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Quantitative Analysis of Free D-threonines: A Novel Exploitation of L-threionine Aldolase

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Senior Thesis

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Almost all proteins found in biological systems are composed of L-configured amino acids. This general finding in nature is essential for the proper folding of proteins; α-helices can only form if all of a protein's constituent amino acids adopt the same stereochemical configuration.[1] From this observation it would appear that amino acids of the D-configuration have no functional purpose in an organism. Despite this, D-amino acids have in fact been found in living organisms. Neurotoxins produced by Conus *gladiator* & Conus *mustelinus* (cone snails) have been found to contain D-valine.[2] In bacteria, D-alanine & D-glutamine have been found in peptidoglycan (a structural component of cell walls), and in mammalian brains, free D-serine has been recently identified as a neurotransmitter.[3, 4] From these examples, it is appears as though D-amino acids are more common in biological systems than one would suspect at first glance. Interestingly, recent evidence has emerged which implicates the presence of these D- amino acids with Alzheimer's disease.

In a 1998 study by Fisher *et al*, samples of cerebrospinal fluid (CSF) from Alzheimer patients and normal subjects were assayed for total free D-amino acid content.[5] These researchers importantly discovered significantly higher levels of free D-amino acids in CSF from Alzheimer patients, compared to normal subjects. However, their study was not complete because it did not investigate free threonine in all of its stereoisomeric forms (only D-threonine content was analyzed). This may be partly due to the fact that studying free threonines requires the ability to separate enantiomers and diastereomers (all in the same mixture). A separation of this type is usually quite technically challenging and often requires the use of specialized and expensive equipment (in the form of chiral chromatography columns). Due to the technical hurdle and time-consuming nature of these separations, the threonine case was understandably left uninvestigated. However, recent work by Serafin *et al* has shown that tandem mass

spectrometry can simplify the process of stereospecific identification of amino acids, especially in the case of free threonine.[6] As mentioned previously, threonine poses a novel challenge for analysis because unlike the other 18 stereogenic amino acids, threonine has four possible stereoisomers (see **Figure 1**).

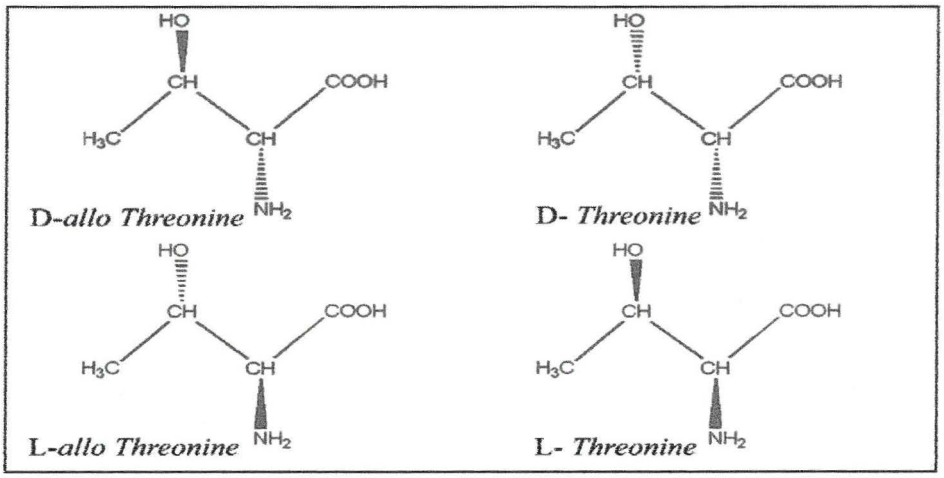

Figure 1: The four stereoisomers of threonine.

Serafin *et al* demonstrated that among these four stereoisomers (**Figure 1**), threonine diastereomers are more likely form a different charged species when ionized and fragmented via collision-induced dissociation (CID).[6] The different charged species from each diastereomer can be seen in the mass spectra and the relative abundance ratio of two key ions can be directly correlated to the abundance of each diastereomer in the original sample.[6] However, one caveat of such a method is a requirement for each sample to contain only threonine diastereomers; enantiomers cannot be quantified via this method because each enantiomeric pair will fragment in exactly the same way. However, given the ever growing and widespread use of mass spectrometry, such a detection method is certainly desirable because it will widen the applicability of our method. Thus, if tandem mass spectrometry (MS/MS) is going to be used for our analysis, a sample can only contain mixtures of L- & L-*allo* threonine or mixtures of D- & D-*allo* threonine.

The above requirement presents an obvious problem if a sample contains a mixture of all four threonine stereoisomers, which could potentially be the case if the sample is an extract from a biological source. We have been unable to find any previous biological analysis of all four threonine stereoisomers and must therefore assume all are present. In order for D-threonines in this mixture to be quantified via MS/MS, the L- & L-*allo* enantiomers need to be removed. Research conducted by Liu *et al* has presented us with a novel solution to this separation problem, which does not involve the use of chiral chromatography columns. In their study of E. *coli* bacteria, a low-specificity L-threonine aldolase (LTA) was identified, which specifically catalyzes the conversion of L- & L-*allo* threonine into glycine and acetaldehyde; D- & D-*allo* threonine were not compatible substrates.[7] Thus, LTA can potentially be used to help solve the problem faced if a sample contains all four threonine stereoisomers; adding LTA will rid the sample of any L-threonines and leave behind only the D-threonines (see **Figure 2**).

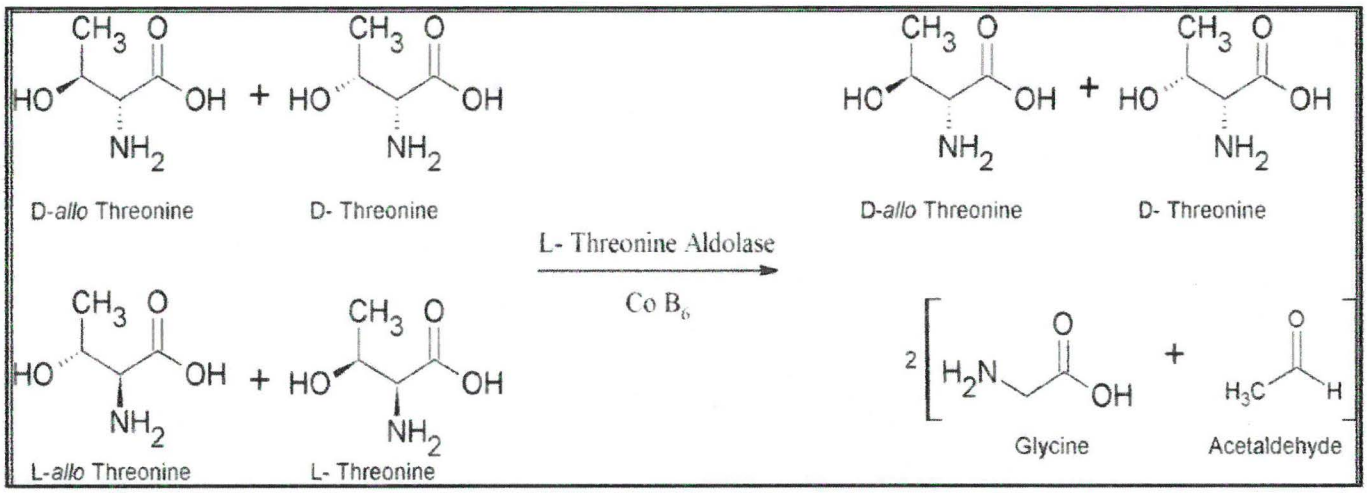

Figure 2: Reaction scheme of proposed method for LTA-assisted threonine separation. A biological sample may contain any or all forms of threonine (left side of reaction arrow). By mixing the sample with LTA, the L- threonines are effectively eliminated from the sample, which leaves only the D- threonines.

As stated before, these D-threonines can then be quantified via MS/MS. Unfortunately, LTA is no longer commercially available and as far as we can tell, no other lab in the country is actively

working with it. Therefore in order to obtain LTA, we have to make it ourselves, which has been a large focus of our research.

In principle, LTA could be obtained from wild-type E. *coli* by growing the bacteria in multi-liter cultures and using traditional "grind and find" biochemical methods to extract and purify the enzyme. While such a process is feasible, it is also time consuming because multiple purification steps are involved. For this reason, we took a different approach to obtain LTA, which takes advantage of the E. *coli* genome having been completely sequenced and published. This means the LTA gene can be precisely cloned, inserted into an expression plasmid, and used to transform a host organism (BL21 (DE3) E. *coli*). The transformed bacteria can then be grown in large-scale culture, from which LTA can be extracted. In our venture to obtain LTA we decided to use a pET expression vector system, which has several important features that allow us to conduct a more precise LTA purification compared to "grind and find." See **Figure 3** for details about our complete expression vector system.

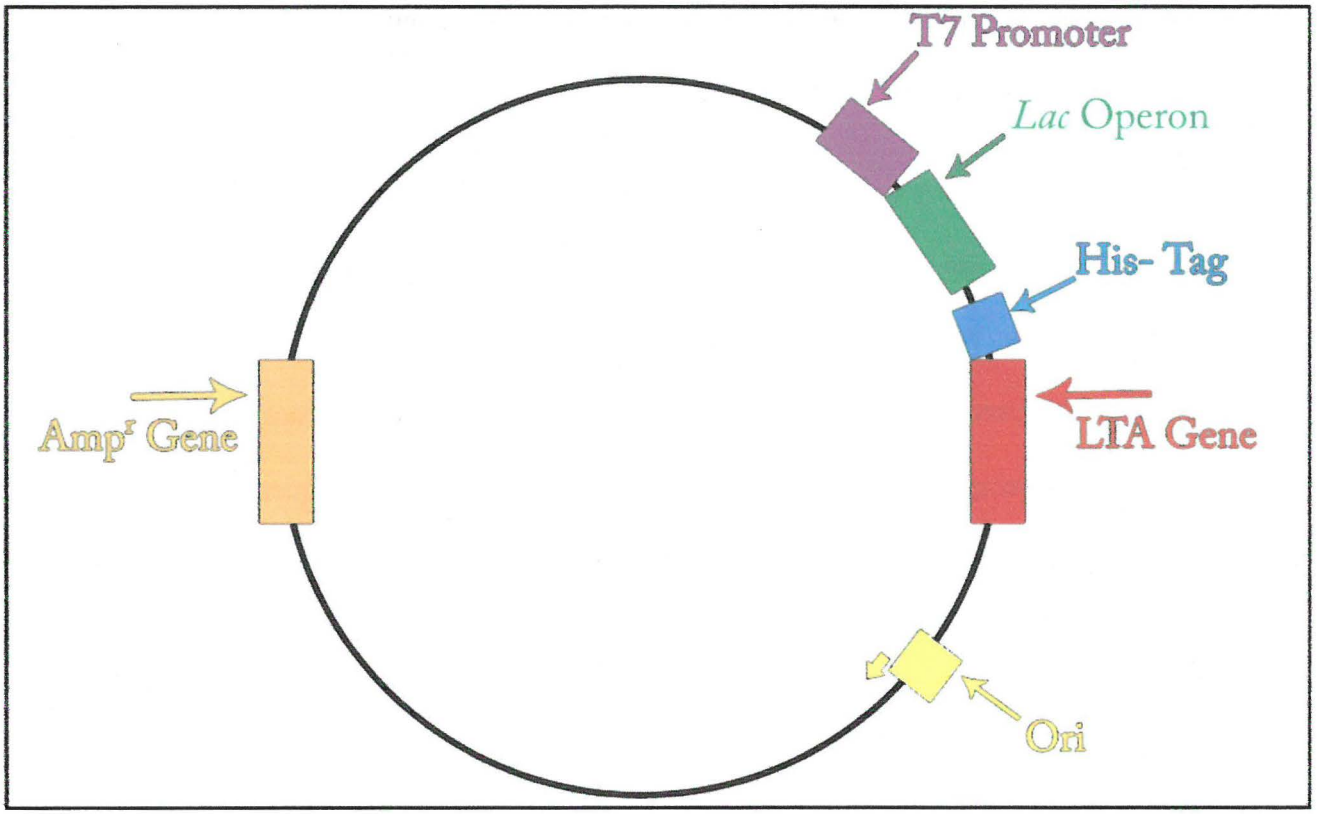

Figure 3: Complete pET plasmid used for purification of LTA (created summer of 2008 by Brian Monk).[11] The gene encoding for L-threonine aldolase is shown in red. The plasmid also contains a his-tag, which simplifies purification (blue). A *lac* operon (green) has been inserted to control production of the enzyme. The bacteriophage T7 RNA polymerase promoter (purple) allows for selective expression of the gene because the T7 promoter is not used elsewhere by the host organism because it is a viral promoter and thus creates a dedicated expression system. Selection of our bacteria containing this plasmid is aided by the ampicillin-resistance (Amp^r) gene (orange) because non-transformed cells will be killed when ampicillin is added to the media. The Ori (yellow) ensures the Plasmid DNA will be replicated *in vivo* as the bacteria go through their reproduction cycle.

The first important feature is a *lac* control system upstream of our cloned gene. When bound to its operon, the *lac* repressor protein prevents transcription of the LTA gene. The repressor can be removed by addition of galactose or a lipid-soluble homolog, isopropyl-thiol-D-galactoside (IPTG). Such a system allows us to precisely control when the LTA gene is expressed and also gives us the option of over-expressing the LTA gene. By over-expressing the LTA gene, we can theoretically obtain more LTA enzyme per bacteria cell, compared to normal expression levels. A second important plasmid feature is the T7 viral promoter site. Because BL21 (DE3) E. *coli* have been genetically engineered to produce the bacteriophage T7 RNA

polymerase, expressing the LTA gene can be carried out with a dedicated polymerase. In addition, the plasmid also adds six histidine residues to the N-terminus end of LTA (also known as a his-tag). Importantly, these six histidine residues allow nickel affinity chromatography to be used as our primary protein purification method. Because of histidine's extremely high affinity for Ni^{2+}, the crude cellular extract from our transformed host can be decanted into a chromatography column containing immobilized nickel ions. His-tagged LTA will associate very strongly with the nickel ions while all of the other cellular proteins and cell debris flow through the column. As a result, LTA can theoretically be purified in extremely high yield after only one purification step.

Once LTA is purified, we will need to perform a kinetics assay and verify that LTA can be used to rid a biological sample of all L-threonines. The reported K_m for LTA is in the 2-4 mM range[7], but the concentration of threonine in our proof of concept sample (blood plasma) is in the 0.1-0.2 mM range.[8] We will need to optimize incubation conditions for LTA in order to ensure sufficient catalytic activity is being achieved at these low substrate concentrations.

Because the presence of free D- threonine has already been established in human CSF,[5] there is precedent for developing a method which can detect D-*allo* threonine as well. It is the ultimate goal of our research to develop such an analysis method. However, in creating our method, we are striving to ensure that it can be carried out without the use of chiral chromatography columns and thus be easily applied in a clinical laboratory setting. If demonstrated to be reproducible and accurate, our analysis method has the potential to greatly simplify the task of measuring free D- threonines in a biological matrix because we will be able to assay both D-forms in the same experiment. Once their existence (or lack thereof) has been established in a laboratory animal, we would like to take samples (in the form of CSF) from

individuals afflicted with degenerative disorders and determine the relative amounts of this free D- amino acid. These data would allow us to determine if there is a correlation between the presence of free D- threonines and a given degenerative disorder.

Experimental

Transformation of Host Cells

The following transformation procedure was adapted from a Novagen® cloning kit user protocol.[9]

Day 1: BL21 (DE 3) E. coli were used as the expression strain for production of LTA. To begin the transformation, a tube of NovaBlue Singles Competent Cells was removed from the freezer and allowed to thaw on ice and resuspended. One µL of the pET expression plasmid[11] (created by Brian Monk) was then added to the competent cells. The mixture was then allowed to incubate on ice for 5 minutes. Following the ice incubation, the cells were transferred to a 42 °C water bath for exactly 30 seconds. The transformed cells were then placed on ice for 2 minutes. Next, 250 µL of SOC media was added to the cells. Twenty µL of the mixture was then spread on LB agar media. Antibiotic was added by top-spreading 200 µL of 50 mg/mL ampicillin. The plate was then incubated overnight at 37 °C.

Day 2: Each distinguishable colony was transferred to liquid culture (37 °C with 250 RPM shaking) by re-suspension in 5 mL of LB media containing 0.5 mg/mL ampicillin. Liquid cultures were maintained until colonies with the LTA gene insert were identified.

Plasmid Insert Verification

After 16 hours in liquid culture, a 1 mL aliquot was removed from each culture tube. Then, using a Qiagen® DNA Mini extraction kit, representative DNA was extracted from each of the former colonies. When DNA extraction was completed, 1 µL of the extract was transferred to a PCR reaction tube with the following LTA gene specific primers: 5'GACGACGACAAGATG3' (forward) and 5'GAGGAGAAGCCCGGTAA3' (reverse). Primers were added to a final concentration of 0.5 µM. The thermo cycler was run according to the following protocol: 94.0 °C for 5 min, 55.5 °C for 45 seconds and 72.0 °C for 2 minutes. The cycle was then repeated for a total of 30 cycles. Amplification products were then transferred to an 0.8 % agarose gel, ran at 120 volts, and stained with ethidium bromide. The presence of amplification products (seen via UV illumination of the gel) was used to identify recombinant cells containing the LTA gene insert.

Storage of Recombinant Cells

Once LTA gene-containing cultures were identified, aliquots were removed and mixed with glycerol to a final concentration of 10 % by volume. The cells were then flash frozen with liquid nitrogen and stored at -70 °C for future use.

Threonine Aldolase Gene Expression Verification & IPTG Expression Gradient

A container of frozen LTA expression strain was thawed and distributed among 8 test tubes containing 5 mL LB media and 0.5 mg/mL ampicillin. Half of these culture tubes were placed in a 30 °C water bath and the other half were placed in a 32 °C water bath. Each was set to 250 rpm. Cell growth was then followed spectrophotometrically by monitoring absorbance at 550 nm. When an absorbance of 0.5 was reached, the following IPTG concentration gradient was setup in separate tubes: 0 mM, 0.1 mM, 0.2 mM and 0.5 mM. However, before addition of IPTG, a 1 mL aliquot was removed from each culture and spun down to collect cells. After 21 hours, an identical aliquot was removed and spun down to collect the cells. Each pellet was then re-suspended in 200 µL LSB. Ten µL of each re-suspension was then loaded onto an 12.5% polyacrylamide gel and run at 120 volts for 1 hour. The separated proteins were then stained with Coomassie blue.

Large Scale Purification of Threonine Aldolase by Nickel Affinity Chromatography

Day 1: A tube of frozen cells was removed from the -70 °C freezer and immediately thawed in a 37 °C water bath. 60 µL of the cell mixture was then transferred to 5 mL of LB media containing 0.5 mg/mL of ampicillin. The culture was then incubated at 37 °C in a shaking water bath (250 rpm) overnight.

Day 2: 1.5 mL of the overnight culture was transferred to a flask containing 250 mL of LB media and ampicillin (0.5 mg/mL). The new culture was then placed into a shaking water bath set at 32 °C and 250 rpm. The culture growth was then monitored spectrophotometrically by monitoring the absorbance of the culture mixture at 550 nm. When an absorbance reading between 0.5 and 1.0 was obtained (usually between 6-9 hours), IPTG was added to the culture to a final concentration of 0.15 mM. The cell culture was then allowed to grow for 18-20 hours.

Day 3 (Part I): Cells were collected from the culture by centrifugation at 10,000 g for 10 minutes. During centrifugation and from this point on, all cellular extracts were kept between 0 and 4 °C at all times. After decanting the supernatant, the pellet was re-suspended with 25 mL of a pH 7.9 solution containing 0.25 M NaCl, 20 mM Tris-HCl, and 5 mM imidazole. Once re-suspended, the collected cells were placed on an ice bath and sonicated with a Branson Sonifer 450. The instrument was set at power level 4 and a duty cycle of 10. The extract was sonicated for a total of 8 minutes, but carried out in 2 minute bursts, followed by 1 minute rest intervals to avoid overheating the extract. During each rest interval, a small aliquot of the suspension was examined via darkfield microscopy at 1000x magnification, in order to monitor the lysing of

cells. After completing the sonication procedure, the suspension was spun at 20,000 g to remove any insoluble material. The post-centrifugation supernatant was then filtered through a 0.45 μm syringe-end filter. Absorbance readings were taken at 260 & 280 nm using a Spectral Solutions Inc CCD Array UV-Vis spectrophotometer to determine the approximate protein concentration of this crude extract.

Day 3 (Part II): The affinity chromatography column was prepared by allowing 2 mL of His-Bind® resin to settle in a 2.5 mL polypropylene chromatography column. The column was then washed with 6 mL of sterile deionized water. Next, 10 mL of 50 mM $NiSO_4$ was passed through the column, followed by 6 mL of a pH 7.9 solution containing 0.25 M NaCl, 20 mM Tris-HCl, and 5 mM imidazole.

Day 3 (Part III): The crude extract was then loaded onto the column and washed with a pH 7.9 solution containing 0.25 M NaCl, 60 mM imidazole and 20 mM Tris-HCl. Another wash was conducted by loading 20 mL of a pH 7.9 solution containing 0.25 M NaCl, 20 mM Tris-HCl, and 5 mM imidazole onto the column. To begin elution of LTA, 12 mL of a pH 7.9 solution containing 1 M imidazole, 0.25 M NaCl and 20 mM Tris-HCl was loaded onto the column. Collection of LTA began after letting the first 2 mL of eluent flow into the waste container. After the second 2 mL volume was collected, the rest of the flow-through was discarded. Collected eluent was then mixed with glycerol to a final concentration of 50 % glycerol by volume. The mixture was then flash frozen with liquid nitrogen and stored at -70 °C.

Note: A small aliquot of the column eluent (~20 μL) was kept aside for analysis via SDS-PAGE (8%) acrylamide.

Dialysis/ Refolding of Purified Threonine Aldolase

Five 0.5 mL aliquots of previously frozen LTA solution were each loaded into a separate segment of dialysis tubing and each placed into a separate NaCl solution. The NaCl concentrations used were 0 M, 0.1 M, 0.25 M, 0.50 M and 0.75 M. Each dialysis solution also contained 50 mM K_2HPO_4 at and 0.1 mM DTT. In addition, the pH of each solution was adjusted to 8.5. All solutions were kept between 0 and 4 °C and allowed to incubate for 3 hours. The enzyme aliquots were then transferred to identical buffer solutions and allowed to incubate for another 3 hours. However in the second buffer solution, DTT was replaced with 20 μM pyridoxal 5' phosphate. At the end of the second 3 hour incubation, each aliquot was mixed with glycerol to a final concentration of 50% by volume and flash frozen with liquid nitrogen. The frozen aliquots were stored at -70 °C for later analysis.

Enzyme Kinetics Protocol

To verify the activity of threonine aldolase, the following kinetics protocol was adapted from a study conducted by Liu *et al.*[7] First, 10 μmol of pyridoxal 5' phosphate and 20 μmol of HEPES buffer (pH adjusted to 8.0) were added to an Ependorf tube. Second, L-threonine was

added to an anticipated final concentration between 2mM and 50 mM (depending on the experiment). If necessary, the volume was adjusted to 120 μL with water. Once all reagents were mixed, 80 μL of a solution containing LTA was added to the mixture to give a final volume of 200 μL. Due to the dynamic nature of protein purification, the exact amount of LTA added to each incubation tube varied between purifications. The enzyme catalyzed reaction was then carried out a 60 °C for 20-60 minutes (depending on the experiment). At the end of the allotted time interval, the reaction was quenched by adding 60 μL of a 30% trichloroacetic acid solution. A blank for zeroing the spectrophotometer was prepared and treated identically to the other samples, except water was added in place of the LTA-containing solution.

Note: Because glycerol was used as a preservative for LTA, it was present in all incubation mixtures at an approximate concentration of 20% (v/v). Also, when samples were run with mouse plasma, 20 μL of the plasma were added in place of a prepared threonine solution (same final volume of 200 μL).

Determination of Acetaldehyde Produced by LTA Catalysis

The amount of acetaldehyde produced by the enzyme-catalyzed reaction was determined by adapting a protocol developed by Paz *et al.*[10] Two hundred μL of the quenched catalysis reaction were mixed with 1000 μL of 0.1 M glycine buffer (pH = 4) and 200 μL of 0.1 % (w/v) N-methyl benzothiazolone hydrazone (MBTH). The final volume was then adjusted to 2000 μL with water. The prepared mixtures were then incubated at 40 °C for 30 minutes. The absorbance of each solution was then read at 306 nm using a Spectral Solutions Inc CCD Array UV-Vis spectrophotometer. Beer's law was then used to determine the amount of acetaldehyde added to the reaction (Ext: 22 L $mmol^{-1}$ mm^{-1}).

Determination of Protein Concentration in Purified Extracts

Overall protein concentration was determined via Bradford assay. 50, 40, 30, 20, 10 and 5 μg BSA standards were prepared and made up to a final volume of 100 μL. The standards were then mixed with 5.0 mL of Bradford reagent and allowed to incubate for 5 minutes. The absorbance of each was then read at 595 nm using a Spectral Solutions Inc CCD Array UV-Vis spectrophotometer. The purified extract was then diluted into the standard range (if necessary) and read alongside the standards.

Precautions Taken to Minimize Damage During Thawing

In order to ensure optimum viability of frozen cells and LTA enzyme, all mixtures being removed from -70 °C storage were immediately buried in ice and then rapidly transported to a 37 °C water bath for rapid thawing. Once thawed, all mixtures were placed back on ice until needed.

Results

Identification/ Verification of Recombinant E. *coli*

Competent BL21 DE3 E. *coli* were transformed with a pET expression plasmid containing the LTA gene insert via a heat shock technique. After plating the transformed cells on Ampicillin-containing media, 26 recombinant colonies were found after an overnight incubation.

Once recombinant colonies were visible, each was transferred to liquid culture to allow for continued propagation. After growing overnight in liquid culture, DNA was extracted from each colony and run in a separate PCR reaction using LTA gene-specific primers. Amplification products from each of these reactions were then loaded onto an agarose gel and visualized **(figure 4)**.

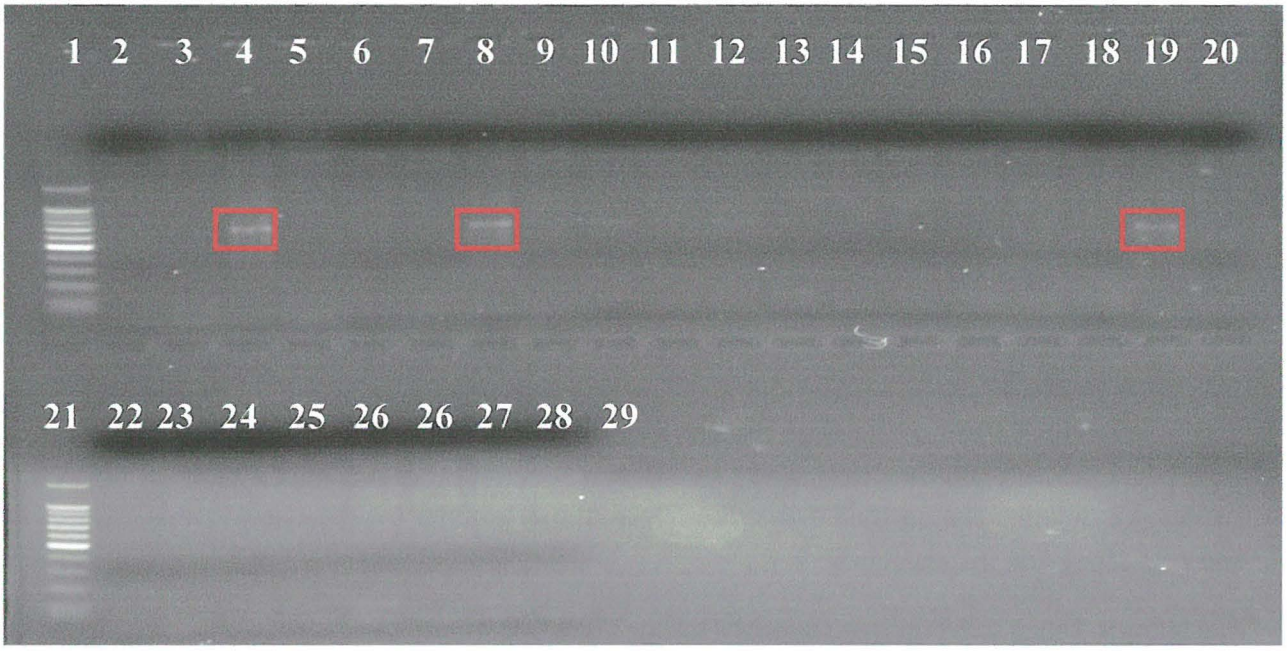

Figure 4: Amplification products from PCR reaction with LTA gene-specific primers. Lane 1: DNA 1000 BP ladder. Lane 2: Negative control from PCR reaction (water blank). Lane 3-20: Amplification products from PCR reaction with extracted DNA from colonies 1-18. Lane 21: DNA BP ladder. Lane 22-29: Amplification products from PCR reaction with extracted DNA from colonies 19-26. Red boxes denote the most significant amplification, determined by ethidium bromide staining/ UV visualization.

Data from this experiment shows successful LTA gene amplification from the PCR with DNA extracts from colonies 2, 6 and 18. While other positive amplification results are definitely visible, the bands from colonies 2, 6 and 18 were the most prominent when visualized under an ultraviolet light source.

To double check the previous positive amplification result, a representative sample from colony 6 (TAEXP 6) was removed from storage and grown to confluency in six separate culture tubes. After extracting DNA from each of these replicate cultures and running a LTA gene-specific PCR reaction, the presence of the LTA gene insert was again visualized on an agarose gel (**Figure 5**).

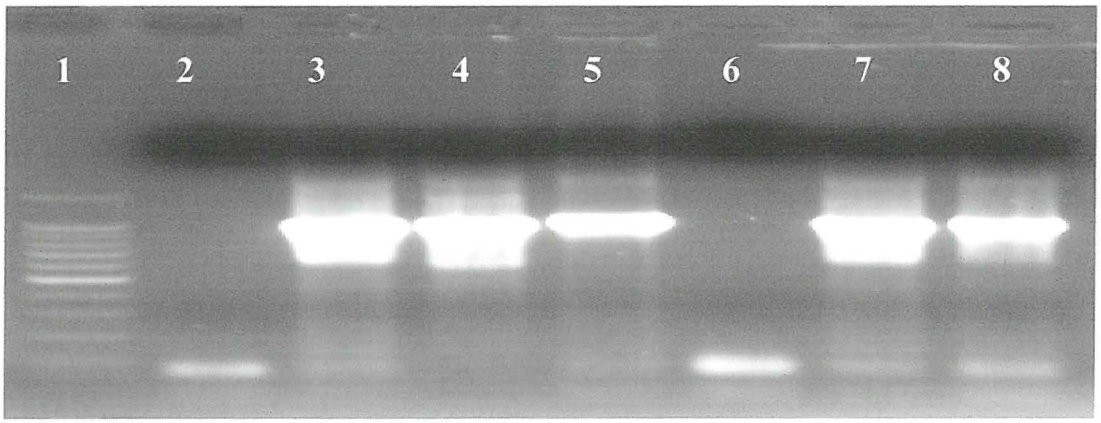

Figure 5: Amplification products from PCR reaction with LTA gene-specific primers. Lane 1: DNA 1000 BP ladder. Lane 2: Negative control from PCR reaction. Lanes 3-8: DNA extracted from representative sample of colony 6. Note: A loading error was encountered when setting up PCR reaction for lane 6.

Data from this experiment again demonstrates that DNA from colony 6 produced successful LTA gene amplification.

Threonine Aldolase Gene Expression Verification & IPTG/ Temperature Gradient

After setting up 2 parallel cultures (one at 30 °C and one at 32 °C), varying concentrations of IPTG were added to different culture tubes in each temperature setting. Pre and post aliquots

were taken from each culture tube and analyzed for overall protein composition via SDS-PAGE. Data from the 30 °C experiment are shown in **Figure 6** and data from the 32 °C experiment is shown in **Figure 7**.

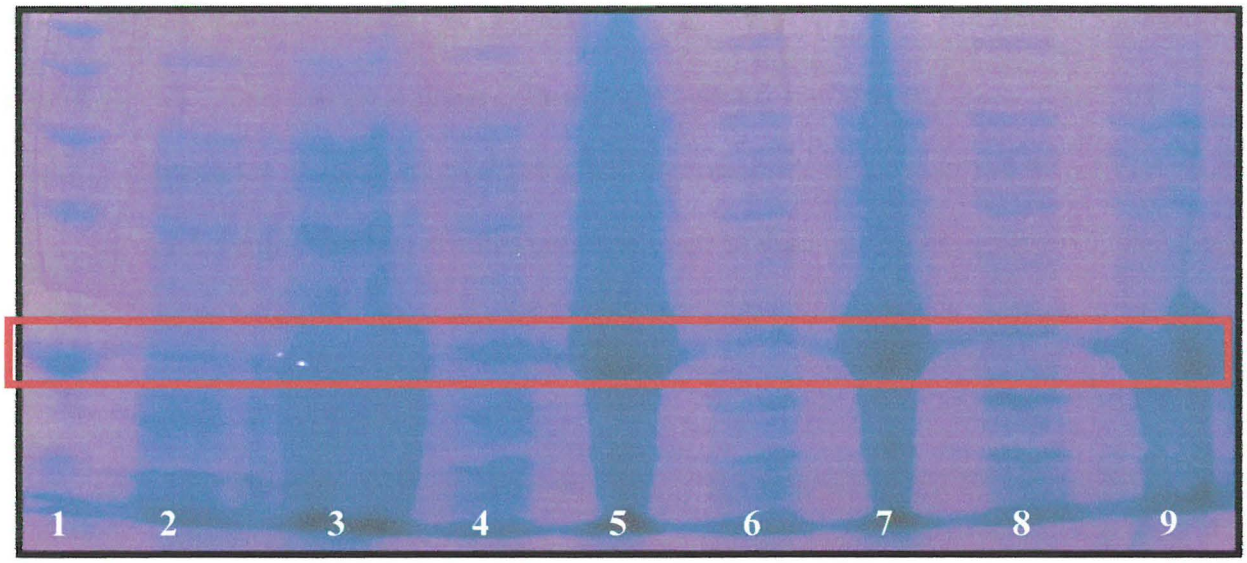

Figure 6: SDS-PAGE analysis of protein extracted from cell cultures at 30 °C during IPTG concentration gradient test. T_i aliquots were taken after approximately 8 hours of growth (before IPTG was added). T_f aliquots were taken after approximately 21 hours of growth. Lane 1: Broad range MW standard. Lane 2: No IPTG T_i. Lane 3: No IPTG T_f. Lane 4: 0.1 mM T_i. Lane 5: 0.1 mM T_f. Lane 6: 0.2 mM T_i. Lane 7: 0.2 mM T_f. Lane 8: 0.5 mM T_i. Lane 9: 0.5 mM T_f. Boxed region in red corresponds to a molecular weight of 35 kDa, which is close to where threonine aldolase should appear (MW~34 kDa).

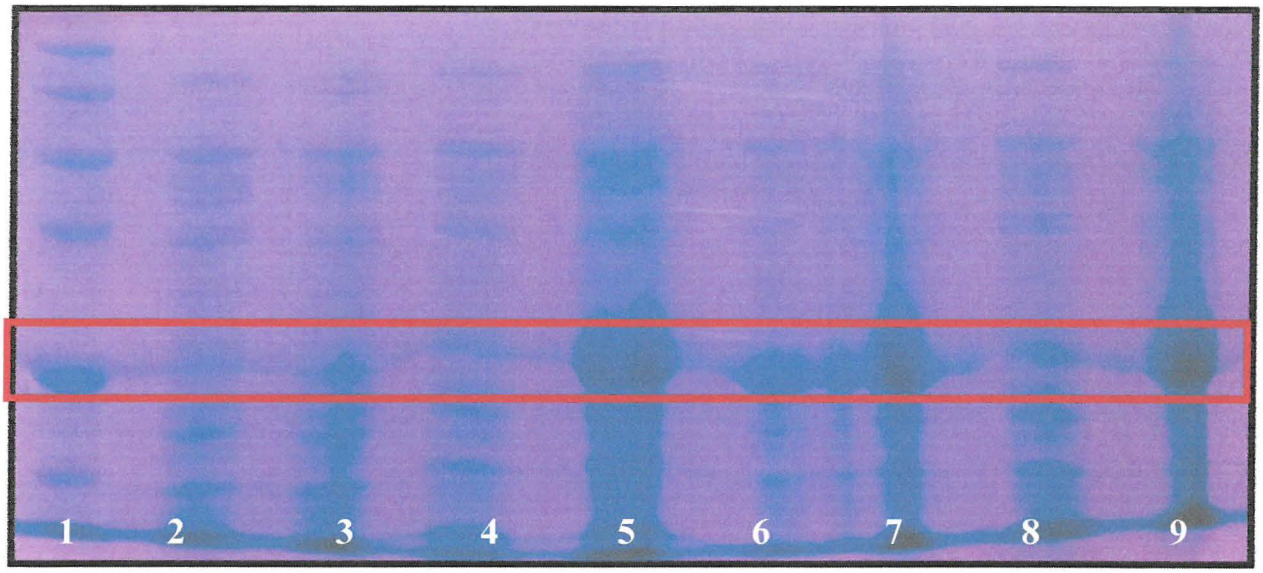

Figure 7: SDS-PAGE analysis of protein extracted from cell cultures at 32 °C during IPTG concentration gradient test. T_i aliquots were taken after approximately 8 hours of growth (before IPTG was added). T_f aliquots were taken after approximately 21 hours of growth. Lane 1: Broad range MW standard. Lane 2: No IPTG T_i. Lane 3: No IPTG T_f. Lane 4: 0.1 mM T_i. Lane 5: 0.1 mM T_f. Lane 6: 0.2 mM T_i. Lane 7: 0.2 mM T_f. Lane 8: 0.5 mM T_i. Lane 9: 0.5 mM T_f. Boxed region in red corresponds to a molecular weight of 35 kDa, which is close to where threonine aldolase should appear (MW~34 kDa).

Data from this experiment shows a distinct band around the 35 kDa region from all cultures exposed to IPTG at all concentrations. This demonstrates the cells are producing a protein with a molecular weight of ~34 kDa following exposure to IPTG. However, protein bands from cultures with 0.1 mM IPTG (**Fig. 6** lane 5 & **Fig. 7** lane 5) appear to be the most prominent. Due to the poor quality of the gel in **Figure 6**, it is difficult to determine which of these two bands (**Fig. 6** lane 3 & **Fig. 7** lane 5) is most prominent.

Examination of Cell Suspensions during Sonication Process

Samples of the cell re-suspension were analyzed during sonication to monitor lysing of cells. Data from these observations are shown in **Figure 8**.

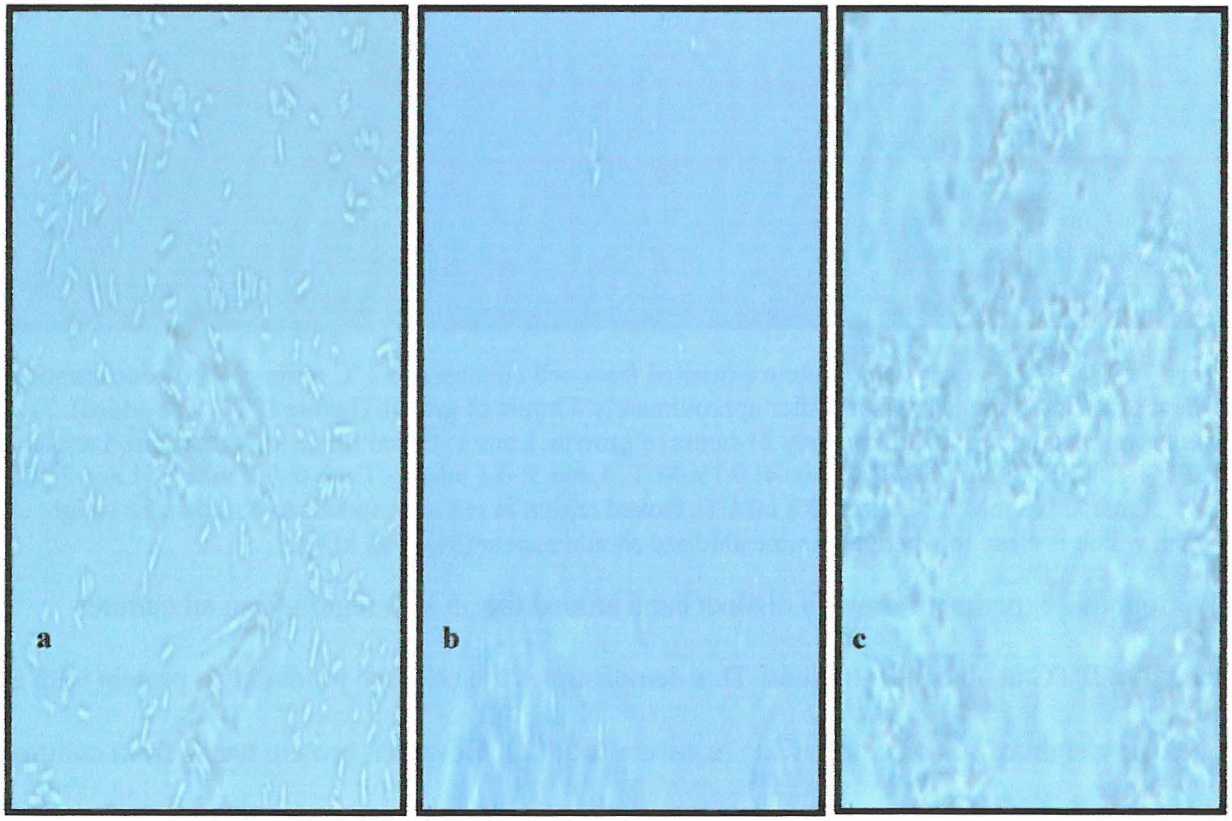

Figure 8: Photos taken of cell re-suspension mixture at various intervals during sonication. 5a: Pre-sonication. 5b: 3 min, 30 sec sonication. 5c: Completed 8 min sonication. All photos were taken using 1000x darkfield microscopy.

Analysis of photos presented in **Figure 8** clearly reveals the majority of cells were successfully lysed following an 8 minute sonication.

Large Scale Purification of LTA

Once collected, a small fraction of affinity chromatography eluent was analyzed via SDS-PAGE. In addition, the post-dialysis solution was also analyzed for protein composition in the same gel. Data from this experiment are shown in **Figure 9**.

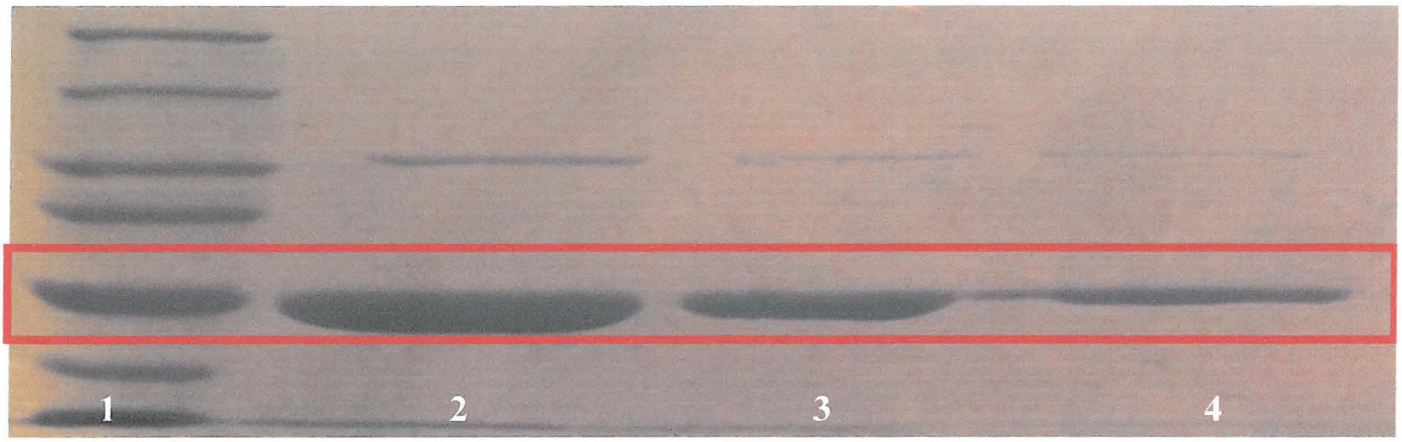

Figure 9: SDS PAGE of affinity chromatography column eluents and dialyzed extracts. Lane 1: Broad range MW standard. Lane 2: Column 1 eluent. Lane 3: Column 2 eluent. Lane 4: Column 2 eluent after dialysis. Boxed region in red corresponds to a molecular weight of 50 kDa. The large bands in this region correspond to the bulk of the purified protein.

Data from **Figure 9** clearly show an extremely prominent band around 50 kDa in all lanes, which indicates the presence of a purified protein. Importantly, lane 3 from **Figure 9** also shows this protein is still present following dialysis. There is also another protein present in the 100 kDa region, though it is clearly not as abundant as the 50 kDa protein.

Determination of Protein Concentration in Column Eluent and Dialyzed Extracts

To determine the amount of purified protein, a Bradford assay was used to determine the protein concentration in the column eluent and in the post-dialysis solutions. BSA was chosen as the protein for making a standard curve (**Figure 10**).

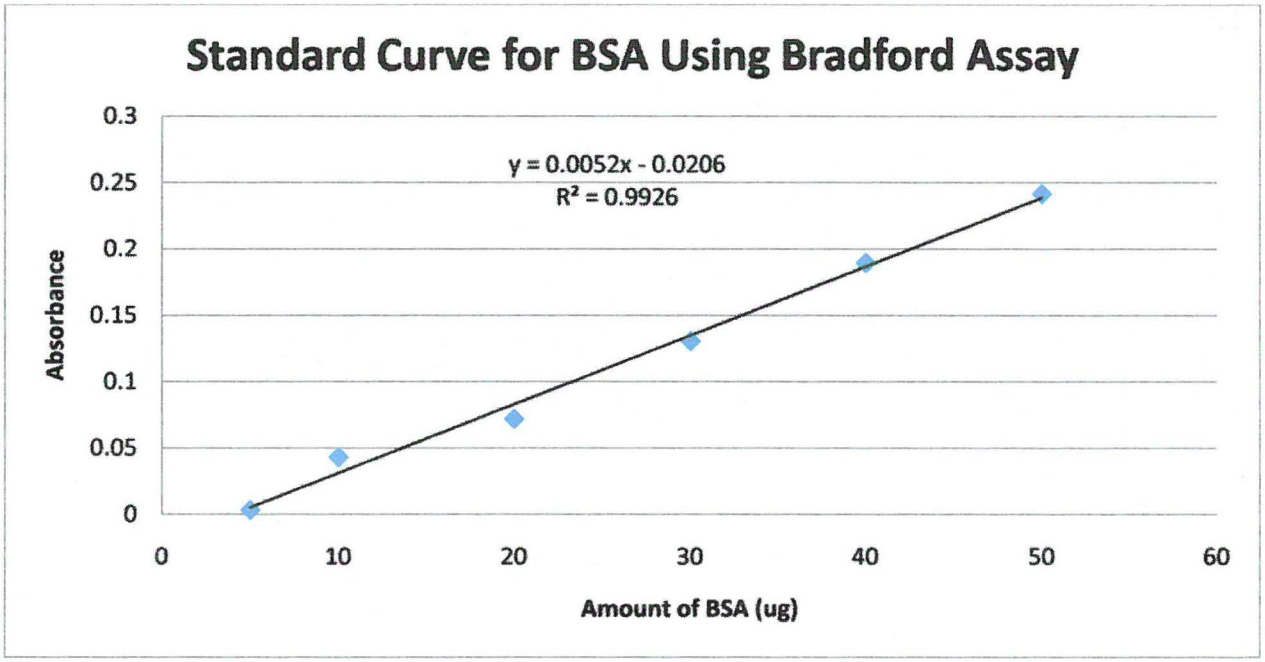

Figure 10: Bradford assay standard curve. 5, 10, 20, 30, 40 and 50 µg standards of BSA were prepared and read at 595 nm.

The high correlation coefficient (0.99) in **Figure 10** demonstrates an accurate standard curve was made for protein amounts between 5 µg and 50 µg. An aliquot from the column eluent and from each of the dialyzed extracts were taken, diluted if necessary, and mixed with Bradford reagent. The determined protein concentrations are shown in **Table 1**.

Table 1: Overall protein concentration in each sample is shown below, as determined via the standard curve in figure 6. Listed NaCl concentrations refer to the specific dialysis treatment conducted. Note: All samples contained 50% (v/v) glycerol.

Sample (M NaCl)	**Protein Concentration (µg/mL)**
0.0	110
0.1	90
0.25	80
0.5	190
0.75	130
Column Eluent (undialyzed)	350

Determination LTA Specific Activity After Dialysis/Refolding Treatment

Following dialysis, each extract was incubated under identical conditions to determine if the refolding process and/ or varying salt concentrations had any effect on overall enzyme activity. By comparing these treated extracts to the undialyzed control, we could determine if these treatments were doing anything to optimize LTA activity. Results from these tests are summarized in **Figure 11**.

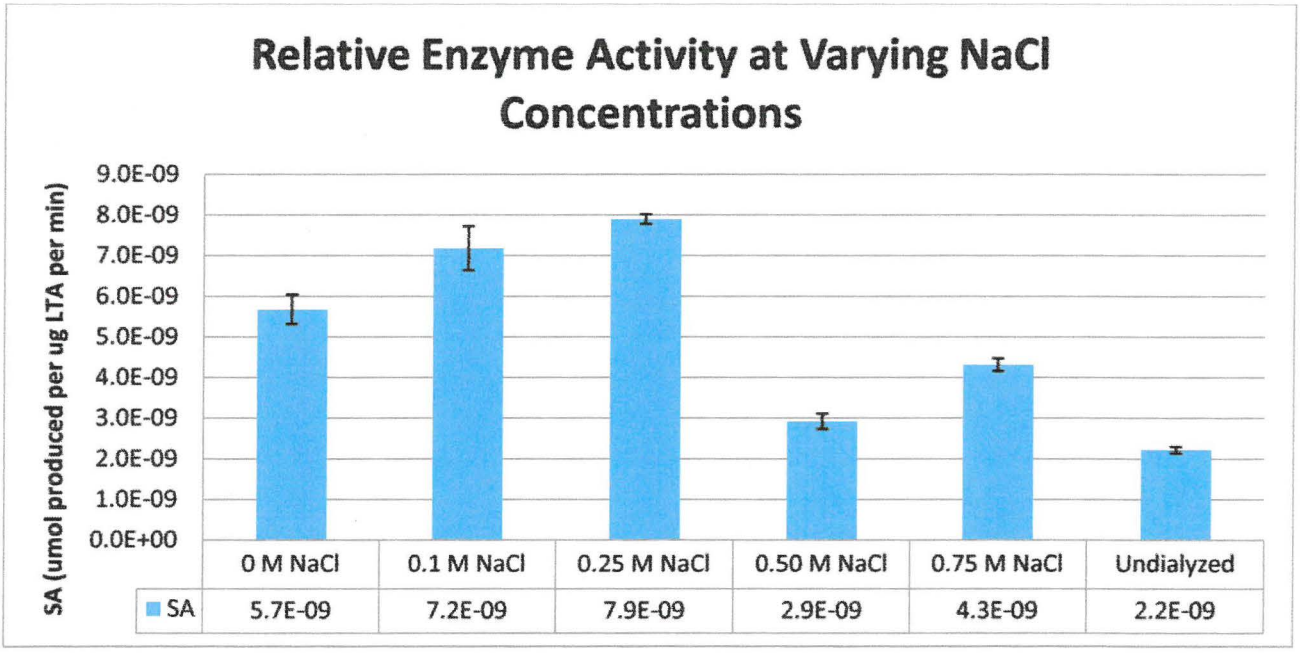

Figure 11: Comparison of averge LTA specific activity values following different refolding treatments. Each enzyme sample was incubated for 60 minutes at 60 °C with a 2.0 mM substrate concentration (L-threonine). Production of acetaldehyde was determined spectrophotometrically using MBTH. All averages were found to be significantly different using ANOVA ($p<0.01$). Note: the dialysis treatment was only performed once. The replicate measurements were taken from three enzyme mixtures subjected to the same dialysis treatment.

Data presented in **Figure 11** clearly demonstrates that refolding in combination with a 0.25 M NaCl concentration during dialysis results in a significantly higher activity ($p<0.01$), compared to any other treatment. Importantly, the activity resulting from the 0.25 M treatment is even higher than the column eluent, despite the treated enzyme being subjected to an addition freeze/ thaw cycle.

Determination of Reaction Progress Over Time

Because the goal of our project is to eliminate threonine enantiomers in a biological sample, we needed to follow the reaction over time to determine when all of the L- threonine isomers had been consumed. To carry out this study, three parallel enzyme incubations were setup with 5 mM L- threonine as the substrate. A reaction was quenched and assayed at an interval over the course of 165 minutes. Data from this experiment is shown in **Figure 12**.

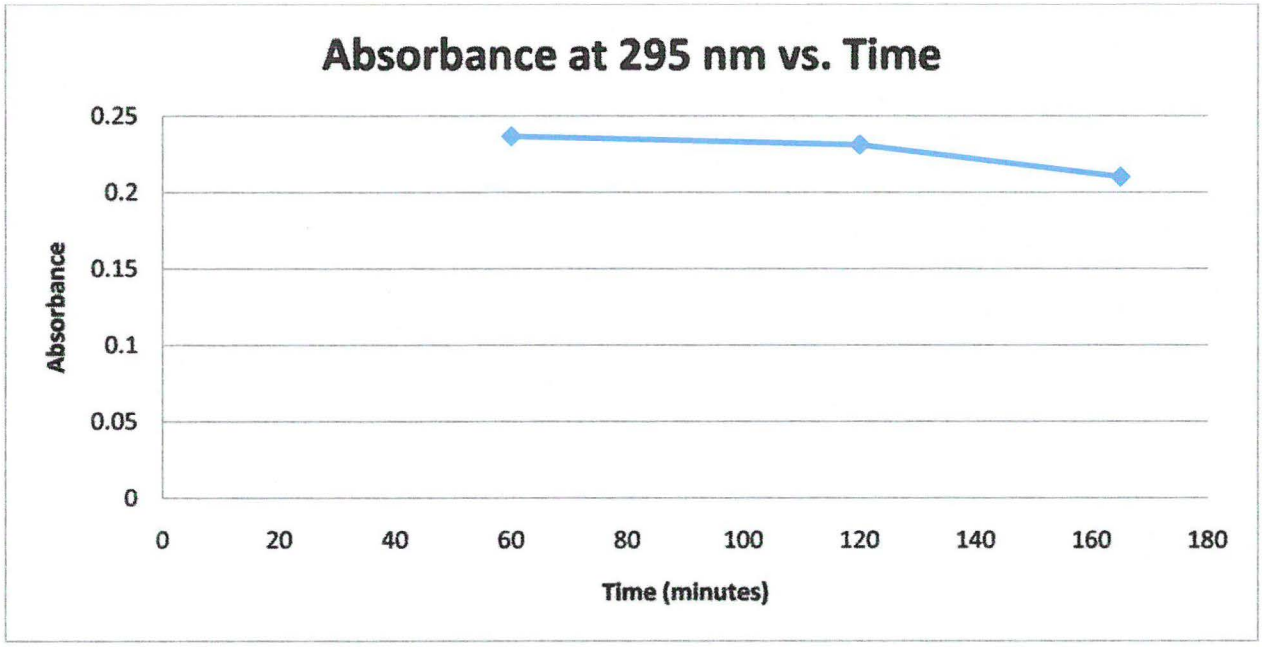

Figure 12: Reaction progress of LTA-catalyzed reaction over time with 5 mM L- threonine as the substrate. Absorbance readings taken according the method of Paz *et al.* Absorption at 295 nm directly relates to the concentration of acetaldehyde via the Beer-Lambert Law.

Results from this experiment show that reactions left incubating for a long period of time (120 minutes and 165 minutes) contain less acetaldehyde in their respective incubation solutions, compared to the 60 minute reaction.

Demonstration of LTA Activity with Substrate from Biological Sample

Mouse plasma was chosen as the biological sample to use as our "proof of concept" method trial. As such, mouse plasma was added to the incubation mixture instead of a prepared threonine solution. Data from this trial is shown in **Table 2**.

Table 2: Absorbance data from LTA-catalyzed reaction (65 minute duration) with mouse plasma as the threonine source. Absorption at 295 nm directly relates to the concentration of acetaldehyde via the Beer-Lambert Law. Note : the observed peak was partially obscured by noise at the neighboring wavelengths (probably from compounds in the blood).

Sample Name	Abs at 295 nm
Incubation with Mouse Plasma	0.6

This experiment clearly shows production of acetaldehyde when mouse plasma was used as the threonine source. Unfortunately, the 295 nm peak could not be completely resolved due to noise.

Discussion

The presence of amplification products after running a PCR reaction with LTA gene-specific primers and recombinant colony DNA clearly demonstrates that we created a strain of BL21 (DE3) E. *coli* with the LTA gene insert. Because the gene is under control of the *lac* operon, we were able to induce expression of this gene by adding IPTG to the cell culture at an optimized concentration. Once grown to confluency, we were able to extract and purify LTA in extremely high purity and in a relatively high yield by using nickel-affinity chromatography. Appropriate sonication conditions were experimentally determined to optimize the early stages of the purification, which ensured minimal damage to the enzyme. We also kept the enzyme between 0 and 4 °C at all times during the purification to further aid in this regard. Once purified, a small sample of the column eluent was assayed via SDS-PAGE, which revealed two distinct protein bands. We assumed the darker band corresponded to purified LTA and disregarded the

heavier protein seen on the top of the gel. To optimize LTA activity, we subjected the enzyme to a refolding treatment with a mild reducing agent and transferred the enzyme to a more stable phosphate buffer system at pH 8.5.[7] In the dialysis process, we decided to setup a concentration gradient with different concentrations of sodium chloride to determine if a particular concentration could afford a higher enzyme activity. After carrying out kinetics assays of LTA at these varying salt concentrations, we found a 0.25 M sodium chloride concentration during dialysis produced optimal specific activity. This result was found to be statistically significant by ANOVA ($p<0.01$). The enzyme catalyzed reaction was also carried out with mouse plasma as the threonine source to demonstrate its viability with a biological sample.

Now that we know the conditions necessary to generate optimal activity of LTA, we can make our purification process more efficient by using chromatography buffers to match these conditions. This should expedite the overall purification process and allow us to move on to the next aspect of our project.

Before we can use our proposed method we need to demonstrate that LTA can catalyze significant conversion of low concentration L/ L-*allo* threonine into glycine and acetaldehyde. If our analysis method is going to work, we need to be certain that all enantiomers are removed from a given sample. Following acetaldehyde production over time produced a surprising result in this regard because the data showed acetaldehyde concentration to be lower when the reaction was allowed to incubate for longer periods of time. Off hand, this means the reaction is actually going in reverse over time, as opposed to converting L- & L-*allo* threonine into acetaldehyde and glycine. However there is another explanation when you consider the properties of acetaldehyde. As it turns out, acetaldehyde is not a terribly stable compound and boils above 20 °C temperatures. Given the fact that our reaction temperature is 60 °C, it is possible that the

acetaldehyde is boiling and/or thermally decomposing. This would explain why its concentration decreased over time during the reaction. If this is the case, we will need to use LC/MS to monitor the reaction progress over time by measuring glycine production instead of acetaldehyde. By using mass spectrometry as a detection method, we will be able more accurately determine if and when the reaction is going to completion. Because such an instrument is not currently available in our laboratory, we could test the acetaldehyde decomposition hypothesis by making a solution of acetaldehyde that matches the expected concentration of a completed reaction and placing it at 60 °C. This mixture could then be incubated and monitored over the course of three hours. Results from such an experiment would allow us to determine if there is actually enough acetaldehyde in solution to undergo significant decomposition and/or vaporization.

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