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Lab Report for Mass Spectrometry Analysis of Urate Oxidase

Abstract:

In our analysis of the sequence and mechanism of urate oxidase we used ESI-TOF and MALDI-TOF in order to determine whether our previous experiments were successful. That is, we used ESI-TOF to limited success when trying to determine the presence of our F182Y mutation in *Bacillus subtilis* urate oxidase because of poor spectral resolution. Our ESI-TOF also did not succeed in determining any new and relevant information regarding the mechanistic pathway from urate to allantoin. Despite these two relative failures, we were once again able to confirm the successful mutation from phenylalanine-182 urate oxidase to tyrosine-182 urate oxidase using MALDI-TOF and a tryptic digest. In order to accomplish this, we related the expected MALDI peaks to the measured peaks in both the wild-type and the mutant. Overall, the mass spectrometry experiments were performed and analyzed adequately; however, our experimental data collection parameters could have used some improvement.

Introduction:

Urate oxidase is a homotetramer that catalyzes the degradation of urate to 5-hydroxyisourate[CITE]. It is important in the purine degradation pathway and is implicated in gout and tumor lysis syndrome[2]. For these reasons, there is scientific motivation, and thus funding opportunities, for research into better understanding the structure and function of the enzyme. Furthermore, we are attempting to build on our prior experiments by confirming a previously performed site-directed mutagenesis experiment and by further analyzing the mechanistic pathway of the reaction.

Specifically, in experiments 1 and 2 we are attempting to determine whether or not the F182Y mutation that our group performed last quarter is present in the *Bacillus subtilis* isomer of urate oxidase. In order to accomplish this, we performed two basic mass spectrometry experiments. The first experiment consisted of an ESI-TOF mass spectrum comparison between wild-type and mutant F182Y urate oxidase. ESI-TOF can best be explained by breaking it down into electrospray ionization and time-of-flight analyzer. The protein sample is first prepared in a mixture of water and acetonitrile. Acetonitrile is a highly volatile solvent that helps to vaporize the protein by reducing surface tension while the solution is placed in a strong vacuum[3]. Acid is typically added to form positive ions which are much less reactive molecular amino acid ions than negative ions[3]. The solution is also usually dialyzed to decrease interactions from various salt ions[3]. These conditions allow for the protein molecules to vaporize and thus be separated. This separation occurs via the time-of-flight analyzer. In this technique, the gaseous molecular ions are accelerated by a constant electric field to a known kinetic energy dependent on their charge and the applied voltage[3]. In this way, ions with the same charge acquire the same amount of kinetic energy, independent of their mass[3]. The acceleration occurs in the direction of a flight

tube. Within this flight tube ions are detected at different flight times. That is, they travel at different velocities because they have the same kinetic energies and different masses[3]. This allows for separation of different fractions over time. We will attempt to identify the residue of interest by analyzing the different peaks within the wild-type and mutant spectra. Specifically we will look for peaks differing in mass by about 16 Da. This value represents the mass difference between the native phenylalanine and the mutant tyrosine. Of course, we will also look out for slightly greater or slightly smaller mass separation, 16 ± 2 Da. To improve the resolution of our spectra we included an in line high pressure liquid chromatography column. This further purified our protein sample and should reduce peak broadening.

We will take a similar approach in our second experiment. There are; however, two key differences. First and foremost, we will be utilizing MALDI-TOF. Matrix assisted laser desorption/ionization (MALDI) is similar in effect to ESI, but is achieved and analyzed differently. In MALDI the protein is mixed with trifluoroacetic acid and a matrix, in our case sinapinic acid, before being hit by a brief laser burst[3]. This high energy radiation explosively evaporates the charged protein molecules[3]. Our protein molecules are now charged and gaseous, and thus can enter the time-of-flight mass analyzer. It is important to note that MALDI analysis typically produces singly charged ions, while ESI analysis can yield many different ion charges[3]. Before the sample is mixed with the matrix, we will treat the protein with trypsin. Trypsin is a common analytical enzyme that hydrolyzes peptide bonds after specific residues, lysine and arginine[4]. We will use the sequences of these fragments to calculate the expected mass of each amino acid sequence[5]. Since the protein sequence of the two urate oxidases differ by one, we can determine the amino acid chain that correlates to the phenylalanine and the tyrosine in their respective proteins. This allows us to definitively determine the presence of our mutation.

Our third experiment is designed to elucidate the mechanistic pathway of the urate to allantoin. We plan on accomplishing this by using ESI-TOF mass spectrometry on the reaction of urate oxidase with urate. We will assign molecule structure based on the measured m/z values and the intensity of said values at various times. We collected the spectra of the following three blanks: the solvent, the solvent and urate, and the solvent and urate oxidase. By doing this we can eliminate many of the irrelevant peaks during our analysis. This greatly simplifies accomplishing the task at hand. We will use previously collected data to confirm or refute our results.

Methods:

Experiment 1:

The data for experiment 1 was collected in two different ways:

The mutant data used dialyzed mutant urate oxidase from 2021 that was diluted 1:1 in H_2O. It was collected in a Waters Xevo G2-XS QTOF mass spectrometer and purified in a Waters BEH C_{18} UHPLC column at 40 ℃. The acetonitrile was added as a gradient from 0 % to

60 %. The spectrometer was set to ESI-TOF, positive ion mode, and a range o f 2000 m/z. The capillary voltage was approximately 0.5 kV.

The wild-type data used dialyzed wild-type urate oxidase from 2012 that was diluted 1:1 with H_2O. It was collected in a Waters Xevo G2-XS QTOF mass spectrometer and purified in a Waters BEH C_{18} UHPLC column at 40 ℃. The acetonitrile was added as a gradient from 0 % to 60 %. The spectrometer was set to ESI-TOF, positive ion mode, and a range o f 4000 m/z. The capillary voltage was 2.0 kV.

Analysis of our data was fairly straightforward due to the use of both Mathematica and MestReNova. In particular, we analyzed the raw instrument data using MestReNova and the converted dx files using Mathematica. We determined potential peak masses in the first experiment using the ESI charge determination equations:

$$m_1 = (M+n)/n$$

$$m_2 = (M+n+1)/(n+1)$$

Experiment 2:

The data for both the mutant and the wild-type were collected after digestion with Trypsin in 10 mM ammonium bicarbonate for 2 hours. Both were spotted with sinapinic acid and collected in the Bruker MALDI Instrument. Analysis of the trypsin fragments was performed automatically.

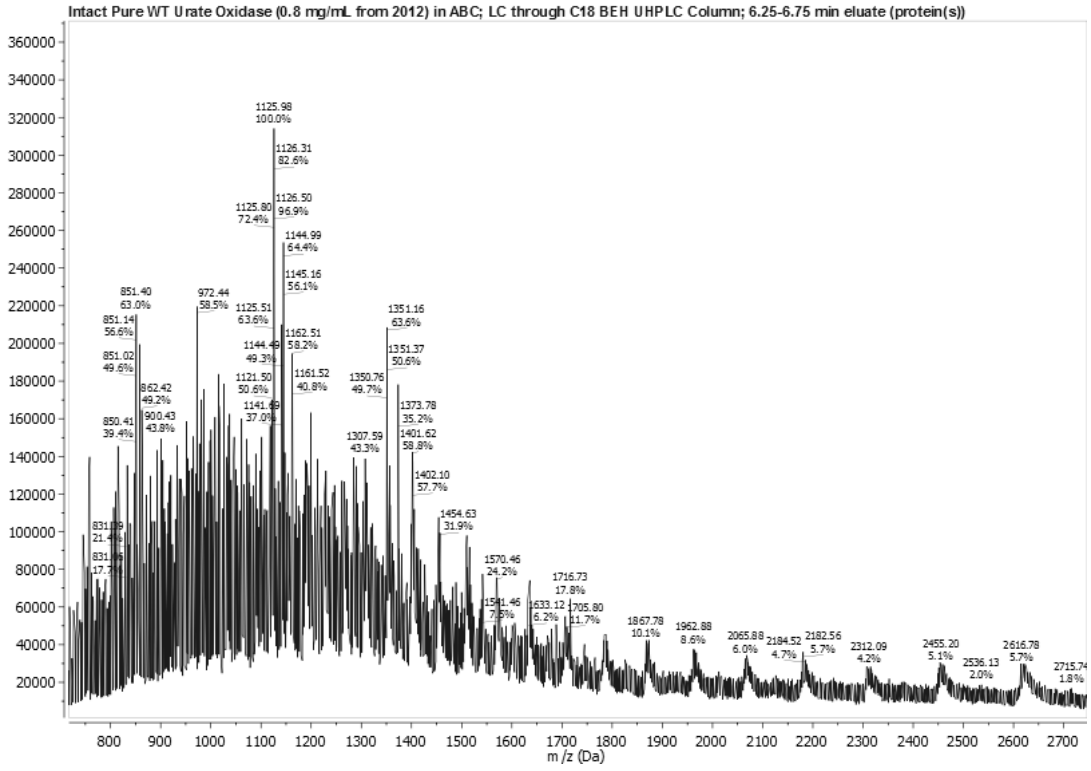
Experiment 3:

400 µL of 20-30 µM urate was mixed with µL of active F182Y urate oxidase. Acetonitrile was added after the reaction had proceeded for two minutes. Negative ion mode was used in the ESI-TOF instrument and spectra were then collected at 4 min, 7 min, 15 min, and 20 min.

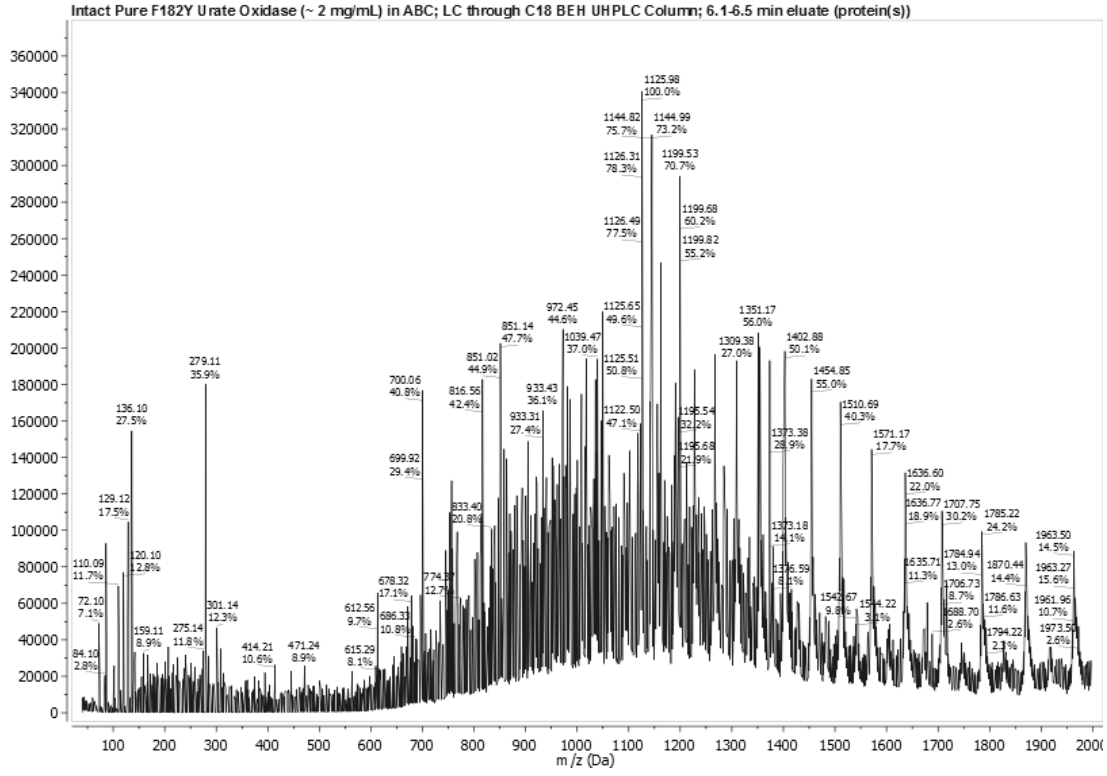
Results:

Experiment 1:

For experiment 1 of our mass spectrometry analysis of mutant urate oxidase, we collected the two ESI-TOF spectra shown below.



a)



b)

Figure 1: ESI-TOF Spectra

The ESI-TOF mass spectrum of the wild-type urate oxidase is given in part a, while the mutant spectrum is given in part b.

Shane Kalani Abbley

Experiment 2

In experiment 2, we obtained the following MALDI-TOF mass spectra and compared their peaks to the expected masses as predicted by the tryptic digest table below.

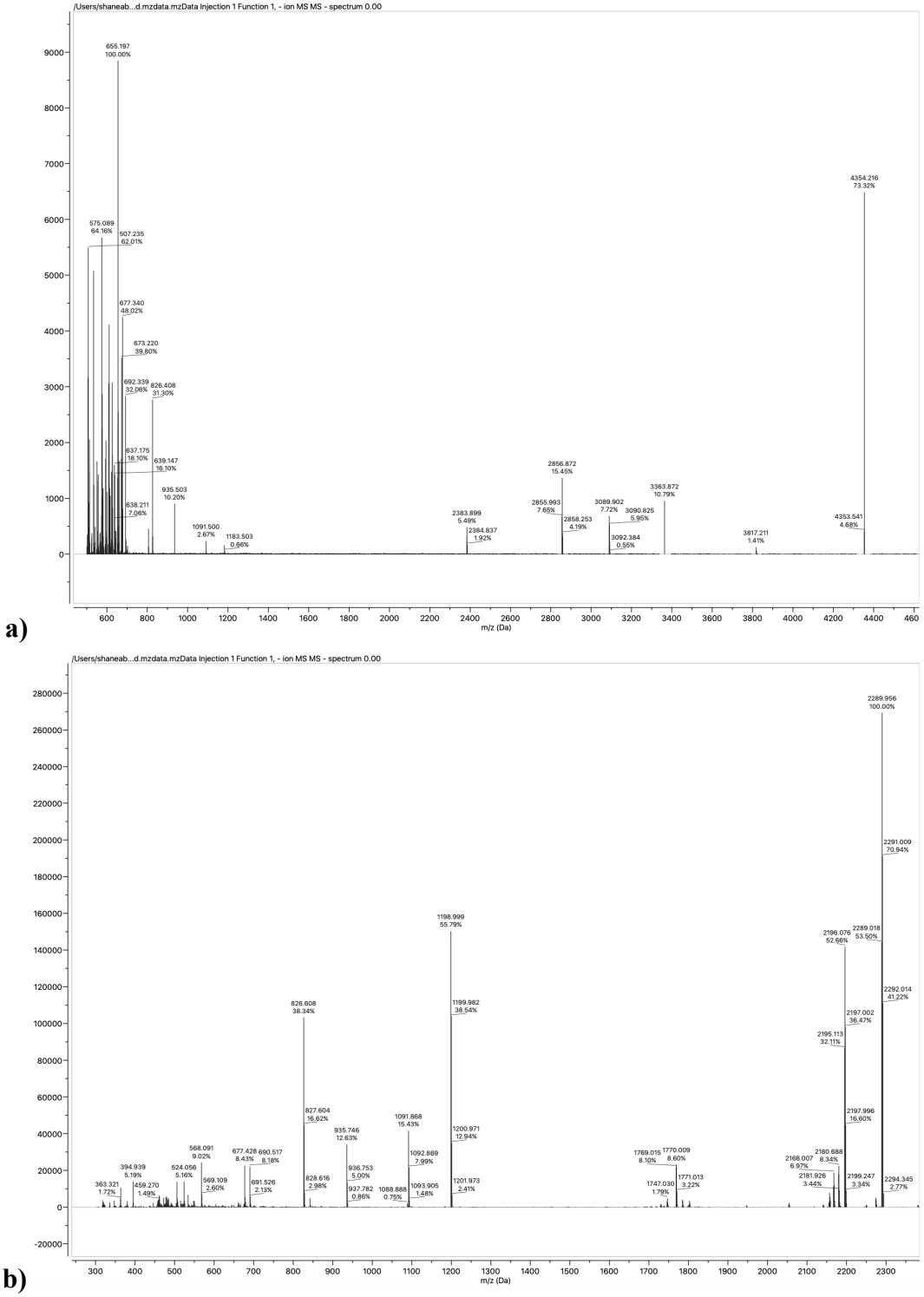


Figure 2: MALDI-TOF Spectra

The MALDI-TOF mass spectra for the wild-type and F182Y mutant are shown in parts a and b, respectively.

Residue Numbers	Tryptic Fragment for WT Urate Oxidase	Monoisotopic MW	Average MW	PI
1-18	MGSSHHHHHHSSGLVPR	1898.88191	1900.05211±0.12517	9.6
18-25	GSHMLEMK	931.42556	932.12438 ±0.07289	6.75
26	R	174.11168	174.20147 ±0.01033	9.75
27-32	TMSYGK	685.31051	685.79171 ±0.05052	8.26
33-39	GNVFAYR	825.41334	825.91083 ±0.0538	8.75
40-49	TYLKPLTGVK	1118.66995	1119.35341 ±0.07543	9.7
50-59	QIPESSFAGR	1090.54072	1091.17472 ±0.06886	6
60-96	DNTVVGVDVTCEIGGEAFLPSFTDGDNTLVVATDSMK	3815.77575	3818.15795 ±0.2544	3.57
97-101	NFIQR	676.36566	676.76455 ±0.04369	9.75
102-122	HLASYEGTTTEGFLHYVAHR	2288.09743	2289.46027 ±0.14691	6.27
123-150	FLDTYSHMDTITLTGEDIPFEAMPAYEEK	3363.52031	3365.69481 ±0.23044	3.95
151-156	ELSTSR	691.35007	691.73147 ±0.04188	6.1
157-160	LVFR	533.33257	533.66351 ±0.03663	9.75
160	R	174.11168	174.20147 ±0.01033	9.75
161-162	SR	261.1437	261.2788 ±0.01517	9.47
163-166	NER	417.1972	417.41811 ±0.02421	6
167-168	SR	261.1437	261.2788 ±0.01517	9.47
169-172	SVLK	445.29003	445.5537 ±0.02961	8.47
173-175	AER	374.19138	374.39328 ±0.02228	6.05
176-196	SGNTITITEQYSEIMDLQLVK	2382.19884	2383.6722 ±0.158	4.14
197-207	VSGNSFVGFIR (F182Y)	1181.61931	1182.32815 ±0.07713	9.72
208-444	DEYTTLPEDGNRPLFVYLNISWQYENTNDSYASDPAR	4352.97744	4355.55057 ±0.27601	3.9
245-252	YVAAEQVR	934.48723	935.03541 ±0.05982	6
253-277	DLASTVFHELETPSIQNLIYHIGCR (C253T)	2855.42761	2857.20242 ±0.19111	5.27
278-281	ILAR	471.31692	471.59456 ±0.03136	9.75

Figure 3: Tryptic Digest Map

This table gives the expected amino acid chains of our sample after digestion of our urate oxidase enzyme with trypsin.

Experiment 3

The data for experiment 3 consists of our first and last ESI-TOF spectra after letting urate oxidase react with urate as well as a chart containing noteworthy peaks from the four measured time frames.

m/z value	139.052	147.048	158.905	159.922	167.049	183.046	184.047	206.116	207.118	227.014
4 min	27,830	17,864	119,122	15,862	65,942	568,328	42,416	313,388	32,293	17,405
7 min	23,628	20,766	109,861	14,510	65,433	522,831	40,205	297,004	33,251	9,278
15 min	14,525	9,432	108,836	8,646	50,288	323,431	25,679	211,824	21,055	7,691
20 min	9,382	6,418	99,667	5,638	48,623	263,829	8,081	214,299	22,825	5,415

m/z value	233.185	247.070	268.989	282.064	283.299	285.977	339.241	340.241	363.083
4 min	8,179	33,450	35,817	37,566	86,616	26,169	17,917	2,048	41,516
7 min	7,733	37,380	32,876	36,390	78,341	25,764	19,773	4,374	44,528
15 min	3,533	25,221	27,661	26,688	119,338	17,237	27,623	4,967	34,957
20 min	6,350	24,113	26,985	24,437	373,381	17,409	105,049	25,830	28,880

Figure 4: Table of Interesting Fragments

This table lists the intensities of unique m/z peaks at the four different times given. Peaks with both similar m/z values and with similar intensities to those seen in the blanks were removed. Peaks with high intensities are highlighted in blue.

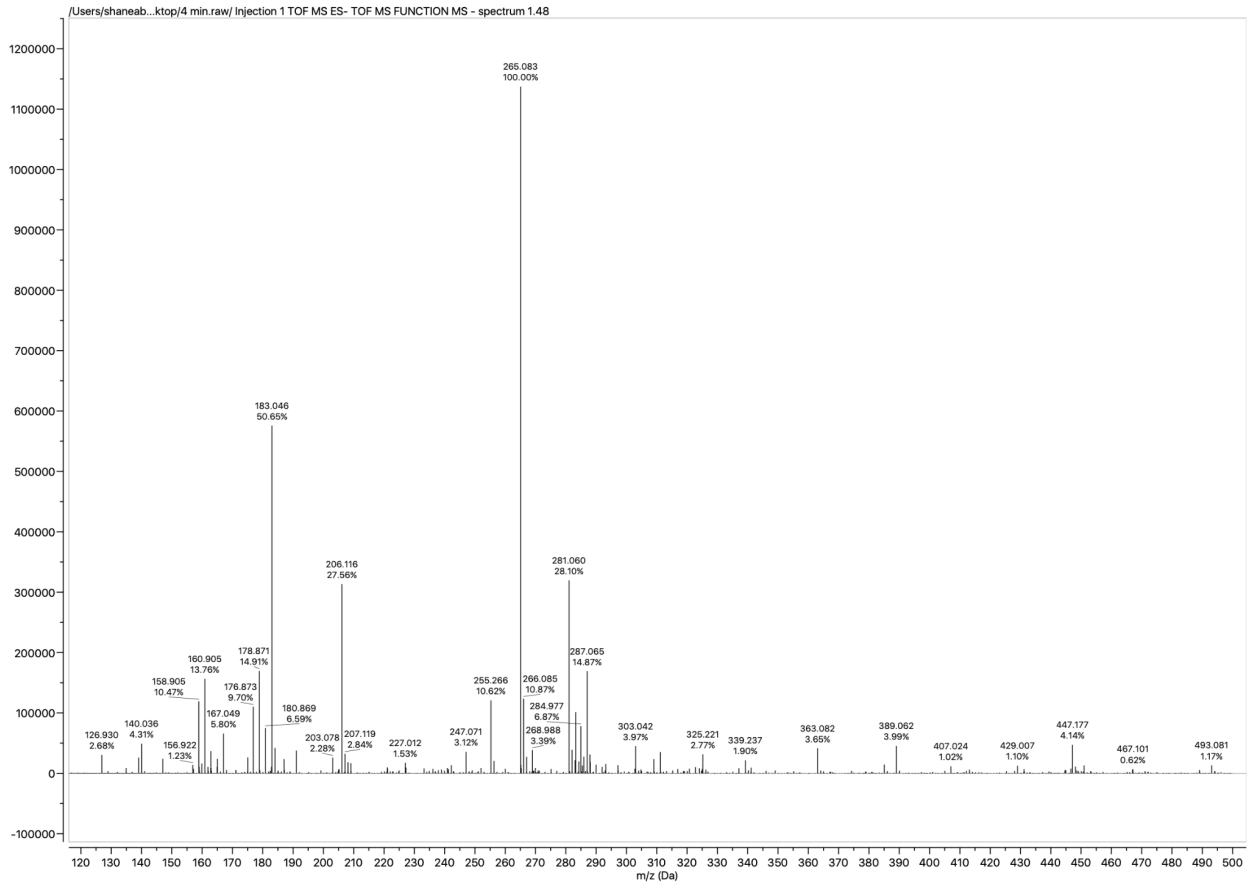


Figure 5: ESI-TOF of Urate Oxidase Reaction (4 min)

This spectrum shows the peaks of the urate oxidase reaction at 4 minutes in the ESI-TOF instrument.

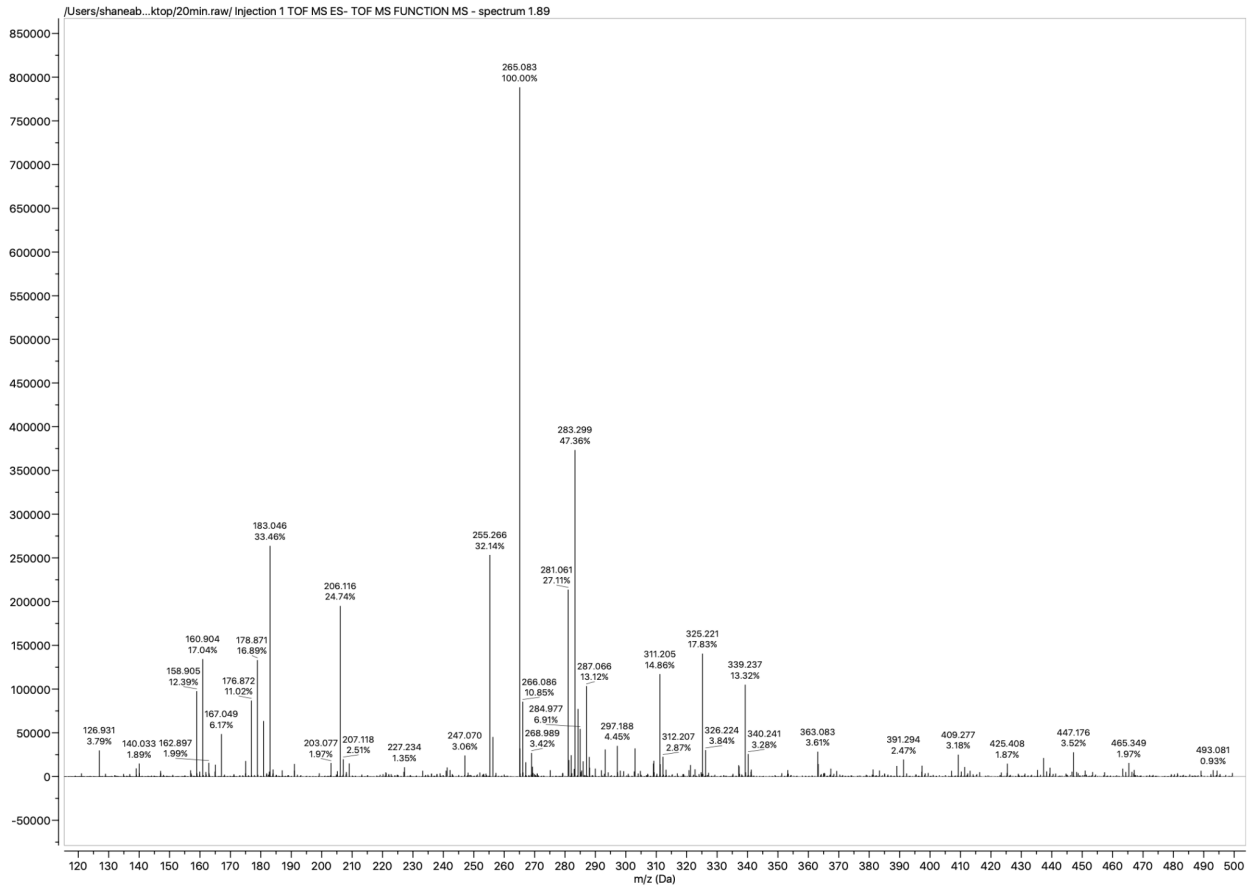


Figure 6: ESI-TOF of Urate Oxidase Reaction (20 min)

This spectrum shows the peaks of the urate oxidase reaction at 4 minutes in the ESI-TOF instrument.

Discussion:

Experiment 1:

We first attempted to determine whether the two spectra clearly represent the two different proteins. It did not appear that this was the case. Our group tried to determine the masses of similar peaks from each spectra that could represent similar fractionations in both experiments. That is, a peak in the wild-type spectra that corresponded to a molecular ion with a mass that was 16 Da less than the same peak in the mutant spectra. We found one at 1633.12 Da (figure 1a) in the wild-type spectra and 1635.71 Da (figure 1b) in the mutant spectra. These peaks corresponded to a charge value of 6 and thus a mass difference of 6 x (1635.71 Da - 1633.12 Da) = 15.54. This is close to what we were looking for; however, conclusions can not be drawn from this for several reasons. First, it was too far off of the desired value of 15.999. Secondly, there were several other peaks of similar differences and similar low intensities. Lastly, this data is inconsistent. It is possible that this peak is just representative of differences in fractionation because the resolution of the spectra is so poor. The main reason that these spectra

are unsuitable for quantitative analysis is that the conditions under which the experiments were performed were not the same. Of the different parameters used, the most significant are the different capillary voltages and the conditions in which the protein was prepared. Not only was the wild-type enzyme nearly 10 years older, but it was also prepared by completely different and likely equally inexperienced undergraduate students. This was a significant disadvantage of our experimental design because it introduced a lot of uncertainty. A properly designed experiment would do two things better. First, it would make the only independent variable be the sequence of the peptide and second, it would utilize MALDI-TOF spectra instead of ESI-TOF spectra. MALDI would be superior because it is easier to interpret the singly-charged cations than the variable charged cations in ESI. This is not because ESI does not provide the information required, but instead because the additional complexity becomes troublesome when the spectral resolution is poor. In order to improve the resolution, I would try using a quadrupole analyzer to separate the molecular ions by a small m/z range in a given data collection window[3]. This could, given a sufficiently small m/z range at a time, result in less peak overlap.

Experiment 2:

Our plan for experiment 2 data analysis was straightforward. We first performed a virtual trypsin digest (figure 3) to acquire the masses and sequences of each of the amino acid segments. We then identified which segment contained our amino acid of interest. We determined that the wild-type amino acid sequence would weigh 1181.62 Da when neutral and that the neutral mutated fragment would weigh 1197.62 Da, ~ 16 Da more. We found peaks directly next to this at 1183 Da (figure 2a) and 1199 Da (figure 2b). The peaks being off by a molecular weight of two is best explained by the addition of two protons each. This experiment does not have the uncertainty as described in the previous experiment because we used MALDI instead of ESI. We were expecting to observe m/z values that correlated directly with the mass of the segments and we did because a majority of the molecular ions had a charge of one. One improvement to this experiment would be to perform another digest with a different protease and measure the spectra again. This is not necessary because the data here is fairly conclusive, but it would strengthen the argument that we achieved the expected mutation.

Experiment 3:

Our results from this experiment were certainly the most convoluted and provided very little insight into the intermediates of the urate to allantoin pathway. That being said, we were able to identify the most critical molecules in the pathway. Allantoin was determined to be located at 158.905 Da and 5-hydroxyisourate was determined to be located at 183.046 Da. Two other possible structures were determined. These were: Guanidino Hydantoin anion + 5-Hydroxy-8-Oxoguanine acid is the likely structure at 339.241 Da and a 5-hydroxyisourate anion that lost a H-CO-NH fragment is probable at 139.052 Da. It is important that we found 5-hydroxyisourate because it is the product of the catalyzed reaction and we know that it has a half life of about 30 minutes[6]. This is not off by too much, but it appears that our half life is

closer to 15 minutes. This is likely because the negative ion mode used in the third experiment created a slightly basic environment in which 5-hydroxyisourate is less stable[7]. The allantoin strangely decreases throughout the reaction. This is likely a result of the reaction of anionic allantoin with other charged molecules, despite its continuous production. This is a challenging experiment to perform in general because of the tight time constraints. The intermediates between 5-hydroxyisourate and allantoin, like 2-oxo-4-hydroxy-4-carboxy-5-ureidoimidazoline (OHCU), were not observed because of the instability and thus short lived nature of the intermediates[8]. In order to encounter such an intermediate a different type of experiment is required, specifically one that is more time sensitive. One possible experimental improvement would be to utilize an Ion Cyclotron Resonance Analyzer because it allows for detection of multiple m/z values in a single timeframe. We also waited at least two minutes for the experiment to proceed before we collected measurements. Doing this allowed all the urate to be consumed, but certainly caused us to miss the intermediates between urate and 5-hydroxyisourate.

Conclusion:

Overall, we successfully used MALDI-TOF spectra to determine the presence of our mutation in conjunction with a tryptic digest. Our ESI-TOF experiments were not as successful. We determined some of the key molecules in the mechanistic pathway between urate and allantoin, but we did not acquire data sufficient to distinguish short lived intermediates. Several improvements would have resulted in spectra better suited for the purposes of this experiment.

References:

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