



## DISCOVERING ZEAXANTHIN IN COMPLEX WITH GSTP1

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### Abstract

Carotenoids are an evolutionary adaptation commonly found in the pigments of brightly colored fruits and vegetables.<sup>12</sup> Due to its photo-protective function in light harvesting complexes within plants, the carotenoid possesses antioxidant properties mammals acquire through dietary means.<sup>3</sup> There is immense interest in understanding the benefits of the antioxidant properties to alleviate chronic diseases, such as Age-related Macular Degeneration (AMD).<sup>3</sup> Zeaxanthin and lutein are localized in the macula of the human eye by binding to their respective carotenoid binding proteins, GSTP1 and STARD3.<sup>1</sup> The protein captures and anchors the carotenoid, thus concentrating the pigments in areas where photo-toxicity risk is high.<sup>2</sup> Although the structure of carotenoid-binding GSTP1 protein is known, the structure of the protein in complex with its respective substrate, zeaxanthin, remains undetermined.<sup>9</sup> Discovering zeaxanthin bound to GSTP1 would potentially reveal a structure that can contribute to a structure-based drug design for AMD and provide valuable comprehension of the bioavailability of dietary-obtained carotenoids.

### Introduction

The earliest function of carotenoids are traced back to membrane-supporting lipids in archaeobacteria.<sup>12</sup> Although some fungi and animals retain this function, the hydrophobic carotenoids evolved from supporting membranes to protecting the reaction centers of plants from light induced oxidative damage.<sup>12</sup> Derivative forms of carotenoids also function as color patterning and signaling hormones within both plants and animals.<sup>12</sup> In contrast to plants and archaea mentioned above, mammals are unable to synthesize carotenoids and therefore rely heavily on the consumption of plants in order to acquire its antioxidant properties.<sup>3</sup> Accumulation of the pigment within mammals has been observed in the macula of the human eye and in the skin of the Honduras bat.<sup>4</sup> This suggests bioavailability of carotenoids in mammals, in which a portion of the ingested pigments are available for utilization in the body.<sup>3</sup> Lutein, zeaxanthin and meso-zeaxanthin are the carotenoid pigments concentrated in the macula of the human eye bound to proteins GSTP1 and STARD3.<sup>2,8</sup> The concentrated carotenoid protects the eye against damage by quenching free radicals induced by light.<sup>1</sup> Degradation of the light protecting pigments in the eye causes irreversible vision damage, known as Age-related Macular degeneration (AMD).<sup>11</sup> AMD is currently the world's leading cause of irreversible blindness among elderly individuals in industrialized nations and has no cure.<sup>10</sup> There is strong association between increasing amounts of lutein and zeaxanthin dietarily with decreased progression of AMD.<sup>3</sup> Because of this close association between the xanthophylls and AMD, I am interested in the structures that form when the binding proteins create complexes with lutein and zeaxanthin.

Prior research has discovered the un-complexed structure of GSTP1 and since has been measured for higher-resolution data (MPH, unpublished data).<sup>9</sup> Our collaborators have also observed the binding event of zeaxanthin to GSTP1 in the primate eye.<sup>2</sup> However what remains unknown is the molecular interactions of the zeaxanthin•GSTP1 complex. Structure determination is met by three main challenges: zeaxanthin•GSTP1 complex formation, crystallization, and crystal analysis.<sup>7</sup> The first challenge of forming the zeaxanthin•GSTP1 complex will be addressed in this report. Due to the highly hydrophobic nature of zeaxanthin, it is difficult for the carotenoid to be readily available to GSTP1 in solution. Synthetic detergents form a micelle to solubilize the carotenoid, thus providing an opportunity for zeaxanthin to bind with GSTP1 in solution.<sup>2</sup> Zeaxanthin exists in only two states: unbound in a detergent micelle or bound to its protein. Size Exclusion Chromatography (SEC) is used to distinguish whether zeaxanthin actively binds to the detergent micelle or GSTP1. Once it is confirmed that zeaxanthin is in detergent micelles and not aggregating in solution, the synthetic detergent must be removed for complex formation. Not only is it necessary to remove the detergent for complex formation, but also for crystallization which is the next step in structure determination. The flexible synthetic detergents compete with the rigid, lattice-packing contacts of the crystal structure. I discovered two models in this report, column interactions and the fog model, which explains the molecular behavior occurring with zeaxanthin, GSTP1, and SEC chromatography.

## Results

The zeaxanthin•GSTP1 complex serves as building blocks to form crystals, which is the reagent needed to achieve the long range goal of structure determination. This complex turns out to be difficult to prepare because of the need for synthetic detergent to solubilize hydrophobic zeaxanthin. The short term goal is to use Size Exclusion Chromatography to distinguish zeaxanthin in a detergent micelle, which ensures the carotenoid is not aggregating in solution and is prepared for complex formation.

SEC separates molecules traveling in a neutral solvent, called the mobile phase, through a porous bead column, called the solid phase, by size.<sup>5</sup> Molecules exiting the column from largest to smallest are detected through the absorbance of light tuned to a specific wavelength.<sup>5</sup> To detect GSTP1, the light was tuned to 280 nm by programming the absorbance detector to look at this channel.<sup>2</sup> GSTP1 along with other molecules including buffer components, zeaxanthin, and detergent are detected at 280 nm. To distinguish zeaxanthin from other molecules, the carotenoid is detected at 452 nm.<sup>2</sup> Zeaxanthin reflects wavelengths of 620 nm, giving an orange visual appearance and as a result the carotenoid is able to absorb blue light at 452 nm, protecting the macula from light induced oxidative damage.<sup>2</sup> Zeaxanthin in a detergent micelle can be identified if zeaxanthin is detected at a different retention time than GSTP1. Similarly, complex formation can be identified post detergent removal when the overlaid graphs from the two channels show a single retention time.

Previous experiments using DDM detergent showed GSTP1 and zeaxanthin in detergent micelle are traveling through the column at the same time, suggesting both are similar in size. The sizes are ~46 kDa and 50 kDa for DDM and GSTP1, respectively, making it impossible to know where zeaxanthin is binding (Figure 1). I screened three alternate detergents, CHAPS, GDN, and sodium cholate, looking for a detergent micelle size with a different retention time than GSTP1. The optimal detergent to use for complex formation is one that can be identified separate from GSTP1. This will allow confirmation that zeaxanthin is not aggregating in solution and is prepared to form a complex with GSTP1.

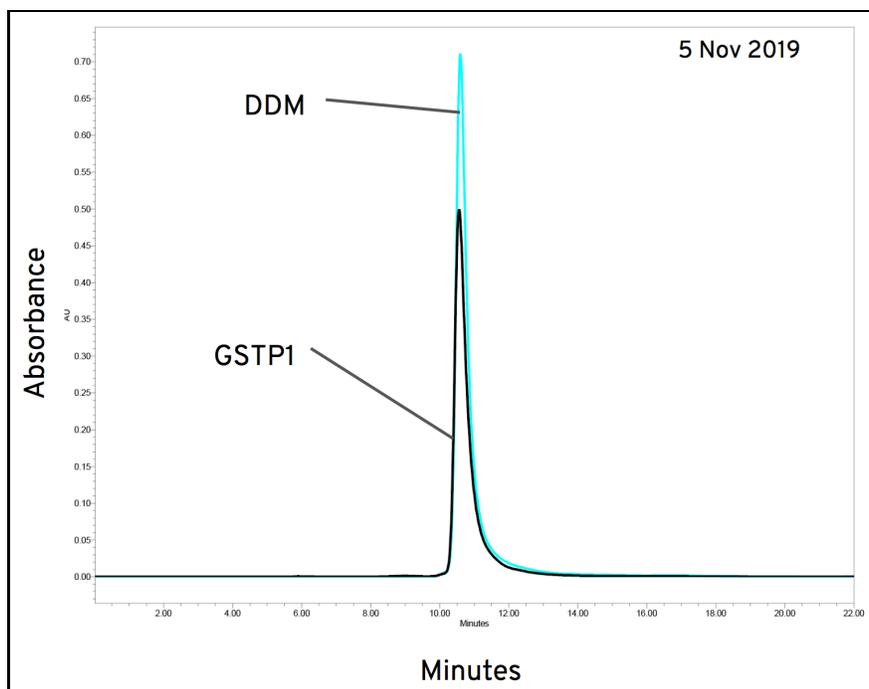


Figure 1. Previous SEC results of zeaxanthin in DDM detergent (blue) and GSTP1 (black) from 2019. The DDM micelle and GSTP1 protein have the same retention time of ~10 to 11 minutes.

As mentioned earlier, the mobile phase is a solvent that molecules travel in through an SEC column. To rule out GSTP1-detergent interactions, I reanalyzed the protein by SEC using a mobile phase containing detergent. By overlapping GSTP1 detected using detergent mobile phase and no detergent mobile phase, a comparison graph was created. The GSTP1 traveling through a detergent mobile phase produced a negative reading in absorbance around 11 minutes indicating that the protein was interacting with the detergent (Figure 2). It is concluded from this comparison that a mobile phase with no detergent is needed for SEC analysis moving forward to eliminate unwanted interactions between the protein and detergent. Further analysis of GSTP1 independently determines that the protein appears in four consistently sized peaks (Figure 3A). As mentioned earlier, GSTP1 is detected at 280 nm where other molecules, like buffer components, also are able to be detected. DTT was used in the buffer solution when purifying GSTP1 and therefore was speculated to be one of the smaller peaks. DTT control and the fourth peak in the protein graph had the same retention time of approximately 13.5 minutes, confirming that the unknown fourth peak is DTT (Figure 3B). Knowing where GSTP1 appears in SEC analysis results, we can now look for synthetic detergent micelles that appear at different retention times in the chromatograms. A synthetic detergent micelle that is distinguishable from GSTP1 will help confirm that zeaxanthin is solubilized and ready for complex formation.

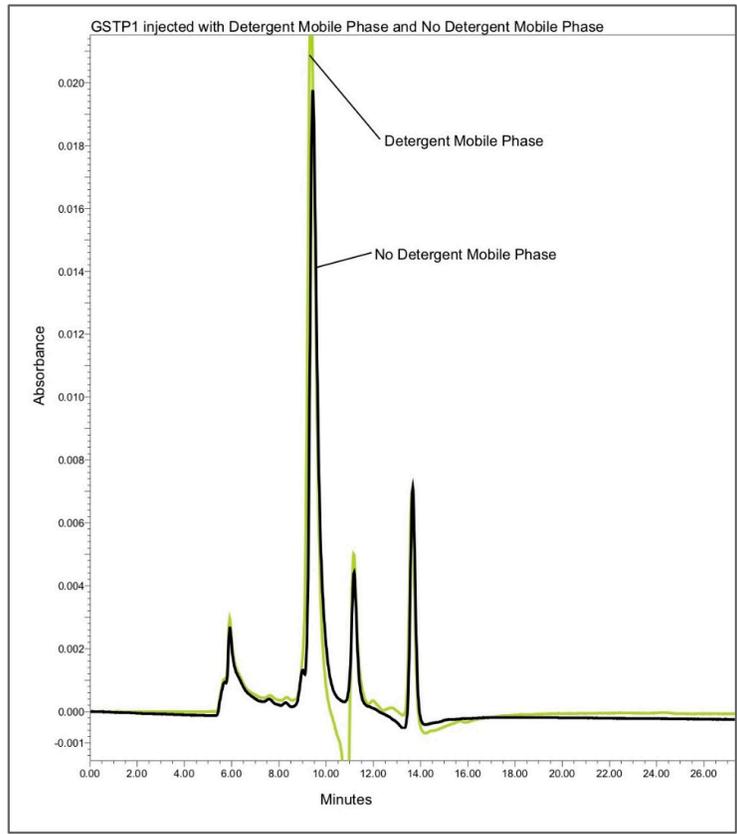


Figure 2. Overlay SEC comparison of GSTP1 using no detergent mobile phase vs. sodium cholate detergent containing mobile phase.

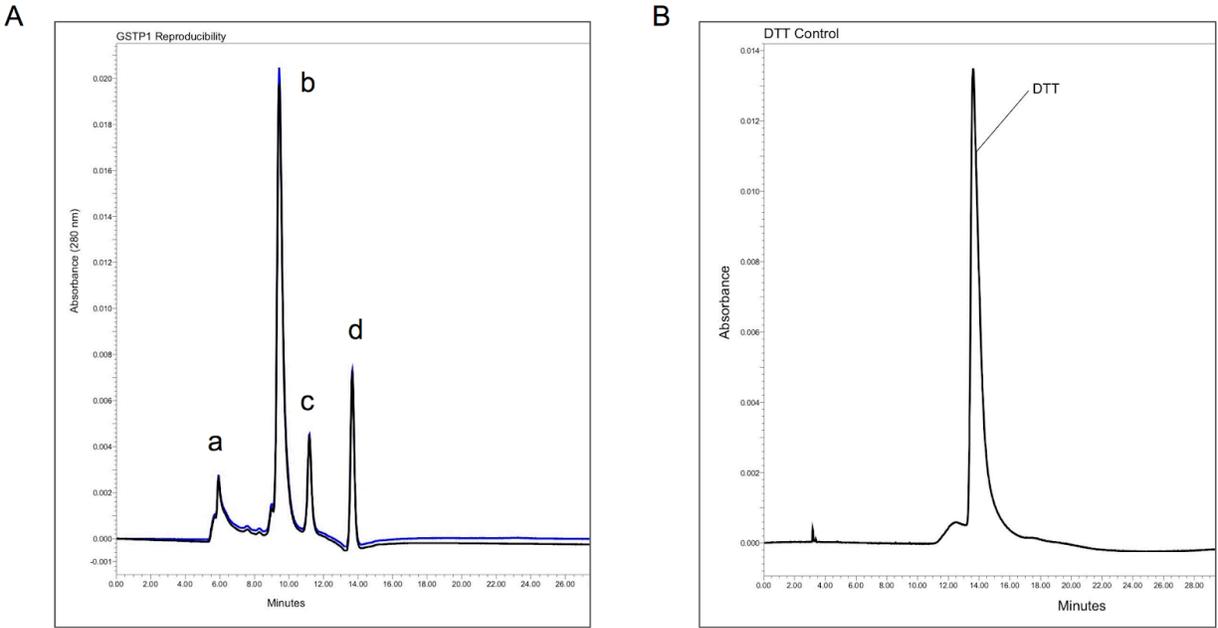


Figure 3. (A) Overlay of two separate GSTP1 SEC results measured with no detergent mobile phase. a. is the large aggregate molecule at ~6 minutes, b. is the main GSTP1 protein in its dimeric state at ~10 minutes, c. is the protein's monomeric form at ~11 minutes, and d. is the DTT at ~14 minutes. (B) DTT control results with no detergent mobile phase. The retention time of isolated DTT is ~14 minutes.

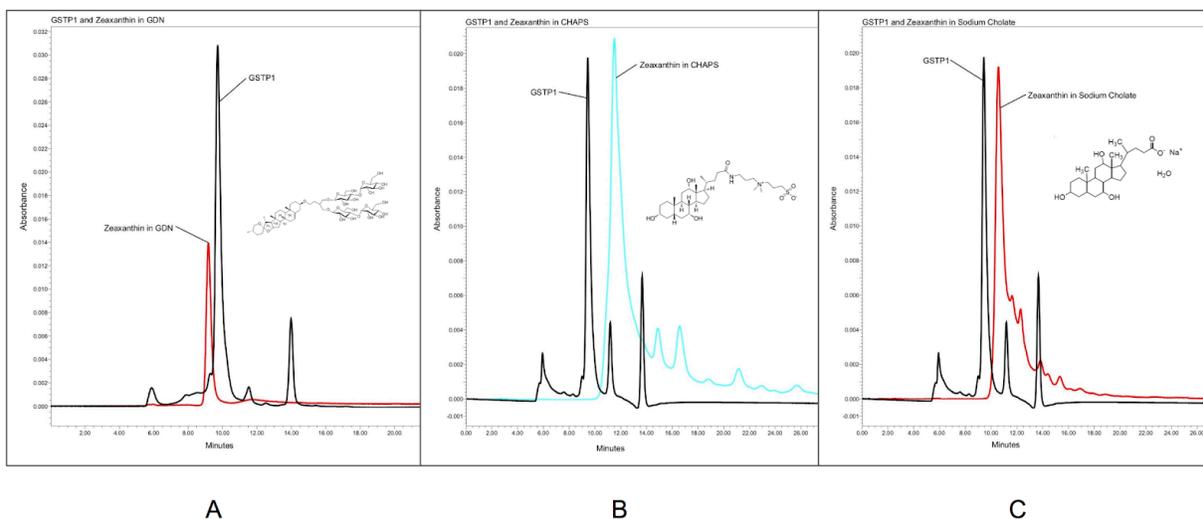


Figure 4. SEC graph results of the detergent assay. All the tests were done using no detergent mobile phase and are compared to GSTP1 (black). Corresponding detergent structures are shown in the blank space of the graph. (A) GDN. Has the most simple shape so this is preferred. (B) CHAPS. (C) Sodium cholate.

Detergent comparison of CHAPS, GDN, and sodium cholate was conducted in order to find the optimal detergent that will host zeaxanthin in a micelle of a different size than GSTP1. The ability to identify zeaxanthin in a detergent micelle will provide confirmation that the carotenoid is ready for complex formation. The results of the detergent comparison show that all three detergents have different retention times than the protein, indicating that all of the tested detergents have micelle sizes different than the size of GSTP1 (see Figure 4 and Table 1). CHAPS and sodium cholate displayed a trailing, saw-tooth pattern in the chromatograms suggesting heterogeneity in micelle size -- a major concern for any crystallography project. The different retention time observed for CHAPS and sodium cholate is evidence that zeaxanthin is in a detergent micelle. Like CHAPS and sodium cholate, GDN solubilized with zeaxanthin with a retention time that differs from the retention time of GSTP1. Interestingly, the retention time for zeaxanthin is earlier than GSTP1, whereas retention time for zeaxanthin in other detergents is later. The peak height and area for zeaxanthin solubilized in GDN was reduced in comparison to the heights and areas observed in CHAPS and sodium cholate. Furthermore, the peak shape for GDN was significantly more symmetrical and lacked trailing peaks featured in the other detergents. This points to high homogeneity in micelle sizes GDN is able to form around zeaxanthin. These results give hope in using GDN as the optimal detergent to solubilize zeaxanthin, preparing the carotenoid to bind with GSTP1.

## Discussion

Understanding the molecular interactions occurring in the zeaxanthin•GSTP1 complex will accelerate a structure-based drug design for AMD as well as provide mechanistic insights for the bioavailability of carotenoids in the human body. The first component in structure determination, the zeaxanthin•GSTP1 complex, remains elusive despite my persistent efforts. The SEC protein analysis and detergent assay provided comprehension on protein behavior, simplifying SEC measurement conditions, and the optimal detergent to use moving forward. Here I present the interpretations and new ideas emerging from these new results.

GSTP1 consistently produced one strong peak along with three small surrounding peaks on the SEC chromatograms (see Figure 3A). Because GSTP1's absorbance was measured at 280 nm, I speculate the three smaller peaks are different sized molecules that are not the protein. The first peak (retention time = 6 minutes) can be attributed to large aggregates of protein, because larger molecules avoid getting caught in the pores of the SEC column and elute the fastest. The strongest peak (retention time = 9.5 minutes) measured by the SEC is the bulk of GSTP1 because the high absorbance indicates high quantity (see Figure 3A). This main peak has the same retention time of ~10 minutes as a previously measured sample of GSTP1 (see Figure 1). Discrepancies in the time the protein was eluted is attributed to the pore size of the column. The SRT SEC-500 column with a pore size of 500-Å used in the previous year elutes later (10.5 minutes) compared to the retention time using the new SRT-C SEC-300 column with a pore size of 300-Å in this report (10.5 minutes). I suspect that GSTP1 persists in two different quaternary structures. GSTP1 exists primarily in its dimeric form (46 kDa) which elutes earlier (retention time = 9.5 minutes) than its monomeric form (23.3 kDa, retention time = 11.5 minutes). The last peak (retention time = 13.5) in the GSTP1 chromatogram has a retention time that is the same as a control sample only containing DDT (see Figure 3). DTT was part of the buffer solution used to keep thiol groups reduced during purification of GSTP1. Analysis of the different molecules detected in the GSTP1 chromatogram suggests the GSTP1 monomer and dimers do not resolve as separate peaks on the column with 500-Å pores. Identifying where and how GSTP1 appears on chromatograms provides a retention time (9.5 minutes) to compare with the retention time, or size, of zeaxanthin in a detergent micelle. Differentiating the protein from the micelle will allow me to confirm zeaxanthin is solubilized and ready to bind with GSTP1.

Since detergents play a large role in solubilizing the zeaxanthin ligand, I analyzed samples of GSTP1 in different mobile phases to see how detergent impacts the protein. The mobile phase serves as a neutral solution that allows the molecules to travel through the porous SEC column and be separated by size. The negative peak registered in the GSTP1 graph can be explained by a "fog model" in which the mobile phase containing detergent creates a baseline that is dense with detergent molecules (see Figure 4). The negative peak, seen in the GSTP1 chromatogram using detergent mobile phase, is created by the bulky protein knocking away detergent molecules in its vicinity, and letting more light through (see Figure 5). Based on this finding, a mobile phase containing no detergents is used to reduce unwanted interaction when analyzing GSTP1 using SEC.

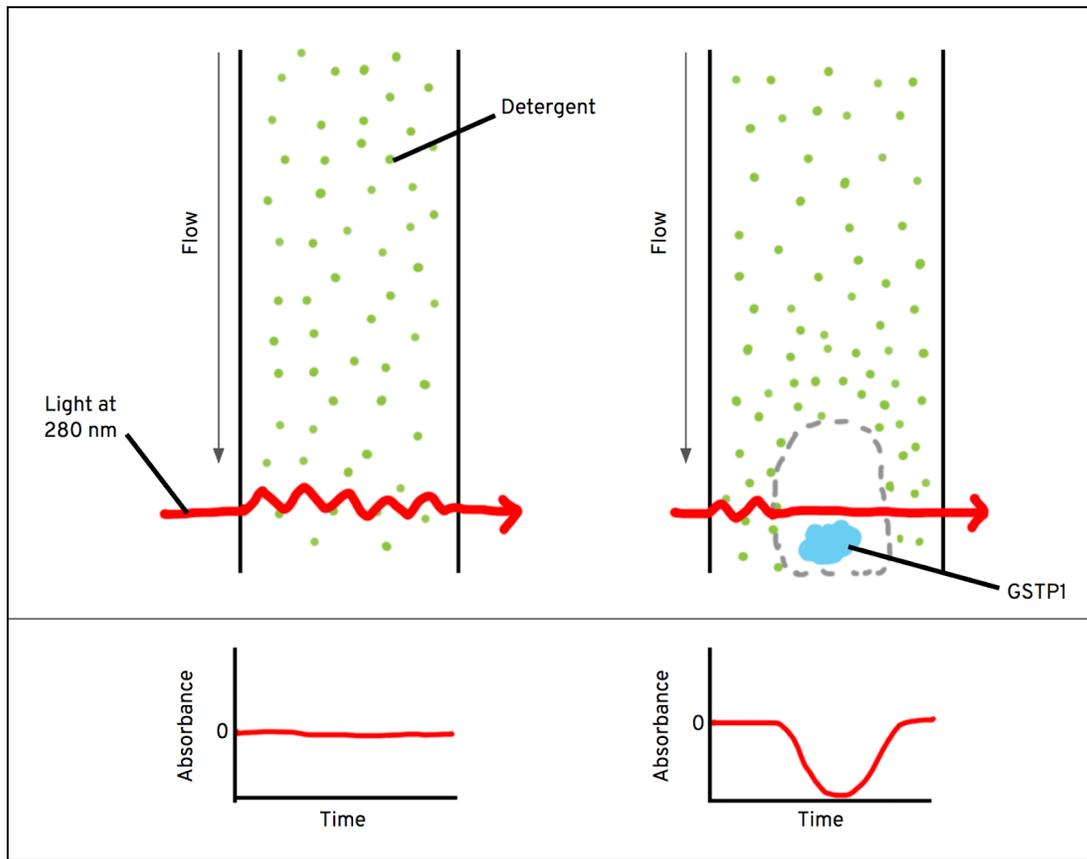
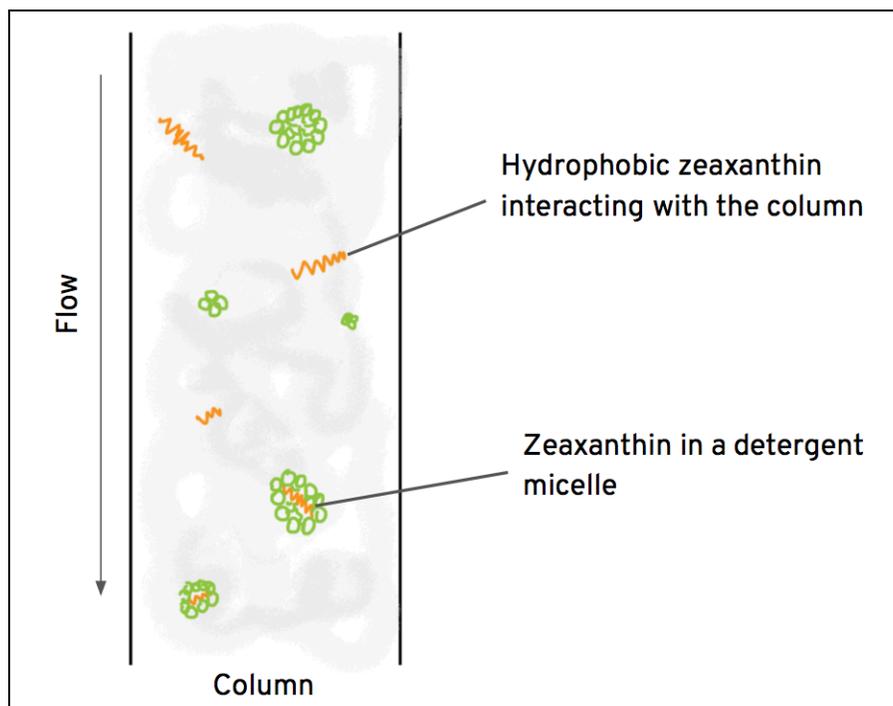


Figure 5. Fog model explaining drop in absorbance when analyzing GSTP1 with mobile phase containing detergent. The left column is the detergent molecules in the mobile phase being detected by light, creating a baseline (as represented by the graph directly below) to compare with molecules that will travel in the mobile phase. The right column models GSTP1 pushing away surrounding detergent molecules and clearing up a space as represented by the grey dashed border. There are no molecules to absorb around GSTP1, creating a sudden drop in absorbance that is read as a negative dip in the graph shown right below.

Different detergents were compared against the analyzed GSTP1 described above in order to find the optimal detergent. The optimal detergent has a micelle size that is differentiable from the size of GSTP1 (46 kDa) and solubilizes zeaxanthin. The distinction in size is crucial in determining whether zeaxanthin is bound to the detergent micelle or its protein using two channel overlay chromatograms produced by SEC. Solubilized zeaxanthin is necessary for readily available binding to GSTP1 in solution. The binding event forms the zeaxanthin•GSTP1 complex that can be crystallized. The crystallization of the complex is the reagent needed for the overarching goal of discovering the structure of zeaxanthin bound to GSTP1. Previous SEC tests using DDM detergent show that its micelle is the same size as GSTP1, ~50 kDa and ~46 kDa respectively, because both molecules elute at the same time (retention time = 11 minutes) (see Figure 1 and Table 1). The detergent assay concludes that GDN, CHAPS, and sodium cholate have micelle sizes that are different from the protein (see Figure 4). Of the three detergents tested, GDN is the optimal detergent (see Figure 4A). GDN appears to have high homogeneity in micelle size, suggesting the detergent forms stable micelles. The saw-tooth trail observed in CHAPS and sodium cholate represent “column interaction” where zeaxanthin that is partially protruding or has completely exited the micelle, directly interacts with the SEC column. Column interaction is not favorable as it causes uncertainties with chromatogram results, blurring the

differentiability of the detergent micelle from GSTP1. The reduced peak height in GDN compared to CHAPS and sodium cholate detergent may be the result of the low critical micelle concentration (18  $\mu$ M) GDN possesses, the concentration of detergent needed to form a micelle in solution. Due to the low critical micelle concentration, zeaxanthin requires a small volume of GDN detergent to become solubilized in solution. A small volume of GDN in solution with zeaxanthin can be effectively removed and is beneficial for crystallization, the next step toward structure determination.



*Figure 6. Model of column interaction. The zeaxanthin is able to protrude out of, or in some cases completely exit the micelle. The hydrophobic properties of zeaxanthin are no longer enclosed in the micelle, allowing the carotenoid to stick against the column wall. This is a proposed mechanism to explain the saw-tooth, trailing chromatography graphs produced by CHAPS and sodium cholate detergent.*

The results of this report address only a small piece in a big puzzle to discover the structure of the zeaxanthin•GSTP1 complex. Structure determination consists of three major steps: complex formation, crystallization, and crystal analysis. Although I did not form the complex, I have uncovered first level molecular interactions of GSTP1 and zeaxanthin with synthetic detergents. Understanding of these molecular interactions revealed from the data I have collected thus far brings me closer to completing the first step of structure determination. What the structure of zeaxanthin in complex with GSTP1 will specifically unveil is unknown, however in many other systems a structural picture opens the door to transitional applications such as drug design. Pharmaceuticals such as HIV protease inhibitors used to treat HIV, have been discovered using structure-based drug design and resulted in the development of an effective treatment for a chronic disease.<sup>5</sup> Similarly, acquiring the structure and molecular interactions of zeaxanthin in complex with GSTP1 can contribute a solution to the irreversible blindness caused by Age-related Macular Degeneration among adult populations.

## Methods

*Over Expression and Purification of GSTP1:* Select for BL21(DE3) E.coli bacteria cells containing a plasmid that encodes for human GSTP1 protein using kanamycin antibiotic. Make a 375 mL media flask by adding 1.9 - 2 mL of 1M glucose, 11.25 mL of KPO<sub>4</sub>, and 1.5 mL of 10 µg/mL Kanamycin to autoclaved 375 mL DI water and 11.25 g 2xyT media. Repeat ratios for a 60 mL media flask. Use a pipette tip to transfer BL21 cells into 2mL culture tubes. Create a control tube. Place culture tubes in a 37 °C shaker overnight. Take a blank cuvette of the media from the 60 mL media flask for spectrophotometry. Start passage one with 300 µL from the 2 mL culture tubes added to the 60 mL media flask. Place in a 37°C shaker for 2.5 to 4 hours. For passage two transfer 10 - 30 mL of culture from the 60 mL flask to the 375 mL media flask. Place the 375 mL media flask into the shaker for 1.5 - 2 hours. Take OD readings every 30 mins - 1 hour using spectrophotometry starting at hour one. Once the OD reading reaches 0.9 - 1.1 at 650 nm, induce the culture with 375 µL of 0.5M IPTG. Return the flask to the 37°C shaker and harvest the cells in 6 to 8 hours after adding IPTG by spinning down chilled 50 mL conical tubes at 5K RPM for 10 minutes in a chilled F13S rotor. To purify the protein, resuspend the pellet in 5 mL of lysis buffer and DTT using a spatula to gently break up the pellet. Sonicate the protein with six 10 second pulses with a 45 second rest. Centrifuge the sonicated sample in a SS34 rotor at 18K RPM for 15 minutes. Filter the sample through a 0.45 µm filter and a 5 mL syringe into a 15 mL conical tube containing a glutathione bead slurry that has been washed with lysis buffer and DTT. Nutate in the cold room for 1.5 to 2 hours. Concentrate the protein by filling a filter column conical tube with 15 mL no detergent mobile phase. Centrifuge to balance and align the filter. Remove the no detergent mobile phase. Add 15 mL of GSTP1 purified protein and pipette up and down on either side of the filter. Centrifuge at 6K RPM for 10 minutes. Remove and place the filtrate in a 50 mL conical tube. Repeat until all purified protein is used. Buffer exchanges the protein by filling and centrifuging a filter column conical tube containing the protein with 15 mL buffer (30 mL no detergent mobile phase and 60 µL DTT) buffer at 6K for 10 minutes. Repeat this two times.

*Mobile Phase:* One liter of no detergent mobile phase used for Size Exclusion Chromatography was created with a 0.2M Tris pH ~7 and 0.15M NaCl (s) ratio in a 1L glass bottle filled to 1L with 18 Mohm water. The detergent mobile phase was made using the same ratios but a final volume of 250 mL. The amount of detergent needed for the mobile phase was calculated based on a working CMC 2-5 times higher than the detergent's natural CMC (Table 1, 2). All mobile phases were sterile filtered 0.45 micron filter paper.

Detergent	Aggregate Number	CMC (mM)	Molecular Weight (g/mol)	Micelle Size (kDa)
DDM	78 - 149	0.17	510.6	50.04
CHAPS	10	4.3 - 10	614.9	6.15
Sodium Cholate	2 - 4.8	14	430.6	1.3
GDN	2	0.018	1229.3	2.5

Table 1. Characterization of DDM, CHAPS, Sodium Cholate, and GDN detergent.

Detergent	Micelle MW (g/mol) <sup>£</sup>	Weight by Volume <sup>¢</sup>	Working Concentration <sup>∞</sup>	Amount needed for 250 mL Mobile Phase <sup>§</sup>
DDM	50038.8	0.0086%	<sup>a</sup> 0.043 g $\approx$ 0.05 g per 100 mL	0.125 g
CHAPS	6149	0.37%	0.73788 g $\approx$ 1 g per 100 mL	2.5 g
Sodium Cholate	1291.8	0.60284%	1.205 g $\approx$ 1.5 g per 100 mL	3.75 g
GDN	2458.6	0.002%	0.0044 g $\approx$ 0.005 g per 100 mL	0.0125 g

Table 2. Detergent Calculations.

<sup>£</sup>Micelle MW = aggregate# / molecular weight.

<sup>¢</sup>wt / v (100 mL) = CMC \* mol.weight \* 0.1L.

<sup>∞</sup>WC = (wt / v) \* 2.

<sup>a</sup>DDM was calculated at a 5X working concentration due to its availability.

<sup>§</sup>Stock = (g/mol)\*(2.5/2.5).

*Zeaxanthin and Detergent “Extract” Solutions:* Add and dissolve 5% of detergent (0.01 g) to 500  $\mu$ L of 18 Mohm water in an eppendorf tube. Add a scoop of 70% pure zeaxanthin using a small spatula. Calculate the amount of zeaxanthin by subtracting the weight of the eppendorf before and after adding zeaxanthin. Wrap the eppendorf tube in tin foil and allow the extract to nutate for 2 hours.

*Prepping HPCL Vials:* GSTP1 was not diluted for any tests. For GSTP1, add 100  $\mu$ L of concentrated GSTP1 to 500  $\mu$ L of no detergent mobile phase. Centrifuge in the cold room at 13K RPM for 8 minutes. Transfer 200  $\mu$ L of the supernatant into an HPLC vial. For the detergent assay, all zeaxanthin + detergent extracts were diluted by adding 100  $\mu$ L of the extract to 500  $\mu$ L of the corresponding detergent mobile phase. Centrifuge at room temperature at 13K RPM for 8 minutes. Transfer 200  $\mu$ L of the supernatant into an HPLC vial.

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## References

1. Bernstein, P. S., Li, B., Vachali, P. P., Gorusupudi, A., Shyam, R., Henriksen, B. S., & Nolan, J. M. (2016). Lutein, zeaxanthin, and meso-zeaxanthin: The basic and clinical science underlying carotenoid-based nutritional interventions against ocular disease. *Progress in retinal and eye research*, 50, 34–66. <https://doi.org/10.1016/j.preteyeres.2015.10.003>
2. Bhosale, P., Larson, A. J., Frederick, J. M., Southwick, K., Thulin, C. D., & Bernstein, P. S. (2004). Identification and characterization of a Pi isoform of glutathione S-transferase (GSTP1) as a zeaxanthin-binding protein in the macula of the human eye. *The Journal of biological chemistry*, 279(47), 49447–49454. <https://doi.org/10.1074/jbc.M405334200>
3. Fraser, P. D., & Bramley, P. M. (2004). The biosynthesis and nutritional uses of carotenoids. *Progress in lipid research*, 43(3), 228–265. <https://doi.org/10.1016/j.plipres.2003.10.002>
4. Galván, I., Garrido-Fernández, J., Ríos, J., Pérez-Gálvez, A., Rodríguez-Herrera, B., & Negro, J. J. (2016). Tropical bat as mammalian model for skin carotenoid metabolism. *Proceedings of the National Academy of Sciences of the United States of America*, 113(39), 10932–10937. <https://doi.org/10.1073/pnas.1609724113>
5. Ghosh, A. K., Sridhar, P. R., Leshchenko, S., Hussain, A. K., Li, J., Kovalevsky, A. Y., Walters, D. E., Wedekind, J. E., Grum-Tokars, V., Das, D., Koh, Y., Maeda, K., Gatanaga, H., Weber, I. T., & Mitsuya, H. (2006). Structure-based design of novel HIV-1 protease inhibitors to combat drug resistance. *Journal of medicinal chemistry*, 49(17), 5252–5261. <https://doi.org/10.1021/jm060561m>
6. Hong, P., Koza, S., & Bouvier, E. S. (2012). Size-Exclusion Chromatography for the Analysis of Protein Biotherapeutics and their Aggregates. *Journal of liquid chromatography & related technologies*, 35(20), 2923–2950. <https://doi.org/10.1080/10826076.2012.743724>
7. Horvath, M. P., George, E. W., Tran, Q. T., Baumgardner, K., Zharov, G., Lee, S., Sharifzadeh, H., Shihab, S., Mattinson, T., Li, B., & Bernstein, P. S. (2016). Structure of the lutein-binding domain of human StARD3 at 1.74 Å resolution and model of a complex with lutein. *Acta crystallographica. Section F, Structural biology communications*, 72(Pt 8), 609–618. <https://doi.org/10.1107/S2053230X16010694>
8. Li, B., Vachali, P., Frederick, J. M., & Bernstein, P. S. (2011). Identification of StARD3 as a lutein-binding protein in the macula of the primate retina. *Biochemistry*, 50(13), 2541–2549. <https://doi.org/10.1021/bi101906y>
9. Oakley, A. J., Rossjohn, J., Lo Bello, M., Caccuri, A. M., Federici, G., & Parker, M. W. (1997). The three-dimensional structure of the human Pi class glutathione transferase P1-1 in complex with the inhibitor ethacrynic acid and its glutathione conjugate. *Biochemistry*, 36(3), 576–585. <https://doi.org/10.1021/bi962316i>
10. Pennington, K. L., & DeAngelis, M. M. (2016). Epidemiology of age-related macular degeneration (AMD): associations with cardiovascular disease phenotypes and lipid factors. *Eye and vision (London, England)*, 3, 34. <https://doi.org/10.1186/s40662-016-0063-5>
11. Roberts, J. E., & Dennison, J. (2015). The Photobiology of Lutein and Zeaxanthin in the Eye. *Journal of ophthalmology*, 2015, 687173. <https://doi.org/10.1155/2015/687173>
12. Vershinin A. (1999). Biological functions of carotenoids--diversity and evolution. *BioFactors (Oxford, England)*, 10(2-3), 99–104. <https://doi.org/10.1002/biof.5520100203>

