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The assessment of selenium bioaccessibility and bioavailability from selenium-rich algae

Laura Ann Walter
Iowa State University

The assessment of selenium bioaccessibility and bioavailability from selenium-rich algae

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Laura Ann Walter

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Manju B Reddy, Major Professor
Christina Campbell
Terri Boylston
Zhiyou Wen

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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NOMENCLATURE

Bioaccessibility: BAC

Bioavailability: BA

Cellular Glutathione Peroxidase: GPx1

Glutathione: GSH

Glutathione Peroxidase: GPx

Hydrogen Peroxide: H_2O_2

Hydrogen Selenide: H_2Se

Oxidized Glutathione: GSSG

Plasma Glutathione Peroxidase: GPx3

Reactive Oxygen Species: ROS

Saccharomyces cerevisiae: S. cerevisiae

Se-adenosylhomocysteine: SAH

Se-adenosylmethionine: SAM

Selenium: Se

Selenocysteine: SeCys

Selenomethionine: SeMet

Se-methylselenocysteine: MeSeCys

Selenoprotein P: Sepp

Sodium: Na, Na^+

Superoxide: O_2^-

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ABSTRACT

Selenium (Se) is an essential trace mineral known for its role as an antioxidant in oxidation-reduction reactions. Due to deficiency in many parts of the world, development of new Se supplementation is increasing in popularity. The objective of this study was to examine the bioaccessiblity (BAC) and bioavailability (BA) from Se-rich algae using an in vitro digestion/Caco-2 cell model. Algal samples were grown in Se-rich media with varying concentrations of either selenite or selenate, then subjected to in vitro digestion. The centrifuged supernatent was applied on the Caco-2 cell monolayer for a 24-h treatment. BAC was tested based on the Se solubility post in vitro digestion and BA by induction of cellular glutathione peroxidase activity (GPx1) in a Se deficient Caco-2 cell model. Algal samples were compared to Se-salts, selenomethionine (SeMet) and Se-yeast commonly used in supplementation. Cells treated with algae grown in selenate had a mean GPx1 activity that was 15-62% ($p<0.05$) of the GPx1 activity relative to the cells with SeMet treatment. Cells treated with algae grown in selenite had a mean GPx1 activity that was 11-34% ($p<0.05$) of the GPx1 activity from SeMet treated cells. Additionally, the Se-yeast treated cells had 134% ($p<0.05$) GPx1 activity, selenate salt treated cells had 214% ($p<0.05$) GPx1 activity, and selenite salt treated cells had 126% ($p<0.05$) GPx1 activity of the SeMet treated cells. The results indicated that the Se-rich algae was not as effective as traditional Se supplementation in increasing GPx1 activity. However, the results provided valuable information to improve future Se BA of algae.

CHAPTER 1. GENERAL INTRODUCTION

Background

Worldwide, the distribution of selenium (Se) among soil and water varies greatly, creating the issue of Se toxicity, and more often, Se deficiency throughout different regions in the world. Some of the Se that enters the environment results from various agricultural and industry practices, and sediment from seleniferous rocks[1]. Worldwide, Se deficiency affects approximately 1 billion humans[2]. It can cause an increased risk of infection, cancer, and increase the risk of diseases such as cardiomyopathy[3]. Deficiency in animals can cause white muscle disease, decreased immunity, and even death[4-6]. Selenium is obtained in the diet through a variety of plant and animal sources, however, because of the environmental inconsistencies, these sources also vary with levels of Se. Recommended dietary intake in the United States for adults is 55 µg/day but some people who live in Se-deficient areas of the world consume less than 15 µg of Se per day[7-9].

Understanding how Se is absorbed and utilized in the body is complex. There are several factors that influence its bioaccessibility (BAC) which is the absorbable fraction of a particular compound. Solubility, pH, and the chemical form of Se are just a few examples that result in most organic forms to be absorbed better than most inorganic forms of Se[10,11]. The route of metabolism and how the body can utilize Se for selenoprotein synthesis, storage, or excretion also varies by chemical form[12,13]. Selenium is ambiguous because BAC is not always a good indicator of bioavailability (BA), which is the absorbed amount of the element that is delivered to tissues for use[14]. For example, some inorganic Se may have a lower BAC than organic Se, but have greater BA compared to organic Se[10].

With the ever-increasing understanding of how Se impacts our health, more efforts are being put into creating more consistent daily intakes among humans and animals[7]. Algae has the ability to accumulate larger concentrations of Se and can be grown in mass quantities[4,15,16]. With wastewaters produced from agricultural and industry practices that can contain high concentrations of Se, there is opportunity for creating products that can increase daily Se consumption. The following research was conducted to determine how Se is absorbed and utilized in a cell model. Using Se-rich algae (Chlorella *sp*.), we examined the BAC fractions of the Se with a Se-deficient Caco-2 cell model and cellular glutathione peroxidase (GPx1) activity as a marker for BA.

Objectives

1. Determine the BAC of Se from Se-rich algae and compare it to the common Se supplements* using in vitro solubility
2. Determine the BA of Se from Se-rich algae and compare it to the common supplements* by using GPx activity following cellular uptake

* SeMet, Na_2SeO_3, Na_2SeO_4, and Se-yeast (as controls)

Thesis Organization

This thesis is organized into four chapters including a general introduction and background, literature review, a manuscript, and an overall conclusion. Both the manuscript in chapter 3 and references at the end of each chapter are formatted based on the requirements of the *Journal of Agricultural and Food Chemistry*.

Author's Role

The work I performed for my master's degree was spent working for Dr. Manju Reddy in her research lab. She provided guidance and insight with all of my cell work, enzyme and protein assays that I performed. We collaborated with Gross-Wen Technologies, LLC who provided all selenium-rich algal samples and ICP-OES analysis. Dr. Reddy oversaw and edited the manuscript for my research that I wrote, which is included in chapter 3 of this thesis.

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CHAPTER 2. LITERATURE REVIEW

Role of Selenium in Human and Animal Health

Selenium has a multitude of important roles in the body making it an essential daily nutrient[1,2]. Its essentiality to health though was only recently discovered as it was once known solely as a toxic element. Now, its main role can be thought of as a secondary antioxidant and important cofactor to a number of proteins known as selenoproteins[3]. The most well-known and studied are the glutathione peroxidase (GPx) enzymes that scavenge free radicals formed during aerobic metabolic reactions, thus preventing oxidative damage[4,5]. The relationship of Se with enzymes extends to many others such as iodothyronine deiodinase enzymes that are important for thyroid hormone synthesis and thioredoxin reductases which are important for a number of redox reactions[4,6].

Research with Se's role in human health has recently expanded to look at how it can affect various diseases. Several clinical trials with humans receiving daily Se supplementation have examined how Se can reduce the incidence of certain cancers (e.g. liver, colon, prostate) and may provide protection against toxicity with certain drugs used with cancer treatment[7]. Monomethylated Se (e.g. methylselenicacid and methylselenol) are thought to be effective chemical forms of Se for their ability to enhance apoptosis of androgen-dependent and androgen-independent cancer cells[8-10]. Regarding cardiovascular health, Se has been shown in some human clinical trials to lower total non-HDL cholesterol and increase HDL cholesterol[11]. In relation to Type 2 Diabetes, low levels of Se in the body have been shown to potentially increase risk of complications due to higher levels of oxidative stress[12,13]. Very high levels of Se in the body, on the other hand, may be associated with an increase of insulin resistance[14]. Overall, the

knowledge regarding Se's necessity to health is rapidly expanding with new research and development of new dietary supplements[3].

Chemical Forms of Selenium

Selenium exists as organic, inorganic, or as its elemental form. Inorganic forms are abundant in the environment and include elemental Se, selenate and selenite[15,16]. Several organic compounds exist as selenoproteins and selenoamino acids. Selenocysteine (SeCys) is identified as the 21st proteinogenic amino acid, which is an amino acid that is incorporated into proteins during translation. Selenocysteine, selenocystine and selenomethionine (SeMet) are predominantly found in plants and animals[15,17,18]. Organic, methylated compounds exist such as methylselenol, dimethylselenide and trimethylselenonium, which are excretory metabolite compounds in humans[15,19]. Seleno-amino acids and methylated Se compounds are found within Se-accumulating plants such as the allium (e.g. garlic) and brassica species (e.g. broccoli and cabbage)[20,21]. Brazil nuts are known to contain large amounts of Se and other good sources of Se are beef, poultry, milk, and grains[19,22].

Deficiency

Selenium is well recognized for its essentiality to toxicity within a small range of concentrations. In animals, when daily Se levels are below adequate health problems including reproductive issues, growth problems, poor immune function, and white muscle disease can occur[16,23,24]. Deficiency of this element in humans was first recognized in the Keshan area of China where a cardiomyopathy known as Keshan Disease was termed as a result of very low levels of Se in the diet. Kashin-Beck Disease is a type of osteoarthropathy also resulting from Se deficiency, causing oxidative damage to bone and cartilage cells[25-28]. This area of China currently averages a daily Se intake of less than 15 μg[26,29]. In comparison, the United States has a

daily average intake of 60-160 μg, Great Britain 34 μg, and Canada 98-224 μg[25]. During times of Se deficiency there is a reduction of selenoproteins synthesized, including the various GPx enzymes and iodothyronine enzymes. It is important to note though that the level of reduction toward different selenoprotein synthesis varies depending on their metabolic importance[30]. Several effects can happen from reduction of these enzymes including reduced protection against free radical cellular damage, reduced immune function, infertility particularly in men, reduced thyroid function, and even an increased risk of certain types of cancer[25,27,29].

Toxicity

Selenium is well recognized for its U-shaped relationship to health and for its ability to quickly reach levels of toxicity[1]. It was first identified for its toxic effect in animals creating the terms ‘alkali disease’ and ‘selenosis’. Signs and symptoms of excessive Se exposure in animals range from extreme weight loss, tachycardia, and neurological issues, to reproductive issues, deformities, and even death[16,31,32]. Toxic effects are particularly known to occur in aquatic life including egg-laying vertebrates[16,31]. In the United States, the tolerable upper intake level for humans is 400 μg/d but in some countries, daily intake far exceeds this[26,32,33]. In the Enshi region of China in 1983, high levels of Se were exposed in the environment from the coal industry, causing toxicity amongst many people. Symptoms included skin lesions, increased white blood cell count, and neurological problems when exposed to levels above 850 μg/day[32]. Other signs and symptoms of toxicity include garlic odor in the breath, brittle hair and nails, skin rashes, and adverse effects on endocrine, nervous and immune systems[34-36].

Supplementation

Current Dietary Recommendations

Recommendations for Se intake varies among different countries. For humans and animals, the recommendations for daily dietary intake are based on the amount needed to achieve adequate GPx activity[29]. The current recommended dietary allowance for adult men and women in the United States is 55 μg/day and deficiency is considered less than 30 μg daily[25,29]. In the United States, recommendations were originally based on those made in China as 40 μg/d for a 60 kg adult Chinese male. Based on weight differences between the two countries and individual variance with weight, the range of 55-70 μg/d was established for adult men and women[37]. The World Health Organization, on the other hand, recommends 70 μg/day for adults in the United States[33]. Based on more evidence of Se intake related to diseases, experts are suggesting that there may be benefit to having a higher daily intake, making supplementation ever more popular[38]. Some researchers suggest that current recommendations are insufficient for optimal expression of certain selenoproteins and as much as 300 μg/d may be beneficial[26,33]. Actual intake can vary greatly amongst different countries due to differences in diets.

Current Supplementation

In more recent years, Se was identified as essential to human and animal health, resulting in the development of Se supplements for humans, and Se supplemented feed for animals[39]. Supplements such as multivitamins and animal feeds often contain inorganic Se as their salts, sodium (Na)-selenate and Na-selenite for their low cost and ability to affectively increase selenoprotein synthesis[19,25,40]. Supplements also often contain SeMet because it is of high bioavailability (BA) and is considered the best form of Se to increase overall Se body status in humans because it can be stored, unlike inorganic Se[39]. Supplements that only contain Se often

have 200 µg of Se per daily dose, which is an amount based on numerous published studies demonstrating the benefits of Se decreasing the risk of certain cancers in humans.[39,41,42].

Selenium-Yeasts

Selenium-enriched yeasts such as *Saccharomyces cerevisiae* (*S. cerevisiae*) are common in supplement form for their low cost and ability to accumulate large quantities of the element[19,43]. As supplements, their popularity is attributed to high BA and effectiveness to increase selenoprotein synthesis which promotes health in humans and animals[44]. This results from organic Se, such as SeMet, being incorporated into body proteins in place of methionine, allowing for that Se to be used at later times of need, as well as increasing Se tissue concentration[45].

High concentrations of Se among many plants results in toxicity due to excessive inorganic Se reacting with thiols and generating reactive oxygen species (ROS), resulting in oxidative damage[46]. However, some plants and organisms such as yeast have the ability to accumulate larger quantities of Se because they metabolize inorganic Se to seleno-amino acids, and methylated Se such as Se-methylselenocysteine (MeSeCys)[19,47,48]. For example, *S. cerevisiae* has the ability to accumulate up to 3,000 µg of Se/g[44]. Other yeasts that are commonly studied for this ability are *Candida utilis* and *Yarrowia lipolytica*[49]. For supplements, S. cerevisiae is fermented in a Se-rich, sugar-based medium often containing Na-selenite[13]. Once inorganic Se is taken up by the yeast cells, it is metabolized and organically bound. The majority of organically bound Se formed is as SeMet[41,49]. Although it is difficult to determine all forms of Se in yeast supplements, many studies have reported that other identified Se compounds include SeCys and methylselenol[47]. The amount of inorganic Se in Se-enriched yeasts should be very minimal to be considered adequate for supplementation purposes[44].

Selenium and Algae

Similar to yeasts, many algae species can absorb larger quantities of Se when grown in proper conditions and metabolize it to organically bound forms[33]. Also, like other plants, algae absorb Se mostly as selenate or selenite and some algae species have been reported to synthesize and utilize selenoproteins[33,50,51]. Cultivating algae is typically done through means of a Na-selenite enriched media, allowing for cell uptake during exponential growth phase of the algae, although selenate is more bioavailable with many algae species[33,52,53]. Some of the most common algae species examined with Se-enrichment include *Chlorella sp.* and *Spirulina plantesis*[33,53]. Traditional methods of growing and harvesting algae involve use of open pond systems and enclosed photobioreactors, often followed by chemical treatment to harvest the algae and reach the appropriate concentration for the final biomass[54].

The method of growing algae in such a way to become Se-rich is increasing in popularity for several reasons. One is that like Se-rich yeasts, Se-rich algae have been shown to have similar beneficial effects on health and allow for incorporation of organically bound Se following absorption in humans and animals[50,52]. Several animal studies involving ewes, chickens, and rats have shown increased GPx activity and increased Se tissue concentration after consumption of Se-rich microalgae. Although in the same studies, the increase in GPx activity was typically not better than with consumption of Na-selenite supplemented in the diet, but enhanced oxidative stability is seen in the meat of broiler chickens[33,53,55]. Another reason is that the method of cultivating algae in Se rich media provides opportunity for minimizing regions of Se-toxic waters that can be the result from agricultural practices, which is a benefit to the environment[52].

Regardless of Se content, however, certain strains of algae offer a variety of health benefits when supplemented in the diet of humans and animals[56]. *Chlorella sp*. is popular for its

higher protein composition of approximately 50%. Approximately 20% of its composition is lipids, including several monounsaturated fatty acids such as oleic acid and palmitoleic acid, and many polyunsaturated fatty acids such as linoleic and linolenic acid[57-60]. *Chlorella sp*. also contains vitamins such as vitamin C and E, and minerals such as iron, zinc, calcium, and phosphorus. The composition of *Spirulina plantesis* is approximately 60% protein and 15% lipids. This algae species is rich in several monounsaturated fatty acids as well as linoleic acid, linoleic acid, docosahexaenoic acid, and several other polyunsaturated fatty acids[56-59]. It contains vitamins such as vitamin E and B12, as well as several minerals including iron, magnesium, zinc, selenium, and calcium[56]. Both algae species contain fiber, all of the essential amino acids, and offer several health benefits from these nutrients[61]. Utilizing algae species such as these for Se enrichment could provide a natural source of Se for humans and animals[56].

Selenium in the Environment

Soil content of Se varies greatly in the environment and its BA in plants is affected by conditions such as soil content, soil pH, and moisture level[34]. It is mainly present in soils as its elemental form or as inorganic Se, including selenate, selenite, and various inorganic salts[62]. Most plants absorb inorganic Se and have the ability to transform it into organic forms[63,64]. Because of the limited ability to absorb much Se, many plants contain less than 25 μg of Se/g of dry weight. Some plants are considered Se-accumulating for their ability to transform inorganic Se into organic Se for incorporation into non-protein amino acids compared to plants that experience adverse effects to incorporating Se into proteins. These plants can accumulate Se at up to 1-15 mg of Se/g of dry weight[64,65]. Some soil can have high levels of Se but the BAC in plants is low because of these varying conditions such as pH and moisture level. Therefore, Se content in soil, typically as selenite, selenate, or elemental Se accounts for many differences of

Se levels in plants and animals, which therefore, affects dietary intake in humans and animals[26,66].

There are several causes to the differences of environmental Se, often due to coal and phosphate mining, and agricultural irrigation practices[16,49]. Much of the naturally occurring Se originates from rock erosion[63]. Because of the uneven distribution of Se in soil and water throughout the world, there are areas of toxic levels of Se, often caused by runoff produced from industry and agricultural water systems[23]. There are even more areas with very low Se in soil, creating several challenges revolving around Se deficiencies[46,68,69].

Selenium's Role in the Animal Feed Industry

Within the animal feed industry, there is the constant challenge of providing livestock with adequate daily amounts of Se. Livestock that rely heavily on foraging are at risk of deficiency if those plants contain low levels of Se[34]. Many feeds do not contain enough Se to meet the range of 0.1-0.3 mg/kg, which is considered adequate for animal health[69]. Se levels in some livestock feed can be as low as 0.03-0.12 mg/kg of dry matter, therefore, supplementation is necessary. Adding Se can bring its concentration to recommended levels of 0.3-0.5 mg/kg of dry matter[24]. Currently, the Food and Drug Administration has regulations set at Se added to feed as either Na-selenite, Na-selenate, SeMet, or Se-yeast from *S. cerevisiae* to not exceed 0.3 ppm[70].

The majority of feeds that are supplemented with Se use inorganic Se-salts (Na-selenite, Na-selenate, calcium-selenite) because of their lower costs and effectiveness in improving animal health[22,33]. Organic compounds such as Se-yeasts are becoming more popular[33,69]. The predominant form of Se in yeasts is SeMet and a benefit of using organic compounds is that it can be stored in the body proteins as well as be utilized for selenoprotein synthesis. Supplementation is used to ensure animals are receiving appropriate amounts of daily Se to avoid

deficiency. Daily supplementation ensures proper growth, reproduction, and high performance in the animals[22,24].

A study conducted at California State University evaluated the effects that Se-rich canola oil seed cake meal would have on the Se milk and blood levels in cattle compared to canola oil not fortified with Se. This method had the benefit of removing excess Se from the environment by utilizing canola, which has the ability to uptake larger amounts of Se. It also increased Se milk concentrations in the cattle compared to the control, and maintained adequate Se blood levels[69].

Absorption

Bioavailability vs Bioaccassibility

Because of the complexity of Se metabolism, it is important to distinguish between bioaccessibility (BAC) and BA. Soluble fraction of a compound following gastrointestinal uptake is termed BAC, which is dependent on factors such as pH and type of food matrix[71,72]. BA refers to the absorbed portion that becomes available to cells for use[25]. Selenium BAC and absorption, therefore, is dependent upon the chemical form consumed[40]. Other terms often used regarding Se absorption and metabolism include non-bioaccessible, which refers to the unabsorbed portion of a compound, and bioactivity, which refers to the portion of a compound that becomes metabolically active (**Figure 2.1**) [71].

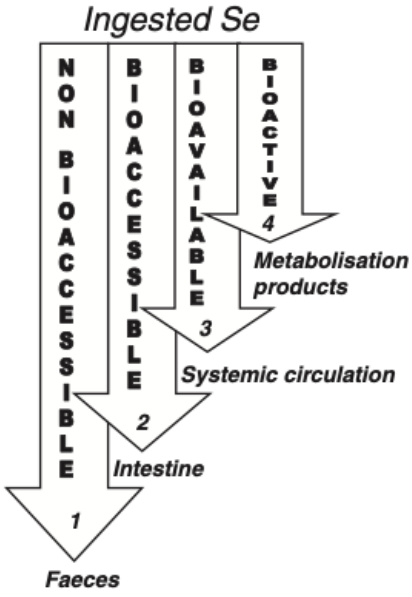

Figure 2.1 Terminology used to describe Se once ingested. (1) Non-bioaccessible refers to Se unabsorbed that is excreted in feces; (2) Bioaccessible refers to Se that is absorbed across intestinal epithelia; (3) Se that reaches systemic circulation and is available for cells to use represents the bioavailable portion; (4) Bioactive Se represents the fraction made into metabolic compounds and utilized[71].

Routes of Absorption

Selenium absorption appears to be unregulated[73]. Most chemical forms of Se are well absorbed within ranges of 60-98%, with the exception of elemental Se being much lower. Selenite absorption is typically around 60% and Selenate around 95%. Selenomethionine is known to be better absorbed with absorption of 95-98%. Methylated versions of Se and SeCys are similar to SeMet[40,74,75]. Some Caco-2 cell uptake studies though have reported Se-methylselenocysteine (MeSeCys) to be better absorbed than SeMet[76].

The subject of Se BA is complex. Although most forms of Se are well absorbed, their BA can be very different from each other. Methylated Se such as MeSeCys is not as effective as increasing Se in body proteins as SeMet but has been shown to induce a higher GPx activity[71,77]. Organic Se species such as SeMet and Se-yeasts have similar responses to GPx activity and are both able to be stored in the body. Inorganic selenate and selenite, however, cannot be stored in

the body and are immediately reduced for selenoprotein synthesis or excretion. Although selenite is not absorbed as well as organic forms, it tends to induce a greater GPx activity and reaches a quicker plateau of Se concentration in blood than organic Se[71]. Inorganic Se is considered more toxic because of the pathway it follows and for its ability to become a prooxidant due to its reactions with thiols when in excess[76,78]. In ruminant animals, it has been reported that Se BAC is lower because the microbial environment reduces much of the Se to the poorly absorbed elemental Se[79].

Absorption of Se occurs throughout the small intestine, and generally increases further down in the small intestine[80]. Much of Se absorption is related to amino acid absorption[75]. Multiple active transporters are utilized for amino acid absorption. One is the Na^+ dependent B^o active transporter for neutral amino acids[81]. Expression of this transporter increases further down the small intestine and has varying affinity for neutral amino acids, with higher affinity for methionine compared to cysteine. Other Na^+ dependent amino acid transports transporters assisting with SeMet uptake include the $b^{o,+}$rBAT and SIT1 transporters. Each of these transporters are expressed in varying degrees in the intestines and kidneys[82].

Another amino acid transporter with higher expression in the jejunum compared to other portions of the small intestine is the ASCT2 transporter. This transporter is Na^+ dependent and transports several amino acids across the epithelial, including methionine[83]. Because of this, SeMet is rapidly absorbed and to a greater extent compared to inorganic Se in all sections of the small intestine. The greatest absorption for SeMet takes place in the jejunum, and lesser amounts in the duodenum and ileum. Selenocysteine has also been shown to mainly utilize passive diffusion but can also the $b^{o,+}$rBAT transporter[82,84]. Due to its molecular size, selenite goes by means of paracellular transport and passive diffusion while selenate can utilize paracellular

transport and Na^+ dependent transporters[85]. Both inorganic species increase their absorption further down the small intestine, with the greatest percentage occurring in the ileum[86].

Absorption Enhancers and Inhibitors

Several factors can influence Se status in humans including food composition, age, current health status, and pharmaceutical drug usage[1]. In several studies analyzing effects of Se BAC and BA in seafood, seaweed, and mollusks, fat emulsification did not affect Se absorption. Carbohydrate content including soluble fiber overall does not affect Se absorption and solubility. How protein affects Se BAC is somewhat conflicting. In the studies involving seafood, the amino acids present in the in vitro digest increased ionic strength and lowered Se solubility[87]. Other studies have suggested that protein can improve Se absorption. Vitamins A and E have been reported to improve Se absorption. Ascorbic acid has been reported to both enhance and inhibit the absorption of Se, depending on the chemical form of Se consumed[47,88,89]. Ascorbic acid may reduce some forms of Se such as selenite to its elemental form, greatly reducing its BAC. The presence of heavy metals, particularly cadmium, zinc, and mercury can greatly limit absorption of Se[22,90]. Differing pH in the small intestine may affect the level of absorption. In the duodenum pH is between 5 and 6, while in the jejunum and ileum it is around 7. Most Se is better absorbed further down the small intestine where the pH is higher[89]. During intestinal epithelial cell uptake, organic Se and selenate compete for absorption with its sulfur analogues while selenite is not affected[79,91].

Metabolism

Relationship with Sulfur

The analogous relationship between Se and sulfur allows for the versatility of Se in plant and animal metabolism[92]. Similarities between Se and sulfur allow for Se to enter metabolic

pathways in place of sulfur, such as the trans-selenation pathway and to be incorporated non-specifically into proteins in place of methionine and cysteine (**Figure 2.2**). In many cases, selenols have metabolic advantage over sulfur containing thiols. Compared to thiols, selenols are more acidic, more nucleophilic, and more easily oxidized[93,94]. These variations make Se compounds more favorable and reactive[95].

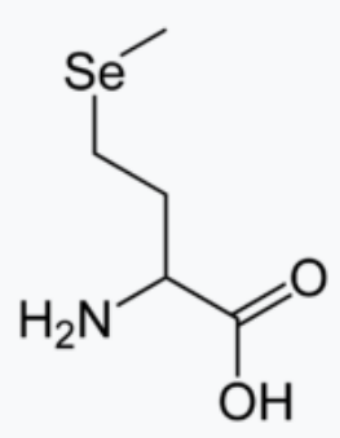

Figure 2.2. The structure of SeMet where the protein methionine contains Se in place of sulfur, allowing for easy and non-regulated incorporation into body proteins (Wikipedia image).

Metabolic Pathways

Once absorbed, Se follows three main regulated routes of metabolism: selenoprotein synthesis, incorporation into proteins as SeMet and methylation for excretion[96-98]. Immediately following intestinal absorption, selenite is rapidly and selectively taken up by red blood cells. Through a spontaneous reaction with glutathione (GSH), selenite is reduced to selenodiglutathione[62,99]. This compound is then reduced to glutathioselenol by GSH where it can then be converted to elemental Se and GSH or reduced by GSH to hydrogen selenide (H_2Se). Selenite can also be reduced to H_2Se by thioredoxin and the thioredoxin reductase enzyme[100]. The H_2Se is transported by albumin to the liver via the hepatic portal vein for further processing into selenoproteins[62,91].

Little is currently known about how selenate is immediately reduced to selenite. It has been suggested that a membrane bound selenate reductase may be used in a manner similar to some microorganisms[68]. Another proposed method for selenate reduction involves ATP sulfurylase which converts selenate to adenosine selenophosphate before reducing it to selenite[101]. Once

selenite and selenate are reduced to H_2Se, selenoprotein synthesis can occur[102]. Excess Se can be methylated and excreted in the form of urinary metabolites[103]. One reaction involves converting H_2Se to methyl selenide by methyl transferase, and then additional methylated Se compounds are formed[104]. These metabolites include methylselenol, which is the active methylated form of Se for biological use and selenosugar B, which can be oxidized to methylselenic acid that can be available to the body during Se deficiency. Other urinary metabolites include 1β-methylseleno-N-acetyl-D-galactosamine, dimethyldiselenonium, and trimethylselenonium[97,105]. Dimethylselenide is an additional metabolite that can be excreted through the breath[100]. Organic Se is also transported by albumin to the liver for metabolism[106]. Because Se is analogous to sulfur, they share similar routes of metabolism. Selenomethionine can be inserted into body proteins rather than methionine, allowing for storage and availability to amino acid pools[107,108]. It can also be converted to methylselenol, which can be further processed for excretion or demethylated to H_2Se for further processing[109]. Similar to the trans-sulfuration pathway, SeMet can enter the trans-selenation pathway to synthesize H_2Se, making Se available for selenoprotein synthesis (**Figure 2.3**)[28]. The initial step of the trans-selenation pathway involves SeMet reducing to Se-adenosylmethionine (SAM) by the enzyme SAM synthetase and to then Se-adenosylhomocysteine (SAH) by methyl transferase. From there, the compound is

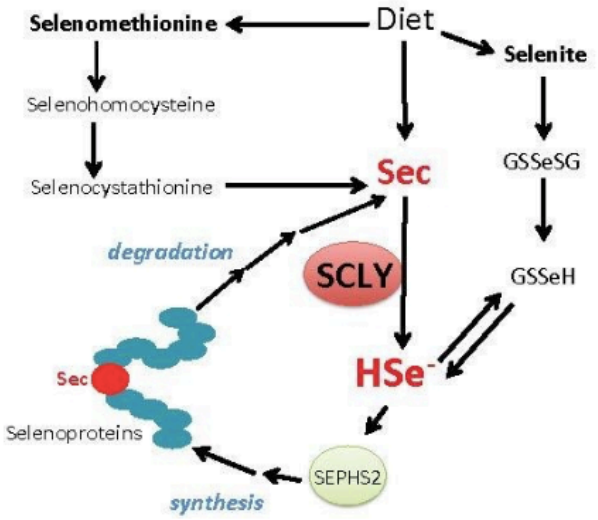

Figure 2.3 SeMet enters the trans-selenation pathway to synthesize selenocysteine (Sec). Sec is reduced to hydrogen selenide (HSe^-) by selenocysteine β-lyase (SCLY), then transformation in selenoproteins is initiated by selenophosphate synthase 2 (SEPHS2). Selenite is reduced to selendiglutathione (GSSESG) then to glutathioselenol (GSSeH). Seale[1]

hydrolyzed to Se-homocysteine by SAH hydrolase and by cystathionine β-lyase, is converted to Se-cystathionine. Cystathionine γ-lyase then converts Se-cystathionine to SeCys which is then reduced to H_2Se by selenocysteine β-lyase[106,110,111]. Both methylselenocysteine and SeMet can be converted to methylselenol by γ-lyase and β-lyase, respectively with the intermediate compound being methylselenic acid[105,111].

Selenoproteins

Selenoproteins are proteins synthesized in the body, all requiring at least one SeCys in their active centers to function. At least 25 selenoproteins have been identified[35]. They have a variety of important roles in the body including thyroid hormone synthesis, free radical scavenging, immune health, reproductive health and fertility, involvement in redox reactions, and even reduce virulence of some infections[112,113]. Among the most well-known and investigated are the GPx enzymes, the majority of which prevent oxidative damage during aerobic metabolic reactions[114]. Selenoprotein P (Sepp) is a major selenoprotein and member of the thioredoxin family[115]. It carries up to 10 selenocysteines and transports Se from the liver to other tissues. It also acts as an antioxidant, reducing phospholipid hydroperoxides and preventing oxidation of low-density lipoproteins and peroxinitrite-mediated oxidation[116]. It represents the largest portion of Se in human serum and serves as an indicator of Se nutritional status in humans[115,117]. Deiodinase enzymes 1 and 2 convert the thyroid hormone thyroxine to the active triiodothyronine and the other serves to deactivate triiodothyronine[118,119]. Thioredoxin reductase is an important enzyme for redox reactions such as those that maintain the redox state of transcription factors[120,121]. Selenophosphate synthetase is essential for the production of selenoproteins by producing selenophosphate from H_2Se[122]. Selenoprotein W acts as an

antioxidant but its role as such is not yet well understood. Selenoprotein T, selenoprotein O, and selenoprotein N also have functions not yet well understood[123,124].

Selenoprotein Synthesis

Selenoprotein synthesis begins with the central compound to Se metabolism, H_2Se. When metabolic demands require selenoproteins, both organic and inorganic Se are transformed into H_2Se[125]. The H_2Se compound is first phosphorylated to selenophosphate by selenophosphate synthase 2. Selenoprotein synthesis is a unique process that utilizes selenophosphate as a substrate and co-translational incorporation of SeCys by the UGA codon[100]. Traditionally, UGA functions as a stop codon but specific secondary mRNA stem loop structures known as mRNA inserting sequences recognize UGA and recode it to allow for its function as a SeCys codon[107,125,126]. Selenocysteine inserting sequence binding proteins recognize this sequence, initiating binding of $tRNA^{[ser]sec}$. Seryl-tRNA synthetase converts $tRNA^{[ser]sec}$ to $Ser\text{-}tRNA^{[ser]sec}$. Next, phosphorylation by phosphoseryl-tRNA kinase generates $P\text{-}Ser\text{-}tRNA^{[ser]sec}$. Selenocysteine synthase uses the selenophosphate as a Se donor, to make $P\text{-}Ser\text{-}tRNA^{[ser]sec}$. The last step utilizes the UGA codon to incorporate SeCys into the polypeptide chain for selenoprotein synthesis[95,110,127].

Homeostasis

Homeostasis of Se is regulated primarily by the liver and achieved through storage in body proteins and excretion through the kidney and respiratory system[73,106,128]. In total, the average human body contains 10-20 mg of Se, with about half of the Se located in the skeletal muscle. Within plasma, Sepp accounts for approximately 53% of total plasma Se and plasma glutathione peroxidase (GPx3) accounts for approximately 39% of plasma Se. Albumin, which transports Se to the liver after absorption accounts for approximately 9% of total plasma Se[116]. Highest tissue

concentrations of Se are seen in the liver, kidneys, testes, and hemoglobin[34]. Selenomethionine can be incorporated into proteins and tissues in place of methionine and to a greater extent in tissues such as skeletal muscle, red blood cells, and the kidneys (**Figure 2.4**) [129].

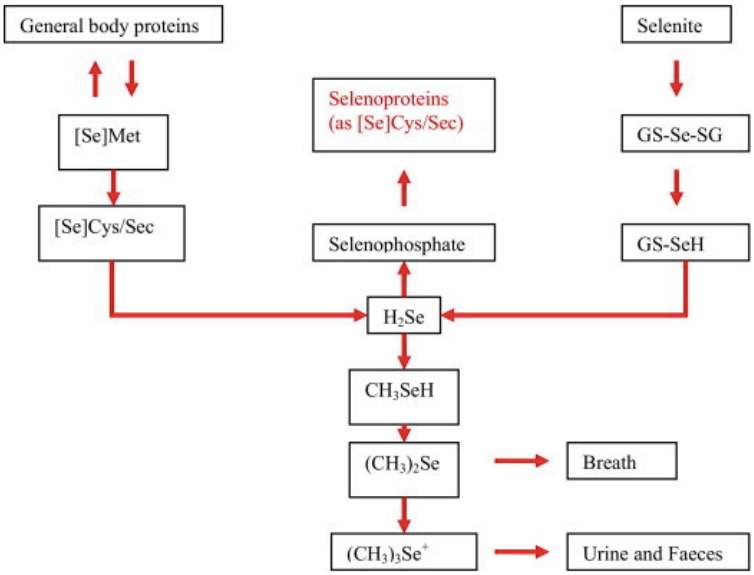

Figure 2.4 Homeostasis of selenium is achieved through storage in body proteins, generation of selenoproteins, and excretion[113]

In times of surplus Se, generation of multi-methylated urinary metabolites and renal excretion is the main route of elimination[130]. In times of Se deficiency, the body tightly regulates and reduces the production of selenoproteins in order of their metabolic importance. Excretion through urine still occurs but mainly with monomethylated Se and through the breath by dimethylselenide[128]. Glutathione peroxidase enzymes drastically decrease in synthesis as well as many other selenoproteins, while type 1 iodothyronine deiodinase is initially affected to a lesser degree[124,131,132]. Selenocysteine β-lyase may be involved with regulating increased Se uptake to the brain[111]. Because Sepp helps to maintain selenoprotein status in the brain and testis, its synthesis and plasma concentrations decrease less than other selenoproteins during Se deficiency[115,133]. Selenoprotein P in fact, has been considered to be a marker of Se status in humans because it carries the majority of plasma Se[131,134]. In several mice studies, during Se

deficiency and or deletion of Sepp, Se concentration in tissues decreases to a greater extent in tissues such as the liver and testis, but less in tissues such as the brain, heart, and kidney[28]. During repletion of Se in the diet, inorganic and organic Se are effective at restoring enzyme activity[135].

Glutathione Peroxidase and Oxidative Damage

Oxidative stress can occur as a result of normal metabolic processes but can also result from environmental stressors such as cigarette smoking. Oxidative stress is a contributor to diseases such as cardiovascular disease, diabetes, cancer and is also a contributor to aging[136-138]. Redox reactions generate ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2), which are damaging to cell membranes and protein function if they are not properly eliminated or are present in excessive amounts[139,140]. One way that H_2O_2 can damage a cell is by reacting with metals nearby, producing a hydroxy radical. The hydroxy radical can cause cell membrane lipid peroxidation and production of toxic byproducts that inactivate important cellular enzymes[141]. Reactive oxygen species do have vital roles though such as secondary messengers in cellular transduction reactions[142]. During inflammatory immune response, superoxide dismutase can turn O_2^- to H_2O_2 in response to invading pathogens, but if left available for too long, can cause tissue damage[140].The GPx family consists of enzymes that act as antioxidants, catalyzing the reduction of hydrogen peroxides using two reduced glutathione molecules (GSH) to two water molecules and oxidized glutathione (GSSG)[143]. They also reduce lipid peroxides to lipid alcohols[140,144]. Oxidized glutathione and NADPH + H^+ then go through a reaction catalyzed by glutathione reductase to reduce GSSG, resulting in the reduced glutathione, GSH, and $NADP^+$[143,145]. **Figure 2.5** displays the mechanism of oxidative damage and how GPx interferes.

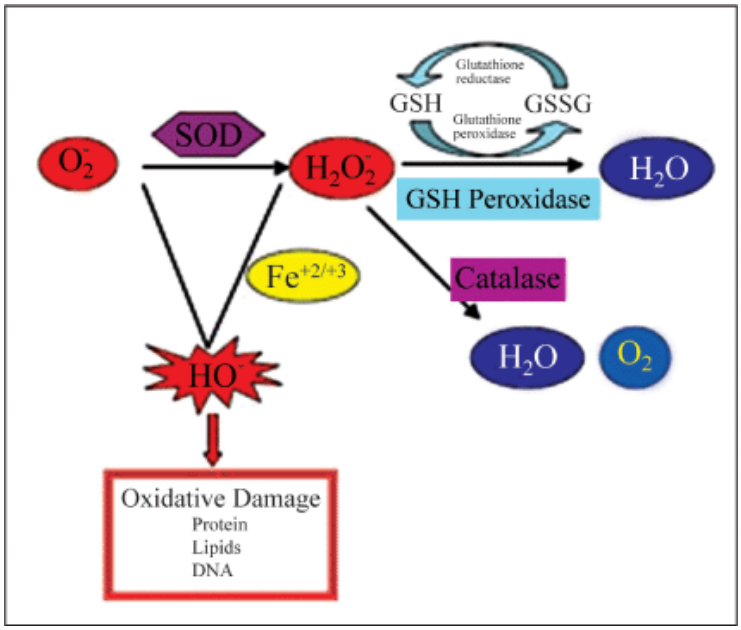

Figure 2.5 Mechanism of oxidative damage caused by ROS. GPx functions to reduce ROS in the form of hydrogen peroxide to water and oxygen. Glutathione reductase follows by reducing cellular GPx[146].

Currently eight GPx enzymes have been identified, most of which require a SeCys at the catalytic sites of the homotetramer compound[147]. Cellular glutathione peroxidase is present throughout the body and its activity is utilized for many studies regarding Se BA. Gastrointestinal GPx is mainly present in the gastrointestinal tract and liver. Plasma glutathione peroxidase, as previously described, is present in the plasma but also several organs[143,148]. Phospholipid GPx is present at high concentrations in the testis, but also located throughout the body as it protects specifically against phospholipid peroxidation[142]. Glutathione peroxidase 5 is seen in sperm nuclei, embryo tissue, and olfactory epithelium. Glutathione peroxidase 6 has been identified, but its function is currently unknown[148]. Glutathione peroxidase 7 and 8 are both thought to act as antioxidants but also are involved with protein folding in the endoplasmic reticulum of cells[62].

Methods of Selenium Bioavailability Assessment

Several methods exist for determining Se BA. For any mineral to be absorbed it has to be in soluble form. Cell models, particularly using Caco-2 cells are used commonly as they can easily be used to examine the absorbable fraction of Se. Caco-2 cells are favorable because they are a colon adenocarcinoma cell line that create a cell monolayer representative of the intestinal lining[149,150]. Many mammalian cells require serum to contain 30-50 nmol/L of Se to maintain their growth. Because serum containing medias can contain trace amounts of Se, studies using these cells often follow a gradual serum reduction method to make the cells Se deficient[18,77]. Following in vitro digestion Se compounds can be directly added to the cell monolayer which allows for a more accurate representation of how the compound would be digested in humans and animals[34]. Following cell uptake of a Se treatment, Se content and enzyme activity in the extract can easily be tested[151]. Many animal models are utilized to examine the Se concentration in blood and organs and enzyme activity, particularly follow restoration of Se-depletion in the diet[71,135]. Level of Se excreted through urine, breath, and feces can also easily be tested. A multitude of studies in rats, mice, chickens, ewes, and cows have been conducted to aid in the understanding Se BA[152-155]. In humans and animals, Se-blood concentration and serum GPx activity can be used to determine BA[153]. For long-term Se status in humans, Se concentration in toenails and hair has shown to be an effective method of assessment. Urine concentrations can be measured to determine the level of excretion of various Se compounds[152,153].

Conclusion

This literature review detailed the necessity of Se in the diets of humans and animals, as well as its importance and consequences of Se deficiency/toxicity. It also explained the absorption and metabolism of different chemical forms of Se, which is important to understand when studying the effects this element has in the body. With inadequate daily intake comes many

health problems including oxidative damage that can lead to chronic disease and increase risk for certain types of cancer. Other notable health problems include thyroid hormone dysfunction, reproductive, and nervous system issues. Toxicity of Se can be equally damaging to health. Se concentration in soil and water greatly varies around the world for a number of reasons, creating inconsistent and often low dietary intake. This creates opportunity for development of new Se supplementation. Algae can remove Se from wastewaters and at the same time, use it for animal feed. It is therefore important to study BA from this novel Se-rich algae. More methods are developing utilizing algae for its ability to grow well while taking in high concentrations of inorganic Se. The following research was designed to find the potential that Se-rich algae has for future animal supplementation.

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CHAPTER 3. ASSESSING THE BIOAVAILABILITY AND BIOACCESSIBILITY OF SELENIUM FROM SELENIUM-RICH ALGAE

A manuscript prepared for the submission to the *Journal of Agricultural and Food Chemistry*

Laura A Walter[*], Martin Gross[†], Zhiyou Wen[*†], Xuefei Zhao[†], and Manju B Reddy[*]

[*]Department of Food Science and Human Nutrition, Iowa State University, Ames, Iowa, 50011, USA

[†]Gross-Wen Technologies, LLC, Slater, Iowa, 50244

Abstract: New methods for selenium (Se) supplementation are emerging. The objective of this study was to test the Se bioaccessibility (BAC) and bioavailability (BA) of Se-rich algae. Algal samples were cultivated in media containing selenite or selenate then compared to Se-salts, Se-yeasts and Se-amino acids from traditional supplementation. BAC was tested based on the Se solubility post *in vitro* digestion and BA by induction of cellular glutathione peroxidase activity (GPx) in a Se deficient Caco-2 cell model. Cells treated with algae grown in selenate had a mean GPx activity that was 15-62% ($p<0.05$) of the GPx activity relative to the cells with SeMet treatment. Cells treated with algae grown in selenite had a mean GPx activity that was 11-34% ($p<0.05$) of the GPx activity from SeMet treated cells. Se-rich algae did not increase GPx activity as well as traditional supplementation forms, but the results give insight for improving Se BA studies.

Keywords: *selenium, algae, glutathione peroxidase, Caco-2 cell model*

Introduction

Selenium (Se) is an essential mineral to human and animal health. It is known for its antioxidant and inflammatory properties, maintaining thyroid hormone metabolism, and even

protective properties against certain forms of cancer[18,107,108]. One of its most studied roles is as a cofactor of cellular glutathione peroxidase (GPx). As all selenoproteins, GPx requires a selenocysteine (SeCys) at its catalytic cite and is essential for removal of reactive oxygen species generated from aerobic metabolic reactions[96,109]. Specifically, GPx catalyzes the reaction of a hydrogen peroxide and two reduced forms of glutathione molecules, resulting in an oxidized glutathione and water[80,89]. Depending on the dose and which chemical form Se is consumed though has an influence on the level of GPx activity.[89]

When assessing Se metabolism, it is important to distinguish bioaccessibility (BAC) from bioavailability (BA), and how the body metabolizes the different chemical forms of this element. Absorbable fraction such as solubility of an element during gastrointestinal digestion is referred to as BAC. Once absorbed, an element can be distributed to cells for use, characterizing its BA[15,110,111]. Selenomethionine (SeMet) is often considered to have the greatest BAC and is less toxic compared to inorganic Se[24]. Inorganic selenite is typically shown to have lower BAC than the organic forms and cannot be stored in the body but inorganic selenate tends to have a BAC comparable to organic Se[15]. However, selenite has been shown to induce a higher cellular GPx activity compared to organic Se because it is immediately reduced to an available form for selenoprotein synthesis[45,77]. Other studies suggest that organic selenized yeast has higher BAC than inorganic Se but also results in a lower GPx activity[112]. Overall, once Se is absorbed, it follows three main routes of metabolism: selenoprotein synthesis, incorporation into body proteins, or excretion.[18,43]

The mechanism of Se absorption is dependent upon the chemical form consumed. SeMet is absorbed to a greater extent throughout the small intestine compared to inorganic forms, particularly in the jejunum using active transporters along with its sulfur analogues[27,28]. Selenate

is absorbed via Na^+-dependent active transporters and increases further down the small intestine[113]. Selenite on the other hand, utilizes passive diffusion which also increases further down the small intestine[26,29,32]. Following intestinal epithelial uptake, inorganic Se is selectively taken up by the red blood cells and immediately reduced to hydrogen selenide (H_2Se) and transported by albumin to the liver for selenoprotein synthesis or Se excretion[6,45]. SeMet follows a longer trans-selenation pathway to generate H_2Se, which is similar to the trans-sulfuration pathway taken by methionine.[43,80] H_2Se is then converted back to SeCys for co-translational incorporation into various selenoproteins by the UGA codon, or methylated for excretion.[18,43,80]

In supplementation, inorganic Se-salts and Se-rich yeasts are commonly used[2,17]. Algae though is being recognized more for its economic advantage and ability to hold high concentrations of Se[114]. Incorporating Chlorella *sp.* into dietary supplements is popular because it is a natural product containing a variety of vitamins (i.e. vitamin C and E), minerals (i.e iron and calcium), and fiber[26,27]. Over 50% of Chlorella *sp.* dry mass is as protein, providing all of the essential amino acids. Approximately 26% of the dry mass is lipids, providing several polyunsaturated and monounsaturated fatty acids such as oleic acid and linoleic acid[28-30].

Gross-Wen Technologies, LLC (GWT) has developed a Revolving Algal Biofilm System (RAB) that produces quantities of algal biomass that are 10 times greater compared to other existing systems such as open-pond systems. The RAB system is cost effective and has the ability to remove nutrients like Se from the effluent generated in food manufacturing[31]. However, no studies are reported to date on the BA of Se from the algal biomass. Current methods for Se BA and BAC include animal and cell models. BAC can be measured with solubility after *in vitro* digestion as a surrogate. GPx activity is often used as a surrogate to Se BA in a cell model because it gives an accurate representation of the intestinal uptake of different

forms of Se[32,33]. The objectives of this study were to assess the BAC and BA of Se from the algae grown in different conditions with varying concentrations of selenite and selenate, and compare them to organic and inorganic Se often used in supplements.

Materials and Methods

Materials

L-Selenomethionine (SeMet), pancreatin and pepsin were purchased from Millipore Sigma (MA, USA); Sodium Selenite (Na_2SeO_3) from Alfa Aesar (MA, USA), and Sodium Selenate (Na_2SeO_4) were obtained from Fisher Scientific (MA, USA). Natural Factors SelenoExcell Selenium Yeast (Yeast) was from Natural Factors (USA). For the cell culture, a human adenoma carcinoma cell line (Caco-2) was obtained from American Type Culture Collection (USA). For the media, fetal bovine serum (FBS) was purchased from Atlanta Biological (GA, USA); Advanced Dulbecco's Modified Eagle Medium (DMEM), Gibco HEPES 1M, Gibco Antibiotic-Antimycotic 100x solution, and Gibco L-Glutamine 200 mM from Life Technologies, Thermo Fisher Scientific (CA, USA). Glutathione Peroxidase Assay Kits were purchased from Cayman Chemicals, (MI, USA). Phosphate Buffered Saline (PBS) and 0.5% Trypsin EDTA were purchased from Life Technologies, Thermo Fisher Scientific (CA, USA).

Preparation of Algal Samples

Se-rich algal samples were cultivated and harvested in the GWT lab at Iowa State University, Ames, IA. One-liter medium reservoirs were used to grow Chlorella *sp.* in a bold basal medium. Each reservoir had 0.12 m^2 of attached material that revolved in and out of the reservoirs and contained a different concentration of either selenate or selenite (10, 30, 50, 100, or 200 ppm). Specified levels of sulfite were also added to the medium (0 or 200 ppm). As a control to the RAB, a conventional bubble column reactor was also used to cultivate algae. Algae

was harvested at varying time frames of either week 1, 2, 3, 4, or 5 of growth and Se content in dry mass was measured post-harvest by inductively coupled plasma optical emission spectroscopy analysis (ICP-OES).

In Vitro Simulated Human Digestion

SeMet, Na_2SeO_3, Na_2SeO_4, Yeast, and Se-enriched algal biomass provided by GWT containing 200 µg Se were subjected to simulated *in vitro* human digestion for assessing soluble Se, as well as Se uptake in the cell culture model for assessing BAC and BA, respectively. Each sample with 200 µg of Se was mixed with Mili-Q water to create a slurry. The slurry was initially adjusted to pH 2 and incubated along with pepsin at 37°C for 1 hour while shaking at 300 rpm to simulate gastric digestion. After adjusting to pH 6 and adding pancreatin, the samples were incubated again at 37°C for 1 hour while shaking at 300 rpm to simulate duodenal digestion. Enzyme deactivation was achieved by boiling the samples for 4 minutes immediately following second incubation as suggested for iron[34]. Finally, samples were centrifuged at 1070 x g for 15 minutes at 37°C before collecting supernatant and storage at -20°C. Se content was measured in duplicates using ICP-OES system to assess percent solubility and intestinal cellular uptake of Se. Although approximately 70 algal samples in total went through *in vitro* digestion and solubility analysis, only a selected number were used for cell treatments. Selection was based on samples that had approximately 10% solubility and above. Table I lists the algal samples that were used for the remainder of the study, including a description of the names and designated letter.

Cell Culture

Caco-2 cells were grown at 37°C with 5% CO_2 and DMEM containing 10% FBS. The cells were maintained by weekly passage by washing with PBS and then with 0.5% Trypsin

EDTA to disassociate the cells after reaching 90% confluency. For the cell uptake experiments, cells were passed to 6-well plates in DMEM with 10% FBS at a cell count of 190,000 cells/mm^3 per plate well. Because serum used in the culture media contains trace levels of Se, the cells were made Se deficient to assure optimal uptake of Se. This was achieved by growing them with DMEM supplemented with 10% FBS for 2 days, followed by 5% FBS for 2 days, and then 0% FBS for 6 days. At the end of 6 days, the cell model became Se deficient as measured by GPx activity (Figure 3.1). We used these Se deficient cells for Se uptake experiments.

For BA experiments, immediately after the serum reduction period, the cells were washed with PBS. They were then treated with 4mL of serum free DMEM and 1mL of in vitro digest supernatants containing 100 nmol/L of Se either from SeMet, Na_2SeO_3, Na_2SeO_4, Yeast, or Se-enriched algal biomass, with duplicates of each treatment. Treatment remained on the cells for 24 h to induce GPx activity prior to harvesting with 1mL of cold buffer (50mM Tris-HCl, pH 7.5, 5mM ethylenediamine tetraacetic acid, and 1mM dithiothreitol) and then sonicated for 4 seconds on ice. The samples were stored at -80°C prior to being thawed and centrifuged at 10,000 x g for 15 minutes at 4°C for protein analysis using the Pierce™ Coomassie Bradford Protein Assay Kit (Thermo Fisher Scientific CA, USA) and GPx analysis. Two uptake measurements were made from each duplicate digestion.

Statistical analysis

The GPx activity between Se deficient and Se sufficient cells was determined using Student t-test. Data of BAC (solubility) and BA (Gpx) are shown as mean ± SD and the differences among groups were determined by ANOVA with Tukey multiple comparison test for BAC and BA. Duplicate wells of each treatment were used in the cell model as well as

duplicates for solubility and GPx testing. Differences among the treatments were considered significant at $P<0.05$ among.

Results and Discussion

Bioaccessibility

BAC is determined by solubility of the *in vitro* digestion. The solubility (%) of the Se-containing samples was calculated based on the fraction of soluble Se concentrations after *in vitro* digestion from 200 µg of Se. Figure 3.2A compares the solubility of the algae samples grown in various concentrations of selenite to SeMet, Na_2SeO_3, and the Yeast supplement as controls. No significant differences were seen in mean solubility between SeMet, Na_2SeO_3, and Yeast ($p>0.05$) with 84.3%, 74.3%, and 81.6% solubility, respectively. The lower solubility from Se-rich algae samples was significantly lower ($p<0.05$) compared to SeMet, Na_2SeO_3, and Yeast. The algae sample A1 had the highest mean solubility of 30.7%, which was significantly different from the other four algae samples ($p<0.05$). Its growth conditions consisted of 30 ppm selenite in media, 0 ppm sulfite, and harvested at week 1 of growth with 0.51% Se in its dry mass. Its solubility was also approximately 36% of SeMet. Compared to the other samples with 30 ppm selenite and 0 ppm sulfite, the growth conditions for A1 had a shorter cultivation time. Based on several previous studies, SeMet is recognized to be more soluble and therefore, have greater BAC compared to most other forms of Se, particularly selenite and elemental Se[1,32]. It is also recognized as the best form of Se to raise overall Se status in the body, as it is able to be stored, methylated, and can increase selenoprotein synthesis. Selenate, although inorganic, is comparable in BAC to organic Se, based on several published studies[15,18,32]. Selenite has lower BAC compared to organic forms of Se such as SeMet, SeCys, and methylselenocysteine[35,36].

Figure 3.2B compares the solubility of the algae samples grown in various concentrations of selenate to SeMet, Na_2SeO_4, and the Yeast supplement. Again, SeMet, Na_2SeO_4, and the Yeast were not significantly different ($p>0.05$) with 84.3%, 87.1%, and 81.6% solubility, respectively. The Se-rich algae samples all had a much lower solubility and were significantly different compared to SeMet, Na_2SeO_4, and Yeast ($p<0.05$). Sample A8 had growth conditions of 200 ppm selenate in media, 0 ppm sulfite, and harvested at week 2 of growth with 0.31% Se in its dry mass. It had the greatest average solubility (28.3%) and was significantly higher compared to sample A9 and A12-A15 ($p<0.05$). Its solubility was approximately 34% of the SeMet. These results could be explained by the higher concentration of selenate added to the growth medium, the shorter cultivation time compared to the other samples, and having no sulfite in the medium.

Algae can successfully absorb larger amounts of inorganic Se and can metabolize it to organic forms of Se. This includes generation of selenoproteins and incorporation into other proteins for storage and later use[37,38]. Some studies report that methylated forms of Se can be generated[24,39]. What is currently not clear though, is exactly what type and how much organic Se is formed following absorption of inorganic Se, creating challenges for assessing the BA[115]. The composition of algae may play a major role in how Se is metabolized and utilized. Fiber and sulfur content, for example, could inhibit Se absorption, particularly organic Se[113]. Lastly, pH of the simulated digestion could affect the results for all of the treatments. The pH of 6 that was used in our *in vitro* digestion protocol was reflective of duodenal pH, which is commonly used for iron[40]. However, Se is absorbed to the greatest extent in the jejunum[17,41]. Perhaps using a slightly higher pH to mimic further down the small intestine could increase absorption of all the

treatments, but a different cell line has to be used for this. Overall, our results for solubility with the SeMet, Yeast, and inorganic Se-salts are consistent with current literature[2,14,32].

Bioavailability

To assess the BA of the Se-rich algae we utilized GPx activity in the cell extracts after Se was taken up by the cell. Figure 3.1 shows the Se-deficient cells had approximately a 70% reduction in GPx activity and which was significantly lower compared to the Se sufficient cells ($P<0.05$). These results were slightly different from the methods used in a published study (Zeng et al., 2008) that showed an 80% reduction in GPx activity in their Se-deficient model[28]. We assumed that not all forms of Se, although having similar BAC, would induce GPx to the same extent. Concentration and chemical form are key factors regarding the ability for Se to induce GPx activity. Using the same concentration of 100 nmol/L of Se in each cell treatment allowed for easy comparison between each sample result. Different concentrations might have a different effect based on the relationship to GPx, therefore, using a different concentration that used in this study may produce different results.

Figure 3A compares the GPx activity of the algae samples cultivated in selenite under different conditions as described in the methods to SeMet, Na_2SeO_3, and Yeast. The Yeast and Na_2SeO_3 had the highest GPx activity of 771 mU/mg cell protein and 725 mU/mg cell protein, respectively, and were not significantly different ($p>0.05$). They were significantly different ($p<0.05$) compared to SeMet, which had a lower GPx activity. SeMet had an average GPx activity of 574.1 mU/mg cell protein. The GPx activity of Na_2SeO_3 was 126% of the SeMet and the Yeast GPx activity was 134% of SeMet. All of the algae samples had a significantly lower ($p<0.05$) GPx activity compared to SeMet, Na_2SeO_3, and Yeast and we couldn't identify any of the algae samples as superior. Sample A1 did appear to have a better overall GPx activity but it

was only significantly different ($p<0.05$) in GPx activity to sample A5, which was cultivated in a higher concentration of selenite and harvested at a later time. The growth conditions for sample A5 included 50 ppm selenite in media, 0 ppm sulfite, and harvested at week 3 of growth with 0.25% Se in dry mass.

Figure 3B compares the GPx activity of the algae samples grown in selenate to SeMet, Na_2SeO_4, and Yeast. The GPx activity of Na_2SeO_4 was 214% of the SeMet GPx activity. All selenate algae samples had a significantly lower ($p<0.05$) GPx activity compared to SeMet, Na_2SeO_4, and Yeast. Sample A6 growth conditions consisted of 200 ppm selenate in media, 0 ppm sulfite, and harvested at week 5 of growth with 0.58% Se in dry mass. It had the highest GPx activity (354.4 mU/mg cell protein) though and was significantly different ($p<0.05$) compared to the other algae samples, except for sample A7 which had the same growth conditions but was harvested at week 3 of growth. In general, the algae grown in the higher concentration of selenate (200 ppm) with no sulfite appeared to have a better BAC than all other algae samples.

It is clear that Se solubility may not be the best predictor of the Se BA. The inorganic selenite and selenate showed higher GPx activity compared to SeMet which could be explained by the direct reduction to H_2Se before incorporation into selenoproteins, compared to the longer pathway that organic Se uses[12,42,43]. Organic Se is not directly transformed to H_2Se and may go through other routes such as incorporation into body proteins in place of methionine[44,45]. No matter the concentration or the form of Se, BA from Se-rich algal samples was very low compared to organic and inorganic Se. Knowing how algae metabolizes the inorganic Se it absorbs is important because it is clear that there is a difference in BA between organic and inorganic Se. As with solubility, considering factors such as algal composition could further the

understanding of its BA Algal biomass could simply be more difficult to digest and finding other ways to improve the digestion could improve the BAC, and perhaps the BA. Because the algae Chlorella *sp.* provides a higher amount of protein including essential amino acids, monounsaturated and polyunsaturated fatty acids, fiber, vitamins, and minerals, it has much to offer in terms of nutritional benefits[26,28,29]. It also has a positive impact on the environment when considering how well it can remove certain nutrients from water sources that may have a negative impact if not removed[34]. Because algae can be cultivated so efficiently and effectively, it is very promising to use for biotechnological purposes, such as creating a natural supplement for humans or animals.

This study helps represents the complexity that exists among Se absorption and metabolism. Se-rich algae is emerging in research for its potential to remove Se from wastewaters and areas in the environment that have high Se concentration. Although this study did not show the bioaccessiblity and bioavailability of Se-rich algae near as great as traditional forms of Se used in supplementation, it offers insight for future research with algae.

Abbreviations Used

Advanced Dulbecco's Modified Eagle Medium (DMEM); Bioavailability (BA); bioaccessibility (BAC; adenoma carcinoma cell line (Caco-2); cellular glutathione peroxidase (GPx); fetal bovine serum (FBS); Gross-Wen Technologies (GWT); hydrogen selenide (H_2Se); inductively coupled plasma optical emission spectroscopy (ICP-OES); Revolving Algal Biofilm System (RAB); sodium selenate (Na_2SeO_4); sodium selenite (Na_2SeO_3); Natural Factors SelenoExcell Selenium Yeast (Yeast); phosphate buffered saline (PBS); selenium (Se); selenocysteine (SeCys); selenomethionine (SeMet).

Author Information

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Table 1. Description of Se-rich algal samples grown in either a selenite or selenate-rich medium

Selenite[a]					Selenate[a]				
selenite (ppm)	sulfite (ppm)	wk of harvest	Se in dry mass (%)	abbrev.	selenate (ppm)	sulfite (ppm)	wk of harvest	Se in dry mass (%)	abbrev.
30	0	1	0.51	A1	200	0	5	0.58	A6
30	0	3	1.00	A2	200	0	3	0.50	A7
30	0	2	0.56	A3	200	0	2	0.31	A8
200	200	3	0.16	A4	100	0	3	0.30	A9
50	0	3	0.25	A5	50	0	5	0.25	A10
					100	0	5	0.40	A11
					100	0	4	0.23	A12
					50	0	2	0.29	A13
					50	0	1	0.13	A14
					50	0	3	0.24	A15

[a] All samples displayed were grown in the Revolving Algal Biofilm system with bold basal medium

Figure 3.1

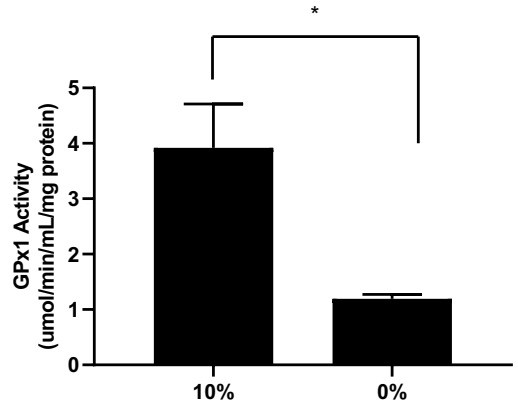

Figure 3.1 A Se deficient cell model. Caco-2 cells grown in DMEM with 10% FBS compared to Caco-2 cells grown in serum free DMEM using a gradual serum reduction method. Cells grown in serum free DMEM have approximately 70% reduction in GPx activity compared to cells grown in DMEM with 10% FBS. * Data represents mean ± SD of duplicates and considered significantly different at $P<0.05$ by Student t-test.

Figure 3.2

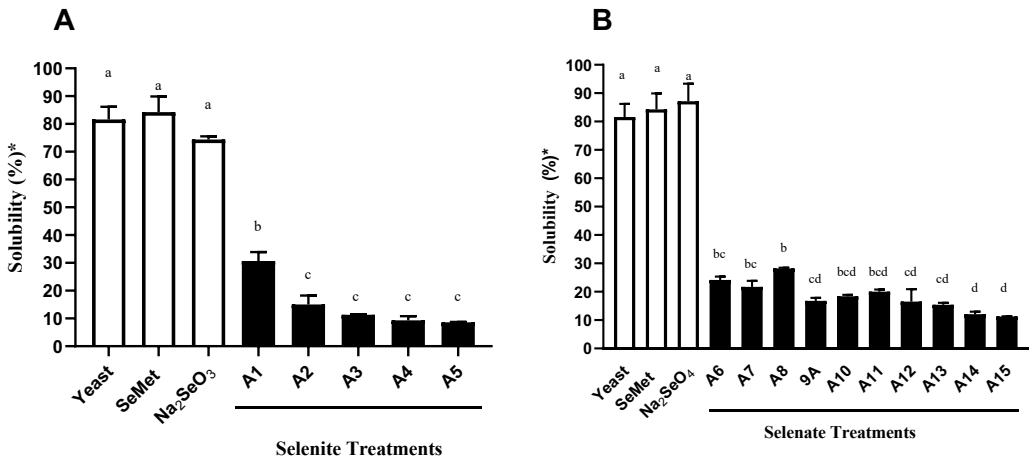

Figure 3.2 (A) Solubility (%) of algal samples grown in selenite medium compared to solubility of SeMet, Na_2SeO_3, and Yeast supplement. (B) Solubility of algal samples grown in selenate medium compared to solubility of SeMet, Na_2SeO_4, and Yeast supplement. Data represents mean ± SD of duplicates and those not sharing the same letter are considered significantly different.

Figure 3.3

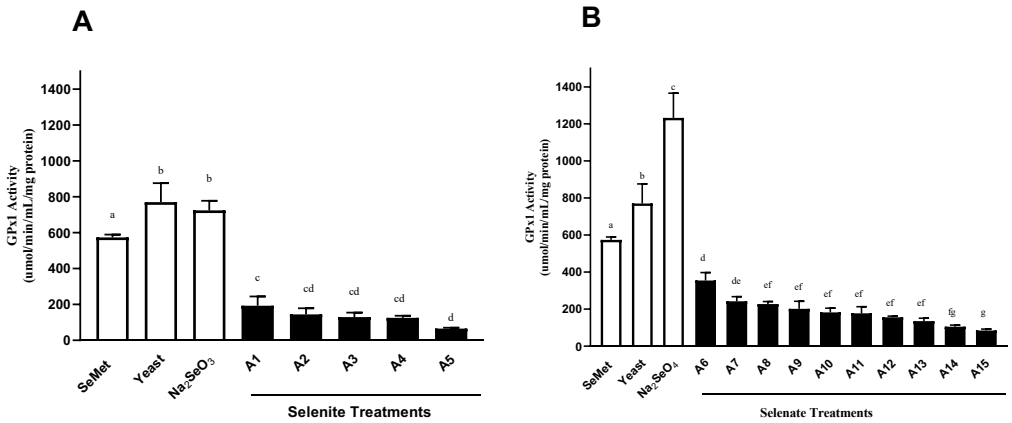

Figure 3.3 (A) GPx activity of algal samples grown in selenite medium compared to GPx activity of SeMet, Na_2SeO_3, and Yeast supplement. (B) GPx activity of algal samples grown in selenate medium compared to GPx activity of SeMet, Na_2SeO_4, and Yeast supplement. Data represents mean ± SD and those not sharing the same letter are considered significantly different ($P<0.05$) (ANOVA with Tukey multiple comparison); n=3 for SeMet, Na_2SeO_3, and A1; n=4 for all other samples. GPx activity shown as mU/mg cell protein or umol/min/mL/mg protein.

CHAPTER 4: GENERAL CONCLUSIONS

This study investigated the BAC and BA of Se from algae grown in media containing varying concentrations of Se as selenite or selenate. The main focus was to find the best growth conditions for algae to have an optimal Se BA, as there is currently no known data about Se BA from algae. The soluble concentration of Se following in vitro digestion allowed for determining the BAC of each sample and the induction of GPx1 activity allowed for evaluation of BA of each sample. The results of the study found that the Se-rich algae had lower BAC and BA when compared to organic and inorganic forms traditionally used in supplementation (SeMet, Yeast, Na_2SeO_3, and Na_2SeO_4). The SeMet, Yeast, Na_2SeO_3, and Na_2SeO_4 were comparable in BAC while all the Se-rich algae samples were significantly lower ($P<0.05$). It was found that Na_2SeO_4 had the highest BA compared to SeMet, Na_2SeO_3, and the Yeast, and the Se-rich algae was significantly lower than all of them ($P<0.05$).

We acknowledge that there were several limitations to the study. Based on the methodology used for in vitro digestion mimicking the duodenal environment, using a methodology that mimics the latter part of the small intestine may produce different results. This could include increasing the pH from 6 to 7 and changing the particle size of the algal samples. Further, a different cell line could be utilized to better represent the latter part of the small intestine. These findings show that the algal samples are not yet promising for having Se BA comparable to current forms of Se used in supplementation. However, certain algae species such as Chlorella sp. have much to offer still in terms of health benefits because of its rich protein, fatty acid, vitamin, and mineral content. Developing a Se-rich algae supplement could provide a natural product for supplementation that not only offers a variety of important nutrients, but also

benefits the environment by acting as a natural source to remove Se from areas of potential toxicity. Overall, this algae has much to offer because of its composition and ability to effectively remove Se from the environment. Future studies can be performed to hopefully improve the algal digestion and perhaps, BA.