Mechanism of Nucleoside Hydrolase Isolated from Alaska Pea Seeds

by

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I dedicate this thesis research to my family, friends, and coworkers who have helped me along this journey. I would not be where I am today without them.

"Never give up, never forget"-David Charles Moore

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Abstract:

Nucleosidases or nucleoside hydrolases are a class of enzymes that hydrolyze nucleosides into a pentose sugar and a nitrogenous base. These enzymes are widely distributed, and are found in parasitic protozoans, plants, bacteria, and fungi. Since this class of enzymes does not occur in humans, drugs based on the inhibition of the enzymes have been created to target susceptible organisms. Similarly inhibition of these enzymes may provide a route for a new class of herbicides. Among the plant species in which these enzymes have been found include yellow lupin, coffee leaves, and tea leaves. The nucleosidases are often present as isozymes, with multiple versions occurring in the same organism. The objective of this study was to isolate and characterize the isozymes found in germinated pea seeds with regard to chromatography behavior, molecular weight, and substrate specificity.

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CHAPTER I: INTRODUCTION

Nucleosides:

Nucleosides are biological compounds that are composed of a sugar pentose residue, and a heterogeneous nitrogen base residue. The structures of these components are used to differentiate types of nucleosides. For example, adenosine, 2'-deoxyadenosine, and 2',3'-dideoxyadenosine all contain adenine as the nitrogenous base, but differ with respect to the number of hydroxyl groups attached to the sugar moiety. Aside from the sugar portion of the molecule, nucleosides also differ with respect to the nitrogenous base that is bound to the sugar moiety. Nitrogenous bases are separated into two main groups, purines and pyrimidines. Purines are composed of two fused heterocyclic rings, while pyrimidines are composed of a single heterocyclic ring. Adenosine, an example of a purine nucleoside, is seen in Figure 1-1. An example of several of these nitrogenous bases is presented in Figure 1-2.
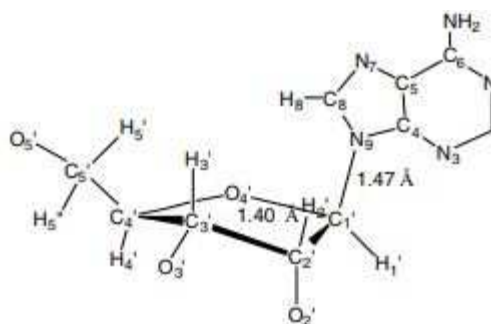
Figure 1-1: Adenosine consists of adenine, a nitrogenous base, linked to a ribose sugar. (*1*)
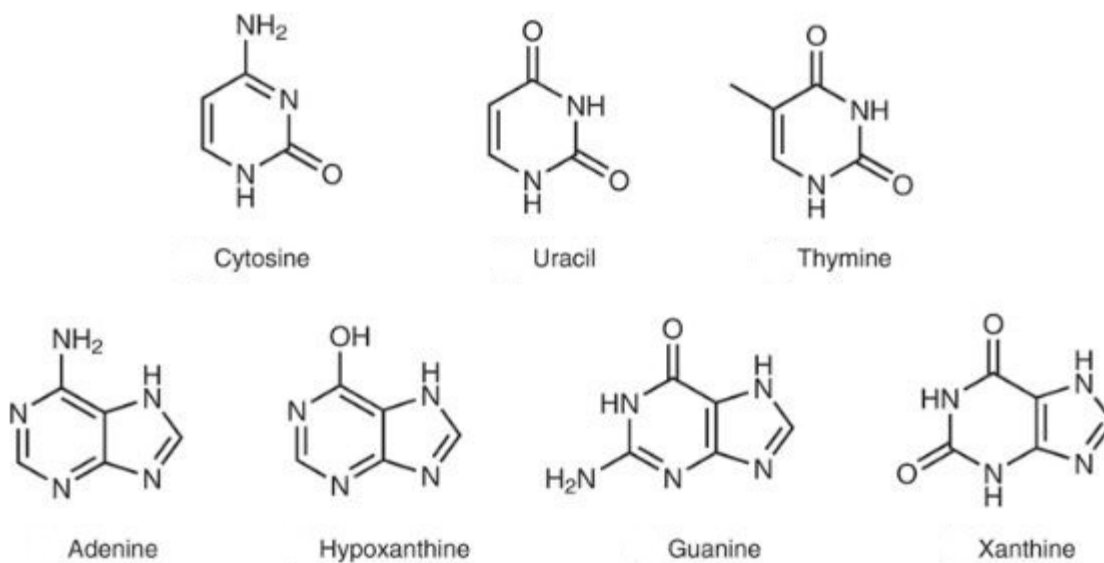
Figure 1-2: Several nitrogenous bases commonly seen in nature (*2*)

There are several different biochemical processes that utilize nucleosides and nucleotides, derivatives of nucleosides that contain a phosphate group. One such process is the utilization of phosphorylated nucleosides such as adenosine triphosphate as energy currency for cellular processes. Another commonly recognized use for nucleosides/nucleotides is the storage and transmission of genetic information in the form of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA). As such, it is important for organisms to maintain a constant supply of nucleosides/nucleotides.

Nucleosidases:

Nucleosidases or nucleoside hydrolases are a class of enzymes that catalyze the degradation of nucleosides into their respective nitrogenous base and sugar moieties, as depicted in Figure 1-3.
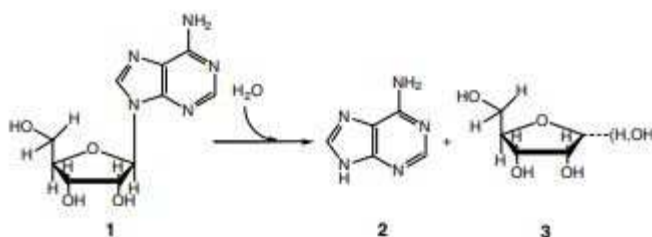
Figure 1-3: Hydrolysis of adenosine, (1) into adenine (2), and ribose (3). While ribose is shown, the sugar may be ribose or 2'-deoxyribose. The initial anomeric configuration of the ribose product is not known (*1*).

Often organisms contain multiple isozymes of nucleosidases, which can be categorized into subclasses based on the characteristics of these enzymes. One such characteristic that can be utilized to differentiate isozymes is the ability of the enzymes to either utilize purines or pyrimidines or both (*3, 4, 5*). For example, nucleosidases that show pyrimidine specificity or no specificity have been referred to as "group I" nucleosidases, while nucleosidases that prefer purines are referred to as "group II" nucleosidases (*4*). In addition, nucleosidases can also be classified based on the specific nucleosides they favor. These include inosine-adenosine-guanosine nucleosidases (IAG-NH), guanosine-inosine nucleosidases (IG-NH) , adenosine nucleosidase, and inosine nucleosidase, depending on the purines the enzymes favor (*3, 5, 6, 7, 8*). Nucleosidases can also be classified based of their organism of origin. For example, nucleosidases isolated from *Crithidia fasciculata, Trypanosoma vivax,* and *Trypanosome brucei brucei* all have unique characteristics such as molecular weight and complexity, being composed of tetramers, and dimers (*5, 9,10*). As more nucleosidases are discovered and characterized, then the classification of these enzymes will need to be refined and expanded to encompass novel characteristics that were previously undiscovered.

Sources of Nucleosidases:

Nucleosidases were initially identified and studied in several species of parasitic protozoans such as *Crithidia fasciculata*, *Trypanosoma brucei brucei,* and *Trypanosoma vivax*. Parasitic protozoans have been identified as the pathogenic agents in several diseases, including malaria and trypanosomiasis (*6*). As such, these enzymes were initially studied as potential drug targets for these organisms (*5, 6, 9, 10*). This is due to the fact these organisms do not possess the ability to synthesis purines or pyrimidine *de novo* (*5, 6*). While these enzymes have also been isolated from bacteria such as *Escherichia coli,* they do not exist in mammalian systems (*4*). As such, these parasitic organisms must rely on salvage pathways in which nucleosidases are a part of to obtain their purines from a host organism, generally mammalian cells, that can produce the nucleic bases on their own (*5, 6*).

It has been discovered that several plant sources contain nucleosidases, including yellow lupin, tea leaves, *Coffea arabica* leaves, and *Arabidopsis thaliana*, among others (*3, 11, 12, 13*) . However, plants possess the ability to synthesize nucleotides *de novo* from precursor compounds and convert them to nucleosides (Figure 1-4).
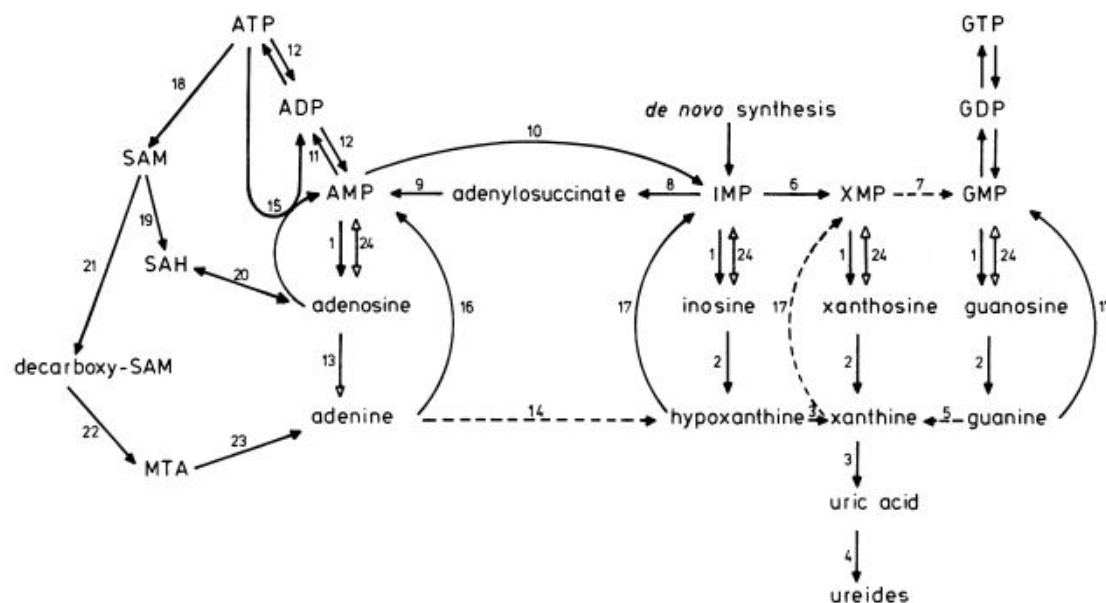
Figure 1-4: Purine synthesis and conversion in plants. 1- general acid phosphatase, 2-inosine nucleosidase, 3- xanthine dehydrogenase, 4- urate oxidase, 5-guanine deaminase, 6-IMP dehydrogenase, 7- GMP synthetase, 8- adenylosuccinate synthetase, 9-adenylosuccinate lysase, 10-AMP deaminase 11- adenylate kinase, 12 – apyrase, 13-adenosine nucleosidase, 14- adenine deaminase, 15- adenosine kinase 16- adenine phosphoribosyltransferas, 17- hypoxanthine-guanine phosphoribosyltransferas, 18- S-adenosylmethionine (SAM) synthetase, 19- methyltransferase, 20- S-adenosylhomocysteine (SAH) hydrolase, 21- S-adenosylmethionine decarboxylase, 22-aminopropyl transferase 23- 5'-methylthioadenosine (MTA) nucleosidase, 24-nucleoside phosphotransferase (*3*). Reprinted with permission of American Society of Plant Biologists.

Because of this system, plant purine synthesis can be observed as a combination of the *de novo* and the salvage pathways. As such, nucleosidases originating from plant-based sources have become a focal point for enzymatic research because of the unique role these enzymes serve in plants' purine and pyrimidine metabolic pathways. Nucleoside hydrolases have been theorized to serve a variety of functions in plants, including generating precursors for caffeine production in tea and coffee leaves, extracellular

signaling, and wound repair (*11,12,13*). There are also nucleosidases that either show specificity to pyrimidines or are non-specific to either pyrimidines or purines. The pyrimidine specific or non-specific nucleosidases are generally found in bacteria that can synthesis purines *de novo* (*4*).

Enzymatic Structures:

Nucleosidases are a diverse class of enzymes, with unique structures from many different organisms. Research has shown that there are considerable differences in the molecular weight and complexity of the enzymes isolated from protozoan sources, bacteria, and plants. Nucleosidases range from 62 kDa monomers isolated from yellow lupin to 143 kDa tetramers isolated from *Crithidia fasciculata* (*3, 5, 6, 7, 8, 9, 11, 12*). Nucleosidases from plant sources tend to be monomers and dimers with lower molecular weights compared to the larger trimers and tetramers found in protozoan sources (*3, 5, 6, 7, 8, 9, 11, 12,*).

A common technique utilized to study the structure of proteins is x-ray crystallography (*4*). This technique results in data that can be interpreted to decipher the three-dimensional structure and changes in the conformation as an enzyme interacts with a substrate. Previous studies by Iovane *et al.* have utilized this technique to study the structure and mechanism of wild-type and several mutated variants of YeiK, a nucleosidase isolated from *Escherichia coli*, as the enzyme interacts with inosine (*4*). The data collected resulted in the image seen in Figure 1-5, and the identification of the α9 portion of the enzyme as the location of the active site (*4*).
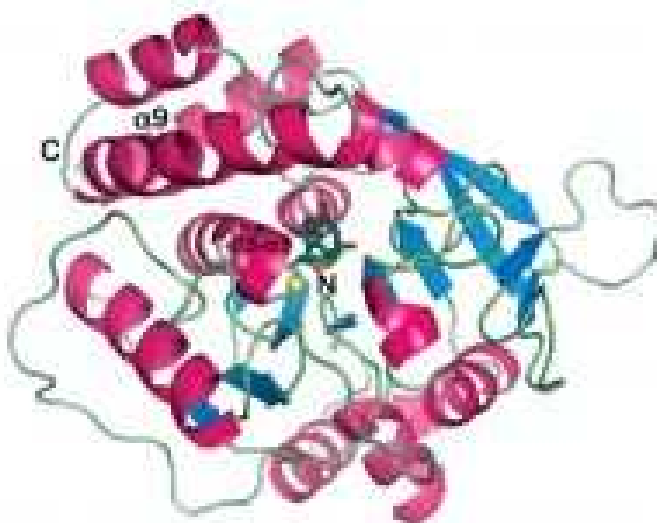
Figure 1-5: Structure of YeiK monomer, where the active site (α9), the Ca^{2+} ion (yellow dot), the C-terminus, and the N-terminus are shown (*4*). This enzyme structure, based on the inosine-uridine nucleosidase from *Crithidia fasciculata*, is composed of eleven α-helices and ten β-sheets separated into three regions, a core of six alternating α-helices and six β-sheets, a lobe consisting of α-helices 7-10 and the C-terminal α-helix 11, and an extension of the core that contains the remaining β-sheets 7-10 that form an interfacial region of the enzyme. (*14*)

There were a few structural characteristics that were of interest in this

nucleosidase. The first was a conserved N-terminal DXDXXXDD sequence that is seen

in many nucleosidases, and appears to be involved in the binding of the Ca^{2+} ion to the

active site of the nucleosidase (*4*). Researchers have also identified a group of enzymes

from yellow lupin seedlings that show a several-fold increase in activity in the presence

of Ca^{2+} ion compared to the absence of the ion (*8*). This research supports the concept

that Ca^{2+} ions are necessary for the activity of these enzymes.

It has also been shown that for IU- nucleosidases, there exists a necessary

catalytic triad consisting of Tyr227, Tyr223 and His 239 residues for purine hydrolysis,

while pyrimidine hydrolysis only require a His 239 residue (*4*). This is because His 239

residue can immediately protonate the O2 atom of the leaving pyrimidine, while a proton transfer from His 239 to Tyr 227 or to Tyr 223 and subsequently Tyr 227 was required to protonate the N7 atom of the leaving purine (*4*). The Ca^{2+} ion acts to polarize the water molecule that acts as a nucleophile to cleave the C-N bond between the ribose and the base as seen in Figure 1-7 (*4*).

Nucleosidase Isozymes:

When nucleosidases where initially discovered in *Crithidia fasciculata*, it was initially believed to contain only a single nucleosidase isozyme (*9*). However, it was later discovered a second nucleosidase isozyme exists that has its own unique substrate specificities, size, and other characteristics when compared to the originally discovered enzyme (*6*). It has also been discovered several isozymes exist in other organisms. Nucleosidases have also been identified in bacteria such as *Escherichia coli*. However, the nucleosidases found in bacteria are generally either pyrimidine specific or have no specificity to either pyrimidines or purines, since bacteria can synthesize purine nitrogenous bases and nucleosides *de novo* (*4*).

One example of this phenomenon is a study that identified three different nucleosidases from tea leaves (*11*). While the nucleosidase appeared as a single homogenous peak through both a DEAE-cellulose column and a Sephadex G-100 gel filtration column, when the pooled fractions were chromatographed on a CM-cellulose column, the enzyme was differentiated into three distinct peaks (*11*), as shown in Figure 1-6. While the three isozymes were similar with respect to their sizes, pH stability, thermal stability, and substrate specificity, there were slight differences with respect to

their optimal pH for activity, with the first and third peak enzyme exhibiting maximum

activity at a pH of 4.0 and the second peak having an optimal pH of 4.5 (*11*).

A.
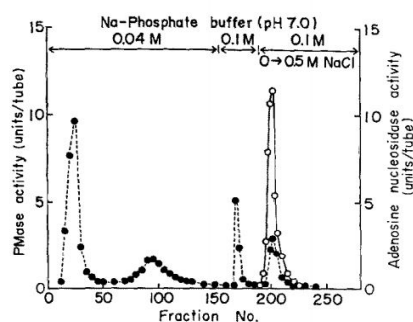
B.
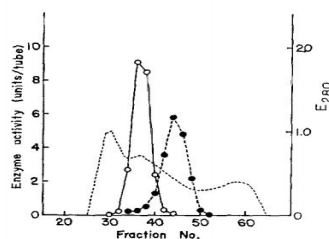
C.
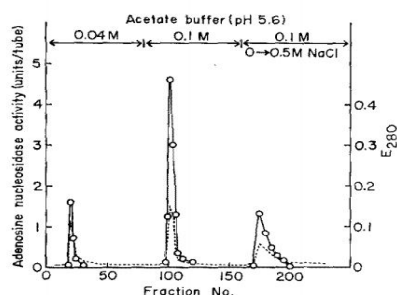
Figure 1-6: **A.** The DEAE elution profile for nucleosidases isolated from tea leaves (*11*). Nucleosidase activity is represented by white dots and phosphomonoesterase (PMase) is represented by the black dots. **B.** The G-100 elution profile for tea leaves nucleosidases after chromatography on a DEAE column, with the nucleosidase activity represented by the white dots, and the absorbance at 280nm represented by black dots (*11*). **C.** The CM-cellulose elution profile after the G-100 chromatography with the three isozymes identified by the three distinct peaks. The nucleosidase activity is represented by the white dots. The absorbance at 280nm was represented by the dashed line (*11*). Reprinted with permission of Japan Society for Bioscience, Biotechnology and Agrochemistry.

However, tea leaves are not the only plants to have multiple nucleosidase isozymes identified. *Arabidopsis,* one of the first identified plant-based nucleosidases sources, was later discovered to contain three unique nucleosidase isozymes including an extracellular variant (*13*). There have also been multiple different isozymes isolated from yellow lupin, including an adenosine nucleosidase, inosine nucleosidase, and even a calcium-stimulated guanosine-inosine nucleosidase (*3, 7, 8*).

Mechanism of Nucleosidases:

There have been several studies addressing the chemical mechanism of nucleosidases. The most widely studied nucleosidase is the IU-nucleosidase isolated from *Crithidia fasciculata* (*4*). Current research on a recombinant YeiK nucleosidase originally isolated from *Escherichia coli* has resulted in a proposed mechanism for these nucleosidases. The β N-glycosidic bond between the ribose and the base was destabilized by induced partial charges between the ribose and the base (*4*). This induced positive charge interacts with a Ca^{2+} polarized water molecule that protonates a negatively charged Asp 11 residue that conducts a nucleophilic attack on the carbocation formed on the ribose (*4*). Simultaneously the partial negative charge on the nitrogen of the C-N bond is delocalized to either the O2 or N7 position atoms of the pyrimidine or purine respectively (*4*). In pyrimidines, the partial negatively charged O2 atom is directly protonated by a His 239 residue for pyrimidine nucleosides (*4*). Conversely, the partial negatively charged N7 atom is indirectly protonated by a His 239 to Tyr 223 proton transfer or a His 239 to Tyr223 to Tyr 227 proton transfer for purine nucleosides (*4*). Ultimately, the stabilization of the partial positive and negative charges results in the

cleavage of the C-N β N-glycosidic bond which hydrolyzes the nucleoside (*4*). This

mechanism is outlined in Figure 1-7.
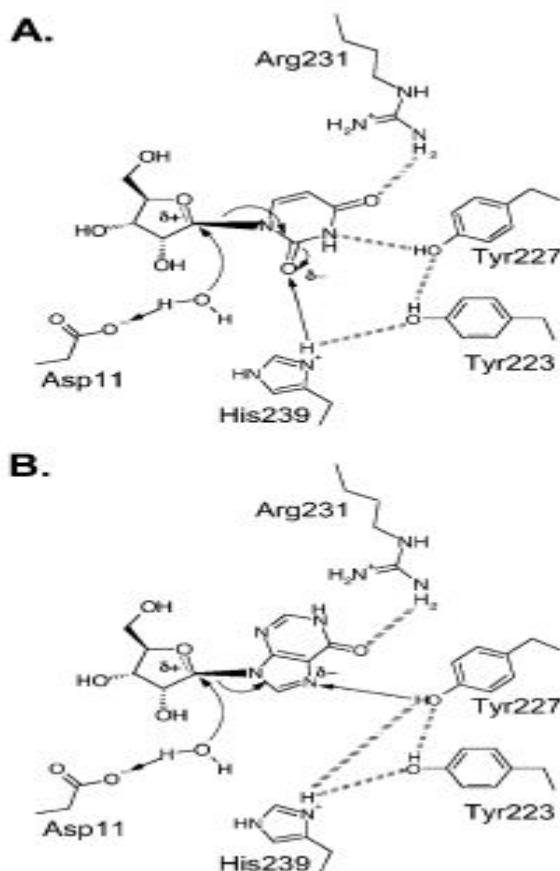
Figure 1-7: Proposed mechanism of nucleosidases for pyrimidines, in this case uridine (A) and purines, in this case inosine (B) for YeiK, a nucleosidase from *Escherichia coli* (*4*). Reprinted with permission from American Chemical Society.

Transition State of Nucleosidases:

Previous research has proposed a transition state for the hydrolysis of adenosine

by adenosine nucleosidase originating from yellow lupin. This research studied the

interaction between the nucleosidase isolated from yellow lupin and several isotopically labeled adenosine derivatives (*1*). The results indicate that adenosine goes through several structural changes as the C1'-N9 bond between the base and the ribose is cleaved including stretching of the C1'-N9 bond from 1.47 Å to 1.92 Å, C1' carbon undergoing nucleophilic attack by a polarized water molecule, conversion of the ribose ring from C3'*endo* conformation to a C2' *exo* conformation, a contraction of the C1'-O4' carbon-oxygen bond, the generation of an oxocarbenium ion between C1' and O4', and protonation of the adenine portion of the nucleoside at N7 (*1*), as exemplified in Figure 1-8.

A. Ground State   B. Transition State
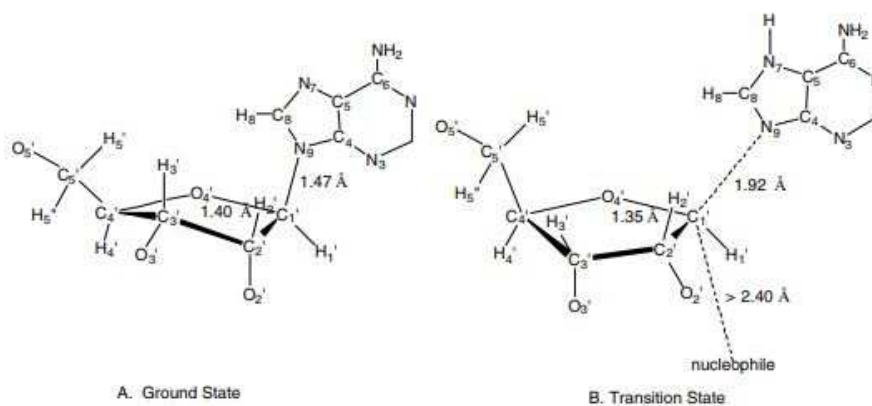
Figure 1-8: The proposed ground state and transition state of adenosine hydrolysis to adenine and ribose via adenosine nucleosidase from yellow lupin (*1*). Reprinted with permission from Phytochemistry.

This transition state resembles the transition state for the recombinant IU-nucleosidase from *Crithidia fasciculata* (*4*).

CHAPTER II: MATERIALS AND METHODS

Equipment:

Protein concentrations were determined at 280nm on a Thermo Scientific Nanodrop Spectrophotometer, while ribose concentrations were determined based on the absorbance at 450nm using a Barnstead-Turner SP-830 spectrophotometer. Preparative scale centrifugation was carried out using a Sorvall Lynx 6000 refrigerated centrifuge equipped with carbon fiber fixed angled rotors. Size exclusion chromatography was carried out on an AKTA Purifier UPC 10 FPLC system equipped with pH and conductivity monitors, dual pumps, and a UV detector. Substrate specificity studies were performed on a Dionex Ultimate 3000 UHPLC system with a quaternary pump, autosampler, column oven, and variable wavelength detector, with a Kinetex 5μm C18 100Å 150mm ×4.6mm LC column.

Materials:

Protease inhibitor cocktail for plant cells that contained AEBSF, bestatin, E-64, leupeptin, pepstatin A, and phenanthroline was obtained from bioWORLD, Dublin, Ohio. Nucleosides and nitrogenous bases were provided by Sigma-Aldrich, with two exceptions. The exceptions were nebularine from Toronto Research Chemicals Inc. located in Ontario, Canada, and erythrouridine which was a gift of Omicron Biochemical located in South Bend, Indiana. SDS supplies of pre-cast RunBlue 10% SDS gels, RunBlue 20× SDS Run Buffer, InstantBlue coomassie staining solution, 2× RunBlue LDS Sample, and RunBlue 10× Sample Reducer were purchased from Expedeon located in Heidelberg, Germany. Molecular weight markers were Precision Plus Unstained

Protein Standards purchased from Bio-Rad, located in Hercules, California, which contained ten recombinant proteins with molecular weights ranging from 10kD to 250kD. SDS-PAGE was run on a Hoefer Inc. Mighty Small II Deluxe Mini Vertical Electrophoresis System. Ammonium sulfate was provided by Fisher BioReagents located in Pittsburgh, Pennsylvania, and protamine sulfate salt from salmon was provided by Sigma-Aldrich. Pierce cation exchange spin columns were obtained from Thermo Scientific located in Waltham, Massachusetts. Methanol for the UHPLC methods was provided by VWR Chemicals located in Radnor, Pennsylvania. All other chemicals were reagent grade.

Germination of Seeds:

Alaska Pea:

Alaska Pea seeds were supplied by Sustainable Seed Company. Seeds ($\approx$100g) were submerged in bleach for five minutes, rinsed with tap water, until there was no longer a bleach smell, and placed onto a raised plastic grate between two moistened paper towels. The seeds were sprayed with tap water and monitored for mold daily for 13 days after rinsing with bleach.

Little Marvel:

Little Marvel seeds were supplied by Ferry-Morse Seed Company. Between 25-30g of seeds were submerged in bleach for five minutes, rinsed with tap water until there was no longer a bleach smell, and placed onto a raised plastic grate between two layers of moistened paper towels. The seeds were sprayed with tap water and monitored for mold daily for 9 days after rinsing with bleach.

Soybeans:

Soybean seeds were supplied from Johnny's Selected Seeds of Winslow ME. Approximately 25-30g of seeds were submerged in bleach for five minutes, rinsed until there was no longer a bleach smell to the seeds with tap water, and placed onto a raised plastic grate between two layers of perforated paper towels. The seeds were sprayed with tap water and monitored for mold daily for 15 days after rinsing with bleach.

Homogenization of Seeds:

Following germination, seeds were homogenized in 500mL of 50mM Tris buffer, pH 7.2, containing 2.05g protamine sulfate, 160mg of dithiothreitol (DTT), and 100μL of protease inhibitor cocktail for plant cell extracts. Seedlings were homogenized at 4□ for 1 min in a Waring Commercial Blender, followed by a 1 min rest. This cycle was repeated 5 times. The homogenized seed extract was centrifuged at 12,000×*g* for 20 minutes at 4 □. The supernatant was strained through cheese cloth to separate any remaining solid particulates.

Salting Out and Resuspension of Pellet:

Once the non-soluble material was separated from the initial lysate, the solution was brought to 50% ammonium sulfate saturation by slowly adding ammonium sulfate to the initial lysate (*15*). The solution was left at 4 □ overnight. The next day, the 50% saturated solution was centrifuged for 20 minutes at 12,000×*g* at 4 □. After centrifugation was complete, the supernatant was separated from the 50% saturated pellet which was resuspended in 50mM Tris pH 7.2. The resuspended pellet was stored at 4 □,

except for the pellet from the Alaska Pea seed which was discarded. The 50% supernatant was brought to 70% saturation by the slow addition of ammonium sulfate to the supernatant. The 70% saturated solution was centrifuged for 20 minutes at $12,000 \times g$ in a 4 □ cold room after overnight incubation. After centrifugation, the 70% supernatant was removed, and the 70% saturated pellet was resuspended in 50mM Tris, pH 7.2. Once resuspended, the 70% pellet was dialyzed three times for a minimum of 12 hours each against 1L of 50mM Tris, pH 7.2 to prepare for loading onto the DEAE column.

Reducing Sugar Assay:

The reducing sugar assay utilizes two main components; a copper reagent, and a neocuproine solution (*16*). The copper reagent was prepared by dissolving 16.0g of Na_2CO_3, 6.4g of glycine, and 0.18g of $CuSO_4 \cdot 5H_2O$ in 400mL of 18MΩ H_2O. The neocuproine solution was prepared by dissolving 0.48g of neocuproine in 400mL of 18MΩ H_2O and adjusting the pH to 3.0 with HCl. To a mixture containing 1000µL of a reducing sugar solution and 100µL of an enzyme containing sample, 250µL each of neocuproine and copper reagents were added. The assay mixture was incubated in a water bath at approximately 95□ for 7 minutes. Once the solutions had cooled, the absorbance of the solutions were measured at 450nm and the amount of reducing sugar present determined by comparison to a calibration curve prepared using known amounts of ribose.
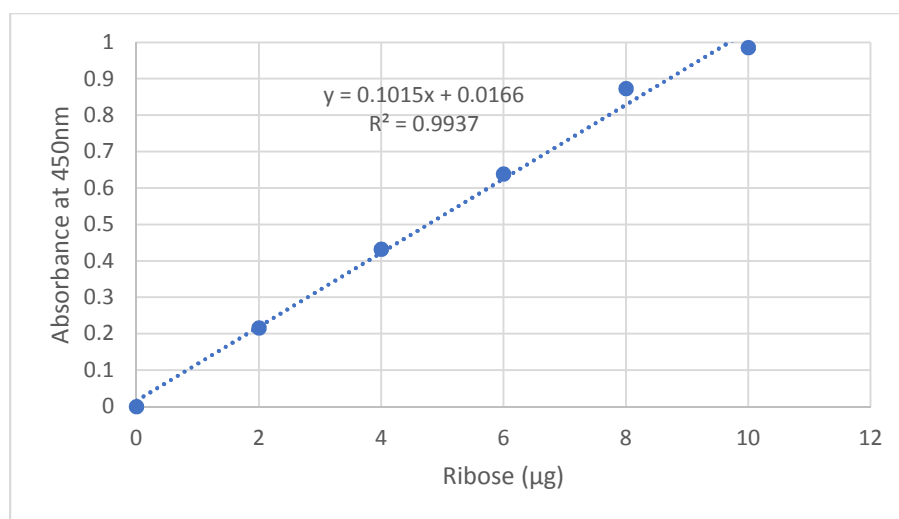
Figure 2-1: Standard curve for reducing sugar assay.

Determination of Protein Concentration:

Protein concentration was determined via measuring absorbance at 280nm on a Thermo Scientific Nanodrop spectrophotometer. This was accomplished by placing approximately 2µL of sample onto the nanodrop stage, closing the top, and measuring the absorbance. If the absorbance values were greater than 1.0, then a 1/10 serial dilution was performed to dilute the sample to generate a value between 1.0 and 0.1. Once an that value was found, and corrected for the dilution, the concentration was determined using the equation below.

$$Abs@280nm \times \frac{\frac{1mg}{mL}}{Abs @ 280nm} = Concentration \left(\frac{mg}{mL}\right)$$

DEAE Chromatography:

A column containing diethylaminoethyl (DEAE) resin from GE Healthcare was packed into a 50cm×2.5cm column from Ace Glass. Prior to loading the sample, the

column was washed with approximately 1L of 1000mM NaCl, followed by approximately 1L of 18MΩ DH$_2$O, and lastly 1L of 50mM Tris, pH 7.2, to equilibrate the column. Once the column was equilibrated, the dialyzed 70% pellets or Alaska pea supernatant were loaded onto the column and a step-wise gradient of 350mL of each of the following NaCl concentrations were used to elute the column; 0mM 100mM, 200mM, 300mM, 400mM, 500mM, 600mM, and 1000mM. Fractions containing approximately 13mL were collected. Once the fractions were collected, the column was cleaned following the protocol described above. Protein concentrations were estimated by absorbance at 280nm and activities were determined by reducing sugar assay. Aliquots (100μL) from each fraction were added to 1000μL of 1mM inosine in 50mM Tris, pH 7.2. The reaction mixture was incubated for 2 hours at room temperature and the amount of reducing sugar produced was determined by reducing sugar assay, per the previously described reducing sugar assay protocol. Related fractions with activity were pooled, concentrated, and stored at 4□ for analysis. Depending on the amounts involved, protein-containing solutions were concentrated using either 3,000 or 10,000 MWCO Amicon Ultracel centrifugal filters or a 50mL Amicon stirred ultrafiltration cell with a 5KD MWCO disk.

Fast Protein Liquid Chromatography (FPLC) Size Exclusion Chromatography:

After equilibrating the HiPrep 26/60 Sephacryl S-100 HR column with two column volumes (640mL) of an elution buffer containing 50mM Tris, 150mM NaCl, pH 7.2. Approximately 2mL of Alaska Pea pool 51-55 was filtered through a 0.22μm filter and loaded onto the size exclusion column followed by 10 mL of elution buffer to rinse the 5mL sample loop. The sample was eluted with 2 column volumes of the elution

buffer, with fractions collected every 10mL. Fractions were assayed with the reducing

sugar assay, and protein concentration was determined via nanodrop spectrophotometry.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE):

Running buffer was prepared by diluting 25mL of RunBlue 20X triethanolamine-

Tricine running buffer concentrate, from Expedeon, to 500mL. A 10% precast gel was

placed into a Hoeffer minigel system, samples were prepared by combining 10µL of the

sample with 3µL of 18 MΩ H_2O, 5µL of 4X RunBlue LDS sample buffer, and 2µL of

RunBlue 10X sample reducer for a total volume of 20µL for each sample. Once the

samples were prepared, they were spun for approximately 8 s, placed into a 70-80☐

heating block for 7 mins, and recentrifuged for approximately 8 s. Once the samples were

recentrifuged, they were loaded onto the 10% pre-cast gel, along with 10µL of molecular

weight markers and run at a constant current of 50mA for almost 3.5 hours. Once the gel

was done running, it was stained with InstantBlue coomassie staining solution overnight

on a rocker, rinsed with 18 MΩ H_2O, and photographed.

Substrate Specificity:

Nucleoside reaction mixtures (1mM nucleoside) were prepared by dissolving

nucleosides in 50mM PIPES, pH 6.6, and placing 1mL of the solution into an HPLC vial.

The reaction was initiated by adding either 10µL or 50µL of the enzymatic pool to the

1mL of 1mM nucleoside solution and monitoring the area of the peaks generated. The

reaction time was approximately 18 hours, or until the reaction was complete. Whichever

was shorter. The nucleosides were eluted from C-18 UHPLC column with a mobile

phase containing varying percentages of filtered 10mM ammonium phosphate, pH 5.4,

and HPLC grade methanol. All substrate specificity analysis and standards were run at

0.6mL/min with an injection volume of 10µL. The column oven was set to 32□ for all runs, and the elution was monitored at either 254nm or 268nm. Once a standard was eluted, without the enzyme, the concentration of the solution was divided by the area under the peak associated with the nucleoside. The nucleoside, elution conditions, base, and retention time for the nucleoside and base are listed below in Table 2-1.

Cation Exchange Spin Column:

Cation spin columns were rinsed with 400µL of 0.45µm filtered 50mM Tris buffer, pH 7.2 at approximately 2,000×*g* to equilibrate. Next, 100µL of 0.45 µm filtered Alaska Pea pool 9-11 (125µL diluted to 1000µL) was added to the column and the column spun at approximately 2,000×*g* to load the column. The spin column was eluted with two rounds of 400µL of 0mM NaCl in 50mM Tris, pH 7.2, followed by elution with 400µL of the following solutions; 100mM, 200mM, 300mM, 400mM, 500mM, 600mM, 1000mM, and 2000mM NaCl in 50mM Tris, pH 7.2. The fractions were collected based on the eluting salt concentration. The activity in each fraction was determined by incubating with 1mL of 1mM inosine for 2 hours at room temperature and measuring the amount of ribose produced using the reducing sugar assay.

Table 2-1: UHPLC conditions for substrate specificity analysis.

UHPLC conditions for substrate specificity analysis.						
Nucleoside	Base	Methano l %	Ammonium Phosphate %	Nucleoside Retention Time '(mins)	Base Retention Time (mins)	Wave- length (nm)
Nebularine (Purine Riboside)	Purine	10%	90%	6.3	5.0	254
5'-Deoxyadenosine	Adenine	10%	90%	13.5	5.1	254
Tubercidin (7-Deazaadenosine)	Adenine	10%	90%	6.9	5.1	254
3-Deazauridine	2,4-dihydroxypyridine	2%	98%	6.8	4.5	268
6-(γ,γ-Dimethylallyl-amino) purine riboside	6-(γ,γ-Dimethylallyl-amino) purine	30%	70%	33.3	31.7	268
6-Chloropurine riboside	6-Chloropurine	10%	90%	13.4	8.7	268
6-Benzylamino purine riboside	6-Benzylamino purine	30%	70%	28.4	26.4	268
Kinetin Riboside	Kinetin	25%	75%	20.1	17.1	268
Erythrouridine	Uracil	2%	98%	5.2	3.6	254
Uracil 1-β-arabino-furanoside	Uracil	2%	98%	7.2	3.5	254
Adenine 1-β-arabino-furanoside	Adenine	2%	98%	20.7	9.3	254
Cordycepin (3'-Deoxyadenosine)	Adenine	2%	98%	53.3	10.0	254
Xanthosine	Xanthine	2%	98%	8.5	5.7	254
Inosine	Hypoxanthine	2%	98%	10	5.0	254
Adenosine	Adenine	10%	90%	8	5.0	254
2'-Deoxyadenosine	Adenine	10%	90%	9.4	5.0	254
Guanosine	Guanine	2%	98%	11.5	5.2	254
Uridine	Uracil	2%	98%	5.5	3.6	254
2'-Deoxyuridine	Uracil	2%	98%	7.8	3.6	254
5-Methyluridine (Ribothymidine)	Thymine	2%	98%	10.8	6.9	254
Thymidine	Thymine	2%	98%	19	6.9	254
Cytidine	Cytosine	2%	98%	4.3	3.1	254
2'-Deoxycytidine	Cytosine	2%	98%	5.9	3.1	254

CHAPTER III: RESULTS

There are several different techniques that have been utilized to purify

nucleosidases and separate them from other cellular proteins once the cells have been

lysed. These purification protocols generally include salting the nucleosidase out of the

initial cell lysate with ammonium sulfate followed by several different chromatography

techniques such as gel-filtration, ion exchange, and affinity chromatography (*4, 6, 12,*).

The plant seeds in this study were homogenized by blending the seedlings with a protease

inhibitor cocktail for plant extract to prevent degradation of the nucleosidase, and

protamine sulfate to precipitate nucleic acids, in 500mL of 50mM Tris, pH 7.2, buffer

solution. The enzyme was salted out by ammonium sulfate precipitation. Once the

enzyme had been precipitated, it was resuspended in 50mM Tris buffer, pH 7.2, dialyzed

against the same buffer, and ultimately loaded onto a DEAE column to separate via ion

exchange chromatography.

Purification of Nucleoside Hydrolase:

Alaska Pea Seeds 70% Ammonium Sulfate Pellet:

Figure 3-1 is the DEAE elution profile for the dialyzed 70% ammonium sulfate

pellet from Alaska Pea seed, showing activity and protein concentration. The pellet was

resuspended in approximately 17mL of 50mM Tris, pH 7.2, and dialyzed to remove

ammonium sulfate before it was loaded onto the column. Based on the elution profile

from the DEAE chromatography, there appeared to be three peaks of activity and protein

that were subsequently pooled. The first peak, 7-11 pool, eluted at 0mM NaCl, and had

the second highest amount of activity. The second peak, pool 28-34, had the largest

amount of activity of the three and was eluted at 100mM NaCl concentration. This peak (Fractions 28-34) had been previously isolated and studied (*17*). The third peak, Fractions 46-48 which eluted at 200mM NaCl concentration, had the least amount of activity relative to the other two peaks and was relatively impure when compared to the other two peaks.

Fractions 7-11 did not interact with the DEAE column and eluted in the wash. To determine if the peak represented a new enzyme or was the result of column overloading, Fractions 7-11 were dialyzed and reloaded onto the column after the column was cleaned and equilibrated with 1L of 50mM Tris, pH 7.2 buffer. The enzyme again eluted at 0mM NaCl. This indicated that the column was not overloaded, and that this isozyme did not interact with the DEAE column.

A.
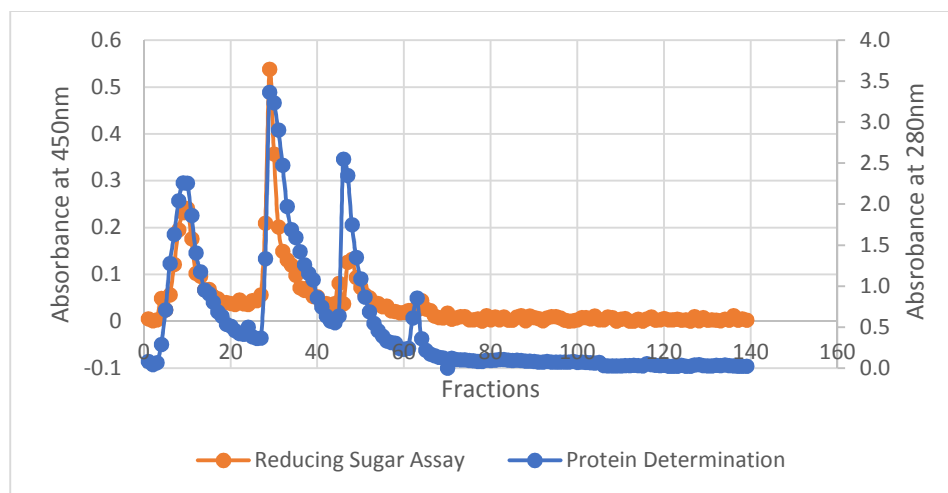
B.
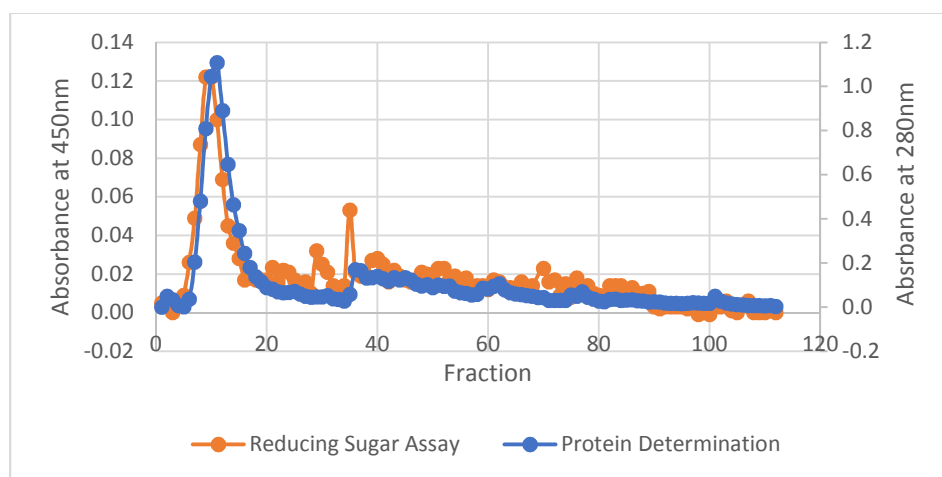
Figure 3-1: A. DEAE elution profile of 70% saturation pellet from Alaska Pea seeds. NaCl concentrations increased in a stepwise manner; 0mM NaCl Fractions 1-16, 100mM NaCl Fractions 17-36, 200mM NaCl Fractions 36-52, 300 mM NaCl Fractions 53-71, 400 mM NaCl Fractions 72-89, 500mM NaCl Fractions 90-106, 600 mM NaCl Fractions 107-123, 1000 mM NaCl Fractions 124-142. Fractions 7-11, 28-34, and 46-48 were pooled. B. DEAE elution profile of Fractions 7-11 reloaded onto the column. NaCl concentrations increased in a stepwise manner; 0mM NaCl Fractions 1-24, 100mM NaCl Fractions 25-48, 200mM NaCl Fractions 49-72, 300 mM NaCl Fractions 73-91, 1000 mM NaCl Fractions 92-112. Fractions 9-11 were pooled.

Alaska Pea Seeds 70% Ammonium Sulfate Supernatant:

The Alaska pea seed supernatant from the 70% ammonium sulfate fractionation

was identified as containing a significant amount of activity based on the reducing sugar

assay. To recover this activity, the supernatant was concentrated by ultrafiltration to a

working volume of approximately 100mL, dialyzed against 50mM Tris, pH 7.2, and

loaded on the DEAE column. The column was eluted with a step-wise NaCl

concentration gradient similar to that used in the chromatography of the 70% pellet.

Fractions 31-35, 38-42, and 51-55 were pooled representing 100mM and 200mM NaCl

respectively, as shown in the elution profile in Figure 3-2.

Figure 3-2: DEAE elution profile for 70% saturation supernatant from Alaska Pea seeds. NaCl concentrations changed in a stepwise manner; 0mM NaCl Fractions 1-20, 100mM NaCl Fractions 21-40, 200mM NaCl Fractions 41-60, 300mM NaCl Fractions 61-80, 400mM NaCl Fractions 81-100, 500mM NaCl Fractions 101-121, 600mM NaCl Fractions 122-141, 1000mM NaCl Fractions 142-161. Fractions 31-35, 38-42, and 51-55 were pooled.

As seen in the elution profile, the supernatant fraction did not contain much of the

0mM NaCl nucleosidase. This is most likely due to a majority of that isozyme

precipitating below the 70% ammonium sulfate saturation point. Another point of difference between the elution profiles of the 70% ammonium sulfate pellet and the 70% ammonium sulfate supernatant is the presence of two peaks in the 100mM NaCl. While it is possible that these two peaks are different nucleosidase isozymes, further characterization would be required to properly determine if these are different forms of the enzyme or not.

Soybean Seeds 70% Ammonium Sulfate Pellet:

Soybean seeds were also germinated, homogenized, and subjected to ammonium sulfate (70%) precipitation. After dialysis and resuspension, the pellet was loaded onto the DEAE column. The column was eluted with a step-wise gradient of NaCl as shown in Figure 3-3 A. The first peak, Fractions 10-20, eluted at 0mM NaCl, and had the second highest amount of activity. The second peak, Fractions 37-43, had the largest amount of activity of the three and was eluted at 100mM NaCl concentration. The third peak, Fractions 63-68 which eluted at 200mM NaCl concentration, had the least amount of activity relative to the other two peaks. However, when an SDS-PAGE analysis was conducted, all of the pools had considerable contamination from other proteins and required additional purification as shown in 3-3B.
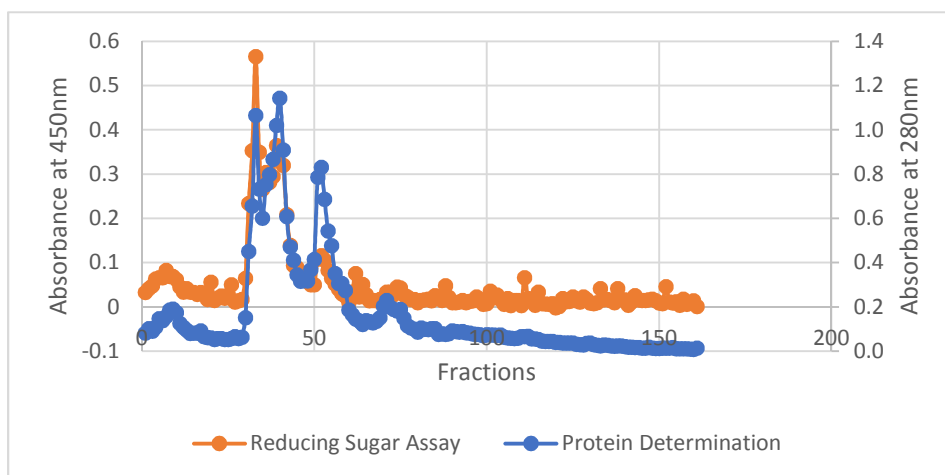
A.
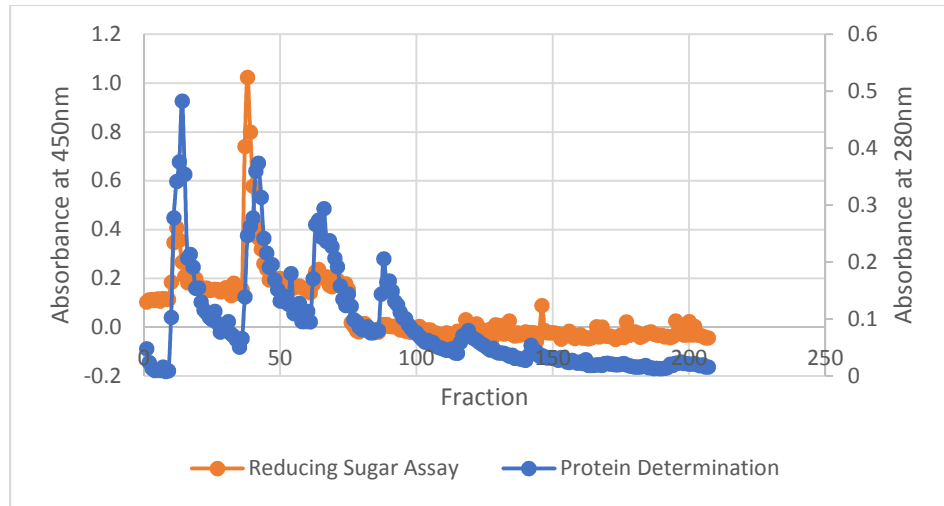
B.

Figure 3-3: A. DEAE elution profile for 70% saturation pellet from soybean seeds. NaCl concentrations changed in a stepwise manner; 0mM NaCl-Fractions 1-25, 100mM NaCl Fractions 26-50, 200mM NaCl Fractions 51-75, 300mM NaCl Fractions 76-104, 400mM NaCl Fractions 105-130, 500mM NaCl Fractions 131-156, 600mM NaCl Fractions 157-182, 1000mM NaCl Fractions 183-207. Fractions 10-20, 37-43. 63-68 were pooled. B. SDS-PAGE gel of Fractions 10-20, 37-43, and 63-68 in the first, second and third lane from the left after the molecular weight ladder.

Little Marvel Pea Seeds 70% Ammonium Sulfate Pellet:

Little Marvel pea seeds were treated as previously described for Alaska pea and soybean seeds. Figure 3-4 shows the DEAE elution profile for the Little Marvel pea seed 70% pellet. The pellet was resuspended in 50mM Tris, pH 7.2, and dialyzed before it was loaded onto the column. Fractions 8-15, 38-50, and 64-66 respectively were pooled. The first peak, Fractions 8-15, eluted at 0mM NaCl, and had the second highest amount of activity, but the highest 280nm readings indicating the largest protein amount. The second peak, Fractions 38-50, had the largest amount of activity of the three and was eluted at 100mM NaCl concentration. The third peak, Fractions 63-68 eluted at the 200mM NaCl concentration, had the least amount of activity relative to the other two peaks. However, when an SDS-PAGE analysis was conducted, all the pools had considerable contamination from other proteins and will require additional purification techniques to isolate the nucleosidases.
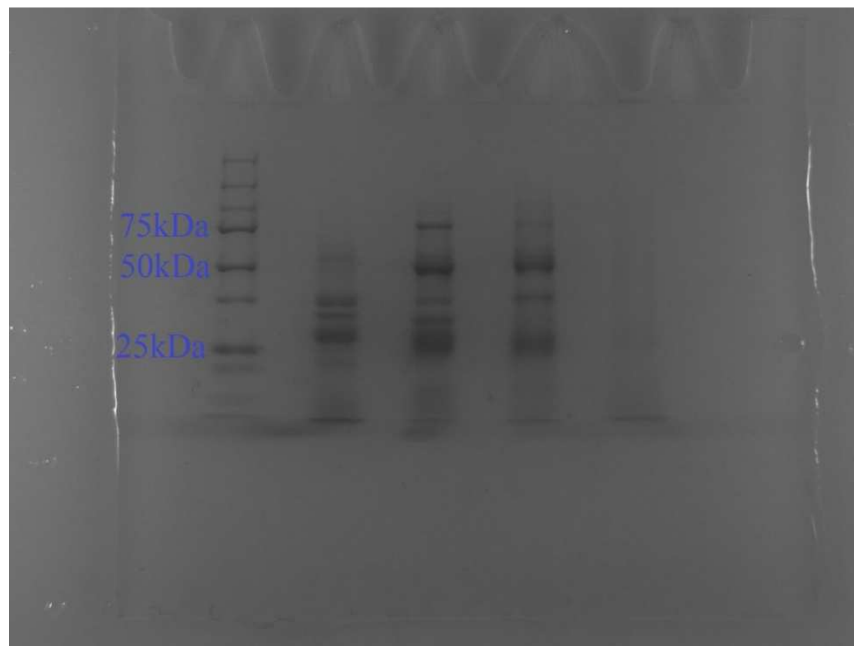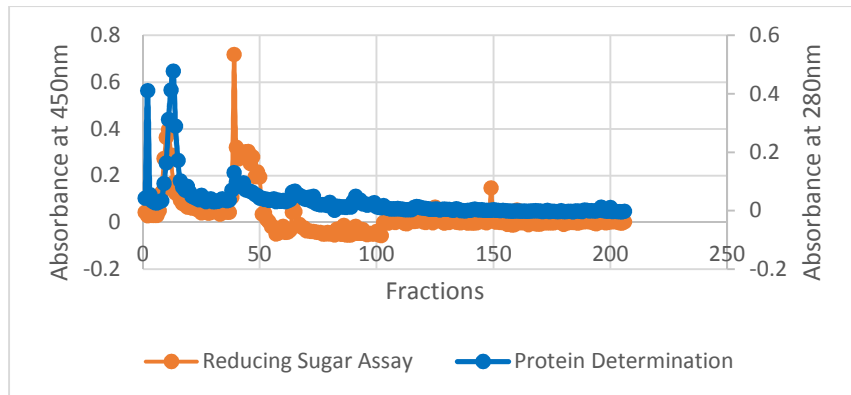
A.

B.
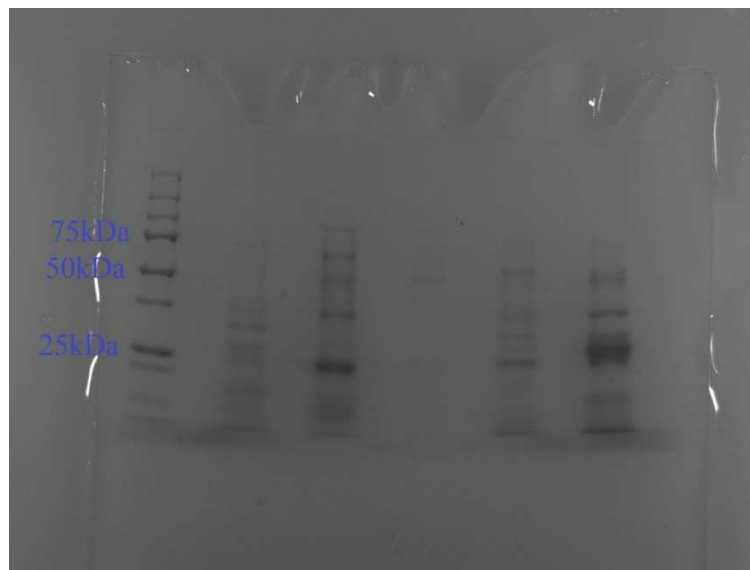
Figure 3-4: A. DEAE elution profile for 70% saturation pellet from Little Marvel seeds. NaCl concentrations changed in a stepwise manner; 0mM NaCl Fractions 1-25, 100mM NaCl Fractions 26-50, 200mM NaCl Fractions 51-76, 300mM NaCl Fractions 77-102, 400mM NaCl Fractions 103-128, 500mM NaCl Fractions 129-154, 600mM NaCl Fractions 155-180, 1000mM NaCl Fractions 181-206. Pooled Fractions 8-15, 38-50, and 64-66 were pooled. B. Gel where the first, second, and third from right to left after the molecular weight marker is Fractions 8-15, 38-50, and 64-66 respectively, followed by two unrelated pools from a Bean Garden isolation attempt.

DEAE Chromatography:

The first conclusion that can be drawn based on the DEAE elution profiles is that there appear to be at least three different isozymes of nucleoside hydrolase in the 70% pellets of each of the plant species studied. The three isozymes appear to elute in the 0mM NaCl concentration, 100mM NaCl concentration, and the 200mM NaCl concentration respectively. The 0mM NaCl isozyme from the Alaska Pea 70% pellet was not the result of overloading the DEAE column, since the 0mM NaCl pool was determined to have no interaction with the column when it was chromatographed a second time. Also, since the amount of Alaska Pea seeds by weight was approximately a quarter of the amount used compared to that used for Little Marvel or soybean, it was unlikely that this would overload the column.

Another interesting trend observed was the contribution each peak made to the total activity of the 70% ammonium sulfate pellets. While each of the 70% pellets' pools eluted at similar NaCl concentrations, the amount of activity each pool contributed to the overall activity in the 70% ammonium sulfate pellet differed. While the 0mM NaCl concentrations were relatively consistent with 32%-33% of the total nucleosidase activity, the same cannot be said about the 100mM and 200mM NaCl concentrations. While the 100mM and 200mM NaCl concentrations pools contributed the most and least activities respectively, their range had considerable variation. The soybean had the least activity present in the 100mM NaCl concentration, approximately 52%, while the Little Marvel had the greatest relative activity with approximately 65% of the total activity from the 70% pellet occurring in the 100mM pool. Conversely, the contributions of the

200mM NaCl pool were highest in the soybean, approximately 15%, while the 200mM

NaCl pool contributed only 3% of the overall activity of the Little Marvel 70% pellet.

The Alaska Pea seed was in between the other two species with 57% and 10% of the total

activity contributed by the 100mM and 200mM NaCl pools respectively. These results

are summarized in Table 3-1.

Table 3-1: Relative percent of activities for each of the pooled from various seed sources
±1%.

70% Pellet Percent Activity	Alaska Pea	Little Marvel	Soybean
0mM NaCl pool	33%	32%	33%
100mM NaCl pool	57%	65%	52%
200mM NaCl pool	10%	3%	15%
Total	100%	100%	100%

Size Exclusion Chromatography:

Due to relatively large amounts of other proteins present in the Alaska Pea seed

supernatant pool 51-55 as determined by SDS-PAGE, it was decided that a Sephacryl S-

100 size exclusion column would be used to further purify the pool. The column utilized

was a GE Healthcare HiPrep 26/60 Sephacryl column 60cm x 26mm with a 320mL bed

volume and a molecular weight range of 10kDa – 100kDa. The mobile phase was 50mM

Tris, pH 7.2, 150mM NaCl filtered through a 0.22µm filter. Based on the elution profile,

Figure 3-5, Fractions 14-15, 20-22, and 30-32 were pooled. The purification was

unsuccessful. Fractions 20-22 and 30-32 had no significant activity or protein yield after

being pool. This resulted in Fractions 14-15 being the only pool to contain usable

amounts of enzyme. Even Fractions 14-15 had a significant loss of activity from that

loaded, approximately 76%, with a recovery of only 37% of protein. (Table 3-2).
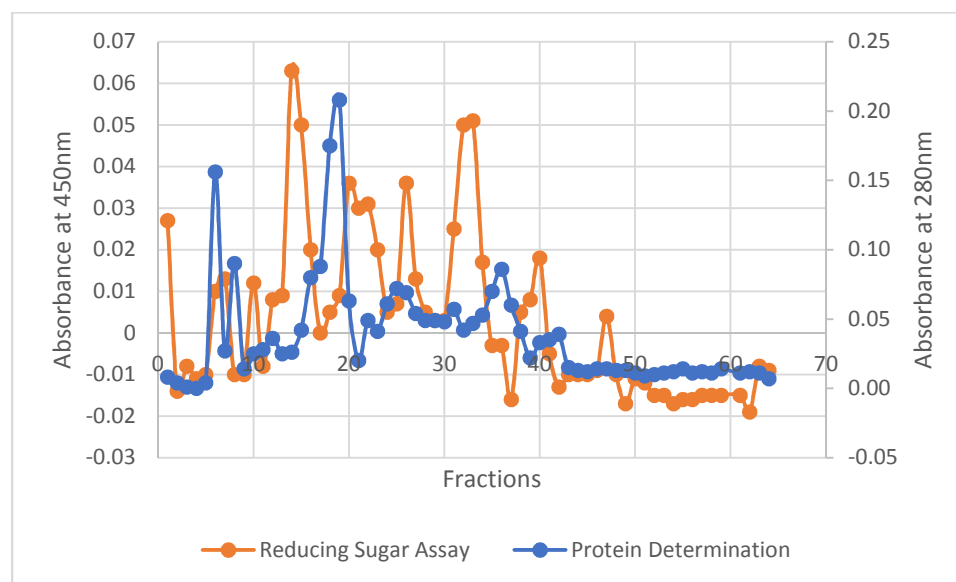

Figure 3-5: Elution profile of size exclusion chromatography of Alaska Pea pool 51-55.

Table 3-2: Protein amount and activity before and after chromatographing Fractions 51-55 from DEAE column through a Sephacryl S-100 column. Amount approximated by Nanodrop spectrophotometer.

Fractions 51-55	Protein Amount (mg)	Activity (Absorbance/mg*min)
Pre S-100	9.03	2.67×10^{-3}
Post S-100	5.71	6.53×10^{-4}
% Recovered	63.27	24.42
% Loss	36.73	75.58

Cation Spin Column:

It was theorized that the Fractions 9-11 pool from the DEAE column contained additional proteins aside from the nucleosidase. However, since the size exclusion column had considerable loss of protein and activity, it was determined to use a different method of protein purification. As such, it was decided that a strong cation exchange column would be utilized to purify and determine if the 9-11 pool had any cationic affinity, since there was no affinity for the anionic DEAE column. The cation exchange column contained a charged sulphonic acid ligand bound to a cellulose membrane which would interact with a protein with a positive charge. The sample, buffer, and the salt fractions used in the elution, dissolved in 50mM Tris, pH 7.2, were filtered through a 0.45μm filter before loading onto the spin column. Based on the strong cation spin column's elution profile, there was no interaction with the cation exchange column with the nucleosidase, since the fraction with the activity elutes in the initial wash. This indicates that there is neither cationic nor anionic affinity with the Alaska Pea seed 9-11

pool nucleosidase. Additional chromatography techniques will need to be tried to purify

this protein.
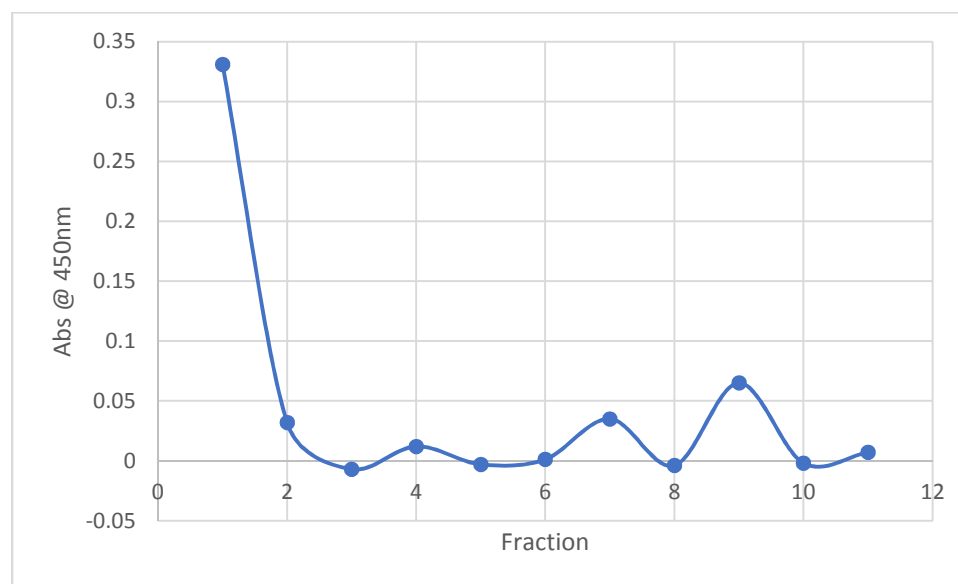
Figure 3-6 : Elution profile of the cation exchange of Alaska Pea pool 9-11, measuring absorbance at 450nm after performing a reducing sugar assay. The conditions for the reducing sugar assay were 100µL of each fraction was incubated at room temperature for 2 hours in 1mL of 1mM inosine, 50mM Tris, pH 7.2. Fraction 1- wash from loading Alaska Pea pool 9-11, Fractions 2 and 3- 0mM NaCl, Fraction 4- 100mM NaCl, Fraction 5-200mM NaCl, Fraction 6-300mM NaCl, Fraction 7-400mM NaCl, Fraction 8- 500mM NaCl, Fraction 9-600mM NaCl, Fraction 10- 1000mM NaCl, Fraction 11- 2000mM NaCl.

SDS-PAGE:

A 10% SDS-PAGE gel was run for approximately 3.5 hours with a constant

current of 50mA. From left to right each lane contained molecular weight markers,

Alaska Pea pool 51-55\S-100 14-15, Alaska Pea pool 28-34, and Alaska Pea 9-11. The

molecular weight markers distance from their well were plotted in Excel, and a

calibration curve relating distance to log of molecular weight were determined by linear regression, as seen in Figure 3-7.

Based on the calibration curve (Figure 3-8), the estimated molecular weights were 14.0kDa for the Alaska Pea pool 51-55,S-100 14-15, 18.8 kDa for the Alaska Pea pool 28-34, and 16.5 kDa for Alaska Pea 9-11. This is dramatically smaller than the average size of nucleosidase monomers. The majority of nucleosidase monomers appear to be between 30kDa-40kDa, with a monomeric nucleosidase from yellow lupin as large as 80kDa (*5, 6, 7, 8,* 9, *12,*). Also, the monomers are approximately half the size of adenosine deaminase nucleosidase II which had a monomeric size estimated to be 25kDa, and 5-7 times smaller than adenosine deaminase nucleosidase I, which was estimated to be a 100kDa monomer (*17*). A possible explanation for these results would be the partial degradation by an uninhibited protease that results cleavage of the native active site from the enzyme. This would result in the pool conserving activity while the enzyme itself would be fragmented. However, further research would be necessary to determine if this is the case.
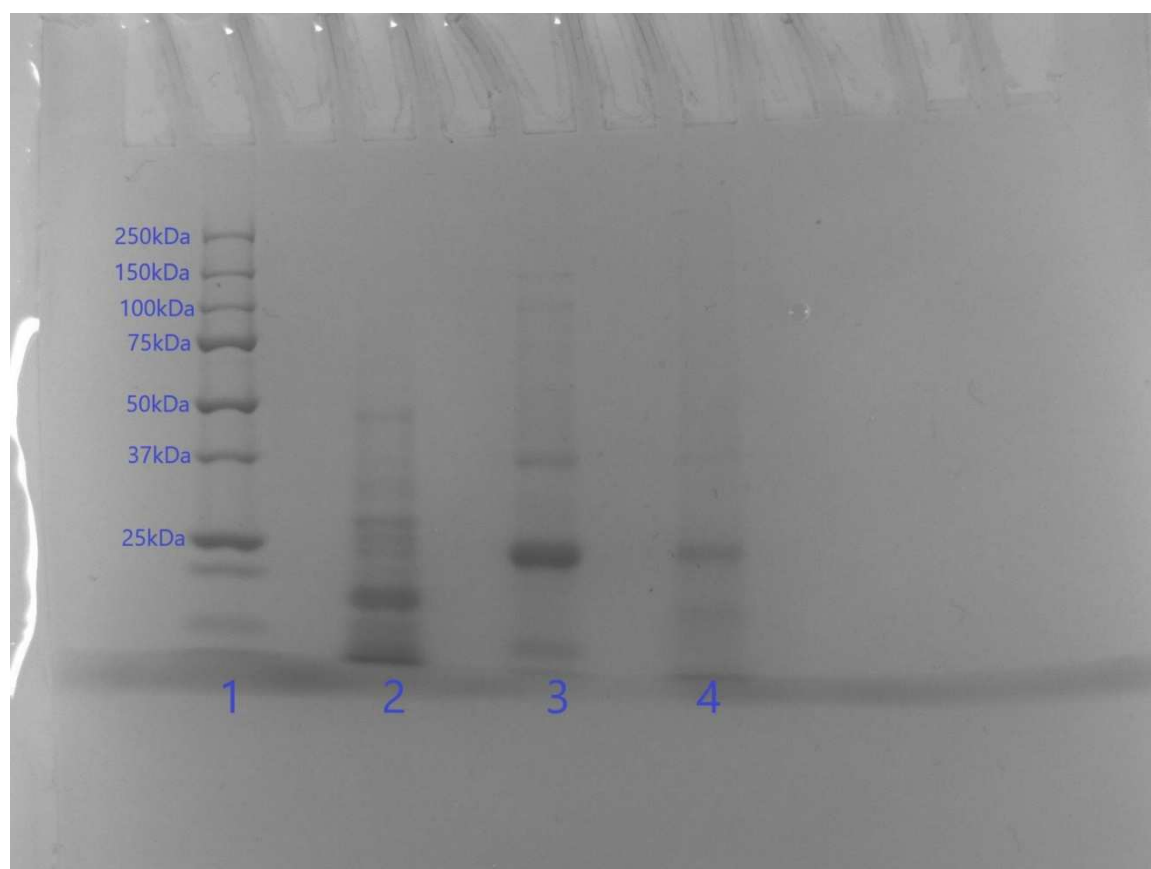
Figure 3-7: SDS-PAGE gel of Lane 1 Bio-Rad unstained molecular weight markers; Lane 2: Alaska pea pools DEAE 51-55, S-100, 14-15; Lane 3: Alaska Pea pool DEAE 28-34; and Lane 4: Alaska Pea pool DEAE 9-11.
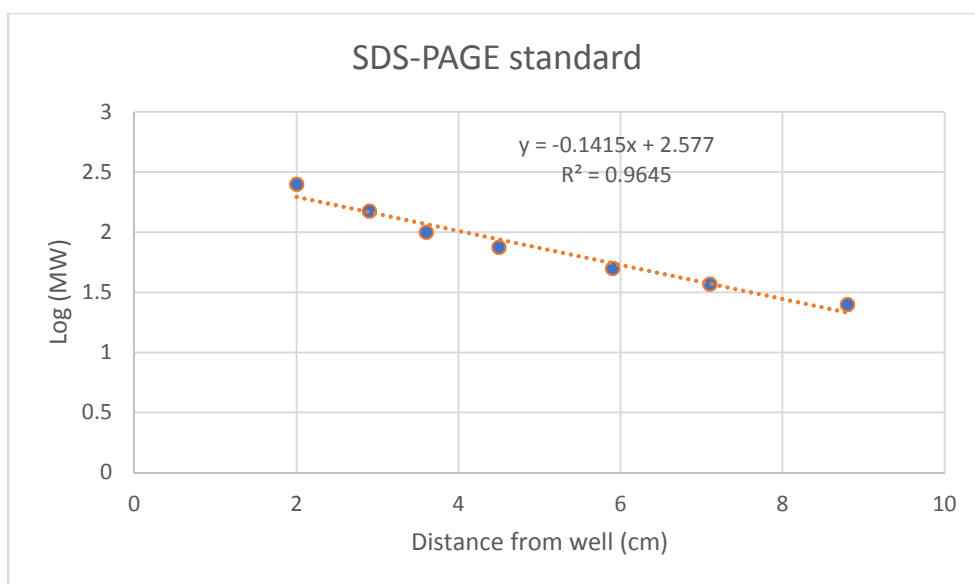
Figure 3-8: Linear plot standards with log of known molecular weights and distance traveled along the gel.

Substrate Specificity:

There are two valuable pieces of information that can be determined by a substrate specificity analysis of an enzyme. The first is the use of substrate specificity to identify and characterize different isozymes based on relative activity to derivatives of a substrate. The second is the determination or reinforcement of a proposed chemical mechanism by determining which atoms in the substrate are involved in the mechanism of the enzyme. In the case of nucleosidases, comparing which nucleosides the enzymes react with and to which degree are vital to further characterization and understanding of these enzymes.

Below is the substrate specificity data for two enzymes isolated from Alaska Pea seeds. Form I represents the enzyme that was eluted at 0mM NaCl concentration, while Form II was eluted at 100mM NaCl concentration. Form II is the main activity peak and

has previously been isolated (*17*). The two pools were assessed for activity via UHPLC

with a wide variety of substrates. Enzyme was added to a 1mM nucleoside solution, with

the exception of guanosine which was 0.5mM, dissolved into a 50mM PIPES, pH 6.6

solution to initiate the reaction. Once the enzyme was added, the peak area of the

nucleoside was monitored over time and a graph was generated where the slope of the

best fit line was the rate in µM/min. That rate was divided by the protein amount in mg to

determine the specific activity.

Table 3-3: Rate of hydrolysis for the standard nucleosides and r^2 values for nucleoside hydrolase from Alaska pea seeds Form I, where N/A indicates nucleosides with no discernable activity.

Form I Nucleosides	Rate(µM/min)	Specific Activity (µM/min*mg)	r-squared
Adenosine	0.053	0.640	0.984
2'-Deoxyadenosine	0.004	0.044	0.534
Guanosine	0.165	1.992	0.990
Uridine	0.551	6.664	0.979
2'-Deoxyuridine	0.013	0.158	0.729
5-Methyluridine	0.037	0.452	0.959
Thymidine	0.018	0.223	0.947
Cytidine	0.062	0.749	0.979
2'-Deoxycytidine	0.014	0.164	0.890
Xanthosine	N/A	N/A	N/A
Inosine	0.205	2.483	0.999

Table 3-4: Rate of hydrolysis for standard nucleosides Alaska Pea seeds and r^2 values for pool Form II.

Form II Nucleosides	Rate (μM/min)	Specific Activity (μM/min*mg)	r-squared
Adenosine	0.292	1.128	0.994
2'-Deoxyadenosine	0.188	0.725	0.748
Guanosine	1.171	4.520	0.963
Uridine	0.163	0.628	0.888
2'-Deoxyuridine	0.003	0.013	0.064
5-Methyluridine	0.138	0.531	0.995
Thymidine	0.009	0.035	0.807
Cytidine	0.156	0.601	0.980
2'-Deoxycytidine	0.004	0.016	0.394
Xanthosine	0.194	0.131	0.998
Inosine	2.448	2.154	1.000

Table 3-5: Rate of hydrolysis for the non-standard nucleosides and r^2 values for Alaska Pea seeds Form I, where N/A indicates nucleosides with no discernable activity.

| Form I Non-Standard. Nucleosides	Rate (µM/min)	Specific Activity (µM/min*mg)	r-squared
Nebularine (Purine Riboside)	0.011	0.128	0.813
5'-Deoxyadenosine	0.007	0.088	0.748
Tubercidin (7-Deazaadenosine)	N/A	N/A	N/A
3-Deazauridine	N/A	N/A	N/A
6-(γ,γ-Dimethylallyl-amino) Purine Riboside	0.013	0.163	0.843
6-Chloropurine Riboside	0.002	0.028	0.241
6-Benzylamino Purine Riboside	0.039	0.466	0.495
Kinetin Riboside (N6-furfuryladenosine)	0.012	0.142	0.806
Erythro-Uridine (Uracil-1-β-erythrofuranoside)	N/A	N/A	N/A
Uracil 1-β-arabino-furanoside	N/A	N/A	N/A
Arabinoadenine (Adenine 1-β-arabinofuranoside)	0.024	0.286	0.433
Cordycepin (3'-Deoxyadenosine)	0.034	0.406	0.810

Table 3-6: Rate of hydrolysis for the non-standard nucleosides and r^2 values for Alaska Pea seeds Form II, where N/A indicates nucleosides with no discernable activity.

Form II Non-Standard Nucleosides	Rate (μM/min)	Specific Activity (μM/min*mg)	r-squared
Nebularine (Purine Riboside)	0.100	0.040	0.990
5'-Deoxyadenosine	0.268	0.207	0.998
Tubercidin (7-Deazaadenosine)	N/A	N/A	N/A
3-Deazauridine	N/A	N/A	N/A
6-(γ,γ-Dimethylallyl-amino) Purine Riboside	0.040	0.062	0.975
6-Chloropurine Riboside	0.179	0.145	0.930
6-Benzylamino Purine Riboside	0.064	0.050	0.940
Kinetin Riboside (N6-furfuryladenosine)	0.084	0.107	0.984
Erythrouridine (Uracil-1-β-erythrofuranoside)	N/A	N/A	N/A
Uracil 1-β-arabino-furanoside	N/A	N/A	N/A
Arabinoadenine (Adenine 1-β-arabinofuranoside)	0.152	0.138	0.990
Cordycepin (3'-Deoxyadenosine)	1.142	0.922	0.996

Adenosine as a substrate tended to produce a unique elution pattern compared to the other substrates. Most of the nucleosides' elution profiles contained one peak corresponding to the nucleoside and one peak corresponding to the product nitrogenous base. However, adenosine appeared to generate one or two additional peaks that did not correspond to either adenine or adenosine. These additional peaks, seen in Figure 3-9, are theorized to be inosine and/or hypoxanthine. This phenomenon has been seen in previous

research of these enzymes, indicating a unique activity with adenosine's interaction with these enzymes (*17*). The appearance of inosine and hypoxanthine is due to the presence of an adenosine deaminase activity which converts adenosine to inosine. The resulting inosine is hydrolyzed to hypoxanthine by the nucleosidase activity.

A.
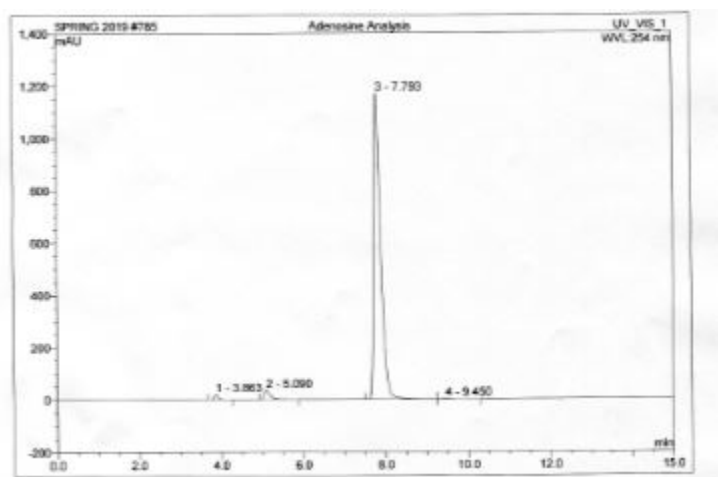
B.
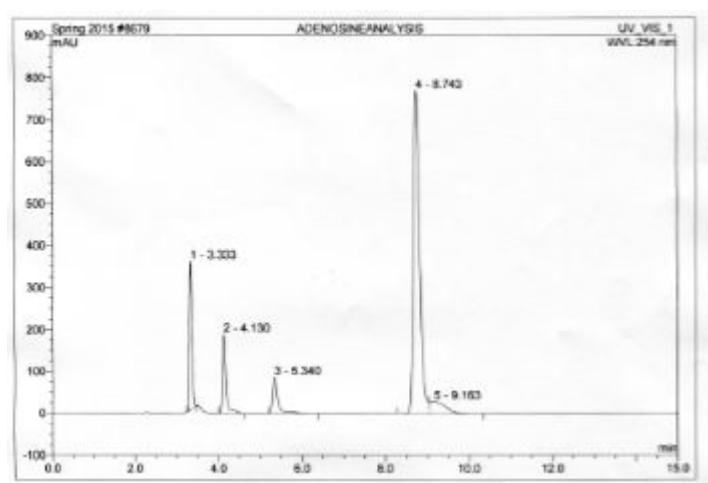
Figure 3-9: The elution profiles of adenosine incubated with Form I (A.) and Form II (B.) nucleosidases. The peak at 7.7 minutes for Form I and 8.7 minutes for Form II is adenosine, and the peak at 5.0 minutes for Form I and 5.3 minutes for Form II is adenine. The additional peaks at 3.8mins for Form I and 3.3 minutes and 4.1 minutes for Form II are theorized to be either inosine or hypoxanthine.

The substrate specificity results indicate that both isozymes studied have

equivalent activity for inosine, but differ with respect to other nucleosides. Form I, a non-

specific nucleoside hydrolase, has the greatest activity with uridine, inosine, and

guanosine respectively. The pattern of activity with respect to the nucleosidase is consistent with that of the inosine-uridine nucleoside hydrolase from *Crithidia fasciculata* (*6*). However, Form II has the greatest activity with guanosine, inosine, and adenosine respectively. As such, the isozymes differ with respect to which nucleosides are the better substrates. Form II, a purine-preferring enzyme, in particular showed a similar pattern of activity to a guanosine-inosine nucleosidase previously isolated from *Crithidia fasciculata (6)*.

Both enzymes showed a substantial decrease in reactivity with the 2'-deoxy substrates when compared to their ribose counterparts. This indicates that the presence of the 2' hydroxyl group increases the reactivity of the substrate, which could aid in the stabilization or formation of the C_1' carbocation indicated in previous studies (*1, 4)*.

Form I and Form II have different activities when it comes to the non-standard nucleosides as well. With this substrate specificity analysis, the activity of the nucleosidases with several nucleoside derivatives, including cytokinins. Unfortunately, the analysis for Form I resulted in relatively low activity, indicating that Form I is not the primary nucleosidase used by Alaska Pea. Cordycepin, 3'-deoxyadenosine, was a good substrate, indicating that a 3'deoxy group has relatively less effect on the enzyme compared to the 2'-deoxy group. Surprisingly, both forms had low 5'-deoxyadenosine specific activity, indicating that the 5'-deoxy functional group plays an important role in the enzyme-substrate interaction. This trend was shared with Form II, where cordycepin had a relatively high activity compared to the 2'-deoxy nucleoside. However, the inversion of the C2' ribose hydroxyl group seen in uracil 1-β-arabinofuranoside, and

adenine 1-β-arabinofuranoside, resulted in a dramatic decrease in activity when compared to the adenosine. This is likely due to interference with specific hydrogen bonding of the ribose motif to amino acids involved with activation of the enzymatic pocket, which can lead to inactivation of the enzyme (*18*). Another trend was the lack of noticeable activity with the 7-deazaadenosine and the 3-deazauridine nucleosides, indicated by "N/A". This is likely due to the disruption of the proton transfer to the N7 of the purine nucleosides and the N3 of the pyrimidine nucleosides, and has been seen in similar compounds (*4, 19*).

Lastly, there were several N6 amino derivatives that were assessed for activity. Between the two enzyme forms, Form I had a higher specific activity for nebularine, which lacks the exocyclic amino group, when compared to Form II. With regards to Form I, the 6-benzylamino purine riboside had a dramatically higher specific activity when compared to nebularine, while the 6-(γ,γ-dimethylallyl-amino) purine riboside had approximately the same specific activity as nebularine and 6-chloropurine riboside which was almost non-existent. However with regards to Form II, the nebularine specific activity was extremely low and comparable to that for 6-(γ,γ-dimethylallyl-amino) purine riboside and the 6-benzylamino purine riboside, while the 6-chloropurine riboside has approximately four times the specific activity when compared to the 6-(γ,γ-dimethylallyl-amino) purine riboside and the 6-benzylamino purine riboside. This may be due to reduced steric hindrance of the N6 atom of the 6-chloropurine compared to the 6-(γ,γ-dimethylallyl-amino) purine or the 6-benzylamino purine. The activity of the enzymes presents a mixed picture with respect to the non-standard derivatives.

In enzymes from parasitic protozoans, the deoxynucleosides are either poor substrates or not substrates (*18*). A series of deoxynucleosides including 2'-deoxy, 3'-deoxy, and 5'-deoxyadenosine were tested for substrate activity. For Form II, all three deoxyadenosines retained significant activity compared to adenosine. This is in contrast to 2'-deoxyuridine, 2'-deoxycytidine, and thymidine, in which the specific activity decreased by at least 95% compared to the corresponding ribopyrimidine. In the case of Form I, the situation is less clear. 2'-deoxy and 5'-deoxyadenosine lost approximately 90% of their activity compared to that of adenosine. In the case of the 2'-deoxypyrimidines, Form I retained lowered but significant activity compared to the corresponding ribopyrimidines.

The mechanism of activation in Alaska pea seed nucleoside hydrolase appears to be the same as that of the parasitic protozoan nucleoside hydrolases. Neither 7-deazaadenosine nor 3-deazauridine are substrates. This is consistent with protonation of the N7 in purines or N3 in pyrimidines being an essential step in the hydrolysis of nucleosides (*4*).

Another point of contact between the enzyme and nucleoside is presence or absence of a group at N6. The best substrates for both Form I and Form II contain a hydrogen bond acceptor, a carbonyl group, in this position. An anomaly is this trend is xanthosine. It is not a substrate for Form I and is a poor substrate for Form II. Nebularine (purine riboside), which lacks an exocyclic group at N6 is a poor substrate. Nebularine has 20% the activity of adenosine in Form I, and is 28 times less active compared to adenosine in Form II. The activities against the cytokinins are difficult to categorize. The

three cytokinins with significant groups at N6, 6-(γ,γ-dimethylallyl-amino) purine, 6-benzylamino purine, and kinetin have lower activities compared to adenosine.

Erythrouracil and erythroadenine are not substrates for either form of nucleoside hydrolase. This indicates a group at 5' position is necessary, but a hydroxyl group is not essential since 5'-deoxyadenosine is a substrate.

The arabinouracils are not substrates for either form, but the arabinoadenines are relatively good substrates for both forms. Changes in configuration at O2' have a profound influence on the exocyclic C4'-C5' bond torsion angles and on the ability of the nucleoside to change base conformation between syn and anti (*20*). This reinforces the importance of the C5' hydroxymethyl group shown by the activities of erythrouridine and 5'-deoxyadenosine.

CHAPTER IV: Conclusions

Our results indicate that plants contain multiple isozymes of nucleoside hydrolase. This is due to the three significant activity peaks that were present in the DEAE elution profiles for each of the plant species studied. Nucleoside hydrolase from Alaska Pea seeds eluted at 0mM NaCl and 100mM NaCl had estimated monomer molecular weights at 16.7kDa and 12.1 kDa respectively. The SDS-PAGE results indicate the rest of the isolated pools required additional purification steps to isolate the nucleosidase from other proteins. Chromatography on a size exclusion column resulted in a large loss of activity. A possibility for this result is the loss of a small molecule or cofactor during the chromatography. Additional research must be carried out.

With regards to the substrate specificity analysis, both Form I and Form II are nucleosidases that have the greatest activity with uridine, inosine, guanosine, and guanosine, inosine, and adenosine respectively. This difference in preference of substrate is further evidence of the two nucleosidases being distinct isozymes of one another.

Consistent with the proposed mechanism, the lack of a nitrogen at N7 of the purines and N3 of the pyrimidines resulted in a loss of enzyme activity. Surprisingly the 5'-hydroxyl group plays a significant role in the hydrolysis of nucleosides.

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