Determination of Multimerization and the Extent of Biotinylation in a Geobacillus stearothermophilus Sulfurylase

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Abstract

In an attempt to advance pyrophosphate sequencing, a next generation sequencing technique, the multimeric state of *Geobacillus stearothermophilus* (Bst) Sulfurylase, an important enzyme in the technique, was investigated along with the extent in which it was biotinylated. The enzyme was determined to be a monomer with evidence of a small percent of enzyme possibly participating in higher order complexes. Between 45% and 57% of the enzyme was biotinylated. The presence or absence of the biotin group did not affect the enzymatic level of activity as evidenced by the extremely similar specific activities determined for both the affinity purified fractions and the non-purified controls. The investigation led to many discoveries and determined ways of improvement moving towards a more efficient technique, such as increasing the percent biotinylation and by changing the storage procedure to increase the enzyme shelf life.

Acknowledgements

Great recognitions go to Bill Lyon, Tim McConnell, and John Leszyc for assisting in making this project and partnership between WPI and Roche Scientific possible.

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Background

Next Generation DNA Sequencing

The ability to rapidly sequence an organism's full genome is an extremely important development that is being made. By decreasing the cost, time and availability of full genomic sequencing the way the health care and research field's function would be revolutionized. With rapid genomic sequencing prenatal assessments could be made, such as determining if children have any conditions allowing the parents and the doctors to take immediate action. Additionally, the knowledge and analysis of a larger number of genomes allows for further understanding of the effects of specific genes allowing for a more competent understanding of our genome to be achieved. Rapid sequencing would also have a large effect on human genetics and genomics, Plants and Agriculture, and Environmental Genomics. Rapid sequencing also allows for a decreased response time and allows one to limit the bacterial outbreaks, allowing the CDC to provide a solution to these outbreaks and minimize repercussions.

Originally DNA sequencing was a manual process that could only identify the sequence of incredibly short regions of DNA. The process involved radiolabeling the fragment of DNA, restricting it at all possible bases and then running the samples on very large gels. The technique was incredibly limited and did not allow for full genomic sequencing. The two original sequencing methods were the Maxam-Gilbert method and the Sanger method. The Maxam-Gilbert method was a sequencing procedure based upon chemical modification and subsequent cleavage of DNA at specific bases. The Sanger plus minus method of DNA sequencing was synthesis based and required that each read start be cloned for production of ssDNA. Although originally the Maxam-Gilbert method was the more popular method, because of its complexity and the dangerous chemicals needed to perform the sequencing, the Sanger method became

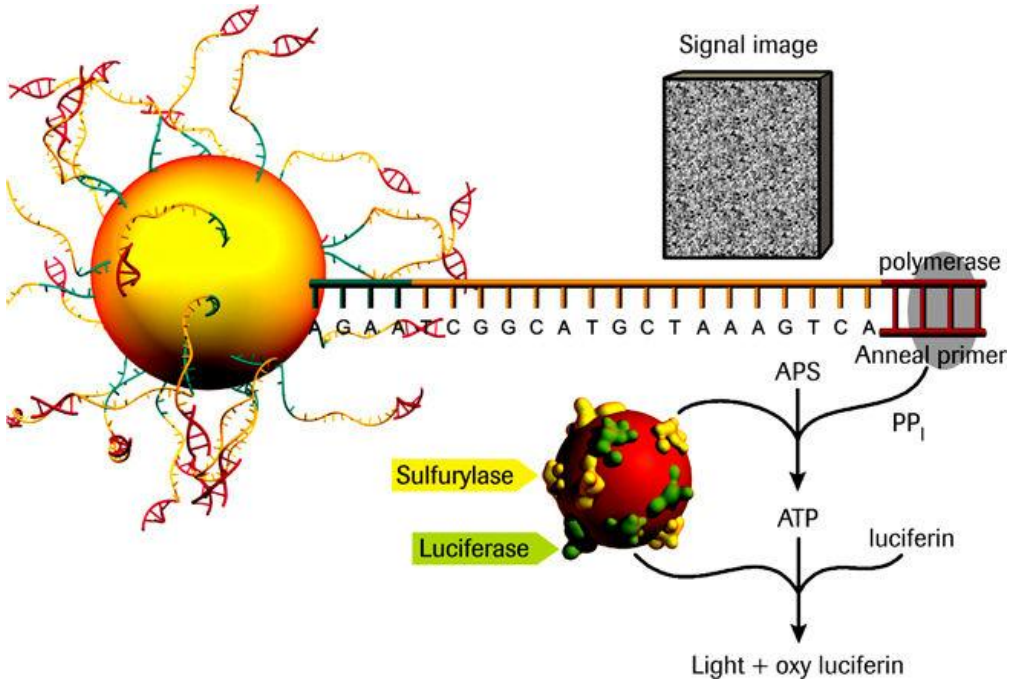
favored over time. When the scientific community came together to sequence the human genome with the Human Genome Project the original thought was that it would take 50 years to complete the process. The capillary sequencing technique was developed and was a higher performance protocol that used all the same principles that the Sanger method did. Instead of running the samples on gels, the samples were loaded into capillary tubes and they were pulled through agarose tubes and when they electrophoresis off the column, the band was read. The full genomic sequencing only ended up taking 11 years, but with this kind of time limitation and cost, sequencing could not be used as a viable form of research or prognosis.

The development of rapid full genomic sequencing is in high demand and new processes are being developed. One of these processes was Ion Semiconductor Sequencing which works by the process of reading the release of hydrogen ions in response to the incorporation of nucleotides onto a template strand via DNA polymerase. When a nucleotide is incorporated both a hydrogen ion is released and an inorganic phosphate. The increase in H+ ion concentration is read by an ISFET ion sensor. This method, although arguably the cheapest and fastest method, does not have the ability to deal with long stretches of the same base on the template strand because it is difficult to quantify the amount of ions released. Nanopore technology also would allow for rapid genomic sequencing. With this process a current is run across a membrane that has an embedded pore. When ions or molecules pass through this pore, a distinct disruption in the electric current occurs. These disruptions could identify different bases. By feeding sequences of DNA through this pore as it travels through one could determine the sequence of that strand live.

Pyrosequencing

454 Life Sciences, a Roche company, developed a next generation sequencing procedure called pyrophosphate sequencing. This technique allows for rapid full genomic sequencing. The

procedure relies on the functionality of two enzymes, Sulfurylase and Luciferase. The technique involves fragmentation of a genome via nebulization into 400bp fragments and subsequent amplification on beads. These beads, each of which has a clonal population of DNA, are then applied to a PicoTiterPlate Device which has 1.6 million wells. The plate is then washed with enzyme beads that have Sulfurylase and Luciferase enzyme conjugated to them. Then the plate is washed with polymerase and an annealing primer that is compatible with the untethered end of the DNA sequence.



The plate is then in turn washed sequentially with triphosphate nucleotides. If the DNA strands on the bead in the well encounter a complementary base it is incorporated and releases an inorganic pyrophosphate (PPi) which in conjunction with APS and Sulfurylase produce ATP which reacts with Luciferase to produce light. This light is recorded and associated with the base that was added and the sequence of the DNA is determined live. Any additional unincorporated nucleotides are degraded by the enzyme apyrase. In an attempt to advance pyrophosphate

sequencing the multimeric state of *Geobacillus stearothermophilus* (Bst) Sulfurylase, an important enzyme in the technique, was investigated along with the extent in which it was biotinylated.

ATP Sulfurylase

ATP Sulfurylase (MgATP: sulfate adenylytranspherase, EC 2.7.7.4), an enzyme that is expressed in all organisms, is responsible for the forward reaction of inorganic sulfate and ATP (MgATP) to form APS and PPi, [1]

This reaction is the first step in the metabolic pathway for the conversion of inorganic sulfate to organic sulfur compounds. In plants these products will be used to create reduced sulfur containing biomolecules, in animals they will be used to create sulfate esters, and in bacteria the APS product will be used as a terminal electron acceptor for anaerobic respiration.[2]

Structure

Many forms of ATP Sulfurylase have been characterized and crystallized already by the scientific community. More commonly, the enzyme has been crystallized as a hexamer, six homologue monomers that form two trimmers and those two trimmers stack onto one another to for the hexamer. The ATP Sulfurylase enzyme from Bacillus stearothermophilus, Roche Scientifics in house bacteria, has yet to be characterized or crystallized. The enzyme being from a thermostable bacteria probably means that, like the Sulfurylase from Thermus thermophilus, it has a more compact active site than other varieties of Sulfurylase, allowing it to maintain activity at higher temperatures.

Materials and Methods

Native and SDS-PAGE

Native Polyacrylamide Gel Electrophoresis was used to separate the Roche Scientific sulfurylase enzyme in order to determine the enzymes molecular weight, if more than one conformation of it existed in solution and if the enzyme was forming muiltimeric complexes. The samples were ran on 9% resolving gels(3ml 30% acrylamide-1%BIS, 3.5ml Common Buffer(Roche Scientific Titanium buffer), 3.4ml ddH_2O, .05mL Ammonium Persulfate, and 7µl TEMED) with a 4% stacking (.65ml 30% acrylamide-1%BIS, 1.25ml Common Buffer(Roche Scientific Titanium buffer), 3.05ml ddH_2O, 25µl Ammonium Persulfate, and 5µl TEMED component the gel had an overall thickness of 1mm. 35ml samples were used per well.

Denaturing Gels were made in the same fashion but also included SDS solution. The 9% resolving gel recipe is the same with the addition of .1ml of a 10% SDS solution and the 4% stacking gel was the same with the addition of 50 µl of a 10% SDS solution.

Sulfurylase Activity Assay

The Sulfurylase activity assay works using the same principles as Pyrosequencing. By pushing the naturally occurring sulfurylase reaction backwards to produce ATP, the ATP can then be quantified and correlated to overall activity by looking at the severity of the reaction with luciferase. The intensity of light generated is proportional to the activity of the sulfurylase.

In this Assay there are three main reagents: 1xAB wash, Sulfurylase Reaction Reagent (SRR), and Luciferase Assay Reagent (LAR). The 1xAB wash (Common Buffer (Roche Titanium buffer) – 49.5ml, 10% Tween 20 – .45ml, DTT 1M – 50µl) is used as the buffer to make an

ATP and sulfurylase ladder to be used as a control. If the assay is used with enzyme bound to beads, the 1xAB wash would be sued to wash those beads.

The Sulfurylase Reaction mixture (Common buffer (Roche Titanium buffer) – 49.5ml, 10% Tween 20 – .45ml, DTT 1M – 50µl, PPi/APS mix – 26µl) serves as a controlled mixture of reagents that will react with sulfurylase to produce ATP. During the assay, once a dilution plate was created, the enzyme samples (20µl) were treated with SRR (80µl) and allowed to react for 30 minutes. Then the samples were reacted with 50µl LAR (Common buffer (Roche Titanium buffer) – 17.8ml, 10% Tween 20 – .18ml, DTT 1M – 20µl, .1M Potassium Chlorate ($KClO_3$) – 2ml, 100mM D-luciferin – 40µl, 3mg/mL UG Luciferase – 20µl) and the luminescence was observed and quantified using a luminometer.

Column Chromatography Assay

Using a G100 Sephadex resin, a 13cm tall column was created. All sulfurylase samples and all size exclusion standards were run through this column and 200ul fractions were collected each time. The fractions that were eluted were analyzed for the presence of protein by both UV spectrometry, and by Bradford Assay. The fractions that contained protein were graphed according to their elution volume over their void volume on the y axis, and their molecular weight on their x axis. The buffer used for all of the columns in this paper was Roche Scientifics in house titanium buffer.

Stability Protocols

A real time stability study, an accelerated stability study and a freeze thaw study was performed on the enzyme. The real time study looked at a sample of enzyme that had been residing at both (-80), (-20), and at room temperature for four weeks. The Accelerated activity assay subjected 3

enzyme samples to 25, 35, and 42 degrees Celsius for 6 hours. The freeze thaw assay looked at a sample of enzyme that had been thawed twice, six times and 11 times. Once all of these samples were collected, a Sulfurylase Activity Assay was performed on them so that their activities could be compared.

Affinity Chromatography Assay

When determining the degree of biotinylation of the Bst Sulfurylase, an enzyme dilution series was made and then the protein concentration was determined via a Bradford Assay. 50µl of streptavidin coated beads were added to one of the two samples (the experimental sample). The beads had a magnetic core and were isolated from the sample via a magnetic tube rack. The supernatant from the experimental sample was transferred to a new tube without the streptavidin coated in all of the biotinylated protein. The dilution of the two samples resided in the center of the linear range of the Bradford assay. This step allowed one to determine an accurate protein concentration. Then the two samples were serial diluted with a 1:15 diction scheme so that the sample would be in the concentration range for the activity assay. They were then tested with the sulfurylase activity assay and their specific activity was calculated.

Results

Investigating other more characterized Sulfurylases served as a starting point for the research into the Bst Sulfurylase. After the sequence analysis/alignment of Roche's ATP Sulfurylase with *Riftia pachyptila, Thermus thermophilius, Saccharomyces cerevisiae, and Penicillium chrysogenum's*[1](figure 1a) Sulfurylase was shown to be more similar to *Riftia pachyptila*(1JHD), and *Thermus thermophiliu*(1V47). When looking further at both of these pre-characterized

[1] Sequences obtained from PDB

enzymes, they both were crystalized as homomeric dimers. Additionally the research into the other sulfurylass allowed one to infer where the catalytic domains are and approximately what the crystal structure might look like.

In order to determine if the Bst Sulfurylase existed in more than one confirmation throughout the pyrophosphate sequencing protocol, Sulfurylase was exposed to an assortment of buffers from the protocol and was then loaded onto a 4-12% non-denaturing gel. The gel (figure 2a) shows no discrepancies in conformation throughout the buffers. The gel was ran with a native mark unstained protein ladder, the molecular weights present to the left of the gel are representative of where the bands of the ladder were. The reason why an estimated molecular weight of 150 kDa for sulfurylase was not accepted was because the rate at which proteins migrate through a polyacrylamide gel is based upon both size and isoelectric point; the proteins in the ladder cannot be accurate for this reason. To see if the enzyme existed in a higher order multimeric state the crude Bst Sulfurylase was exposed to a twofold dilution series of a NP40 detergent solution, and then loaded onto a non-denaturing gel. The gel (figure 2b) showed that sulfurylase underwent no change regardless of the concentration of the NP40 solution. These findings show that the enzyme primarily exists as a monomer.

The molecular weight of the Bst Sulfurylase and the presence of multimeric complexes were examined using a size exclusion column chromatography experiment. If the molecular weight of the enzyme was known, one would be able to determine if the eluted species are consistent with that of a monomer or if the smallest species that was eluting was much larger than a monomer could possibly be. Initially three standards were run on a G100 Sephadex column, Alkaline Phosphatase (86kDa), Lysozyme (30Kda) and DNAse (14.7kDa). Their elution point was determined via both Bradford and UV spec. The three standards were chosen because they

resided in the range of the expected molecular weight of sulfurylase as a monomer and because they were measurable by the G100 Sephadex column. A sample of crude Sulfurylase was run on the same column as the standards. With the linear regression performed on the standards, an equation was produced that allowed one to calculate the molecular weight of a compound with respect to its elution volume/void volume. The graph shows the elution volume over the void volume on the y axis plotted against the molecular weight on the x axis (figure 3a). The molecular weight of the enzyme was determined to be 77.4kDa. The Sulfurylase fractions that eluted from the column were also examined by an activity assay and the assay shows that there are two peaks in activity before the majority of the enzyme elutes off the column (figure 3b). These two peaks (6 and 8) may represent small concentrations of Sulfurylase existing in higher order complexes. This assay showed the possibility that the sample separates into three species, possibly a monomer, a dimer and a trimer although the monomer would be present in vast majority.

The extent that the enzyme is biotinylated allows one to identify if there is a flaw in the manufacturing process of Bst Sulfurylase. A low biotinylation would lead to low concentrations of protein that are binding to the beads, and then leading to low activity during the protocol. In order to determine the extent of which the Bst Sulfurylase was biotinylated, an Affinity Chromatography Assay was performed. Knowing that 454 life science uses streptavidin coated beads to pull down the biotinylated Sulfurylase in their pyrophosphate sequencing procedure the beads were used to pull down the Sulfurylase. First a preliminary investigation was done into the reduction of activity when samples of enzymes were introduced to excess streptavidin beads (figure a). The study looked at the difference in using three different types of beads that Roche Scientific uses in its pyrophosphate sequencing technique (enzyme, packing, and enrichment

beads). The control, a sample of enzyme without beads, was used as a reference for relative activity. The three types of beads showed on average a 57% reduction in activity which should be directly proportional to the percent biotinylation. A more detailed analysis of biotinylation following affinity purification with enzyme beads and a control (no beads) was performed (figure 4b). A serial dilution of supernatant was assayed for activity and protein concentration. The samples were normalized against an ATP standard and the assay was run according to pyrophosphate sequencing conditions. The first two dilutions (bars 1, 2, 5 and 6) showed such high activity that they were not within the machines range of sensitivity, (represented by the red bar) and were not used. The 4^{th} dilution (bars 4 and 8) were so close to background that the reduction determined from those bars was not used as well. The Third dilution was with in the sensitivity range for the machine and its data was used to calculate the rest of the results referenced. This assay determined the reduction in activity to be 55% and the reduction in protein concentration to be 50% along with percent biotinylation (based off of activity) being 55%. The concentration of protein in each sample and the quantity of ATP produced per minute was observed. The specific activity of both the control (mixture) and the unbiotinylated enzyme samples were determined to be 3.42 uMATP mg^{-1} min^{-1} and 3.57 uMATP mg^{-1}. Samples of the enzyme were also analyzed by LC/MS/MS on tryptic fragments of Bst Sulfurylase in order to determine the level of biotinylation. A peptide was detected that correlated to a mass shift for biotin (226.1 AMU). The biotinylation site was determined to be at the 67^{th} amino acid, a Lysine. John Leszyc, the LC/MS/MS technician, made the observation that the occurrence of the peptide shift that is associated with the biotinylation of the enzyme was not consistent with the pull down data previously observed. He observed that a much smaller percent of the enzyme sample

appeared to be biotinylated. This discrepancy is possibly the result of non-specific binding of enzyme onto the beads that were used during the pull down assay.

Lastly the stability of Bst Sulfurylase was analyzed by an activity assay. Specifically the thermo-stability and freeze thaw stability of the samples was observed. The samples were tested in three different ways, by a real time stability study an accelerated stability study and by freeze thawing samples of protein. All of the samples were examined in respect to the control, enzyme that remained at -80 and that never had been thawed (figure 6a). All of the activities seen in this figure are graphed relative to the sample that remained at -80. The stability experiment showed that the Bst Sulfurylase enzyme is incredibly thermo stable and maintains activity over a long period of time at temperatures up to room temperature. The assay also showed that the enzyme does not maintain its functionality if it has been thawed more than 2 times. The effects of different pH were also examined with a relative activity assay (figure 6b). The experiment showed that the enzyme functions best at pH 5.5. All of the activities seen in this figure are graphed relative to the sample that remained at -80.

Discussion

In 454 Life Sciences pyrophosphate sequencing the manufactured Sulfurylase likely exists in a monomer form. According to the size exclusion column, the enzyme was determined to have a molecular weight of 77.4 kDa. However, one cannot exclude the possibility that small amounts of Sulfurylase are forming higher order complexes. The mass spectrometry data showed that the Sulfurylase has a mass shift at the 67^{th} amino acid, a lysine. Although this is consistent with the presence of biotinylated protein, surprisingly the affinity purification experiments and the mass spectrometry data showed that a shockingly large amount of the enzyme was unbiotinylated. An

obvious area for improvement would be to modify the manufacturing procedure in order to achieve a higher level of biotinylated enzyme. Additionally, Roche may be reassured that the specific activities between the affinity purified enzyme and the un-purified control were so similar that one can deduce the biotinylation of the enzyme does not affect its activity. The enzyme showed a high level of stability when it was under most temperature conditions. The enzyme also showed that it does not hold up to freeze thaw cycles. This can be remedied by not freezing the enzyme. Additionally, by decreasing the pH of the overall reaction, the activity of the Sulfurylase could be increased but because this protocol requires the use of several different enzymes the adjustment of the reactions pH must be done so as to not negatively affect the other enzymes. Within the Pyrophosphate sequencing technique there are a large improvements that could be made. These improvements in the procedure could potentially decrease the cost of the assay and the run time of the assay which would allow for Pyrophosphate sequencing more available to the scientific community. That being understood, this approach to rapid full genomic DNA sequencing requires a high quantity of reagents and enzymes to work, Procedures that are less dependent on reagents will be able to reduce their cost in a much more drastic way than this process could. An example of a new generation sequencing technique that has the potential to become even more accessible than 454's pyrosequencing is Oxford Millipore's Nanopore technology. Although there is no current data for this system the company claims to be ready to release their product in the fall of 2012. The system is incredibly elegant and allows for the direct application of lysed cells rather than separate amplification and read steps. Next generation rapid sequencing is an incredibly competitive field currently and this level of competition is insuring that eventually these techniques will eventually be highly accessible.

Figures

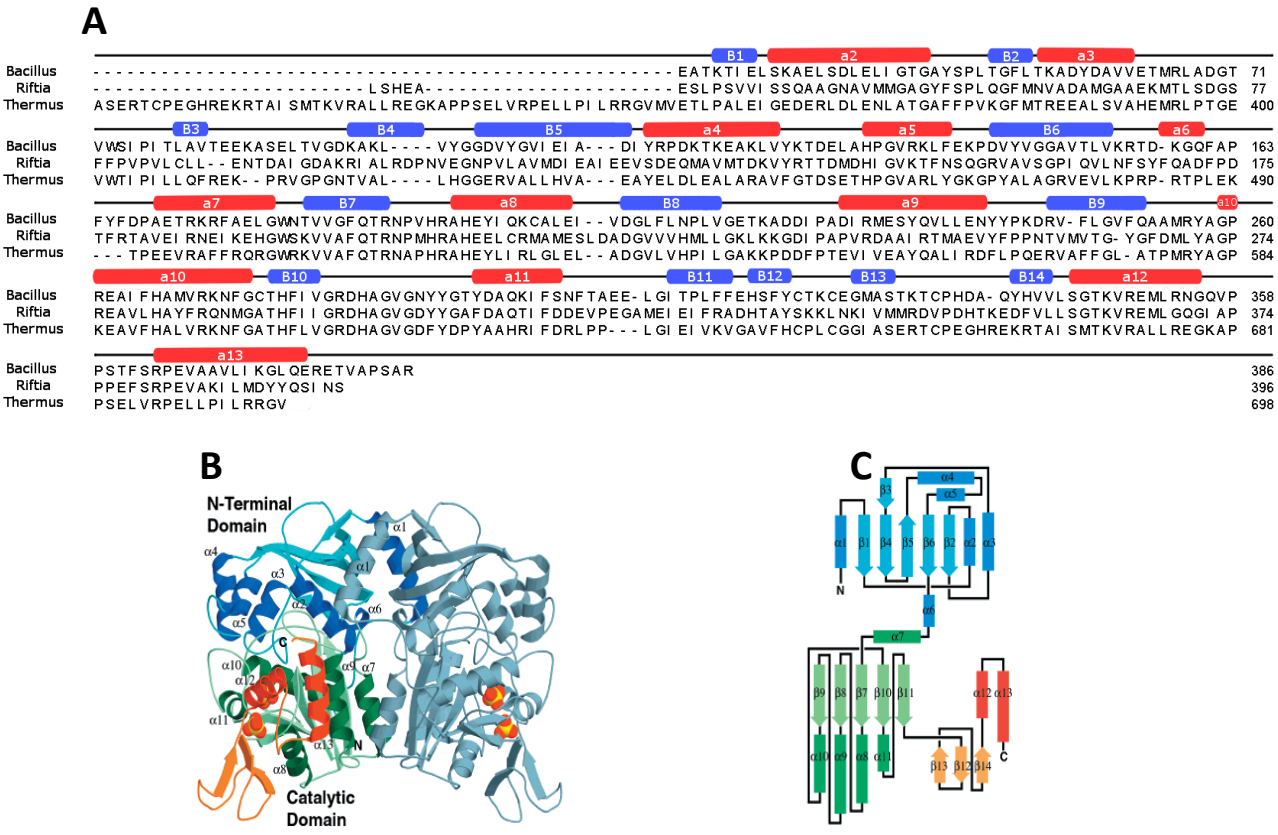


Figure 1: Sulfurylase sequence analysis and structure. (A) Sequence analysis of the *Geobacillus stearothermophilus*, Riftia, Thermus Thermophilius Sulfurylase. The secondary structure here was determined in reference to the paper by Beynon et al.. The beta sheets and alpha helixes are denoted by blue and red bands respectively. (B) A ribbon diagram of the Riftia Sulfurylase enzyme with the domains denoted by color. The catalytic domain is shown in green and red representing the two sub domains. Sulfate ions bound near the catalytic pocket are shown. (C) A schematic topology drawing is also from the paper referenced in B. The coloring matches that in B.

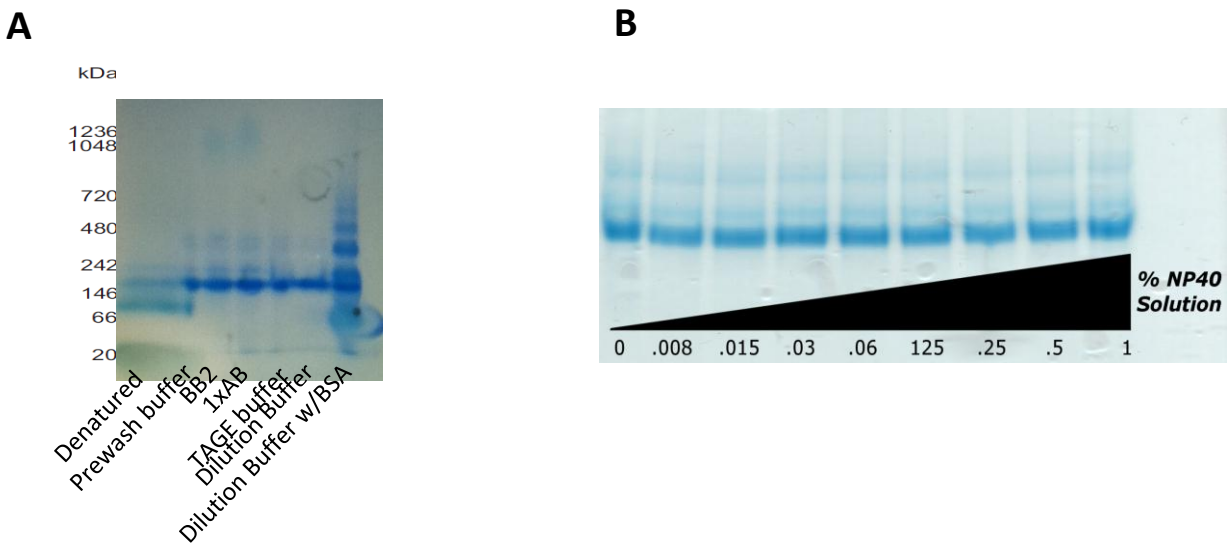


Figure 2: Bst Sulfurylases multimeric state (A) a 4-12% non-denaturing gel loaded with Bst Sulfurylase samples in various buffers used in pyrophosphate sequencing. The gel was run with a "NativeMark" unstained protein standard, the entire gel was visualized using a Coomassie blue protein stain. (B) 40ul samples of crude Bst Sulfurylase (333ug/ml) were exposed to a twofold dilution series of an NP40 detergent then loaded into a non-denaturing gel. No disturbance of higher order complexes occurred.

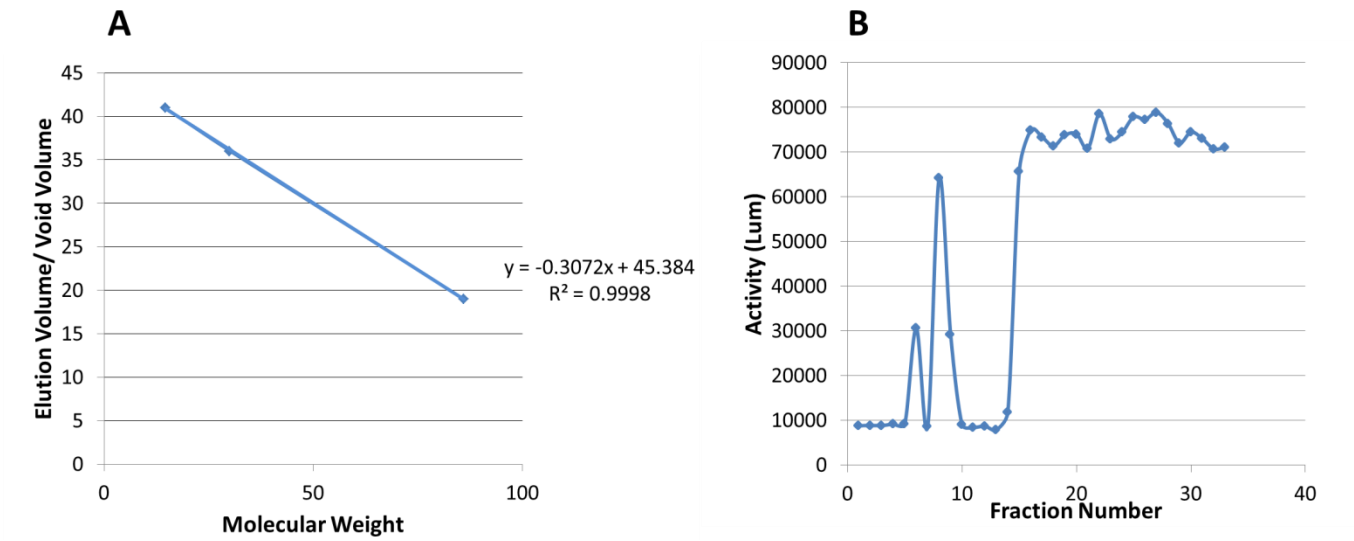


Figure 3: Multimerization and Molecular Weight of Bst Sulfurylase (A) a linear regression of several size exclusion standards was performed on a G100 Sephadex column with Alkaline Phosphatase (86kDa), Lysozyme (30Kda) and DNAse (14.7kDa). In the same column a 200ul sample of 333ug/ml Bst Sulfurylase eluted with a Ve/Vo of 22 and Molecular Weight of 77.4 kDa. Samples were identified with both a Bradford assay and UV spec. (B) on the same column as A a 50ul sample (333ug/ml) of Bst Sulfurylase was eluted from a G100 Sephadex column and 200ul fractions were collected. This assay shows the possibility that the sample separates into three species, possibly a monomer, a dimer and a trimer although the monomer would be present in vast majority.

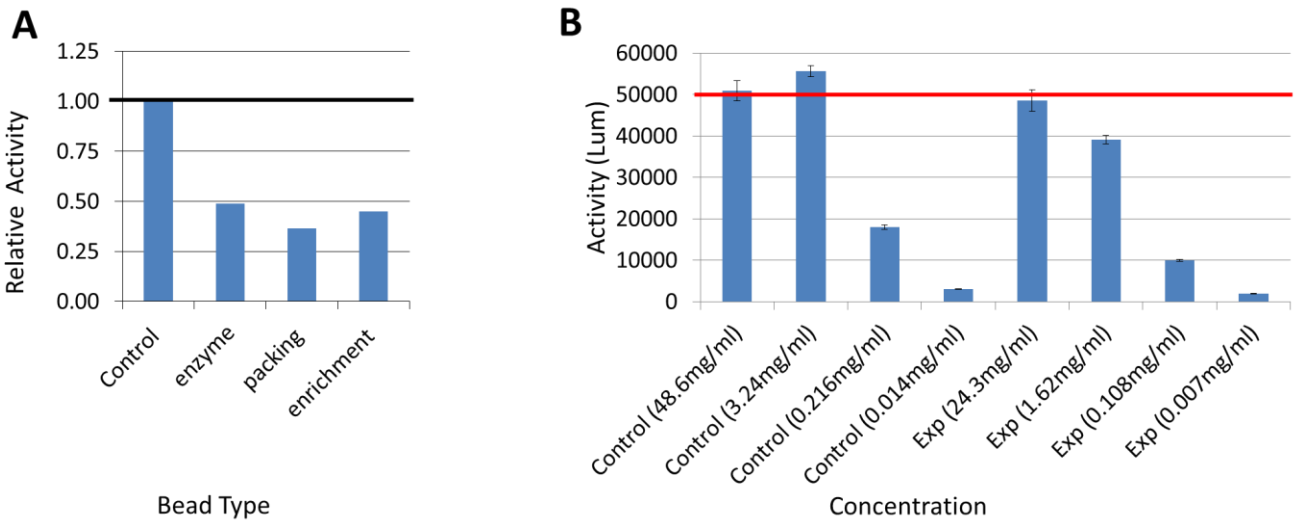


Figure 4: Percent Biotinylation of Sulfurylase (A) a relative activity assay between a control sample of Sulfurylase and samples depleted of biotinylated protein. Three types of streptavidin coated beads were used to pull down the biotinylated protein, enzyme, packing and enrichment beads. The supernatant of these samples containing only unbiotinylated protein and the control were assayed using an activity assay. The percent biotinylation was determined to be 57%. (B) A detailed analysis of biotinylation following affinity purification with enzyme beads and a control (no beads). A serial dilution of supernatant was assayed for activity and protein concentration. The samples were normalized against an ATP standard and the assay was run according to pyrophosphate sequencing conditions. The dilutions that were too close to the machines detection threshold (the red bar) were not taken into consideration in addition to samples with signals close to background levels. This assay determined the reduction in activity to be 55% the reduction in protein concentration to be 50% and percent biotinylation (based off of activity) to be 45%. The concentration of protein in each sample and the quantity of ATP produced per minute was observed. The specific activity of both the control (mixture) and the unbiotinylated

enzyme samples were determined 3.42 uMATP mg^{-1} min^{-1} and 3.57 uMATP mg^{-1} min^{-1} respectively.

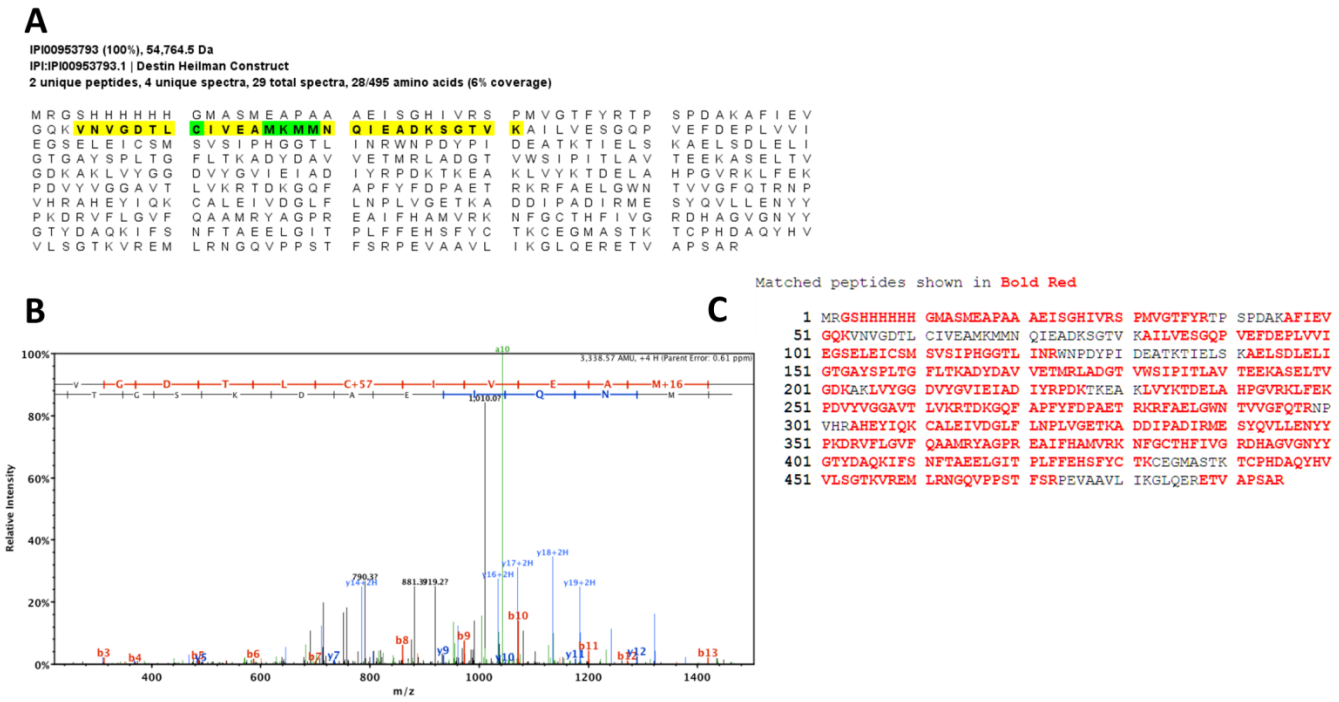


Figure 5: Mass spectrometry Data for sulfurylase (A) LC/MS/MS was conducted on tryptic fragments of Bst sulfurylase. A peptide was detected that correlated to a mass shift for biotin (226.1 AMU) this fragments is denoted in yellow. The green denotes modified amino acids and additionally it shows where the enzyme is biotinylated. Low amounts of the protein appeared to be biotinylated. The biotinylation site was determined to be at the 67th amino acid, a Lysine. (B) This is the mass spectrum of Bst Sulfurylase the shift corresponds to biotinylated peptide mass (C) This shows all the aspects of the Sulfurylase sequence that were matched when the mass spectrometry occurred. The trypsinization normally cuts at lysine except when it is biotinylated. The occurrence of sample that was found that didn't cut at the 67th amino acid implying that the enzyme was biotinylated was incredibly low.

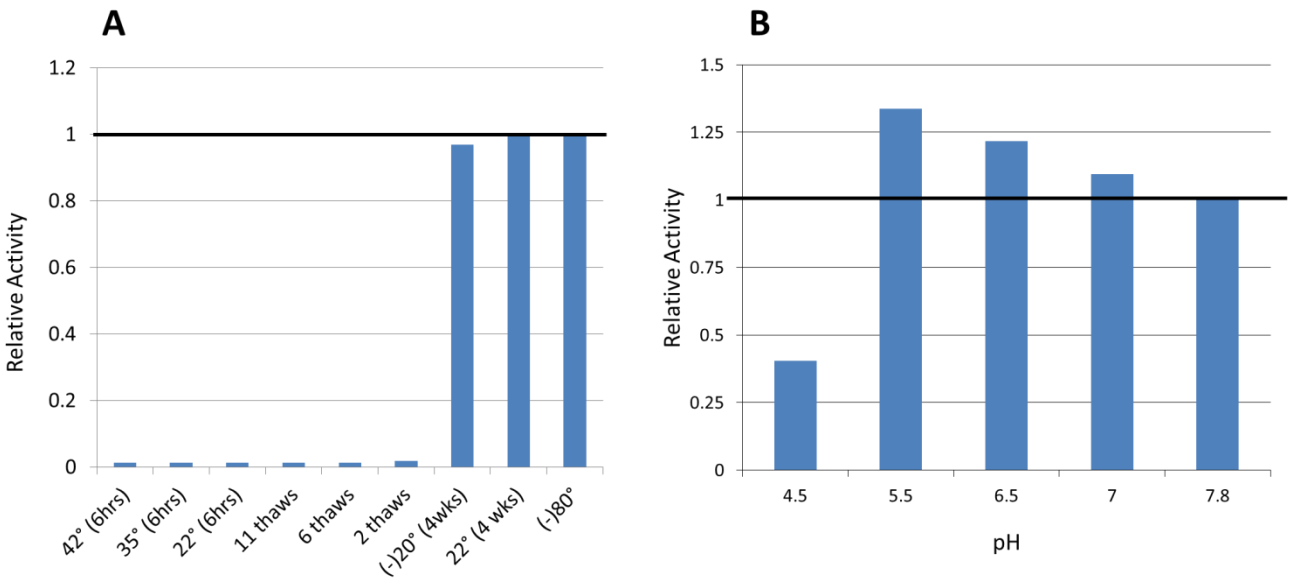


Figure 6: Bst Sulfurylase Stability Study (A) The stability of Bst Sulfurylase was analyzed by an activity assay. Specifically the thermo-stability and freeze thaw stability of the samples was observed, the samples were subjected to various conditions. No activity was seen in the three accelerated time points (42° (6hrs), 35° (6hrs) and 22° (6hrs)). Additionally, no activity was seen for the samples that experienced 2, 6 and 11 freeze thaw cycles. The actual time experiment (four weeks at RT, -20 and -80) showed little reduction in activity. (B) The effects of pH on the activity of Bst Sulfurylase were also assessed with a relative activity assay. The effects of pH (4.5, 5.5, 6.5, 7 and 7.8) on Bst Sulfurylase were determined. All activity is in reference to the activity of the enzyme at pH 7.8. A subsequent gel was then run with enzyme under the same conditions as B.

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