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A Spectroscopic and *in silico* Study of the Interactions of Sinigrin and Allyl Isothiocyanate with common Metal Ions

Bernard Jude M. Gutierrez[1,*], Jaime Raul O. Janairo[1] and Marissa G. Noel[1]
[1]*Chemistry Department, De La Salle University*
**Corresponding Author: bernardjude.gutierrez@gmail.com*

Abstract: Glucosinolates are a family of compounds present in many agriculturally-important plants, primarily in members of Brassicaceae vegetables such as cabbages and broccoli. Their hydrolysis products, the isothiocyanates, have been found to have medicinal properties, such as anti-inflammatory and anti-cancer effects. While the metabolic fate of glucosinolates and their related isothiocyanates are properly documented, their interactions with many chemical species *in vivo* remain poorly understood, especially in cases where such interactions may inhibit the bioavailability and effectivity of these compounds. The interactions between a glucosinolate, sinigrin, and its isothiocyanate, allyl isothiocyanate, with three metal ions common in the human body, Fe(III), Zn(II) and Cu(II) are explored using both *in vitro* and *in silico* techniques. The results show that there is little to no indication these ions for compounds with sinigrin or allyl isothiocyanates that can inhibit their bioavailability.

Key Words: sinigrin; allyl isothiocyanate; bioavailability

1. INTRODUCTION

Glucosinolates are a family of glucoside compounds derived from amino acids with a central carbon bound to a thioglucose group and a sulfated aldoxime. They are secondary metabolites to plants of the Brassicaceae family such as mustard, cabbage, radish, etc. When in conjunction with the enzyme myrosinase, which is also found in plants containing them, glucosinolates undergo a hydrolytic reaction cleaving off the glucose group, forming, among other compounds, isothiocyanantes (Halkier and Gershenzon, 2006). Many of these isothiocyanates have been found to have biological activity, in particular, anti-inflammatory, antibacterial and anticancer effects (Johnson, 2002).

There have been numerous studies linking into the biological activity in these compounds, but there have been few studies dedicated to looking at the interaction of glucosinolates, myrosinase, and isothiocyanates *in vivo* and the potential effects of these interactions on their bioactivity and bioavailability.

There have also been several reports of isothiocyanates conjugating with other chemical species such as estrogen derivatives and heavy metals, but these studies have focused mainly on more exotic metabolites and not on compounds that are more commonly found in the human body. It is therefore pertinent to ask the question if the availability of glucosinolates and isothiocyanates are affected by chemical species that are abundant in the body.

A previous unpublished work has hypothesized due to the changes in the UV spectra of sinigrin, a common glucosinolates, and its isothiocyanates, allyl isothiocyanates, when mixed with copper(II), zinc(II) and iron(III) that there might be conjugation or complexation at work whenever these chemicals come in contact. This study seeks to give insight on the dynamics between glucosinolates and isothiocyanates with these metal ions it might interact with in the human body.

2. METHODOLOGY

Lyophilized sinigrin obtained from Sigma Aldrich was reconstituted in ultrapure water to a final concentration of 0.5μM. This sample was divided into two portions. The first portion underwent desulphonation by passing it though a DEAE sephadex A-25 anion exchange resin with sulfatase. This process involved expanding the resin in acetate buffer at pH 4.0 for 30 minutes, rinsing the resin with deionized water, and then depositing it into a column where 75 μL sulfatase was added, as well as an aliquot of sinigrin and then incubated for at least 12 hours. The desulfated sinigrin was then eluted out with water, and the resulting eluant was collected. Along with the non-desulfated glucosinolate, it was mixed with solutions of $CuCl_2$, $ZnCl_2$ and $FeCl_3$ to produce samples of varying mole fractions of sinigrin, desulfated sinigrin and metal ions. The samples were then analyzed using UV-vis spectroscopy using a wavelength scan from 600 nm to 190 nm. The same procedure was done with allyl isothiocyanates for comparison.

Samples consisting of equimolar amounts of allyl isothiocyanates, sinigrin with metal ions were analyzed using ESI-TOF Mass Spectroscopy. The ESI mode was set to positive for samples containing allyl isothiocyanates and negative for those containing sinigrin. The cone voltages were set to 30V and -35V respectively, while the collision energy was set to 0 eV to minimize fragmentation. The source temperature was set to 100°C in both sets. The m/z values that were inputted for the MS^2 targeted MS set-up were the theoretical atomic masses of the resulting compounds plus or minus 10 amu.

Computational interaction between sinigrin and the enzyme myrosinae was determined with Vina Autodock using PyRx 0.8 software. The model for myrosinase used was taken from the RCSB Protein Databank, using the crystal structure elucidated by Burmeister et al (2000) from myrosinase from *Sinapis alba*. Then, sinigrin modified by attaching metal ions to either its glucose end or sulfate end, as well as modelled theoretical dimers were tested to find their binding affinities. Similarly, interactions between wild-type and metal-conjugated allyl isothiocyanates were also tested with glutathione S-transferase derived from *Arbidopsis thaliana* as elucidated by Reinemeier et al (1996). Active sites were determined through the Catalytic Site Atlas. The Autodock procedures wa carried out through a Lamarckian Genetic Algorithm using 250,000 energy evaluations. The complexes were then visualized and analyzed using PyMol.

Nine dimers between allyl isothiocyanates and sinigrin with the three metals were modeled and their thermodynamic properties calculated *in silico* using Avogadrio for modelling and General Atomic and Molecular Electronic Structure System (GAMESS) for quantum mechanical computations. Dimers were chosen because higher level complexes were too difficult to model with the algorithms used in this study. These computations were broken down into two fundamental steps: Single-Point Energy (SPE) Computation at the B3LYP level of theory and Vibrational Frequency Computation using the B3LYP model. The GAMESS output was then analyzed by McMolPlt in order to comput the formation Free Energies of the compounds using the methods discussed by Ochterski (2000) and Jensen (2010).

3. RESULTS AND DISCUSSION

3.1. UV-Vis Spectroscopy.

It was observed that the different UV spectra exhibit no appreciable changes in the max, nor do they show any changes to the peaks in the spectrum itself. There are, however, marked changes in the absorption at λmax. Both hyperchromic and hypochromic shifts were observed, although no apparent trend can be found. This can be due to several factors, namely, a relative increase in the amount of chromatophores available to the instrument (J.C. & Griffin, 1981). The Hyperchromic and Hypochromic shifts may also be due to solvent effects or increases in the conjugation of a molecule. (Yadav, 2005). While the latter might be indicative of complexation, considering that allyl isothiocyanate as a ligand would conjugate much more if it is chelating a metal ion, the results are not consistent. It is also likely that the changes in the absorbance would be due to solvent effects such as increases or decreases in ionic strength of the solution or electrostatic interactions between the metal and the supposed ligand changing the spectrometric properties of the sample.

Another observation that can be made is the lack of any isosbestic points in the spectra. Isosbestic points are usually found in systems wherein a physical or chemical change has occurred due to the mixing of two reagents being analyzed. The presence of an isosbestic point is usually attributed to the

interaction of two, and only two reacting species (Nowicka-Jankowska, 1971). While isosbestic points are not a confirmation that a reaction has taken place, the presence of one is indicative of the stoichiometry of the reaction remained unchanged during the course of a reaction, as is the case in the formation of complexes. (McNaught & Wilkinson, 1987). The lack of any isosbestic point is something to consider regarding whether or not complexation has indeed occurred.

3.2. Targetted Mass Spectroscopy

Table 1 summarizes the intensities of the peaks of the associated molecules, as well as the signal-to-noise ratios of the peaks of interest, which was used as a measure of the quantitation of species that might have the mass-to-charge ratios predicted.

Table 1. Theoretical Binding Affinities from Docking Experiments

Sample	Expected Molecule	m/z	Relative Intensity	S/N
AITC	Monomer	100.22	70.24	8695.8
AITC Cu	Dimer	262.32	34.18	524.6
AITC Zn	Dimer	262.30	7.04	2682.5
	Tetramer	460.50	12.31	206..6
AITC Fe	Hexamer	651.17	2.94	156.0
	Tetramer	450.27	0.93	117.0
	Dimer	253.36	8.09	3074.0
Sinigrin	Monomer	358.02	100.00	13002.3
Sinigrin Cu	Dimer	779.98	0.06	28.0
Sinigrin Zn	Dimer	781.61	0.06	16.0
Sinigrin Fe	Dimer	770.98	0.1	8.3

It should be noted that none of the test mixtures had appreciable Signal to Noise Ratios (S/N) nor Relative Intensities in the mass-to-charge ratios where the complexes were expected to reside. While this in itself does not definitely state that these compounds did not indeed exist in the sample, the literature cited that the chemical species involved, allyl isiothiocyanate in particular, forming relatively stable conjugated systems with other chemicals suggests that there should have been more considerable signals in the m/z where the complexities were expected to be found. The pure molecules have S/N ratios orders of magnitude higher than the expected complexes further diminishes the credence of the hypothesis (Petkovic et al, 2001).

In all mass spectra, a very prominent peak at m/z=358 was always present. This is consistent with the molecular weight of sinigrin, and the fact that it remains present in all three samples is testament to the fact that it goes unreacted.

3.2. In silico *modelling*

It was observed that the modelled compounds with the metal conjugates have a higher binding affinity to the enzymes tested, but not by much. For example, the sinigrin-Fe molecule has a mean binding affinity to myrosinase of -7.1 kcal/mol as compared to -6.3 kcal/mol for the control, or an 11% increase. ANOVA analysis of these changes changes reveals that some, but not all, of these changes are statistically significant. For myrosinase, there is no statistically significant change in binding affinity for most of the ligands, except for the one with the iron-bound sulfate group. For glutathione S-transferase, the ligands that are more similar with respect to each other tend to have insignificant differences in binding affinity, but vary in between groups.

Table 2. Theoretical Binding Affinities from Docking Experiments

Enzyme	Ligand	Mean Binding Affinity, kcal/mole	% Difference from Control
Myrosinase	Sinigrin (control)	- 6.378	-
	Sinigrin-Cu (g)	- 6.622	3.833
	Sinigrin-Zn (g)	-6.656	4.355
	Sinigrin-Fe (g)	-6.967	9.233
	Sinigrin-Cu (s)	-7.122	2.265
	Sinigrin-Zn (s)	-6.667	4.527
	Sinigrin-Fe (s)	-6.522	11.672
Glutathione S-transferase	AITC (control)	-3.044	-
	AITC-Cu	-3.322	9.124
	AITC-Zn	-3.344	9.854
	AITC-Fe	-3.122	2.554
	AITC-Cu dimer	-3.956	29.27
	AITC-Zn dimer	-3.689	21.168

Looking at the enzyme-ligand complexes for myrosinase, as shown in Figures 1-3, it is observed that the compounds reside in almost the exact same spot within the active site. It should be noted that there is very little, if at all, observable differences in the conformation of the ligands, although, the one with iron has a noticeably different conformation, which can explain the slight increase in the binding energy of the compound as compared to normal sinigrin. Observations of these two complexes indicate that the modified sinigrin rotated close to 180° perpendicular to the z-axis, perhaps due to residues in that area of the active site being more conducive to the pendant ion atom present in the

glucose moiety of the molecule. The conformation of this compound within the binding site seems to have rotated close to 180º along the Z axis, allowing a different set of amino acid residues to interact with it. Looking at the different residues these ligands interact with, as seen in Figures 4-5 below, it is evident why there is an increase in the binding affinity to myrosinase.

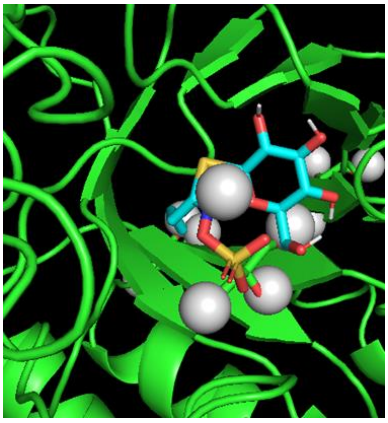

Figure 1. Simulation of sinigrin binding to myrosinase.

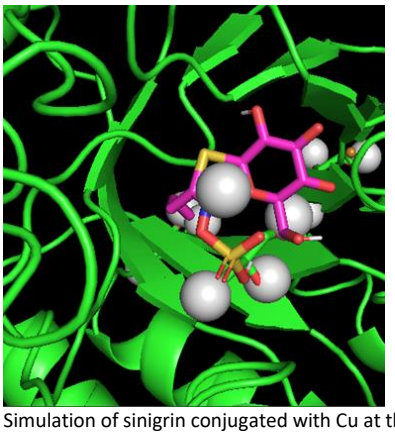

Figure 2. Simulation of sinigrin conjugated with Cu at the glucose end binding to myrosinase.

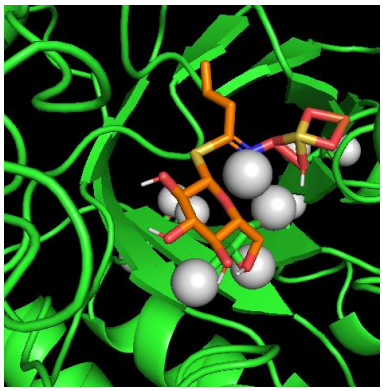

Figure 3. Simulation of sinigrin conjugated with Fe at the sulfate end binding to myrosinase.

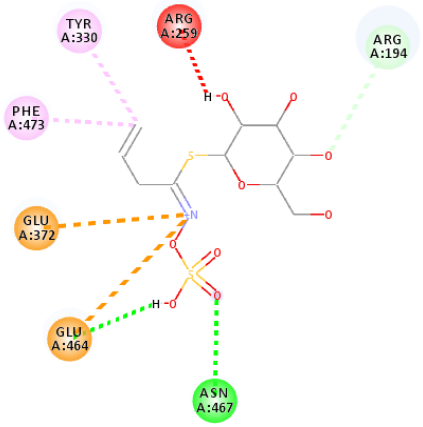

Figure 4. Interactions of normal sinigrin with myrosinase.

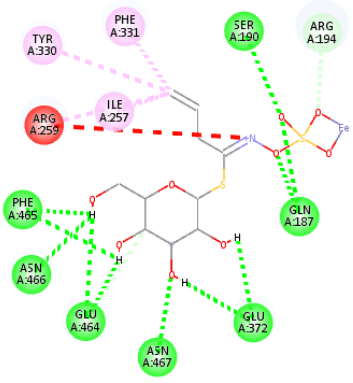

Figure 5. Interactions of Fe-conjugated sinigrin with myrosinase.

It seems that the addition of the iron enhanced the sulfate oxygens' ability to hydrogen

bond with neighboring serine and glutamine residues. The change of conformation also opened up additional H-bonds with residues Glu-372, Asn-467, Glu-464, Asn-466, and Phe-465. While it did lose van-der-waals interaction with Phe-473, it gained three more residues to interact with.

It is observed that the modified allyl isothiocyanate tested here adopt a variety of conformations, possibly due to the size of the binding site compared to the substrate, which also explains the low binding affinity seen across the board. It also explains the fact that the observed binding energies is higher for the dimers, since these would have more opportunities to bind to the residues around the active site. In the regular interaction between AITC and glutathione S-transferase, the allyl carbon interacts through pi alkyl interactions with Ile-222, Ala-118 and Tyr-178. There exists a pi-sulfur interaction between Phe-123 and the substrate, while Ala-10 has a hydrogen bond with it. In contrast, a very different set of residues interacts with the dimer. They are almost all pi-alkyl interactions, with a carbon-hydrogen interaction contributed by Ser-115.It is possible, that since these residues are closer, that the interaction is not "forced", and therefore would be stronger, as noted in the docking data. Figures 6 and 7 demonstrate this.

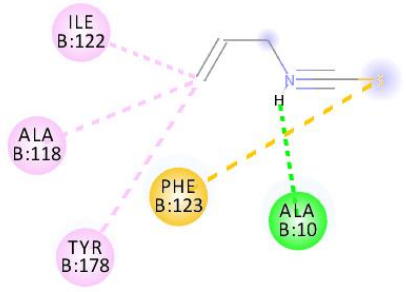

Figure 6. Interactions of normal AITC with GST

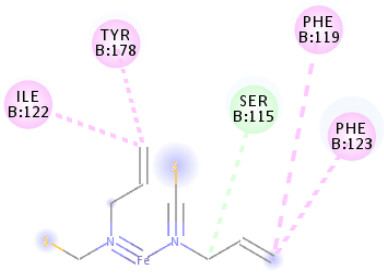

Figure 7. Interactions of Fe-AITC dimer with GST

It would seem that the modifications are not enough for the enzyme not to recognize the substrate. The impact of any conjugation between metal and the glucosinolate would therefore be benign. The same cannot be said for the isothiocyanate, though. Since glutathione S-transferase is the enzyme responsible for removal of isothiocyanate and related species from the body, the increased affinity of the compounds means that these molecules would have reduced bioavailability.

Something to consider, though, is if these hypothetical molecules could form in the first place. Table 3 summarizes the result of the computations for the modelling of the complexes.

Molecule	ΔE (kcal/mol)	ΔG (kcal/mol)	Boltzmann Probability
A	5105.19	5080.34	0
B	5380.67	5355.81	0
C	N/A	N/A	N/A
D	719.22	621.89	0
E	741.72	644.38	0
F	1113.68	1016.34	0
G	1736.37	1639.03	0
H	11012.24	10914.91	0
I	9962.64	9865.30	0

Compound C, a dimer between allyl isothiocyanate and iron was not successfully modelled as the SCF did not converge even after multiple iterations. That is to say, the software was unable to find the lowest energy conformer of the molecule, and hence, the thermodynamic properties of that molecule based on its structure through DFT could not be determined. Nevertheless, data was able to be gathered from the remaining compounds. As can be seen here, extremely high Gibbs' free energies were calculated for the formation of these complexes. This is likely mainly due to the loss of entropy from the system with the net loss of particles from three to one during the reaction, and that the reaction would not be sufficiently exothermic enough to compensate. Indeed, very positive energy changes are observed, which is indicative of the high amounts of energy needed to form these complexes. This leads to Boltzmann probabilities equaling infinitesimally low numbers, approaching zero.

4. CONCLUSIONS

Given that the mass spectroscopy results have not been able to detect significant amounts of the expected compounds, and that no drastic changes in the UV-vis spectra were observed, as is expected when complexation occurs, it is therefore reasonable

to suggest that no such covalent interaction exists between sinigrin, allyl isothiocyanates, and Cu(II), Zn(II) and Fe(III) ions. Therefore, there should be no impediment to the bioavailability of these compounds in the presence of the metal ions found in the human body. However, due to the hyperchromic and hypochromic shifts occurring at the λ_{max} of the mixtures, it can be inferred that some sort of electrochemical, non-covalent interaction exists. This sort of interaction begs further study and expansion for the research into this field is recommended.

5. ACKNOWLEDGMENTS

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