Fall 12-15-2011

The Involvement of 26S Proteasome Complex in Selenium Toxicity

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Year 2011

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Biology

Submitted in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science

In the Honors Program at Coastal Carolina University

December 2011

Melissa Sabbagh

Honors 499

Senior Thesis

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Abstract

Plants that hyperaccumulate elements like selenium to potentially toxic levels may use proteasome complexes to reduce toxicity. The 26S proteasome complex may be a pathway that these plants take to rid themselves of selenium toxicity by destroying damaged proteins caused by selenium. A method to test this hypothesis is to use a hyperaccumulator of selenium *Stanleya pinnata* and nonhyperaccumulators *Populous tremula* and *Arabdopsis thaliana* to evaluate their proteasome abundance without and with selenium. To compare these species western blots were made to show the differences in proteasome abundance. Also to compare the amount of oxidized and an ubiquinated protein in samples without selenium versus with selenium a proteasome inhibitor MG132 was used in another set of samples. The results showed that selenium does upregulate the 26s proteasome complex in *Stanleya Pinnata* and *Populous tremula* but not in *Arabdopsis thaliana* .

Introduction

Selenium (SeO_4) is an important element and micronutrient to animal and plants (Zhu et al., 2009). It is found under sulfur in the periodic table therefore its structure and functions are similar to each other. Plants that accumulate more than 1000 μg/g of selenium become classified as hyperaccumulators (Na and Salt, 2010). It is thought that selenium may become toxic at high concentrations because plants mistake it for sulfur and incorporate it into their proteins (Hanson et al., 2009). Plants that are found in seleniferous soil may accumulate more selenium than needed which can cause toxicity within the plant (Hanson et al., 2009). It is also thought that plants may intentionally accumulate more selenium as a defense mechanism against herbivores (Hanson et al., 2009). An example of selenium used as a defense mechanism would be an experiment showing hyperaccumulator plants of selenium surviving better than low accumulators against predators like the black-tailed prairie dog (Freeman et al., 2009). Some plants, like *Stanleya pinnata*, also known as desert prince's plume, are hyperaccumulators of selenium and seem to be more resistant to selenium toxicity than other plants for unknown reasons. Resistance may be conferred through protein complexes known as proteasomes, specifically the 26S proteasome (Kurepa and Smalle, 2008).

The 26S proteasome complex is made up of two major sub-particles: the 20S proteasome and the 19S regulatory particle (Kurepa et al., 2008). The 26S proteasome is a large proteasome that has more than thirty subunits and is known to destroy denatured proteins in cells (Hendil et al., 2009). The 26S proteasome destroys ubiquitinated proteins, which are proteins that are denatured or misfolded (Ikai et al.,1991) . Ubiquitin is a small protein that attaches itself to denatured proteins and directs them to a proteasome, in this case the 26S proteasome. The 19S

regulatory particle is the one that recognizes the ubiquitin- tagged protein (Suty et al., 2003). This ubiquitin and proteasome pathway is a method for plants to control the amount and activity of their proteins (Viestra, 2003).

The free 20S proteasome can destroy denatured proteins that are not tagged with ubiquitin. The 26S proteasome is ubiquitin- dependent; it is not very effective at degrading oxidized proteins compared to the 20S proteasome because oxidized proteins are not ubiquinated (Davies, 2001). A positive correlation exists between plants that have a hyperaccumulation of an element like cadmium and a higher activity of 20S proteasome because of the oxidative stress caused by the excess amount of that element (Kurepa and Smalle, 2008). Heavy metals like cadmium are known to form free radicals which are the primary cause of oxidative stress (Polge et al., 2009). A side effect of oxidative stress includes structural damage to DNA, proteins, and other biomolecules (Kaur et al., 2007). It may be possible that the 20S proteasome is upregulated by oxidized proteins in plants (Djebali et al., 2008). Therefore, if oxidative stress is caused by a hyperaccumulation of an element then the 26S proteasome complex including its subunits, particularly the 20S proteasome would be upregulated.

Selenoproteins are the proteins that contain the amino acid selenocysteine (Fu et al., 2002). These proteins are found in all three domains of life which means plants also contain selenoproteins (Heras et al., 2011). The existence of the twenty first amino acid selenocysteine, explains why organisms need trace amounts of selenium (Lobanov et al., 2009). The main difference between the amino acids cysteine and selenocysteine is the substitution of sulfur to selenium (Fu et al., 2002). Furthermore the selenocysteine in the selenoprotein can be replaced by cysteine, so they are interchangeable (Castellano, 2009). These selenoproteins may be the

ones that the plant tags with ubiquitin to be destroyed when there is a high concentration of selenium to avoid toxicity.

In these experiments the abundance of the 26S proteasome complex was estimated in hyperaccumulating and nonhyperaccumualting plants exposed to selenium. To infer the abundance of the 26S proteasome I used the proteasome inhibitor Mg132 and then measured the amount of ubiquitinated proteins in hyperaccumulators of selenium versus nonhyperaccumulators.

Methods

Two experiments were conducted. The first one included plant samples that were treated with or without selenium from *Populous tremula, Arabdopsis thaliana,* and *Stanleya pinnata* to compare the effects of selenium on the activity of the 26S proteasome complex. The second experiment included plant samples from *Populous tremula* and *Arabdopsis thaliana* which were treated with or without selenium and MG132. The second experiments purpose was to compare the amount of ubiquinated proteins.

BSA standard Curve

A BSA standard curve was made to determine the amount of protein needed in each of the wells of the western blot. Six centrifuge tubes were labeled and 900 μL of Bradford Rx were added to each one. Then 100 μL of H_20 were added to the first centrifuge tube as a control. To the remaining five tubes, 98 μL, 96 μL, 92 μL, 88 μL, and 84 μL of water were added. The volume of each experimental tube was brought to 100 μL with a known protein (BSA). A spectrophotometer was used to measure absorbancies of each tube at 595nm. A standard curve was made from the measured absorbancies and was used to calculate the amount of protein in samples obtained from the plants.

26S proteasome complex

The nonhyperaccumulators: *Populous tremula, Arabdopsis thaliana*, and the hyperaccumulator *Stanleya pinnata* were the three species used in the first experiment. Individuals of each species were split into two treatment groups: one that received a high amount of selenium and another that had received no selenium. A 500 mg sample of leaves was

acquired from each of the six plants; samples were labeled, wrapped with aluminum foil, and placed in liquid nitrogen to slow down any chemical reactions.

Protein Extraction Protocol

We mixed 1 mL of protein buffer with 1 μL of PMSF (Proteanase inhibitor to prevent the protein degradation) in a centrifuge tube and added 0.5μL (5 mM Beta-mercaptoethanol). We ground each sample of plant material with a mortar and pestle and, used liquid nitrogen to prevent denaturation of proteins. We transferred each sample to a centrifuge tube, added 100μL of the protein buffer mix and then ground the samples with a micropestle. Samples were kept on ice so they would stay cold during the transfer step. We centrifuged each sample for 10 minutes at top speed in a refrigerator. We then removed the supernatant from each tube and transferred it to a new tube. Excess supernatant was kept for future experiments and temporarily stored ; on ice.

We then measured the absorbancies of the supernatant. A blank was made with 900 μL of Bradford RX and 100 μL of water. In separate cuvets, 2 μL of each supernatant were added to 98μL of water.The absorbancies were measured at a wavelength of 595 nm, and the amount of protein in each sample was calculated using the equation from the standard curve.

Western Blot

We transferred 25 μL of each of the individual samples to a corresponding centrifuge tube. Then to each of those tubes we added 25 μL of a protein buffer that contains glycerol (to

keep the protein from floating out of the well). We loaded each sample into a well in the native gel; the, amount needed was determined by the BSA curve. We loaded the last well with 6 μL of protein ladder. The electrical current was run for about an hour at 24 amps. The gel was removed and placed on nitrocellulose film and then parchment paper and sponge squares were applied to both sides and placed into. We transferred this entire stack into a container with buffer and added a stirring rod. The stack containing the gel was placed close to the positive end with the side that had the nitrocellulose paper facing the negative end of the container so that the protein samples on the gel would transfer onto the nitrocellulose paper. We kept the electric current running at 40 volts for 4 hours.

I removed the nitrocellulose film, placed it in a rectangular petri dish containing a milk blocking solution. The petri dish was then placed on a tilter for at least 30 minutes.

Detecting the abundance of the 26S proteasome

We added 10 μL of the 20S antibody with 10mL of milk in the petri dish with the nitrocellulose paper and left it on the tilter once more for 1-2 hours. We then washed the paper four times with Tris-buffered saline and Tween 20 (TBST) in five-minute intervals. Then 10 μL of rabbit antibody were added in 10 mL of TBST and left for an hour on the tilter. The paper was washed with TBST four times and then again with TBS without tween. In a darkened room, the 20S proteasome was detected on the paper by adding 10 mL of alkaline phosphate buffer with 40μL of Nitroblue tetrazolium (NBT) and 75μL of 5-bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP).

MG132

We repeated the experiment with individuals of *Populous tremula* and *Arabdopsis thaliana* that were treated with or without Se with the addition of MG132 the proteasome inhibitor. For the control samples, we substituted MG132 with DMSO.

Results

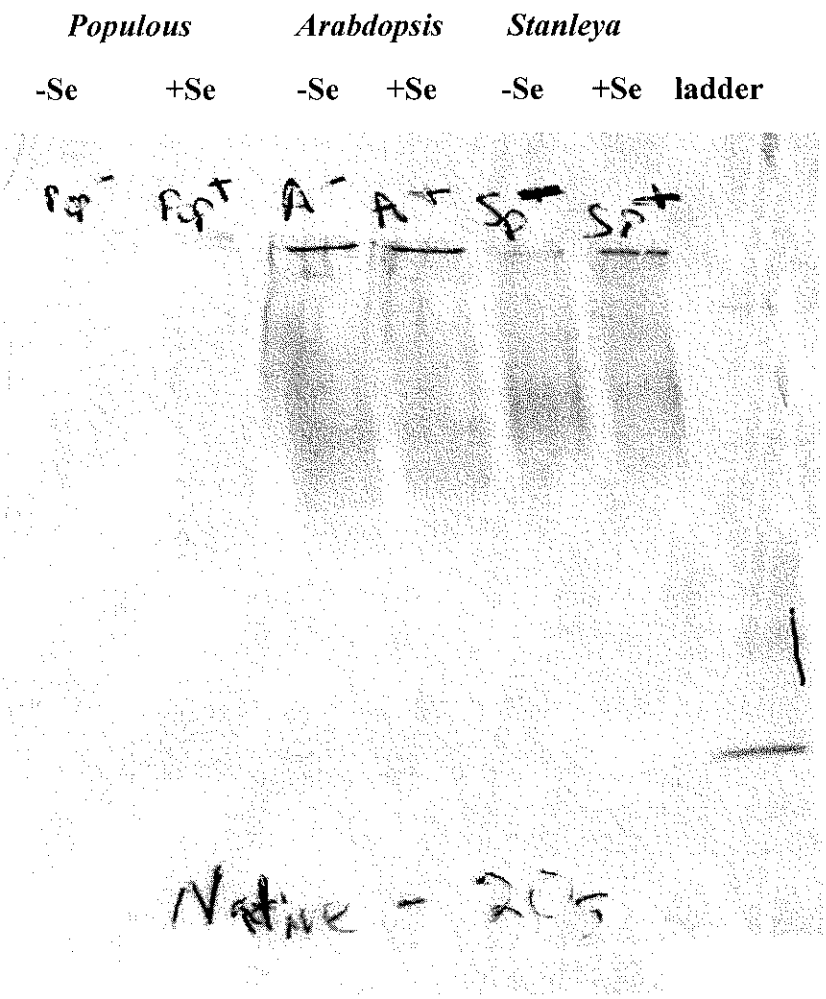

Figure 1: Native gel detecting the 26S proteasome in *Populous tremula, Arabdopsis thaliana*, and *Stanleya pinnata* treated without selenium and with selenium.

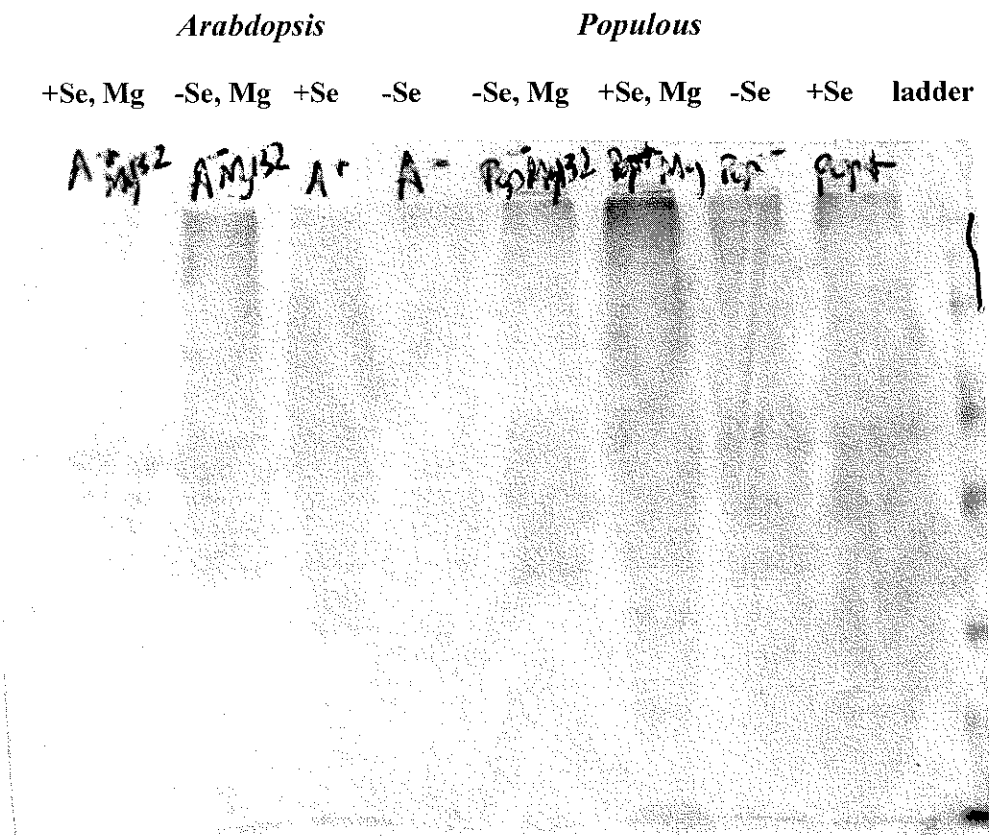

Figure 2: SDS page gel showing ubiquinated proteins in the samples of *Arabdopsis thaliana* and *Populous tremula* that had been treated with a proteasome inhibitor (MG132) with and without selenium.

Figure 1 shows that *Populous tremula* has slightly more 20S proteasome in the sample with selenium than the sample without selenium. While the *Arabdopsis thaliana* samples look the same regardless of the level of selenium to which they were exposed. The sample of *Stanleya pinnata* with selenium has a distinctly darker line, that the sample without selenium which means there was more 26S proteasome that was detected in that sample.

Figure 2 shows that there are more ubiquinated proteins in the *Arabdopsis thaliana* sample treated with MG132 without selenium compared to the sample treated with MG132 with selenium. The *Arabdopsis thaliana* sample treated with selenium and DMSO has more ubiquinated proteins than the one without selenium. The *Populous tremula* sample treated with MG132 and selenium is darker than the one without selenium which means it has more ubiquinated proteins. The *Populous tremula* sample treated with selenium and DMSO has more ubiquinated proteins than the sample without selenium.

Discussion

The results in figure 1 show that in hyperaccumulators like *Stanleya pinnata,* the 26S proteasome is upregulated by the presence of selenium. The nonhyperaccumulater *Populous tremula* showed the same result as *Stanleya* but the difference between plants treated without or with selenium is not as obvious. However, the *Arabdopsis thaliana* samples do not show any difference in the abundance of the 26S proteasome; but further experiments would be necessary to explain this result.

The results in the second experiment shown in figure 2 illustrate the amount of ubiquinated proteins in the plant samples *Populous tremula* and *Arabdopsis thaliana* because in contrast to the first experiment the proteasome inhibitor MG132 was used. Since the proteasome is inhibited what is shown in figure 2 is a representation of the amount of ubiquinated proteins found in those plant samples. The *Populous tremula* samples support the hypothesis that more ubiquinated proteins are found in samples treated with selenium. Since the lane with the sample of *Populous tremula* that is treated with MG132 and selenium is darker it can be inferred that there are more ubiquinated proteins versus the sample treated with only MG132. However, the *Arabdopsis thaliana* samples treated with MG132 did not support the hypothesis; this may be caused by human error from mislabeling the gel or it might just be that selenium affects this plant differently. Future experiments can clarify whether or not it was human error or if there is another pathway that *Arabdopsis thaliana* uses to deal with higher amounts of selenium.

In conclusion, these experiments showed that the *Populous tremula* and *Stanleya pinnata* samples supported the hypothesis that the 26S proteasome is upregulated by high amounts of selenium. This may mean that proteasome complexes like the 26S are how hyperaccumulators

are able to reduce selenium toxicity, more experiments are necessary to further support this hypothesis.

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