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Effects of Selenium on the Freshwater Alga Chlamydomonas reinhardtii with and Without the Proteasome Inhibitor MG132

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EFFECTS OF SELENIUM ON THE FRESHWATER ALGA *CHLAMYDOMONAS REINHARDTII WITH AND WITHOUT THE PROTEASOME INHIBITOR MG132*

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Abstract

Selenium is a required nutrient in animal life but has not been shown to be needed in plants. The model plant cell *Chlamydomonas reinhardtii* may be an exception as it appears to increase its growth rate in the presence of selenium. Because selenium has the same valence state as sulfur it may be incorporated into proteins as the modified amino acid selenocysteine. This incorporation of selenocysteine into protein may disrupt the disulfide bridges that give proteins the correct conformation. Misfolded proteins can wreak havoc on a cell. This stress can be measured through several parameters. Chlorophyll levels, glutathione levels, growth, and peroxidized lipid levels are just a few things that could be affected. This study is interested in the affects selenium has on those levels, and how they are affected when the proteasome is inhibited.

Introduction

Selenium is an element found in varying concentrations throughout nature. Animals require selenium in trace amounts and all selenium used by animals is acquired from plant sources (Ellis and Salt 2003). Interestingly, plants have not been found to require selenium (Fu et al. 2002). Selenium is taken up into plants by mistake because it shares a valence state with sulfur, a required element in plants (Pilon-Smits and LeDuc 2009). This uptake of selenium may be toxic to plants because of possible substitution of selenium for sulfur in cysteine, creating selenocysteine which is incorporated into proteins, not allowing sulfide bridges to form (Sabbagh and Van Hoewyk 2012).

Selenium levels in the environment can be a cause for concern. Although selenium is required by animals in small amounts, too much may cause protein misfolding by disruption of disulfide bridges. Transgenic plants may be created to accumulate selenium as a way to "cleanse" the environment (Pilon-Smits and LeDuc 2009), but it is important to understand the physiological mechanisms involved with selenium metabolism if these plants are to be created.

Selenium stress can induce physiological changes in plants that can be measured. Parameters often measured to gauge the level of stress plants are experiencing include chlorophyll content and glutathione levels. *Chlamydomonas reinhardtii* is a commonly studied model plant system. Because *Chlamydomonas* is an algae it exhibits growth similar to other commonly studied microbes so cell count is often considered measuring growth.

Chlorophyll content can be an indication of cell number because if more cells are in the sample then chlorophyll content will likely increase. Chlorophyll measurements can also indicate changes in the photosynthesis system in the algae. Selenium is likely to affect this system because of selenium's ability to replace sulfur in biomolecules. In the photosynthetic system as well as in other plastid systems iron-

sulfur clusters play an important role and the replacement of sulfur with selenium is likely to wreak havoc on the cell (Geoffroy et al. 2007).

A cells glutathione levels often are affected by stress. Glutathione is responsible for reducing hydrogen peroxides and other reactive oxygen species that may damage a cell (Leisinger et al.1999) Reactive oxygen species (ROS) are always present in aerobic cells as a result of living in a oxygen rich environment but under certain circumstances, particularly when a cell is under stress ROS levels can increase (Dayer et al. 2008).

Cell count is a simple test to determine the growth capabilities of cells. According to Morlon et al. 2005 the final cell count decreases in *Chlamydomonas reinhardtii* when exposed to selenite treatments. This is because it affects the metabolism of the cell though the exact mechanism is not completely known.

Alternative oxidase (AOX) levels may be affected by the introduction of selenium. AOX is an electron carrier involved in plant respiration and it is generally accepted that AOX serves to reduce reactive oxygen species in stressed plants (Tarasenko et al. 2012). Selenium may also increase lipid peroxidation. When lipids are oxidized, the products are toxic to the organism. One oxidative particle can start a chain reaction of lipid peroxidation, which causes havoc on the cell, especially the lipid membranes (Marnett 1999).

One pathway that destroys misfolded proteins is the ubiquitin-proteasome pathway (Lecker et al. 2006). Because selenium may cause proteins to misfold, the effect of selenium on the ub-proteasome pathway should be studied. Selenium may be incorporated into proteins as selenocysteine, which would disrupt the disulfide bridges in the proteins. This would cause a misfolded protein, which would be tagged by ubiquitin and sent to the proteasome for degradation (Kampen et al. 1996). If algae

are exposed to selenium causing proteins to become misfolded, the proteasome being a protein may also become misfolded, decreasing its ability to remove other misfolded proteins.

Selenium treatments will likely inhibit proteasome activity resulting in an increased number of ubiquinated proteins, which can be detected using western blotting techniques. Glutathione levels should increase with exposure to selenium. Growth and chlorophyll levels are likely to be decreased in the presence of selenium.

Materials and Methods

Chlamydomonas reinhardtii Growth and Treatments

Batch cultures of algae (*Chlamydomonas reinhardtii*) were grown in sterile tris-acetate phosphate media (TAP) and tris-phosphate media (TP). Flasks were prepared with varying concentrations and combinations of selenite, selenate, selenocysteine, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), and Mg132 (a proteasome inhibitor). The flasks were inoculated with algae, which were allowed to grow for a few days on a shaker platform in an incubator.

Counting *Chlamydomonas reinhardtii*

The algae were quantified using three techniques: turbidity, hemocytometry, and chlorophyll content. Turbidity was measured by using a spectrometer at an absorbance of 550nm. Hemocytometry was carried out using a hemocytometer and a microscope. Chlorophyll content was determined using a spectrophotometer at an absorbance of 652nm. Chlorophyll concentration measurements also offer insight into the change in the functioning of the photosynthetic system. When using the spectrophotometer cuvettes with each sampler were briefly vortexed immediately before the reading was taken. Vortexing the samples was done to insure that the sample did not settle which could create a decrease in detection. Algae were quantified in order to determine their growth in response to the different treatments.

Glutathione Content

Glutathione levels were determined using an assay. The samples were placed in the spectrometer and measured at an absorbance value of 280nm. The absorbance was measured several times over seven minutes to determine glutathione levels by a change in absorbance. If the absorbance changed quickly

there was a high amount of glutathione present. Glutathione is an antioxidant produced by plants to scavenge reactive oxygen species.

Immunoblotting

The accumulation of ubiquinated proteins was determined using western blotting. Proteins were extracted from samples of alga grown in control media, selenocysteine, selenocysteine plus MG132, and Mg132 only. The concentrations of the protein extracts were determined by performing a Bradford assay. The proteins were loaded onto an acrylamide gel and underwent electrophoresis. The proteins were then transferred to a nitrocellulose membrane and a polyvinylidene diflouride (PVFD) membrane. The membranes were exposed to the primary and secondary antibodies and then were developed. Immunoblotting is used to determining the presence and relative quantity of a specific protein.

TBARS Assay

Algal samples were sonicated to lyse cells. Five hundred microliters of each sample were added to 500 µL of 20% TCA and vortexed. The samples were spun at 5000 rcf for five minutes. Five hundred microliters of the supernatant of each sample were then added to 500 µL of TBA and heated 95°C. Caution must be taken as this step creates gas which can build up in the tube and the TCA is corrosive so you should make sure the tube is able to vent. The samples were allowed to cool and were then centrifuged at 5000 rcf. The absorbance was measured at 532 and 600nm. This assay was conducted to determine the relative amounts of lipid peroxidation.

Results

Cell count of *Chlamydomonas* shows a similar trend to the chlorophyll count. In lower selenium concentrations the number of cells increased compared to the no selenium treatment. If selenium levels are too high then the cell count drops (Figure1)

Cell content decreases very quickly in the presence of the redox reagent DTT but decreases even more quickly when selenocysteine and DTT were present. This is shown in Figure2 as a measurement of cells counted under the microscope and in Figure3 as a measurement of turbidity.

Selenium seems to increase the production of chlorophyll in *Chlamydomonas* when it is exposed in lower concentrations. As the Selenium concentrations rise the chlorophyll concentrations are negatively affected (Figure4).

Chlorophyll content decreases in the alga when selenocysteine and DTT are combined and added compared to when only DTT is added (Figure5).

Chlorophyll concentrations are also affected by the species of selenium they are exposed to and the presence of a proteasome inhibitor Mg132. Figure6 shows when Mg132 is added chlorophyll concentration decreases compared to the DMSO control. When selenate is added the chlorophyll concentration decreases but increases when added with Mg132. Chlorophyll concentration increases when selenocysteine is added but decreases when it is added with Mg132.

Alternative oxidase levels were down regulated in the presence of selenium. The amount of peroxidized lipids increased for the control, selenite-treated alga, and selenocysteine-treated alga from 6 hours to 24 hours (Figure7). The alga treated with selenocysteine experienced a much greater increase in peroxidized lipids than alga in the other two treatments.

There was an attempt to gather data on the glutathione levels and ub-proteins but this was not successfully done due to the nature of the procedure that is required to collect those data sets.

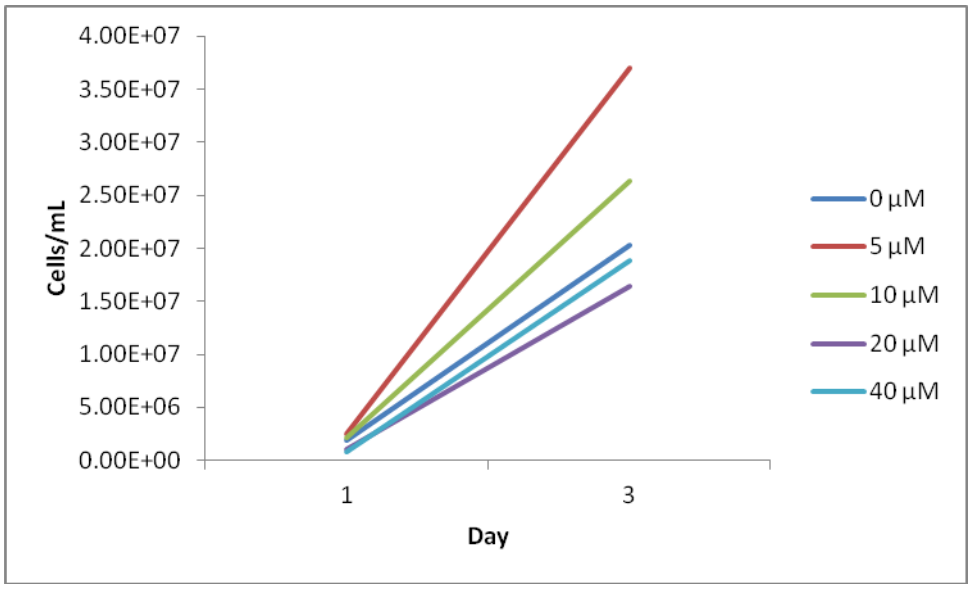

Figure1. *Chlamydomonas reinhardtii* cell density before and after three days of growth in increasing concentrations of selenium.

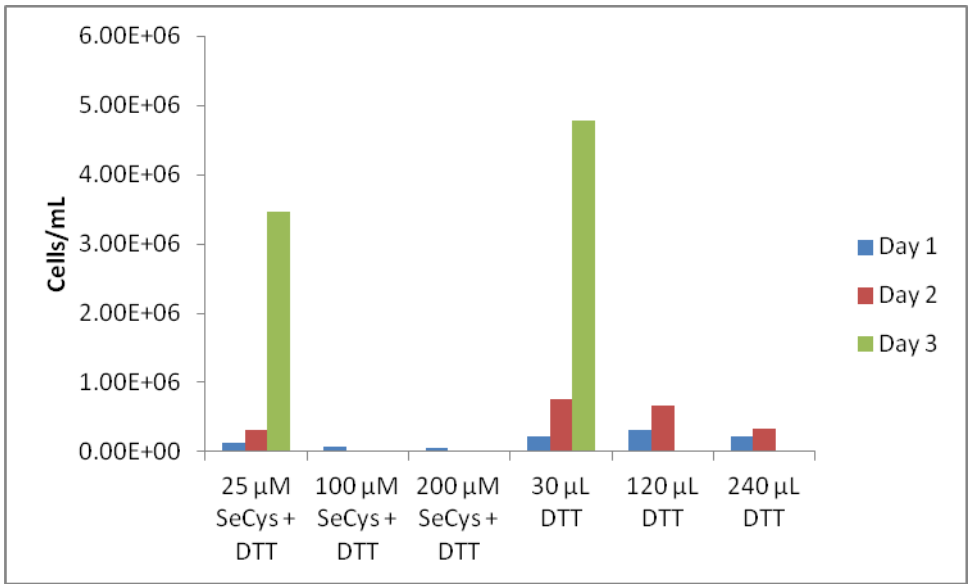

Figure2. *Chlamydomonas reinhardtii* cell density during three days of growth in increasing concentrations of SeCys and DTT.

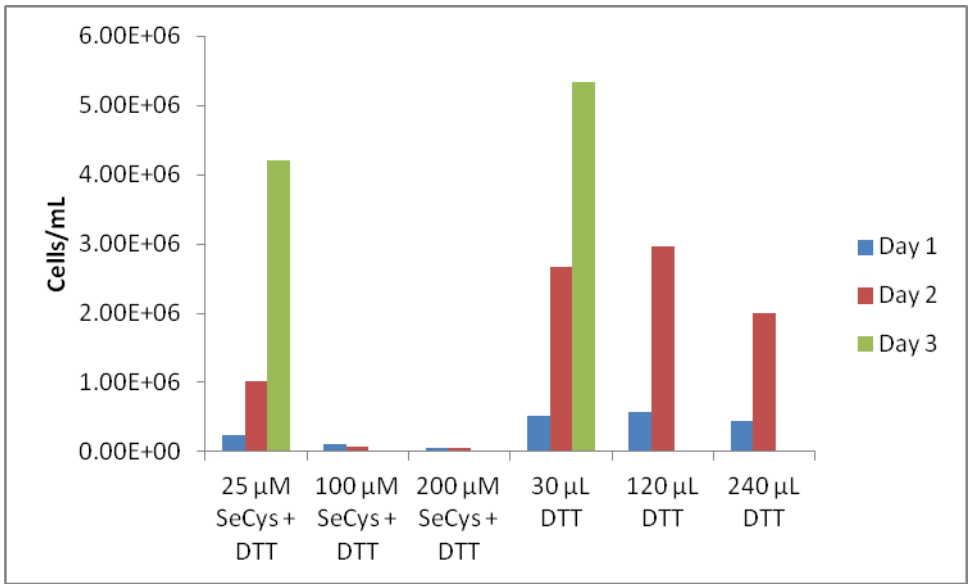

Figure3. *Chlamydomonas reinhardtii* cell density, based on turbidity, during a three day growth period in increasing concentrations of SeCys and DTT.

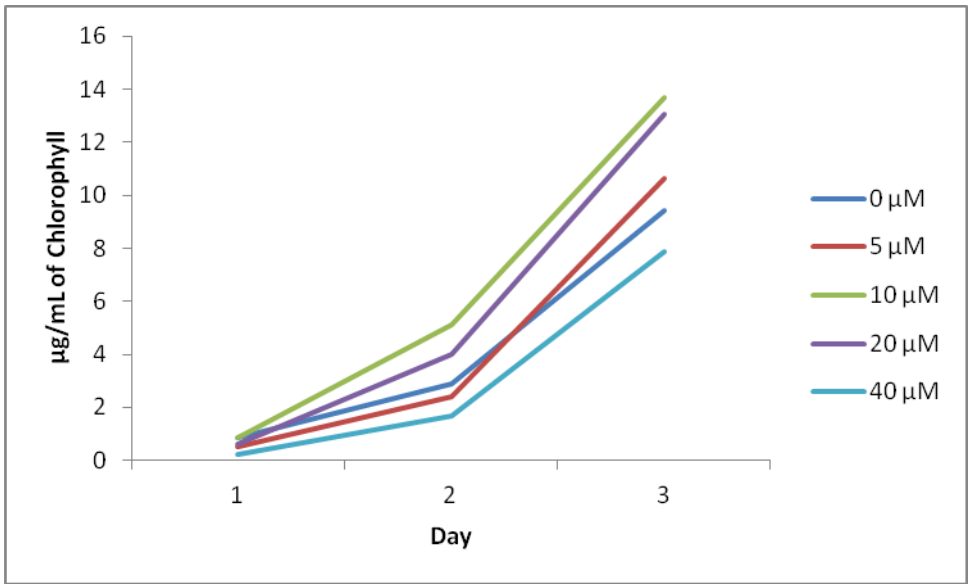

Figure4. Increase in chlorophyll concentration of *Chlamydomonas reinhardtii* grown in increasing concentrations of selenium over three days.

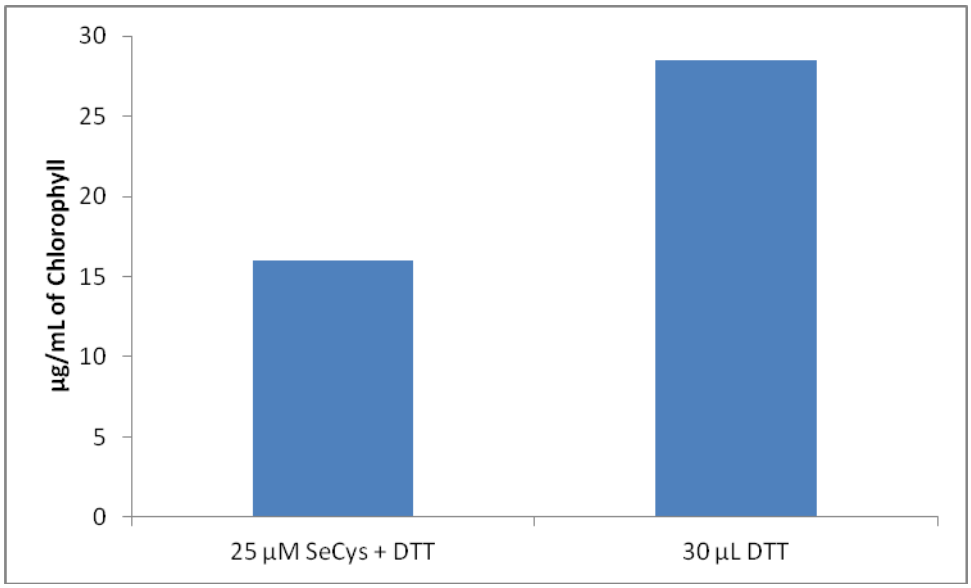

Figure5. Chlorophyll content of *Chlamydomonas reinhardtii* after three days of growth in SeCys + DTT and just DTT.

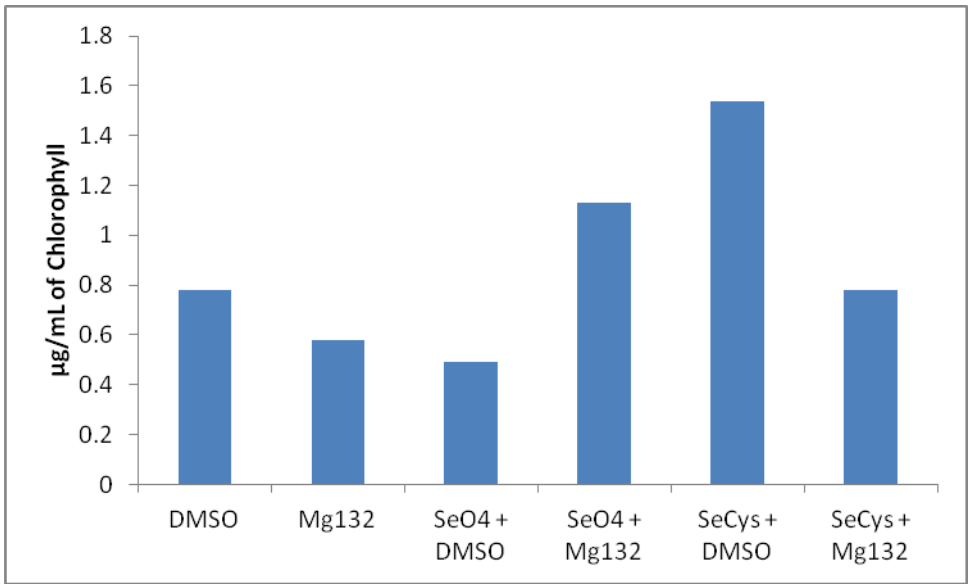

Figure6. Chlorophyll concentration in *Chlamydomonas reinhardtii* grown in different treatments of DMSO, Mg132, SeO4, and SeCys.

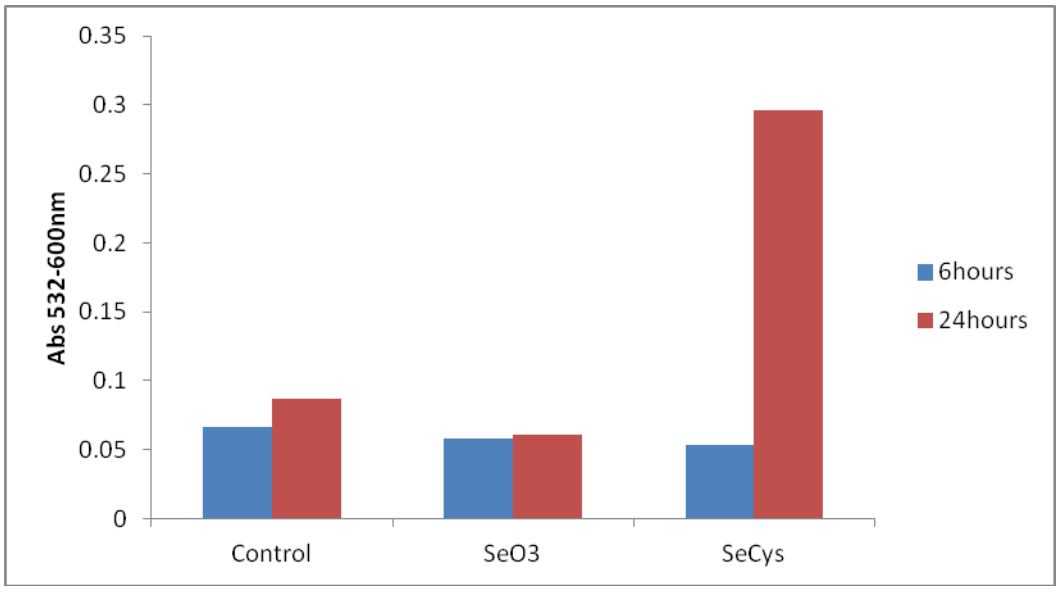

Figure7. The change in lipid peroxidation in the control, selenite-treated alga (SeO3), and selenocysteine-treated alga (SeCys) from 6 to 24 hours. The relative lipid peroxidation is detected at the absorbance value of 532nm – 600nm.

Discussion

The ubiquitin-proteasome pathway is an important mechanism to remove misfolded proteins. Because selenium may cause protein misfolding by being incorporated into proteins and disrupting disulfide bridges, the proteasome may also be affected by selenium since it too is a protein. If the proteasome is misfolded then it will not be able to remove other misfolded proteins. The ability of selenium to incorporate itself into biomolecules may also cause other measurable effects in the cells of *Chlamydomonas reinhardtii* including possible changes in quantity of chlorophyll and glutathione.

The data collected here does not completely support the earlier hypothesis that growth and chlorophyll levels will decrease. There seems to be a small concentration range of selenium that will cause growth and chlorophyll to increase. Data on ub-protein and glutathione levels was inconclusive.

Chlamydomonas reinhardtii growth increases with the addition of selenium in the concentration of 5µM. This is interesting because this indicates that the alga use selenium. However when higher concentrations of selenium are introduced, 100µM of selenocysteine + DTT, we see no cell growth. This indicates that selenium in high concentrations become toxic to the algal cells. This cell death is not solely caused by DTT, which is a molecule that also disrupts disulfide bonds, because we can see a greater decrease in cell growth in a 25µM SeCys + DTT flask as in a control flask with just DTT. The turbidity readings follow the same trend as those taken by using the hemocytometer but the turbidity readings indicate more cells. This occurs because turbidity measurements using the spectrophotometer are affected by floating debris in the sample while with the hemocytometer only cells are counted.

Chlorophyll levels were determined and it was found that chlorophyll concentrations increase with increasing concentrations of selenium. This can be attributed to the increases in cells that also occurred when selenium was added. However, chlorophyll levels are highest in the 10 and 20 µM

selenium treatments. This is interesting because at those concentrations cell count did not increase. This may indicate that chlorophyll content was higher in those cells exposed to the higher concentrations of selenium. When selenium in the form of selenocysteine was introduced to the alga along with DTT the levels of chlorophyll follow the same pattern as the cell count.

In the experiment where the proteasome inhibitor Mg132 is used we find that SeO_4 + Mg132 has a higher concentration of chlorophyll compared with just the selenate treatment. This could be because selenate is a more difficult selenium species to be incorporated into biomolecules. It is known that the proteasome is responsible for degrading misfolded proteins so when it is inhibited or damaged misfolded proteins may accumulate (Valas and Bourne 2008) When the selenium is incorporated it may be at a low rate so that when the proteasome is inhibited and those molecules aren't destroyed the cell ramps up its production of chlorophyll to try and make up for the problem. When SeCys + Mg132 is added to the cells the chlorophyll levels respond in an opposite manner, they decrease compared to the cells exposed only to SeCys. This may be because selenocysteine is much easier to incorporate into biomolecules because it is already in a usable form to build proteins. This likely makes the number of misfolded proteins greater so that when the proteasome is inhibited the buildup of faulty proteins is so great that the alga chlorophyll levels also drop because it cannot handle that level of stress. It is interesting however that selenate on its own decreases chlorophyll content and selenocysteine increases chlorophyll content. Oddly the addition of either selenium species increases the chlorophyll concentration when the proteasome is inhibited.

The increase of lipid peroxidation in the selenocysteine treatment was the greatest likely because selenocysteine can be used directly in a protein causing misfolding. Other selenium species must be transformed into more usable forms before they can be incorporated. Lipid peroxidation would increase as the misfolding of proteins increases.

Though data on glutathione levels were not successfully collected in this study, other studies show an increase in glutathione levels in *Chlamydomonas* grown in selenium. Takeda et al. 1997 shows that there was 2.6 fold increase in the levels of glutathione detected. This might occur for two reasons. The first is because the selenium is inducing a stress response which is a cause of increased glutathione levels. The second reason is that some glutathione in *Chlamydomonas* requires selenium as part of their formation (Novoselov et al. 2002); more selenium might make it possible to make more glutathione.

This study in *Chlamydomonas* is important because selenium intake by algae can affect the entire aquatic food chain (Fourniera et al. 2010). This may also be helpful in developing transgenic algae to clean contaminated environments. It is even more important to focus on the ub-proteasome pathway because it is also important for human health. Studies show that that the ub-proteasome pathway may be involved in mental process such as synaptic plasticity (Hegde 2010) and in some cancers (Mani and Gelmann 2005). If this study were to be continued it should focus on replicating these experiments in high volumes to determine if trends emerge that do not show up in a few test.

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