PURIFICATION AND CHARACTERIZATION OF ACETYLCHOLINESTERASE
FROM TRITONIA TETRAQUETRA HEMOLYMPH

A University Thesis Presented to the Faculty
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In Partial Fulfillment
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Master of Science in Chemistry

By
Eric Ureno
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Abstract

Acetylcholinesterase plays many important neuronal functions; it is responsible for the termination of impulse transmissions in the brain, and at the neuromuscular junction, and may have a role in Alzheimer’s disease. Tritonia tetraquetra, a marine mollusk, has long been used as a model for neurological studies, due to its simple and easily accessible nervous system. T. tetraquetra feed on toxic coral, particularly sea pens. While it is believed butyrylcholinesterase typically helps prevent toxicity, T. tetraquetra does not contain butyrylcholinesterase, which could indicate an isoform of acetylcholinesterase might fill this capacity. A purification protocol was developed to isolate an acetylcholinesterase isoform from the hemolymph T. tetraquetra. Several purification methods were tested, including affinity chromatography, anion exchange chromatography, and hydrophobic interaction chromatography. The most successful purification step involved the preparation of an affinity chromatography column, using procainamide as a bait molecule. Procainamide was determined to be an inhibitor for the acetylcholinesterase isoform with an IC50 value of $5.44 \times 10^{-5}$ M.
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# Table of Contents

Abstract ................................................................................................................................... iii

Acknowledgements ............................................................................................................... v

List of Figures ......................................................................................................................... viii

List of Tables ........................................................................................................................... x

Glossary ..................................................................................................................................... xi

Chapter 1: Introduction ......................................................................................................... 1
  1. Acetylcholinesterase .......................................................................................................... 1
  2. *Tritonia tetraquetra* ......................................................................................................... 2
  3. Research Goal ................................................................................................................... 2

Chapter 2: Materials and Methods ....................................................................................... 3
  1. Materials .......................................................................................................................... 3
  2. Preparation of Stock Solutions ......................................................................................... 4
    2.1 Preparation of Colorimetric Activity Assay Stock Solutions ........................................... 4
    2.2 Preparation of Affinity Column Stock Solutions ............................................................ 5
    2.3 Preparation of Hydrophobic Interaction Chromatography (HIC) Stock Solutions .......... 5
    2.4 Preparation of Anion Exchange Chromatography Stock Solutions ............................... 6
    2.5 Preparation of SDS-PAGE Stock Solutions ................................................................... 6
  3. Preparation of Affinity Column ......................................................................................... 6
    3.1 Preparation of Stock Solutions ..................................................................................... 6
    3.2 Resin Preparation ........................................................................................................ 8
  4. Colorimetric Activity Assay ............................................................................................. 9
  5. Bradford Protein Assay ..................................................................................................... 10
  6. SDS-PAGE ..................................................................................................................... 11

Chapter 3: Results .................................................................................................................... 12
  1. Amicon Ultracentrifugation ............................................................................................. 12
  2. Anion-Exchange Chromatography .................................................................................. 14
    2.1 DEAE A-50 ................................................................................................................... 14
    2.2 DEAE Sepharose CL-6B ............................................................................................ 17
  3. Hydrophobic Interaction Chromatography (HIC) .......................................................... 19
  4. Procainamide Affinity Chromatography ........................................................................... 24
  5. Free Sulphydryl Curve .................................................................................................... 41
  6. Enzyme Kinetics .............................................................................................................. 42
    6.1 Michaelis-Menten Kinetics (Km) ............................................................................... 42
    6.2 Enzyme Inhibition (IC50) ......................................................................................... 49
<table>
<thead>
<tr>
<th>Chapter 4: Discussion</th>
<th>54</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Purification</td>
<td>54</td>
</tr>
<tr>
<td>2. Michaelis-Menten Kinetics (Km)</td>
<td>57</td>
</tr>
<tr>
<td>3. Enzyme Inhibition (IC50)</td>
<td>58</td>
</tr>
<tr>
<td>4. Molecular Weight Determination (SDS-PAGE)</td>
<td>59</td>
</tr>
</tbody>
</table>

References                                                                                   60

Appendix                                                                                      61
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetylcholinesterase mechanism</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>DEAE A-50 anion-exchange gradient</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>DEAE A-50 anion exchange AChE activity and Bradford assays</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>DEAE Sepharose CL-6B anion-exchange gradient</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>DEAE Sepharose CL-6B anion exchange AChE activity and Bradford assays</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
<td>HiTrap Phenyl HP AChE activity and Bradford assays</td>
<td>21</td>
</tr>
<tr>
<td>7</td>
<td>HiTrap Phenyl FF High Sub AChE activity and Bradford assays</td>
<td>21</td>
</tr>
<tr>
<td>8</td>
<td>HiTrap Phenyl FF Low Sub AChE activity and Bradford assays</td>
<td>22</td>
</tr>
<tr>
<td>9</td>
<td>AChE activity and Bradford assays for optimized gradient for HiTrap Phenyl HP</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>Procainamide affinity column gradient</td>
<td>25</td>
</tr>
<tr>
<td>11</td>
<td>AChE activity and Bradford assays for initial procainamide affinity column</td>
<td>26</td>
</tr>
<tr>
<td>12</td>
<td>4-20% tris-glycine SDS-PAGE gel of initial procainamide affinity run</td>
<td>27</td>
</tr>
<tr>
<td>13</td>
<td>Procainamide affinity column gradient</td>
<td>29</td>
</tr>
<tr>
<td>14</td>
<td>AChE activity and Bradford assays for second procainamide affinity run</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>4-20% Tris-glycine SDS-PAGE gel of second procainamide affinity run</td>
<td>32</td>
</tr>
<tr>
<td>16</td>
<td>Procainamide affinity column gradient for initial run on new resin</td>
<td>34</td>
</tr>
<tr>
<td>17</td>
<td>AChE activity and Bradford assays for initial procainamide affinity column run on new resin</td>
<td>35</td>
</tr>
<tr>
<td>18</td>
<td>Procainamide affinity column gradient for the second run on new resin</td>
<td>37</td>
</tr>
</tbody>
</table>
Figure 19. AChE activity and Bradford assays for second procainamide affinity column run on new resin.................................................................38

Figure 20. Procainamide affinity column gradient for the third run on new resin ..........40

Figure 21. AChE activity and Bradford assays for third procainamide affinity column run on new resin........................................................................40

Figure 22. Free sulphydryl curve........................................................................42

Figure 23. Michaelis-Menten plot........................................................................44

Figure 24. Lineweaver-Burk plot ........................................................................46

Figure 25. Eadie-Hofstee plot .............................................................................47

Figure 26. Hanes-Woolf plot ..............................................................................48

Figure 27. IC50 graph .........................................................................................51

Figure 28. Non-reduced 4-20% Tris-glycine SDS-PAGE gel.................................52

Figure 29. Reduced 4-20% Tris-glycine SDS-PAGE gel.......................................53
List of Tables

Table 1. Amicon ultracentrifugation purification table .................................................. 14
Table 2. DEAE A-50 binding capacity ............................................................................. 15
Table 3. DEAE A-50 elution gradient ............................................................................. 15
Table 4. HiTrap Phenyl HP elution gradient .................................................................. 23
Table 5. Samples and volumes concentrated in 100 kDa Amicon centrifugal filters...... 24
Table 6. Summary of pooled fractions from initial procainamide affinity run.............. 26
Table 7. Procainamide affinity column elution gradient for second screening run........ 29
Table 8. Summary of pooled fractions from second procainamide affinity chromatography run ......................................................................................................................................... 31
Table 9. Procainamide affinity column elution gradient for initial run on new resin...... 33
Table 10. Purification table for initial procainamide affinity column run on new resin .. 35
Table 11. Procainamide affinity column elution gradient for second run on new resin... 36
Table 12. Purification table for second procainamide affinity column run on new resin. 38
Table 13. Procainamide affinity column elution gradient for third run on new resin ..... 39
Table 14. Purification table for third procainamide affinity column run on new resin .... 41
Table 15. Substrate concentrations used for Michaelis constant (Km) determination ..... 43
Table 16. Summary of Michaelis constant (Km) and values ......................................... 48
Table 17. Procainamide concentrations and run setup for IC50 determination............. 50
Table 18. IC50 Calculations  ............................................................................................. 50
Glossary

AChE: Acetylcholinesterase

ATCh: Acetylthiocholine

BChE: Butyrylcholinesterase

BGG: Bovine Gamma Globulin

DTNB: 5,5’-Dithiobis-(2-Nitrobenzoic Acid)
Chapter 1: Introduction

1. Acetylcholinesterase

Cholinesterases are a class of carboxylic ester hydrolases that hydrolyze choline esters (Figure 1)\(^1\). Vertebrates have been shown to contain two forms of cholinesterases – acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) – while butyrylcholinesterase has not been demonstrated in invertebrates\(^2\). Acetylcholinesterase is predominantly found in neural synapses, while butyrylcholinesterase is mainly found in blood plasma. In addition to neural synapses, acetylcholinesterase is also present in muscles, liver, kidney, and erythrocytes\(^1,3\). Acetylcholinesterase plays an important role in the nervous system, terminating impulse transmissions at cholinergic synapses by hydrolyzing the neurotransmitter acetylcholine into acetic acid and choline\(^3,4\).

Figure 1. Acetylcholinesterase mechanism. Acetylcholinesterase hydrolyzes acetylcholine into acetic acid and choline.
2. *Tritonia tetraquetra*

*Tritonia tetraquetra*, formerly known as *Tritonia diomedea*, is a marine mollusk that has a simple nervous system that is easily accessible, making it a desirable target for studying neuronal function. *Tritonia tetraquetra* contains a small number of large, re-identifiable neurons in its simple nervous system, making it ideal for neuronal studies. *T. tetraquetra* is found in the soft coral beds of the northwest coast of North America. They prey on pennatulaceans, or sea pens, and might be prey to sea stars if their escape swim response fails.

Although inconclusive thus far, it is believed that BChE plays a role in protecting against toxins that would otherwise inhibit AChE. Since sea slugs have not been shown to contain BChE, AChE in sea slug hemolymph might play a similar role, allowing them to feed on toxic sea pens. Sea pens are believed to have an anti-acetylcholinesterase toxin in them, which should interfere with *Tritonia tetraquetra’s* physiology in the blood. Given their continued ability to consume sea pens, perhaps *Tritonia tetraquetra* has developed a specific resistance to these toxins, which could potentially be found in a specific isoform of acetylcholinesterase.

3. Research Goal

The goal of this research was to find a method to purify acetylcholinesterase from *Tritonia tetraquetra* hemolymph and obtain enzyme kinetics and molecular weight information. To accomplish this, several purification methods were explored and optimized, in order to obtain the best purity and percent recovery. Once a purification
method was established, purified sample was analyzed for various enzyme kinetic parameters, such as Km and IC50, as well as molecular weight using SDS-PAGE gels.

Chapter 2: Materials and Methods

1. Materials

The following chemicals were purchased from Sigma-Aldrich: acetylthiocholine iodide, 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), sodium acetate, and L-glutathione reduced. The following columns and resins were purchased from GE Healthcare Life Sciences: HIC phenyl and butyl 1 mL column screening kit, DEAE Sepharose CL-6B, DEAE Sephadex A-50, and CNBr-activated Sepharose 4B. Monobasic sodium phosphate, ammonium sulfate, trichloroacetic acid, and sodium chloride were purchased from Thermo Fisher Scientific. Sodium bicarbonate was purchased from Mallinckrodt Chemicals. N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) was purchased from Acros Organics.

The following supplies were purchased from Life Technologies: 4-20% Tris-glycine gels, Pierce Bovine Gamma Globulin Standard Pre-diluted set, and SeeBlue Prestained Protein Standard.

Sodium hydroxide and hydrochloric acid solutions were prepared by the technical staff of the chemistry stock room.
Dr. Murray, Biology professor at CSUEB, and his staff provided the hemolymph. The hemolymph was drained from the body of live or freshly-killed slugs in coordination with experiments done on other parts of their bodies.

Most aqueous solutions were prepared with deionized water (ddH$_2$O) obtained from a Milli-Q device.

2. Preparation of Stock Solutions

2.1 Preparation of Colorimetric Activity Assay Stock Solutions

A 0.1 M sodium phosphate solution, at both pH 7 and pH 8, was prepared by dissolving 12.0 g of monobasic sodium phosphate in 800 mL ddH$_2$O. The pH was adjusted to either 7 or 8 using 1 M sodium hydroxide. The final volume was then adjusted to 1 L with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.

A 10.3 mM DTNB solution was prepared by dissolving 410 mg of DTNB in 80 mL of 0.1 M sodium phosphate, pH 7.0 in a volumetric flask. Once fully dissolved, the volume was adjusted to 100 mL with 0.1 M sodium phosphate, pH 7.0.

A 10.7 mM acetylthiocholine iodide solution, which is used as the substrate, was prepared by dissolving 308 mg of acetylthiocholine iodide in 80 mL of 0.1 M sodium phosphate, pH 8.0. Once fully dissolved, the volume was adjusted to 100 mL with 0.1 M sodium phosphate, pH 8.0.

A 1.0 mM glutathione solution was prepared by dissolving 31 mg of reduced glutathione in 80 mL of 0.1 M sodium phosphate, pH 8.0. Once fully dissolved, the volume was adjusted to 100 mL with 0.1 M sodium phosphate, pH 8.0.
2.2 Preparation of Affinity Column Stock Solutions

A 20 mM sodium phosphate, pH 7.4 solution was made by dissolving 4.8 g of monobasic sodium phosphate in 1800 mL ddH$_2$O. The pH was adjusted to 7.4 with 1 M sodium hydroxide. The final volume was then adjusted to 2 L with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.

A 20 mM sodium phosphate, 1 M sodium chloride, pH 7.4 solution was made by dissolving 2.4 g of monobasic sodium phosphate and 58.44 g of sodium chloride in 800 mL ddH$_2$O. The pH was adjusted to 7.4 with 1 M sodium hydroxide. The final volume was then adjusted to 1 L with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.

2.3 Preparation of Hydrophobic Interaction Chromatography (HIC) Stock Solutions

A 1.7 M ammonium sulfate, pH 7.4 solution was prepared by dissolving 224.62 g of ammonium sulfate in 800 mL ddH$_2$O. Once dissolved, the pH was adjusted to 7.4 with ammonium hydroxide. The final volume was then adjusted to 1 L with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.

A 3 M sodium chloride, pH 7.4 solution was prepared by dissolving 135.32 g of sodium chloride in 800 mL ddH$_2$O. Once dissolved, the pH was adjusted to 7.4 with ammonium hydroxide. The final volume was then adjusted to 1 L with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.
2.4 Preparation of Anion Exchange Chromatography Stock Solutions

A 20 mM Tris, pH 8.0 solution was prepared by dissolving 2.422 g of trizma base in 800 mL ddH$_2$O. Once dissolved, the pH was adjusted to 8.0 with 1 M hydrochloric acid. The final volume was then adjusted to 1 L with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.

A 20 mM Tris, 1 M sodium chloride, pH 8.0 solution was prepared by dissolving 2.422 g of trizma base and 58.44 g of sodium chloride in 800 mL ddH$_2$O. Once dissolved, the pH was adjusted to 8.0 with 1 M hydrochloric acid. The final volume was then adjusted to 1 L with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.

2.5 Preparation of SDS-PAGE Stock Solutions

The SDS-PAGE running buffer was prepared by dissolving 10.57 g of Tris base, 65.8 g of glycine, and 3.5 g of sodium dodecyl sulfate (SDS) in 3.2 L ddH$_2$O. Once dissolved, the final volume was adjusted to 3.5 L with ddH$_2$O.

A 5% (m/v) trichloroacetic acid solution was prepared by dissolving 1.01 g of trichloroacetic acid in 1 mL of ddH$_2$O. This yielded a final volume of 1.5 mL. A volume of 75 µL of this would be added to 200 µL sample portions to yield a 5% (m/v) trichloroacetic acid solution.

3. Preparation of Affinity Column

3.1 Preparation of Stock Solutions

A 1 mM hydrochloric acid solution was made by adding 333 µL of 6 M hydrochloric acid to 2 L of ddH$_2$O.
A 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3 solution was made by dissolving 4.20 g of sodium bicarbonate and 14.61 g of sodium chloride in 400 mL of ddH$_2$O. Once dissolved, the pH was adjusted to 8.3 with 1 M sodium hydroxide. The final volume was then adjusted to 500 mL with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.

A 0.1 M Tris-HCl, pH 8.0 solution was made by dissolving 7.878 g of Tris-HCl in 400 mL of ddH$_2$O. Once dissolved, the pH was adjusted to 8.0 with 1 M sodium hydroxide. The final volume was then adjusted to 500 mL with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.

A 0.1 M acetic acid/sodium acetate, pH 4.0 solution containing 0.5 M sodium chloride was prepared by making 500 mL of a 0.1 M acetic acid solution and 500 mL of a 0.1 M sodium acetate solution. The 0.1 M acetic acid was prepared by adding 2.87 mL of glacial acetic acid (17.4 M) into a final volume of 500 mL ddH$_2$O. The 0.1 M sodium acetate solution was prepared by dissolving 4.10 g of sodium acetate in a final volume of 500 mL ddH$_2$O. 0.1 M acetic acid solution was added to the 0.1 M sodium acetate until the pH was 4.0. Based on the final volume, enough sodium chloride was added to yield a 0.5 M sodium chloride solution. In this case, 29.22 g of sodium chloride was added to a final volume of 1 L. Finally, the solution was filtered through a 0.22 µm filter.

A 20 mM sodium phosphate, 1 M sodium chloride, 10% (v/v) ethanol, pH 6.8 solution was prepared by dissolving 1.2 g of monobasic sodium phosphate and 29.22 g of sodium chloride to 400 mL of ddH$_2$O. After fully dissolved, the pH was adjusted to 6.8 with 1 M sodium hydroxide. After adjusting the pH, 50 mL of ethanol was added, and
then the final volume was brought up to 500 mL with ddH$_2$O. Finally, the solution was filtered through a 0.22 µm filter.

3.2 Resin Preparation

To prepare the resin for the affinity column, 10.0 g of CNBr-activated Sepharose 4B was weighed out and suspended in 1 mM HCl. Next, the resin was placed on a sintered glass filter (porosity G3) and washed with 2 L of 1 mM HCl, applied in several smaller portions.

Next, the coupling ligand, in this case 6-aminohexanoic acid, was prepared. A solution of 6-aminohexanoic acid was prepared by dissolving 0.66 g of 6-aminohexanoic acid in 50 mL of 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3. The washed CNBr-activated Sepharose 4B resin was split evenly into two 50 mL falcon tubes and 25 mL of 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3 was added to each falcon tube. This was rotated end-over-end on a nutator for one hour at room temperature.

The mixtures from the two falcon tubes were then transferred to a sintered glass filter and washed with 100 mL 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3. After washing, the resin was transferred to a beaker and covered with 0.1 M Tris-HCl, pH 8.0 and was left to incubate at room temperature for two hours.

After the two-hour incubation, the resin was transferred back to a sintered glass filter and washed with three cycles of alternating pH. Each cycle of the wash consisted of a wash with 100 mL of 0.1 M acetic acid/sodium acetate, pH 4.0 solution containing 0.5 M sodium chloride followed by a wash with 100 mL of 0.1 M Tris-HCl, pH 8.0. One final wash with 100 mL of 0.1 M acetic acid/sodium acetate, pH 4.0 solution containing
0.5 M sodium chloride was done after the three cycles. Lastly, the resin was washed once more with ddH$_2$O, adjusted to pH 4.5, in order to remove any sodium chloride.

One gram of procainamide was dissolved in 70 mL ddH$_2$O adjusted to pH 4.5. Next, the resin and dissolved procainamide were combined in a beaker and mixed with a stir rod. Next, N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC) was added slowly to a final concentration of 0.1 M; this was added in portion wise, waiting for the EDC to dissolve before adding more. This was left mixing end-over-end on a nutator for 24 hours.

After 24 hours of end-over-end mixing, the resin was transferred back to a sintered glass filter and washed with three cycles of alternating alkaline and acidic pH with a high salt concentration. Each cycle consisted of a wash with 100 mL of 0.1 M sodium bicarbonate, 0.5 M sodium chloride, pH 8.3 followed by a wash with 100 mL of 0.1 M acetic acid/sodium acetate, pH 4.0 solution containing 0.5 M sodium chloride. After the three cycles, the resin was washed with 200 mL ddH$_2$O. The resin was then stored in 20 mM sodium phosphate, 1 M sodium chloride, 10% (v/v) ethanol, pH 6.8 at 4°C until it was packed into the column.

4. Colorimetric Activity Assay

The activity of acetylcholinesterase was determined using a colorimetric activity assay based on the Ellman’s reagent (DTNB). Briefly, the hydrolysis of acetylthiocholine catalyzed by acetylcholinesterase produces thiocholine, which reacts with DTNB to yield yellow-colored 5-thio-2-nitrobenzoate (TNB). Monitoring the formation of TNB kinetically shows us the rate of hydrolysis of acetylthiocholine by acetylcholinesterase.
Prior to preparing the assay plate, the microtiter plate reader was turned on, in order to allow the instrument to warm up for at least 30 minutes. Using the included software, a program was made to read the plate at 412 nm for 30 minutes with one-minute intervals between readings.

The activity assay was performed in 96-well, 400 µL flat-bottom microplates. To set up the plate for the activity assay, 250 µL of assay buffer (0.1 M sodium phosphate, pH 8.0) was first added to each well, followed by 10 µL of DTNB color reagent (10.3 mM DTNB), followed by 30 µL of sample; this was mixed thoroughly by aspirating up and down several times with the pipettor when adding the sample. Blanks were performed in the same manner, but instead adding 280 µL of assay buffer and no sample. Just before reading the plate, 30 µL of ATCh substrate (10.7 mM acetylthiocholine iodide) was delivered using a multichannel pipettor, in order to minimize time delay between addition of substrate and initial reading. These solutions were also mixed thoroughly by aspirating up and down several times with the multichannel pipettor when adding the substrate. The plate was then placed into the microtiter plate reader and read for 30 minutes, using one-minute intervals between each reading.

For fractions from varying chromatography methods, all samples were added undiluted. For pooled and concentrated samples, some samples have been diluted; this will be clearly referenced in the results section.

5. Bradford Protein Assay

The Bradford protein assay is based on the binding of Coomassie Brilliant Blue G-250 dye to proteins. Coomassie Brilliant Blue G-250 exists in two different color
forms, red and blue; upon binding to protein, the red form is converted to the blue form\(^9\). This shift in color causes a shift in maximum absorption from 365 nm to 595 nm\(^9\). This method of determining protein concentration is useful for early stage research, when the extinction coefficient of a protein is unknown.

The assay was conducted in 96-well, 400 \(\mu\)L flat-bottom microplates. The traditional method calls for 250 \(\mu\)L of the Bradford reagent to be added to each well, followed by 5 \(\mu\)L of protein sample. For analyzing fractions from purification runs, the samples were too dilute to be accurately detected in this manner, so the volumes were adjusted to 125 \(\mu\)L of Bradford reagent and 125 \(\mu\)L of sample from each fraction for these steps. These results were used strictly for comparison between fractions within the run and not for quantitative purposes, thus no protein standards were used.

For crude hemolymph, as well as concentrated pools and final purified materials, the traditional mixing ratio of 250 \(\mu\)L of Bradford reagent plus 5 \(\mu\)L of sample was followed. Bovine gamma globulin (BGG) standards with concentrations of 125, 250, 500, 750, 1000, 1500, and 2000 \(\mu\)g/mL were used. These standards were employed to generate a standard curve to determine the protein concentration of the samples. After addition of all reagents and samples, the plate was incubated at room temperature for 15 minutes and then read in a microplate reader at 595 nm.

6. SDS-PAGE

SDS-PAGE was utilized to determine the molecular weight distribution of the crude material, in-process material, and final purified material. 4-20\% Tris-glycine gels were used, as these provide a broad separation range for varying size of proteins, which
was particularly important for the crude material. All samples were diluted 1:1 with 2x loading buffer, either without 2-mercaptoethanol for non-reduced samples, or with 2-mercaptoethanol for reduced samples. All samples were at 1 mg/mL prior to the 1:1 dilution with loading buffer, unless otherwise noted; initial procainamide screening run concentrations were unknown, but loaded volumes will be stated. All reduced samples were heated at 95°C for 5 minutes, to help the sample fully reduce. Loading volumes per well varied from 10-20 µL, and will be explicitly noted for each gel. 10 µL of SeeBlue pre-stained standard was used as the molecular weight marker for each gel. Blanks were prepared by diluting non-reducing 2x loading buffer 1:1 with ddH₂O. The gels were run in a mini-gel box at 125 V for 90 minutes. The gels were then removed and placed in Coomassie Blue stain overnight, or about 18 hours. After staining, the gels were destained in ddH₂O for two hours, or until the gel background was significantly lighter than the bands. The gels were then imaged using a Bio-Rad densitometer, utilizing Image Lab software for analysis.

Chapter 3: Results

1. Amicon Ultracentrifugation

Amicon ultracentrifugation was initially tried as the first step in the purification process, since it was believed that with acetylcholinesterase being such a large protein, a 100 kDa cut-off membrane might remove smaller proteins while retaining the larger acetylcholinesterase. Additionally, this step would not only help in purifying the sample,
but would also concentrate the sample and allow for easy and efficient dialysis of the sample into a matrix that is compatible with the ionic strength required to load the sample onto an affinity column. By concentrating the sample, it would also allow the sample to be loaded in smaller volumes for the same amount of material, which would aid in preventing further diffusion of the protein in the column.

Prior to loading the crude hemolymph into the Amicon filter, the hemolymph was centrifuged in a centrifuge tube at 15,000 rpm for ten minutes to help separate out any cell debris, and any other material that could potentially clog the filter membrane. Additionally, each Amicon filter was filled with 15 mL ddH₂O and centrifuged at 3,000 rpm to remove the storage solution from the membrane. After completion of the two previous steps, the supernatant was decanted into two Amicon 15 mL 100 kDa centrifugal filters. The filters were placed in a swinging bucket rotor and spun at 4,000 rpm for 40 minutes. Additional time was added as needed to concentrate the sample further and dialyze it into 20 mM sodium phosphate, pH 7.4. The sample was ultimately concentrated down to a total of 1 mL.

Based on the activity and Bradford results, this step did not prove to be very effective as a purification step, and was instead more useful as just a concentration and dialysis step. Given the negligible change in specific activity, this step did not help in separating out smaller proteins in the eluate; rather most seemed to be retained. The sample took over two hours to fully concentrate, which could be an indication of membrane polarization. The activity and Bradford seem to support this claim, showing about a 38% loss in protein and a 40% loss in activity (Table 1).
Table 1. Amicon ultracentrifugation purification table.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Total Protein (mg)</th>
<th>Activity/mL</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
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<th>Activity Recovery (%)</th>
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<td>4716</td>
<td>101400</td>
<td>2875</td>
<td>100.0</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Amicon 100 K Filter (retentate)</td>
<td>1</td>
<td>21.96</td>
<td>60780</td>
<td>60780</td>
<td>2768</td>
<td>62.3</td>
<td>60.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Amicon 100 K Filter (Eluate)</td>
<td>55</td>
<td>2.75</td>
<td>109</td>
<td>5980</td>
<td>2176</td>
<td>7.8</td>
<td>5.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

2. Anion-Exchange Chromatography

2.1 DEAE A-50

Prior to doing an anion-exchange chromatography run, the binding capacity of the prepared resin was tested. To determine the binding capacity, 500 µL of resin was added to each of six microfuge tubes. Subsequently, to each of the six tubes, 100, 200, 400, 600, 800, and 1000 µL of crude hemolymph was added. The tubes were then mixed end-over-end for 30 minutes. After 30 minutes, the tubes were spun down in a microcentrifuge to separate the resin from the aqueous layer. The activity assay was subsequently performed, taking 30 µL of the aqueous layer from each tube. The binding capacity was determined to be the tube with the highest volume of hemolymph added to 500 µL of resin before an increase in activity was detected. Based on the results from Table 2, the optimum binding capacity was determined to be 200 µL of crude hemolymph per 500 µL of resin (Table 2). The label “Max V [412]” assigned by the plate reader software represents the absorbance increase at 412 nm in units of mOD (x10⁻³ optical density units) per one minute of time. The data generated by the plate reader software is left as is,
and is not corrected for significant figures, in order to show the raw data. Based on the limitations of the plate reader, pipettors, and other measurement equipment used, three digits would be considered significant.

Table 2. DEAE A-50 binding capacity, determined by AChE activity assay.

<table>
<thead>
<tr>
<th>Well ID</th>
<th>Name</th>
<th>Well</th>
<th>Max V [412]</th>
<th>Count</th>
<th>Mean</th>
<th>Vmax - Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLK</td>
<td>Blank</td>
<td>A11</td>
<td>0.709</td>
<td>2</td>
<td>0.676</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blank</td>
<td>A12</td>
<td>0.642</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPL1</td>
<td>100uL</td>
<td>A1</td>
<td>1.006</td>
<td>1</td>
<td>1.006</td>
<td>0.33</td>
</tr>
<tr>
<td>SPL2</td>
<td>200uL</td>
<td>A2</td>
<td>1.486</td>
<td>1</td>
<td>1.486</td>
<td>0.81</td>
</tr>
<tr>
<td>SPL3</td>
<td>400uL</td>
<td>A3</td>
<td>26.657</td>
<td>1</td>
<td>26.657</td>
<td>25.981</td>
</tr>
<tr>
<td>SPL4</td>
<td>600uL</td>
<td>A4</td>
<td>62.435</td>
<td>1</td>
<td>62.435</td>
<td>61.759</td>
</tr>
<tr>
<td>SPL5</td>
<td>800uL</td>
<td>A5</td>
<td>74.428</td>
<td>1</td>
<td>74.428</td>
<td>73.752</td>
</tr>
<tr>
<td>SPL6</td>
<td>1000uL</td>
<td>A6</td>
<td>79.927</td>
<td>1</td>
<td>79.927</td>
<td>79.251</td>
</tr>
</tbody>
</table>

After the column was packed, 1 mL of crude hemolymph was loaded for each ion exchange run, and 3 mL fractions were collected. The loading buffer used was 20 mM Tris, pH 8.0 and the elution buffer was 20 mM Tris, 1 M sodium chloride, pH 8.0. The elution gradient is outlined below in Table 3.

Table 3. DEAE A-50 elution gradient.

<table>
<thead>
<tr>
<th>Start Volume (mL)</th>
<th>End Volume (mL)</th>
<th>% Elution Buffer</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>50</td>
<td>110</td>
<td>0 - 100 (linear gradient)</td>
<td>2.0</td>
</tr>
<tr>
<td>110</td>
<td>150</td>
<td>100</td>
<td>2.0</td>
</tr>
</tbody>
</table>

After the run was complete, all the fractions, shown in Figure 2, were tested for activity and protein concentration; Figure 3 shows these plotted on the same graph.
Although there is an increase in activity around fractions 28-36, there is still varying activity prior to that, indicating the acetylcholinesterase did not bind as tightly as anticipated to the resin. Additionally, during the elution step, the resin would compress significantly, to about \( \frac{1}{4} \) of its initial volume in the column; this would reverse once the resin was rinsed out with the loading buffer. After several more runs with similar results, it was discovered that the A-50 resin is not the best resin for column chromatography, so a resin better suited for column chromatography was sought, leading to the use of DEAE Sepharose CL-6B.

Figure 2. DEAE A-50 anion-exchange gradient, conductivity, absorbance, and fractions. The red line shows the conductivity in mS/cm, the blue line is the absorbance, the blue circles indicate the start and end of the fraction collection, and the green circles at the bottom indicate the fraction number, with every five fractions numbered above the green circles for legibility. The box inside the chromatogram shows the absorbance to be 0.0331 A.U. and conductivity to be 0.92 mS/cm at 21.97 minutes.
2.2 DEAE Sepharose CL-6B

After preparing and packing the DEAE Sepharose CL-6B resin, the same 20 mM Tris, pH 8.0 as the loading buffer and 20 mM Tris, 1 M sodium chloride, pH 8.0 as the elution buffer as was used for the DEAE A-50 column. The gradient and steps used were identical to the DEAE A-50 column and can be found in Table 3, with the fractions outlined in Figure 4.

Based on the activity and Bradford assay results shown in Figure 5, this anion exchange chromatography run had very poor separation between acetylcholinesterase and all other proteins. Throughout the first 23 fractions, there was an unstable fluctuation of AChE activity, with a somewhat distinguishable peak from fractions 24-30. However, the Bradford assay shows virtually no proteins eluting until fractions 24-30; this shows that
most proteins, including acetylcholinesterase, eluted at the same time, indicating there was no significant separation.

Figure 4. DEAE Sepharose CL-6B anion-exchange gradient, conductivity, absorbance, and fractions. The red line shows the conductivity in mS/cm, the blue line is the absorbance, the blue circles indicate the start and end of the fraction collection, and the green circles at the bottom indicate the fraction number, with every five fractions numbered above the green circles for legibility.
Figure 5. DEAE Sepharose CL-6B anion exchange AChE activity and Bradford assays. Higher adsorption value for Bradford assay (blue) indicates higher protein content in the fraction. Higher adsorption value for AChE activity (red) indicates higher amount of AChE enzyme in the fraction.

Given the results from the two anion-exchange resins, it seems anion exchange may not be a suitable purification method for this enzyme. Perhaps further development work on buffer salt and pH is needed, but given the poor results, this was bypassed in favor of other purification methods.

3. Hydrophobic Interaction Chromatography (HIC)

As part of the purification screening process, hydrophobic interaction chromatography (HIC) was tested to determine if this method was suitable to separate acetylcholinesterase from other proteins in the hemolymph. Three GE Healthcare HIC columns were initially screened: HiTrap Phenyl HP, HiTrap Phenyl FF HS (high sub), and HiTrap Phenyl FF LS (low sub). Initially, 1 M ammonium sulfate was used;
however, when performing the activity assay, the absorption values of all fractions, as well as the blanks, increased very rapidly. Having previously screened HIC on an HPLC using ammonium sulfate, this was unexpected. One possible cause for this could be linked to the grade of ammonium sulfate used; the previous screening was conducted in a pharmaceutical lab, with very high-grade chemicals. The ammonium sulfate was remade and tested as a blank to ensure no contamination or error, and the same phenomenon occurred again. Because of this, a 3 M sodium chloride buffer was used instead as the loading buffer. The loading buffer was 20 mM sodium phosphate, 3 M sodium chloride, pH 7.4 and the elution buffer was 20 mM sodium phosphate, pH 7.4.

All initial screening was performed using a linear gradient of 0% to 100% elution buffer. The sample was prepared by taking 500 uL of crude hemolymph and diluting it 1:1 with 500 uL of 20 mM sodium phosphate, 3 M sodium chloride, pH 7.4, in order to get the ionic strength closer to that of the loading buffer, without the need for dialysis.
Figure 6. HiTrap Phenyl HP AChE activity and Bradford assays. The left axis corresponds to the AChE activity (red) and the right axis corresponds to the Bradford assay (blue), while the x-axis corresponds to the fraction number.

Figure 7. HiTrap Phenyl FF High Sub AChE activity and Bradford assays. The left axis corresponds to the AChE activity (red) and the right axis corresponds to the Bradford assay (blue), while the x-axis corresponds to the fraction number.
Of the three columns screened, only the HiTrap Phenyl HP (Figure 6) gave a decent and somewhat well-defined peak for the activity and Bradford assays. The HiTrap Phenyl FF High Sub column (Figure 7) did not separate out the acetylcholinesterase from any of the other proteins, and based on the assay results shown in Figure 7, all proteins, including acetylcholinesterase, eluted at the same time, peaking at about fraction 38. For the HiTrap Phenyl FF Low Sub (Figure 8), it seems as though the acetylcholinesterase did not bind to the resin very well, as there is a big spike in AChE activity beginning at fraction 5. While this sort of phenomenon can occur if a column is overloaded, the remaining acetylcholinesterase on the column started to elute around fraction 25, which is where most other proteins also began to elute. Moving forward, the HiTrap Phenyl HP
column was used and efforts were made to optimize the elution gradient for best separation and yield.

In an attempt to increase the efficiency and yield of the HIC method, the elution gradient was changed to that outlined in Table 4. The hope was to get the AChE to elude in fewer fractions, giving a more well-defined peak in the activity assay plot, as well as get the majority of other proteins to elute after most of the acetylcholinesterase.

Table 4. HiTrap Phenyl HP elution gradient.

<table>
<thead>
<tr>
<th>Start Volume (mL)</th>
<th>End Volume (mL)</th>
<th>% Elution Buffer</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>10 - 40 (linear gradient)</td>
<td>1.0</td>
</tr>
<tr>
<td>35</td>
<td>50</td>
<td>100</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Figure 9. AChE activity and Bradford assays for optimized gradient for HiTrap Phenyl HP. The left axis corresponds to the AChE activity (red) and the right axis corresponds to the Bradford assay (blue), while the x-axis corresponds to the fraction number.
The results from this adjusted gradient did yield a much more narrow and defined peak in the AChE activity plot (Figure 9), indicating that the acetylcholinesterase was eluted in fewer fractions. With this new gradient, fractions 40-44 would likely be pooled to continue to a second chromatography purification step, while with the previous gradient, the activity ranged from fractions 25-40. Despite the more narrow elution range for acetylcholinesterase, it seems there are many other proteins eluting at the same time, based on the Bradford assay. However, since other purification steps would follow, such as an affinity step, this could still be a very useful purification step, as the Bradford assay also shows a large amount of proteins eluting after the main AChE activity peak.

4. Procainamide Affinity Chromatography

Prior to loading the sample onto the procainamide affinity column, four tubes of crude hemolymph were pooled and concentrated to a total volume of 1 mL and dialyzed into 20 mM sodium phosphate, pH 7.4 using 100 kDa Amicon centrifugal filters as outlined in Table 5.

Table 5. Samples and volumes concentrated in 100 kDa Amicon centrifugal filters for procainamide affinity column testing.

<table>
<thead>
<tr>
<th>Tube #</th>
<th>Sample</th>
<th>Beginning Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Td Tofino 9/30/10</td>
<td>~23</td>
</tr>
<tr>
<td>2</td>
<td>Td Tofino 10/13/10</td>
<td>~27</td>
</tr>
<tr>
<td>3</td>
<td>Td Tofino 10/11/10</td>
<td>~45</td>
</tr>
<tr>
<td>4</td>
<td>Td Tofino 7/15/10</td>
<td>~48</td>
</tr>
</tbody>
</table>

To test the purification ability of the procainamide column, a 300 µL aliquot from the concentrated pool was diluted to 1 mL in 20 mM sodium phosphate, pH 7.4 and
loaded onto the column. This initial run used a linear elution gradient, from 0% elution buffer, or 100% loading buffer (20 mM sodium phosphate, pH 7.4), to 100% elution buffer (20 mM sodium phosphate, 1 M sodium chloride, pH 7.4); gradient and fractions are shown in Figure 10.

![Figure 10. Procainamide affinity column gradient, conductivity, absorbance, and fractions. The red line shows the conductivity in mS/cm, the blue line is the absorbance, the blue circles indicate the start and end of the fraction collection, and the green circles at the bottom indicate the fraction number, with every five fractions numbered above the green circles for legibility.](image-url)
Figure 11. AChE activity and Bradford assays for initial procainamide affinity column. Higher adsorption value for Bradford assay (blue) indicates higher protein content in the fraction. Higher adsorption value for AChE activity (red) indicates higher AChE enzyme in the fraction.

Based on the results from the activity and Bradford assays in Figure 11, six pools were made and concentrated using 100 kDa Amicon centrifugal filters. All six concentrated pools, shown in Table 6, were diluted to a total volume of 1 mL using 20 mM sodium phosphate, pH 7.4.

Table 6. Summary of pooled fractions from initial procainamide affinity chromatography run.

<table>
<thead>
<tr>
<th>Pool</th>
<th>Fractions</th>
<th>Initial Volume (mL)</th>
<th>Final Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42-46</td>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>47-55</td>
<td>54</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>56-62</td>
<td>42</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>63-70</td>
<td>48</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>26-36</td>
<td>66</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>2-14</td>
<td>78</td>
<td>1</td>
</tr>
</tbody>
</table>
After concentrating the six pools, each of them was run on a 4-20% tris-glycine SDS-PAGE gel. A volume of 10 μL from each pool was used and added to 10 μL of 2x loading buffer with 2-mercaptoethanol. Each sample was incubated at 85°C for 2 minutes to fully reduce and denature the samples. 15 μL of each sample with loading buffer was loaded into each well. The gel was run at 125 V for 90 minutes. The gel was then stained overnight in a Coomassie Blue stain and then destained for two hours using ddH2O.

Figure 12. 4-20% Tris-glycine SDS-PAGE gel of initial procainamide affinity run. Lanes 1 and 10 are blanks. Lanes 2 and 9 contain Seeblue pre-stained molecular weight standard. Lane 3 corresponds to pool 1. Lane 4 corresponds to pool 2. Lane 5 corresponds to pool 3. Lane 6 corresponds to pool 4. Lane 7 corresponds to pool 5. Lane 8 corresponds to pool 6. Reference Table 6 for corresponding fraction information for each pool.

As can be seen on the gel in Figure 12, it seems the protein of interest (acetylcholinesterase) is located in pools 1 and 2, showing a molecular weight of roughly
120 kDa. Pools 3, 4, and 6 contain no visible proteins, while pool 5 contains all the proteins that were separated out and eluted before the acetylcholinesterase peak. Based on the Bradford assay results, as well as the gel results, it was determined that proteins did not begin eluting until roughly fraction 24, where the gradient was roughly 20% of the elution buffer. This indicates that to elute the proteins we hope to remove, a step gradient might be useful. Also, based on the AChE activity assay results, the acetylcholinesterase begins to elute around fraction 40, when the gradient is at roughly 50% elution buffer. These results show that a step gradient from 0% to 20% elution buffer might be useful in optimizing the procedure, while a linear gradient from 20% to 50% elution buffer, and then a step gradient to 100% elution buffer might help better separate out the other proteins and elute acetylcholinesterase in a much more narrow peak.

Given the results from the initial procainamide screening run above, the elution gradient was adjusted to that listed in Table 7. Based on the initial conductivity shown in Figure 13, it seems the column was not properly rinsed out and equilibrated with loading buffer, and still contained high salt from the end of the previous run; this led to the loss of acetylcholinesterase in the earlier fractions. After the column equilibrates with the loading buffer, around fraction 4, the AChE activity drops back down to a relatively stable baseline, as shown in Figure 14. Using this new gradient with the two step gradients yields a much more defined and narrow AChE activity peak, indicating that the change worked to better elute acetylcholinesterase over fewer fractions, helping not to diffuse and dilute it as much.
Table 7. Procainamide affinity column elution gradient adjusted for second screening run.

<table>
<thead>
<tr>
<th>Start Volume (mL)</th>
<th>End Volume (mL)</th>
<th>% Elution Buffer</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>60</td>
<td>240</td>
<td>20 - 50 (linear gradient)</td>
<td>4.0</td>
</tr>
<tr>
<td>240</td>
<td>420</td>
<td>100</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Figure 13. Procainamide affinity column gradient, conductivity, absorbance, and fractions. The red line shows the conductivity in mS/cm, the blue line is the absorbance, the blue circles indicate the start and end of the fraction collection, and the green circles at the bottom indicate the fraction number, with every five fractions numbered above the green circles for legibility.
Figure 14. AChE activity and Bradford assays for second procainamide affinity column run. Higher adsorption value for Bradford assay (blue) indicates higher protein content in the fraction. Higher adsorption value for AChE activity (red) indicates higher AChE enzyme in the fraction.

Several varying pools were made for the next step; these are outlined in Table 8. Prior to making pools 1-4, 200 µL of each fraction was taken to make the TCA pools listed in Table 8, each containing five fractions for a total of 1 mL per pool. These pools were not concentrated in any way, rather these were used to try a procedure using trichloroacetic acid (TCA), which is thought to allow low concentration samples to be run on gels without the need to concentrate them, thus saving an additional step. The remaining 5.8 mL of each fraction was used to make pools 1-4.
Table 8. Summary of pooled fractions from second procainamide affinity chromatography run.

<table>
<thead>
<tr>
<th>Pool</th>
<th>Fractions</th>
<th>Initial Volume (mL)</th>
<th>Final Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13-27</td>
<td>87</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>30-42</td>
<td>75.4</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>46-52</td>
<td>40.6</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>53-60</td>
<td>46.4</td>
<td>1</td>
</tr>
<tr>
<td>TCA 1</td>
<td>1-5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 2</td>
<td>6-10</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 3</td>
<td>11-15</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 4</td>
<td>16-20</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 5</td>
<td>21-25</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 6</td>
<td>26-30</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 7</td>
<td>31-35</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 8</td>
<td>36-40</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 9</td>
<td>41-45</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 10</td>
<td>46-50</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 11</td>
<td>51-55</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 12</td>
<td>56-60</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 13</td>
<td>61-65</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TCA 14</td>
<td>66-70</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

TCA pools 10-12 were used to test the TCA method and to be run on SDS-PAGE gels. We wanted to add 5% m/v trichloroacetic acid to each pool. To achieve this, 1.01 g of trichloroacetic acid was weighed out and placed in a microfuge tube, along with 1 mL of water. This yielded a final volume of 1.5 mL, so 75 µL of this solution was added to each pool. The TCA treated pools were then placed on ice for 30-60 minutes to precipitate out the proteins and centrifuged for 10 minutes. The supernatant was discarded and the pellet was rinsed out with acetone several times, while centrifuging for 10 minutes each time before discarding acetone. The pellet was then resuspended in 10 µL 20 mM sodium phosphate, pH 7.4 and 10 µL 2x loading buffer was added directly.
The four concentrated pools, as well as TCA pools 10-12 were run on a 4-20% Tris-glycine gel; 20 µL of each sample were loaded into each well. Based on the gel in Figure 15, most proteins seemed to be removed, with the majority being in the concentrated pools 1 and 2. Pools 3 and 4 contain the acetylcholinesterase, which is shown at about 250 kDa, but another band around 36 kDa can be seen. The pools using trichloroacetic acid seemed to not work very well, as the bands are very faint.

Figure 15. 4-20% Tris-glycine SDS-PAGE gel of second procainamide affinity run. Lanes 1 and 10 are blanks. Lane 2 contains Seeblue pre-stained molecular weight standard. Lane 3 corresponds to pool 1. Lane 4 corresponds to pool 2. Lane 5 corresponds to pool 3. Lane 6 corresponds to pool 4. Lane 7 corresponds to TCA pool 10. Lane 8 corresponds to TCA pool 11. Lane 9 corresponds to TCA pool 12. Reference Table 8 for corresponding fraction information for each pool.
Due to a contamination issue, a fresh batch of resin was needed and was prepared on February 21, 2015 and 20 mL were packed into a column on March 6, 2015. The same size column was used for all experiments before and after this new batch of resin.

Prior to starting a new affinity run, a new batch of crude hemolymph was concentrated in a 100 K Amicon ultracentrifugation filter and dialyzed into 20 mM sodium phosphate, pH 7.4. The tube was labeled as “Tetraquetra blood 4/17/14” and had a total volume of 22.5 mL after centrifuging at 15,000 RPM to remove cell debris. One mL of this was set aside for assay comparison to the starting material, and the rest was concentrated down to a total volume of 1 mL.

For the first procainamide affinity run on the newly prepared resin, 200 µL of concentrated hemolymph was diluted with 400 µL of 20 mM sodium phosphate, pH 7.4, giving a total volume of 600 µL. The gradient shown in Table 9 was used, with the fractions shown in Figure 16.

Table 9. Procainamide affinity column elution gradient for initial run on new resin. Flow was slowed to 1.5 mL/min after fraction 17 and then to 1 mL/min after fraction 23, due to the volume of buffer above the resin rising quickly. It seemed to stabilize and stay consistent at 1 mL/min.

<table>
<thead>
<tr>
<th>Start Volume (mL)</th>
<th>End Volume (mL)</th>
<th>% Elution Buffer</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60</td>
<td>0</td>
<td>4.0</td>
</tr>
<tr>
<td>60</td>
<td>240</td>
<td>20 - 50 (linear gradient)</td>
<td>4.0</td>
</tr>
<tr>
<td>240</td>
<td>420</td>
<td>100</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Figure 16. Procainamide affinity column gradient, conductivity, absorbance, and fractions for initial run on new resin. The red line shows the conductivity in mS/cm, the blue line is the absorbance, the blue circles indicate the start and end of the fraction collection, and the green circles at the bottom indicate the fraction number, with every five fractions numbered above the green circles for legibility. The box inside the chromatogram shows the absorbance to be 0.0162 A.U. and conductivity to be 42.69 mS/cm at 143.26 minutes.
Figure 17. AChE activity and Bradford assays for initial procainamide affinity column run on new resin. Higher adsorption value for Bradford assay (blue) indicates higher protein content in the fraction. Higher adsorption value for AChE activity (red) indicates higher AChE enzyme in the fraction.

Table 10. Purification table for initial procainamide affinity column run on new resin.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Total Protein (mg)</th>
<th>IU/mL</th>
<th>Total Activity (IU)</th>
<th>Specific Activity (IU/mg)</th>
<th>Protein Recovery (%)</th>
<th>Activity Recovery (%)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Hemolymph</td>
<td>21.5</td>
<td>35.3</td>
<td>0.13</td>
<td>2.75</td>
<td>0.08</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Amicon 100 K Filter (retentate)</td>
<td>1</td>
<td>22.0</td>
<td>1.65</td>
<td>1.65</td>
<td>0.08</td>
<td>62.3</td>
<td>60.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Amicon 100 K Filter (Eluate)</td>
<td>55</td>
<td>2.75</td>
<td>0.00</td>
<td>0.16</td>
<td>0.06</td>
<td>7.8</td>
<td>5.9</td>
<td>0.8</td>
</tr>
<tr>
<td>Procainamide Affinity Column</td>
<td>0.57</td>
<td>2.09</td>
<td>0.14</td>
<td>0.08</td>
<td>0.04</td>
<td>5.9</td>
<td>2.9</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Based on the activity and Bradford assays shown in Figure 17, it seems as though the majority of proteins, including acetylcholinesterase, eluted at the same time. There was another peak in the Bradford assay around fractions 4-7, which indicated some other proteins eluting. Based on the AChE activity assay results, fractions 17-20 were pooled
and concentrated. This pool was then tested for AChE activity and Bradford protein concentration on the same plate as the crude hemolymph and Amicon filter retentate and eluate, to collect data for a purification table, shown in Table 10. Given that most proteins eluted together, this was not a favorable purification, as indicated by a purification factor of 0.5. It is also possible that some acetylcholinesterase activity was lost due to enzyme damage during purification and sample storage.

Examining the point at which all proteins, including acetylcholinesterase, eluted, it seems as though it occurred when the step was made to 20% elution buffer. Because of this, the gradient was adjusted for the following run, to slowly increase from 0-10% elution buffer and then hold at 10% elution buffer, before making a step to 100% elution buffer to elute acetylcholinesterase. The gradient used for the second run is shown in Table 11, and the conductivity, absorbance, and fractions shown in Figure 18.

### Table 11. Procainamide affinity column elution gradient for second run on new resin.

<table>
<thead>
<tr>
<th>Start Volume (mL)</th>
<th>End Volume (mL)</th>
<th>% Elution Buffer</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>60</td>
<td>110</td>
<td>0 - 10 (linear gradient)</td>
<td>1.0</td>
</tr>
<tr>
<td>110</td>
<td>118</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>118</td>
<td>260</td>
<td>100</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 18. Procainamide affinity column gradient, conductivity, absorbance, and fractions for the second run on new resin. The red line shows the conductivity in mS/cm, the blue line is the absorbance, the blue circles indicate the start and end of the fraction collection, and the green circles at the bottom indicate the fraction number, with every five fractions numbered above the green circles for legibility. The program was paused after fraction 12 to adjust the gradient to that outlined in Table 11. After the “P” for pause located above, the fraction number is n+14; therefore, fraction 1 after “P” is actually fraction 15.
Figure 19. AChE activity and Bradford assays for second procainamide affinity column run on new resin. Higher adsorption value for Bradford assay (blue) indicates higher protein content in the fraction. Higher adsorption value for AChE activity (red) indicates higher AChE enzyme in the fraction.

Table 12. Purification table for second procainamide affinity column run on new resin.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Total Protein (mg)</th>
<th>IU/mL</th>
<th>Total Activity (IU)</th>
<th>Specific Activity (IU/mg)</th>
<th>Protein Recovery (%)</th>
<th>Activity Recovery (%)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amicon 100 K Filter (retentate)</td>
<td>0.2</td>
<td>5.06</td>
<td>1.42</td>
<td>0.28</td>
<td>0.06</td>
<td>100</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Procainamide Affinity Column</td>
<td>0.203</td>
<td>0.912</td>
<td>0.54</td>
<td>0.11</td>
<td>0.12</td>
<td>18.0</td>
<td>38.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

For the second run, 200 μL of concentrated hemolymph was diluted 1:1 with 20 mM sodium phosphate, pH 7.4 to a total of 400 μL. This was loaded onto the resin bed and run with the elution gradient shown in Table 11.

With the new adjusted gradient, the AChE activity assay and Bradford assay in Figure 19 show a much better separation of other proteins from the main AChE activity.
Based on the AChE activity assay results, fractions 29-31 were pooled and concentrated and tested again for AChE activity and Bradford protein concentration to determine the purification fold. As can be seen in Table 12, the purification factor was 2.2, significantly better than the 0.5 obtained in the previous run. Given that the Bradford protein concentration peak was still decreasing and not fully back down to a minimal value when the 100% elution buffer step occurred, perhaps holding at 10% elution buffer for a longer period of time would help elute more proteins prior to the acetylcholinesterase. The ramp rate from 0-10% elution buffer and the hold at 10% elution buffer were changed for the next run, as shown in Table 13.

Table 13. P rocainam id e affinity column elution gradient for third run on new resin.

<table>
<thead>
<tr>
<th>Start Volume (mL)</th>
<th>End Volume (mL)</th>
<th>% Elution Buffer</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>60</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>60</td>
<td>160</td>
<td>0 - 10 (linear gradient)</td>
<td>1.0</td>
</tr>
<tr>
<td>160</td>
<td>202</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>202</td>
<td>302</td>
<td>100</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Figure 20. Procainamide affinity column gradient, conductivity, absorbance, and fractions for the third run on new resin. The red line shows the conductivity in mS/cm, the blue line is the absorbance, the blue circles indicate the start and end of the fraction collection, and the green circles at the bottom indicate the fraction number, with every five fractions numbered above the green circles for legibility.

Figure 21. AChE activity and Bradford assays for third procainamide affinity column run on new resin. Higher adsorption value for Bradford assay (blue) indicates higher protein content in the fraction. Higher adsorption value for AChE activity (red) indicates higher AChE enzyme in the fraction.
Table 14. Purification table for third procainamide affinity column run on new resin.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (mL)</th>
<th>Total Protein (mg)</th>
<th>Total Activity (IU)</th>
<th>Specific Activity (IU/mg)</th>
<th>Protein Recovery (%)</th>
<th>Activity Recovery (%)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amicon 100 K Filter (retentate)</td>
<td>0.4</td>
<td>6.85</td>
<td>1.52</td>
<td>0.61</td>
<td>100</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Procainamide Affinity Column</td>
<td>0.173</td>
<td>0.507</td>
<td>1.05</td>
<td>0.18</td>
<td>7.4</td>
<td>30.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>

For the third purification run on the new resin, 400 µL of concentrated hemolymph was diluted 1:1 with 20 mM sodium phosphate, pH 7.4. This was loaded onto the resin bed and run with the elution gradient shown in Table 13, with fractions shown in Figure 20.

With the adjusted gradient based on the previous results, the Bradford and AChE activity assay results in Figure 21 show even better separation of other proteins from the main AChE activity peak. From this, fractions 39-41 were pooled and concentrated and then tested again for AChE activity and Bradford protein concentration against the material that was loaded. This adjusted gradient yielded a purification factor of 4.1 (Table 14), or twice that of the previous run, which serves to show that the adjustments in gradient proved to be very effective. This material was then utilized for further characterization, which included enzyme kinetics and SDS-PAGE.

5. Free Sulfhydryl Curve

The plate reader software reports all raw data with the label Vmax in units of mOD/min. The label Vmax is not to be confused with the enzyme kinetics parameter v_max. In order to convert the Vmax readings from the plate reader into meaningful
units, μmol/min in this case, a free sulfhydryl curve is needed (Figure 22). Since the substrate used is acetylthiocholine iodide, cleavage of the substrate results in the formation of a free sulfhydryl group. Known amounts of free sulfhydryl (-SH) are added in the form of reduced glutathione in the absence of enzyme to determine the change in absorbance per unit of free sulfhydryl for each spectrophotometer or plate reader.

Figure 22. Free sulfhydryl curve. The Vmax readings from the plate reader are divided by the slope from this curve, to give units of μmol/min.

6. Enzyme Kinetics

6.1 Michaelis-Menten Kinetics (Km)

The Michaelis-Menten model is based on the theory that the enzyme, E, and its substrate, S, react reversibly to form an enzyme substrate complex, ES, which then...
breaks down to yield the enzyme, E, and product, P. This is shown by the equation

\[ E + S \rightleftharpoons ES \rightarrow E + P \]. From this, the Michaelis constant, \( K_m \), is derived to be

\[ K_m = \frac{(k_{-1} + k_2)}{k_1} \]. The Michaelis-Menten equation is

\[ v = \frac{v_{max}[S]}{K_m[S]} \].

To determine the Michaelis constant (\( K_m \)), the 4.1 fold purified acetylcholinesterase from the final procainamide affinity run was used. Substrate (acetylthiocholine iodide) concentration was varied from 6.63 mM to 0.02 mM in solution, as outlined in Table 15. The final purified material was diluted 10-fold from 2.93 mg/mL to 0.293 mg/mL for this assay.

Table 15. Substrate concentrations used for Michaelis constant (\( K_m \)) determination.

<table>
<thead>
<tr>
<th>Total well volume (uL)</th>
<th>Substrate volume/well (uL)</th>
<th>Initial [Substrate] (mM)</th>
<th>[Substrate] in Soln. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>320</td>
<td>30</td>
<td>0.21</td>
<td>0.02</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>1.06</td>
<td>0.10</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>2.12</td>
<td>0.20</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>4.24</td>
<td>0.40</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>4.60</td>
<td>0.43</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>10.6</td>
<td>0.99</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>17.7</td>
<td>1.66</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>21.2</td>
<td>1.99</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>26.2</td>
<td>2.45</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>35.4</td>
<td>3.32</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>53.0</td>
<td>4.97</td>
</tr>
<tr>
<td>320</td>
<td>30</td>
<td>70.7</td>
<td>6.63</td>
</tr>
</tbody>
</table>
Michaelis-Menten plot. Also known as a substrate saturation curve, this graph shows that the velocity ($v$) approaches a limit ($v_{\text{max}}$) at which the enzyme is oversaturated with substrate, and is no longer dependent on substrate concentration. At low concentrations of substrate, the velocity is proportional to the substrate concentration, and behaves as a first-order reaction. As the enzyme becomes oversaturated with substrate, the enzyme begins to behave as a zero-order reaction, in which the rate is independent of the substrate concentration.

To determine the Michaelis constant ($K_m$), several different linear fit models exist. These models were developed since linear models are much easier to calculate than non-linear models like the Michaelis-Menten saturation curve (Figure 23). However, there are now computer models that can perform the calculations from the Michaelis-Menten saturation curve. The activity results and values used for the linear fit models can be found in Table A1. A summary of the $K_m$ and $V_{\text{max}}$ values for all four fit models can be found in Table 16.
The Lineweaver-Burk model (Figure 24) is known as the double-reciprocal plot, since it takes the reciprocal of both sides of the Michaelis-Menten equation to yield:

\[ \frac{1}{v} = \left( \frac{K_m}{V_{max}} \right) \left( \frac{1}{[S]} \right) + \frac{1}{V_{max}}. \]

Graphing using this equation will give the x-intercept = \(-\frac{1}{K_m}\), the y-intercept = \(-\frac{1}{V_{max}}\), and the slope = \(\frac{K_m}{V_{max}}\). However, this model has a disadvantage in that it gives a non-uniform distribution of error.

The Hanes-Woolf model (Figure 26) is based on the Michaelis-Menten model, but has an advantage in that it doesn’t overemphasize the data obtained at low substrate concentrations\(^1\). Multiplying both sides of the Michaelis-Menten equation by \([S]\) and simplifying yields the equation:

\[ \frac{[S]}{v} = \left( \frac{1}{V_{max}} \right) [S] + \frac{K_m}{V_{max}}. \]

Graphing using this equation will give the x-intercept = \(-K_m\), the y-intercept = \(\frac{K_m}{V_{max}}\), and the slope = \(\frac{1}{V_{max}}\).

The Eadie-Hofstee model (Figure 25) is another linear model based on the Michaelis-Menten equation, using the equation:

\[ v = -K_m \left( \frac{v}{[S]} \right) + V_{max}. \]

This model has an advantage over the Lineweaver-Burk model in that it tends to give more realistic views of errors, but also has the disadvantage that \(v\) appears on both axis, which is generally more subject to error than substrate concentration. Graphing using this equation will give the x-intercept = \(\frac{V_{max}}{K_m}\), the y-intercept = \(V_{max}\), and the slope = \(-\frac{1}{K_m}\).
Figure 24. Lineweaver-Burk plot. The x-axis is $1/(\text{substrate concentration})$ in molar, and the y-axis is $1/\text{initial velocity}$ in $\mu\text{mol/min}$. 

$$y = 0.0813x + 184$$

$$R^2 = 0.997$$
Figure 25. Eadie-Hofstee plot. The x-axis is the initial velocity in µmol/min divided by the substrate concentration in molar, and the y-axis is the initial velocity in µmol/min.
Figure 26. Hanes-Woolf plot. The x-axis is the substrate concentration in molar, and the y-axis is the substrate concentration in molar divided by the velocity in μmol/min.

Table 16. Summary of Michaelis constant (Km) and v_max values based on various enzyme kinetic models.

<table>
<thead>
<tr>
<th>Plot</th>
<th>Km (M)</th>
<th>V max (μmol/min)</th>
<th>Coefficient of Determination (R²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Michaelis-Menten</td>
<td>2.08E-04</td>
<td>4.17E-03</td>
<td>0.992</td>
</tr>
<tr>
<td>Lineweaver-Burk</td>
<td>4.41E-04</td>
<td>5.42E-03</td>
<td>0.997</td>
</tr>
<tr>
<td>Eadie-Hofstee</td>
<td>3.00E-04</td>
<td>4.40E-03</td>
<td>0.905</td>
</tr>
<tr>
<td>Hanes-Woolf</td>
<td>1.99E-04</td>
<td>4.08E-03</td>
<td>0.999</td>
</tr>
</tbody>
</table>

Given the data and graphs established from the AChE run with varying substrate concentrations, the Michaelis constant (Km) was determined from the Lineweaver-Burk plot, since the Eadie-Hofstee plot yielded a poor correlation. Extrapolating from the equation of the linear regression line on the Lineweaver-Burk plot, the slope, which is

\[ y = 245x + 0.0488 \]

\[ R^2 = 0.999 \]
equal to $\frac{K_m}{v_{\text{max}}}$ is $8.13 \times 10^{-2}$ (M min)/µmol, and $\frac{1}{v_{\text{max}}}$ is $184.35$ min/µmol.

Dividing $\frac{1}{v_{\text{max}}}$ by $\frac{K_m}{v_{\text{max}}}$ gives us $\frac{1}{K_m}$, which equals $2267.528$ 1/M. Dividing 1 by $\frac{1}{K_m}$ gives us $K_m$, which turns out to be $4.41 \times 10^{-4}$ mol/L for acetylcholinesterase in *Tritonia tetraquetra*. For the Eadie-Hofstee model, $-$Km is equal to the slope of the linear fit, giving a value of $3.00 \times 10^{-4}$ mol/L. For the Hanes-Woolf model, $-$Km is equal to the x-intercept, or where y=0, giving a value of $1.99 \times 10^{-4}$ mol/L.

6.2 Enzyme Inhibition (IC50)

The half maximal inhibitory concentration (IC50) was determined using the AChE activity assay; the assay was altered slightly by decreasing the buffer volume by 50 µL and replacing it with 50 µL of varying concentrations of the inhibitor procainamide as outlined in Table 17. Due to the solubility of procainamide, higher volumes of inhibitor were needed for the three highest procainamide concentrations; the volume of procainamide listed was subtracted from the buffer in the assay to keep a consistent volume.
Table 17. Procainamide concentrations and run setup for IC50 determination.

<table>
<thead>
<tr>
<th>[Procainamide] (mM)</th>
<th>Volume procainamide in assay (uL)</th>
<th>Buffer Volume (uL)</th>
<th>Enzyme Volume (uL)</th>
<th>DTNB Volume (uL)</th>
<th>ATCh Substrate volume (uL)</th>
<th>Total well volume (uL)</th>
<th>[Procainamide] In assay (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.184</td>
<td>250</td>
<td>0</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>144</td>
</tr>
<tr>
<td>0.184</td>
<td>200</td>
<td>50</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>115</td>
</tr>
<tr>
<td>0.184</td>
<td>150</td>
<td>100</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>86.2</td>
</tr>
<tr>
<td>0.184</td>
<td>50</td>
<td>200</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>28.7</td>
</tr>
<tr>
<td>0.184</td>
<td>50</td>
<td>200</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>2.87</td>
</tr>
<tr>
<td>0.184E-01</td>
<td>50</td>
<td>200</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>2.87E-01</td>
</tr>
<tr>
<td>0.184E-02</td>
<td>50</td>
<td>200</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>2.87E-03</td>
</tr>
<tr>
<td>0.184E-03</td>
<td>50</td>
<td>200</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>2.87E-04</td>
</tr>
<tr>
<td>0.184E-04</td>
<td>50</td>
<td>200</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>2.87E-05</td>
</tr>
<tr>
<td>0.184E-05</td>
<td>50</td>
<td>200</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>2.87E-06</td>
</tr>
<tr>
<td>0.184E-06</td>
<td>50</td>
<td>200</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>2.87E-07</td>
</tr>
<tr>
<td>0.184E-07</td>
<td>50</td>
<td>200</td>
<td>30</td>
<td>10</td>
<td>30</td>
<td>320</td>
<td>2.87E-08</td>
</tr>
</tbody>
</table>

Table 18. IC50 Calculations. The 100% reference is the Vmax of the sample without inhibitor minus the blank mean. The % of reference column lists the “slope-blank” column divided by the 100% reference blank times 100%.

<table>
<thead>
<tr>
<th>[Procainamide] in assay (mM)</th>
<th>Slope</th>
<th>Slope - Blank</th>
<th>% of Reference</th>
<th>Log (Procainamide in M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Reference</td>
<td>131</td>
<td>1.54E+00</td>
<td>1.00E+00</td>
<td>-0.84</td>
</tr>
<tr>
<td>Blank</td>
<td>0.539</td>
<td>1.65E+00</td>
<td>1.11E+00</td>
<td>-0.94</td>
</tr>
<tr>
<td></td>
<td>86.2</td>
<td>1.78E+00</td>
<td>1.24E+00</td>
<td>-1.06</td>
</tr>
<tr>
<td></td>
<td>28.7</td>
<td>4.72E+00</td>
<td>4.18E+00</td>
<td>-1.54</td>
</tr>
<tr>
<td></td>
<td>2.87</td>
<td>2.50E+01</td>
<td>2.44E+01</td>
<td>-2.54</td>
</tr>
<tr>
<td></td>
<td>2.87E-01</td>
<td>5.77E+01</td>
<td>5.72E+01</td>
<td>-3.54</td>
</tr>
<tr>
<td></td>
<td>2.87E-02</td>
<td>6.89E+01</td>
<td>6.84E+01</td>
<td>-4.54</td>
</tr>
<tr>
<td></td>
<td>2.87E-03</td>
<td>9.29E+01</td>
<td>9.24E+01</td>
<td>-5.54</td>
</tr>
<tr>
<td></td>
<td>2.87E-04</td>
<td>1.16E+02</td>
<td>1.16E+02</td>
<td>-6.54</td>
</tr>
<tr>
<td></td>
<td>2.87E-05</td>
<td>1.23E+02</td>
<td>1.23E+02</td>
<td>-7.54</td>
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<td></td>
<td>2.87E-06</td>
<td>1.23E+02</td>
<td>1.23E+02</td>
<td>-8.54</td>
</tr>
<tr>
<td></td>
<td>2.87E-07</td>
<td>1.28E+02</td>
<td>1.28E+02</td>
<td>-9.54</td>
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<tr>
<td></td>
<td>2.87E-08</td>
<td>1.26E+02</td>
<td>1.26E+02</td>
<td>-10.5</td>
</tr>
</tbody>
</table>
Figure 27. IC50 graph. Log of the procainamide concentration in M is plotted on the x-axis, and the relative activity, or the % of reference, is plotted on the y-axis.

To determine the IC50, the “trend” formula was used for the points bracketed in the graph in Figure 27. The formula was =TREND(Log (Procainamide in M), % of reference, 50), where the “Log (Procainamide in M)” and “% of reference” correspond to the data for those points in the respective columns in Table 18; the original activity data is shown in Table A2. This gave a trend value of -4.26424. The IC50 was then calculated by taking $10^{(\text{trend value})}$, giving an IC50 value of $5.44 \times 10^{-5}$ M.

7. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was utilized to compare the starting crude hemolymph to the in-process sample, as well as the final purified sample. SDS-PAGE separation yields
information on the molecular weight distribution of proteins in the sample. Both reduced (Figure 29) and non-reduced gels (Figure 28) were performed for the crude hemolymph, the 100 kDa Amicon filter retentate, and the final purified sample, at 5 and 10 µg loads. All samples were diluted to 1 mg/mL and loaded at either 10 µL (5 µg) or 20 µL (10 µg).

Figure 28. Non-reduced 4-20% Tris-glycine SDS-PAGE gel. Lanes 1, 3, 6, and 9 are blanks. Lanes 2 and 12 are the SeeBlue molecular weight standard. Lane 4 is 5 µg of crude hemolymph. Lane 5 is 10 µg of crude hemolymph. Lane 7 is 5 µg of Amicon filter retentate. Lane 8 is 10 µg of Amicon filter retentate. Lane 10 is 5 µg of final purified material. Lane 11 is 10 µg of final purified material. The apparent molecular weights of the SeeBlue molecular weight standard are listed in red on the left side of the gel.
Figure 29. Reduced 4-20% Tris-glycine SDS-PAGE gel. Lanes 1, 3, 6, and 9 are blanks. Lanes 2 and 12 are the SeeBlue molecular weight standard. Lane 4 is 5 µg of crude hemolymph. Lane 5 is 10 µg of crude hemolymph. Lane 7 is 5 µg of Amicon filter retentate. Lane 8 is 10 µg of Amicon filter retentate. Lane 10 is 5 µg of final purified material. Lane 11 is 10 µg of final purified material. The apparent molecular weights of the SeeBlue molecular weight standard are listed in red on the left side of the gel.

From both the reduced and non-reduced gels, it is clearly evident that the amount of lower molecular weight proteins is decreasing, as the bands further down the gel are more faint in the final purified material than either of the other two samples analyzed.

Unfortunately, the 100 kDa Amicon filter retentate samples tend to show a decrease in the intensity of the higher molecular weight material, which would most likely correspond to acetylcholinesterase; this indicates that the enzyme is likely sticking to the membrane during the ultracentrifugation step. Also, there was no decrease in the
intensity of the bands at lower molecular weight, indicating that these were not eluting through the membrane, which would be due to polarization of the membrane.

Unfortunately, the final purified material still shows several bands on the SDS-PAGE gel. This indicates that several purification steps need to be combined to purify the sample even further. Perhaps combining the procainamide affinity column with the HiTrap Phenyl HP column would yield a more favorable purification.

Chapter 4: Discussion

1. Purification

In order to purify acetylcholinesterase from *Tritonia tetraquetra* hemolymph, a variety of purification techniques were tested, including separation by size, charge, and affinity. Due to the expected large size of acetylcholinesterase, separation by size using a 100 kDa cutoff membrane was tested. Although acetylcholinesterase is much larger than 100 kDa, the hemolymph seems to have polarized the membrane, causing very slow elution of the sample through the membrane and also causing acetylcholinesterase to stick to the membrane. This step yielded no increase in purity and thus was predominantly used to concentrate and dialyze the sample prior to other purification steps.

The next purification step attempted was an anion exchange chromatography method. Anion exchange separates proteins by charge, using a positively charged resin, which binds negatively charged proteins. The proteins can be eluted using either a salt or
pH gradient. When using a salt gradient, a low salt concentration is used initially and the salt concentration is increased to displace the protein, since the negative ions of the salt compete with the protein bound to the resin. When using a pH gradient, a pH which gives the protein an overall negative charge is used, and then the pH is decreased to give the protein a positive charge, causing it to be displaced from the positively charged resin.

Initially, a DEAE-A50 resin was used to screen whether this could be a suitable purification step. Based on the AChE activity assay, this resin did not seem to bind acetylcholinesterase very well, which led to an unsatisfactory purification. Additionally, the resin would severely compress during the elution process, which indicated this resin may not be suitable for column chromatography.

A second anion exchange resin, DEAE Sepharose CL-6B, was used to pack a column to test anion exchange chromatography a second time. The activity and Bradford results from this column also gave unsatisfactory separation, as it seemed acetylcholinesterase didn’t tightly bind to the resin and was eluting in early fractions, and all proteins, including the remaining acetylcholinesterase, seemed to elute at the same time. Given the results from these two anion exchange resins, anion exchange chromatography was no longer pursued as a purification step.

After anion exchange, hydrophobic interaction chromatography (HIC) was tested. Hydrophobic interaction chromatography uses a hydrophobic resin and separates proteins based on the presence of hydrophobic areas on the surface of proteins. HIC is different from most other chromatography methods in that the sample is loaded at a high salt concentration and eluted with a low salt concentration. The high salt concentration
promotes a hydrophobic effect, and causes the hydrophobic regions of the protein to bind to the hydrophobic resin. Reducing the salt concentration reduces the hydrophobic interactions, causing the proteins to be displaced from the hydrophobic resin.

Different phenyl sepharose columns from GE Healthcare were tested, including HiTrap Phenyl FF low sub, HiTrap Phenyl FF high sub, and HiTrap Phenyl HP. The HiTrap Phenyl HP has a smaller particle size, at 34 µm, compared to the particle size of 90 µm for the HiTrap Phenyl FF columns. Additionally, the Phenyl HP and Phenyl FF low sub have a substitution of 25 µmol phenyl per mL of gel, while the Phenyl FF high sub has a substitution of 40 µmol phenyl per mL of gel. Of the three columns, the HiTrap Phenyl HP gave the best separation. Despite this, other proteins were still eluting at the same time as acetylcholinesterase, but it seemed as though many other proteins were still eluting after the main AChE activity peak. Perhaps this could still be a useful purification step if followed up by an affinity purification step.

The final purification method attempted was an affinity column. Affinity column chromatography is the most specific form of purification used in this study, as it uses an inhibitor specific for acetylcholinesterase, and does not rely on general characteristics of proteins. A strong or weak inhibitor can be attached to a solid support resin. For weaker inhibitors, the sample is loaded at a low salt concentration and eluted with a high salt concentration. For stronger inhibitors, a high salt concentration may not be sufficient to displace the protein from the inhibitor on the resin, and a high concentration of the same or stronger inhibitor may be needed in the mobile phase. While a stronger inhibitor
would, in theory, be better for purification, fractions with inhibitor cannot be tested for activity, and thus would have to be dialyzed in order to determine the activity.

The affinity column used here was generated using the acetylcholinesterase inhibitor procainamide. While procainamide is not a strong acetylcholinesterase inhibitor, it is still strong enough to bind acetylcholinesterase to it and allow it to be eluted off with a high salt wash, instead of the need for a high concentration of inhibitor in the elution buffer. This advantage allows for much easier screening of fractions, as there’s no need to dialyze the fractions prior to testing for AChE activity. This method, once optimized, gave the best separation of all the attempted purification steps. The procainamide affinity column was used to purify acetylcholinesterase from hemolymph. The purified sample was used in the enzyme kinetic experiments.

2. Michaelis-Menten Kinetics (Km)

The Michaelis-Menten parameters $K_m$ and $v_{\text{max}}$ characterize the enzyme kinetic efficiency of an enzyme. The Michaelis constant $K_m$ can provide information on the binding affinity of a substance, provided that the catalytic step with the rate constant $k_2$ is slower than the dissociation step of the ES complex with the rate constant $k_{-1}$. In this case, $K_m = \frac{(k_{-1} + k_2)}{k_1} = \frac{k_{-1}}{k_1}$, and a small $K_m$ value then indicates high substrate binding affinity.

The final purified material was tested for Michaelis-Menten kinetics against acetylthiocholine iodide. This was conducted using the AChE activity assay and varying the concentration of the substrate (acetylthiocholine iodide), while maintaining all other
reagents in the assay constant. The Michaelis constant (Km) was derived from several different fit models, with the Michaelis-Menten model giving a Km of $2.08 \times 10^{-4}$ mol/L. A study, conducted by Wallace, et al., on *Aplysia californica* (sea hare gastropod) determined the Km to be 0.79 mM, or $7.9 \times 10^{-4}$ mol/L for hemolymph against acetylthiocholine. Another study, conducted by Lopez, et al., utilizing *Electrophorus electricus*, or electric eel, determined the Km to be 0.52 mM, or $5.2 \times 10^{-4}$ mol/L. The determined Michaelis constant for *Tritonia tetraquetra* in this study is within range of what has been observed for other similar species.

3. Enzyme Inhibition (IC50)

Since procainamide was used to create an affinity column, the half maximal inhibitory concentration (IC50) was determined for this cholinesterase inhibitor using the final material for which 4.1-fold purification was achieved. The IC50 value was determined to be $5.44 \times 10^{-5}$ M. A study conducted by Talesa, et al., tested fully soluble *Helix pomatia* (garden snail) hemolymph cholinesterase against procainamide and determined the IC50 to be $10^{-4}$ M. This shows a two-fold difference in IC50 values; given that the cholinesterases are from different species, slight differences can be expected. When comparing IC50 values, a smaller value is indicative of a stronger inhibitor, since a smaller amount of the inhibitor is required to inhibit the activity of the protein or enzyme.

The determined IC50 for procainamide against the final purified material here shows that procainamide is not a strong inhibitor against acetylcholinesterase, which shows why we were able to elute the enzyme with only high salt. Using a stronger
inhibitor, such as tacrine, to create an affinity column would allow for a better purification, but would also require eluting the enzyme with a high concentration of inhibitor; this would then require dialysis of the fractions in order to be able to test them for activity. A study by Kaya et al., showed the IC50 of tacrine to be $5.0 \times 10^{-8}$ M for bovine erythrocyte AChE.\(^3\)

**4. Molecular Weight Determination (SDS-PAGE)**

SDS-PAGE allows us to look at how the purity of the sample is improving each step, by monitoring the protein bands that are being removed or decreased relative to the desired protein. In addition to purity information, SDS-PAGE also provides estimated molecular weight information. While the purification of acetylcholinesterase from *Tritonia tetraqueta* has not been published before, previous work has been done on *Aplysia californica*, another marine mollusk, making it a closely-related species. Bevelaqua, et al., determined that acetylcholinesterase from *Aplysia californica* hemolymph to be 250,000 Da.\(^{14}\) Additionally, *Electrophorus electricus* acetylcholinesterase purified and sold by Sigma-Aldrich is documented to have a molecular weight of 280,000 Da.

Looking at the final purified material in the non-reduced gel, it appears there are two bands right at the 250 kDa molecular weight marker. There is one slightly thinner and fainter band right at 250 kDa and one a bit broader and darker immediately above it; one of these bands would likely be acetylcholinesterase.
References


Appendix

Table A1. AChE activity results and calculations for Michaelis-Menten determination. The question marks shown for SPL 12 on the last row correspond to this being out of the range of the plate reader, thus the Max V was not determined. The 1/[S], 1/Vo, and Vo/[S] values were used in determining the Michaelis-Menten constant in Chapter 3, Section 6.1 using various linear-fit models.

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Table A2. AChE activity results from IC50 determination. The concentration of procainamide, an acetylcholinesterase inhibitor, was varied to determine the half maximal inhibitory concentration (IC50), as outlined in Chapter 3, Section 6.2.

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