Inhibition of Xanthine Oxidase by Flavonoids using UV-based Assay and Extending to NMR Method

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Abstract In this experiment, the inhibitory effects of flavonoids on the enzyme xanthine oxidase were measured using a UV absorption assay and DOSY-NMR analysis. First the UV assay was used to determine the rate of xanthine oxidase producing uric acid from xanthine under the presence of various flavonoids. The flavonoid chrysin was found to have strong inhibitory effects, naringenin and catechin showed slight signs of inhibition, and naringin and hesperetin showed almost no signs of inhibition. The next step was to measure the change in diffusion rate of the flavonoid in the presence of xanthine oxidase using DOSY-NMR analysis as an indication of binding. Chrysin was found to have a significant change in diffusion rate based on xanthine oxidase concentration, while catechin showed minimal change in diffusion rate. It was concluded that the NMR analysis was a viable means to confirm or clarify primary analysis of flavonoid inhibition, but further research needs to be put forth to determine the full extent to which NMR can reveal information about flavonoid inhibition.

Introduction

Xanthine oxidase (XO) is an oxidative enzyme found in most animals, but for mammals it is found almost exclusively in the liver and intestine.[1] It functions primarily as the final enzyme in the breakdown of purine compounds, converting the compound xanthine into uric acid. As a side effect of this reaction, however, XO will commonly produce either a superoxide radical $O_2^{\bullet-}$ or hydrogen peroxide H_2O_2.[2] The reaction may appear as in Figure 1.[1]

The overabundance of the superoxide radical and hydrogen peroxide, classified as reactive oxygen species, can lead to detrimental effects on an individual. The oxidative stress caused by these species has been known to

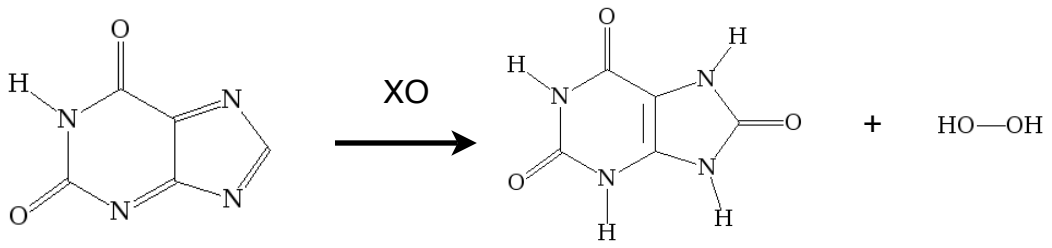


Figure 1. Conversion of xanthine to uric acid and hydrogen peroxide by xanthine oxidase

contribute to such processes as inflammation, cancer, and aging.[2] As a result, an increasing amount of research has been put into finding inhibitors to XO to develop therapeutic agents.[3]

Flavonoids are a classification of organic compounds found commonly in plants as a yellow pigment.[4] Flavonoids bear a similar structure to the compound flavone (Figure 2).[4] This family of organic compounds has be shown to exhibit many biological activities including antibacterial, antioxidant, and anticancer effects.[2][4] Flavonoids have also been used as non-steroidal anti-inflammatory drugs (NSAIDS) for the purposes of reducing swelling, reducing fever, and reducing pain.[5]

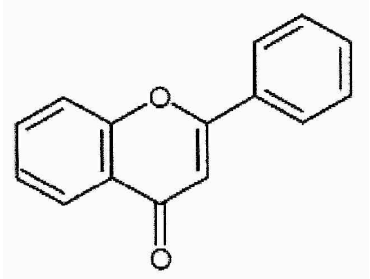


Figure 2. Structure of Flavone

Studies have already shown that some flavonoids function as an inhibitor of xanthine oxidase.[2] Because of this, flavonoids were chosen as the class of inhibitors used for this experiment.

The most common and well-documented method for measuring xanthine oxidase activity is done through ultraviolet absorption through continuous spectrophotometric rate determination. The uric acid produced by the enzyme oxidation of xanthine absorbs ultraviolet light at 290 nm.[6] By mixing the xanthine and the XO in a spectrophotometer cuvette and measuring the absorption, a concentration of uric acid can be detected. By measuring the change of uric acid concentration over time, the rate of the reaction, and therefore the enzyme activity, can be characterized.

Nuclear magnetic resonance (NMR) spectroscopy is a well-developed field for studying and determining the structure of compounds. Its noninvasive approach makes it a preferred method for many experiments. Although NMR spectroscopy is most often used to determine the structure of

compounds, the diffusion-ordered NMR spectroscopy (DOSY) method can be used to determine the diffusion rate of a molecule.[7] Since the diffusion rate of molecule is largely determined by the size of the molecule, a change in diffusion rate of a small molecule can be indicative of its binding to a larger molecule, which is the principle behind using DOSY NMR analysis for detecting ligand binding to protein.[7] This method of testing for protein-ligand interactions removes the need for any chemical reaction to take place.

Materials and Methods

Chemicals. Monobasic and dibasic potassium phosphate, DMSO, deuterated DMSO, deuterium oxide. The flavonoids hesperetin, naringin, naringenin, chrysin, catechin hydrate, as well as xanthine, and the enzyme xanthine oxidase were purchased from Sigma Chemical Co.

UV Assay. A 50mM potassium phosphate buffer was made to a pH of 7.5 at 25˚C. A 0.15 mM stock solution of xanthine was made by dissolving the xanthine into deionized water. A stock solution of 0.62 unit/mL xanthine oxidase was made by dissolving the enzyme in the phosphate buffer. One unit of xanthine oxidase is approximately 0.125 mg. For each flavonoid, a 7.7 mM stock solution was made by dissolving the flavonoid in DMSO.

A multi-well plate was used in an ultraviolet spectrophotometer. Each well would 200 µL of liquid. Each test and control was carried out with four different concentrations of xanthine. The usual set of four wells were arranged as follows:

Well	µL Stock Xanthine Oxidase	µL Stock Flavonoid (Buffer for Control)	µL Stock Xanthine	Final Concentration Xanthine (mM)
1	64.4	2.6	133	0.1
2	64.4	2.6	66.5	0.05
3	64.4	2.6	33.3	0.025
4	64.4	2.6	16.6	0.012

For wells 2 - 4, the remainder of the well was filled with the phosphate buffer solution to reach 200µL total liquid in the well. The final concentration of xanthine oxidase in each well was approximately 0.2 unit/mL, and the concentration of flavonoid in each well was 0.1 mM.

The UV spectrometer was set up to record the absorbance of the wells at 290 nm over a period of 5 minutes. The reaction within the wells was

started immediately before beginning the absorption recording by adding the xanthine oxidase to the wells simultaneously with a multi-tipped pipet.

DOSY-NMR Spectroscopy. A phosphate buffer was created as described above, but deuterium oxide was used in place of water. This D_2O phosphate buffer was used to dissolve xanthine oxidase to create an approximately 4.6 µM stock solution. After examining the results from the UV assay, chrysin and catechin were chosen to provide a comparison between an inhibiting and a noninhibiting flavonoid. 1 mM stock solutions of the flavonoids chrysin and catechin hydrate were created using deuterated DMSO. Three solutions were created for each flavonoid and placed into NMR tubes with the following amounts of solutions:

Tube	µL Stock Flavonoid	µL Stock XO	Final Concentration XO
1	100	0	0 µM
2	100	15	0.1 µM
3	100	30	0.2 µM

Each NMR tube was filled to 700 µL with the D_2O phosphate buffer.

All NMR experiments were conducted at temperatures of 298 ± 1 K. Automated z-gradient shimming was performed by using deuterium spin echoes. The initial 1H NMR spectra for all of the samples were obtained at 90° pulse (10.8 µs), 16 scans, 8K data points, and spectral width (SW) was set as 5000Hz and centered at 5 ppm. DOSY experiments were performed by using the one-shot pulse sequence within VNMRJ program based on the sequence and directions found at nmrwiki.org.[8]

Twenty experiments consisting of 16 scans were performed for each measurement. The 20 experiments were arrayed with varying diffusion-encoding gradients of 500, 3475, 4890, 5978, 6897, 7707, 8439, 9113, 9741, 10330, 10888, 11418, 11925, 12411, 12878, 13330, 13766, 14190, 14600, and 15000 Gradient units. The maximum gradient strength of the Performa II gradient system is 75 Gauss/cm with the equivalent of 32768 Gradient units. The relaxation delay was 3 seconds, the total diffusion-encoding gradient pulse duration δ equaled 2 ms.[9]

Data analysis was performed using MestReNova Version 6.2. Data sets were transformed into 16K data points after an initial multiplication of data with an e-function weighing by 1.0Hz. Phase correction, and base line correction using a Whittaker smoother function was performed and

subsequently the intensity of two flavonoid peaks chosen to plot against the gradient level change. A line fitting assuming mono exponential decay was performed and correspondingly the diffusion constant obtained as the power of the e-function y= B* exp(-x*G). For the data analysis, the two diffusion constants were averaged together. Diffusion constants are reported in cm^2/sec

Results

After conducting the UV-Assay on each flavonoid with varying concentrations of xanthine, the results appeared as in Table 1.

Table 1. UV Assay Results

Flavonoid	Xanthine (mM)	Rate (Abs/min) $*10^{-3}$	% Difference from Control (Abs/min)
No Flavonoid	0.1	54.428	0%
(control)	0.05	35.788	0%
	0.025	17.528	0%
	0.012	6.982	0%
Hesperetin	0.1	52.439	3.65%
	0.05	36.007	0.61%
	0.025	18.49	5.49%
	0.012	8.401	20.32%
Chrysin	0.1	5.302	90.26%
	0.05	4.579	87.21%
	0.025	-22.45	228.08%
	0.012	-9.689	238.77%
Naringenin	0.1	43.028	20.95%
	0.05	30.395	15.07%
	0.025	16.147	7.88%

Flavonoid	Xanthine (mM)	Rate (Abs/min) $*10^{-3}$	% Difference from Control (Abs/min)
	0.012	7.95	13.86%
Catechin	0.1	46.306	14.92%
	0.05	31.344	12.42%
	0.025	17.949	2.40%
	0.012	8.743	25.22%
Naringin	0.1	51.224	5.89%
	0.05	35.879	0.25%
	0.025	14.194	19.02%
	0.012	7.36	5.41%

Based on the results of the UV assay, the tested flavonoid can be divided into three tiers. The first tier would show almost no appreciable signs of inhibition. This would include the flavonoids naringin and hesperetin. The second tier would show some noticeable signs of XO inhibition, but would not cause a drastic change in the performance of the enzyme. The second tier would include the flavonoids naringenin and catechin. The third tier would demonstrate a drastic change in the performance of XO and provide clear indication of inhibition. Chrysin fell into this third category. From these results, chrysin and catechin were chosen to be compared using DOSY analysis.

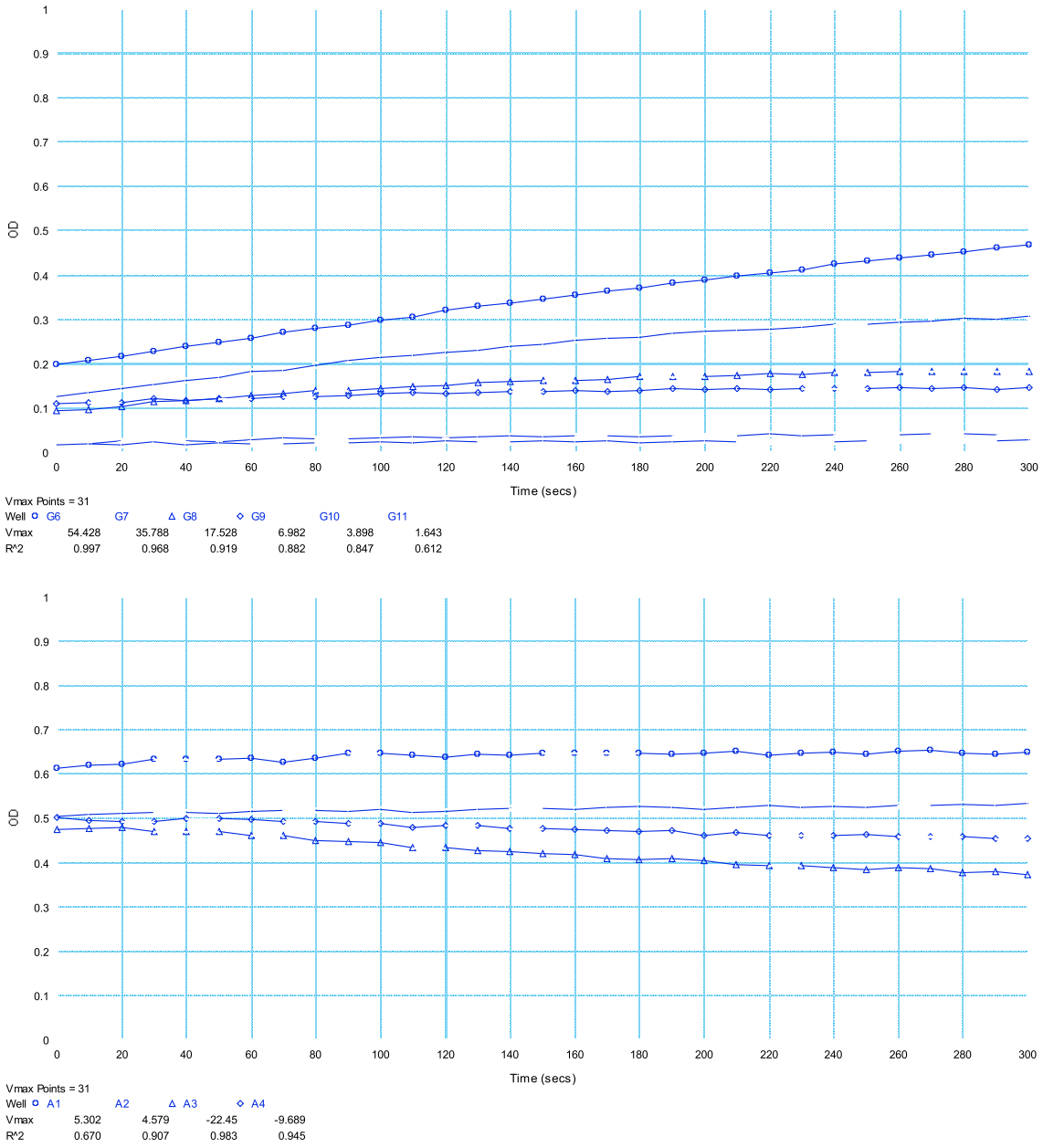


Figure 3. The absorption control experiment of xanthine oxidase (top) compared to the trial with chrysin inhibitor (bottom). The significant decrease in slope in the chrysin experiment is indicative of inhibition of xanthine oxidase.

The DOSY-NMR spectroscopy yielded the diffusion rates for chrysin and catechin with varying concentrations of xanthine oxidase in solutions. The results from the experiments appeared as in Table 2.

Table 2. DOSY-NMR results for chrysin and catechin

Flavonoid	Xanthine Oxidase (µM)	Diffusion Rate (cm^2/s) $*10^{-6}$	% Change (cm^2/s)
Chrysin	0	5.1 ± 0.1	0%
	0.1	2.4 ± 0.1	52%
	0.2	2.1 ± 0.1	59%
Catechin	0	2.0 ± 0.1	0%
	0.1	1.96 ± 0.09	6%
	0.2	1.98 ± 0.09	6%

The DOSY NMR analysis showed that chrysin had a significant change in diffusion rate after the addition of xanthine oxidase. Increasing the concentration of XO also led to a detectable decrease in diffusion rate for chrysin. For the catechin solution, the addition of XO caused a detectable decrease in diffusion but falls within the error range to indicate no significant change in diffusion. Increasing the concentration of XO in the catechin solution did not generate a significant change in diffusion rate. The results from the DOSY-NMR analysis seemed to have confirmed that results from the UV assay, indicating that chrysin exhibits significant binding to xanthine oxidase and that catechin has a appreciably lower affinity for the enzyme.

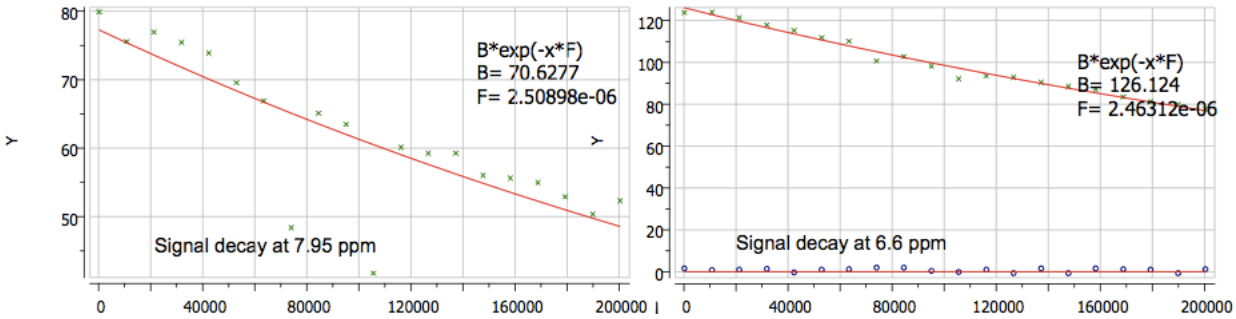
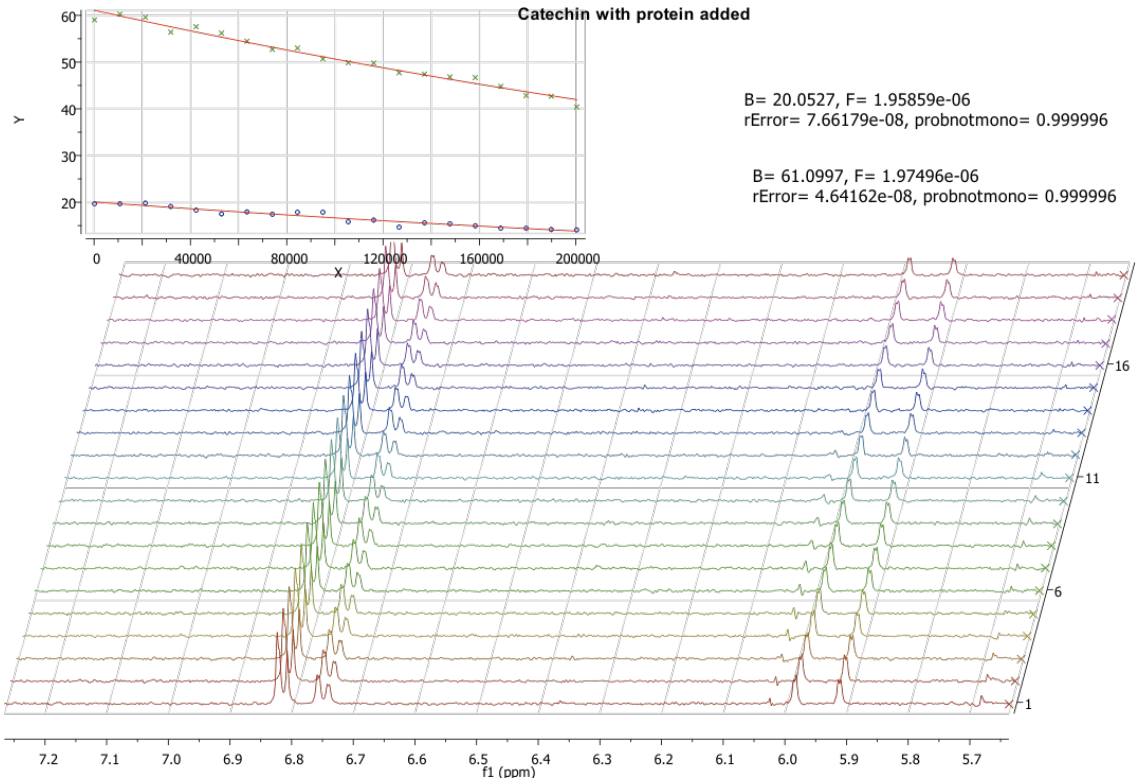


Figure 4. Plots of intensity vs. gradient strength, showing chrysin with 0.1 µM enzyme at 7.95 ppm (left), at 6.6 ppm (right). Catechin with 0.1 µM enzyme is shown with twenty spectra (next page).



Discussion

The UV assay is a well established and effective method for detection of xanthine oxidase activity. With the addition of flavonoids into the mixture, however, complications arose that would make detailed analysis difficult. The flavonoids tested in this experiment exhibited their own absorption at 290 nm. Furthermore, it would appear that in some cases, most notably in the case of chrysin (see Figure 3), that the flavonoid would lose absorption over time, perhaps due to binding with the protein. The altered absorption readings created by the flavonoids led to many complications in generating reliable and clear data from the UV assay. After much trial and error, the method of using a constant flavonoid concentration over a range of xanthine concentrations was determined to be the most effective method. The results of this experiment ranked chrysin a stronger inhibitor than naringenin which was in turn stronger than catechin. These results matched previous analysis of these flavonoids as XO inhibitors and reaffirm the accuracy of the UV method employed.[2]

The complications generated from the UV assay indicate that false positive or inaccurate results may be a strong possibility when attempting to generate data from a UV assay containing flavonoids. To combat such complications, it would be advantageous to have a second method to verify

any results. This is the very reason for developing the DOSY NMR method of analyzing enzyme inhibition.

The preparations for the NMR analysis were notably easier due to the fact that no chemical reaction needed to take place. There was no need to add xanthine to the solution or track a chemical reaction. Also, since the DOSY-NMR analysis does not measure the rate of a reaction, the experiment was much less time-sensitive. The UV assay required the enzyme to be added to the wells immediately prior to the measurement in order to record the initial velocity.[10] The most notable advantage of the NMR method over the UV assay is that the flavonoids were clearly detectable and trackable, whereas the flavonoid UV absorption caused difficulties in differentiating flavonoid concentration from uric acid concentration. The drawback of the NMR, however, is that it required a bare minimum concentration of flavonoid to be detectable, limiting the ability to analyze low concentration conditions. The enzyme xanthine oxidase contains eight iron atoms in the form of ferredoxin iron-sulfur clusters.[11] It was an initial concern that the paramagnetic nature of the iron would interfere with the detection of the flavonoid, but this did not appear to hinder the detection.

The DOSY-NMR method employed in this experiment was limited only to detecting ligand binding to enzyme. The diffusion rates were analyzed as an indication of ligand binding to a protein, and the degree of change of diffusion indicated the strength of that binding, however this experiment made no attempt to characterize the type of inhibition occurring. Enzyme inhibitors can act in a number of different ways to decrease the activity of the enzyme. Types of inhibitors include competitive, noncompetitive, and uncompetitive.[10] Through analysis of the change in rate of reaction caused by an inhibitor, it can be classified into one of these categories. Perhaps in future experiments, an attempt to characterize the type of inhibition occurring may be undertaken using NMR analysis.

Conclusion

The goal of this experiment was to test flavonoids for inhibition of xanthine oxidase through a UV assay and confirm those results with DOSY-NMR analysis. The data gathered from the two methods agreed with each other and seemed to confirm that the NMR method was a valid approach to detecting enzyme inhibition. This experiment was limited to only detect binding between the ligand and enzyme using DOSY. The next stage of research should focus determining whether or not NMR analysis would also be capable of detecting the type of inhibition caused by the flavonoid.

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