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## Dehydrin-like proteins in desiccation tolerance in intertidal seaweeds

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DEHYDRIN-LIKE PROTEINS IN DESICCATION TOLERANCE IN  
INTERTIDAL SEAWEEDS

A thesis presented

by

Jonathan W. Wong

to

The Department of Biology

In partial fulfillment of the requirements for the degree of  
Master of Science

in the field of

Biology

Northeastern University  
Boston, Massachusetts  
April, 2009

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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements  
for the degree of Master of Science in Biology  
in the Graduate School of Arts and Sciences of  
Northeastern University, April, 2009

## ABSTRACT

The intertidal red alga *Porphyra* occurs higher locally in New England than any other intertidal seaweed and is emerged twice a day which can last up to 8 hours per tide. It experiences a drastic change of water content during its emergence, which is very different from what happens in desiccation tolerant land plants. It is very likely that *Porphyra* may possess unique mechanisms in desiccation tolerance. Though the primary focus of past studies in desiccation tolerance in intertidal seaweeds has been the role of ROS metabolism, past investigations on two species of *Porphyra* have suggested that ROS metabolism is not involved. Dehydrin-like proteins are more likely to be involved in the desiccation tolerance in *P. umbilicalis* and not in *P. yezoensis*. In this study, the presence and role of these dehydrin-like proteins were evaluated in *P. umbilicalis* and *P. yezoensis*. The results of this study indicate that a dehydrin-like protein was present in the desiccation tolerant *P. umbilicalis* but not in the desiccation sensitive *P. yezoensis*. These constitutively expressed dehydrin-like proteins were extremely hydrophilic under dehydrative conditions and thermally stable. During desiccation, *P. umbilicalis* fractions are able to suppress protein aggregation and inactivation of citrate synthase. Although these dehydrin-like proteins are present in *P. umbilicalis*, a survey of three other intertidal algae revealed that their occurrence may not be common in intertidal algae.

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## INTRODUCTION

### **I. Harmful effects and damages caused by desiccation**

Drought is a central problem for terrestrial life and a leading cause of agricultural failure and human famine. Furthermore, its impact is expected to intensify with climate change. Drying to equilibrium with even moderately dry air is instantly lethal to most species of animals and plants as water maintains the structure of membranes and intracellular macromolecules (Alpert 2005). The removal of water from cells of desiccation-sensitive organisms results in irreversible aggregation of essential macromolecules and disintegration of organelles (Tweddle et al 2003).

Metabolism is believed to stop by the time a cell has dried to about 10% water content, due to leaving too little water to form a monolayer around proteins and membranes (Clegg 1973, Billi and Potts 2002). Drying to 10% absolute water content is roughly equivalent to equilibration with air of 50% relative humidity at 20°C and a drop in water potential to -100 Megapascals (MPa) (Alpert 2005).

A number of problems can arise as cells start to lose water. For example, as water is removed, the packing density of the head groups of the phospholipids in cellular membranes and thylakoids increases. This leads to increased van der Waals interactions among the hydrocarbon chains (Crowe et al 1992). Consequently, the phase transition temperature,  $T_m$ , increases significantly and dry lipids turn into a gel phase at room temperature. Then, when these dry lipids are rehydrated they undergo a phase transition back to the liquid state and become leaky, releasing their contents (Hoekstra et al 2001; França et al 2007).

In addition, during desiccation cell membranes and proteins become more susceptible to attack by reactive oxygen species (ROS). ROS attacks on lipids cause extensive peroxidation and de-esterification of membrane lipids, making them more fluidized and permeable (Senaratna et al 1987, Crowe et al 1989). ROS attacks on proteins results in oxidation of the functional groups of amino acids, making them susceptible to proteolysis and inactivation or to having reduced activity upon rehydration (Rossib et al 2003, França et al 2007).

Lastly, the removal of intracellular water causes the cytoplasmic volume to decrease and the cytoplasm to become more viscous, thereby increasing the chances for molecular interactions. In the absence of water, proteins are forced to compensate for the loss of hydrogen bonding by bonding to other molecules, resulting in denaturation and aggregation (Hoekstra et al 2001, Wolkers et al 2002, Rebecchi et al 2007). Upon rehydration, many proteins are in an unfolded state and have either reduced activity or none at all (Hoekstra et al 2001).

On the other hand, desiccation tolerant organisms are able to tolerate drought by drying to as little as 10% water content without dying (Tweddle et al 2003, Alpert 2006). The number of organisms with this ability is quite rare. For example, of the approximately 265,000 known vascular plant species, only about 330 species are desiccation tolerant (Porembski and Barthlott 2000). As a result, the mechanism of desiccation tolerance is of great interest in plant biology and agriculture.

## II. Proposed mechanisms of desiccation tolerance in land plants

Most studies on desiccation tolerance have focused on physiologically tolerant species such as the resurrection plant *Craterostigma plantagineum*, an Angiosperm, the moss *Tortula ruralis*, and a few ferns (Ingram and Bartels 1996). In addition, desiccation tolerance has been studied in a number of agricultural crops especially *Zea mays* and *Oryza sativa* (Close 1996). From past studies on these and other plants, there are three mechanisms for desiccation tolerance that have been proposed: 1) ROS defense, 2) repression of membrane phase transition, and 3) vitrification.

The first mechanism, ROS defense, was studied in desiccation tolerant plants that scavenge or prevent the formation of free radicals to avoid damage to membranes and proteins. For example, the angiosperms *Craterostigma wilmsii* and *Xerophyta viscosa* up-regulate the expression of reactive oxygen scavenging enzymes such as catalase and superoxide dismutase (Ingram and Bartels 1996, Sherwin and Farrant 1998). However, these enzymes can only function under conditions of sufficient water. In the dried state, antioxidants can alleviate such oxidative stress (Vertucci and Farrant 1995). For example, the angiosperm *Myrothamnus flabellifolius* substantially increases levels of antioxidants such as glutathione and ascorbate in response to desiccation (Kranmer et al 2002, Moore et al 2005).

The second mechanism, repression of membrane phase transition, is the ability to depress the rapid phase transition temperature,  $T_m$ , which occurs during desiccation and rehydration. In the absence of adequate water, desiccation tolerant plants are able to use water substitutes to satisfy the hydrogen bonding requirement of polar groups on proteins and do not pass through a phase transition during

rehydration (Crowe et al 1998, Hoekstra et al 2001, Wolkers et al 2002). For example, *Craterostigma pumilum* utilizes non-reducing sugars such as trehalose and sucrose as a water substitute. These non-reducing sugars are able to preserve labile proteins such as phosphofructokinase and hen egg white lysozyme, by hydrogen bonding of their –OH groups to polar residues in the protein (Carpenter et al 1987, Crowe et al 1992, Lins et al 2004). Further studies of the sugar trehalose have revealed that it is the most efficient sugar for preserving dry membranes. That is, lower concentrations of trehalose are able to preserve membranes than other non-reducing sugars (Crowe et al 1992).

However, studies on some other desiccation tolerant plants revealed that certain proteins might also play a role in desiccation tolerance (Close 1996, Ingram and Bartels 1996). For example, studies of the plants *Arabidopsis thaliana*, *Hordeum vulgare*, and *Zea mays*, have identified a number of proteins that accumulate in response to dehydration. Among these induced proteins, dehydrins have been the most commonly observed (Close 1996, Rampino et al 2006, Rorat 2006). Immunolocalization experiments have revealed that dehydrins are localized in various cell compartments, particularly near the plasma membrane. In addition, *in vitro* experiments show the ability of dehydrins to interact with proteins as a novel chaperone (Close 1996, Hoekstra et al 2001, Herzer et al 2003, Rorat 2006, Kovacs et al 2008).

The third mechanism, vitrification, occurs when the cytoplasm dries to 10% water content and exists in a so-called “glass state” (Bryant and Wolfe 1992). The glass state is defined as an amorphous metastable state that resembles a solid, brittle

material, but with the retention of the disorder and physical properties of a liquid.

In this glass state, the rates of molecular diffusion and chemical reactions are significantly reduced. This glass state is dependent upon water content, temperature, and its chemical composition (Bryant and Wolfe 1992). The critical function of glasses has been implicated to provide stability to macromolecules and limiting lateral stresses in membranes during desiccation (Bryant and Wolfe 1992, Hoekstra et al 2001). Though vitrification is necessary for desiccation tolerance, it alone is not sufficient (Crowe et al 1992, 1998). That is, a specific interaction of a molecule or molecules such as dehydrins in conjunction with vitrification is believed to be required (Crowe et al 1992, 1998; Wolkers et al 1999).

### **III. Protective effects of dehydrins in desiccation**

Dehydrins have been discovered in a wide range of photosynthetic organisms, including vascular plants, ferns, and cyanobacteria. Dehydrins in vascular plants were found in conditions that include low temperature, vegetative tissue dehydration (by evaporation or use of osmolytes), and embryogenesis (Close 1996, Campbell and Close 1997). Proteins similar to dehydrins have also been found in algae, cyanobacteria, and rotifers. The concentration of these proteins is often correlated to a state of relative desiccation tolerance (Campbell and Close 1997, Li et al 1998, Tunnacliffe et al 2005).

All dehydrins have one or more copies of a conserved lysine-rich sequence (called the K-segment), EKKGIMDKIKEKLPGEKLP, usually near the C-terminus, which is a defining characteristic amongst a large group of proteins in the LEA (Late

Embryogeneis Abundant) family. In general, they are hydrophilic with various molecular masses, are very stable during prolonged exposures to 75° to 100° C (ie >10 min), and are primarily localized near the plasma membrane (Rorat 2006). Their function may be to protect macromolecules and membranes, as well as to prevent the accumulation of macromolecules during reduced water conditions in vascular plants and anhydrobiotes (Dure 1993; Bray 1993; Close 1997; Goyal et al 2003, 2005b). Additionally, proteins immunologically related to dehydrins have been found in a variety of other organisms, including cyanobacteria (Close and Lammers 1993) and a Brown fucoid alga (Li et al 1998), by utilizing antibodies directed against the K-segment, thus suggesting that these proteins have been conserved during evolution. Despite this, direct evidence on the *in vivo* roles of dehydrins has yet to be explained (Close 1996, 1997).

#### **IV. Desiccation tolerance in seaweeds**

Compared to the many studies conducted on desiccation tolerance in land plants, there have been relatively few studies conducted on seaweeds. Of all the potentially adverse environmental conditions intertidal seaweeds are exposed to, desiccation is thought to be the most important, with periods of exposure up to eight hours occurring twice a day (Bell 1993, Davison and Pearson 1996).

Generally speaking, past work has shown a correlation between desiccation tolerance of intertidal seaweeds and their intertidal distribution. For example, Dring and Brown (1982) showed that high intertidal seaweeds *Pelvetia canaliculata* and *Fucus spiralis* had a better rate of photosynthetic recovery after desiccation than the

lower intertidal seaweed *Laminaria digitata*. Similarly, Brown (1987) found better recoveries in two other high intertidal seaweeds, *Bostrychia arbuscula* and *Apophloea lyallii* in New Zealand, than in lower intertidal seaweeds. Abe et al. (2001) tested 18 intertidal seaweeds in Japan and found that the highest intertidal seaweed, *Porphyra dentata*, could better recover its photosynthetic activity after being desiccated in 30% relative humidity than all the other species. Also, *Porphyra linearis*, another high intertidal species collected from the Israeli Mediterranean coast, was shown to fully recover from 24 hours of desiccation at 5% relative humidity in just 10 minutes (Lipkin *et al.* 1993). Thus desiccation tolerance has been observed in intertidal seaweeds from around the world. However, past studies have not fully explained the mechanism involved on the tolerance to desiccation in seaweeds (Davison and Pearson 1996).

A primary focus of past studies in desiccation tolerance in intertidal seaweeds has been the role of ROS metabolism. Reactive oxygen metabolism has been shown to play a role in stress tolerance in several intertidal seaweeds, however the specific protective mechanism involved differed amongst species. Additionally, differences have been observed amongst the same species collected at different intertidal heights. For example, studies of the high intertidal red alga *Mastocarpus stellatus* revealed increased levels of catalase and glutathione reductase, whereas studies of the green alga *Ulva rigida* and species of the brown alga *Fucus* revealed increased levels of ascorbate peroxidase in response to exogenously produced H<sub>2</sub>O<sub>2</sub> (Collén and Pedersén 1996; Collén and Davison 1999a, 1999b). Also, specimens of the green alga *Ulva lactuca* collected from a higher intertidal zone produced less H<sub>2</sub>O<sub>2</sub> and lipid

hydroperoxides than specimens collected from lower intertidal zone (Ross and Van Alstyne 2007).

In conclusion, despite numerous studies showing the desiccation tolerance of intertidal seaweeds, scientists still know very little about the actual mechanism behind desiccation tolerance in seaweeds. Most reports have suggested a relationship between intertidal height and antioxidative activity, but these studies usually measured antioxidant activity in the hydrated or partially dehydrated states. Since they did not test specimens after rapid and extreme desiccation, ROS defense may not explain the survival of intertidal seaweeds under such extreme stress. Lastly, desiccation tolerance is a complex phenomenon and several protection mechanisms, not just ROS defense, have been proposed for land plants. However, these hypotheses have not been tested in intertidal seaweeds such as *Porphyra*.

## **V. Past observations in two species of *Porphyra* undergoing occurring desiccation**

The intertidal alga *Porphyra* is the highest intertidal seaweed in New England. It is emerged twice a day and can be out of water up to 8 hours per tide, thus it experiences a drastic change of water content during its emergence. This is very different from what happens in land plants where natural drying occurs very slowly. For example, it can take days for resurrection plants to develop desiccation tolerance (Black and Pritchard 2002). Therefore, it is very likely that *Porphyra* may possess unique mechanisms in desiccation tolerance. In addition to its unique habitat, the presence of a congeneric species at low tidal height, as well as its simple construction,

and culturability make *Porphyra* an ideal model for desiccation studies.

For the past several years, two species of *Porphyra* have been used in the Cheney lab to study desiccation tolerance in intertidal seaweeds. Specifically, they have shown that the high intertidal species, *P. umbilicalis*, is much more tolerant to desiccation than the low intertidal species, *P. yezoensis* (Yen-Chun Liu, PhD thesis, *in preparation*). Although both species lose water at a similar rate, Liu has shown that *P. umbilicalis* is completely viable after a 3-hour desiccation period, while *P. yezoensis* was no longer viable (Yen-Chun Liu, PhD thesis, *in preparation*).

Further examination into the consequences of desiccation showed that *P. yezoensis* after a 3-hour desiccation period had severely impaired photosynthesis, amino acid leakage, and electron transport chain disruption. These results imply that the membranes of *P. yezoensis* are sensitive to desiccation. In addition, transmission electron microscopy (TEM) micrographs show direct evidence that the membranes of *P. yezoensis* were disrupted and fused. Meanwhile, *P. umbilicalis* was not affected by the 3-hour desiccation and its membranes were perfectly normal and intact in TEM micrographs (Yen-Chun Liu, PhD thesis, *in preparation*).

Since membrane damage was observed as a result of desiccation, analysis of ROS defense was done in both species to see if it was playing a role in causing the membrane damage in *P. yezoensis*. If ROS is responsible, desiccation of *P. yezoensis* in the dark should alleviate such damage since photosynthesis is considered the major cause of ROS formation (Smirnoff 1993, Buchanan et al 2000). However, desiccation of *P. yezoensis* in the dark still showed amino acid leakage and low viability similar to levels when desiccated in light. Additionally, levels of

hydroperoxides caused by ROS attacks on lipids did not increase in either species.

Therefore, ROS defense cannot explain the difference in desiccation tolerance between the two species, leaving repression of phase transition and vitrification as the only two possible tolerance mechanisms to be tested (Yen-Chun Liu, PhD thesis, *in preparation*).

Additional studies by Liu showed that repression of phase transition was not important in desiccation tolerance in *P. umbilicalis* and that neither sucrose nor trehalose are present in cold-water extracts from either species. However, Fourier transform infrared spectroscopy (FTIR) spectra of *P. yezoensis* and *P. umbilicalis* showed that the membranes of both species were in a glass state. FTIR analyses along with electron paramagnetic resonance (EPR) data of both species showed that the dried *P. umbilicalis* had a slower shift of OH stretching band, suggesting that *P. umbilicalis* cytoplasm forms a more stable glass state when dried. Therefore, vitrification appears to play an important role in desiccation tolerance of *P. umbilicalis*. However the exact molecular mechanism is unknown and is the subject of this investigation.

## CHAPTER 1: OBJECTIVES

### Objectives

The overall objective of this study was to investigate the presence and role of proteins immunologically related to dehydrins (herein referred to as dehydrin-like proteins) in desiccation tolerance in *P. umbilicalis* and *P. yezoensis*. In addition, a survey was conducted to see how common these dehydrin-like proteins are in other intertidal algae.

The specific objectives of this study were the following:

1. To determine the presence of dehydrin-like proteins in two species of *Porphyra*, *P. umbilicalis* and *P. yezoensis*.
2. To characterize the hydrophilicity and thermal stability of extracts containing these dehydrin-like proteins.
3. To test the ability of fractions containing these proteins to prevent macromolecule aggregation.
4. To test the ability of fractions containing these proteins to protect macromolecules.
5. To determine the presence of dehydrin-like proteins in the intertidal brown alga *Fucus vesiculosus* and two green alga *Spongomorpha aeruginosa*. and *Monostroma pulchrum*.

## CHAPTER 2: MATERIALS AND METHODS

### I. General culture methods

For all experiments, a strain of *Porphyra yezoensis* (called U-51 from Japan) and a strain of *Porphyra umbilicalis* collected from the high intertidal zone of the Cape Cod Canal, MA were used. Axenic cultures of blades (thallus, haploid) of each species were maintained separately.

Monocultures of blades were grown, maintained, and reproduced asexually via monospores in the laboratory in enriched seawater (seawater containing 1.2% ESS, by volume), as described in the Appendix. ESS contains a mixture of metals, vitamins, and phosphorus and nitrate sources. Blade cultures were maintained in 0.5 and 1.0 L aerated flasks at 15°C, at a light intensity of 25-30  $\mu\text{E}/\text{m}^2/\text{s}$  on a 12:12 (L:D) cycle. ESS and seawater were sterile filtered to 0.2  $\mu\text{m}$ . Culture media was changed weekly.

### II. Preparation of cell extracts

#### A. Preparation of cell extracts from *P. umbilicalis* and *P. yezoensis*

Blades were collected from lab cultures and immediately dried with paper towels and dry weight was measured. The dried plant material was frozen in liquid nitrogen and then ground using a mortar and pestle. 250 mg powders were resuspended (1:3) with a buffer that contained 75 mM Tris, 1 mM EDTA, 1% SDS, 0.25%  $\beta$ -mercaptoethanol, pH 8.3 (herein referred to as extract buffer). The mixed material was sonicated using a Sonifer Cell Disruptor 350 (Branson Sonic Power Co.,

Danbury, CT, USA) on a pulsed setting for 1 minute on ice, and then placed on a DS-500 orbital shaker (VWR Scientific, Bridgeport, NJ, USA) for 1 hour.

Crude cell extracts were centrifuged at 10,000 rcf for 10 minutes. The insoluble pellet was discarded while protein concentrations were determined from the supernatant using a Bradford assay using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA).

#### B. Preparation of cell extracts from *Arabidopsis thaliana*

Extracts of *A. thaliana* (Col ecotype) seeds were prepared using 50 mg of dry material and ground up with a mortar and pestle. The powder was resuspended (1:2) in dH<sub>2</sub>O. Crude cell extracts were centrifuged at 10,000 rcf for 10 minutes. The insoluble pellet was discarded while protein concentrations were determined from the supernatant using the Bio-Rad Bradford Protein Assay (Bio-Rad, Hercules, CA, USA).

### III. Antibody selection

A BLAST search indicated that the K-segment is found in all dehydrins. The dehydrin antibody was chosen based on literature searches on past work on vascular plants and cyanobacteria. All published work indicated that the commercially available anti-dehydrin (StressGen, Victoria, Canada, Cat. PLA-100) which recognizes the K-segment is the best one available.

#### IV. Western blot analysis

Crude cell extracts and fractions from ethanol precipitation ( $20 \mu\text{g}\cdot\text{lane}^{-1}$ ) were mixed with Laemmli buffer (4% SDS, 20% glycerol, 10%  $\beta$ -mercaptoethanol, 0.004% bromphenol blue, 125 mM Tris-HCl, pH 6.8; Laemmli 1970), boiled at  $100^{\circ}\text{C}$  for 10 minutes, then separated electrophoretically in ready-made 4-20% gradient gels (Bio-Rad). The EZ-Run pre-stained RecA protein ladder was used as a molecular weight marker for comparison (Fisher Scientific). Electrophoresis was done at 100 V for 1.5 hours using a mini-gel apparatus (Bio-Rad). Proteins in gels were transferred onto  $0.2 \mu\text{m}$  Immobilon P<sup>sq</sup> membrane (Millipore, Burlington, MA, USA) using a Mini Trans Blot Cell (Bio-Rad). Protein were transferred for 1 hour at 0.35 A in Towbin buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol [pH 8.3]; Towbin et al. 1979) at  $4^{\circ}\text{C}$ . Membranes were blocked in 5% non-fat dry milk in Tris-buffered saline Tween-20 (TBST: 10 mM Tris, 192 mM NaCl, 0.05% Tween-20, pH 7.5) for 2 hours or overnight at  $4^{\circ}\text{C}$ . The membranes were then incubated with anti-dehydrin at 1:1000 dilution in TBST overnight at  $4^{\circ}\text{C}$ , washed 4 times with TBST (10 minutes each wash), then incubated with anti-rabbit IgG horseradish peroxidase conjugate (Sigma) at 1:10000 dilution in TBST for 1 hour, then washed more 4 times with TBST (10 minutes each wash). Immunoreactive proteins were detected by using Immobilon Western Chemiluminescent HRP Substrate (Millipore) reagent for 5 minutes, then put into a plastic protector in an autorad cassette. A piece of x-ray film (Kodak, Rochester, New York) was put over the blot, and the exposure time varied from 1 second to 10 minutes, according to the brightness of the bands. Protein extracts from *A. thaliana* (Col ecotype) seeds were used as a positive control.

## V. Hydrophilic characterization

1 mL of crude cell extracts from *P. yezoensis* and *P. umbilicalis* were mixed with 4 mL of 95% ethanol, then centrifuged at 2,000 rpm to remove precipitated proteins (ethanol precipitation). The supernatant was evaporated using an aquarium pump to boil off the ethanol. The pellet that remained was resuspended in 100  $\mu$ L of dH<sub>2</sub>O. Protein concentrations were determined using a Bradford assay using the Bio-Rad Bradford Protein Assay (Bio-Rad). Ethanol-precipitated fractions (herein referred to as fractions) and an untreated sample (as a control) were analyzed with western blots and anti-dehydrin as described previously and by Coomassie Brilliant Blue G-250 Stain (Bio-Rad).

## VI. Thermal-stability experiments

Aliquots (0.5 mL) of the cell extracts from *P. umbilicalis* were heated at 45°, 55°, 75°, or 95 °C in a heat block for 15 minutes; cooled on ice for 15 minutes; and centrifuged at 10,000 rpm for 15 minutes to remove precipitated proteins. To test for any effect of extraction buffer on the *P. yezoensis* or *P. umbilicalis* extracts, extracts of both algae were prepared as previously described but using dH<sub>2</sub>O instead of extract buffer. Supernatants and an unheated aliquot of the same sample (as a control) were analyzed with western blots and anti-dehydrin as described previously.

## VII. Macromolecular aggregation experiments

Crude cell extracts and fractions were prepared as previously described. Aliquots (0.12 mg) of porcine heart citrate synthase (herein referred to as CS) (Sigma, Cat. No. C3260) were mixed with fractions of *P. yezoensis* and *P. umbilicalis*

(0.4 mg total protein content determined by Bradford assay) in 100  $\mu$ L volume were dialyzed against  $\text{dH}_2\text{O}$  using mixed cellulose ester 0.2  $\mu\text{m}$  membranes (Millipore, Cat. No. VSWP02500) for 30 minutes to remove buffer salts. The mixtures, as well as dialyzed CS alone (as a control), were desiccated in an incubator set at room temperature with a dehumidifier overnight in a 48-well cell culture plate (BD Biosciences, Bedford, MA, USA). Desiccated samples were rehydrated in 170  $\mu\text{L}$   $\text{dH}_2\text{O}$  and subjected to another cycle of desiccation under the previously mentioned conditions. After rehydration to 170  $\mu\text{L}$  volume with  $\text{dH}_2\text{O}$ , aggregation of CS was monitored by reading absorbance at 340 nm using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) at 0.5-minute intervals for 30 minutes.

### **VIII. Macromolecular protection experiments**

To assay citrate synthase (CS) activity, 1 mL of substrate buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 100  $\mu\text{M}$  DTNB [5,5'-dithiosbis-(2-nitrobenzoic acid)], 150  $\mu\text{M}$  acetyl-CoA) was mixed with the rehydrated aliquots from the aggregation assay and undesiccated, dialyzed CS (as a control) in the same 48-well cell culture plate. Changes in absorbance at 412 nm were measured in a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments) every 10 seconds for 3 minutes at 25°C.

### **IX. Statistical analysis**

Statistical tests were performed on macromolecular protection data using SPSS (2009), which included a t-Test of sample means. Significance was evaluated at the 0.01 level.

## **X. Intertidal algae survey**

Young blades of *Fucus vesiculosus*, and adult plants of *Spongomorpha aeruginosa* and *Monostroma pulchrum* were collected off rocky intertidal zones at Northeastern University's Marine Science Center (East Point, Nahant, MA). Crude cell extracts were made from the 0.5 - 1.5 cm apical tips of *Fucus vesiculosus* as described in Li et al (1998). Crude cell extracts of whole plants of *Spongomorpha aeruginosa* and *Monostroma pulchrum* were prepared similarly. An aliquot of each extract was dialyzed against dH<sub>2</sub>O using mixed cellulose ester 0.2 µm membranes (Millipore) for 30 minutes to remove unknown compounds that may interfere with protein concentration by Bradford assay, as mentioned in Li et al (1998). Electrophoresis and western blots of crude cell extracts and dialyzed aliquots were run as previously mentioned.

## CHAPTER 3: RESULTS

### I. Presence of Dehydrin-Like Proteins

To determine the presence of dehydrin-like proteins in two species of *Porphyra*, blades of *P. umbilicalis* and *P. yezoensis* were removed from culture flasks and dried immediately. I tested for the presence of the K-segment using the antibody specific for dehydrins and found, by western blots, a 24 kD band for dehydrins in *A. thaliana* seed extracts and a 17 kD band for dehydrin-like proteins in *P. umbilicalis* (Figure 1). The 17 kD band was consistently present among replicates in *P. umbilicalis*, meanwhile no bands were detected in *P. yezoensis* extracts.

### II. Hydrophilic Characterization

If these proteins in *P. umbilicalis* were dehydrin-like, they should remain in the supernatant after ethanol precipitation due to their highly unfolded and hydrophilic structure. A comparison of the crude cell extracts to ethanol-precipitated fractions of *P. umbilicalis* and *P. yezoensis* were done on gels stained for Coomassie blue (Figure 2, left). An examination of lanes with crude cell extracts revealed the presence of many proteins while not many were present in ethanol-precipitated fractions. 15 kD and 17 kD bands were present in fractions of both *P. umbilicalis* and *P. yezoensis*. After running aliquots of the same fractions for western blot analysis (Figure 2, right), a dehydrin-like protein can be detected at 17 kD in *P. umbilicalis* but not in *P. yezoensis*. These results were observed consistently in four experimental replicates.

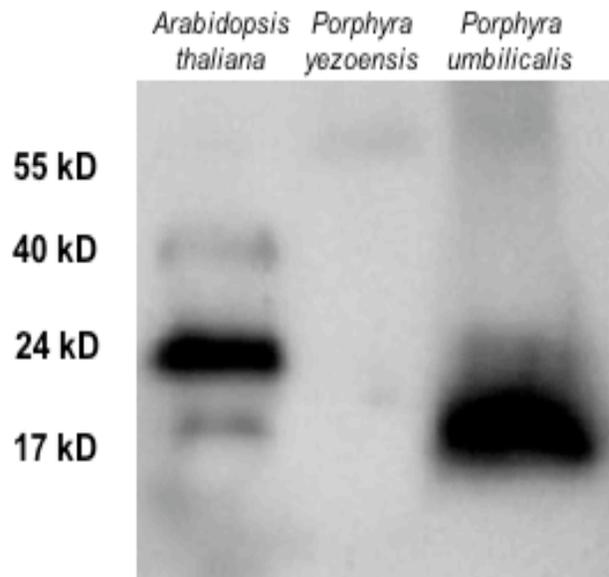


Figure 1. The presence of dehydrin-like proteins in *P. umbilicalis* as detected using the antibody specific for the K segment of dehydrins. The western blot also shows the presence of dehydrins in *Arabidopsis thaliana* (Col ecotype) (as a control) but absent in *P. yezoensis*.

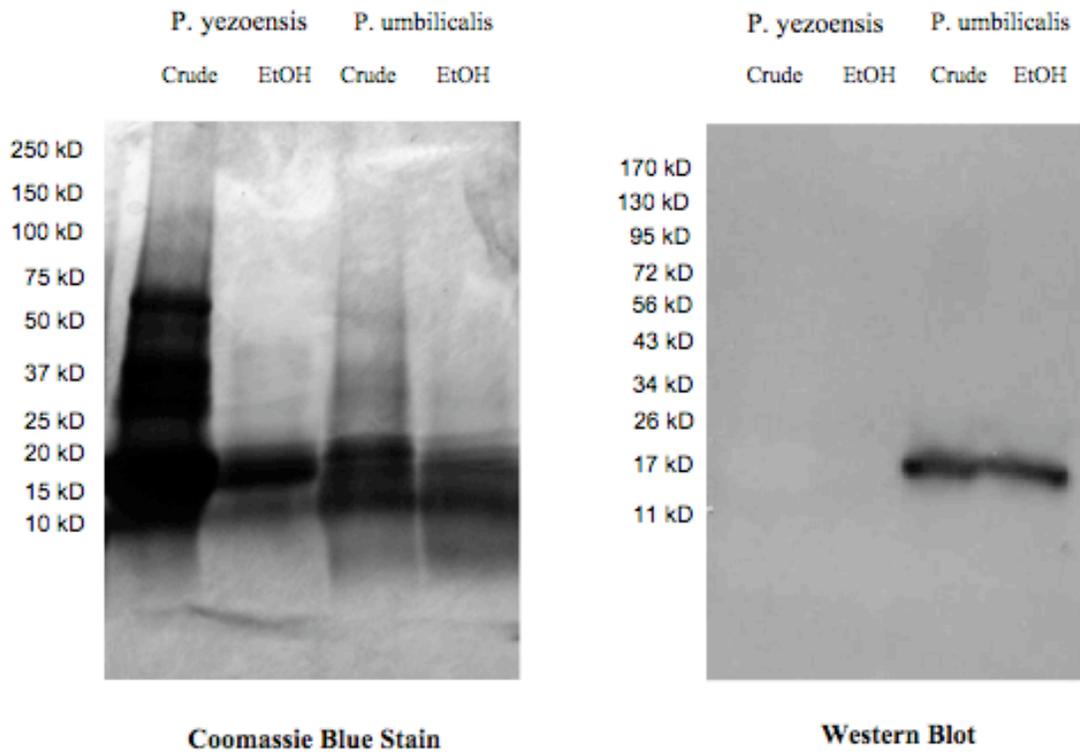


Figure 2. (left) Coomassie blue stain of a 4-20% Tris-HCl gel with crude cell extracts and ethanol-precipitated fractions from *P. yezoensis* and *P. umbilicalis*. When the same extracts are run on another gel and transferred to a PVDF membrane for Western blot (right), a dehydrin-like protein can be detected as a 17 kD band in both crude cell extracts and ethanol-precipitated fractions of *P. umbilicalis* and not in *P. yezoensis*.

### III. Thermal Stability

To determine if these dehydrin-like proteins were thermally stable and remain soluble at 75°-100°C for a prolonged period, crude cell extracts of *P. umbilicalis* were heated at increasing temperature increments (45°, 55°, 75°, then 95°C) for 15 minutes, then immediately placed on ice for 15 minutes. Western blots revealed that amount of dehydrin-like protein (17 kD) remained the same when an extract prepared in buffer was heated at the indicated temperatures, in comparison to an unheated aliquot of the same sample (Figure 3A). The same results were observed with extracts prepared in dH<sub>2</sub>O (Figure 3B). Dehydrins (24 kD) from *A. thaliana* remained soluble during prolonged exposure to 95°C, whether in extraction buffer or dH<sub>2</sub>O (data not shown). These experiments were repeated twice and the same results were obtained.

### IV. Macromolecular Aggregation

To determine if these dehydrin-like proteins are able to play a chaperone-like role during desiccation, 0.12 mg of citrate synthase (CS) was mixed with fractions of *P. umbilicalis* and *P. yezoensis* (0.4 mg total protein content each) and dialyzed against dH<sub>2</sub>O to remove buffer salts. After two cycles of desiccation and rehydration to 170 µL volume, CS showed marked aggregation with an apparent increase in absorbance at 340 nm for 30 minutes (Figure 4, red line). However, absorbance started to decrease after 30 minutes. Aggregates were observed to be so large that they did not stay in suspension and deposited on the walls of the wells. Thus, the cutoff time limit of the experiment was set at 30 minutes.

The desiccated mixture of CS and *P. umbilicalis* protein fraction (Figure 4, green line) was able to prevent aggregation when compared to desiccated CS alone (Figure 4, blue line) and desiccated mixture of CS and *P. yezoensis* protein fraction (Figure 4, yellow line). Interestingly, the desiccated mixture containing CS and *P. yezoensis* protein fraction was able to prevent some degree of macromolecular aggregation when compared to desiccated CS alone, though not as much as the desiccated mixture of CS and *P. umbilicalis* protein fraction.

## V. Macromolecular Protection

To determine if the dehydrin-like proteins found in *P. umbilicalis* are able to protect macromolecules when subjected to water stress by desiccation, activity of CS was assayed by mixing 1 mL of substrate buffer with the rehydrated samples from the aggregation assay and undesiccated, dialyzed CS (as a control) in the same 48-well cell culture plate. Due to limits of the plate reader, readings could not be taken every 1.5 seconds as per the protocol described by Goyal et al (2005), but were taken every 10 seconds for three minutes to observe any differences in activity. Results are expressed as a percentage of control activity after one minute. This experiment contained three well replicates per condition and was repeated twice.

Changes in absorbance at 412 nm were observed within the first minute of the assay (Figure 5). Desiccated CS mixed with fractions of *P. umbilicalis* displayed 49.55%(+/- 0.11%) enzymatic activity compared to undesiccated CS (Figure 5, Figure 6B, green and blue respectively). 0% activity was seen in desiccated CS alone (Figure 5, Figure 6B, red) or CS mixed with *P. yezoensis* (Figure 5, Figure 6B, yellow).

T-Test analysis showed that fractions from *P. umbilicalis* were able to prevent aggregation and preserve enzyme activity significantly more ( $p < 0.01$ ) than desiccated CS alone and desiccated CS mixed with the *P. yezoensis* fraction (Figure 6A). Although *P. yezoensis* fractions were able to prevent aggregation ( $p < 0.01$ ) compared to desiccated CS alone, they were not able to preserve CS enzyme activity under desiccation.

## VI. Intertidal algae survey

To determine how common the presence of dehydrin-like proteins are in intertidal algae, a survey was conducted on the brown alga *Fucus vesiculosus* and two green algae *Spongomorpha aeruginosa* and *Monostroma pulchrum*. Adult blades of *Fucus vesiculosus*, *Spongomorpha aeruginosa*, and *Monostroma pulchrum* were collected and crude cell extracts of were prepared. Aliquots were dialyzed against dH<sub>2</sub>O to remove potential interfering compounds and then protein concentrations were determined by Bradford assay.

After loading 20 ug/lane for each extract and dialyzed aliquots, proteins were transferred to membranes. Dehydrin-like proteins could not be detected by western blotting in *F. vesiculosus*, *Monostroma pulchrum*, and *Spongomorpha aeruginosa*, however both 24 and 17 kD bands were detected in *A. thaliana* and *P. umbilicalis* (Figure 7).

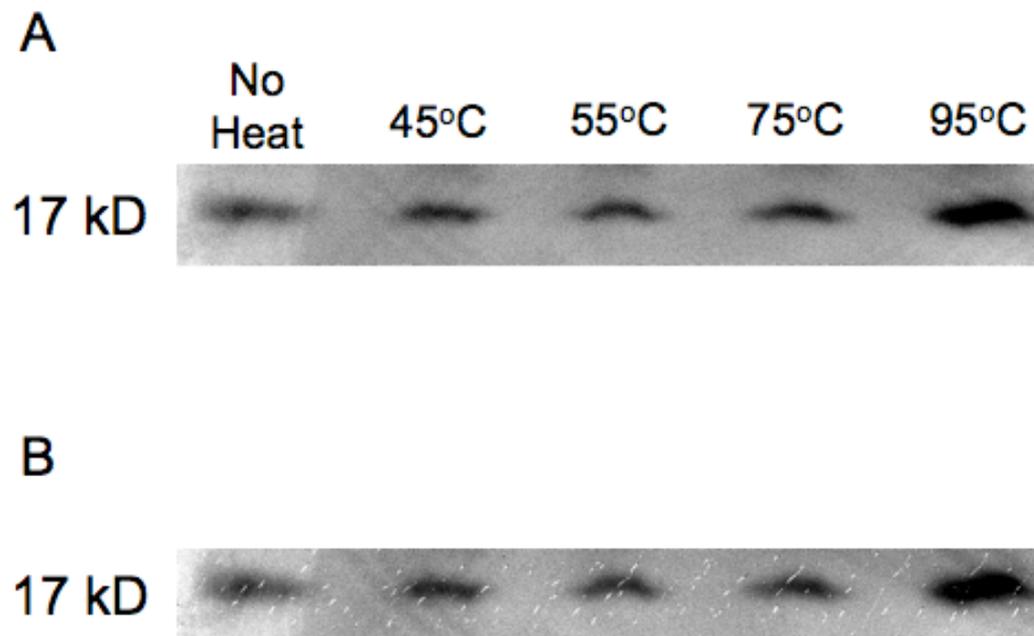


Figure 3. (A) *P. umbilicalis* extract prepared with extract buffer. (B) *P. umbilicalis* extract prepared with dH<sub>2</sub>O. Dehydrin-like proteins in crude cell extracts of *P. umbilicalis* were thermally stable and remained soluble after heating at the indicated temperatures for 15 minutes then rapidly cooling on ice for 15 minutes. No difference in stability was seen in extracts prepared with extract buffer or dH<sub>2</sub>O.

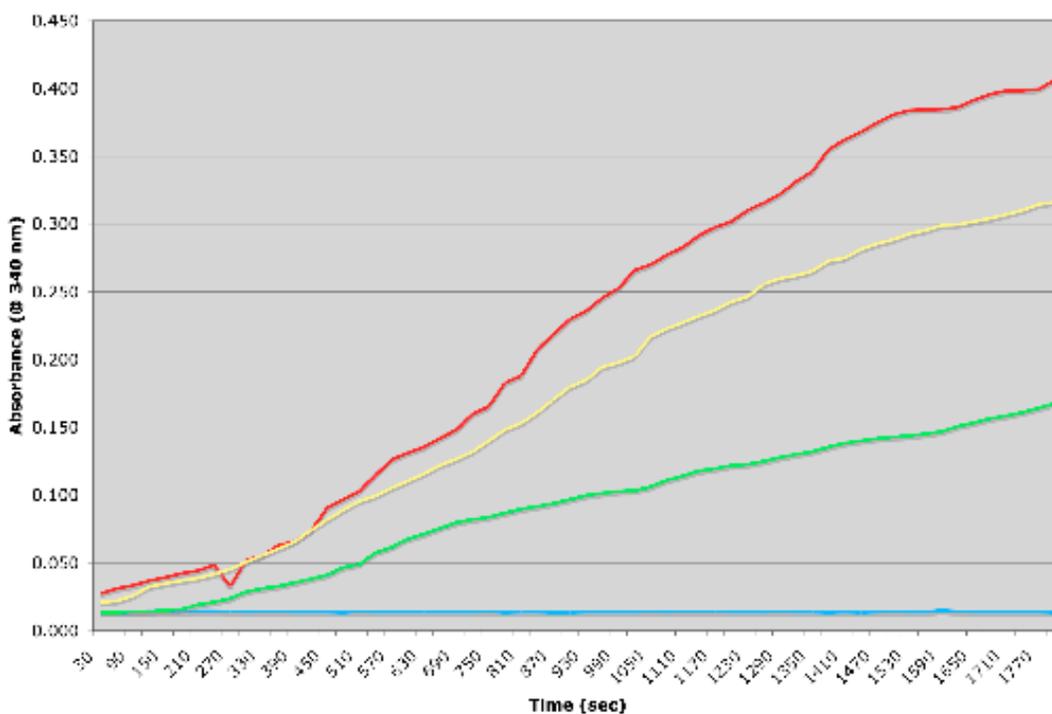


Figure 4. The aggregation kinetics of desiccated citrate synthase was determined at room temperature in the presence or absence of ethanol-precipitated fractions of *P. yezoensis* and *P. umbilicalis*. Absorbance was measured at 340 nm with readings taken every 30 seconds. Undesiccated CS (0.12 mg) (—), desiccated CS alone (0.12 mg) (—), desiccated CS with *P. yezoensis* (0.12 mg + 0.4 mg, respectively) (—), and desiccated CS with *P. umbilicalis* (0.12 mg + 0.4 mg, respectively) (—).

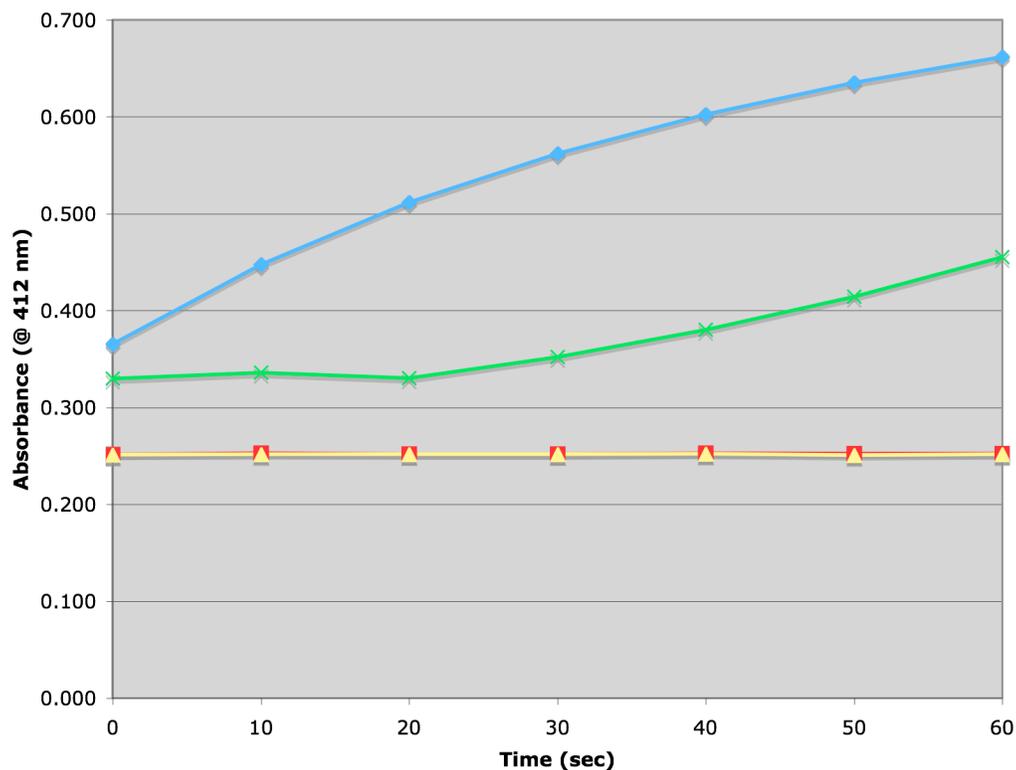
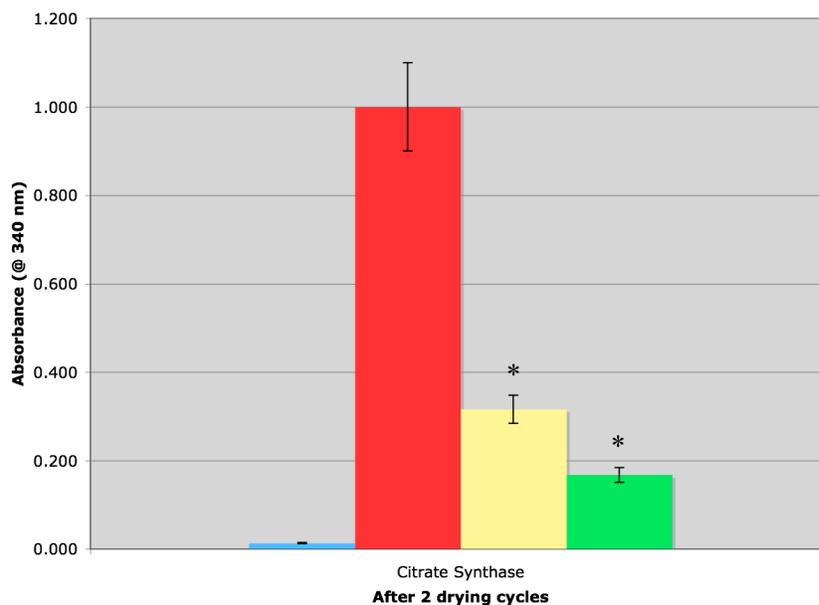


Figure 5. The effect of desiccation on citrate synthase was determined at room temperature in the presence or absence of ethanol-precipitated fractions of *P. yezoensis* and *P. umbilicalis*. Absorbance was measured at 412 nm with readings taken every 10 seconds. Undesiccated CS (0.12 mg) (—), desiccated CS alone (0.12 mg) (—), desiccated CS with *P. yezoensis* (0.12 mg + 0.4 mg, respectively) (—), and desiccated CS with *P. umbilicalis* (0.12 mg + 0.4 mg, respectively) (—).

A



B

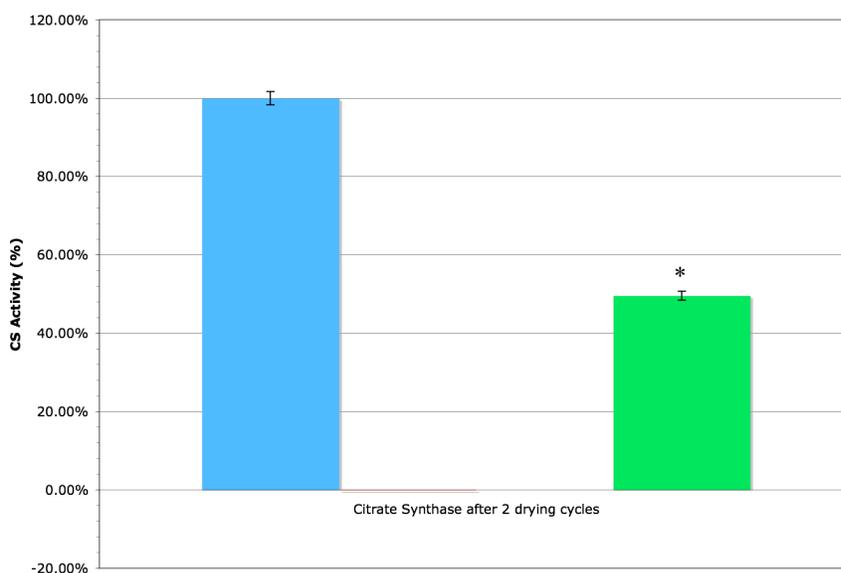


Figure 6. (A) Aggregation and (B) activity of 0.12 mg of citrate synthase before desiccation (blue bar), after desiccation (red bar), in presence of 0.4 mg total protein content from *P. yezoensis* ethanol-precipitated fraction (yellow bar), and 0.4 mg total protein content from *P. umbilicalis* ethanol-precipitated fraction (green bar). Aggregation is measured by absorbance at 340 nm. Enzyme activity is assayed according to standard methods and results are expressed as a percentage of control activity. \*  $p < 0.01$  shown above the bar represents results significantly different from those for citrate synthase alone using t-test.

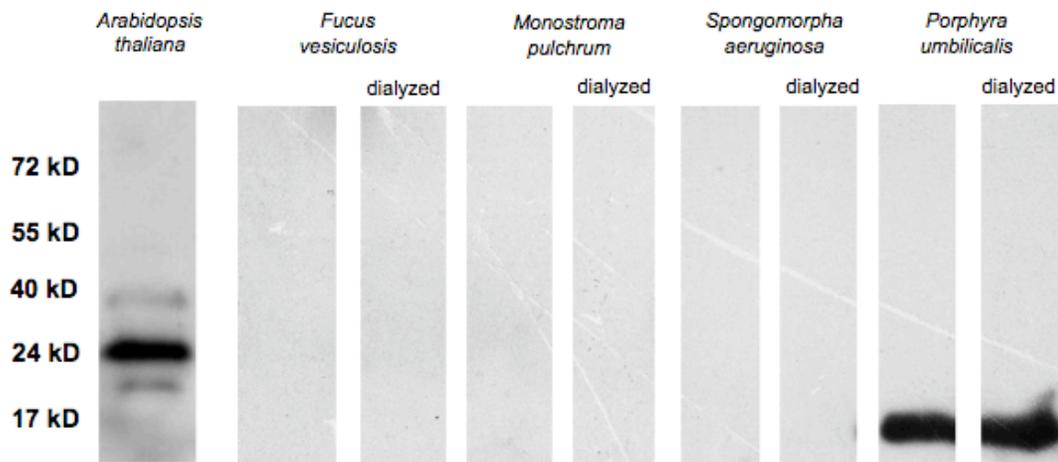


Figure 7. Survey of intertidal algae using crude cell extracts and dialyzed aliquots using anti-dehydrin. Though a 24 kD band for dehydrins and a 17 kD band for dehydrin-like proteins in *A. thaliana* and *P. umbilicalis* were detected, no bands could be seen in *F. vesiculosus*, *Monostroma pulchrum*, and *Spongomorpha aeruginosa*.

## CHAPTER 4: DISCUSSION

The main objective of this study was to determine whether dehydrin-like proteins are found in *Porphyra umbilicalis* and play a role in desiccation tolerance. These factors would then be characterized in terms of hydrophilicity, thermal stability, and ability to prevent aggregation and to preserve enzyme activity after desiccation. The final goal of this study was to the expression of dehydrin-like proteins in other intertidal algae.

For the past several years, studies in the Cheney lab have shown that the high intertidal species, *P. umbilicalis*, is much more tolerant to desiccation than the low intertidal species *P. yezoensis*. In addition, work in the Cheney lab has shown that ROS defense and membrane phase transition cannot explain the difference in desiccation tolerance between the two species (Yen-Chun Liu, PhD thesis, *in preparation*). Liu's results suggest that vitrification plays an important role in desiccation tolerance of *P. umbilicalis*. However, the molecules involved in facilitating this mechanism were unknown. It was hypothesized that dehydrin-like proteins could play a role in this vitrification mechanism. The present study confirms that a dehydrin-like protein appears to be involved. This study shows that dehydrin-like proteins are expressed in *P. umbilicalis* but not in *P. yezoensis*. This is especially remarkable since these two seaweeds have been grown under laboratory conditions and have not been exposed to a natural tidal cycle for more than two years.

The constitutive expression of these dehydrin-like proteins in *P. umbilicalis* is a very interesting finding. It is similar to findings reported in past work on vascular

plants. Constitutively expressed dehydrin-like proteins have been found in a species of pea (*Pisum sativum*), in the bark tissues of some woody plants, and in the desiccation tolerant moss *Tortula ruralis* (Bewley et al 1993, Robertson and Chandler 1992, Wisniewski et al 1996). Bewley et al (1993) proposed that the constitutive expression of some protective molecules might be an evolutionary strategy for nonvascular plants that experience frequent and/or unpredictable periods of drought and that lack morphological or physiological attributes to limit water loss. *P. umbilicalis* in New England experiences severe emersion twice a day, lasting up to 8 hours per tide. It lacks structural adaptations to limit water loss and loses 95% water content within an hour (Yen-Chun Liu, PhD thesis, *in preparation*). Therefore, constitutive expression of these dehydrin-like proteins could be what confers the protection required for survival under these intertidal conditions.

In the current study, these dehydrin-like proteins in *P. umbilicalis* were found to be extremely hydrophilic after ethanol-induced dehydration. Ethanol can have a direct effect of substituting for water by displacing hydrogen-bonded water within proteins itself as a form of dehydration (Klemm 1990). Therefore, when adding more than the amount of ethanol required to produce 50% denaturation of proteins at 25°C, proteins stabilized by a hydrophobic interior in the presence of water will become very disorganized and denatured (Herskovits et al 1970). The irreversible denaturation of these proteins results in reduced protein solubility and low melting temperature, causing them to precipitate out of solution (deWit and Klarenbeek 1984).

There is a likely explanation for why dehydrin-like proteins remain in solution when *P. umbilicalis* extracts were mixed with a large volume of ethanol. Dehydrins found in vascular plants are usually rich in hydrophilic amino acids and have charged and polar amino acids dominating a large proportion of their peptide sequences (Oliveira et al 2007). In addition, most dehydrins lack cysteine and tryptophan residues (Ingram and Bartels 1996). This residue composition in turn inhibits the formation of an extensive hydrophobic core typical for a folded protein. These properties give dehydrins a disordered structure with low secondary structure content allowing water to become more tightly bound than other disordered proteins (Bokor et al 2005, Mouillon et al 2006). The presence of dehydrin-like proteins in *P. umbilicalis* after the ethanol-induced dehydration imply that these proteins possess a very hydrophilic amino acid sequence that allows it to remain soluble under conditions of very little water.

Another result of the present study revealed that these dehydrin-like proteins are remarkably heat stable. The extract buffer did not confer the stability observed as the same results were seen in *P. umbilicalis* extracts prepared with water. Though the exact amino acid sequence of these dehydrin-like proteins found in *P. umbilicalis* is unknown, the thermal stability of these dehydrin-like proteins is promising and agrees with known characteristics of dehydrins found in vascular plants (Reid and Walker-Simmons 1991, Close et al 1989).

An explanation for this heat stability is based on *in vitro* studies utilizing the hydrophilic amino acid-rich portion of dehydrins. These portions of dehydrins demonstrate their heat stability, as they fail to coagulate upon boiling (Mouillon et al

2006, Oliveira et al. 2007). In addition, the amino acid sequences of *Arabidopsis thaliana* dehydrins have been shown to contain very few hydrophobic amino acids, which coincides with their thermal stability (Oliveira et al 2007).

To better understand the mechanistic role these dehydrin-like proteins may play in desiccation tolerance of *P. umbilicalis*, a standard assay for chaperone activity and prevention of aggregation by desiccation was conducted in this study. It revealed that *P. umbilicalis* fractions were capable of suppressing aggregation and inactivation of citrate synthase. An unexpected result in this study was that *P. yezoensis* fractions were also able to prevent aggregation. However, the degree of protection was much lower than that of *P. umbilicalis* fractions, and was not able to prevent inactivation of citrate synthase.

The result that *P. umbilicalis* protein fractions can display possible chaperone activity during desiccation is intriguing. *In vitro* studies of dehydrins in the past have demonstrated their ability to act as non-classical molecular chaperones under water stress conditions caused by desiccation (Brown et al 2004, Goyal et al 2005). Dehydrins *in vitro* have been demonstrated to bind to non-native proteins to maintain them in a folding competent state and prevent irreversible aggregation (Ellis 2004, Goyal et al 2005). In addition, the K segment of dehydrins in vascular plants has been proposed to interact with hydrophobic patches of proteins undergoing denaturation resulting from low water content, which would also interfere with protein aggregation (Close 1996). Therefore, the expression data and the protection and aggregation results provide insight about the function of these dehydrin-like proteins in *P. umbilicalis*.

The characteristic of *P. yezoensis* fractions suppressing aggregation of citrate synthase, but not inactivation is probably due to the presence of some other molecule other than dehydrin. Though dehydrin-like proteins were not detected in *P. yezoensis* fractions, there could be another molecule or molecules that remained in the fractions after dialysis with dH<sub>2</sub>O. For instance, species of *Porphyra* produce sugars called floridosides as photosynthetic products from the Calvin cycle, though their function is unknown (Majak et al 1966, McLachlan et al 1972). It is possible that these floridosides remained in the *P. yezoensis* fractions and as an artifact of macromolecular crowding, prevented aggregation of citrate synthase to an extent during desiccation.

Recent findings on *P. umbilicalis* have found that ROS metabolism does not play a role in its desiccation tolerance nor are the sugars sucrose and trehalose present in this seaweed (Yen-Chun Liu, PhD thesis, *in preparation*). When desiccated, *P. umbilicalis* forms a more stable glass state than that of the sensitive seaweed *P. yezoensis* (Yen-Chun Liu, PhD thesis, *in preparation*). The mechanistic explanation of this more stable glass state could be explained by the preliminary characterization of these dehydrin-like proteins in *P. umbilicalis* fractions. These proteins could act as structural supports, by spatially cross-linking with other macromolecular constituents that crowd as a result of insufficient water content during the formation of this glass state (Mouillon et al 2008).

To determine how common these dehydrin-like proteins are in other intertidal algae, this study conducted a survey that included a brown alga, *Fucus vesiculosus* and two green algae, *Monostroma pulchrum* and *Spongomorpha aeruginosa*. Li et al

(1998) reported a 35 kD band for dehydrin-like proteins in *F. vesiculosus* from Maine. However after repeated attempts, expression of dehydrin-like proteins in *F. vesiculosus* could not be detected.

There are several possible explanations for the apparent lack of dehydrin-like proteins in these three algae that were surveyed. First an examination of Li et al (1998) figures 3 and 4, showed a weak band for vegetative tissue and a stronger band for reproductive receptacles. I did not look at receptacles in this survey due to the presence of large amounts of mucilage material that we felt would have made protein extraction much more difficult (D. Cheney, personal communication). Our feeling is that Li et al (1998) must have used other “cleaning” methods not described in their paper to get a band at 35 kD. Another explanation is that our antibody must somehow be different from theirs. As for the two green algae that were surveyed, these two species grow in intertidal regions off of the rocky shores of Nahant, MA. However they only grow in relatively moist conditions (D. Cheney, personal communication). Therefore, they may truly lack dehydrin-like proteins. In addition, a literature search for *Spongomorpha* sp, revealed that this alga synthesizes a sulphated heteropolysaccharide composed of arabinose, xylose, galactose, and glucose (Rao et al 1991). Though this polysaccharide has not been tested, other non-reducing sugars like trehalose and sucrose have been demonstrated to play a role in desiccation tolerance of land plants (Carpenter et al 1987, Crowe et al 1992, Lins et al 2004). Therefore, *Spongomorpha* sp. and *Monostroma pulchrum* may possess a different mechanism of desiccation tolerance devoid of dehydrin-like proteins.

My first suggestion for future research in this area would be to identify and sequence the dehydrin-like protein at 17 kD for *Porphyra umbilicalis* so that they can be compared with those reported for land plants. Also an immunoprecipitation of the dehydrin-like protein should be done to see if it is conferring that activity. In addition, it is recommended that a cDNA library be constructed to clone it and see if it can confer desiccation tolerance to other sensitive plants. I would also recommend that *Fucus vesiculosus* and other intertidal algae be reexamined for dehydrin-like proteins by using genomic or cDNA sequencing to see if they might express them. Although this study did not reveal dehydrin-like proteins in the two green algae that were tested, there are others that could be surveyed including the high intertidal, green alga *Prasiola crispa* found in Maine which demonstrated to be remarkably desiccation tolerant and may possess a similar mechanism facilitating this tolerance (Jacob et al 1992).

## CHAPTER 5: CONCLUSIONS

The major findings of this study were as follows:

- 1.) Dehydrin-like proteins were found in the desiccation tolerant *P. umbilicalis* and not in the desiccation sensitive *P. yezoensis*.
- 2.) Dehydrin-like proteins in *P. umbilicalis* are extremely hydrophilic and remained soluble after ethanol-induced dehydration.
- 3.) Dehydrin-like proteins in *P. umbilicalis* crude cell extracts were very heat stable and remained soluble after heating to 95°C for 15 minutes.
- 4.) Fractions of *P. umbilicalis* containing these dehydrin-like proteins were able to suppress protein aggregation of the labile enzyme citrate synthase after two cycles of desiccation.
- 5.) The same fractions of *P. umbilicalis* that suppressed protein aggregation were also able to maintain 49.55% activity of citrate synthase.
- 6.) A survey of the intertidal brown alga *F. vesiculosus* and two green algae *Spongomorpha aeruginosa* and *Monostroma pulchrum* did not find dehydrin-like proteins in crude cell extracts of these species.

## Appendix

### ESS Stock Solution\*

|                                      |           |
|--------------------------------------|-----------|
|                                      | Mg/L      |
| Sodium nitrate                       | 6000      |
| Sodium glycerophosphate              | 800       |
| Ferric sequestrene                   | 1.0       |
| PII Metal Stock Solution (see below) | 4 mL/1 L  |
| ESS Vitamin Mix (see below)          | 10 mL/1 L |

### *PII Metal Stock solution (100X)*

|                                 |           |
|---------------------------------|-----------|
| Zinc chloride                   | 10.9 mg/L |
| Manganese chloride tetrahydrate | 144 mg/L  |
| Cobalt chloride tetrahydrate    | 4 mg/L    |
| Iron III chloride hexahydrate   | 49 mg/L   |
| EDTA                            | 0.9 mg/L  |
| Boric acid                      | 1.14 mg/L |

### *ESS Vitamin Mix (100X)*

|                        |                |
|------------------------|----------------|
| Vitamin B-12           | 1.0 mg/100 mL  |
| Biotin                 | 1.0 mg/100 mL  |
| Thiamine hydrochloride | 100 mg/100 mL  |
| Nicotinic acid         | 100 mg/100 mL  |
| Calcium pantothenate   | 100 mg/100 mL  |
| p-Aminobenzoic acid    | 10 mg/mL       |
| Meso-inositol          | 1000 mg/100 mL |
| Thymine                | 100 mg/mL      |

\*ESS stock solution was diluted to 1.2% in filter-sterile seawater for use as algae media.

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