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# DETERMINING THE MOLECULAR STRUCTURE OF THE ZEAXANTHIN-GSTP1 COMPLEX

Markell Kolendrianos (Martin P. Horvath, PhD.)
School of Biological Sciences

## Abstract

For my undergraduate research project, I worked to understand the biochemistry of a protein that helps protect our eyes from the toxic effects of light. The macula of the primate eye is enriched in xanthophyll carotenoids, photoprotective antioxidants.[2] The protein GSTP1 has been identified as the xanthophyll-binding protein for the carotenoid zeaxanthin in the retina.[3] However, the structure of zeaxanthin in complex with GSTP1 remains unknown. Synthetic detergents are utilized as a mechanism expected to mobilize the hydrophobic zeaxanthin to become available for binding with GSTP1 in aqueous solution. Size exclusion chromatography (SEC) is utilized to distinguish between zeaxanthin hosted in a detergent micelle or in complex with GSTP1 (Figure 2). SEC results indicate the detergent GDN and its characteristics make it the most optimal for complex distinction and future structural analysis. Results are discussed in a micelle-to-protein transition state model (Figure 1). Structure determination of the zeaxanthin-GSTP1 complex through crystallization will reveal the molecular interactions critical for the protection of the eye and elucidate its role in the prevention of AMD, the leading cause of vision loss among elderly in developed nations.[2,4]

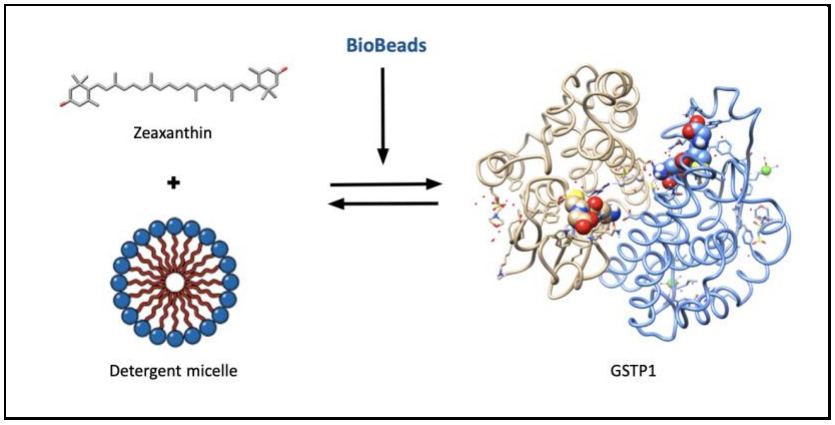


Figure 1. Micelle-to-protein transition state model.

## Introduction

Xanthophyll carotenoids are fat-soluble, pigment molecules that act as potent antioxidants capable of stabilizing free radicals. [2,4] Excessive free radicals contribute to chronic diseases proposing significant physiological relevance of these compounds. [4,8] Humans and other primates are unable to synthesize xanthophylls, therefore the protective functions are obtained dietarily. [1] The macula of the primate eye is enriched in xanthophyll carotenoids.[1,5] Through the means of high-affinity binding proteins, xanthophylls act as efficient localized, short-wavelength (blue) light-absorbers stabilizing free radicals and reactive oxygen species.[3,11] Thus, providing

essential protection against phototoxicity and ocular diseases, such as age-related macular degeneration (AMD).[1] AMD is a chronic ocular disease caused by the deterioration of the macula, the center of the retina.[1,3] The accumulated xanthophyll carotenoids in the macula provide the protective layer necessary to reduce deterioration. Research suggests supplemental and dietary intake of xanthophyll carotenoids help decrease the incidence of AMD, the leading cause of vision loss among elderly in developed nations.[5,10] The structure of GSTP1 has been recognized as a carotenoid-binding protein in the retina.[3] GSTP1 directs the physiological interactions of the antioxidant zeaxanthin.[3,11] The structure of zeaxanthin in complex with GSTP1 remains unknown. Determining the structure of the zeaxanthin-GSTP1 complex through crystallization will reveal the molecular interactions critical to the protection of the eye. To prepare the zeaxanthin-GSTP1 complex for crystallization, it must be formed in solution. The reagents needed include purified GSTP1, zeaxanthin, a carotenoid mobilizing detergent, and a means of detergent removal. Optimizing these conditions to form the zeaxanthin-GSTP1 complex is the first step leading to structure determination to elucidate its role in ocular protection and ultimately aid in the prevention of AMD.

**Results**

*Challenge & approach.*

In order to crystallize the zeaxanthin-GSTP1 complex for structure determination, it must first be formed in solution. Creating the complex has proven to be difficult due to the hydrophobic nature of zeaxanthin and the binding protein requiring a hydrophilic environment. In order to overcome this challenge, synthetic detergents are utilized as a mechanism expected to mobilize the hydrophobic zeaxanthin to become available for binding with GSTP1 in aqueous solution. The various detergents analyzed include DDM, CHAPS, GDN, and sodium cholate. Each detergent hosts zeaxanthin in a micelle, an aggregate of molecules in a colloidal solution, with a characteristic size and critical micelle concentration (CMC) specific to that detergent (Table I). Removal of the detergent was also tested to prevent potential issues that may occur during the ultimate goal of crystal formation.

Establishing the optimal detergent is imperative to distinguish complex formation through Size Exclusion Chromatography (SEC), a method by which molecules are separated by size (kDa) and measured through the absorbance of light and retention time (min).[6] Separation of components is realized by running a mobile phase through a porous column. Large compounds pass through the pores of the column faster than smaller molecules indicating an earlier retention time.[6] The xanthophyll zeaxanthin is expected in two forms, hosted in the detergent micelle or complexed with GSTP1 (Figure 2). GSTP1 is capable of being detected at 280 nm (the protein standard characteristic to its size; ~46 kDa).[2] Zeaxanthin absorbs short-length (blue) wavelengths which is efficiently detected at 452 nm.[2] SEC is applied to differentiate between the two forms based on different retention times. Detergent comparison is applied to determine the most compatible to utilize for complex formation. Complex distinction can be identified through comparing SEC analysis of GSTP1 to zeaxanthin utilizing a detergent removal method. Experimental approach to optimize the appropriate conditions zeaxanthin-GSTP1 complex formation includes protein expression in bacterial cells, protein purification, detergent solubilization of zeaxanthin, and detergent removal utilizing BioBeads (Figure 6). Results are discussed in terms of a micelle-to-protein transition state model (Figure 1).

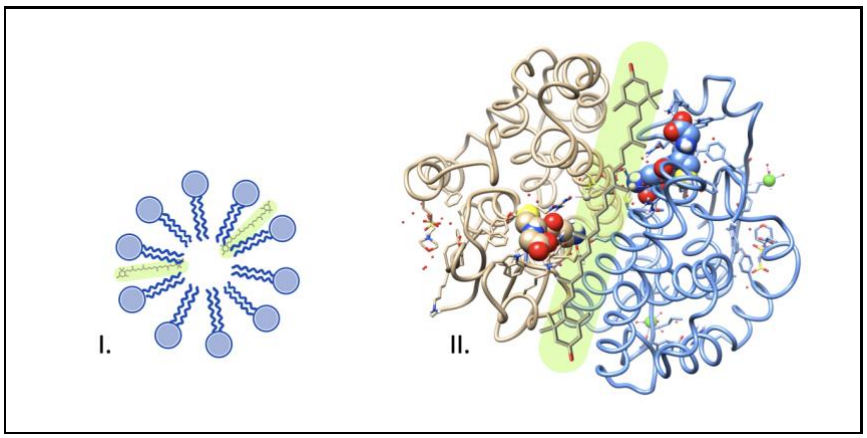


*Figure 2.* Model of zeaxanthin in expected forms: I.) hosted in a detergent micelle or II.) complexed with GSTP1. This is a hypothesis not yet verified by experiment.

*GSTP1 overexpression & purification.*

GSTP1 has successfully been expressed in bacterial cells and purified in high yield. Pure, concentrated GSTP1 was analyzed through a UV quartz spectrometer. Using absorbance readings of different wavelengths characteristic to proteins and aggregates (280 nm & 350 nm, respectively) and reciprocal extinction coefficient of GSTP1 at 280 nm (1/0.029 $\mu M^{-1}$), concentration was calculated. Sample absorbance readings at respective wavelengths were 0.091 and 0.0300. Concentration was calculated yielding 25.83 µM. Through calculating concentration, mg/mL of GSTP1 was determined by multiplying the molecular weight of GSTP1 (23 kDa per monomer) by concentration (µM) and 1/1000 (µL/mL), yielding 0.6 mg/mL. In 7 mL, there is 4.2 mg of pure, concentrated GSTP1 suggesting highly sufficient protein yield from the overexpression and purification procedure. SEC analysis of the GTSP1 were used to further confirm purification and concentration. SEC absorbance of GSTP1 was effectively reproduced. The GSTP1 sample consistently produced four peaks. The first peak was aggregate molecules at ~6.00 min. The second peak is GSTP1 in its dimeric form (46 kDa) at ~10.00 min. The third peak is monomeric GSTP1 (23 kDa) at ~11.00. The fourth peak is DTT at ~14.00 min, confirmed through DTT Control SEC analysis. (Figure 3*)*. Different mobile phases were analyzed to determine how detergents impact the protein. GSTP1 injected with sodium cholate detergent containing mobile phase produced a negative absorbance reading. Compared to that of the GSTP1 sample injected with no detergent mobile phase, the conclusion was made to utilize no detergent mobile phase (*Figure 4*).

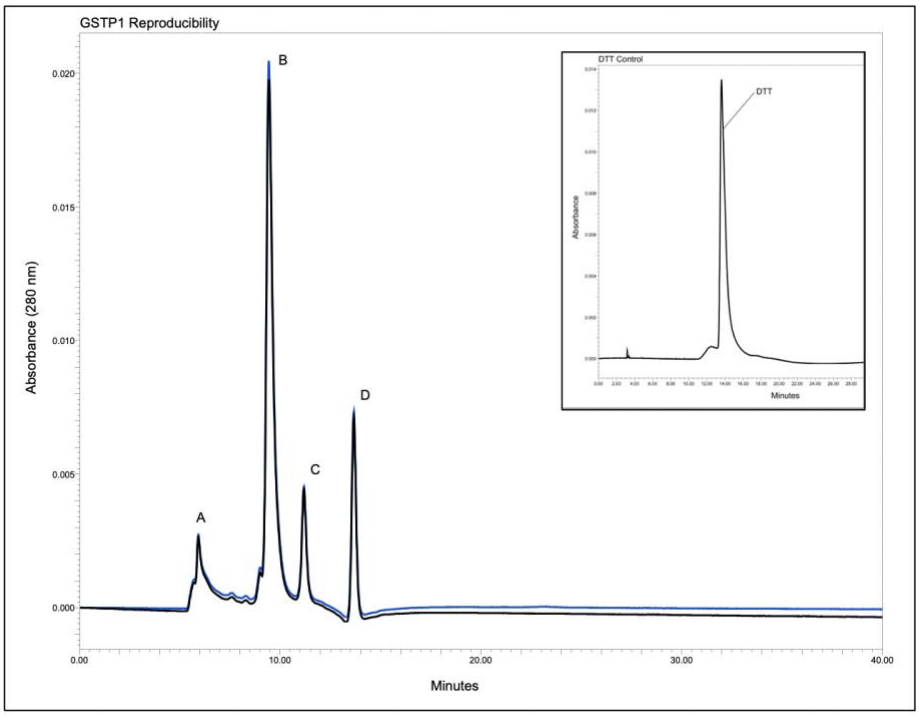


Figure 3. SEC results of GSTP1. Overlay of two different injections representing reproduced protein sample. A) aggregate molecules at ~6.00 min. B) Dimeric GSTP1 (46 kDa) at ~10.00 min. C) Monomeric GSTP1 (23 kDa) at ~11.00 min. D) DTT at ~14.00 min, confirmed through DTT Control SEC analysis.

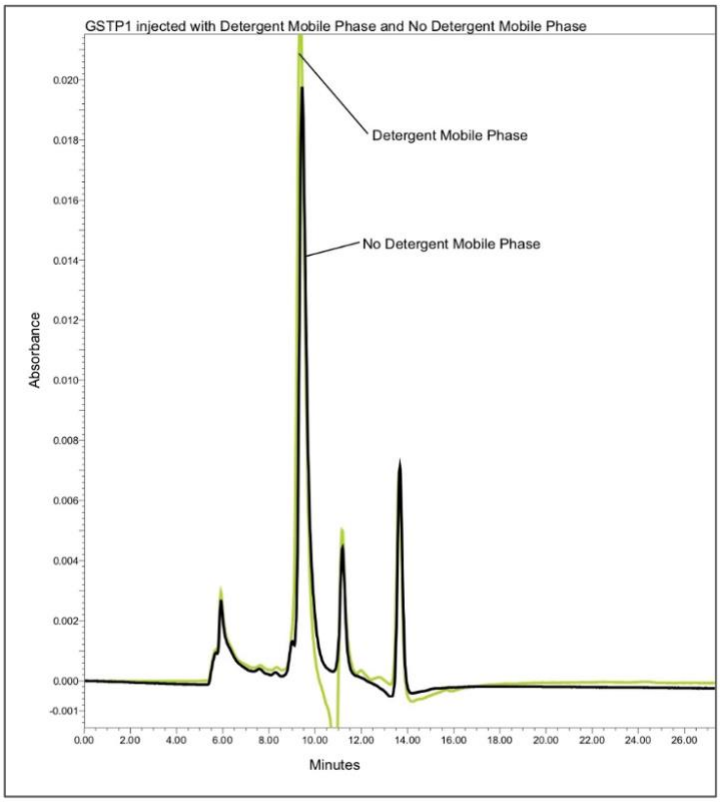


*Figure 4.* SEC results of GSTP1 injected with sodium cholate detergent mobile phase compared to no detergent mobile phase.

*Detergent-solubilization of zeaxanthin.*

The use of synthetic detergents is expected to aid in solubilizing the xanthophyll. The various detergents analyzed include DDM, CHAPS, GDN, and sodium cholate. Each detergent hosts zeaxanthin in a micelle with a characteristic size and critical micelle concentration (CMC) specific to that detergent (Table I). SEC analysis was utilized to compare the mobilization efficiency of each detergent. SEC analysis of the detergent-solubilized zeaxanthin samples (detected at 452 nm) were contrasted to that of GSTP1 (detected at 280 nm) to distinguish the most compatible zeaxanthin-hosting micelle (Figure 2) by producing different retention times. SEC results of zeaxanthin solubilized in DDM detergent and GSTP1 indicated the same retention time (~10 minutes), suggesting incompatibility for complex distinction (*Figure 5*). The detergents CHAPS (~11-12 minutes) and sodium cholate (~11 minutes) produced different retention times than GTSP1, however absorbance readings are distributed. In contrast, the GDN micelle has an earlier retention time (~9 minutes) in comparison to the other detergents, as well as to GSTP1. Size Exclusion Chromatography analysis has provided the conclusion that GDN may be the most compatible detergent to utilize moving forward. Successful testing for detergent removal was conducted using BioBeads, nonpolar polystyrene adsorbents (Figure 6).

*Table I.* Detergent characterization of DDM, CHAPS, GDN, and Sodium cholate.

| Detergent | Molecular weight (g/mol) | Micelle size (kDa) | CMC (mM) | Aggregate number |
|---|---|---|---|---|
| DDM | 510.6 | 50.04 | 0.17 | 78-149 |
| CHAPS | 614.9 | 6.15 | 4.3-10 | 10 |
| GDN | 1229.3 | 2.5 | 0.018 | 2 |
| Sodium cholate | 430.6 | 1.3 | 14 | 2-4.8 |

*Table II.* Formulas used to calculate the amount of detergent needed for 250 mL mobile phase. Detergents were calculated at a working concentration 2x the CMC. **DDM was calculated at a 5x CMC working concentration due to its availability.

| Detergent | Micelle MW (g/mol) = Aggregate #/ Molecular Weight | Weight by Volume wt./v (100 mL) = CMC * MW* 0.1L | Working Concentration = (wt./v) * 2 | 250 mL Mobile Phase = (g/mol) *(2.5/2.5) |
|---|---|---|---|---|
| DDM | 50038.8 | 0.01% | ** 0.043 g ≈ 0.05 g per 100 mL | 0.125 g |
| CHAPS | 6149 | 0.37% | 0.73788 g ≈ 1 g per 100 mL | 2.5 g |
| GDN | 2458.6 | 0.002% | 0.0044 g ≈ 0.005 g per 100 mL | 0.0125 g |
| Sodium Cholate | 1291.8 | 0.60% | 1.205 g ≈ 1.5 g per 100 mL | 3.75 g |

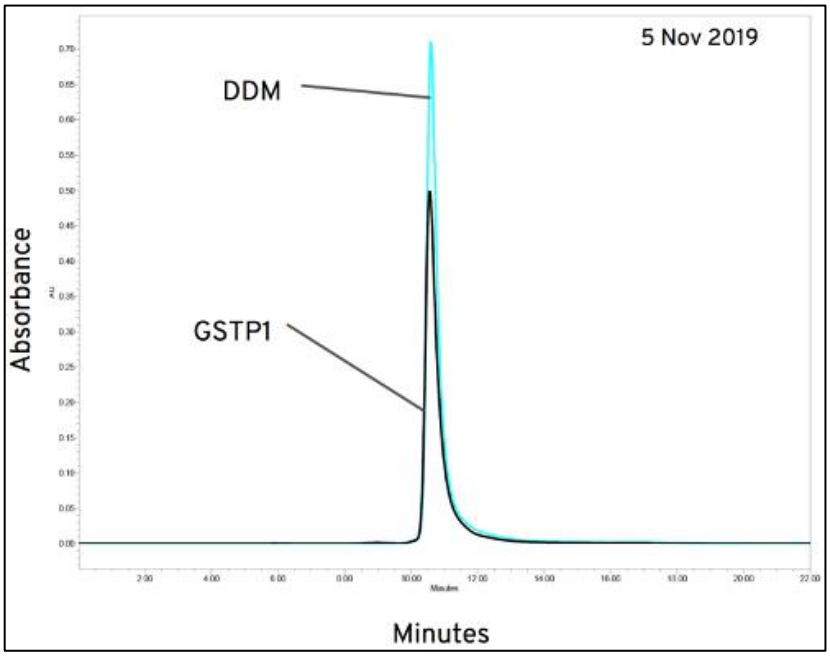


*Figure 5.* SEC results of zeaxanthin solubilized in DDM (452 nm) compared to GSTP1 (280 nm). 2019. Note: a different SEC column (SRT-C SEC-500) was utilized with DDM detergent samples (2019) than samples containing CHAPS, GDN, and sodium cholate (2020).

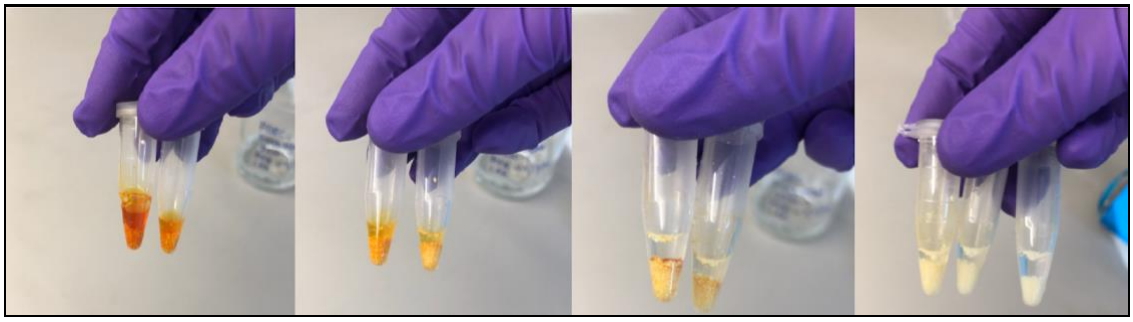

*Figure 6.* 70% pure zeaxanthin diluted in various amounts of DDM detergent to test the efficiency of detergent removal by BioBeads.

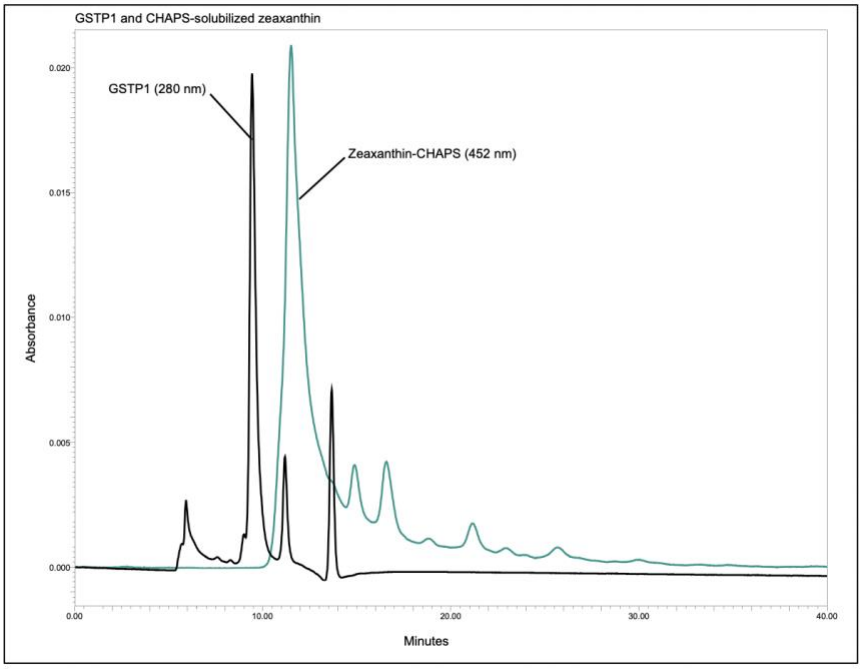


*Figure 7.* SEC results of GSTP1 (280 nm) compared to CHAPS-solubilized zeaxanthin (452 nm). 2020.

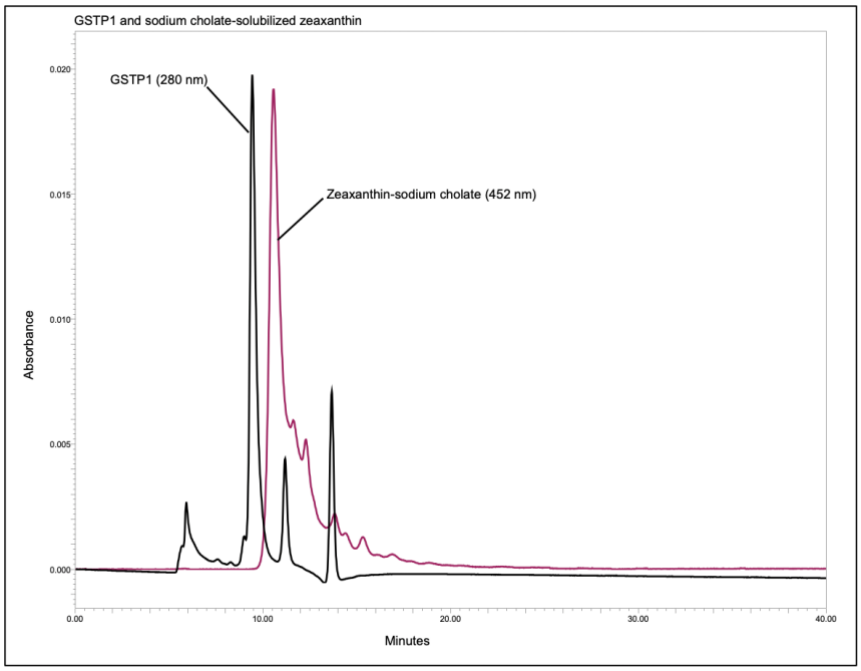


*Figure 8.* SEC results of GSTP1 (280 nm) compared to sodium cholate-solubilized zeaxanthin (452 nm). 2020.

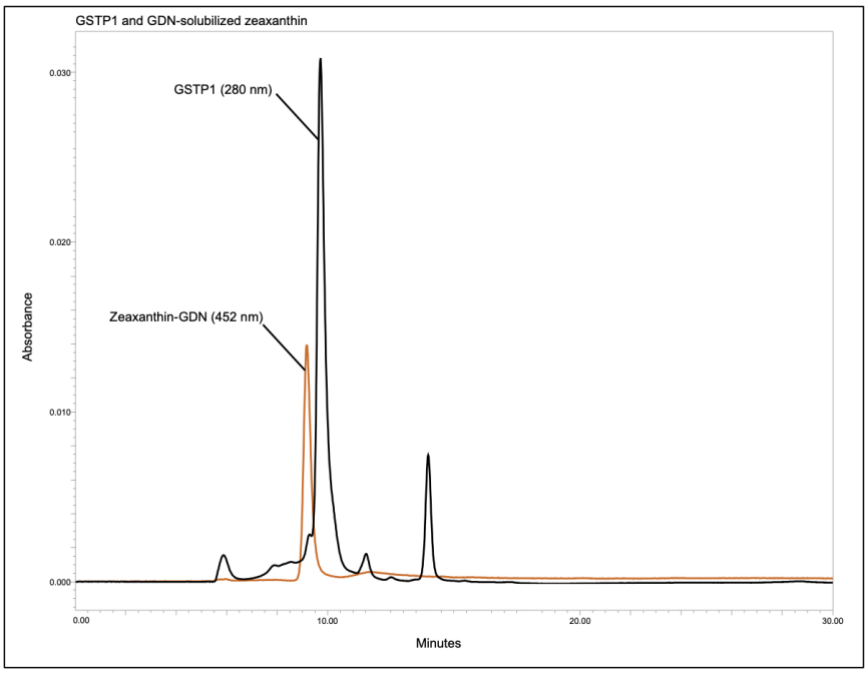


*Figure 9.* SEC results of GSTP1 (280 nm) compared to GDN-solubilized zeaxanthin (452 nm). 2020.

**Discussion**

Determining the molecular structure of the zeaxanthin-GSTP1 complex will reveal more clearly its biochemical function critical to ocular protection. Structural determination remains unknown. Optimizing the complex in solution is a challenging and dexterous feat. Despite the challenges and persistent efforts, many new insights have been realized through SEC analysis.

It is now apparent that GSTP1 quaternary structure change is visible through SEC analysis. These changes may be linked to size-exclusion column interaction. Experimental measures of the DDM-solubilized zeaxanthin and GSTP1 samples (Figure 5) utilized a different column (SRT-C SEC-500) than protein samples compared to zeaxanthin solubilized in CHAPS, GDN, and sodium cholate (SRT-C SEC-300) (Figures 7, 8, & 9). It is plausible that the monomer and dimers are unable to project as separate peaks on the SEC-500 column (500Å pores) as they do on the SEC-300 (300Å pores). This indication is related to the size of GSTP1 in its various structural forms, the 23 kDa monomer and the 46 kDa dimer to that of the pore sizes of the columns (300Å and 500Å). Based on this new insight on quaternary structure change visibility, I hypothesize there is an activation energy barrier that must be overcome for zeaxanthin binding.

Different mobile phases were analyzed to determine how detergents impact GSTP1 as they have an immense effect on the ligand, zeaxanthin. SEC results of GSTP1 injected with a detergent containing solution indicated detergent interactions occurring with the protein in contrast to no detergent mobile phase. The negative peak indicates light is not being absorbed in the detergent containing solution. Further analysis of GSTP1 samples will be injected with a mobile phase without detergent to deter absorbance reading interactions.

The hydrophilic environment required for complex formation contrasts the molecular properties of the fat-soluble zeaxanthin. To overcome this challenge, synthetic detergents act as a carotenoid mobilization mechanism. Mobility efficiency is characteristic to the size of the detergent micelle and its critical micelle concentration (CMC), the amount of detergent needed to form a micelle (Table I & II). As discussed, zeaxanthin is expected to be hosted in a detergent micelle or complexed with GSTP1 (Figure 2). Distinguishment between the two forms is analyzed through retention time. SEC results of zeaxanthin solubilized in DDM detergent and GSTP1 indicated the same retention time (Figure 5). DDM has a micelle size of 50.04 kDa, which is vastly similar to the molecular size of GSTP1 (46 kDa), suggesting incompatibility for complex distinction. The detergents CHAPS and sodium cholate produced different retention times than GTSP1, however absorbance readings are highly distributed. The variability of the peak characteristics of CHAPS and sodium cholate prose difficulty in detergent removal and crystal formation. In contrast, the GDN micelle had an earlier retention time than GSTP1 and the other detergents, suggesting compatibility for distinguishing complex formation. Characteristics of the absorbance peak of GDN-solubilized zeaxanthin also supports this notion of compatibility. The overall shape of the GDN peak is symmetrical. The peak height and area were substantially reduced in comparison to the other detergents, suggesting fewer micelles which is supported by its small micelle size (2.5 kDa) and low CMC (0.018 mM) (Table I). These properties of GDN may provide the ideal condition to mobilize zeaxanthin and efficiently remove it with BioBeads. The homogeneity of the GDN micelle makes it the most optimal for complex distinction and future structural analysis.

Moving forward, I am aiming to minimize the time and/or concentration of the GDN detergent in solution to produce the carotenoid-protein complex. GDN detergent removal will be conducted with BioBeads, as the adsorbent properties have successfully removed DDM, a much larger molecule (Table I). I hypothesize that the binding of zeaxanthin may be linked to the quaternary structure of GSTP1, indicating a potential energy barrier to overcome. I plan to analyze the impact of activation energy input through temperature and sonication. Once I have

determined the optimal conditions to form the zeaxanthin-GSTP1 complex in solution the next steps will be crystallization and structure determination, the overarching goal of my research.

Determining the structure of the zeaxanthin-GSTP1 complex through crystallization will reveal the molecular interactions critical for the protection of the eye, creating a molecular picture that explains how humans and other primates adapted to day-time hunting and gathering even in the face of blinding photo-toxicity. This discovery will allow for a better understanding of the retinal accumulation of carotenoids driven by xanthophyll-binding proteins and their role in the prevention of chronic ocular diseases, such as AMD.

**Procedural methods**

*GSTP1 protein overexpression.* BL21(DE3) E. coli bacteria cells containing the plasmid that encodes for the human glutathione S-transferase pi gene (GSTP1) were cultivated with shaking in 2xYT medium supplemented with 1M glucose, 1M of KPO4, and 40 µL/mL kanamycin. 2 mL cell cultures (and 2 mL control) were grown for 13-15 hours. 60 mL and 375 mL cultures were expanded at 37˚C. Protein overexpression process included two passages. Passage one was the transfer of 300 µL of the 2 mL culture to the 60 mL shaking at 37˚C for 2.5-4 hours. Passage two was the transfer of 10-30 mL of the 60 mL culture to the 375 mL shaking for 1.5-2 hours. At an $OD_{650nm}$ of 0.9-1.1, 375 µL of 0.5M IPTG was added. Induction proceeded shaking at 37˚C for 6-8 hours then chilled on ice prior to harvesting cell cultures by centrifugation at 5K RPM. The cell pellets were stored at -20˚C.

*GSTP1 purification.* Frozen cells were submerged in 5 mL lysis buffer + DTT consisting of 50 mM Tris pH 8.0, 100 mM NaCl, 0.5 mM EDTA, and 10 mM DTT. Upon thawing, cell pellets were lysed by sonication on ice. The lysate was centrifuged at 18K RPM. The sample was 0.45 µm filtered into a 6 mL glutathione bead slurry that was washed with 18 Mohm water and lysis buffer + DTT, respectively. The sample was set to nutate in the cold room for 1.5-2 hours. Centrifugation at a slow speed and subsequent washing with the addition of lysis buffer + DTT and supernatant removal was conducted. 100 µL elutions were obtained using elution buffer consisting of 40 mL lysis buffer + DTT and 0.12 g reduced glutathione. The pure protein was concentrated through a filter column conical tube and centrifuged at 6K RPM. In preparation for Size Exclusion Chromatography Analysis (SEC), repeated (3x) 15 mL buffer exchange was implemented with SEC mobile phase consisting of 1M Tris pH 7.5 and 0.15M NaCl. Protein concentration was determined through absorbance reading by way of UV quartz spectrometer and use of GSTP1 extinction coefficient (calculation formula: (A280-A350) *(770/70) *(1/0.029 $\mu M^{-1}$)). 70 µL of concentrated protein was added to 700 µL of SEC mobile phase. UV quartz spectrometer was baselined with the SEC mobile phase and sample absorbance was analyzed.

*Detergent solubilization of zeaxanthin.* 70% pure zeaxanthin was combined in the various detergents, CHAPS, DDM, GDN, and sodium cholate to form extract solutions. 5% of the detergent (0.01 g) was dissolved in 500 µL of 18 Mohm water. An average of 10 mg zeaxanthin was added to each detergent solution and set to nutate for 2 hours. 100 µL zeaxanthin-detergent extracts were diluted in 500 µL of the corresponding detergent mobile phase consisting of 1M Tris pH 7.5 and 0.15M NaCl. Each zeaxanthin-detergent dilution was centrifuged at 13K RPM.

*Detergent removal.* Testing for detergent removal was conducted using BioBeads, nonpolar polystyrene adsorbents. DDM was the only sample analyzed (Figure 6).

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