cultivated as a commercial source of carrageenan (Bixler, 1996). Carrageenans are widely used in the food and pharmaceutical industries due to their precious value as stabilizers, viscosity modifiers and gelling agents. *E. cottonii* is a good source of kappa-carrageenans, which can form strong gels in the presence of potassium ions and are widely employed as a suspending, thickening and gelling agent in certain food such as chocolate, milk, ice cream, convenience foods, puddings, etc. Pharmaceutical applications of kappa-carrageenan include suspension of barium sulfate and antibiotics (Parker, 1974). It also contains high amounts of dietary fibres, minerals, vitamins, antioxidants, polyphenols, phytochemicals, proteins and polyunsaturated fatty acids (Matanjun *et al.*, 2009).

E. cottonii belongs to the genus that is known in commerce as “*cottonii*”. The term “*cottonii*” refers to the seaweed that produces kappa carrageenan (Santos, 1989). *Eucheuma* species are hardy, multibranched, non-rooting algae which grow attached by holdfasts to dead coral. *Eucheuma* species reproduce both vegetatively and by sporulating. They can be sectioned anywhere and re-growth will occur in all sections (Parker, 1974). This feature makes *Eucheuma* species adaptable to different environments in various forms. Due to morphological plasticity and lack of defined morphological characteristics, the

taxonomic classification of the *Eucheuma* species is confused with the *Kappaphycus* species, with the expansion of commercial cultivation that triggered the misapplication of commercial and scientific names. Until very recently, Liu *et al.* (2013) showed that the 18S rDNA sequence can separate *Kappaphycus* from *Eucheuma* species. Nevertheless, further identification of *Eucheuma* and *Kappaphycus* at the inter-species level is still unclear.

Genomics concerns the generation and analyses of nucleotide sequences of the full or near-full genome, as well as cDNA collections. One step beyond the raw sequence data which is provided by genomics, transcriptomics, offers a better view of the active components in the cell by providing information on the presence and relative abundance of RNA transcripts. Proteomics is complementary to genomics and transcriptomics as it provides additional information on protein expression, protein–protein interaction, organization of proteins in multi-protein complexes and their localisation in tissues (Jamers *et al.*, 2009).

The earlier proteome studies mostly revolved around the microbial and animal world. The proteomics of the kingdom Plantae is relatively rare. This is particularly true for the proteome study of marine algae, which is presumably due to the difficulty in the extraction of algae proteins. The cell walls of algae, especially those of multicellular macroalgae, are often heavily impregnated with polysaccharides such as cellulose, pectin, mannan, glucomannan, xyloglucan, carrageenan, etc. (Popper *et*

al., 2011). This makes protein extraction of macroalgae extremely tough, as co-extraction of polysaccharides may result in a smearing effect that deteriorates the resolution of gel analysis. Furthermore, due to the inherent endogenous proteolytic activity of macroalgae, the protein yields of macroalgae are often very low, resulting in a poor resolution gel image and insufficient protein samples for subsequent analysis.

In any proteome study, the most critical step is sample preparation and extraction. As mentioned above, proteomics analysis of algae tissues is typically more problematic and troublesome than of other biological systems. In this regard, several protocols have been developed for protein extraction from macroalgae, which included urea extraction (Contreras *et al.*, 2008), Tris-buffer extraction, TCA/acetone extraction and phenol extraction (Wong *et al.*, 2006; Contreras *et al.*, 2008; Nagai *et al.*, 2008). Among these, phenol extraction proved to be the most efficient protocol for protein extraction from algae tissues (Wong *et al.*, 2006; Contreras *et al.*, 2008; Nagai *et al.*, 2008).

In the present study, three phenol based extraction protocols; (i) phenol/lysis buffer extraction, (ii) phenol (TRI reagent)/chloroform extraction, and (iii) phenol/dense SDS extraction were utilised for protein extraction from *E. cottonii*. The aim of this study was to compare the effectiveness of protein extraction protocols by producing a good quality one dimensional gel electrophoresis (1DE) profile of *E. cottonii* and to enhance protein

yields of *E. cottonii*. A high quality gel based protein profile produces a gel image with clear protein bands and without significant smearing. As of this writing, no proteomic work has been carried out on *E. cottonii*. Hence, this study is important in assisting in the choice of a suitable protein extraction protocol for up-scaling the proteomic analysis of *E. cottonii* and other carrageenan-rich seaweed species in the near future.

MATERIALS AND METHODS

Sample Preparation

Seaweed samples (*E. cottonii*) were delivered in dried form from the east coast of Sabah to UCSI University. In order to minimise possible variations in protein contents in different samples, all of the seaweed samples were harvested at the same time from the ocean. Within 48 hours of harvest, all the seaweed samples were air-dried at 35°C for about three days. The dried *E. cottonii* samples were kept in a cabinet until used. Prior to protein extraction, the seaweed samples were carefully chosen and those with suspected endoparasitic infections and others with unknown defects or infections were thrown. Then, each seaweed sample was rinsed with tap water thrice and with ultrapure water (Merck Millipore) another three times. By using a pestle and a mortar, the seaweed sample (whole plant) was ground into fine powder in liquid nitrogen. Finally, about 5 g of the resulting powder was placed in a 50mL falcon tube for each subsequent extraction.

Protein Extraction Protocols

In this study, a total of three protein extraction protocols; (i) phenol/lysis buffer extraction, (ii) phenol (TRI reagent)/chloroform extraction and (iii) phenol/dense SDS extraction, were carried out to compare 1DE protein profiles of *E. cottonii*. Each protein extraction protocol was done in triplicates.

Phenol/lysis Buffer Extraction

This protocol was based on Contreras *et al.* (2008) with some modifications. About 5g of *E. cottonii* powders were suspended in 15 mL of lysis buffer (1.5% w/v polyvinylpyrrolidone [PVP], 0.7 M sucrose, 0.1 M potassium chloride, 0.5 M Tris-HCl pH 7.5, 250 mM EDTA, 20 µL/mL complete protease inhibitor cocktail, 2% v/v 2-mercaptoethanol and 0.5% w/v CHAPS) and homogenised for 15 minutes. An equal volume of Tris-HCl saturated phenol, pH 7.9, was added, and the mixture was re-homogenised for another 15 minutes. The mixture was centrifuged at 10000 g for 15 minutes and the upper phenol phase was transferred into a new 50 mL falcon tube. The lower phase was re-extracted using an equal volume of Tris-HCl saturated phenol, pH7.9. The mixture was centrifuged at 10000 g for 15 minutes, and again the upper phenol phase was transferred into the previous 50 mL falcon tube. For protein precipitation, five volumes of 0.1 M methanolic ammonium acetate were added to the phenol phase and the mixture was incubated at -20°C overnight. After that, the mixture was centrifuged at 10000 g for

15 minutes, the supernatant was discarded, and the protein pellet was rinsed in 0.1 M methanolic ammonium acetate at -20°C for 20 minutes. The resulting protein pellet was rinsed with 4 volumes of 80% v/v ice-cold acetone and once in cold acetone containing 20 mM DTT.

TRI Reagent / Chloroform Extraction

This protocol was based on Wong *et al.* (2006) and the manufacturer's recommendation with some modifications. About 5 g of seaweed powders was added into 15 mL of TRI reagent. The mixture was homogenised at room temperature for 10 minutes. Three (3) mL of chloroform was added. The mixture was shaken vigorously for 15 seconds and incubated at room temperature for 10 minutes. The mixture was centrifuged at 10000 g for 15 minutes. The upper aqueous phase was discarded. Six (6) mL of 100% ethanol was then added. The mixture was mixed by inversion and incubated at room temperature for 5 minutes. The mixture was centrifuged at 8000 g, 4 °C for 5 minutes. The pinkish organic layer was transferred into a new 50 mL falcon tube and 22.5 mL of isopropanol was then added for protein precipitation. The organic layer-isopropanol mixture was incubated at room temperature for about 45 minutes. The mixture was centrifuged at 10000 g, 4°C for 15 minutes. The supernatant was removed and the protein pellet was washed with 25 mL of 95% ethanol containing 0.3 M guanidine hydrochloride and 2.5% v/v glycerol and centrifuged at 10000 g, 4°C for 2 minutes. The washing process was

repeated twice. Finally, the protein pellet was rinsed once in 25 mL of 100% ethanol.

Phenol/SDS Buffer Extraction

This protocol was based on Nagai *et al.* (2008) with some modifications. About 5 g of seaweed powders was added to 15 mL of SDS buffer (0.1M Tris-HCl buffer, pH8.0, 30% w/v sucrose, 2% w/v SDS and 5% v/v 2-mercaptoethanol). The mixture was homogenised for about 15 minutes. An equal volume of Tris-HCl saturated phenol (pH7.9) was added, and the mixture was re-homogenised for another 15 minutes. The mixture was centrifuged at 1000 g for 15 minutes, and the upper phenol phase was transferred into a new 50 mL falcon tube. The lower phase was re-extracted using an equal volume of Tris-HCl saturated phenol (pH7.9). The mixture was centrifuged at 10000 g for 15 minutes, and once again, the upper phenol phase was transferred into the previous 50 mL falcon tube. For protein precipitation, five volumes of 0.1 M methanolic ammonium acetate were added to the phenol phase and the mixture was incubated at -20°C overnight. After that, the mixture was centrifuged at 10000 g for 15 minutes, the supernatant was discarded, and the protein pellet was rinsed in 0.1 M methanolic ammonium acetate at -20°C for 20 minutes. The resulting protein pellet was rinsed with 4 volumes of 80% v/v ice-cold acetone and once in cold acetone containing 20 mM DTT.

Protein Quantification

All the protein extracts were solubilised in 40 mM Tris-HCl. The concentration of each sample was estimated using the Bradford microassay (Quick Start Bradford Protein Assay, Biorad). In this study, bovine serum albumin (BSA) was used as a protein standard in the estimation of protein content in different extracts of *E. cottonii*. A series of protein standards (1.25 - 10µg/mL) was prepared in triplicate. Each protein sample was diluted 100 fold (triplicates): 1.5µL stored sample with 148.5µL ultrapure water. Then, 150µL of each protein sample and the BSA standard were mixed with 150µL of Bradford reagent (Biorad) in a final volume of 300µL. All the mixtures were incubated at room temperature for about five minutes. The absorbance value for each mixture was measured at 595nm using a spectrophotometer (Techne 6320D). Table 1 shows the absorbance values of BSA standards in five different concentrations.

TABLE 1
Absorbance value of BSA standards at 595nm

BSA protein standards	
Concentration (µg/mL)	Absorbance value at 595nm (A_{595})
1.25	0.054±0.002
2.5	0.100±0.003
5	0.206±0.002
7.5	0.294±0.002
10	0.370±0.002

Results are means ± SD (n = 3)

A calibration curve was plotted using Microsoft Excel. Using the calibration curve, the protein concentration of each sample was determined. The absorbance value at 595 nm is directly proportional to the amount of solubilised protein in each sample. For protein quantification, 100-fold dilution was carried out for each of the protein sample.

Fig.1 presents a calibration graph obtained using a series of BSA standards. BSA is commonly used as a protein standard because it is inexpensive and readily available in pure form (Kruger, 2002). Due to the ubiquity of BSA as a protein standard, it allows the results of this study to be compared directly to those of many previous studies.

One Dimensional Gel Electrophoresis (1DE)

The gel unit with glass sandwich set was assembled in casting mode with 1.0 mm

spacers in place. 13% separating gel (3.1 mL of distilled water, 4.3 mL of 30% acrylamide /bis, 2.5 mL of 1.5 M Tris-HCl, pH8.8, 0.1 mL of 10% w/v SDS, 50 µL of 10% w/v ammonium persulfate and 5 µL of TEMED) was prepared. The solution was gently swirled in the beaker for proper mixing, and applied into the glass sandwich as soon as possible. One (1) mL of ultrapure water was immediately applied on top of the gel to prevent the formation of curved meniscus. The ultrapure water was then decanted when the separating gel polymerised. A 4% stacking gel (6.1 mL of distilled water, 1.3 mL of 30% acrylamide/bis, 2.5 mL of 0.5 M Tris-HCl, pH6.8, 0.1 mL of 10% w/v SDS, 50 µL of 10% w/v ammonium persulfate and 10 µL of TEMED) was prepared. The solution was gently swirled for proper mixing and was then applied into the glass sandwich as soon as possible. The Teflon comb was inserted carefully and time was allowed for gel polymerization. When the gel

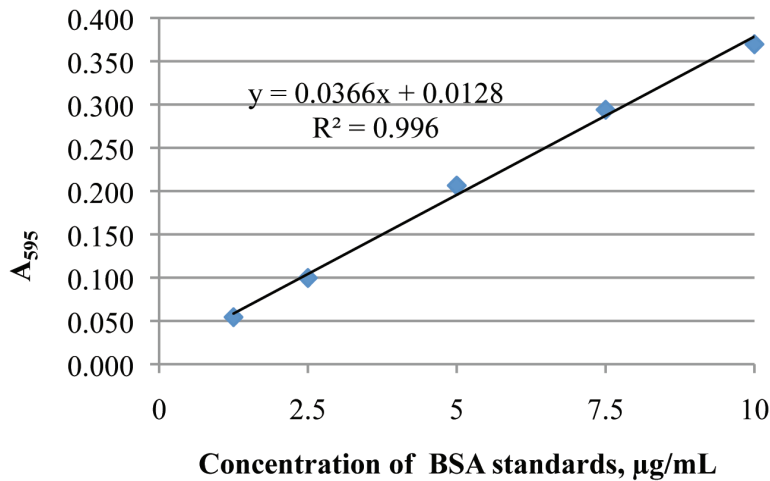


Fig.1: A calibration graph of BSA standards for protein quantification of *E. cottonii* extracts

polymerised, the Teflon comb was removed. The wells were immediately rinsed with running buffer. The gel was removed from its casting stand and was assembled into the gel electrophoresis slab unit. The wells and chamber were filled with running buffer. 10 µg of each protein sample was mixed with 25 µL of SDS sample buffer. Then, all of the samples and 7 µL of NEB protein marker (2-212 kDa) were heated at 95°C for four (4) minutes. About 40 µL of sample mixture and 7 µL of protein marker were loaded into the wells. The gel electrophoresis system was connected to a power source, and a constant voltage of 200 V was applied for about 45 minutes until the tracking dye reached the end of the separating gel. The gel was removed carefully and rinsed with distilled water thrice. The gel was then soaked in 50 mL of Bio-Safe Coomassie staining solution (Biorad) and gently rocked on a shaker for 1 hour. Finally, the gel was rinsed several times with 200 mL of distilled water for 30 minutes each until the background was clear. The gel image was viewed and captured using GS-800™ Calibrated Densitometer (Biorad).

RESULTS AND DISCUSSION

Comparison of Protein Yields

Bradford microassay (Kruger, 2002) was utilised to determine total protein contents in various extracts of *E. cottonii* with three extraction methods. Bradford assay is a rapid and relatively accurate colorimetric method to estimate protein concentration in proteomic studies. This assay was first described by Bradford in 1975 (Bradford,

1976) and it is still widely used as a key protocol in protein quantification. In general, the Bradford assay relies on the binding of the dye Coomassie blue G250 to protein. The dye exists in three appearances: it is red in colour when in cationic forms, remains green in colour when it is neutral, and turns blue when it is in anionic form. Ordinarily, the dye was purchased in protonated red cationic form with maximal absorption of 470nm. However, when the dye bound to proteins in protein assay, it was converted to a stable unprotonated blue complex with a maximal absorption of 595nm (Georgiou *et al.*, 2008). Thus, the protein concentration in a particular sample can be quantified by measuring the absorbance of the solution at 595 nm.

All of the protein pellets, obtained through the three different extraction methods, were dissolved in 40 mM Tris-HCl, pH7.5 buffers, as mentioned by Contreras *et al.* (2008). This was performed because certain buffering components such as mercaptoethanol, tris, SDS and others are known to be incompatible with Bradford reagent (Campion *et al.*, 2011). Table 2 reveals the total protein yields of extracts from *E. cottonii*, using three different protein extraction methods: (a) Phenol/lysis buffer extraction, (b) TRI reagent/chloroform extraction, and (c) Phenol/SDS buffer extraction.

Among the three phenol based extraction protocols with different combinations of chemicals, the phenol/lysis buffer gave slightly higher protein yields (0.027±0.000 mg/g) than the phenol/

TABLE 2

The concentration ($\mu\text{g/mL}$), mass (μg) and total protein yields (mg/g) of *E. cottonii* extracted with three different methods.

Protein extraction from <i>E. cottonii</i>			
	Concentration ($\mu\text{g/mL}$)	Mass (μg)	Total protein yields (mg/g)
Phenol/lysis buffer extraction	675.410 \pm 11.375	135.082 \pm 2.275	0.027 \pm 0.000 ^a
TRI reagent/chloroform extraction	448.330 \pm 22.793	89.666 \pm 4.559	0.018 \pm 0.001 ^c
Phenol/SDS buffer extraction	597.086 \pm 38.067	119.417 \pm 7.613	0.024 \pm 0.002 ^b

Results are means \pm SD (n = 3). For total protein yields, values followed by the same letter (a-c) are not statistically different at $P < 0.05$.

SDS buffer (0.024 \pm 0.002 mg/g) (Table 2). The TRI reagent/chloroform extraction showed the lowest protein yields, i.e. about 0.018 \pm 0.001 mg/g (Table 2). TRI reagent is a mixture of phenol and guanidine thiocyanate in a monophasic solution. It is a commercial reagent that is used in the simultaneous isolation of RNA, DNA and proteins from different biological samples and most commonly used for the isolation of total RNA. Wong *et al.* (2006) successfully established the proteomics of *Gracilaria changii* (red algae) by using TRI reagent in combination with chloroform. With reference to protein extraction from *G. changii*, the protein yields of *E. cottonii* are slightly higher. As shown in Table 2, the protein yields of *E. cottonii* were 0.018 \pm 0.001 mg/g as compared to 0.005 mg/g from *G. changii* (Wong *et al.*, 2006). In this study, it was assumed that the protein contents of *E. cottonii* are much higher than in *G. changii*. However, this is not conclusive as more work needs to be carried out in order to verify the claim. Protein contents in seaweeds may also vary due to growing environments, harvesting seasons, etc. (Parker, 1974).

In this study, the phenol/lysis buffer showed the highest protein yields (0.027 \pm 0.000 mg/g) of the three protein extraction protocols. This is probably because the inclusion of a protease inhibitor cocktail may have reduced the loss or degradation of seaweed proteins during the extraction process. In addition, the water-soluble properties of polyvinylpyrrolidone (PVP) in the lysis buffer may have enhanced the solubility of seaweed proteins (Wang *et al.*, 2006), resulting in higher readings in Bradford's protein quantification. Conversely, the protein extracts of *E. cottonii* from the TRI reagent/chloroform extraction showed relatively weak solubility in 40 mM Tris-HCl and thus contributed to the lowest protein yields (0.018 \pm 0.001 mg/g).

Bradford protein assay was employed as a protein quantification tool in this study. The readings relied on the extent that Coomassie Brilliant Blue G-250 dye binds to the proteins. In other words, the spectral change that accompanies protein binding was used to determine the protein concentration. Generally, all dye binding assays suffer from the limitation of potential interference from

any non-protein substance that can also form a complex with a dye, or otherwise, modify the binding interaction between the dye and the protein (Berkelman, 2008). For instance, the guanidine hydrochloride used in the washing steps of the TRI reagent/chloroform extraction may compete with the dyes for the proteins in certain degrees, thus leading to underestimation of the protein content (Kruger, 2002).

Nevertheless, the Bradford assay is commonly applied for protein quantification in many other studies (Berkelman, 2008). This is because the Bradford assay is relatively easy to use and fairly free from interference by most commonly used biochemical reagents, except for certain chaotropic solubilising agents, detergents, reductants, buffers, or carrier ampholytes (Berkelman, 2008). In order to avoid any possible interference by other harsh solubilising agents, all protein pellets obtained from the three extraction protocols were dissolved only in 40 mM Tris-HCl, pH 7.5 buffers (Contreras *et al.*, 2008). However, the protein pellets obtained from the TRI reagent/chloroform extraction are partially soluble in 40mM Tris-HCl buffer.

As compared to bacteria, animals and other terrestrial plants, the protein yield of macroalgae like *E. cottonii* was very low. This may due to the nature of low protein content in macroalgae species (Wang *et al.*, 2006). Furthermore, BSA, which is a common protein standard in many protein assays suffers from the disadvantage of exhibiting an unusually large dye response in the Bradford assay. This may cause

underestimation of the protein content in a sample (Kruger, 2002). These are the two major drawbacks in the protein quantification of this study, but no other studies have addressed these issues.

Comparison of the Three Extraction Protocols Based on the 1DE patterns

One dimensional gel electrophoresis (1DE) is commonly known as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE is a relatively easier and cheaper protocol for proteomic analysis, and hence, is widely adopted in different disciplines such as forensic science, molecular biology, biochemistry, ecology, agricultural sciences and others. A SDS-PAGE separates protein based on their electrophoretic mobility, and is commonly used to estimate the relative molecular mass of protein subunits in order to determine the relative abundance of major proteins in a sample and to determine the distribution of proteins among fractions. Fig.2 shows the SDS-PAGE protein profiles of *E. cottonii* with three different protein extraction methods: (a) Phenol/lysis buffer extraction, (b) TRI reagent/chloroform extraction, and (c) Phenol/SDS buffer extraction.

In this study, three different protein extraction protocols were compared on seaweed *E. cottonii* to determine the method that best shows resolved proteins clearly and fewer interfering substances. At the first glance, the qualitative comparisons of protein extracts from *E. cottonii* using the three different extraction methods revealed that all three methods shared almost similar

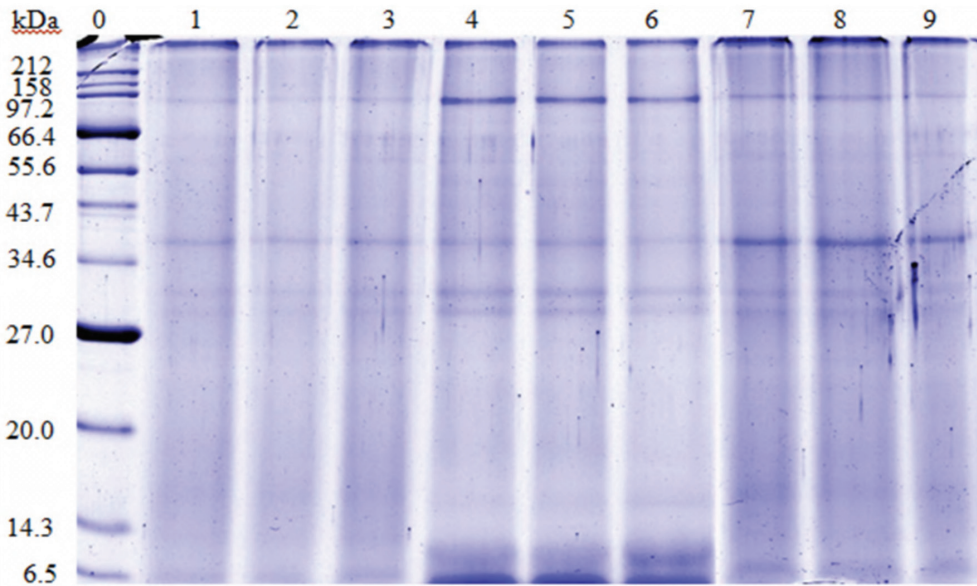


Fig.2: SDS-PAGE separation of *E. cottonii* proteins extracted using different methods. Lane 0: NEB protein marker (2-212 kDa), lanes 1-3: 10 µg of protein sample from phenol/lysis buffer extraction (triplicates), lanes 4-6: 10 µg of protein sample from TRI reagent/chloroform extraction (triplicates), lanes 7-9: 10 µg of protein sample from phenol/SDS buffer extraction (triplicates).

protein profiles. Intense protein bands were observed from 27 kDa to 158 kDa (Fig.2). All three extraction methods utilised phenol as part of their extraction reagents.

However, the different extraction methods produced protein bands in different intensities. TRI reagent/chloroform extraction gave the most intense protein band at about 97.2 kDa. Phenol/SDS buffer extraction produced an intense protein band at about 40 kDa. Phenol/lysis buffer extraction produced diverse protein bands, which resolved in the ranges of 27 kDa to 34.6 kDa, 34.6 kDa to 43.7 kDa, 55.6 kDa to 66.4 kDa and 97.2 kDa (see Fig.2). All the three phenol based extraction methods produced fairly good one dimensional electrophoresis (1DE) protein profiles of *E. cottonii* with almost no smearing observed.

As compared to the higher level terrestrial plants, macroalgae proteomics are relatively less studied. Most of the protein extraction protocols utilised in macroalgae proteomics were adapted from the proteomics of other higher level terrestrial plants. Pavokovic *et al.* (2012) stated that phenol extraction was the most effective method for the removal of unwanted interfering substances. Phenol extraction was utilised in the extraction of other macroalgae species: *Scytosiphon gracilis* and *Ectocarpus siliculosus* (Contreras *et al.*, 2008), *Gracilaria changii* (Wong *et al.*, 2006) and *Ecklonia kurome* (Nagai *et al.*, 2008). By comparing the TCA/acetone and phenol extraction protocols used by Carpentier *et al.* (2005) and Saravanan and Rose (2004), respectively, an observation

that both of the methods were efficient in extracting proteins from recalcitrant tissues was ruled. Phenol extraction was found to be the most efficient method of removing the interfering substances, and it resulted in the highest quality gels with less background and fewer smearing effects. Phenol based extraction minimises protein degradation that is often encountered in sample preparation that occurs because of endogenous proteolytic activity. Saravanan and Rose (2004) also pointed out that the phenol method yielded a greater number of glycoproteins.

Phenol extraction is based on the separation of macromolecules into organic and aqueous phases. Phenol is a simple aromatic alcohol that contains only a polar hydroxyl group that is bound to an aromatic ring. It exhibits weak acidic properties and is partially miscible with water. When saturated with water, the aqueous layer contains about 7% phenol and the organic layer contains about 28% water (Faurobert *et al.*, 2007). During the extraction, phenols interact with proteins mainly via hydrogen bonding, which causes protein denaturation and causes the phenols to become solubilised in the organic phenolic phase. The aqueous phase preferentially dissolves nucleic acids, carbohydrates and cell debris, while the phenolic phase carries proteins and lipids. In both phenol/lysis buffer extraction and phenol/SDS buffer extraction, proteins from the phenolic phase were then precipitated with methanol and ammonium acetate.

TRI reagent solution is a combination of phenol and guanidine thiocyanate in a

monophasic solution. It was first invented by Chomczynski and Sacchi (1987) for the isolation of RNA from cultured cells in most animal tissues. TRI reagent was then utilised in protein extraction from *Gracilaria changii* (Wong *et al.*, 2006). Chloroform mixed with phenol is more efficient at denaturing proteins than the individual reagent alone (Perry *et al.*, 1972). In this study, the mixtures of phenol to chloroform used were 5:1 (v/v). At acidic pH, a 5:1 ratio caused the absence of DNA from the upper aqueous phase; this further enhanced the purity of protein extracts later. Purified phenol has a density of 1.07 g/cm³ and formed the lower phase when mixed with water (1.00 g/cm³) later. Chloroform ensured phase separation of the two liquid because chloroform is miscible with phenol and it has a higher density (1.47 g/cm³) than phenol. As a result, this procedure facilitated the removal of proteins containing pink coloured organic phase with minimal cross contamination of nucleic acids from the aqueous phase.

According to a general rule, a solute dissolves best in a solvent that shares a similar chemical structure. Therefore, the overall solvation capacity of a solvent depends primarily on its polarity. The protein extracts of *E. cottonii* may contain varying proportions of charged and uncharged domains, which construct hydrophobic and hydrophilic regions. In the presence of phenol in the TRI reagent, the hydrophobic cores interact with phenol, causing precipitation of proteins and glycoproteins and further partitioning at the interphase and

the pink coloured organic phase. This may explain the intense band close to 97.2 kDa (Fig.2) that was observed in the TRI reagent/chloroform extraction, as glycoproteins usually have higher molecular weight than single protein subunits. In other words, TRI reagent/chloroform extraction yielded a higher amount of high molecular weight glycoproteins as compared to the phenol/lysis buffer extraction and phenol/SDS buffer extraction.

As discussed earlier, the protein extracts obtained from the TRI reagent/chloroform extraction were sparingly dissolved in 40 mM Tris-HCl, pH 7.5 buffers. At this stage, it was speculated that the protein extracts obtained with TRI reagent/chloroform were more hydrophobic and hence less likely to dissolve in water soluble Tris buffers. However, with the addition of SDS as a protein denaturation and solvation agent prior to 1DE, the protein extracts of *E. cottonii* from TRI reagent/chloroform extraction were well dissolved in SDS-denaturing buffer. SDS is a long-chain hydrocarbon with a negatively charged hydrophilic head and a long hydrophobic hydrocarbon tail. SDS is an anionic detergent known to dissociate and denature proteins. It acts at a very low concentrations and causes the unfolding of proteins by inducing conformational changes to alpha helices (Rao & Prakash, 1993). As a result, the protein extracts obtained from TRI reagent/chloroform extractions displayed well resolved, distinctive protein bands, resembling the protein profiles obtained from the phenol/lysis buffer extraction and

phenol/SDS buffer extraction. Ten (10) µg of the protein extracts were loaded for 1DE for all three protein extraction protocols from *E. cottonii*.

As shown in Fig.2, all the three extraction methods shared similar 1DE protein profiles. Each extraction method succeeded in producing well-resolved protein bands. In other words, there was no smearing or tailing as observed in other non-phenol based extraction protocols in a range of other macroalgae species (Contreras *et al.*, 2008; Nagai *et al.*, 2008). This proved that the phenol based protein extraction protocols applied in this study had successfully removed other non-protein interfering substances from *E. cottonii*. In addition, all the three phenol based extraction methods resulted in high molecular weight proteins, specifically of protein bands of about 97.2 kDa that were intensely observed in the *E. cottonii* sample with TRI reagent/chloroform extraction and protein bands of about 39 kDa that were strongly observed in the *E. cottonii* sample with phenol/SDS buffer extraction. Overall, three distinctive protein bands in a range of 27 kDa to 43.7 kDa and a protein band of about 97.2 kDa were clearly observed in the *E. cottonii* sample with the three different phenol based protein extraction protocols. Other protein bands in a range of 43.7 kDa to 66.4 kDa were barely detected in the *E. cottonii* sample with the three phenol based protein extraction protocols used in this study. Nonetheless, the limitations of 1DE protein profiles restrict the identification of protein groups or protein types, which are

represented by each of the well resolved protein bands in the protein profile of *E. cottonii* with three different phenol based protein extraction protocols.

CONCLUSION

This study is an initial proteome study of *E. cottonii*. To date, no other proteomics on *E. cottonii* have yet been reported. Three phenol based protein extraction protocols were compared for their suitability in 1DE proteome studies of *E. cottonii*. Of the three extraction methods, the phenol/ lysis buffer protocol yielded the highest protein concentration (0.027 ± 0.000 mg/g), followed by the phenol/SDS buffer protocol (0.024 ± 0.002 mg/g), and the TRI reagent/ chloroform protocol (0.018 ± 0.001 mg/g). All the three extraction protocols utilised in this study produced good quality 1DE protein profiles of *E. cottonii* with distinctive protein bands. The 1DE profile of *E. cottonii* from the three extraction protocols showed similar patterns, and high molecular weight protein molecules ranging from 27 kDa to 158 kDa in different intensities were observed. Among the three phenol based protein extraction protocol, the phenol/ SDS buffer protocol was recommended for preliminary proteome study of *E. cottonii* as it is the cheapest method, relatively easy to start up and yet gave good protein yields, as well as produced comparable good quality 1DE protein profiles of *E. cottonii* as in the other two protein extraction protocols in this study. In conclusion, proteomics is increasingly seen as an important tool in marine biology, and it is

particularly important to enhancing a deep understanding of marine seaweed biology. Proteomics is an effective research tool when used in addition to other “omics” technologies. This study may facilitate other downstream studies of *E. cottonii* and other carrageenan rich seaweed species. Seaweed proteomics is particularly valuable to aquaculture sectors. This study shows that “*E. cottonii*” proteomics is vital to reaching the goals of aquaculture of high productivity and a better quality product.

ACKNOWLEDGEMENTS

The authors are thankful to Asst. Prof Dr. Crystale Lim Siew Ying and Asst. Prof Patrick Nwabueze Okechukwu, who are lecturers at UCSI University and the laboratory staff of UCSI University, including Mr. Chua Kim San, Ms. Tan Ting Ting, Ms. Vasuki A/P Sengalarian, Ms. Khong Mei Li and Mr. Anbalakan A/L M. Govindan for their assistance and technical supports. We also thank the Faculty of Science, UCSI University, for providing the necessary facilities for the present study.

REFERENCES

- Berkelman, T. (2008). Quantitation of protein in samples prepared for 2D electrophoresis. *Methods in Molecular Biology*, 424, 43 – 49.
- Bixler, H. J. (1996). Recent developments in manufacturing and marketing carrageenan. *Hydrobiologia*, 326(327), 35 – 57.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, 248 – 254.

- Campion, E. M., Loughran, S. T., & Walls, D. (2011). Protein quantitation and analysis of purity. *Methods in Molecular Biology*, 681, 229 – 258.
- Carpentier, S. C., Witters, E., Laukens, K., Deckers, P., Swennen, R., & Panis, B. (2005). Preparation of protein extracts from recalcitrant plant tissues: an evaluation of different methods for two-dimensional gel electrophoresis analysis. *Proteomics*, 5(10), 2497 – 2507.
- Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*, 162(1), 156 – 159.
- Contreras, L., Ritter, A., Dennett, G., Boehmwald, F., Guitton, N., Pineau, C., Moenne, A., Potin, P., & Correa, J. A. (2008). Two dimensional gel electrophoresis analysis of brown algal protein extracts. *Journal of Phycology*, 44, 1315 – 1321.
- Faurobert, M., Pelpoir, E., & Chaïb, J. (2007). Phenol extraction of proteins for proteomic studies of recalcitrant plant tissues. *Methods in Molecular Biology*, 355, 9 – 14.
- Georgiou, C. D., Grintzalis, K., Zervoudakis, G., & Papapostolou, I. (2008). Mechanism of Coomassie brilliant blue G-250 binding to proteins: a hydrophobic assay for nanogram quantities of proteins. *Analytical and Bioanalytical Chemistry*, 391(1), 391 – 403.
- Jamers, A., Blust, R., & Coen, W. D. (2009). Omics in algae: paving the way for a systems biological understanding of algal stress phenomena? *Aquatic Toxicology*, 92, 114 – 121.
- Kruger, N. J. (2002). The Bradford method for protein quantitation. *The Protein Protocols Handbook*, 1, 16 – 20.
- Liu, C. L., Huang, X. H., & Liu, J. G. (2013). Assessment of introduced *Kappaphycus* (Solieriaceae, rhodophyta) species relationships in China with molecular markers. *Acta Oceanologica Sinica*, 32(2), 59 – 65.
- Matanjun, P., Mohamed, S., Mustapha, N. M., & Muhammad, K. (2009). Nutrient content of tropical edible seaweeds, *Eucheuma cottonii*, *Caulerpa lentillifera* and *Sargassum polycystum*. *Journal of Applied Phycology*, 21, 75 – 80.
- Nagai, K., Yotsukura, N., Ikegami H., Kimura, H., & Morimoto, K. (2008). Protein extraction for 2-DE from lamina of *Ecklonia kurome* (laminariales): Recalcitrant tissue containing high levels of viscous polysaccharides. *Electrophoresis*, 29, 672 – 681.
- Parker, H. S. (1974). The culture of the red algal genus *Eucheuma* in the Philippines. *Aquaculture*, 3, 425 – 439.
- Pavokovic, D., Kriznik, B., & Rasol, M. K. (2012). Evaluation of protein extraction methods for proteomic analysis of non-model recalcitrant plant tissues. *Croatica Chemica Acta*, 85(2), 177 – 183.
- Perry, R. P., La Torre, J. L., Kelley, D. E., & Greenberg, J. R. (1972). On the liability of poly (A) sequences during extraction of messenger RNA from polyribosomes. *Biochimica et Biophysica Acta*, 262, 220 – 226.
- Popper, Z. A., Michel, G., Herve, C., Domozych, D. S., Willats, W. G. T., Tuohy, M. G., Kloareg, B., & Stengel, D. B. (2011). Evolution and diversity of plant cell walls: from algae to flowering plants. *Annual Review Plant Biology*, 62, 567 – 590.
- Rao, K. S. & Prakash, V. (1993). Interaction of sodium dodecyl sulphate with multi-subunit proteins. *The Journal of Biological Chemistry*, 268(20), 14769 – 14775.
- Santos, G. A. (1989). Carrageenans of species of *Eucheuma* J. Agardh and *Kappaphycus* Doty (Solieriaceae, Rhodophyta). *Aquatic Botany*, 36, 55 – 67.
- Saravanan, R. S. & Rose, J. K. (2004). A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. *Proteomics*, 4(9), 2522 – 2532.

- Wang, W., Vignani, R., Scali, M., & Cresti, M. (2006). A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. *Electrophoresis*, 27(13), 2782 – 2786.
- Wong, P. S., Tan, L. J., Navi, H., & Abubakar, S. (2006). Proteomics of the red alga, *Gracilaria changii* (Gracilariales, Rhodophyta). *Journal of Phycology*, 42, 113 – 120.

