Profiling of lectin production in wild-type and in vitro cultivated Kappaphycus alvarezii

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ABSTRACT
Kappaphycus alvarezii is widely cultivated around the world as the demand for seaweed-based products now exceeds the supply of seaweed raw material. Protein profiling of macroalgae is important to understand the changes that occur during growth which can in turn lead to improved culture strains. A Proteomic study was carried out by comparing protein profiles of different growth stages of in vitro and wild-type K. alvarezii. Protein profile analysis was done using polyacrylamide gel electrophoresis followed by identification using MALDI-TOF/TOF-MS. Additional band of low molecular weight observed in 12-weeks cultures, with similar intensity and size observed in wild-type, was indicated to be similar to lectin ESA-2 from Eucheuma serra. The production of lectin may indicate that the explants are ready to be transferred to the natural environment. Thus, the presence of lectin is a potential biomarker that can determine the status of seaweed cultures for introduction to the wild.

Keywords: Lectin; Kappaphycus alvarezii; In vitro cultivation; Protein profiling; Polyacrylamide gel electrophoresis; Biomarker
1. **INTRODUCTION**

Algae are one of the most commercially important marine plants. Macroalgae, also known as seaweed are cultivated artificially as the demand for seaweed-based products now exceeds the supply of seaweed raw material that are found naturally (Reddy et al. 2008). *Kappaphycus alvarezii* is one of the major sources of phycocolloids which serve as thickening, stabilizing and emulsifying agent also known as carrageenans and agars. In addition to the benefit it brings to the economy, seaweed farming also contributes to the ecosystem by reducing global warming, as soil conditioners to improve growth of land-based crops and as an alternative livelihood for coastal dwellers (Azanza & Ask 2001; Bindu & Levine 2011; Yunque et al. 2011). Farms of *K. alvarezii* are located at numerous places including Malaysia while the Philippines is the largest global producer of seaweed (Vairappan 2006).

The seaweed farming industry faces many challenges such as the seasonal changes that are unsuitable for seaweed growth, inconsistent yield and quality of carrageenan, natural disasters, pests and susceptibility to diseases as well as infection. The technical level of alga culture is still very much behind higher plants (Minocha 2001; Reddy et al. 2008) and this has driven the need for intensified research. Postgenomic approaches such as proteomics and transcriptomics are able to provide better understanding on how organisms physiologically adapt to abiotic and biotic stresses (Qureshi et al. 2007; Contreras et al. 2008; James et al. 2009; Tran et al. 2009; Kosova et al. 2011; Mahong et al. 2012). The protein functions and functional networks of protein including the biochemical pathways in plants can be better elucidated by proteomic analysis (Hirano et al. 2004). The most common method used for protein separation is by SDS-PAGE. SDS-PAGE is an extremely useful method particularly for estimation of protein sizes, protein quantitation and comparison of protein composition of different samples (Hamdan & Righetti 2005).

The common goal of protein profiling in proteomics analysis is to determine the protein function and to explore the functional network of proteins. Studies have shown that the most abundant class of proteins in plants was involved in metabolic processes. High amount of proteins were involved in protein synthesis, protein degradation and signal transduction (Hirano et al. 2004). The application of proteomic tools has helped to discover functions of numerous important proteins in a number of higher plants, including *Arabidopsis thaliana*, *Oryza sativa*, tobacco, barley, maize and *Zea mays*. Proteomics enable comparison between species and strains of healthy and diseased plants as well as identification of species-specific proteins. It is important for detection of disease-associated proteins that could lead to better understanding of biosynthetic pathways for useful crop improvements (Wong et al. 2006).

Proteomic studies in algae are still in the early stage, with only few species of microalgae being studied. Most studies done on algae were related to stress and effects of different environments on protein expression (Wang et al. 2004; Wong et al. 2006; Tran et al. 2009; Contreras et al. 2010; Mahong et al. 2012; Yotsukura et al. 2012). There have been limited reports of proteomic analysis of *K. alvarezii*. Although most current research done is focused on stress-induced protein expression to understand biochemical pathways, the protein profile during growth is equally important to understand the changes that occur during growth which can lead to improved varieties and optimization of culture conditions. The objective of this study was to profile the production of short peptides in wild-type and *in vitro* cultivated *K. alvarezii*.

2. **MATERIALS AND METHODS**

2.1. Plant Material and Culture Conditions

*K. alvarezii* explants were obtained from Semporna, Sabah, Malaysia. Axenic explants were obtained according to the protocol reported by Yong et al. (2011). Healthy, disease-free explants were chosen and surface sterilized using 3% povidone iodine and 15% ethanol for 30 seconds each. This is followed by immersion in 100 mg/l penicillin G and 100 mg/l kanamycin sulphate for 24 hour. Axenic explants were
cultured in 50% Provasoli Enriched Seawater (PES) medium (Provasoli 1968) supplemented with 2.5 mg/l 1-naphthaleneacetic acid (NAA) as plant growth regulator. The temperature was 25°C to 30°C, under cool white fluorescent tube light and 12:12 light and dark photoperiod. Explants were transferred into fresh medium every week for 20 weeks. Wild-type *K. alvarezii* samples were taken from the same location, and were not subjected to any *in vitro* culturing conditions.

2.2. Analysis of Protein Profile by SDS-PAGE

Explants were weighted and homogenized with pre-frozen mortar and pestle. Homogenized sample was placed in microcentrifuge tube and sample buffer added. The ratio of sample weight to sample buffer is 3 to 1. Approximately 1 g of sample was added with 330 ml sample buffer. Sample buffer consisted of 0.5 M Tris-HCl (pH 6.8), 20.2% (v/v) glycerol, 0.0001% (w/v) bromophenol blue, 0.04% (w/v) SDS, 0.03% (w/v) dithiothreitol (DTT) and 0.04% (v/v) 2-mercaptoethanol. This was followed by centrifugation at 10,000 x g for 2 min. Supernatant was collected and boiled at 95°C for 5 min. Prepared sample was kept in ice before loading into gel.

Gel was made according to Laemmli (1970). An 11.25% separating gel containing 1.5 M Tris-HCl (pH 8.8), 20% SDS, 10% ammonium persulphate and TEMED was prepared. A 4.8% stacking gel was prepared using 0.5 M Tris-HCl (pH 6.8), 20% SDS, 10% ammonium persulphate and TEMED. The electrophoresis running buffer consisted of 25 mM Tris base, 192 mM glycine and 3 mM SDS. Electrophoresis was accomplished at 150V for 9 hours using Hoefer SE600 Ruby Standard Vertical Electrophoresis Unit (Amersham Biosciences).

Gel was stained with 0.5% Coomassie Brilliant Blue R-250 in 45% (v/v) methanol, 10% (v/v) acetic acid for 1 hour and destained for 12 hours with 20% (v/v) methanol and 10% (v/v) acetic acid. Gel was washed with distilled water until background was clear. Gel viewed and photographed with Alpha Innotech Alphaimager HP MultiImage II. The protein molecular marker used was bovine serum albumin (BSA) 66kDa from Amresco.

2.3. Protein Identification by MALDI-TOF/TOF-MS (Proteomics International)

The cut target protein band was sent for identification by Proteomics International. Protein was trypsin digested based on Bringans *et al.* (2008), followed by analysis with MALDI-TOF/TOF-MS and identification using Mascot sequence matching software on Ludwig NR database (Methods by Proteomics International Pty Ltd). Serum albumin, found in *Bos taurus* was used as the control.

3. RESULTS

Protein separation of *K. alvarezii* explant produced discrete bands composed of dark and light ones. Figure 1 shows the separation profile of *in vitro* cultivated explants on 8th week, 12th week, 16th week and 20th week to be compared to the profile of the wild-type. The protein profiles were observed to be very similar among the *in vitro* cultivars but additional prominent low molecular weight protein band was found on 16th and 20th week. An additional band of similar size that was found on the 16th and 20th week of culture was also prominent in the wild-type (as indicated as target protein in Figure 1). However, the overall protein profile of the wild-type was observed to be slightly different from the *in vitro* cultivars.

According to the identification based on Mascot Search (Matrix Science) as shown in Table 1, the most possible or similar protein hit of the band isolated was lectin ESA-2 which was found in red algae, *Eucheuma serra*. The highest protein score of matched peptides was taken. Protein score above 52 is considered significant, indicating that the match did not happen at random or by chance. The number of peptides that matched the protein are 10. The coverage of peptide sequences against lectin ESA-2 was 52%.
The protein candidate, lectin ESA-2 has the molecular weight of approximately 27kDa which is relatively low. The peptide sequence coverage is not too high (52%), indicating an acceptable result. This is because a high sequence coverage is likely due to abnormally short protein described in database, resulting in irrelevantly high sequence coverage. It is more accurate to look into the localization of matching peptides in protein sequence than the sequence coverage (Sommerer et al. 2006). As shown in Figure 2, the matched peptides were localized all over the protein sequence. Thus, this further indicates that lectin ESA-2 is a valid candidate.

4. DISCUSSION

Protein separation via one-dimensional SDS-PAGE has proven to be able to produce promising results. The distinctive additional band found in the wild-types and in vitro cultures on week 16 and 20 indicated that the particular protein was expressed after the 12th week of cultivation. It is considered a growth-induced protein since all other parameters and culture conditions were kept constant throughout the experimental period. When compared to the gel obtained from separation of wild-type K. alvarezii, an additional band of similar intensity and size with those found in week 16 and 20, was also found. Darker bands indicate higher amount of protein due to stoichiometric reaction with the stain. The additional bands observed were relatively dark compared to the other bands. This also showed that the protein was expressed in relatively high amount after the 12th week. The slight difference in protein profile of the wild-type explant from the in vitro cultivars may be due to the different environment that induced different protein expressions. However, the exact mechanisms or factors need to be investigated for more conclusive explanations. During the first stage of in vitro cultivation which is before week 12, the protein was not expressed. Expression was stopped or suppressed when cultivated in the in vitro environment. This indicates that the additional protein expressed is possibly linked to survival, stress or defence in the non-sterile natural environment. The exact function can only be known by identification of the particular protein.

Lectins are carbohydrate-binding proteins found in nature from prokaryotes to corals, algae, fungi, plants, invertebrates and vertebrates (Hung et al. 2009). In plants, lectins have been found in the roots, sap, fruit, seeds, flowers, barks, stem and leaves. Some plants have more than one lectin while some synthesized as isoforms. Some lectins are glycoproteins. Hundreds of plant lectins have been identified and characterized (Doyle 1994). Lectins have been found in many algal species such as Kappaphycus striatum, Gracilaria ornata, Bryothamnion seaforthii, Bryotarmniaon triquetrum, Palmaria palmata and Ulva lactuna but of different types (Doyle 1994; Pinto et al. 2009). The types of lectin that has been found in K. alvarezii were KAA-1, KAA-2 and KAA-3, also known as ECA in Eucheuma cottonii (another name for K. alvarezii) (Hung et al. 2009). On the other hand, high amount of lectin isoforms KSA-1, KSA-2, and KSA-3 were identified in Kappaphycus striatum, a species which showed to be more resistant to epiphytes, ‘ice-ice’ infestation compared to other species (Hung et al. 2011). Eucheuma species has been found to produce high amount of isolectins with similar molecular weight, homologous between species. This also suggest similar function which is to defend against pathogen infections. It was proposed that the whole genus of Eucheuma is a valuable source of lectin (Kawakubo et al. 1999). Thus, it is not surprising that lectin was found in this study.

Marine algal lectins were found to be small molecules, has great stability due to disulfide bridges and show high specificity for complex carbohydrates and glycoconjugates such as the ones present on the cell surface membrane (Pinto et al. 2009). The lectin isolated in this study is ESA-2, which bounds specifically to high-mannose type N-glycans. This lectin protein recognises the branched oligomannosides of the N-glycans (Hori et al. 2007). Algal lectins have been proven to possess hemaaglutinin ability. A study done have shown that KAA lectins strongly agglutinate trypsin- and papain- treated erythrocytes of sheep and
rabbit (Hung et al. 2009). KAA-2 is also a high-mannose specific lectin and has the potential to inhibit influenza virus infection (Sato et al. 2011). The lectin found in *Eucheuma cottonii* has the ability to recognize and bind to carbohydrates, including viruses, bacteria, fungi and parasites (Holdt & Kraan 2011). The ability to defend against pathogens may be similar to higher plants, marine mussels and invertebrates (Liao et al. 2003). However, the presence of lectin was only found after 12 weeks of *in vitro* cultivation. This may suggest that the explants develop defensive mechanism by expressing lectin after 12 weeks of cultivation. The ability of lectin to bind to pathogens may decrease the susceptibility of *K. alvarezii* to infection and subsequently increase its survival rate in the wild. Lectin production was suppressed when first introduced into the *in vitro* environment because lectin production was not found before the 12th week of cultivation. The production of lectin protein *in vitro* may act as a biomarker to indicate adaptation to the wild where explants are ready to be transferred to the natural environment.

5. **CONCLUSION**

This study has demonstrated that the production of short peptides is suppressed during the early stages of *in vitro* culture. However it is evident that the production is restored after the explants stabilize *in vitro*, indicating the presence of underlying mechanisms of adaptation. This finding will benefit the seaweed farming industry by development of potential biomarker for the assessment of the status of seaweed cultures *in vitro*.

**REFERENCES**


FIGURES

![Figure 1](image.png)

**Figure 1.** One-dimensional SDS-PAGE electropherogram of *in vitro* cultivated and wild-type *Kappaphycus alvarezi* (11.25%) Each lane was loaded with 50 µl of sample solution and gel stained with Coomassie Brilliant Blue R-250.
Figure 2. Peptide sequence of protein lectin ESA-2. Peptides from sample that matched the protein are shown in bold.

TABLES

Table 1. Properties of protein identified based on Mascot Search (Matrix Science).

<table>
<thead>
<tr>
<th>Protein Hit</th>
<th>Lectin ESA-2 (Eucheuma serra)</th>
</tr>
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<tbody>
<tr>
<td>*Protein score</td>
<td>623</td>
</tr>
<tr>
<td>Number of matched peptides</td>
<td>10</td>
</tr>
<tr>
<td>Peptide sequence coverage</td>
<td>52%</td>
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<td>Accession number</td>
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</table>

*Protein score above 52 (>52) indicate identity or extensive homology (p>0.05).
**Protein scores are derived from ions scores as a non-probabilistic basis for ranking of protein hits (Mascot Search, Matrix Science).