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The Catalytic Effect of D68N Mutant in Thermococcus Thioreducens Inorganic Pyrophosphatase

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Abstract

Inorganic Pyrophosphatase (IPPase) from *Thermococcus thioreducens* catalyzes the hydrolysis of Inorganic Pyrophosphatase to produce Orthophosphate. Even though IPPase is a universal enzyme, the mechanism of catalysis is not completely known. Aspartate 68 (D68) is one of 13 conserved residues in the active site of IPPase across all domains of life. The function of D68 in the mechanism of catalysis is not clear. To elucidate the of D68, a recombinant mutant of IPPase containing and Asparagine in place of Aspartate at position 68 (D68N) was isolated and purified. The catalytic activity of the D68 mutant will be compared with that of the wild-type. By coupling molecular modeling and enzymatic studies of D68N mutant, the structural and functional role of Asp 68 can be further understood.

Introduction

Inorganic Pyrophosphatase (IPPase) is an important enzyme found universally throughout all organisms. The importance of this enzyme is in its ability to regulate the concentration of inorganic pyrophosphate by hydrolyzing organic pyrophosphate. Inorganic pyrophosphate is a byproduct of many biosynthesis reactions like protein synthesis and if left unregulated would lead to an imbalance of the cell's physiology. IPPase is present in organisms in three different nonhomologous categories: Family I, Family II, and Family III. Even though the enzymes perform the same type of catalysis, there are dissimilarities in both structure and mechanism. For the purpose of this study, Family II is studied to effectively decipher the mechanism through mutagenesis.

Family II is structurally and mechanistically different from the other Families and is only found in archaebacteria and bacterial lines including human pathogenic bacteria (Tommi et al. 2013), (Baykov et al. 2017). The distribution of sequenced Family IPPase II has revealed 499 in Firmicutes and 228 found in Proteobacteria (Baykov et al. 2017). Also, due to only being recently identified in the past 20 + years, some mechanistic properties are still ambiguous, but the structure is known. Family II IPPase is a homodimer and the protein's active site is in a conserved region of Asp-His-His motif that holds the crucial metal ions of Mn^{2+}. This conserved region is conserved motif found in the "DHH phosphoeterase superfamily" that consisted of multiple other enzymes like single-stranded DNA exonuclease (Baykov et al. 2017).

The current uncompleted mechanism begins with a water molecule on the DHH domain laying a nucleophilic attack on the electrophilic phosphate group (Baykov et al. 2017). This is done by the unique center made by the water and the three metal ions causing a rearrangement of the water molecule rearranging 1 Å from the binding of the electrophilic phosphate substrate. This stabilizing rearrangement could only be done with metals. The whole mechanism is done cleanly without an intermediate, which is common throughout the other two families

It is suggested that the enzyme's use of Mn^{2+}, with its higher affinity to the active site, instead of Mg^{2+} is the reason for the higher rate of catabolism kinetically at kcat $\cong 2000s^{-1}$ (Tommi et al. 2013). Due to the differences in the structure of Family II IPPase, it can be safely be assumed that the mechanism of the catalysis is different. It is suggested because of the various metal ion coordination differences; the hydrolysis is done with a "dissociative mechanism" rather than an

associative mechanism. This mechanism is done by an electrophile with influencing the hydroxide ion of a water molecule for a nucleophile attack of the substrate.

The stabilization by the second metal is poorly understood and this is the area being studied by using point mutation of various parts of the conserved active site. Particularly the 68th amino acid, aspartate (D68), coordinates with the pyrophosphate to activate the substrate into an electrophile to be stabilized by the metal ion in question. By mutating the aspartate, the occupation and importance of the amino acid to the mechanism with the metal ion stabilization will be better understood. This study focuses on the mutation of D68 to an asparagine (D68N) switching the original negative side chain to a positive side chain in hopes of observing a change in the mechanism through an enzyme activity assay.

IPPase Family II is found in some pathogenic bacteria and by understanding the catlysism and possible inhibition of enzyme, it would be possible to manipulate. By manipulating the enzyme with the mechanism and function, it might become increasingly possible to bring about another defense against pathogenic diseases in this era of antibiotic resistant bacteria.

Materials and Methods

Transformation

Firstly, a plasmid that contains both antibiotic resistance against chloramphenicol and carbenicillin as well as the DNA sequencing for *Thermococcus thioreducens* Inorganic Pyrophosphatase (IPPase) is added to a solution of competent E. coli cells. This DNA sequence would also contain the antibiotic resistance that would ensure growth of the expressing bacteria. These cells are kept in a -80 ℃ freezer and must thaw over ice for one-time use. The DNA sequence is added in a 1:25 volume: volume ratio with the cells and allowed to incubate on ice for 20 minutes. Once these cells are incubated, they are placed in a 42 ℃-heat shock to activate the cells for DNA acceptance. Then the cells are suspended in 1 mL LB Miller broth which will supply the nutrients necessary for the cell's growth. This growth takes place in a 37 ℃-water bath for 45 minutes. This now turbid cell solution then has 1 mL poured over an agar plate that contains 50 µg/mL carbenicillin and 30mg/mL chloramphenicol. Cells were then allowed to grow over night on the plates.

Small Scale Inoculation

Upon formation of cell colonies, production of IPPase must be screened for. Half the colony was inoculated into 25 mL LB Miller media with the same antibiotic concentration as the agar plate. Once the cells were grown, protein was roughly purified through heat cut and protein expression of the different colonies were portrayed through an SDS-PAGE. When overexpression of IPPase at the 21 kDa range was present, the other half of the colony would be used for a large scale inoculation.

Large Scale Inoculation

Half of the colonies were inoculated each into 125 mL volumetric flasks containing 50 mL of sterilized LB miller broth, 50 mg/mL chloramphenicol, and 100 mg/mL carbenicillin. The flasks

were left to incubate at 37 ℃ in a shaker overnight. After 21 hours, 10 mL of the now turbid solution is transferred to a 4000 mL volumetric flask containing 1000 mL of sterilized LB miller, 50 mg/mL chloramphenicol, and 100 mg/mL carbenicillin. The flasks were left to be incubated at 37 ℃ in a shaker. The absorbance was checked at every hour at 600 nm to determine the moment before the bacteria start their exponential growth estimated to be at an absorbance of 0.300. After 3 hours and 20 minutes, bacteria solution reached desired absorbance. At this time, the temperature was dropped to 30 ℃ and the operon IPTG was added for an overall concentration of 0.5 mM. The solution was left to shake overnight.

Lysing Bacteria

The turbid solution transferred into four 250 mL centrifuge bottles and balanced. The solution was centrifuged down at 6000 rcf at 25℃ for 7 minutes. After centrifuging, bacterial pellets were collected and weighed into no more than 10 g pellets and plastic wrapped. The 10g mass was found to be the optimal size for protein concentration while not overloading a 40 mL anion exchange column. The pellet was stored at -80℃ overnight. The pellet was unwrapped frozen and resuspended into 50 mL lysis buffer of 25mM NaCl, 2 mM EDTA, 1 mM PMSF, 0.1 mg/mL lysozyme, and 50 mM HEPES pH 7.5. Once resuspended, the bacteria were further lysed using sonication for 10 minutes, 10 seconds on and 20 seconds off cycles until solution is homogeneous. The sonicated solution is transferred into round bottom centrifuge tubes and centrifuged for 1 hour at 4 ℃ and 6000 rcf. The supernatant was collected, and the cell debris pellet disposed.

Heat Cut

The supernatant was transferred into a 125 mL volumetric flask, where a DNase stock solution was added at 500 µL per 100 mL. Then, the solution was allowed to incubate at room temperature for 30 minutes. Afterwards, the flask was placed in a 72 ℃-water bath for 45 minutes. The heat cut solution is transferred into round bottom centrifuge tubes and centrifuged for 1 hour at 4 ℃ and at 6000 rcf. The supernatant is collected into conical tubes and the pellet disposed.

Anion Exchange Column

A 160 mL Q-sepharose column was charged with a resolving buffer consisting of 50 mM HEPES buffer pH 7.5, 50 mM NaCl, 1 mM PMSF, and 1 mM EDTA at a 2 mL/ minute flow rate. Once the column was charged the protein sample was loaded onto the column and a linear gradient buffer of 10 times column volume switch was started. This buffer switch consists of a change from the resolving buffer to an identical 1 M NaCl concentration elution buffer. During this linear gradient, fractions of a 10 mL volume were collected and tested in a nanodrop as well as an SDS-PAGE for protein concentration. These tested fractions would later be combined and concentrated for size exclusion chromatography.

Size Exclusion

The samples are concentrated down by spinning down the solution in a 10 kDa concentrator tube that is spun down in a centrifuge at 4 ℃, 5000 rcf for 20 minutes. The protein sample is

concentrated down to a volume of 500 µL before being carefully pipetted evenly onto a 120mL S100 size exclusion column. A 25 mM HEPES 100mM NaCl buffer was ran through the column to both charge it before the sample was loaded, and run with the sample both at 1 mL/min. Once the concentrated sample was loaded onto the column 10 mL fractions were collected and tested on the nanodrop for protein concentration.

Enzyme Activity Assay

For the assay, multiple reagents were made. Reagent A was made with 50mM Tris-HCl solution brought to basic pH of 9.0 with concentrated 15 M NaOH. Reagent B is a 10 mM Sodium Pyrophosphate solution that was made with Pyrophosphate tetrasodium. Reagent C is a 10 mM Magnesium Chloride solution. Reagent D is a 10% Ammonium molybdate solution in 10N Sulfuric acid. This solution was made by diluting concentrated sulfuric acid 36N to 10N and dissolving 5g of Ammonium molybdate in 10N sulfuric acid diluting to 50 mL. The last reagent, reagent E, dissolves 5g of Ferrous sulfate in 10 mL of Reagent D and diluting to 100mL with deionized water.

First, the standard curve was made using a 96 well plate. Deionized water was added in decreasing increments of 1µL starting at 150 µL and ending the 11^{th} well with 140 µL. the substrate reagent, 10nmol/µL Sodium Pyrophosphate, was added at increasing increments of 1 µL starting the first well at 0 µL ending the 11^{th} well at 10 µL. The last Reagent added was Reagent D with 150 µL in each plate to total each well at 300 µL. Afterwards, the wells were incubated at room temperature for 10 minutes. After the incubation, the well plate was assayed with a spectrophotometer and read at an Absorbance of 660nm. The data was recorded and graphed.

The samples were constructed in Eppendorf tubes before being transferred to 96 well plate as well using the viable fractions from the size exclusion. The solution was arranged with 40 µL of the Reagent A added in 0.5 mL Eppendorf tubes followed by 10 mL of Reagent B and 10 mL of Reagent C. Lastly, 1 µL of the enzyme sample from the size exclusion fraction was added and the solutions were incubated for 30 minutes in a 72ºC water bath. The blank was made similarly but with 41 µL Reagent A and no enzyme sample. After the incubation, 145 µL of deionized water was added to the wells along with 5 µL of the incubated sample and 150 µL of Reagent E for a final volume of 300 µL in each well plate. The plate was incubated at room temperature for 10 minutes before being assay with a spectrophotometer at an Absorbance of 660 nm. The value were recorded and the Specific Activity was calculated.

Results

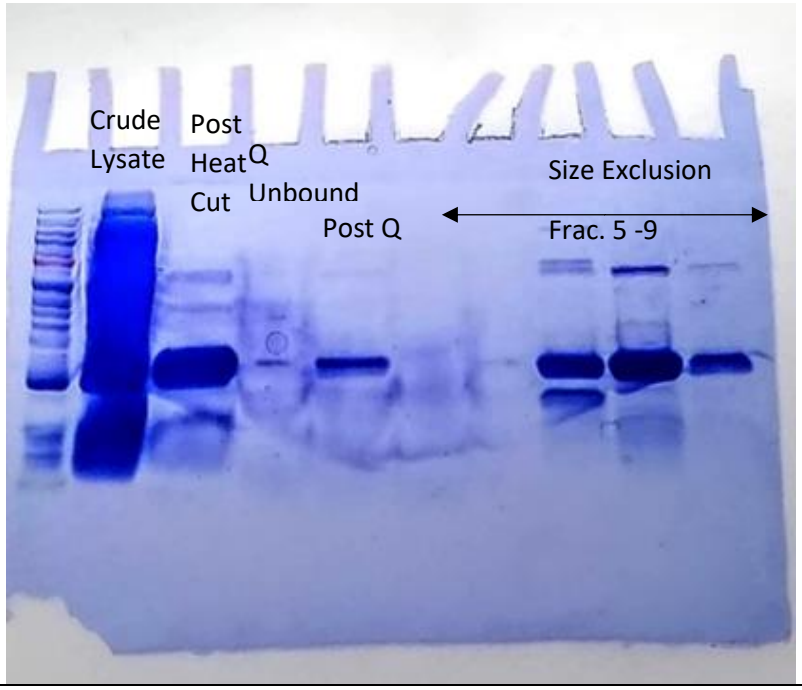

Figure 1 SDS – PAGE for Wild Type IPPase containing protein expression for Crude Lysate, Post Heat Cut, Unbound from Anion Exchange, Post Anion Exchange, and Size Exclusion Fractions

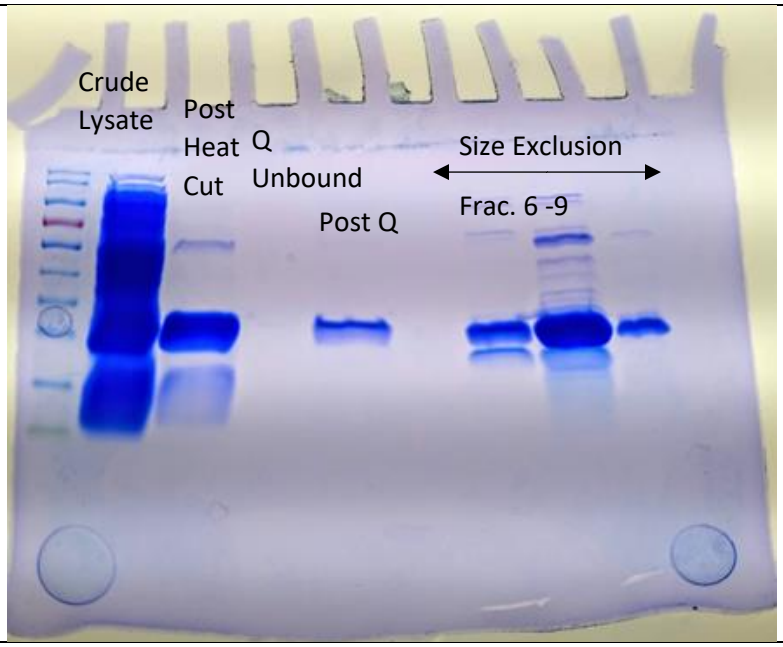

Figure 2 SDS – PAGE for D68N IPPase containing protein expression for Crude Lysate, Post Heat Cut, Unbound from Anion Exchange, Post Anion Exchange, and Size Exclusion Fractions

In an SDS - PAGE, protein is denatured and run through the gel. Tt IPPase is found in the 21 kDa region. In both **Figure 1 and 2**, the protein purification process appear qualitatively to be working with the band for IPPase becoming more concentrated

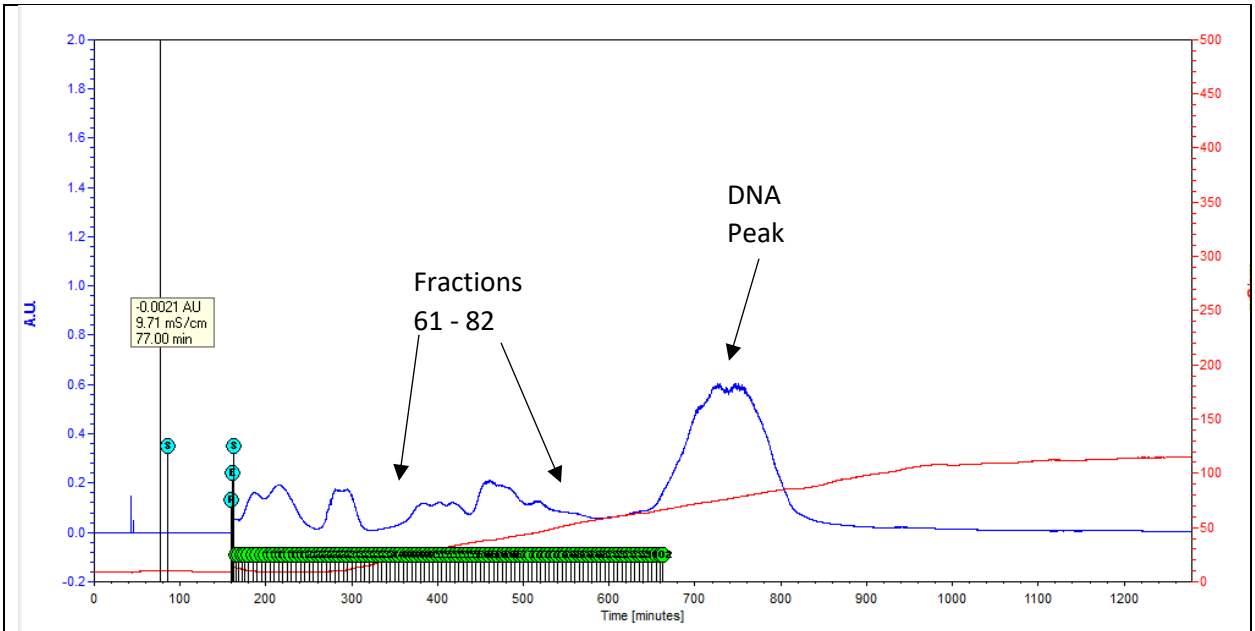

Figure 3 Anion Exchange purification of Wild Type IPPase portraying the Fractions that were eventually combined and the separation of the DNA contamination.

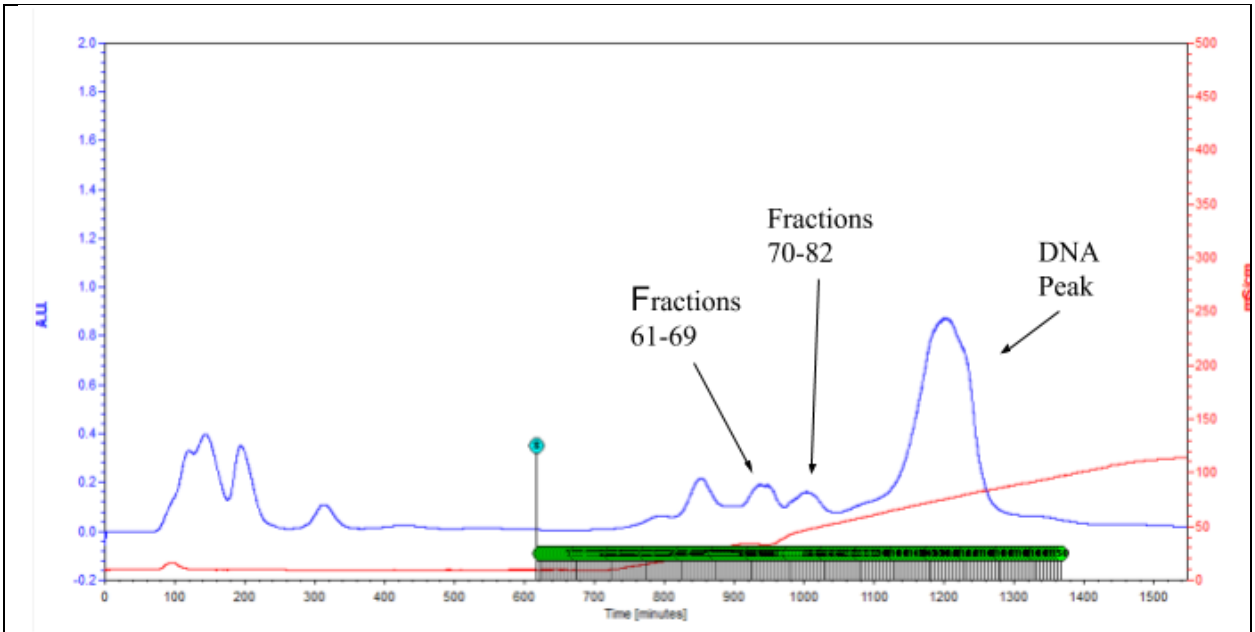

Figure 4 Anion Exchange purification of D68N IPPase portraying the Fractions that were eventually combined and the separation of the DNA contamination.

With the peaks being cut off due to machine limitations, nanodrop readings and SDS-PAGE of each fractions was necessary for determining which fractions would be conserved for size exclusion. A high A280/260 ratio would signify high protein presence. A280 refers to absorbance of UV light that is emitted by tryptophan amino acids at a wavelength of 280 nm, while A260 refers to an absorbance due to nucleotides. Those with high IPPase (21 kDa) expression were concentrated down for size exclusion gel filtration.

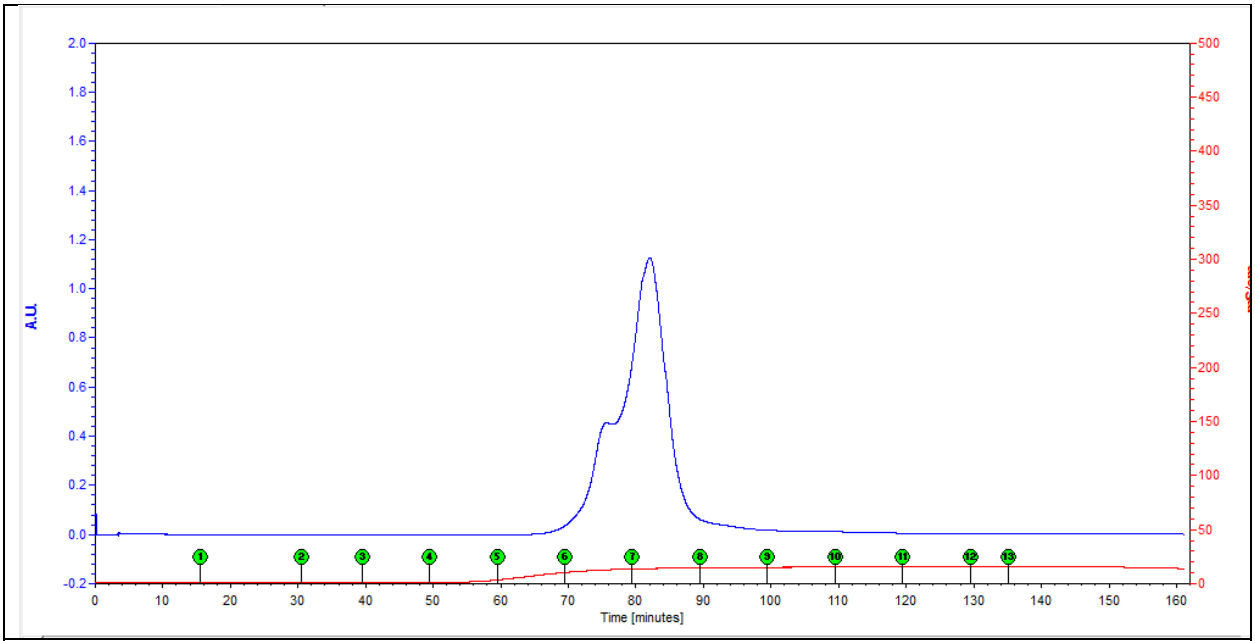

Figure 5 Size Exclusion Purification Wild Type IPPase

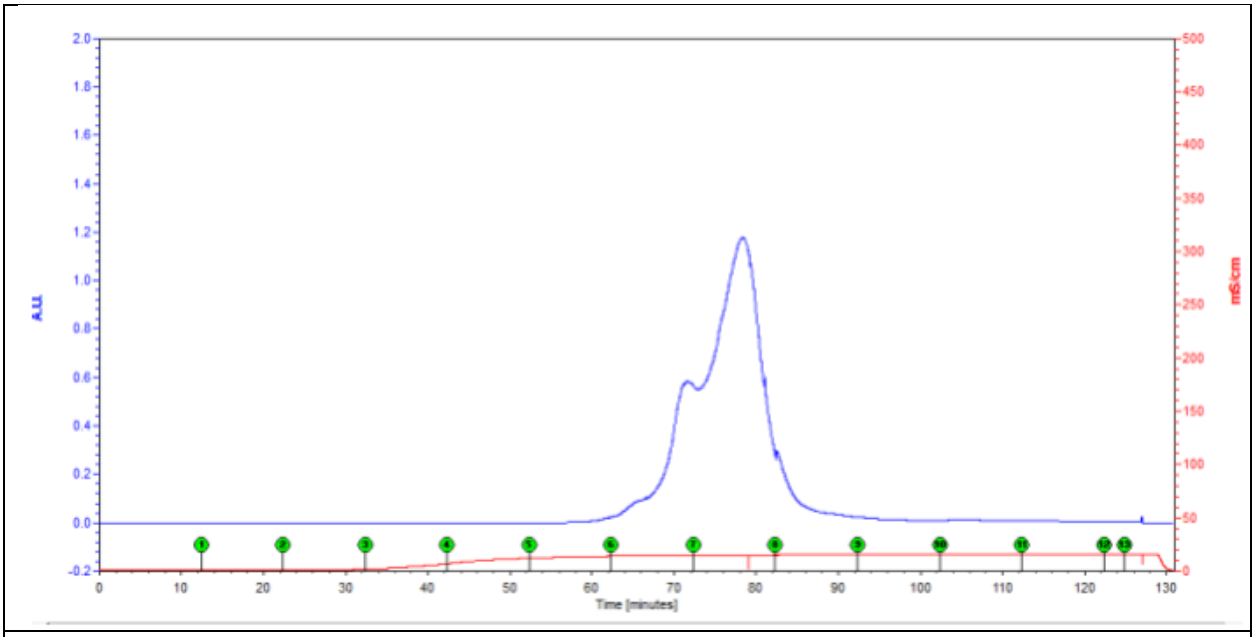

Figure 6 Size Exclusion purification D68N IPPase

An S100 chromatography column separates proteins based on weight up to 100 kDa, allowing larger proteins to flow through and be collected in earlier fractions, while smaller proteins would pe trapped in the gel and take longer to elute off. Nanodrop and SDS-PAGE of the fractions determined where the IPPase had eluted off. Both **Figure 5 and 6** have peaks eluting at the same time identifying the exact time protein will likely elute off.

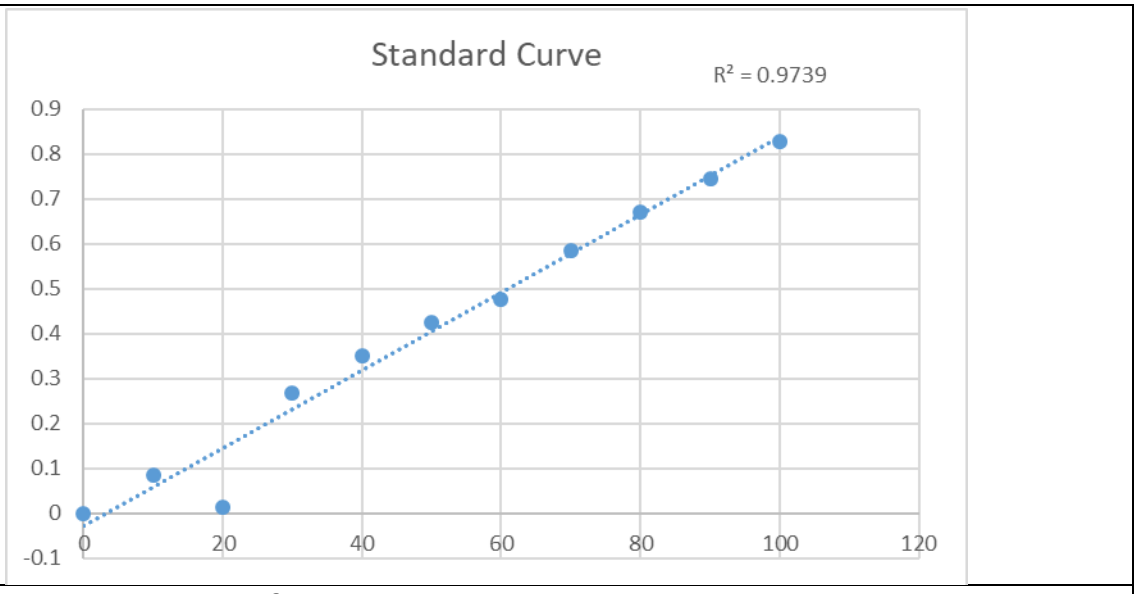

Figure 7 Standard Curve of Enzyme Activity Assay

Samples	**Blank**	**WT Fraction 7**	**WT Fraction 9**	**D68N Fraction 7**	**D68N Fraction 8**	**D68N Fraction 9**
A_{660}	0.000	0.141	0.141	0.150	0.134	0.145
Concentration (µmol)	-	19.709	19.709	20.756	18.895	20.174
Specific Activity (µmol/min)	-	120.227	120.227	126.610	115.262	123.0

Table 1 Values of Enzyme Activity Assay

Discussion

In this study, an enzyme activity assay was used as the determinant of the possible activity or inactivity of the mutated D68N IPPase compared to the Wild Type IPPase. As seen in **Table 1**, there is a difference in activity between the mutant and the Wild Type, but their cannot be any hard conclusions on the activity of the enzyme, There appears to be a change catalysys of the substrate, Inorganic Pyrophosphatase, but there is not a clear sign of complete inactivity of the D68N enzyme. Without the inactivity, it maybe possible that the D68 amino acid is not critical in the stabilization of the metal ion meaning that another study must be made mutating another amino acid in the conserved active site to distinguish the mechanism of the hydrolyzation of inorganic pyrophosphatase with IPPase.

Reference

Baykov, Alexander A., Viktor A. Anashkin, Anu Salminen, and Reijo Lahti. 2017. “Inorganic Pyrophosphatases of Family II—two Decades after Their Discovery.” *FEBS Letters* 591 (20): 3225–34. https://doi.org/10.1002/1873-3468.12877.

Tommi, Kajander, Kellosalo Juho, Goldman Adrian, Alexander Gabibov, Vladimir Skulachev, Felix Wieland, and Wilhelm Just. 2013. “Inorganic Pyrophosphatases: One Substrate, Three Mechanisms.” https://doi.org/10.1016/j.febslet.2013.05.003.