



Short communication

Stuck between a ROS and a hard place: Analysis of the ubiquitin proteasome pathway in selenocysteine treated *Brassica napus* reveals different toxicities during selenium assimilation

Aleksandar Dimkovikj^a, Brian Fisher^a, Kim Hutchison^b, Doug Van Hoewyk^{a,*}^a Biology Department, Coastal Carolina University, Conway, SC 29526, USA^b Department of Soil Science, North Carolina State University, Raleigh, NC 27695, USA

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ABSTRACT

During the selenium assimilation pathway, inorganic selenate and selenite are reduced to form selenocysteine (Sec). Tolerance to selenium in plants has long been attributable to minimizing the replacement of cysteine with selenocysteine, which can result in nonspecific selenoproteins that are potentially misfolded. Despite this widely accepted assumption, there is no evidence in higher plants demonstrating that selenocysteine induces toxicity by resulting in malformed proteins. In this study, we use *Brassica napus* to analyze the ubiquitin–proteasome pathway, which is capable of removing misfolded proteins. Sec rapidly increased proteasome activity and levels of ubiquitinated proteins, strongly indicating that selenocysteine induces protein misfolding. Proteasome inhibition increased the amount of selenium in protein in Sec-treated plants. Collectively, these data provide a mechanism that accounts for Sec toxicity. Additionally, Sec did not cause oxidative stress as judged by examining levels of superoxide using fluorescent microscopy. Therefore, the cellular response to Sec is different compared to selenite, which was recently shown to increase antioxidant metabolism in response to elevated mitochondrial superoxide that ultimately impaired proteasome activity. Therefore, plants must contend with two divergent modes of cytotoxicity during selenium assimilation. Selenite can result in oxidative stress, but increased flux of selenite reduction can yield Sec that in turn can cause protein misfolding.

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Introduction

In humans, selenium (Se) has been shown to have anti-carcinogenic properties (Rayman, 2000), and is an essential trace element required for the synthesis of selenocysteine (Sec). This amino acid is needed to make 25 specific selenoproteins in humans (Papp et al., 2007). In contrast, higher plants do not make specific selenoproteins and it has yet to be demonstrated that they have a requirement for Se, despite it being beneficial to some plants (Pilon-Smits et al., 2009) and the recent discovery of a tRNA^{Sec} in cranberry (Fajardo et al., 2014). Still, crops supply humans most of their required dietary intake of Se. Thus, the development of crops with fortified levels of Se is desirable. In particular, *Brassica* crops have shown promise in their ability to accumulate Se and synthesize chemopreventive molecules (Ávila et al., 2014). However, Se biofortification can be limited because an excess of the trace

element in plant tissue is typically toxic to most crops. Therefore, elucidating the cellular responses to Se toxicity may yield new varieties of *Brassica* crops with elevated levels of Se and nutraceutical properties.

Se toxicity in plants occurs through two different modes (Van Hoewyk, 2013). It is well established that inorganic selenium, such as selenate and selenite, deplete glutathione and cause oxidative stress (Hugouvieux et al., 2009; Łabanowska et al., 2012). Recently, *Brassica napus* (canola) plants treated with selenite rapidly accumulated mitochondrial superoxide; in response to selenite, roots reconfigured primarily metabolism – including antioxidant metabolism – to counter the effects of oxidative stress (Dimkovikj and Van Hoewyk, 2014). Additionally, both selenate and selenite can be metabolized by sulfur assimilatory enzymes and assimilated into Sec, as extensively reviewed elsewhere (Zhu et al., 2009). The synthesis of Sec is also problematic for plants, as it can compete with cysteine (Cys) during translation and produce nonspecific selenoproteins (Brown and Shrift, 1981). A Cys to Sec replacement in polypeptides likely alters protein structure, such as disulfide bond length, which in turn can yield misfolded proteins that impair cellular processes.

* Corresponding author. Tel.: +1 843 349 2431.

E-mail address: doughv@coastal.edu (D. Van Hoewyk).

Misfolded proteins can be cytotoxic if not removed by the ubiquitin-proteasome pathway (UPP). In this conserved eukaryotic pathway, both regulatory and misfolded proteins are first tagged with the small protein ubiquitin, which is dependent upon ubiquitin activating (E1), ubiquitin conjugating (E2), and ubiquitin ligase (E3) enzymes (Smalle and Vierstra, 2004). Poly-ubiquitinated proteins are then delivered to the 26S proteasome, which consists of the 20S proteolytic core capped with either one or two ATP-dependent regulatory lids. In contrast to the 26S proteasome, the free 20S proteasome lacks regulatory lids, and is primarily involved in the proteolysis of oxidized proteins in an ATP and ubiquitin independent manner (Davies, 2001). Thus, the 20S proteasome is associated with oxidative stress caused by the accumulation of reactive oxygen species (ROS), whereas the 26S proteasome removes misfolded proteins as a result of heat stress or amino acid analogs, such as canavanine or azetidine-2-carboxylic acid which can replace arginine and proline, respectively, in proteins (Kurepa et al., 2008; Peng et al., 2001). Although the 26S proteasome has been implicated in response to selenate in the Se hyperaccumulating plant *Stanleya pinnata* (Sabbagh and Van Hoewyk, 2012), the direct effects of selenocysteine on the UPP have not been investigated in higher plants.

Treating plants with excess selenate and selenite – the two most common forms of selenium that are taken up by plants – can lead to both ROS accumulation and ultimately to the formation of Sec. Thus, it is hard to decipher if ROS or nonspecific selenoproteins drives toxicity during selenate and selenite treatment. The objective of this study is to determine if selenocysteine, the downstream product of selenate and selenite assimilation, exerts cytotoxicity by inducing the formation of misfolded proteins rather than ROS accumulation. The divergent modes of toxicity during the assimilation of selenate to Sec in plants are discussed, as well as strategies to mitigate Sec toxicity.

Methods and materials

Brassica napus seeds were sown on soil prior to being transferred after 7 d into 3 L of Hoagland's media. Aerated and hydroponically-grown plants were grown in a growth chamber (14 h light/10 h dark cycle, 24 °C) for another 14 d before being transferred into 1 L of media containing 0, 20, and 50 μM Sec, unless otherwise stated. These concentrations for Sec were selected because in preliminary experiments it restricted root growth by 30% and 52%, respectively, after three days compared to untreated samples. To obtain Sec, selenocystine (Sigma, USA) was reduced to Sec at 37 °C for 16 h in a solution containing 0.05 M dithiothreitol (DTT). Therefore, control plants without Sec contained 50 μM DTT to account for the DTT that was also present in samples treated with Sec.

To determine proteasome activity, root tissue from 6 plants was harvested 0, 4, and 24 h after treatment with or without Sec and ground in liquid nitrogen, as described previously (Valentine et al., 2014). Briefly, non-denatured proteins were extracted in proteasome extract buffer (50 mM potassium-phosphate buffer – pH 7.4, 5% glycerol, 10 mM ATP, and 5 mM beta-mercaptoethanol), and protein concentration was determined using the Bradford assay. Chymotrypsin activity of the proteasome was determined fluorometrically ($\text{Ex}_{360}/\text{Em}_{410}$) in a reaction containing 5 μL of protein extract and 95 μL of reaction buffer containing 50 μM of the fluorogenic peptide Suc-LLVY-AMC with or without 10 μM of the proteasome inhibitor MG132 dissolved in 0.1% DMSO. Activity was determined after 30 min as the fluorescence per microgram of protein in reactions, and is the difference in reactions with or without MG132 to account for non-proteasomal proteolysis. Proteasome activity is reported as the percentage relative to control samples at 0 h.

Levels of ubiquitinated proteins were determined in 3 pooled plants growing in 100 mL of media containing 50 μM of MG132 treated with or without Sec for 4 and 24 h. Briefly, 50 μg of denatured protein were separated on an 8% SDS-PAGE gel, and high molecular weight poly-ubiquitinated proteins were detected using an ubiquitin antibody raised in mouse as described (Valentine et al., 2014). In a parallel experiment, 10 μg of protein from each sample were loaded onto a 15% SDS-PAGE gel to detect levels of free ubiquitin (8 kDa).

The amount of Se in protein was analyzed in the root tissue of plants treated with 50 μM Sec for 4 h containing either 0.1% DMSO or 50 μM MG132 dissolved in 0.1% DMSO. Proteins were extracted and precipitated with 10% trichloroacetic acid, as previously described (Valentine et al., 2014). Proteins were digested in nitric acid at 95 °C overnight. Filtered samples were analyzed using inductively coupled plasma-mass spectroscopy (ICP-MS, Varian 820, Collision Reaction Interface).

Accumulation of mitochondrial superoxide was estimated in roots treated with or without Sec or selenite for 24 h using the fluorescent probe Mitosox Red (Molecular Probes, Invitrogen). Briefly, 10–15 root tips (0.8–1.2 cm) were allowed to incubate in 1 mL of Hoagland's media containing 5 μM Mitosox Red ($\text{Ex}_{510}/\text{Em}_{580}$) for 15 min on a rotating platform, as described (Dimkovikj and Van Hoewyk, 2014). Roots were washed 3x in Hoagland's media before fluorescence was visualized using a TRITC filter on an Olympus BX51 fluorescent microscope. Cell viability was assessed in separate roots as describe above, except that the root tips were incubated with the fluorescent probe fluorescein diacetate (20 μM). Green fluorescence of fluorescein diacetate ($\text{Ex}_{495}/\text{Em}_{515}$) is dependent upon cell membrane integrity, and was detected using a FITC filter.

Results

To determine whether or not Sec toxicity caused protein misfolding, the ubiquitin-proteasome pathway was analyzed in *B. napus* roots. Proteasome activity in root tissue was examined after treatment with or without Sec for 0, 4, and 24 h. At 4 h, activity increased 1.9 and 2.4 fold in plants treated with 20 and 50 μM Sec, respectively, compared to control (Fig. 1). Proteasome activity in Sec-treated plants decreased to roughly 1.7 fold at 24 h, but was still higher than control plants. Delivery of misfolded proteins to the 26S proteasome is ubiquitin-dependent, in contrast to the 20S proteasome which removes oxidized proteins. Therefore, it was desirable to determine if increased proteasome activity in Sec-treated roots coincided with increased levels of ubiquitinated proteins. Sec-treated plants resulted in the accumulation of ubiquitinated proteins; these high-molecular weight proteins were most apparent after 4 h in plants treated with 50 μM Sec (Fig. 2). Levels of free ubiquitin were run on a separate gel, and did not differ greatly between treatments; thus, the absence of ubiquitinated proteins in the control groups could not be explained by a lack of free ubiquitin which tags proteins for 26S proteasome degradation. The amount of Se in protein was measured in the root tissue of Sec-treated plants growing with or without MG132. If Cys to Sec replacement results in selenoproteins that can be removed by the proteasome, then proteasome inhibition was expected to increase the amount of Se in protein. After 4 h of Sec treatment, the amount of Se in protein in MG132 treated plants increased by more than 60% compared to control plants (Fig. 3).

The fluorescent probe MitoSox Red was recently used to demonstrate that selenite induces the accumulation of mitochondrial superoxide in *B. napus* (Dimkovikj and Van Hoewyk, 2014). However, in this study, wide-field microscopy did not reveal a difference in red fluorescence between plants treated with or without Sec

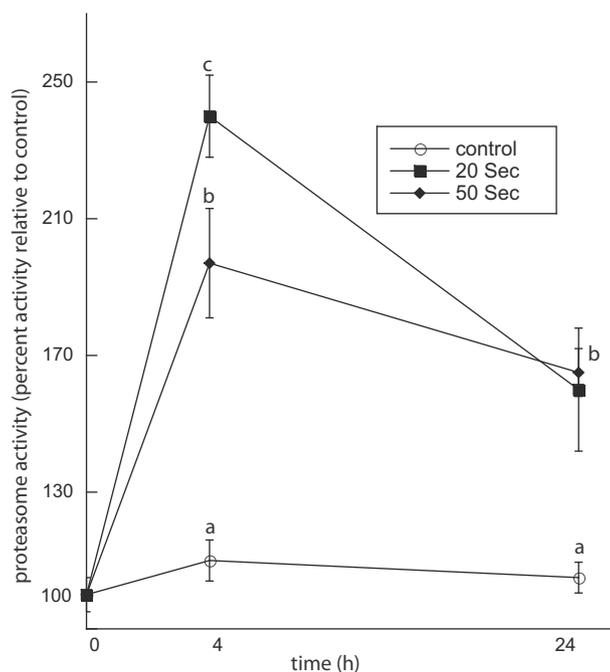


Fig. 1. Proteasome activity in *Brassica napus* treated with selenocysteine (Sec). Values for proteasome activity represent percent increase in activity relative to control plants at 0 h at each time interval. Data are the mean of six individual plants and standard deviation, and are similar to two other experimental replicates. Lowercase letters represent a significant difference in activity at each time point ($p < 0.05$).

(Fig. 4a). In contrast, selenite (which served as a positive control) exhibited bright fluorescence. To determine if the absence of mitochondrial superoxide in Sec-treated roots was a result of impaired membrane integrity in non-viable cells, the fluorescence of fluorescein diacetate was examined. Green fluorescence in the root tips was apparent among all treatments, indicating that the roots tips in Sec-treated plants contained viable cells (Fig. 4b).

Discussion

In this study, the UPP was examined in order to determine if Sec resulted in misfolded proteins that most likely result from a Cys to Sec substitution. The proteasome can selectively remove

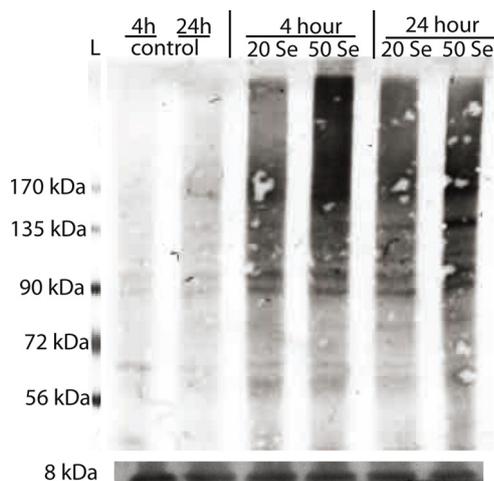


Fig. 2. The accumulation of ubiquitinated proteins (top) and free ubiquitin (bottom) in 3 pooled *Brassica napus* plants grown in the presence of 50 μM MG132 dissolved in 0.1% DMSO and treated with or without selenocysteine (Sec) at 4 and 24 h. Image is representative of two experimental replicates. L, ladder; kDa, kilodalton.

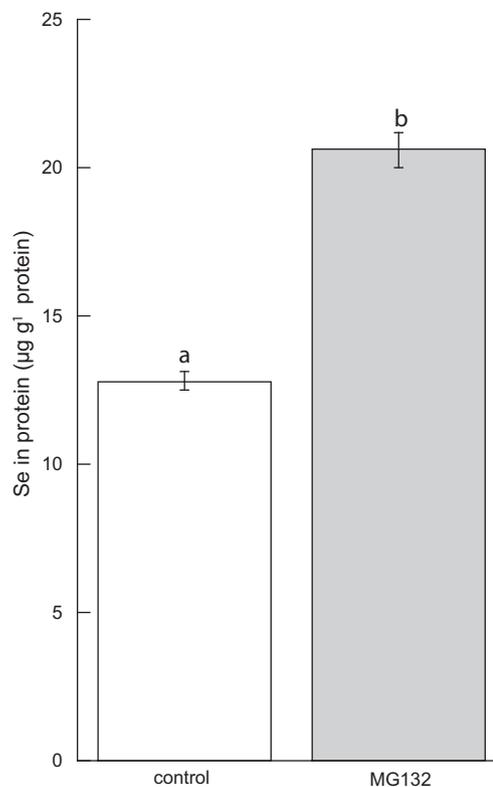


Fig. 3. The effect of proteasome inhibition on the content of selenium in root protein. Plants were grown in the presence of 50 μM SeCys and treated with or without MG-132 for 4 h. Shown are the mean and standard error ($n = 4$). Lowercase letters indicate significant difference between groups ($p < 0.05$).

misfolded proteins containing Sec, as judged by the increased Se in protein in proteasome-inhibited plants. Additionally, in response to Sec, *B. napus* roots increased proteasome activity, which was associated with elevated levels of ubiquitinated proteins. Although proteasome activity and ubiquitinated proteins in Sec-treated roots were dose-dependent at 4 h, they were not time-dependent. Protein aggregation can impair the UPP in human cells (Bence et al., 2001). Thus, a decrease in proteasome activity at 24 h in Sec treated plants could occur as a result of the accumulation of protein aggregates containing a Cys to Sec substitution. Collectively, however, these data demonstrate that the 26S ubiquitin dependent proteasome is involved in a Sec response, likely in an attempt to remove malformed selenoproteins containing Sec. To our knowledge, this is the first time that Sec has been shown to directly increase proteasome activity in any organism.

As modeled in Fig. 5, this study demonstrates a direct link between Sec and protein misfolding, and reveals the differences between the cytotoxic effects of inorganic selenium and Sec. Although selenate and selenite result in ROS accumulation, their direct mode of toxicity is unclear because these inorganic metabolites can also be assimilated into Sec. Plants likely respond to selenite and Sec differently in order to mount a successful stress response. Selenite treatment in *B. napus* increased antioxidant metabolism after 24 h, as judged by increased levels of NADPH and activity of glucose-6-phosphate dehydrogenase (Dimkovikj and Van Hoewyk, 2014), which diverts glucose into the oxidative-pentose phosphate pathway. In that same study, selenite intriguingly decreased proteasome activity and levels of ubiquitinated proteins. The UPP becomes impaired as ROS accumulate during severe oxidative stress in human cells (Huang et al., 2013; Shang and Taylor, 2011) and *Chlamydomonas* (Valentine et al., 2014), and inhibition of the UPP during selenite treatment was explained

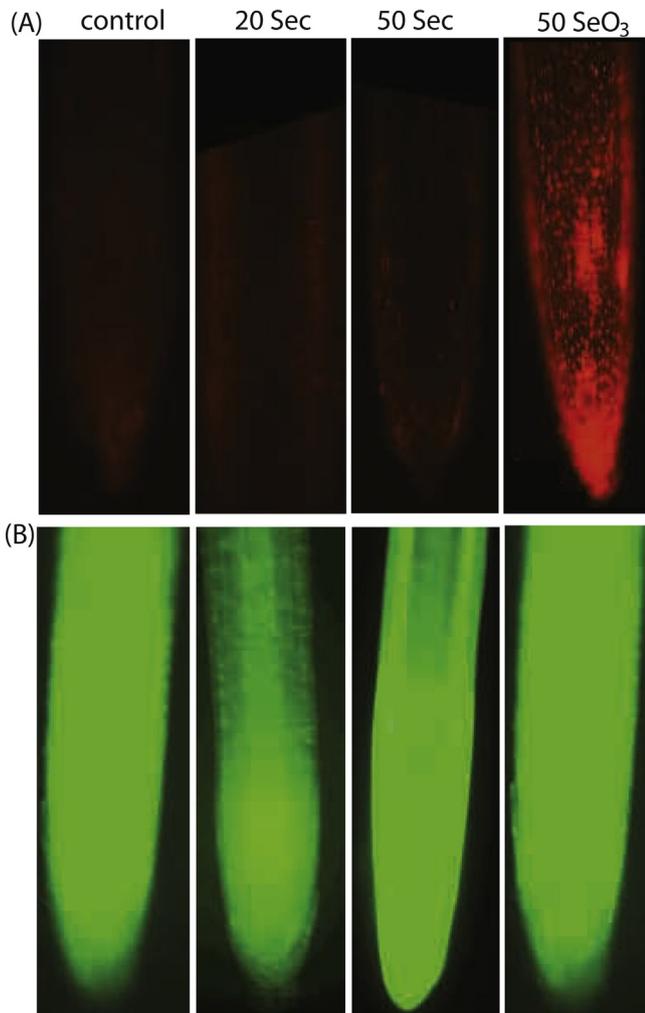


Fig. 4. Visualization of mitochondrial superoxide and cell viability. Root tips of *B. napus* plants were excised and incubated with the fluorescent probe MitoSox Red to gauge accumulation of mitochondrial superoxide (A) or the probe fluorescein diacetate to determine cell viability in (B). Epifluorescence is representative of 3 experimental replicates.

by elevated levels of mitochondrial superoxide. Clearly the toxicity of Sec differs from selenite, because proteasome activity and ubiquitinated proteins increased. Although it was not measured, it is unlikely that Sec stimulates antioxidant metabolism as it did for selenite, because Sec did not appear to induce oxidative stress.

Our results are in agreement with studies in mammalian systems that also demonstrate the divergent modes of Se toxicity. In human cell lines, selenite, but not Sec, was also shown to result in accumulation of mitochondrial superoxide (Wallenberg et al., 2010) and impair mitochondrial structure (Wallenberg et al., 2014). Additionally, treating human cells with Sec resulted in an increase of Se in protein, and this was associated with an unfolded protein

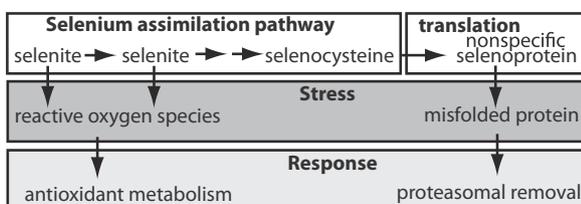


Fig. 5. A model depicting the divergent modes of selenium toxicity as selenate is reduced to selenocysteine via the selenium assimilation pathway.

response that was characterized by increased protein ubiquitination and ER stress. In contrast, selenite induced ER stress, which can result when nascent polypeptides do not fold correctly and thus cannot be secreted (Liu and Howell, 2010).

Although Sec in protein is toxic, decreasing the flux of selenium assimilation is not likely a viable option to increase Se tolerance. For example, APR2 regulates both the assimilation of sulfur (Loudet et al., 2007) and selenium (Grant et al., 2011; Chao et al., 2014) in Arabidopsis. Intriguingly, *apr2-1* mutants had decreased Se in protein, but were still Se sensitive due to the accumulation of selenate that caused an accumulation of superoxide. Preventing the accumulation of Sec in protein has long been associated with tolerance in Se-hyperaccumulating plants (Brown and Shrift, 1981), which can occur when Sec is methylated into methyl-Sec (Neuhierl and Bock, 1996). It should be noted that in contrast to Sec, selenomethionine is not considered as toxic when it is incorporated into protein. For example, about 85% of the Se in the seeds of the Se-hyperaccumulator *Lecythis minor* is found in proteins; however, nearly all the Se in protein is in the form of selenomethionine (Németh and Dernovics, 2015). The lack of Sec in the protein of *L. minor* supports the findings that the random replacement of Cys with Sec is detrimental. Whether or not exclusion of Sec from proteins in *L. minor* or other Se-hyperaccumulating plants is partially due to an unusual tRNA^{Cys} that effectively discriminates against Sec is not yet known.

Conclusion

Treating plants with Sec increased proteasome activity and levels of ubiquitinated proteins in the roots *B. napus* plants, but did not induce oxidative stress as determined by the absence of mitochondrial superoxide. Inhibition of the proteasome increased the amount of Se in protein, indicating that Sec in protein results in misfolded selenoproteins that are targeted for proteasome degradation. These data indicate that Sec directly causes protein misfolding, and that the ubiquitin proteasome pathway can respond to Sec stress. This work highlights the divergent toxicities of Sec and inorganic selenium, such as selenite that results in an accumulation of reactive oxygen species and increased antioxidant metabolism. Therefore, plants must effectively cope with both selenite-induced oxidative stress and protein misfolding caused by Sec.

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