Food Science: Copper Chelation and its relation to Wilson's Disease in *C. elegans*

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Abstract | The purpose of this study is to determine whether certain foods have chemical elements that can mimic the effects of common drugs that are used in the treatment of Wilson's Disease (WD) in which there is a buildup of copper in the body. *C. elegans* is used as a model organism for testing with its cua-1 gene that corresponds to ATP7A/B in humans. Varying copper levels and levels of copper-sequestering molecules can affect the development of *C. elegans* since some copper is needed for its development [1]. In this experiment, garlic will be used for its various sulfur-containing molecules that may mimic the copper-sequestering properties of some WD medications.

Introduction

C. elegans has been used as a model organism since the mid-1960's [2]. As a result of this, it has a ton of useful information on it at the tips of our fingers. Much research on this worm and its model gene cua-1 as a homolog to the WD gene ATP7A/B has been conducted. Because of this, this research too makes use of the cua-1 gene as a homologue for the study of Wilson's Disease.

What is Wilson's Disease?

Wilson's disease is a disorder in which the body holds on tightly to copper ions so that an excess buildup of copper in the body causes issues like liver cirrhosis or psychological illness [3]. This has to do with mutations to the ATP7B gene specifically; ATP7A is related to Menkes Disease which is another copper-related disease [4]. There are many different kinds of mutations in the ATP7B gene that can cause Wilson's disease [5,6], but all of these mutations produce the same effect in which the protein that is produced called copper-binding ATPase 2 or Wilson's Disease protein (WND) holds on tightly to copper ions which causes copper levels to increase [7]. Normally, the protein should transfer Cu+ ions from its six copper-binding domains to ceruloplasmin in the Golgi apparatus to be sent into the bile and later expelled from the body, but this tight binding hinders that handoff [8].

Though all of the mutations and mechanisms for WD are not fully understood, enough research has been done that the mechanism can be understood to the point that various medicines can be used to help those who suffer from this disease. The main type of treatment involves copper chelation therapy in which the ingested medicine is able to chelate copper more

strongly than WDN so that the copper can be removed and expelled [9]. Therefore, the question arose as to whether a diet with compounds that act similarly to the copper-binding compounds in these chelation therapy medicines could possibly help to release copper from the body.

Methods

Picking the Food:

The main mechanism of treatment for WD involves copper chelation therapy. What this entails is having another negatively charged compound steal the positively charged copper ions out of WDN. Research has shown that the affinity of copper-chelating drugs varies by four orders of magnitude and correlates positively with the number of sulfur atoms in the compounds in the drug and negatively with the number of atoms between the SH groups [10]. The researchers of this article then went on to predict whether α-lipoic acid is able to help treat WD based on its structure.

With this information, some research was done to find some high-sulfur foods [11]. It was found that many vegetables in the Brassica genus tend to have high sulfur contents due to their glucosinolates which is why cabbage was considered for use in this study [12]. This Choi 2014 study reported that the average total amounts of GSLs per gram of the outer and inner portions of dried red or green cabbages ranged from 8.55μM-13.5μM. This gives an average of 11μM per gram of dried cabbage. On top of that, Adelanwa 2015 found that green and red cabbages on average have a water content of about 90% [13]. Therefore, this should give a total of 1.22μM GSLs per gram of cabbage that is not dried.

Furthermore, members of the genus Allium also tend to have high levels of sulfurous compounds [14]. Because of this, garlic was chosen as the food that was actually used in this test as it has around 33 sulfur-containing compounds that are naturally found in it [15].

The Ingredients:

This test used a few different ingredients. The first was $CuCl_2$ or copper (I) chloride. This is a salt that ionizes when dissolved in water. That can be exploited to add extra copper to the plates with the worms.

The second ingredient is garlic, probably something like *Allium sativum* which is usually the common garlic found in grocery stores [16]. This will be the independent variable as the garlic concentrations will be varied to see whether the growth rate, brood size, and survival rate will change.

Two heads of garlic were put in a food processor and processed until a paste was formed. This was strained through a cheesecloth, squeezed, rinsed with some water, and squeezed again. The amount of water added during rinsing was accounted for. The resulting garlic juice was then reduced too the correct volume without the rinsing water through boiling. This would have gotten rid of a lot of volatile sulfur compounds, but many other sulfurous compounds should still remain even after heating [18].

Table 1: Eggs, L1, and L2 for BCS and Garlic Plates

Garlic %	Eggs (Garlic)	L1 (Garlic)	L2 (Garlic)	BCS (μM)	Eggs (BCS)	L1 (BCS)	L2 (B
100	21	6	0	300	57	12	
50	42	5	0	200	125	12	
25	67	12	3	150	150	15	
12.5	60	38	0	100	108	16	
6.25	48	13	0	50	66	20	
3.125	46	14	4	25	98	10	

The third ingredient is bathocuproinedisulfonic acid disodium salt (BCS). This salt contains bathocuproinedisulfonic acid which is a big molecule that is able to chelate copper. This will act as a control to compare the garlic against.

Worm Preparation:

Two different types of *C. elegans* will be used in this test. The first will be the N2 worms which are the wild type. They will act as a control group. The second type of worm has a mutation in the cua-1 gene. These worms are from the strain VC194 and have the specific mutation called gk107. The corresponding phenotype is slow-growing and otherwise superficially wild type [17]. All of the worms were grown on agar plates with OP50 *E. coli* as the food source.

Before plating, the cua-1 worms were pseudo-synchronized so that the eggs all hatched around the same time. This was done by placing one or a couple of adult hermaphroditic worms on a plate and incubating at 18C. They should lay eggs which should then all hatch at around the same time. This will result in a bunch of L1 worms on the plate that are similar in age and size. Chunks could be taken from this plate and transferred to other plates for testing.

Methods for Part 1:

Chunks were taken from the pseudo-synchronized plate, and twelve new plates were made. The number of eggs and the number of L1 worms were counted (Table 1). This was done so that the number of worms and eggs later on could be counted to see whether it had changed. Each of the plates was labeled, and BCS or garlic was added. The BCS was added in amounts of 300, 200, 150, 100, 50, and 25μM to the plates. This was done by created the correct molarity of BCS in water and pipetting 100μL of it onto the plate. The garlic was added to the plates in percentages. The pure garlic that was made previously was considered to be 100%. This was diluted with water to 50, 25, 12.5, 6.25, and 3.125% respectively. 100μL of each percentage was pipetted to the plates in the same manner as the BCS. The worms were observed every few days for a week. This whole process was repeated once more.

Methods for Part 2:

The second part of this included brood sizes and worm sizes. Plates were prepared in the same method as before with pseudo-synchronized L1 cua-1 worms. The difference was that instead of chunking, one or two L1 worms were picked for each plate so that there were no eggs on these new plates. The "mother" plate served as a control for worm size. A plate with 300µM BCS was made as another control along with a plate with 250µM $CuCl_2$. This was done for N2 worms as well. The plan was to check the worms every day for three days, but instead, they were all observed after 4 days. The process was then repeated with an even higher concentration of BCS at 0.71mM. A garlic plate was not made for this portion of the test as it did not seem necessary because all the previous garlic plates showed similar growth rates as BCS.

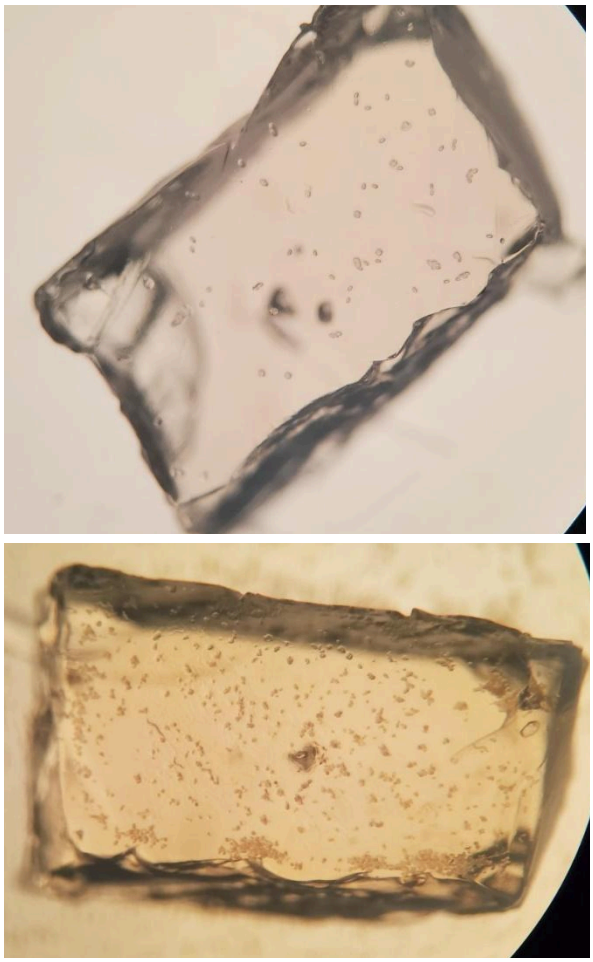
Results

Part 1:

The first part of this test dealt with garlic and BCS versus the normal cua-1 worms. The first trial did not follow the same pattern as Chun et. al. which a fair portion of this experiment was based after[1]. Instead of there being varying growth rates, arrest at L3, or death, all of the worms grew to adulthood and laid many eggs that also hatched. All of the worms were incubated at 18C which should have been the temperature that allowed for a single generation about every week. What was observed (qualitatively) was that more worms seemed to be on the BCS plates than the control cua-1 plates.

The results were not as clear for the garlic plates since the tiny garlic granules were about the same size as the eggs (Figure 1). Therefore, only the worms could be assessed for the garlic plates. Many of the worms were found outside of the garlic on the outer edges of the plates (Figure 2). There were some worms that stayed in the garlic. The numbers of worms seemed to be similar to the control cua-1 plates. None of the worms or eggs were quantitatively counted as there were too many to do so.

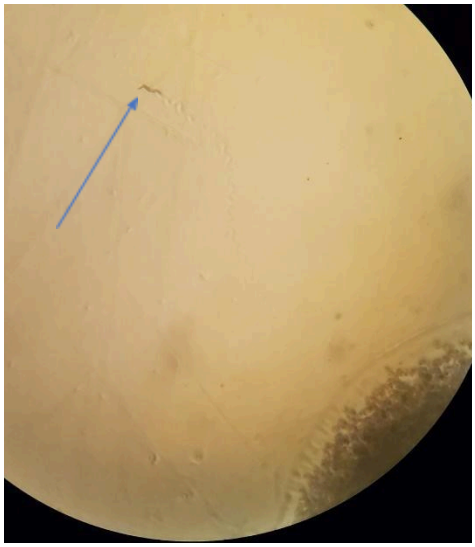
Figure 1: Eggs and Garlic Granules



The top picture shows a chunk with eggs on it. The bottom picture shows a chunk with eggs and garlic on it. Notice how the eggs are indecipherable from the garlic.

The second trial yielded similar results as the first.

Figure 2: Worms Running from Garlic



This figure shows a worm (indicated by the arrow) retreating from the garlic on the bottom-right corner.

Part 2:

The second part dealt with the relative worm sizes and brood sizes. It was found after 4 days that the worms were all about the same sizes. The eggs could not be quantitatively assessed as very many were laid. The N2 and cua-1 worms all seemed to have reached adulthood and grown quite large regardless of the BCS or $CuCl_2$ concentration. Again, the BCS plates seemed to have more worms than the other plates, but this was only qualitatively assessed. The second trial yielded the same results as the first.

Discussion

What does the data mean?

The data does not help at all for understanding the working of garlic for copper chelation. The reason for this is that BCS was to be used as the control to compare against, and the BCS did not really show any clear results comparable to Chun et. al.[1] in any manner. The worms all survived quite well at any concentration of garlic, BCS, or copper. There was no arrest at L3 either.

Why were the results different?

Chun et. al. used *C. elegans* as well and even focused in on the cua-1 gene. The difference between the worms in this study and the worms in that study is that the worms in Chun et. al. had a different type of mutation. That mutation is called ok904. It is a complex mutation with a 1610bp deletion and a 7bp insertion [19]. This mutation is supposed to be embryonic lethal and causes arrest at L3. This is a lot different than gk107 which was already mentioned to cause slower growth along with the worms being superficially wild type [17]. Worm Base does not even have anything to say on the phenotype of gk107 as of yet [20]. All that Worm Base says is that the mutation is a simple 1639bp deletion. Having two different types of mutations supposedly can cause different phenotypes even if the mutations deal with the same gene (and in this case, the same proteins as well).

Are there any results worth exploring further?

Though none of the results showed any large differences, something here may still be worth exploring. The qualitative assessment of a larger number of worms on

the BCS plates might be something. It is possible that the BCS was chelating the copper out of the cua-1 proteins and allowing for faster generation. *C. elegans* generally have the ability of holding 10-15 eggs at a time [21]. As mentioned previously, the temperature of 18C is supposed to allow for a single generation of worms about once a week. This is not what was seen in the second part of this test. Two adult worms were placed on a BCS plate, and when checked after 4 days, there was an uncountable number of worms on the plate. This may suggest that the copper chelation really did help them grow faster—even faster than N2 worms somehow.

The garlic did not seem to follow the same pattern. Not only did it seem like most of the worms did not like being in the garlic, but the results were not significantly different than the normal cua-1 worms. The only difference between any of the parts of any of the tests was the number of worms present of the BCS plates. This would seem to suggest that whether it dealt with copper chelation or not, the garlic does not perform in the same manner as BCS in that particular respect. That being said, the BCS and the garlic both do not perform the same function on these gk107 worms as the BCS does on ok904 worms.

1. Chun, H., Sharma, A. K., Lee, J., Chan, J., Jia, S., & Kim, B. (2016). The intestinal copper exporter CUA-1 is required for systemic copper homeostasis in Caenorhabditis elegans. *Journal of Biological Chemistry, 292*(1), 1-14. www.ncbi.nlm.nih.gov/pmc/articles/PMC5217669/
2. Nigon, V. M. & Felix, M. A. (2005). History of research on C. elegans and other free-living nematodes as model organisms. *WormBook*. www.ncbi.nlm.nih.gov/books/NBK453431/
3. Zero To Finals. (2019, March 14). *Understanding Wilson's Disease* [Video]. YouTube. www.youtube.com/watch?v=O7Vr-r9ef6g
4. ATPase, Cu(2+)-Transporting, Beta Polypeptide; ATP7B. (2021, February 4). *Online Mendelian Inheritance in Man*. Accessed February 24, 2022. omim.org/entry/606882
5. Gupta, A., Bhattacharjee, A., Dmitriev, O. Y., Nokhrin, S., Braiterman, L., Hubbard, A. L., Lutsenko, S., & MacLennan, D. H. (2011). Cellular copper levels determine the phenotype of the Arg^{875} variant of ATP7B/Wilson disease protein. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(13), 5390–5395. www.jstor.org/stable/41125709
6. Roy, S., McCann, C., Ralle, M., Ray, K., Ray J., Lutsenko, S., & Jayakanthan, S. (2020). Analysis of Wilson disease mutations revealed that interactions between different ATP7B mutants modify their properties. *Scientific Reports*, 10. doi.org/10.1038/s41598-020-70366-7
7. Terada, K., Schilsky, M., Miura, N., & Sugiyama, T. (1998). ATP7B (WND) protein. *The International Journal of Biochemistry & Cell Biology, 30*(10), doi.org/10.1016/S1357-2725(98)00073-9
8. Fatemi, N. & Sakar, B. (2002). Molecular mechanism of copper transport in Wilson's Disease. *Environmental Health Perspectives, 110*, 695-698. www.jstor.org/stable/3455074
9. Mohr, I. & Weiss, K. H. (2019). Current anti-copper therapies in management of Wilson Disease. *Annals of Translational Medicine, 7*(2). www.ncbi.nlm.nih.gov/pmc/articles/PMC6531644/
10. Smirnova, J., Kabin, E., Jarving, I., Bragina, O., Tougu, V., Plitz, T., & Palumaa, P. (2018). Copper(I)-binding properties of de-coppering drugs for treatment of Wilson disease: α-Lipoic acid as a potential anti-copper agent. *Scientific Reports*, 8. doi.org/10.1038/s41598-018-19873-2
11. Florin, T., Neale, G., Goretski, S., & Cummings, J. (1993). The sulfate contents of foods and beverages. *Journal of Food Composition and Analysis, 6*(2), 140-151. doi.org/10.1006/jfca.1993.1016
12. Choi, S., Park, S., Lim, Y. P., Kim, S., Park, J., & An, G. (2014). Metabolite profiles of

glucosinolates in cabbage varieties (Brassica oleracea var. capitata) by season, color, and tissue position. *Horticulture, Environment, and Biotechnology,* 55, 237-247. doi.org/10.1007/s13580-014-0009-6

13. Adelanwa, E. B. & Medugu, J. M. (2015). Variation in the nutrient composition of red and green cabbage (Brassica oleracea) with respect to age at harvest. *Journal of Applied Agricultural Research*, 7, 183-189. www.researchgate.net/publication/320058505_VARIATION_IN_THE_NUTRIENT_COMPOSITION_OF_RED_AND_GREEN_CABBAGE_Brassica_oleracea_WITH_RESPECT_TO_AGE_AT_HARVEST
14. Boyhan, G. (2008). Sulfur: Its role in onion production and related alliums. *American Society of Agronomy, Crop Science Society of America, & Soil Science Society of America, 50*. acsess.onlinelibrary.wiley.com/doi/pdf/10.2134/agronmonogr50.c12#:~:text=Sulfur%20plays%20an%20important%20role,a%20specific%20species%20of%20Allium.
15. Omar, S. H. & Al-Wabel, N. A. (2010). Organosulfur compounds and possible mechanism of garlic in cancer. *Saudi Pharmaceutical Journal, 18*(1), 51-58. www.ncbi.nlm.nih.gov/pmc/articles/PMC3731019/#:~:text=Garlic%20contains%20approximately%2033%20sulfur,)%2C%20and%20minerals%20(selenium%2C
16. Alleman, G. (2021, February, 19). *How to plant and store garlic*. HowStuffWorks. recipes.howstuffworks.com/food-facts/how-to-plant-and-store-garlic1.htm#:~:text=Garlic%20Varieties&text=Allium%20sativum%20is%20the%20most,often%20called%20%22culinary%22%20garlic.
17. *VC194*. Caenorhabditis Genetics Center. University of Minnesota. cgc.umn.edu/strain/VC194
18. Kazuki, A., Hori, Y., & Myoda, T. (2019). Volatile compounds of fresh and processed garlic (Review). *Experimental and Therapeutic Medicine*, 19, 1585-1593. doi.org/10.3892/etm.2019.8394
19. Bernstead, R. ok904. *WormBase*. Oklahoma Research Foundation. wormbase.org/species/c_elegans/variation/WBVar00092177#027-456-3
20. Moerman, D. gk107. *WormBase.* C. elegans Reverse Genetics Core Facility. Accessed May 1, 2022. wormbase.org/species/c_elegans/variation/WBVar00145514#027-456-3
21. Shafer, W. R. (2005). Egg-laying. *WormBook.* NCBI. www.ncbi.nlm.nih.gov/books/NBK19787/#:~:text=elegans%20hermaphrodites%20are%20self%2Dfertile%2C%20producing%20first%20sperm%2C%20which,uterus%20at%20any%20given%20time.