IN-DEPTH CHARACTERIZATION OF THE PURIFICATION OF ADENOSINE NUCLEOSIDASE FROM ALASKA PEA SEEDS

By

Fiezah M. Alahmry

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Thesis Committee:

Dr. Paul C. Kline, Chair

Dr. Mary B. Farone

Dr. Justin M. Miller

I dedicate this research to my dear parents, my husband, and my family.

I love you all

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I would like to certainly express my thank to my advisor, Dr. Paul C. Kline, for his guidance, support, and encouragement throughout this project. I also wish thank my committee members, Dr. Mary B. Farone and Dr. Justin M. Miller, for their advice and insightful comments.

In addition, I would like to thank my beloved husband for all his support, encouragement and love without whom none of my accomplishments would have been possible. Also, I would like to express my thank to my sisters for their encouragements and love. Thank you all for your endless support, concern, love, and prayers.

ABSTRACT

Alaska pea seeds were tested for the presence of multiple enzyme activities from dry seeds and at different germination time. The maximum activity was reached at 48 hours of germination. Uridine nucleosidase had the highest activity of 67 µmol/min and the lowest activity was adenosine nucleosidase with 33 µmol/min. However, the dry seeds in all three nucleosidases had the lowest activity.

Adenosine nucleosidase is an enzyme catalyzes the hydrolysis reaction of adenosine to adenine and ribose. The enzyme was purified from Alaska pea seeds five days after germination. Ammonium sulfate precipitation with an increment of 10% was the initial purification step. Further purification was carried out using DEAE and Mono Q chromatography. The enzyme was purified after using only one column which was Mono Q ion exchange column with high pure protein. This is due to the ammonium sulfate fractionations during the purification of the enzyme in which the purity of protein enhanced by increasing salt concentration. The molecular weight of the purified enzyme was determined by SDS-PAGE 26,000 daltons with purification fold of 4.2 and a yield of 1.7%. The specific activity for the enzyme was 2.9 $x10^{-2}$ µmol/min/mg. The desired activity was achieved at 4 °C and an optimum pH value of 7.2. The activity of the enzyme was examined based on the change of the factors that carried out during the purification procedure.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Nucleotides and Nucleosidases

Nucleotides are nitrogenous compounds found in the cells of all living organisms. Nucleotides consist of a nitrogenous base (either purine or pyrimidine), a pentose (five carbons) sugar (ribose or deoxyribose) and one to three phosphate groups. Furthermore, a nucleoside is a structure contained in the nucleotide and consists of a nitrogenous base and sugar (Figure 1) *(3)*.

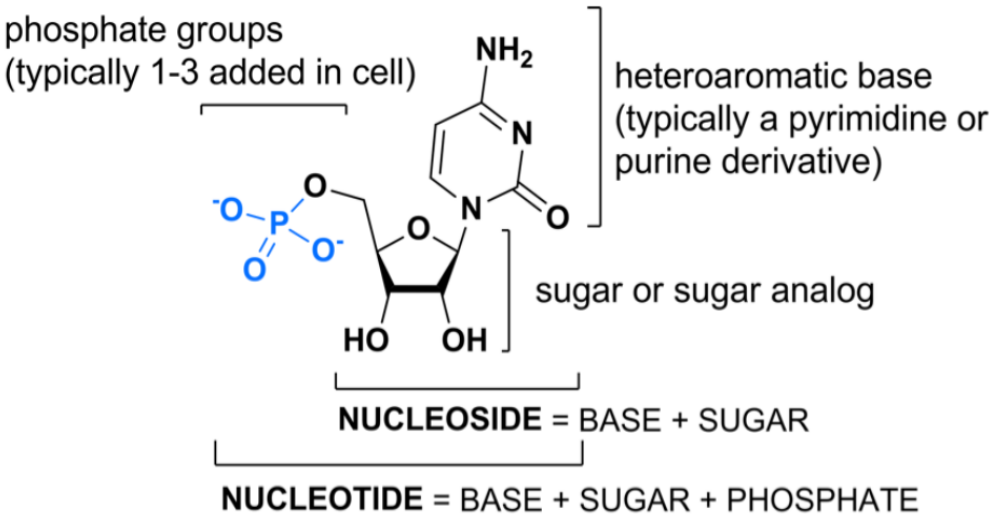


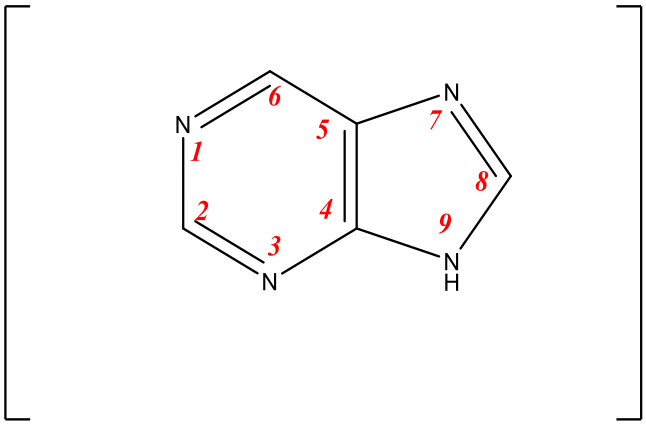
Figure 1. Structure of a nucleotide and nucleoside *(3)*.

Nucleotides are generally named after the nitrogenous bases mentioned above contained in their structure. For example, they can be synthesized via either *de novo* synthesis or via the salvage pathway *(4).* The *de novo* process is instrumental in the generation of nucleoside monophosphates such as adenosine monophosphate and uridine monophosphate from multiple small molecules *(4).* To synthesize nucleotides in plants through the *de novo* process, PRPP and simple molecules such as amino acids, tetrahydrofolate, and carbon dioxide can be used. In the salvage pathways, on the other hand, preformed nucleosides and nucleobases are used. Salvage pathways of nucleotide metabolism have been shown to consume less energy compared to the *de novo* pathways *(5).*

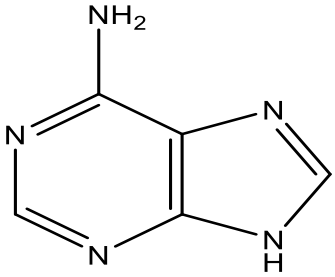
Purine and Pyrimidine Bases

Nitrogenous bases consist of two groups, purines and pyrimidines. Pyrimidines consist of a single six-membered ring containing-nitrogen, whereas purines contain a six-membered nitrogen containing ring fused with a five-membered nitrogen-containing ring *(6).* Common purine and pyrimidine bases include adenine, guanine, cytosine, thymine, uracil, and hypoxanthine. The purine bases include adenine (6-amino purine), hypoxanthine (6-oxy purine), and guanine (2-amino-6-oxy purine) (Figure 2). These bases are incorporated to form the nucleosides adenosine, inosine and guanosine respectively *(44).*

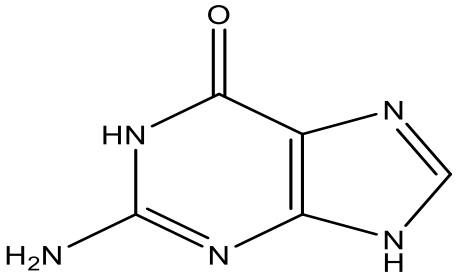
The general structure of the pyrimidines is illustrated in Figure 3. Pyrimidine bases include uracil (2, 4-dioxy pyrimidine), thymine (2, 4-dioxy-5-methyl pyrimidine), and cytosine (2-oxy-4-amino pyrimidine) as shown in Figure 3.



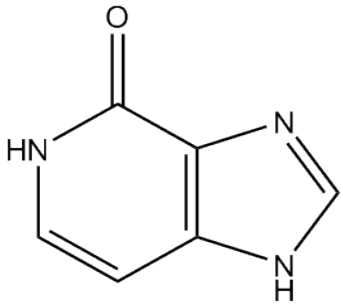
Purine ring



Adenine (**A**)



Guanine **(G)**



Hypoxanthine (**H**)

Figure 2. General structure of purines. The numbering system for the purine bases is shown.

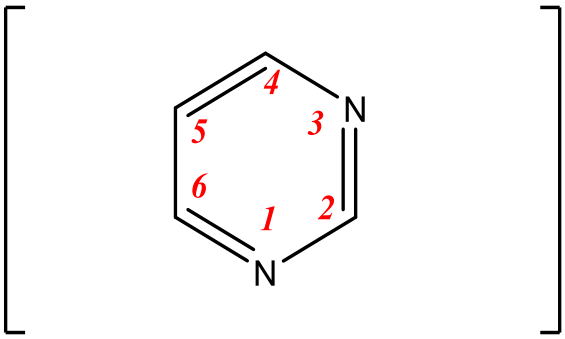
These bases are used to synthesize the nucleosides uridine, thymidine, and cytidine respectively *(7).* The processes and enzymes involved in the synthesis will be discussed and illustrated in greater detail under the metabolism of purines and pyrimidines.

Pyrimidines and purines are involved in many essential biochemical processes in higher plants besides being the building blocks from which nucleic acids are synthesized *(2).* The two act as sources of energy, precursors in the formation of various vital substances such as phospholipids, sucrose, and polysaccharides along with a number of secondary metabolites *(4).*

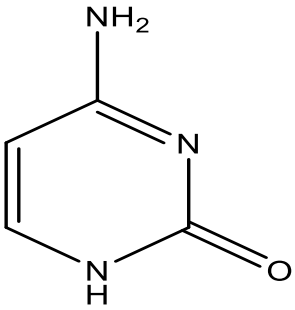
Purine and Pyrimidine Metabolism

Purines and pyrimidines are found in nucleotides and are considered components of the nucleic acids, DNA and RNA *(7).* Nucleoside metabolism occurs in the cells of all living organisms. The process occurs in a number of pathways that can broadly be categorized into four distinct pathways: the *de novo* synthesis, salvage pathways, nucleotide degradation and phosphotransfer pathways *(4).* It is believed that *de novo* synthesis is often most important in cells growing and dividing, while non-growing cells may obtain their nucleotides by salvaging nucleosides *(4).*

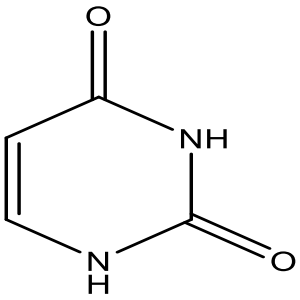
Due to the fact that nucleotides and their synthetic intermediates can act as precursors in other types of syntheses, such as the production of hormones and secondary metabolites, nucleotide metabolism is recognized to be more complicated than amino acid metabolism. For example, the synthesis and degradation reactions of



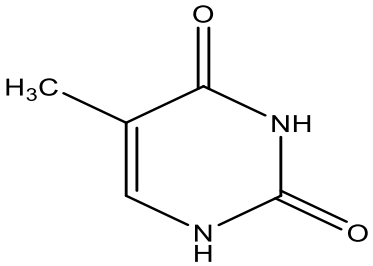
Pyrimidine ring



Cytosine (**C**)



Uracil (**U**)



Thymine (**T**)

Figure 3. General structure of pyrimidines. The numbering system for pyrimidine bases is shown.

sucrose and UDP-glucose are directed by the co-substrates UTP and UDP *(4).* UDP-glucose is used as precursor for important synthetics reactions of cellulose, phospholipids, and glycoproteins. In addition, a wide range of synthetic reactions are catalyzed by the UDP-glycosyltransferases family (UGTs) such as hormones and secondary metabolites synthesis in which UDP-glucose functions as a glucosyl donor *(4).*

The *de novo* biosynthetic pathway (Figure 4) utilizes small molecules such as the amino acids, glutamine, glycine, and aspartate along with N^{10}-formyl tetrahydrofolate (N^{10}-formyl-THF), 5-phosphoribosyl-1-pyrophosphate (PRPP), and carbon dioxide to synthesize purine nucleotides. Figure 4 depicts the pathway for two purine nucleotides, adenine monophosphate (AMP) and guanine monophosphate (GMP) from 5-phosphoribosyl-1-pyrophosphate (PRPP). The most important aspect of this pathway that utilizes ATP, a product of the pathway, as an energy source. Ten enzyme-catalyzed reactions are required to synthesize the common intermediate, inosine monophosphate (IMP). Additional reactions then convert (IMP) to adenine monophosphate (AMP) and guanosine monophosphate (GMP).

The biosynthetic process commences with the formation of phosphoribosylamine (PRA) from PRPP and glutamine in a reaction catalyzed by PRPP aminotransferases (ATase) (Figure 4) *(7).* This is the first committed step in purine biosynthesis. Next, using an amide bond to attach glycine to PRA, glycine amide ribonucleotide (GAR) is produced by GAR synthetase *(4).* Transformylation of GAR by GAR transformylase (GART) follows this reaction and uses N^{10}-

formyltetrahydrofolate (N^{10}-formyl-THF), to form formylglycinamide ribonucleotide (FGAR) *(7).* Formylglycinamidine ribonucleotide synthetase (FGAMS) catalyzes the reaction that follows using ATP and glutamine to form formylglycinamidine ribonucleotide (FGAM), which then undergoes ring closure, carried out by AIR-synthase, to form 5-aminoimidazole ribonucleotide (AIR) *(4).*

The second imidazole ring is provided by reactions that involve carbon dioxide, aspartate, and N^{10}-formyl-THF. AIR is carboxylated forming 4-carboxy aminoimidazole ribonucleotide (CAIR) *(7).* Addition of aspartate and an ATP results in the formation of N-succinyl-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR). Adenylosuccinate lyase (ASL) catalyzed the next step and results in the release of fumarate to form 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR).

The final carbon of the purine ring is supplied by N^{10}-formyl-THF. In this step, 5-formaminoimidazole-4-carboxamide ribonucleotide (FAICAR) is formed. Next, (FAICAR) is dehydrated to allow ring closure and forms the first purine, inosine monophosphate (IMP) *(4).* After forming (IMP), the purine pathway splits into two sub-pathways. The first pathway is responsible for the formation of adenosine monophosphate (AMP) and the second one for the formation of guanosine monophosphate (GMP).

Catabolism of purines is an integral part of the metabolism of nitrogen in plants. Animals do not have the enzyme uricase and therefore uric acid is the ultimate product of purine metabolism. The major source of nitrogen waste excreted is uric

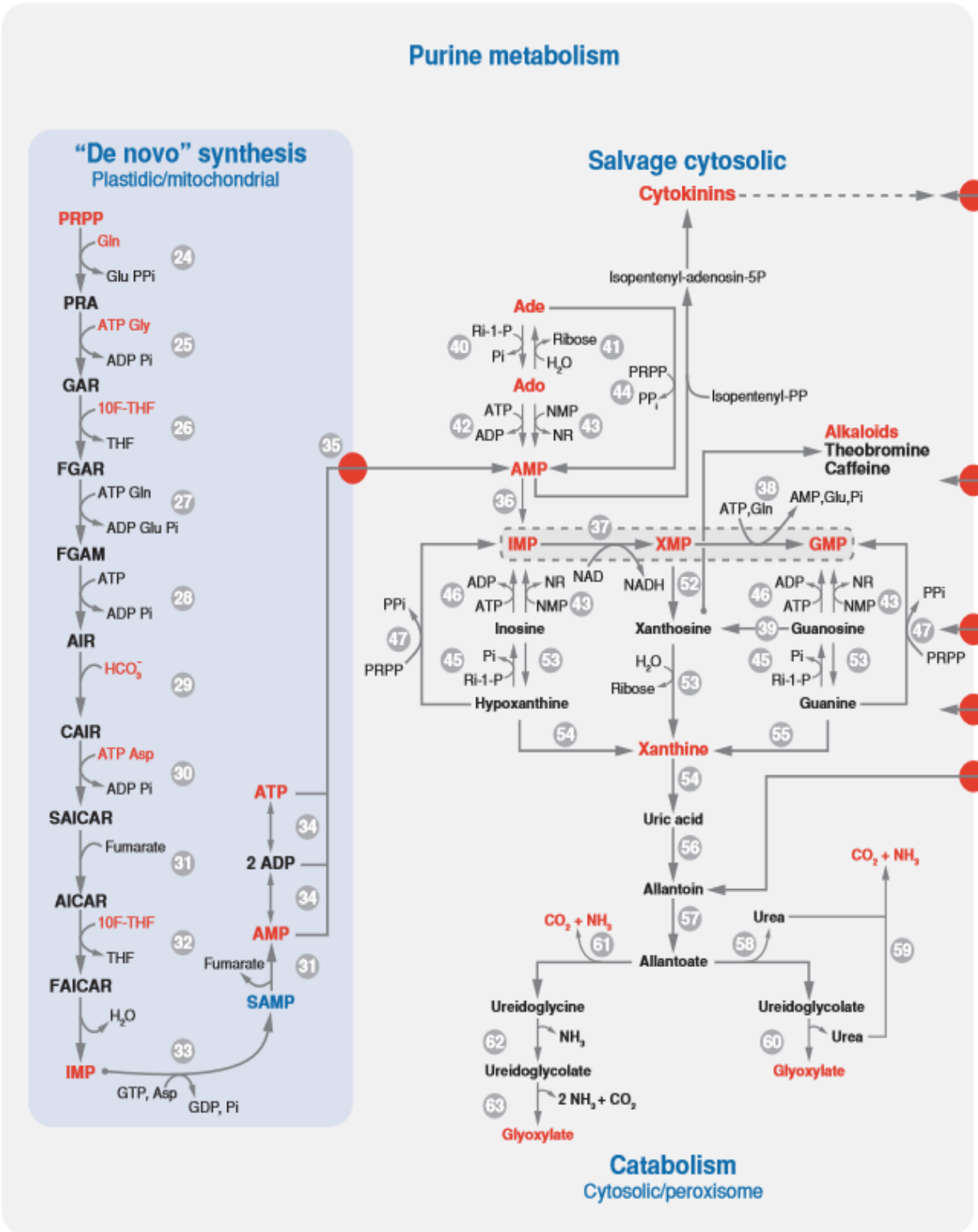

Purine metabolism
"De novo" synthesis
Plastidic/mitochondrial
PRPP
Gln
Glu PPi
PRA
ATP Gly
ADP Pi
GAR
10F-THF
THF
FGAR
ATP Gln
ADP Glu Pi
FGAM
ATP
ADP Pi
AIR
HCO3-
CAIR
ATP Asp
ADP Pi
SAICAR
Fumarate
AICAR
10F-THF
THF
FAICAR
H2O
IMP
GTP, Asp
GDP, Pi
ATP
2 ADP
AMP
Fumarate
SAMP
Salvage cytosolic
Cytokinins
Isopentenyl-adenosin-5P
Ade
Ri-1-P
Pi
Ribose
H2O
Ado
ATP
ADP
NMP
NR
PRPP
PPi
Isopentenyl-PP
Alkaloids
Theobromine
Caffeine
AMP
ATP,Gln
AMP,Glu,Pi
IMP
XMP
GMP
NAD
NADH
ADP
ATP
NR
NMP
Inosine
Xanthosine
Guanosine
PPi
PRPP
Nucleoside
Base
Hypoxanthine
Guanine
Xanthine
Uric acid
Allantoin
CO2 + NH3
Urea
Allantoate
Ureidoglycine
NH3
Ureidoglycolate
2 NH3 + CO2
Glyoxylate
Ureidoglycolate
Urea
Glyoxylate
Catabolism
Cytosolic/peroxisome

Figure 4. Purine metabolism pathway. The metabolic components are; 5-phosphoribosyl-1-pyrophosphate (*PRPP)*, 2. Glutamine (*Gln*), 3. Glutamate (*Glu*), 4. 5-phosphoribosylamine (*PRA*), 5. Pyrophosphate (*PPi*), 6. Glycine (*Gly*), 7. Glycinamide ribonucleotide (*GAR*), 8. N^{10}-formyl tetrahydrofolate (*N^{10}-formyl-THF*), 9. Formylglycinamide ribonucleotide (*FGAR*), 10. Formylglycinamidine ribonucleotide (*FGAM*), 11. 5-aminoimidazole ribonucleotide (*AIR*), 12. 4-carboxy aminoimidazole ribonucleotide (*CAIR*), 13. Aspartate (*Asp*), 14. N-succinyl-5-aminoimidazole-4-carboxamide ribonucleotide (*SAICAR)*, 15. 5 aminoimidazole-4-carboxamide ribonucleotide (*AICAR*), 16. 5-formaminoimidazole-4-carboxamide ribonucleotide (*FAICAR),* 17. Inosine monophosphate (*IMP),* 18. Adenylosuccinate (*SAMP*), 19. Adenosine monophosphate (*AMP*), 20. Xanthosine monophosphate (*XMP*), 21. Guanosine monophosphate (*GMP*), 22. Adenine (*Ade*), and 23. Adenosine (*Ado*) *(4).* Reproduced with permission of Ann. Rev. Plant Biol. (2006).

acid. Nitrogen is an important element in plants and thus rather than excrete it, plants tend to store it *(4).* The purine nucleotides in plants are degraded oxidatively via uric acid and allantoin to form NH_3 and CO_2. The two products are consequently absorbed by glutamine oxoglutarate aminotransferase (GOGAT) pathway *(4).*

Once adenosine monophosphate (AMP) is converted from inosine monophosphate (IMP), two paths materialize for catabolizing adenine nucleotides. In the initial pathway, not shown in Figure 4, the dephosphorylation of IMP to inosine is catalyzed either by phosphatases or 5'-nucleotidases. Hydrolysis of inosine to hypoxanthine by inosine/guanine nucleosidase is the next step. Subsequently, xanthine dehydrogenase (XDH) converts hypoxanthine to xanthine. The second pathway involves the conversion of IMP to XMP by inosine-5′-monophosphate dehydrogenase (IMPDH) as illustrated in Figure 4. Thereafter, 5'-nucleotidases convert the XMP to xanthosine. The xanthosine is then acted upon by inosine/guanine nucleosidase to form xanthine *(4).*

Both adenine degradation pathways rely on adenosine monophosphate deaminase (AMPD). This is because of the absence of both adenosine deaminase and adenine deaminases in plants *(4).* Some contradictory evidence exists pertaining to the existence of adenosine deaminase (ADA) in plants. According to Brawerman and Chargaff, ADA is present in plants after their analysis of a sample of malt diastase *(31).* Fiers *et al*. later reported that barley rootlets extracts also contained the enzyme *(32).* Singhabahu *et al*. have also showed the expression of a human adenosine deaminase in tobacco plant *(33).*

Plants have also been shown to express guanosine deaminase *(42).* This

implies that the dephosphorylation process to produce guanosine can degrade nucleotides of guanine. Xanthine can thus be produced by the deamination of guanosine to xanthosine or conversion to guanine by inosine/guanine nucleosidase occurs *(4).*

Uric acid is formed by degradation of xanthine. Uric acid is transformed to allantoin, which is subsequently broken down to allantoate *(4).* Allantoin and allantoate in tropical legumes are instrumental in the transport of compounds and nitrogen storage. Ureidoglycine and ureidoglycolate can be synthesized from the conversion of allantoic acid *(4).* The ultimate purine metabolism products, NH_3 and CO_2, and glyoxlate can consequently be achieved after ureidoglycine and ureidoglycolate have been metabolized.

The purine *de novo* pathway is energy-intensive and produces a single AMP and GMP each from the hydrolysis of five and seven ATPs respectively. In contrast, the purine salvage pathway is energy efficient as it hydrolyzes one ATP as illustrated in Figure 4. This pathway recycles pools of plant nucleotides. This is achieved through the inter-conversion of nucleosides, nucleotides, and purine bases emerging from the metabolism of plants *(4).* Using PRPP as the source of ribose phosphate helps to recover the monophosphate forms of adenine and guanine by APRTase and HGPRTase. Another mechanism recycles the purine bases and synthesizes AMP and GMP by utilizing adenine and inosine/guanosine phosphorylase *(4).* Adenosine, inosine, and guanosine can be converted to IMP, XMP, and GMP through catalysis by adenosine and inosine/guanine kinases or nucleoside phosphotransferases.

The salvage pathway, unlike the *de novo* pathway, utilizes a single ATP. Nucleosides and nucleotides that are used in other anabolic and catabolic processes

are also processed. Several enzymes take part in these processes. For example, adenine and hypoxanthine/guanine phosphoribosyltransferases are instrumental in the transformation of adenine and guanine to the corresponding monophosphates *(4)*.

The pyrimidine *de novo* biosynthetic pathway, also known as the orotate pathway, involves synthesis of uridine monophosphate (UMP) from carbamoyl phosphate (CP). Addition of aspartate and 5-phosphoribosyl-1-pyrophosphate are required *(7).* This pathway involves six reactions as shown in Figure 5 *(4)*. In the first step, carbamoylphosphate synthase (CPSase) along with the combination of ATP, carbonate, and an amino group produces carbamoylphosphate (CP). Next, aspartate transcarbamoylase (ATCase) facilitates the condensation reaction of carbamoylphosphate (CP) with aspartate to form carbamoylasparate (CA) *(4).*

Dihydroorotase (DHOase) facilitates the formation of a pyrimidine ring resulting from the cyclization of carbamoylaspartate. The dihydroorotate (DHO) undergoes oxidation by dihydroorotate dehydrogenase (DHODH) to form orotate (OA), which is then condensed with PRPP and catalyzed by orotate phosphoribosyltransferase (OPRTase) to yield orotidine 5-monophosphate (OMP) and pyrophosphate. The final step is the formation of uridine-5'- monophosphate (UMP) as a result of decarboxylation of OMP catalyzed by orotidylate decarboxylase (ODCase) *(4).*

The *de novo* pyrimidine nucleotide reactions occur in various subcellular locations in plants *(4).* The plastid hosts the three initial steps. OA is synthesized from DHO in the inner membrane of the mitochondria in a dehydrogenase reaction. The penultimate and ultimate reactions of the *de novo* pathway are also carried out in the

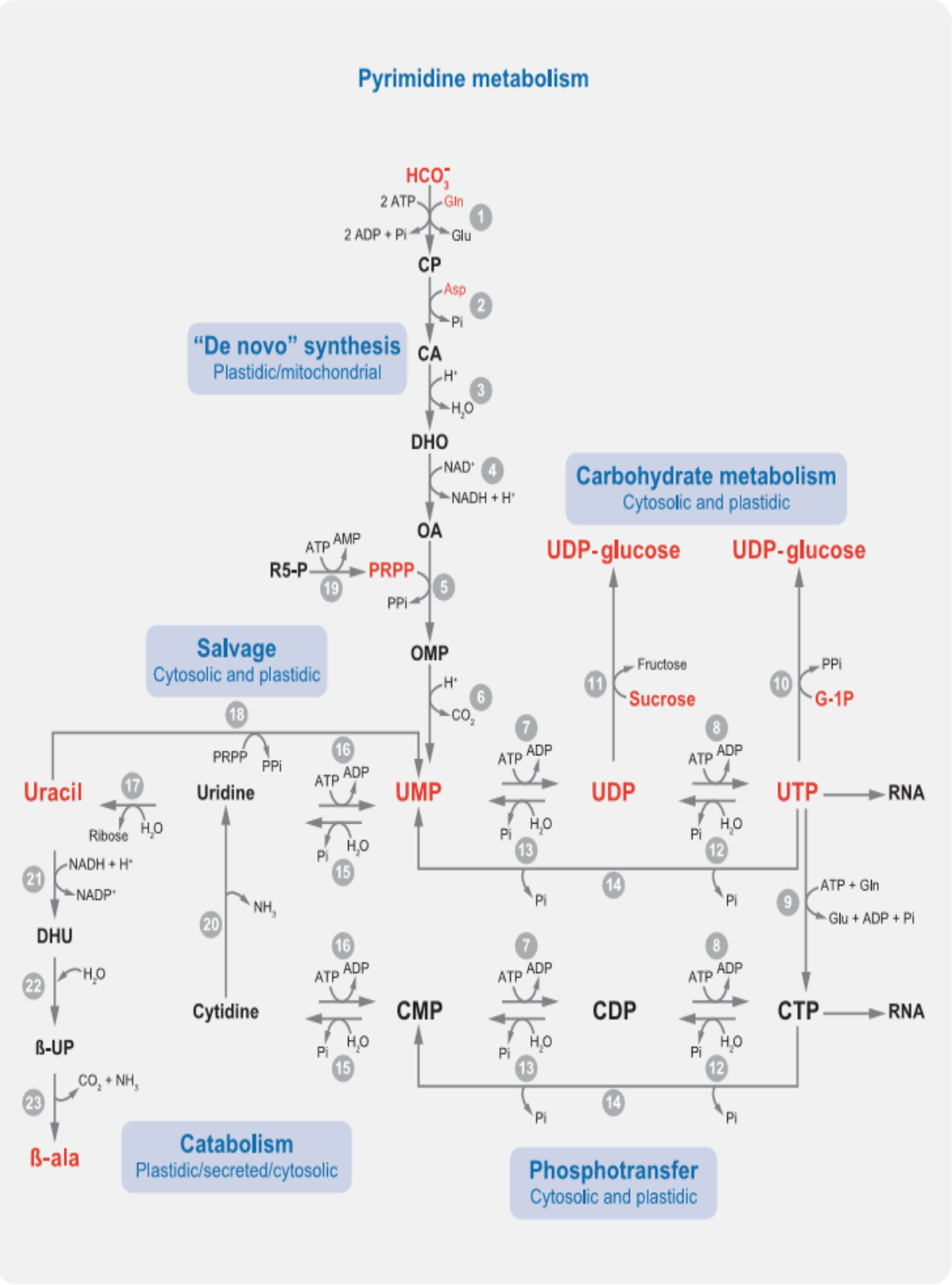

Pyrimidine metabolism
HCO3-
2 ATP
Gln
2 ADP + Pi
Glu
CP
Asp
Pi
"De novo" synthesis
Plastidic/mitochondrial
CA
H+
H2O
DHO
NAD+
NADH + H+
Carbohydrate metabolism
Cytosolic and plastidic
OA
ATP AMP
R5-P
PRPP
PPi
UDP-glucose
UDP-glucose
Salvage
Cytosolic and plastidic
OMP
Fructose
Sucrose
PPi
G-1P
CO2
PRPP
PPi
Uracil
Ribose
H2O
Uridine
UMP
UDP
UTP
RNA
NADH + H+
NADP+
NH3
ATP + Gln
Glu + ADP + Pi
DHU
H2O
Cytidine
CMP
CDP
CTP
RNA
ß-UP
CO2 + NH3
ß-ala
Catabolism
Plastidic/secreted/cytosolic
Phosphotransfer
Cytosolic and plastidic

Figure 5. Pyrimidine metabolism. The metabolic components shown are: 1. 5-phosphoribosyl-1-pyrophosphate (*PRPP)*, 2. Ribose-5-phosphate (*R5-P)*, 3. Glutamine (*Gln*), 4. Glutamate (*Glu*), 5. Adenosine triphosphate (*ATP)*, 6. Adenosine diphosphate (*ADP*), 7. Inorganic phosphate (*Pi)*, 8. Carbamoyl phosphate (*CP)*, 9. Carbamoyl aspartate (*CA*), 10. Dihydroorotate (*DHO*), 11. Orotic acid (*OA*), 12. Orotidine 5-monophosphate (*OMP*), 13. Uridine monophosphate (*UMP*), 14. Uridine diphosphate (*UDP*), 15. Uridine triphosphate (*UTP*), 16. Uridine diphosphoglucose (*UDP-glucose*), 17. Cytosine monophosphate (*CMP*), 18. Cytosine diphosphate (*CDP*), 19. Cytosine triphosphate (*CTP)*, 20. Dihydrouracil (*DHU*), 21. β-ureidopropionate (*β-UP)*, 22. β-alanine (*β-ala*), 23. Pyrophosphate (*PPi)*, 24. Glucose-1-phosphate (*G-1P)*, 25. Aspartate (*Asp*), and 26. Adenosine monophosphate (*AMP*) *(4).* Reproduced with permission of Ann. Rev. Plant Biol. (2006).

plastid. The pathway is regulated through feedback and feedforward loops that act on ATCase and CPSase *(4).* These reactions are examples of allosteric regulation and were originally observed in microbes. The feedback inhibition of UMP and the feedforward activation using PRPP facilitate the regulation mentioned above. Inhibition of the feedback process by UMP on CPSase can be prevented by using ornithine *(4).*

When a phosphate group is removed by 5'-nucleotidases, it leads to catabolism of pyrimidine nucleotides to the corresponding nucleosides (Steps 17, 20- 23 of Figure 5). The nucleosides can be transformed to free pyrimidine bases through the removal of the ribose group. This reaction is catalyzed by number of nucleosidases with different substrates *(4*). Uracil and thymine are degraded through a reductive pathway. Dihydrouracil dehydrogenase (PYDI), dihydropyrimidinase (PYD2), and β-ureidopropionase (PYD3) are used to catalyze three-step reactions involved in the reductive pathway (steps 21-23 Figure 5) *(4).* Eventually, the pathway forms β alanine through the release of NH_3 and CO_2 (Figure 5).

The pyrimidine *de novo* process is energy intensive. Consequently, cells have a pyrimidine salvage pathway that allows them to reuse the preformed nucleosides and nucleobases. Thymidine kinase and uridine kinase are specific nucleoside kinases utilized to salvage thymine and uracil/cytosine nucleosides to their respective nucleotides *(4).* Among the pyrimidine bases, only uracil can be salvaged directly into UMP using PRPP by UPRT (uracil phosphoribosyltransferases). Currently, only a single cDNA of UPRT has been reproduced from *Arabidopsis (4).* The UPRT isolated in the cytosol has a structure similar to phosphoribosyltransferases from other organisms. The *Arabidopsis*

sequence of the genome showed that families of small genes could encode both synthetases of UPRT and PRPP. The cytosol and plastids housed various family members. All isoforms were determined to be expressed differentially based on the confirmation of the expression data *(4).* Consequently, the results indicate that the pyrimidine salvage pathway occurs in the cytosol and plastids *(4).*

Despite the importance of purine and pyrimidine nucleotides, only a few studies have been published on purine and pyrimidine metabolism in higher plants compared to small living organisms and animals *(7).*

Nucleoside Hydrolases

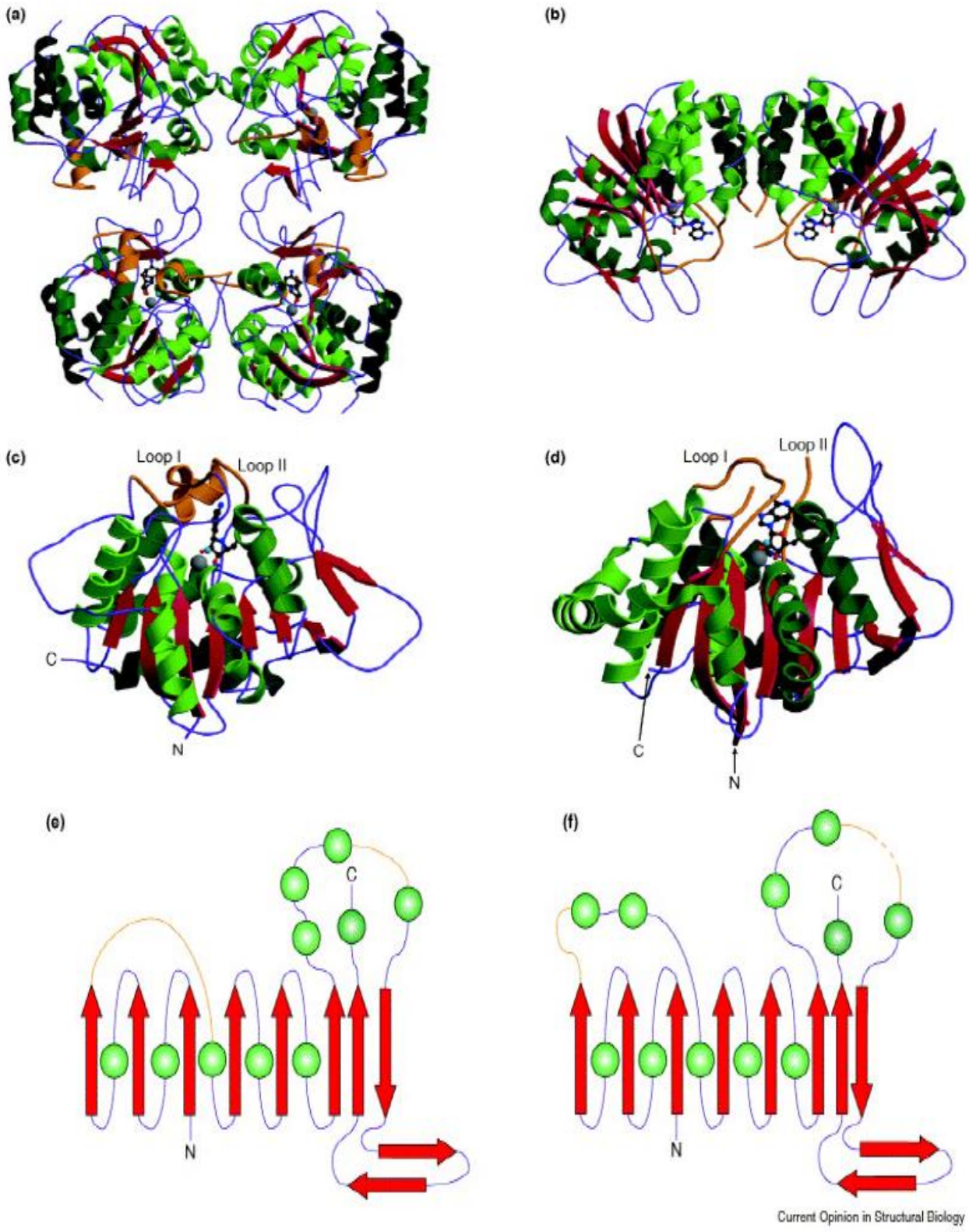
Nucleoside hydrolases (NHs) are enzymes that are found widely in nature *(8).* These enzymes hydrolyze the N-glycosidic bond found in both purine and pyrimidine nucleosides to form the respective bases and ribose. They exist in living organisms such as bacteria *(9),* yeast *(10),* protozoa *(11),* insects *(12)* mesozoa *(13)* and plants *(14)*. Genes that contain the characteristic NH fingerprint sequence DXDXXXDD also exist in amphibians and fish *(6)*. However, mammals do not contain any nucleoside hydrolases *(43).*

There are a number of classifications of nucleoside hydrolases. For example, in protozoa there are four groups of NHs that are classified depending on their specificity; the purine non-specific inosine-uridine favoring nucleoside hydrolases (IU-NH), purine-specific inosine, adenosine, guanosine favoring nucleoside hydrolases (IAG-NH), the pyrimidine-specific uridine-cytidine preferring

nucleosidehydrolases (CU-NH), and 6-oxo-purine-specific inosine-guanine preferring nucleoside hydrolase (IG-NH) *(8).*

Protozoa utilize the salvage pathways to synthesize purines due to the low amount of energy the process requires as opposed to *de novo* pathways which demand the expenditure of an amount of energy which is not sustainable by protozoa *(7).* The salvage pathway also allows for the reuse of ribose and the purine bases *(7).*

The IU-NH from *C. fasciculata* crystallizes as a homotetramer (Figure 6a) whereas IAG-NH from *T. vivax* is a homodimer (Figure 6b) in the crystal. Both compounds have similar architecture and topology with a single globular domain as shown in (Figure 6c, d). Eight-stranded mixed β sheets make up the α/β core of the NH monomer. Of these strands, there are seven parallel α helices, and an anti-parallel strand (Figure 6e, f) *(6).* Loop I and II are flexible and sandwich the active site (Figure 6c, d). These loops are capable of changing the conformation of the active site. A Ca^{2+} ion is found at the bottom of each active site *(15).*



Current Opinion in Structural Biology

Figure 6. Comparison of different nucleoside hydrolase structures from common fold. Quaternary nucleoside hydrolase structures, tertiary structures and topologies of IU-NH of *C .fasciculata* (a, c, e) an IU-NH (*Cf* NH in complex with pAPIR; PDB code 2MAS) and (b, d, f) of IAG-NH of *T. vivax*. β PDB code 1HP0 strands are shown in red while α helices are in green. A gradient from light to dark green when going from the N towards C terminus represent the sequence of different helices. Two recurring loops next to the active site are presented in yellow. The active sites of the enzymes are occupied by Ca^{2+} ions (grey sphere) and a nucleophilic water molecule (blue sphere). The ball and stick models represent the ligands. Reprinted from reference 7 with permission of Elsevier.

Adenosine Nucleosidase

Adenosine nucleosidase is an essential enzyme that converts adenosine to adenine and ribose as shown in Figure 7 *(16)*. Adenosine nucleosidase is involved in the purine salvage pathway. This reaction takes place inside the cotyledon of a seed and the adenine produced is made available to other parts of the germinating seed. Adenosine nucleosidase is vital in cell differentiation, controlling the level of cytokinins, cell division, and development in higher plants *(17)*.

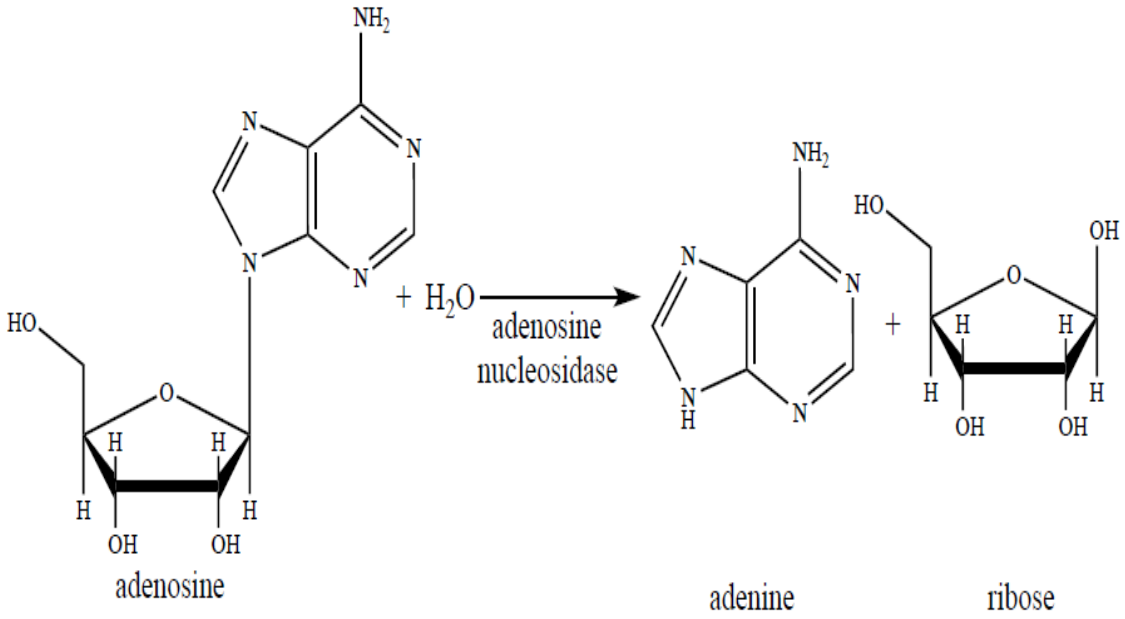


Figure 7. Catalytic hydrolysis of adenosine to adenine and ribose by adenosine nucleosidase

Predominantly, recycling adenine in plants can be correlated to the amount of adenosine nucleosidase *(18).* Adenosine nucleosidase was purified from soybean leaves in 1955 by Miller and Evans *(8).* In plants, the purine salvage pathway cannot be successfully accomplished without the presence of adenosine nucleosidase. Adenosine nucleosidase can play a key role in the growth of plants by controlling the level of cytokinins *(19).*

Most common metabolic pathways depend on the adenosine series, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) *(7).* Furthermore, adenosine is converted to adenosine derivatives in order to allow for the production of nucleotides which are essential during the germination of plant *(20).* According to Liu, adenosine nucleosidase levels were three times greater in apple and cherry trees *(18).* Adenosine nucleosidase has been purified from different types of plants such as barley leaves *(19)* spinach beet leaves *(21),* tea leaves *(22),* tomato roots and leaves *(23),* wheat germ *(24),* Jerusalem artichoke shoots *(25),* yellow lupin seeds *(17),* and Alaska pea seeds *(26).*

Adenosine nucleosidase from barley leaves was purified by Guranowski *et al. (19).* The substrates of ANase were adenosine N1-oxide, purine riboside, and 2'-deoxyadenosine. The research group utilized gel filtration chromatography as one method to determine the molecular weight at 66,000 daltons. The enzyme was a dimer which was confirmed by SDS-PAGE with 33,000 daltons subunit. The optimum pH for citrate and

ethanesulphonic acid was of 4.7 and 5.4 respectively. The range of K_m value for adenosine was between 0.8 to 2.3 µM.

Poulton and Butt were able to partially purify adenosine nucleosidase from spinach beet leaves *(21).* Adenosine was the only substrate for adenosine nucleosidase, and had a K_m value of 11 µM. The optimum pH was 4.5. Additionally, there was similar activity with adenosine N-oxide in which oxidizing adenosine results in the formation of a nitrogen monoxide bond and hence produces adenosine N-oxide.

Three types of adenosine nucleosidase I, II, and III were purified by Imagawa *et al.* from tea leaves *(22).* Type I and III optimum pHs were 4.0, whereas type II was around 4.5. All three types had an approximate molecular weight of 68,000 daltons. Moreover, substrate specificity was comparable in all three types of enzyme. The two naturally occurring substrates that had the ability to undergo hydrolysis by the three adenosine nucleosidase were adenosine and 2'-deoxyadenosine. Compared to adenosine, the hydrolysis rate was estimated to be three to four times greater for 2'-deoxyadenosine.

Chen and Kristopeit examined wheat germ cells and were able to partially purify adenosine nucleosidase *(24).* The molecular weight was found to be 59,000 ± 3000 daltons. The optimum pH was 4.7, and the K_m values for cytokinin nucleoside and adenosine were 2.3 µM and 1.43 µM respectively.

Purine nucleosidase was successfully purified from Jerusalem artichoke shoots by Le Floch *et al. (25).* The activities for adenosine and inosine-guanosine nucleosidase were exhibited in the initial extract. The K_m values for adenosine, guanosine and inosine were respectively 17 µM, 8.5 µM, and 2.5 µM. The substrate specificity was studied to

distinguish between the two enzymes. The hydrolysis rate for either inosine substrate or guanosine substrate were not affected by the addition of adenosine. On the other hand, the hydrolysis rate of inosine and guanosine were inhibited by the addition of 6-mercaptopurine riboside while adenosine showed no effect on the hydrolysis rate.

Burch and Stuchbury were partially able to purify adenosine nucleosidase from tomato roots and leaves *(23).* Also, they were able also to determine two distinguishable types of adenosine nucleosidase called R1 and R2 in the tomato roots. The optimum pH for R1 was 5.0 and 6.0 for R2. In addition, one type of adenosine nucleosidase known as Lf was found in the tomato leaves, and the optimum pH was 6.0. The K_m values for the three enzymes R1, R2, and Lf were 25 µM, 9 µM and 6 µM respectively. The molecular weight for the major enzyme in root, R2, was estimated to be 68,000 daltons. Lastly, a competitive inhibitor for the three adenosine nucleosidases were cytokinin ribosides.

Abusamhadneh *et al.* determined adenosine nucleosidase activity from yellow lupine seeds *(17).* The molecular weight of the enzyme was 177,000 daltons determined by size exclusion chromatography. The optimum pH was 7.5 and K_m value for adenosine was 4.7 µM. The enzyme showed activity with some nucleosides such as adenosine, 2'-deoxyadenosine, cytidine, guanosine, inosine and thymidine. Adenosine as substrate had the highest activity among the nucleosides.

Adenosine nucleosidase was purified by Altawil from Alaska pea seeds. The SDS-PAGE was utilized to determine the subunit molecular weight which is 36,000 daltons *(26).*

Shamsuddin purified a second form adenosine nucleosidase from Alaska pea seeds *(27).* The enzyme was determined to belong to the non-specific inosine-uridine nucleoside hydrolases (IU-NHs). The molecular weight was 26,103 daltons. A yield of 1.3 % with 4-fold purification was accomplished. The K_m value using adenosine as the substrate was 137 ± 48 µM, and the V_{max} was 0.34 ± 0.02 µM/min. A summary of the adenosine nucleosidase molecular weight for previously listed plants is shown in Table 1.

Table 1. Native molecular weight of adenosine nucleoside for different types of plants.

Plants	Molecular weight (Da)
Barley leaves	66,000
Wheat germ	59,000
Tea leaves	68,000
Tomato root and leaves	68,000
Yellow lupin	177,000

Purpose of the Study

Cytokinins are plant hormones that are generally made of either adenosine derivatives or adenine derivatives. The most essential physiological function of cytokinins is to induce cell division on plants. The exogenous cytokinins have been studied and determined to improve flowering and increase crop yields *(18).* Adenosine nucleosidase along with cytokinins metabolizing enzymes may be involved in controlling the cytokinin levels in plants. The purpose of this study was to optimize and improve the purification steps of adenosine nucleosidase from Alaska pea seeds (*Pisum sativum L).* Also, the activity of nucleosidase enzymes at different germination times of the seeds were examined in this study.

CHAPTER II

MATERIALS AND METHODS

Equipment and Instrumentation

A Sorvall LYNX 6000 Superspeed Centrifuge from Thermo Scientific and an Eppendorf 5810R centrifuge were utilized to concentrate the samples to the desired volume. Diethylaminoethyl chromatography on a (DEAE) Sepharose column and Fast Flow Mono Q column were carried out on an AKTA Fast Protein Liquid Chromatography (FPLC) system from GE Healthcare.

High performance liquid chromatography (HPLC) was performed on a Dionex UltiMate 3000 Standard LC System equipped with Kinetex EVO C18 (150 x 4.6 mm) column. UV-Vis spectroscopy was carried out on a Hitachi U-2900 spectrophotometer.

Materials and Reagents

Alaska (Wilt Resistant) pea seeds were obtained from Ferry-Morse Garden Seed Company. Protamine sulfate salt, DL-Dithiothreitol (DTT), protease inhibitor cocktail for plant cell and tissue extracts, uridine, adenosine, inosine, cytidine nucleosides and their corresponding bases uracil, adenine, hypoxanthine, and cytosine were purchased from Sigma-Aldrich.

Protein assay standard II, Bio-Rad protein assay dye reagent, Precision Plus Protein™

Unstained Standards, 10x Tris/Glycine/SDS buffer, and 2x Laemmli sample buffer were obtained from Bio-Rad Inc. Potassium phosphate and neocuproine hydrochloride monohydrate were obtained from Acros. Ammonium phosphate monobasic was obtained from J.T. Baker Chemical Co. Ammonium sulfate, Tris base for molecular biology and Lonza 15 % Gold Precast Gels were obtained from Fisher Scientific. GelCode ™ Blue Safe Protein Stain for SDS-PAGE gels and silver stain kit were obtained from Thermo Scientific. ELGA PURELAB Ultra was used to obtain deionized water with 18.2 MΩ-cm purity. All other chemicals were reagent grade.

Measurement of Enzyme Activity

Throughout the purification steps**,** the enzyme activities for three different nucleosides were measured by using high performance liquid chromatography (HPLC) and reducing sugar assay.

High Performance Liquid Chromatography (HPLC) Assay

The extracts from dry seeds and at different germination times were assayed for adenosine, inosine, and uridine activity by HPLC. The total activity for adenosine, inosine, and uridine in each step was determined by the disappearance of the nucleosides to the base at room temperature.

Three nucleoside activities, adenosine nucleosidase, inosine nucleosidase, and uridine nucleosidase, were determined by HPLC for non-germinated and germinated seeds.

Adenosine nucleosidase activity was determined after each ammonium sulfate precipitation step and after the purification of the same enzyme. The reaction mixtures consisted of 1 mM adenosine, inosine, or uridine in 10 mM Tris pH 7.2. In order to initiate the reaction, 100 µL enzyme for germinated seeds, or 20 µL of enzyme from ammonium sulfate steps, or 100 µL enzyme after columns were added to separate HPLC vials. The control consisted of 1 mL of individual nucleoside in 10 mM Tris buffer pH 7.2 and water equivalent to the amount of enzyme added to reaction mixture. The reaction mixture was incubated at room temperature. The amount of nucleoside and base were quantified by HPLC on a Kinetex EVO C18 (150 x 4.6 mm) column. The mobile phase were 98% 10 mM ammonium phosphate pH 5.4 and 2% methanol. The temperature of the column oven was 30.0 °C. The analysis was monitored at 254 nm. The amount of nucleoside and base were measured based on their respective peak areas and the activity determined by the disappearance of the nucleosides.

Reducing Sugar Assay

Adenosine nucleosidase activity was tested after each purification step using a colorimetric reducing sugar assay. To clean and dry test tubes, 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 was added. To initiate the reaction, 100 µL of enzyme from the corresponding fraction was added to each tube. The reactions were incubated at 30 °C for 4 hours. The reaction was stopped by adding 300 µL copper reagent and 300 µL neocuproine to the test tubes followed by vortexing.

The copper reagent consisted of 4% Na_2CO_3, 1.6% glycine, and 0.045%

$CuSO_4.5H_2O$ dissolved in 400 mL DI water, and the neocuproine reagent was 0.12% neocuproine dissolved in 400 mL water with the pH adjusted to 3.0 using HCl. The samples were incubated at 95 °C for 7 min. After the samples cooled, the absorbance at 450 nm was measured. The amount of ribose was determined by comparison to a standard calibration curve (Figure 8). The fractions exhibiting highest enzyme activity from DEAE and Mono Q columns were identified and labeled as pool #1 and pool #2 respectively. These two fractions were dialyzed against 2 L of 10 mM Tris buffer pH 7.2. The dialysis was performed two times over two days replacing the buffer after 24 hours.

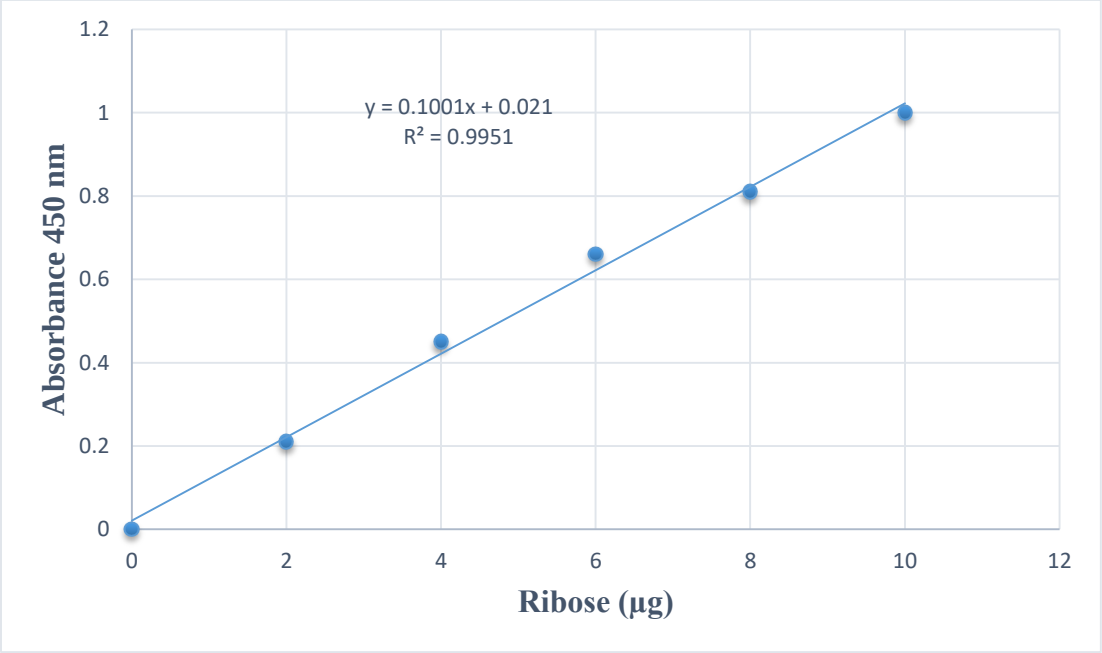


Figure 8. Standard reducing sugar assay calibration curve. The activity of enzyme was measured by the appearance of ribose as the nucleoside was hydrolyzed.

Determination of Protein Concentration

Bio-Rad Assay

The Bio-Rad assay is one method for determining protein concentration in an unknown sample. The assay mixture was composed of 895 µL DI water, 5 µL of diluted sample (1:10 dilution) and 200 µL of dye reagent. The samples were mixed and the absorbance at 595 nm was measured. The protein concentration was determined by comparing the results to a standard Bio-Rad calibration curve (Figure 9). Protein assay standard II was used to prepare the calibration curve. Bio-Rad assay was carried out after each ammonium sulfate precipitation step and on the pooled fractions, pool #1 and pool #2, from both DEAE and Mono Q columns respectively. The protein concentration of fractions from the DEAE (pool #1) and the Mono Q (pool #2) columns was determined by measuring the absorbance of each fraction at 280 nm.

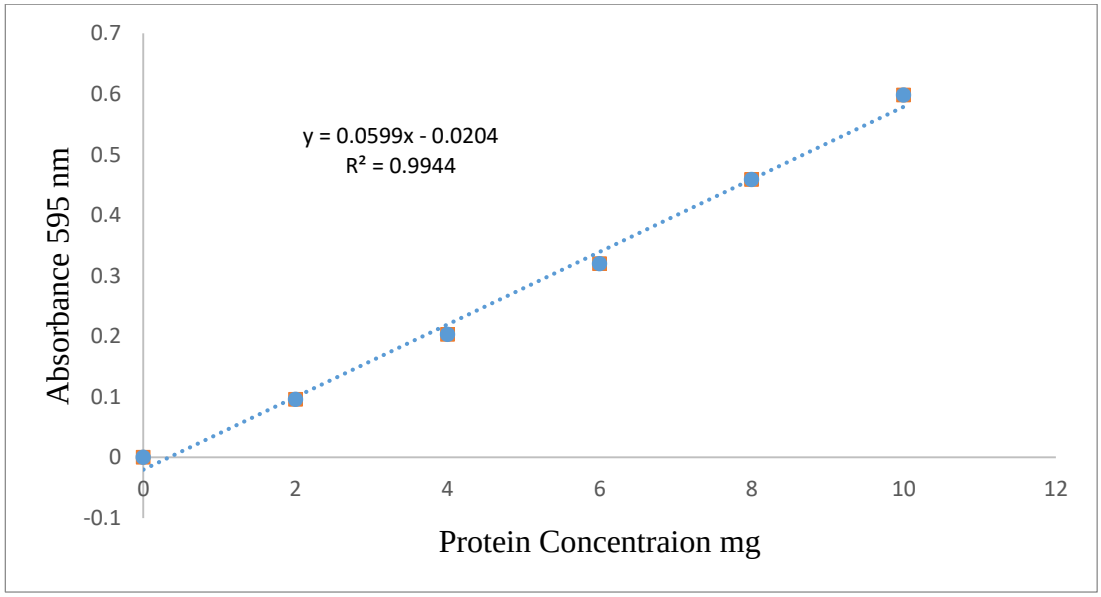


Figure 9. Standard protein calibration curve determined at 595 nm.

Determination of Activity at Different Germination Period

Twenty grams (20 g) of Alaska pea seeds were soaked in 30 mL of bleach for 20 minutes. The seeds were washed extensively with tap water and placed on a moistened paper towel to allow germination to take place. One gram (1 g) of dry seeds was homogenized in 10 mL of 10 mM Tris buffer pH 7.2 and blended five times for 1 min followed by one minute rest. The solution was centrifuged using Sorvall LYNX 6000 centrifuge at 20,000 *xg* for 30 minutes at 4 °C. The extract (7 mL) was labeled and stored in the cold room.

In addition, approximately one gram (1 g) of Alaska pea seeds was homogenized at 4 hours, 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours of germination in 10 mL of 10 mM Tris buffer pH 7.2. The seeds were blended five times for 1 min with one minute rest and then centrifuged at 20,000 *xg* for 30 minutes. The extract (7 mL) was labeled and stored in the cold room for overnight. The extract was used to determine the activity at various germination time.

Preparation of Enzyme Extract

Twenty grams (20 g) of Alaska pea seeds were soaked in 25 mL of bleach for 20 minutes to eliminate any surface bacteria. The seeds were thoroughly washed with deionized water five times and placed on a moistened paper towel to allow germination at room temperature. The next preparation steps were performed in a cold room at 4 °C. After five days of germination, the seeds were placed in a blender and homogenized in 50 mL 10 mM Tris buffer, pH 7.2. In addition, 10 mg of dithiothreritol, 0.5 g protamine sulfate,

and 300 µL protease inhibitor cocktail were added to the blender. The seeds were homogenized 5 times for 1 minute followed by 2 minute rest period.

The resulting solution was filtered through a double layer of cheesecloth and centrifuged at 15,000 *xg* for 20 minutes at 4 °C. The supernatant volume was measured, and the precipitated pellet was resuspended in 5 mL 10 mM Tris buffer, pH 7.2. Both the supernatant and the resuspended pellet were covered with foil and kept in the cold room.

Enzyme Purification

Ammonium Sulfate Precipitation

An incremental increase of 10% ammonium sulfate was performed at each step. The initial supernatant volume (35 mL) was used to calculate the amount of ammonium sulfate needed to increase the saturation level to 20%. Thus, a total of 3.95 g of ammonium sulfate was gradually added to the supernatant *(30).* The solution was slowly stirred while ammonium sulfate was added and kept in the cold room overnight.

The solution was centrifuged at 15,000 *xg* for 20 minutes at 4°C. The supernatant was measured (35 mL), and the pellet was resuspended in 5 mL 10 mM Tris pH 7.2 and kept in the cold room. To increase the saturation to 30%, a total of 2.00 g of ammonium sulfate was added to the supernatant. The solution was left overnight in the cold room. After centrifugation, the 30% supernatant was separated from the pellet, and the pellet resuspended in 5 mL 10 mM Tris pH 7.2. Ammonium sulfate (2.1 g) was added to 34 mL supernatant to bring the saturation level to 40%. The solution was left overnight at 4 °C.

The 40% sample was centrifuged and the supernatant and pellet separated. The resuspended pellet (5 mL) was stored in the cold room overnight. The supernatant (34 mL) was increased to 50% ammonium sulfate saturation through slow addition of 2.14 g of solid ammonium sulfate. The solution was stored in the cold room overnight. In order to obtain 60% saturation, the solution was centrifuged and the supernatant and pellet separated. Total of 2.1 g ammonium sulfate was added to the resulting supernatant (32 mL). The pellet was resuspended in 5 mL 10 mM Tris pH 7.2 and stored in the cold room. The same procedure was repeated to bring the solution to 70% saturation. The resuspended pellet was labeled and stored in the cold room. After each step of ammonium sulfate precipitation, a total of 1 mL of the initial, 20%, 30%, 40%, 50%, 60% and 70% supernatants were saved and stored in the cold room at 4 °C.

Ion Exchange Chromatography

Diethylaminoethyl (DEAE) Sepharose Column

The 70% resuspended pellet had the highest nucleosidase activity. The sample (10 mL) was dialyzed two times against 1 L of 10 mM Tris buffer pH 7.2. After filtering the sample, the first 5 mL was loaded onto a DEAE HiPrep 16/10 FF Sepharose column. The flow rate was 2 mL/min and the pressure limit 0.80 MPa. Bound protein was eluted with a stepwise gradient from 50 mM to 450 mM sodium chloride (NaCl) in 10 mM Tris buffer pH 7.2. The column was first washed with 1 L of 10 mM Tris buffer pH 7.2 followed by a stepwise increase of salt concentration (NaCl). The salt concentration used were 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, and 450 mM.

A total of 82 fractions of 5 mL fraction size were collected and stored in the cold room. The protein concentration was determined by measuring the absorbance at 280 nm and activity was measured by reducing sugar assay at 450 nm. Fractions that showed high activity were pooled and designated as (pool #1).

Mono Q Column

The second 5 mL sample was loaded onto a pre-equilibrated HiPrep Mono Q 16/10 FF column and eluted with a flow rate of 3 mL/min at 0.50 MPa pressure limit. The same stepwise gradient was used to elute the column as was used for the DEAE column. The fraction size was 5 mL. The 280 nm absorbance was measured for all fractions to determine protein concentration, and the activity determined by reducing sugar assay.

Determination of Enzyme Purity by Gel Electrophoresis

SDS-PAGE

Denaturing electrophoresis was carried using a 15 % Lonza Precast gel, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and was performed on pool #1 from the DEAE column and pool #2 from the Mono Q column. Sample (10 µL) and 10 µL 2x Laemmli sample buffer were added to an Eppendorf tube. The sample was centrifuged for 30 seconds and incubated at 95 °C for 5 minutes. The sample was centrifuged for 30 seconds and then loaded onto the gel along with the Precision Plus Protein™ Unstained Standards. The gel ran for 60 minutes at 118 volts. After

completion, the gel was placed in plastic container and washed 2 × for 5 minutes with water.

Due to the greater sensitivity provided, a silver stain protocol was performed to stain the gel. The gel was fixed 2 × for 15 minutes in 30% ethanol and 10% acetic acid. For one minute, the gel was sensitized with 50 µL sensitizer in 25 mL water, followed by 2 × 1 minute water washes. The gel was stained for 30 minutes in a solution of 0.5 mL enhancer and 25 mL silver stain. After 30 minutes, the gel was developed for 2-3 minutes in 0.5 mL enhancer and 25 mL developer solution. To stop the stain, 5% acetic acid was added for 10 minutes. A molecular weight calibration curve was constructed using the Precision Plus Protein™ Unstained molecular weight marker. The molecular weight of the samples (Daltons) was determined by comparing the distance the band traveled to a calibration curve. By visual inspection, the purity of the enzyme was observed to be 95% pure.

CHAPTER III

RESULTS AND DISCUSSION

Adenosine nucleosidase is an essential enzyme involved in the hydrolysis of adenosine to adenine and ribose. Adenosine nucleosidase plays a key role in the purine metabolism pathways in plants. Adenosine nucleosidase, was selected for the study because of the hypothesis that it is involved in the synthesis and degradation of cytokinins. Cytokinins are hormones found in plants and are derived from adenosine. The specific physiological role of this enzyme is unclear.

While cytokinins are not substrates of adenosine nucleosidase, the enzyme may be control their activity by controlling adenosine availability. Cytokinins control a number of plant processes. For instance, they are involved in the differentiation and growth of the cell. These hormones also play a role in determining apical dominance, leaf formation and senescence, and the growth of auxiliary buds *(18).* Cytokinins are also an important component in the culture medium used to grow cells of plants. Without these hormones, division of the cells in the culture would not be possible and thus no growth would occur. Consequently, an understanding of cytokinin metabolism is tied to a better grasp of adenosine nucleosidase. The purpose of this study was to optimize the purification steps and purify adenosine nucleosidase from Alaska pea seeds (*Pisum sativum L*)**.** The activity of the enzyme was determined based on the change of the factors carried out during the purification procedure.

Activity at Different Germination Period

Three nucleoside activities were examined at different germination times of Alaska pea seeds. This was done before the purification of adenosine nucleosidase. The three nucleosides were adenosine, inosine, and uridine. The total activity was measured for dry seeds and at different germination times 4, 24, 48, 72, 96, and 120 hours. A total of one gram (1 g) of 20 g of seeds from various germination times were homogenized separately in 10 mL of 10 mM Tris buffer pH 7.2. In order to remove large particles, the samples were filtered using cheesecloth and then centrifuged for 30 minutes. The resulting extracts were tested for the presence of adenosine, inosine, and uridine nucleosidase activity. All activities were measured by HPLC assay by observing the disappearance of the nucleosides (adenosine, inosine, and uridine).

The total nucleosidase activity was measured by the disappearance of nucleosides and the appearance of respective base using a reversed phase HPLC. The chromatograms for adenosine nucleosidase (Figure 10), inosine nucleosidase (Figure 11), and uridine nucleosidase (Figure 12) were observed to show hydrolase activity in which adenosine, inosine and uridine converted to adenine, hypoxanthine, and uracil. However, the disappearance of adenosine during the germination time was due to both hydrolase and deaminase activities. The chromatogram (Figure 10) showed the presence of multiple peaks which implies the existence of two different metabolizing activities/enzymes. These enzymes are adenosine nucleosidase and adenosine deaminase. The first, adenosine nucleosidase, converted adenosine to adenine and inosine to hypoxanthine, while the second, adenosine deaminase, converted adenosine to inosine as indicated in

Figure 10. The deaminase activity was observed only when using adenosine as substrate at different germination period. Inosine and uridine nucleosidase were converted to their relative bases by nucleosidase activity (Figure 11 and 12). All chromatograms showed an unidentified peak at 1.9 minutes. A comparison of the total activity of adenosine nucleosidase, inosine nucleosidase, and uridine nucleosidase is shown in Figure 13**.** Table summarizing the total activity of adenosine nucleosidase (Table 2), inosine nucleosidase (Table 3) and uridine nucleosidase (Table 4) are shown.

Based on the calculation of the total activity (Tables 2, 3, and 4), dry seeds exhibited the lowest total activity for all three nucleosides compared to activity at different germination periods. The initial extract from dry seeds was observed to have multiple nucleosidase and deaminase activities. These activities were adenosine nucleosidase, converting adenosine to adenine, adenosine deaminase, converting adenosine to inosine, inosine nucleosidase, converting inosine to hypoxanthine, and uridine nucleosidase, converting uridine to uracil. The highest nucleosidase activity in ungerminated seeds was uridine nucleosidase with an activity of 12.6 µmol/min. On the other hand, adenosine nucleosidase had the lowest activity of 0.35 µmol/min followed by inosine nucleosidase with an activity of 1.64 µmol/min. In contrast, no activity was detected for inosine nucleosidase in dry seeds from yellow lupin *(28).* Altawil determined several activities in dry Alaska pea seeds such as nucleosidase, deaminase, and phosphorylase activities *(26).* The adenosine nucleosidase activity in dry Alaska pea seeds was measured to be 4.37 µmol/min.

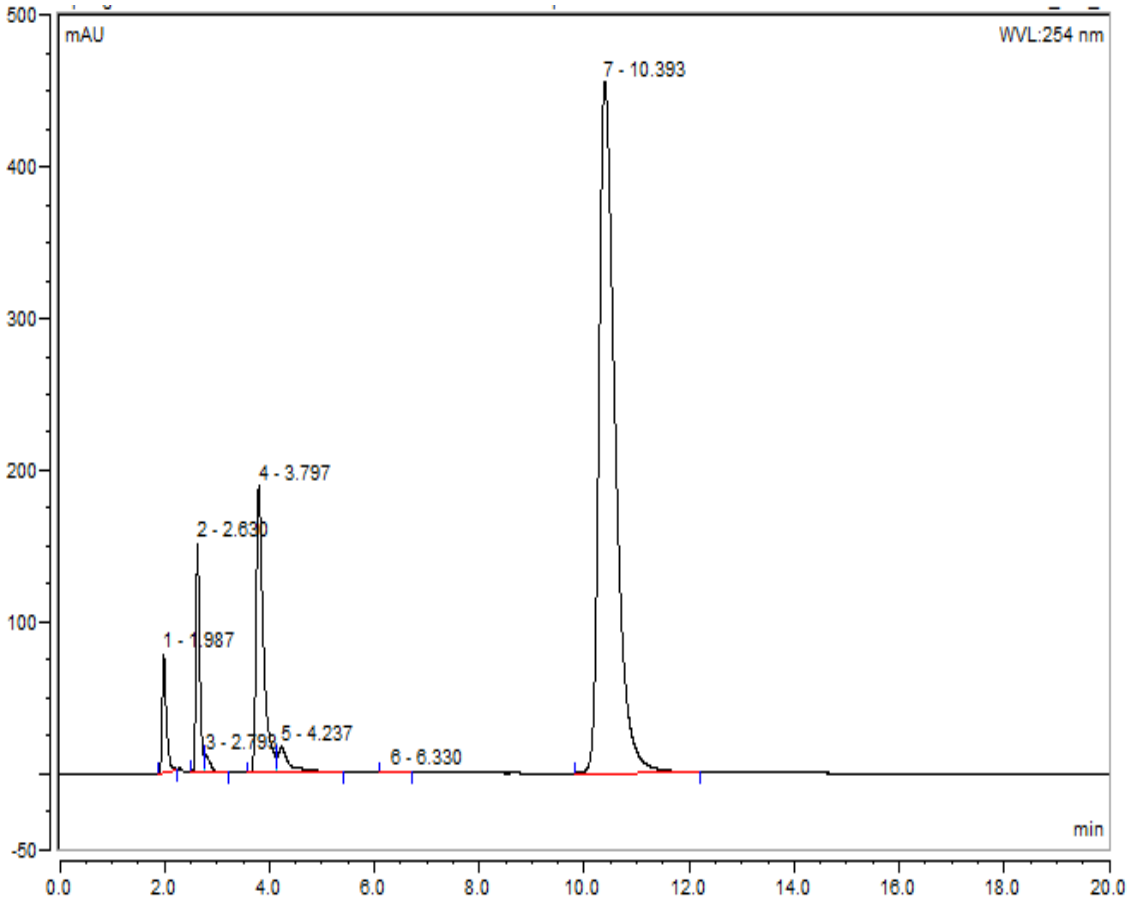


Figure 10. HPLC analysis for adenosine nucleosidase after 48 hours of germination of seeds. The reaction mixture consisted of 1 mL of 1 mM adenosine solution in 10 mM Tris pH 7.2 plus 100 µL of the initial extract. After 8 hours since adding enzyme, the chromatogram showed adenosine (10.393 min), inosine (3.79 min) and hypoxanthine (2.63 min). The peak at 1.987 min is unidentified.

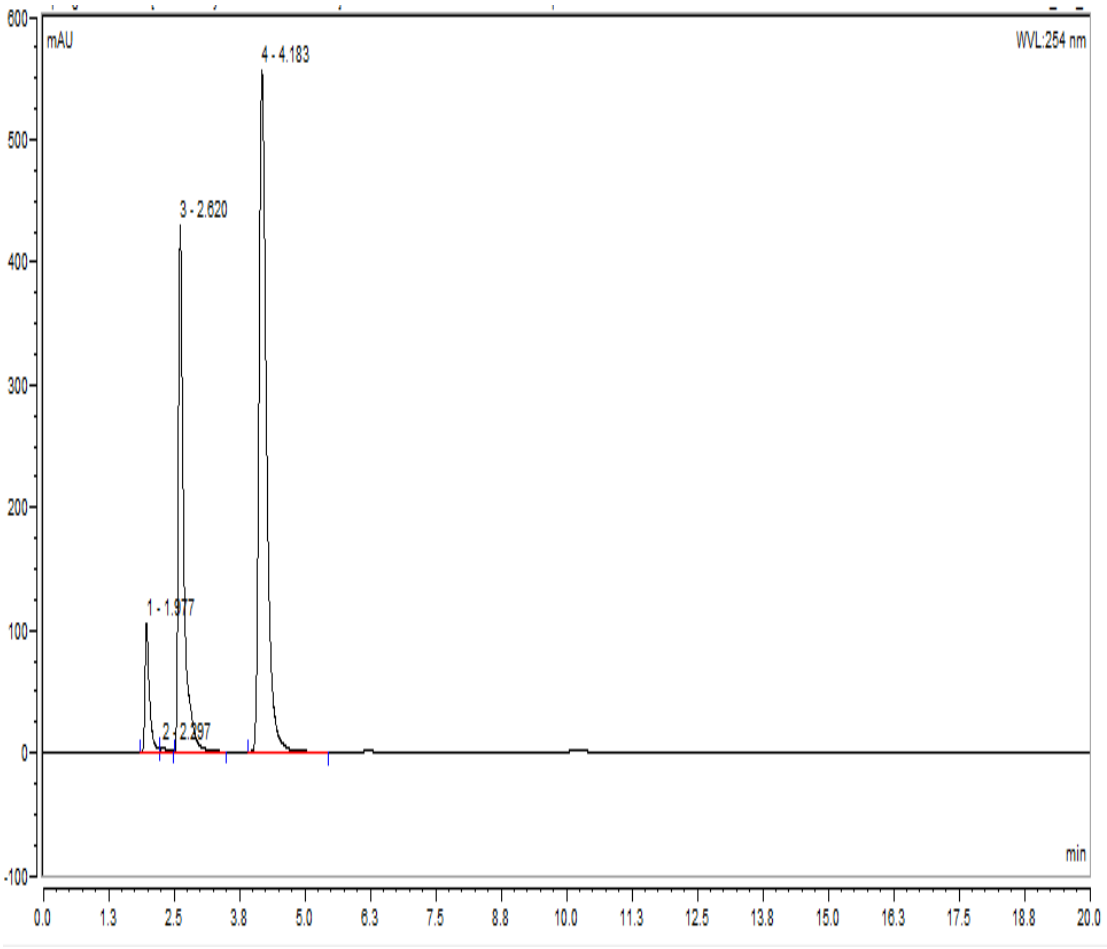


Figure 11. HPLC analysis for inosine nucleosidase after 48 hours of germination of seeds. The reaction mixture consisted of 1 mL of 1 mM inosine solution in 10 mM Tris pH 7.2 plus 100 µL of the initial extract. After 9 hours since adding enzyme, the chromatogram showed inosine (4.18 min), and hypoxanthine (2.62 min). The peak at 1.977 min is unidentified.

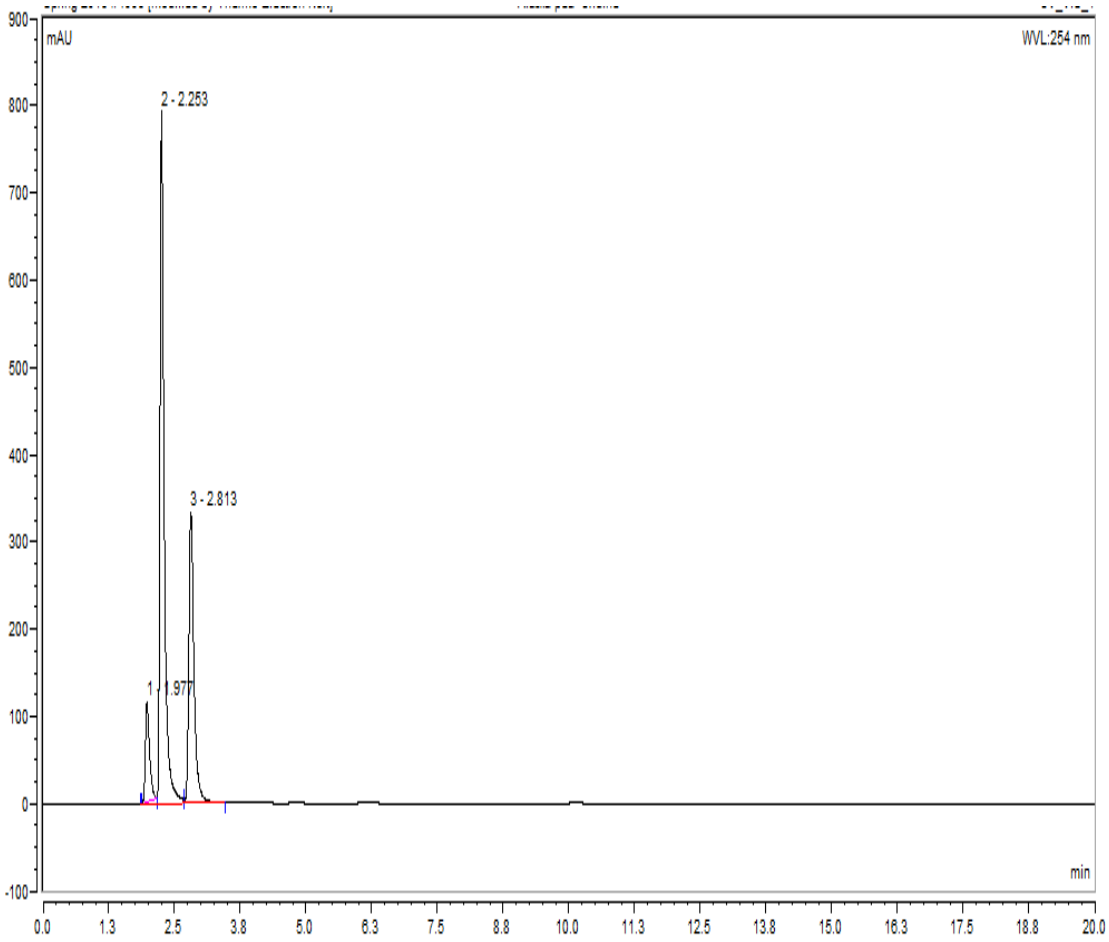


Figure 12. HPLC analysis for uridine nucleosidase for seeds from 48 hours of germination. The reaction mixture consisted of 1 mL of 1 mM uridine solution in 10 mM Tris pH 7.2 plus 100 µL of the initial extract. After 8 hours since adding enzyme, the chromatogram showed uridine (2.81 min), and uracil (2.25 min). The peak at 1.977 min is unidentified.

A gradual increase in the total activity for adenosine, inosine, and uridine nucleosidase was observed as the seeds germinated from 4 hours up to 48 hours of germination. A similar increase in the peroxidase activity in dry pea seeds was observed with the activity continue to increase after the 5^{th} day of germination *(34).* Adenosine, inosine, and uridine nucleosidases activities reached the maximum activity at 48 hours of germination with an activity of 33 µmol/min, 50 µmol/min, and 67 µmol/min respectively. After 48 hours, the total activity of these nucleosidases was observed to decrease. Adenosine nucleosidase had its maximum activity in yellow lupin after 120-144 hours of seed germination *(28).* Inosine nucleosidase activity was observed during the germination of yellow lupin seeds *(29).*

Table 2. Summary of adenosine nucleosidase activity from Alaska pea seeds at different periods of germination. The activity was measured using HPLC.

	Total Volume (mL)	Activity (µmol/min)	Total Activity (µmol/min)
Seeds before germination	7	5.0×10^{-3}	0.4
4 hour after germination	7	6.5×10^{-2}	4.6
24 hours after germination	7	0.18	12.6
48 hours after germination	7	0.47	33.0
72 hours after germination	7	0.15	10.5
96 hours after germination	7	0.13	9.5
120 hours after germination	7	0.13	9.3

Table 3. Summary of inosine nucleosidase activity from Alaska pea seeds over various germination times. The activity was measured using HPLC.

	Total Volume (mL)	Activity (µmol/min)	Total Activity (µmol/min)
Seeds before germination	7	2.3×10^{-2}	1.6
4 hour after germination	7	2.5×10^{-2}	1.8
24 hours after germination	7	0.23	16.5
48 hours after germination	7	0.71	50.0
72 hours after germination	7	0.48	33.6
96 hours after germination	7	0.46	32.0
120 hours after germination	7	0.45	31.5

Table 4. Summary of uridine nucleosidase activity from Alaska pea seeds at different periods of germination. The activity was measured using HPLC.

	Total Volume (mL)	Activity (µmol/min)	Total Activity (µmol/min)
Seeds before germination	7	0.18	12.6
4 hour after germination	7	0.36	25.2
24 hours after germination	7	0.39	27.3
48 hours after germination	7	0.96	67.2
72 hours after germination	7	0.84	58.8
96 hours after germination	7	0.82	58.4
120 hours after germination	7	0.82	58.3

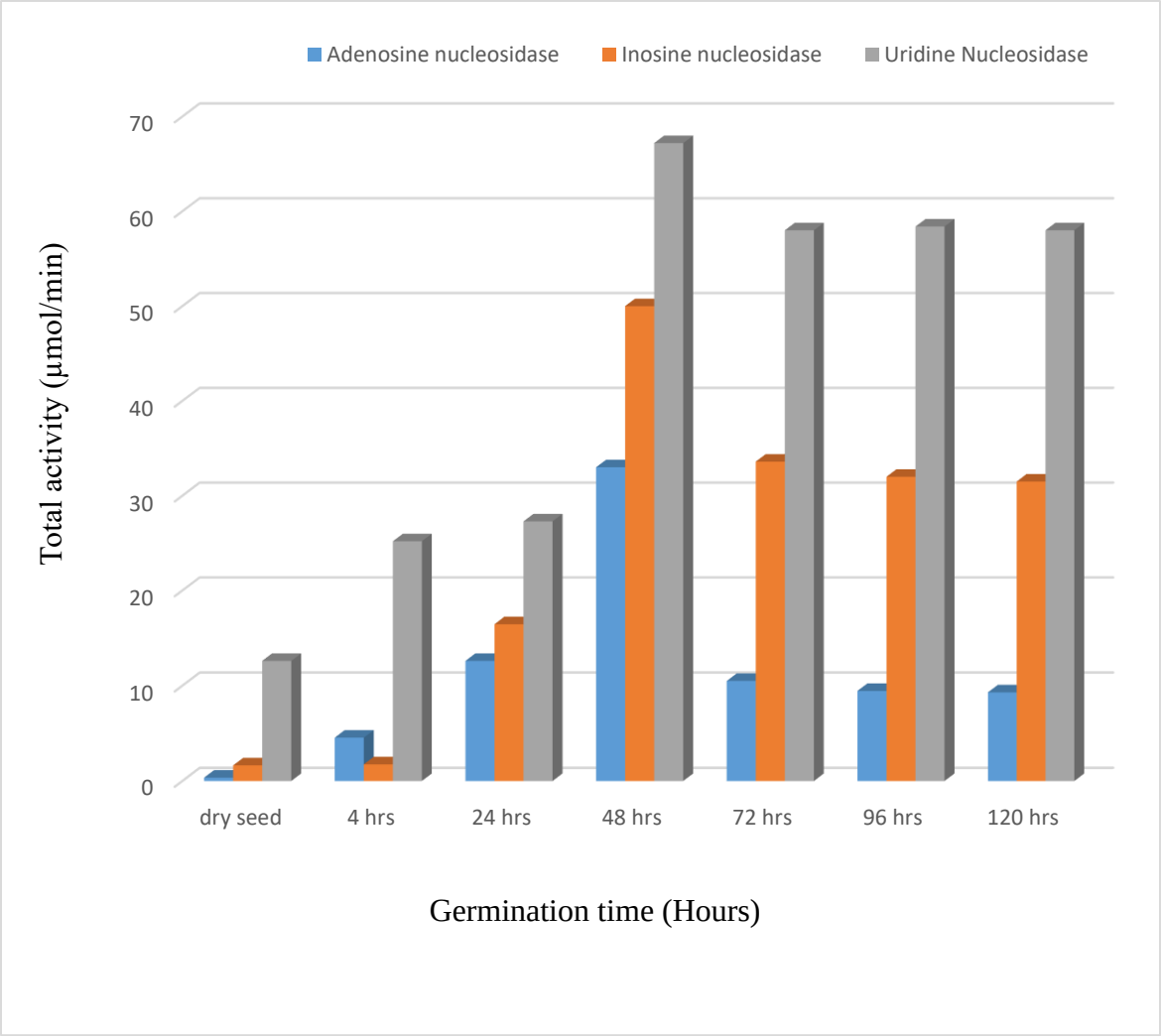


Figure 13. A comparison of the total activity (µmol/min) for adenosine nucleosidase, inosine nucleosidase, and uridine nucleosidase in un-germinated seeds and at different germination times (4hr, 24hr, 48hr, 72hr, 96hr, and 120hr).

Purification of Adenosine Nucleosidase

Improving the purification scheme for adenosine nucleosidase from Alaska pea seeds was the main goal of this project. Seeds (20 g) were homogenized after five days of germination at room temperature. The sample was filtered using a double layer of cheesecloth and then centrifuged to remove insoluble large particles. The initial extract and initial resuspended pellet were assayed by HPLC for the presence of adenosine, inosine, and uridine activities using 1 mM of each nucleoside as substrates.

The enzyme activities present in the initial extract and initial resuspended pellet in the presence of adenosine were adenosine nucleosidase, adenosine deaminase, and inosine nucleosidase (Figure 14a, 14b). Inosine nucleosidase and uridine nucleosidase activities were also observed in both the initial extract and resuspended pellet when inosine and uridine were used as substrates. Inosine nucleosidase converts inosine to hypoxanthine while uridine nucleosidase converts uridine to uracil. The specific activity based on the loss of adenosine for the initial sample and initial resuspended pellet were 6.9 $x10^{-3}$ and 1.3 $x10^{-3}$ µmol/min/mg respectively. Inosine nucleosidase specific activity from initial supernatant and initial resuspended pellet were 7.4 $x10^{-3}$ µmol/min/mg and 2.7 $x10^{-3}$ µmol/min/mg respectively. The specific activity for uridine nucleosidase in the initial supernatant was 9.3 $x10^{-3}$ µmol/min/mg while the resuspended pellet was 4.0 $x10^{-3}$ µmol/min/mg.

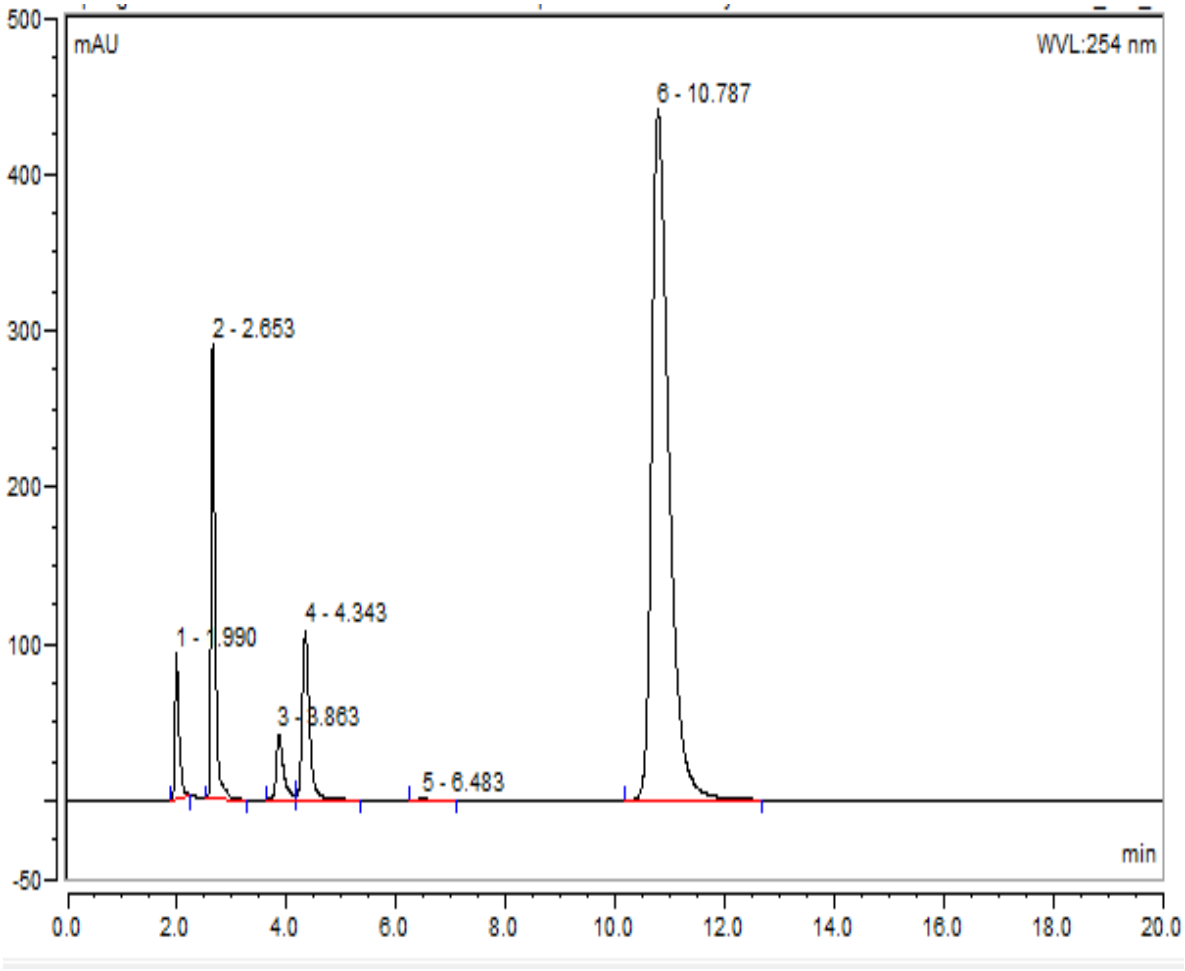


Figure 14a. Adenosine activity analysis by HPLC of the initial extract. The reaction mixture consisted of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 plus 20 µL of initial extract. After 8 hours following enzyme addition, the chromatogram showed five peaks; adenosine (10.78 min), adenine (4.34 min), inosine (3.86 min) and hypoxanthine (2.65 min). The peak at 1.99 min was unidentified.

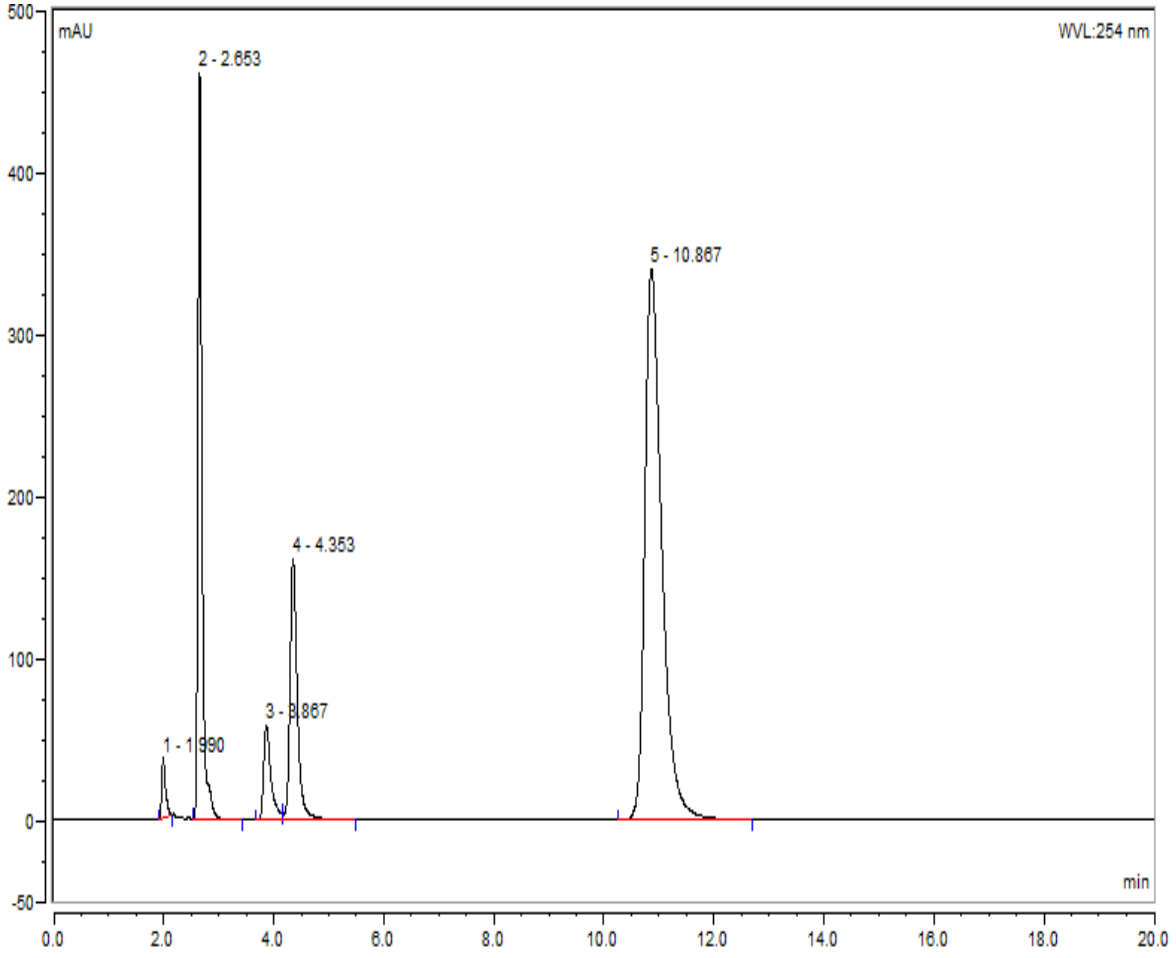


Figure 14b. Adenosine activity analysis by HPLC of the initial resuspended pellet. The reaction mixture consisted of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 plus 20 µL of initial extract. After 10 hours following enzyme addition, the chromatogram showed five peaks; adenosine (10.86 min), adenine (4.35 min), inosine (3.86 min) and hypoxanthine (2.65 min). The peak at 1.99 min was unidentified.

The next purification step was ammonium sulfate fractionation. Ammonium sulfate precipitation is a classical method widely used for protein fractionation *(37).* Ammonium sulfate fractionation was carried out between 20-70% saturation in 10% increments. The various fractions were tested for activity using adenosine, inosine, and uridine as substrates to compare the results. Ammonium sulfate was gradually added to bring the saturation level to 20% after centrifuging the initial sample. The solution was kept in the cold room at 4°C for overnight. After centrifugation of the 20% sample, the supernatant volume was measured to calculate the amount of ammonium sulfate needed to bring the saturation level to 30%. Ammonium sulfate was added, and the solution was stored in the cold room overnight.

The same ammonium sulfate precipitation procedure described above was performed to obtain 30%, 40%, 50%, 60%, and 70% saturation levels. The pellet after each step was resuspended in 5 mL of a 10 mM Tris buffer pH 7.2. The supernatants and resuspended pellets from each precipitation step (20%- 70%) were assayed for adenosine, inosine, and uridine activities by HPLC. The highest specific activity among these nucleosides in the supernatants and resuspended pellets from 20%-70% was determined for uridine nucleosidase followed by inosine nucleosidase and lastly adenosine nucleosidase. The protein solubility in solution increases within the addition of ammonium sulfate (<0.15 M), an effect known as salting in *(35).* At higher ammonium sulfate concentrations, the protein solubility decreases hence precipitation takes place. This phenomenon is known as salting out *(36).* Adenosine nucleosidase was precipitated at 70% fractionation. This observation was determined based on the comparison of the specific activity for both supernatants and

resuspended pellets from different ammonium sulfate% and 70% ammonium sulfate precipitation. The specific activities for 60% supernatant and resuspended pellet were 8.2 $x10^{-3}$ µmol/min/mg and 2 $x10^{-3}$ µmol/min/mg respectively. The specific activities for 70% supernatant was 5.3 $x10^{-3}$ µmol/min/mg and resuspended pellet was 7.3 $x10^{-3}$ µmol/min/mg. As a result, the activity of the adenosine nucleosidase was split after 70% cut. The HPLC chromatogram for 60% supernatant and resuspended pellet (Figure 15a, 15b) shows multiple enzyme activities. Additionally, the chromatogram for 70% supernatant (Figure 16a) and resuspended pellet (Figure 16b) again shows several enzyme activities. The result profiles confirm the presence of two enzyme activities; adenosine nucleosidase and adenosine deaminase.

Inosine nucleosidase and uridine nucleosidase had similar results to adenosine nucleosidase in which the precipitation takes place at 70% saturation. The 60% and 70% resuspended pellet inosine nucleosidase specific activities were 3.0 $x10^{-3}$ µmol/min/mg and 1.1 $x10^{-2}$ µmol/min/mg where the 60% and 70% resuspended pellets of uridine nucleosidase had specific activities of 3.4 $x10^{-3}$ and 9.1 $x10^{-3}$ µmol/min/mg respectively. Proteins with low molecular weight such as interleukin-1β require a high concentration of salt in order to precipitate. On the other hand, the precipitation for large complex proteins take place at a low salt concentration < 20% saturation *(35).* Summary tables for the precipitation steps for adenosine, inosine, and uridine nucleosidases are given in Table 5, Table 6 and Table 7. Comparison of the specific activities of adenosine, inosine, and uridine nucleosidases for both supernatant and resuspended pellet are shown in Figure 17, Figure 18 and Figure 19 respectively.

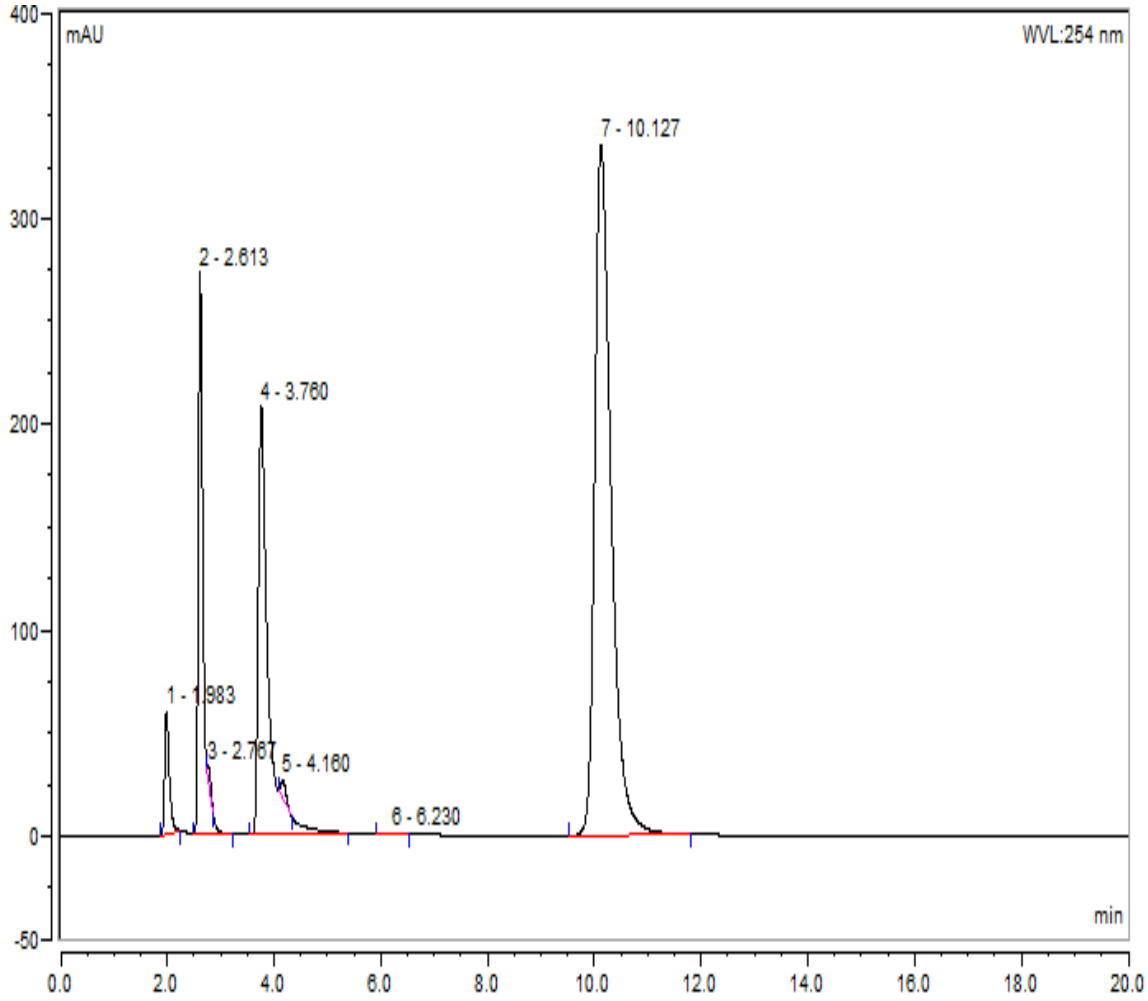


Figure 15a. HPLC analysis for 60% saturation supernatant after 7 hours following enzyme addition. The reaction mixture consisted of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 plus 20 µL of 60% sample. The chromatogram shows multiple peaks; adenosine (10.12 min), inosine (3.76 min), and hypoxanthine (2.61 min). The peak at 1.98 min was unidentified.

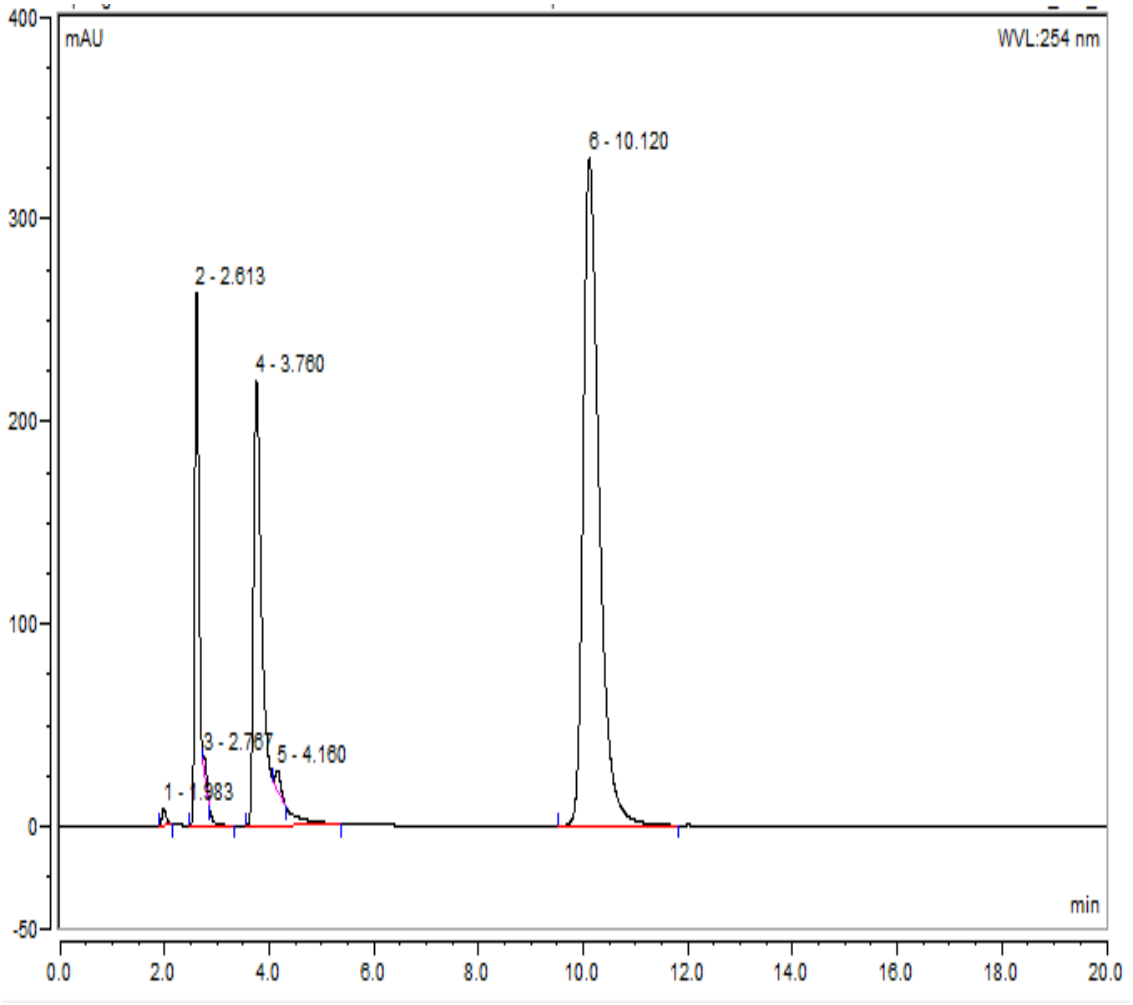


Figure 15b. HPLC analysis for 60% saturation for resuspended pellet after 7 hours following enzyme addition. The reaction mixture consisted of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 plus 20 µL of 60% resuspended pellet. The chromatogram shows multiple peaks; adenosine (10.12 min), inosine (3.76 min), and hypoxanthine (2.61 min).

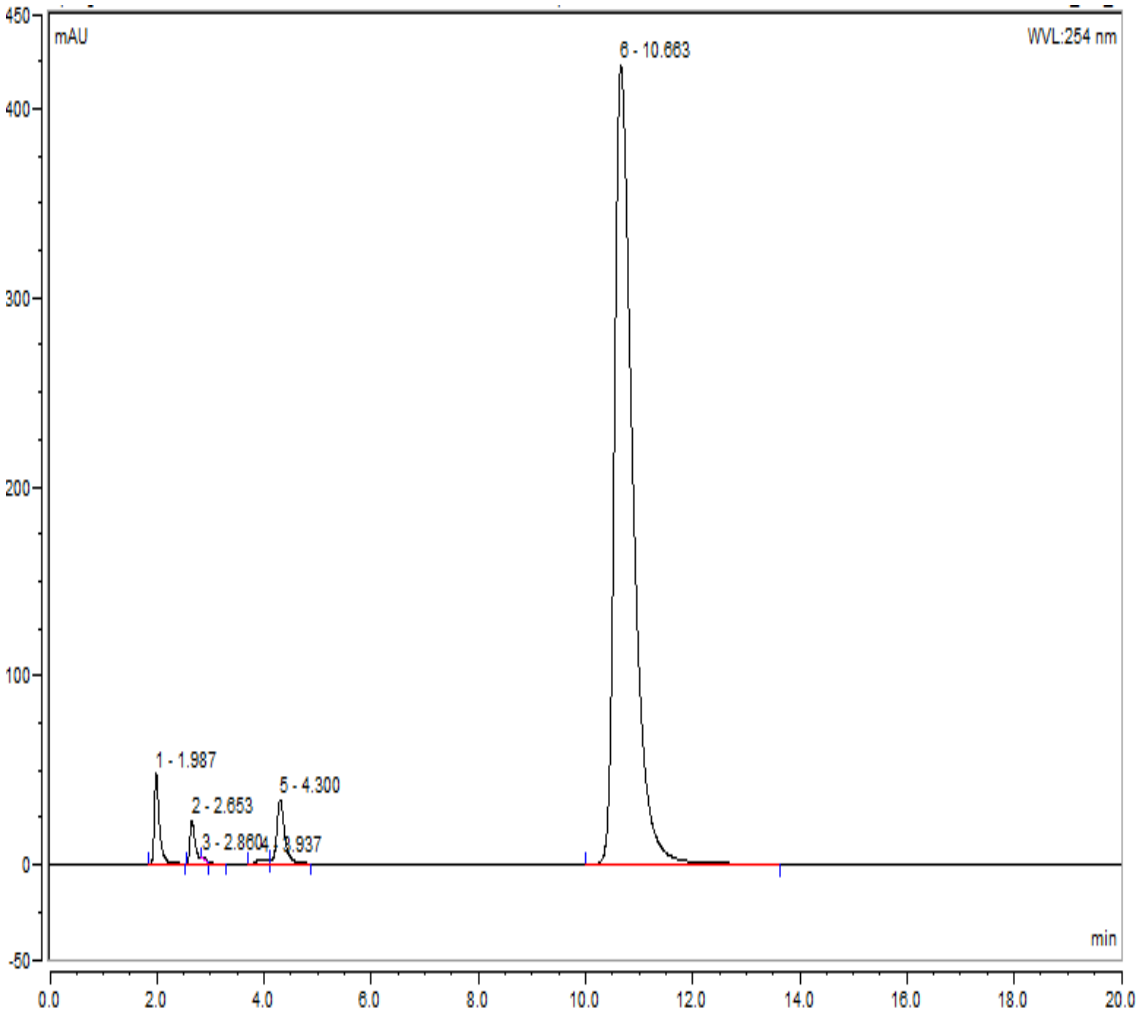


Figure 16a. HPLC analysis for 70% saturation supernatant after 12 hours following enzyme addition. The reaction mixture consisted of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 plus 20 µL of 70% sample. The chromatogram shows multiple peaks; adenosine (10.66 min), adenine (4.30 min), and hypoxanthine (2.65 min). The peak at 1.98 min is unidentified.

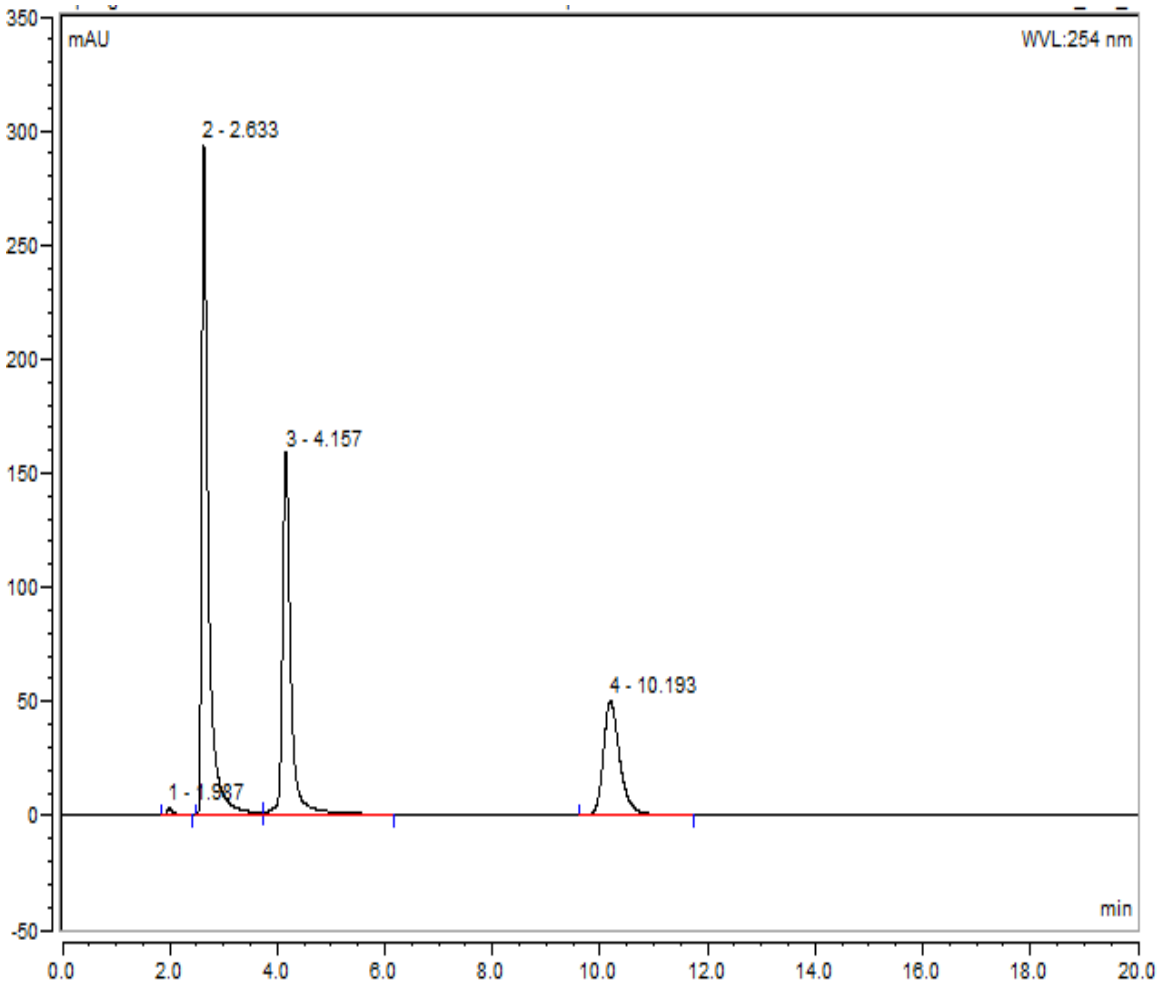


Figure 16b. HPLC analysis for 70% saturation for resuspended pellet after 16 hours following enzyme addition. The reaction mixture consisted of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 plus 20 µL of 70% resuspended pellet. The chromatogram shows multiple peaks; adenosine (10.19 min), adenine (4.15 min), and hypoxanthine (2.63 min).

Table 5. Summary table for the ammonium sulfate fractionations of uridine nucleosidase from Alaska pea seeds.

	Total Volume (mL)	Total protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg)	Purification Fold	% Recovery
Initial Supernatant	35	245	1.7	6.9×10^{-3}	1	100
Initial Pellet	5	18	0.02	1.3×10^{-3}	1	100
20% Supernatant	35	231.6	1.74	7.5×10^{-3}	1.10	102.3
20% Pellet	5	12	0.02	1.8×10^{-3}	1.40	88.1
30% Supernatant	34	221	1.68	7.6×10^{-3}	1.10	99.0
30% Pellet	5	14.5	0.02	1.4×10^{-3}	1.11	84.3

Table 5 (cont.).

40% Supernatant	34	202	1.59	7.8×10^{-3}	1.13	93.5
40% Pellet	5	13	0.01	1.4×10^{-3}	1.12	78.1
50% Supernatant	33	185	1.50	8.1×10^{-3}	1.17	88.2
50% Pellet	5	12	0.01	1.5×10^{-3}	1.20	76.0
60% Supernatant	32	166	1.37	8.2×10^{-3}	2.00	80.6
60% Pellet	5	9	0.01	2.0×10^{-3}	1.54	72.0
70% Supernatant	32	140	0.75	5.3×10^{-3}	0.77	44.1
70% pellet	5	2	0.01	7.3×10^{-3}	5.60	68.1

Table 6. Summary table for ammonium sulfate fractions of inosine nucleosidase from Alaska pea seeds.

	Total Volume (mL)	Total protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg)	Purification Fold	% Recovery
Initial Supernatant	35	245	1.82	$7.4x10^{-3}$	1	100
Initial Pellet	5	18	0.05	$2.7x10^{-3}$	1	100
20% Supernatant	35	231	1.75	$7.5x10^{-3}$	1.02	96.1
20% Pellet	5	16	0.03	$2.4x10^{-3}$	0.90	78.0
30% Supernatant	34	221	1.72	$7.7x10^{-3}$	1.05	94.5
30% Pellet	5	14	0.03	$2.6x10^{-3}$	0.97	76.0

Table 6 (cont.).

40% Supernatant	34	202	1.61	$8.0x10^{-3}$	1.08	88.4
40% Pellet	5	13	0.03	$2.6x10^{-3}$	0.96	70.0
50% Supernatant	33	185	1.49	$8.1x10^{-3}$	1.09	82.1
50% Pellet	5	12	0.03	$2.6x10^{-3}$	0.09	64.2
60% Supernatant	32	166	1.41	$8.4x10^{-3}$	1.13	77.4
60% Pellet	5	9	0.03	$3.0x10^{-3}$	1.1	60.3
70% Supernatant	32	140	1.00	$7.1x10^{-3}$	1.00	55.0
70% pellet	5	2	0.02	$1.1x10^{-2}$	4.37	54.0

Table 7. Summary table for the ammonium sulfate fractionations of uridine nucleosidase from Alaska pea seeds.

	Total Volume (mL)	Total protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg)	Purification Fold	% Recovery
Initial Supernatant	35	245	2.38	$9.3x10^{-3}$	1	100
Initial Pellet	5	18	0.08	$4.0x10^{-3}$	1	100
20% Supernatant	35	231	1.86	$8.1x10^{-3}$	0.86	78.0
20% Pellet	5	16	0.04	$2.8x10^{-3}$	0.70	58.1
30% Supernatant	34	221	1.79	$8.0x10^{-3}$	0.86	75.2
30% Pellet	5	14	0.04	$3.0x10^{-3}$	0.75	55.0

Table 7 (cont.).

40% Supernatant	34	202	1.67	$8.2.x10^{-3}$	0.88	70.2
40% Pellet	5	13	0.04	$3.1x10^{-3}$	0.77	52.5
50% Supernatant	33	185	1.54	$8.3x10^{-3}$	0.89	65.0
50% Pellet	5	12	0.03	$3.2x10^{-3}$	0.81	49.1
60% Supernatant	32	166	1.28	$7.7x10^{-3}$	0.82	54.2
60% Pellet	5	11	0.03	$3.4x10^{-3}$	0.85	47.5
70% Supernatant	32	140	1.03	$7.3x10^{-3}$	0.78	43.2
70% pellet	5	2	0.02	$9.1x10^{-3}$	1.25	47.7

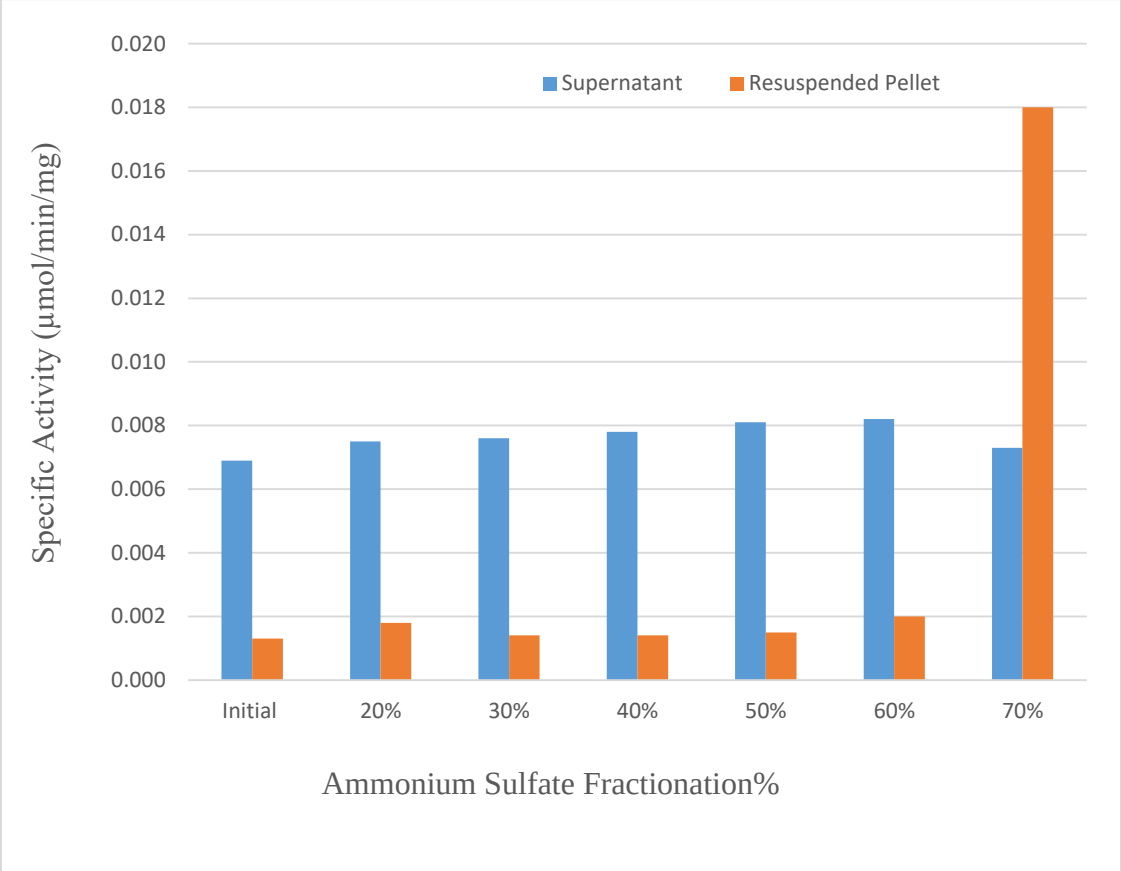


Figure 17. A comparison of the specific activity (µmol/min/mg) for adenosine nucleosidase for both supernatant and resuspended pellet at different percentage of ammonium sulfate fractionation.

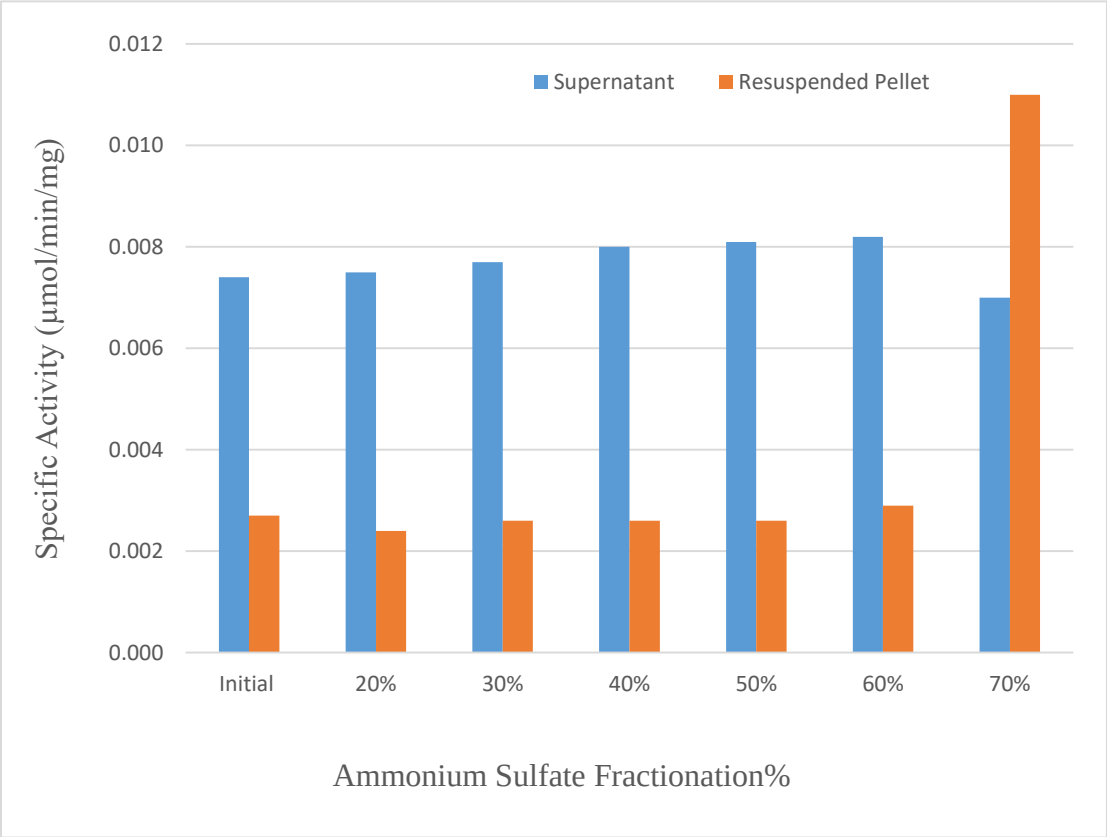


Figure 18. A comparison of the specific activity (µmol/min/mg) for inosine nucleosidase for both supernatant and resuspended pellet at different percentage of ammonium sulfate fractionation.

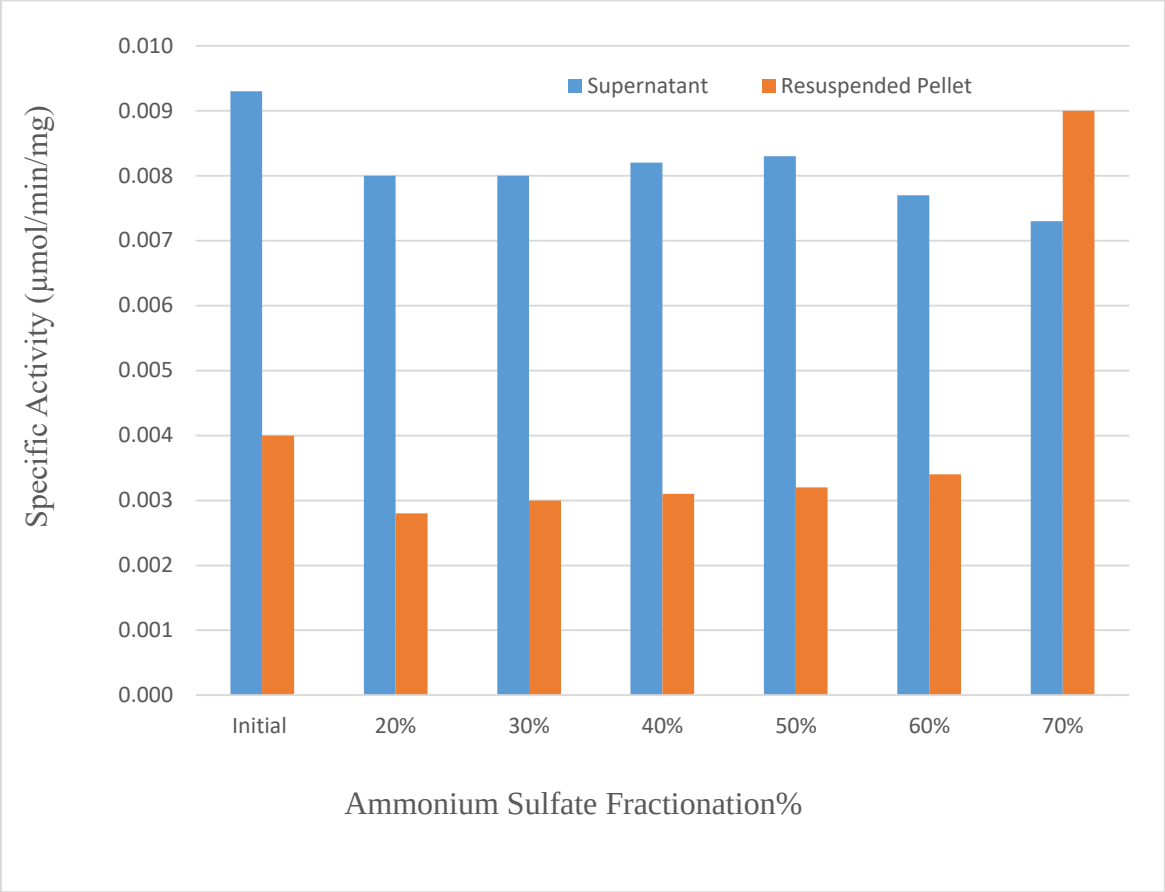


Figure 19. A comparison of the specific activity (µmol/min/mg) for uridine nucleosidase for both supernatant and resuspended pellet at different percentage of ammonium sulfate fractionation.

After dialyzing the resuspended 70% pellet against 1 L of 10 mM Tris buffer pH 7.2, the sample was loaded onto an ion exchange DEAE column. Ion-exchange chromatography is an essential technique in most protein purifications. This type of chromatography separates molecules based on the differences in their charge. The separation principle depends on the reversible interaction between charged medium in the chromatography and the oppositely charged molecule. The ability of molecules, when loaded onto to the column, to bind to the chromatography medium is dependent on the conditions selected *(38).* Cellulose and Sephadex are examples of support materials for most ion exchange columns *(39).* In DEAE chromatography, a low concentration buffer is used to remove protein that did not bind to the column. The bound proteins are eluted by increasing the ionic strength of salt in the buffer *(40).* The protein can be eluted by either using a linear gradient or a stepwise gradient in which the ionic strength of the salt is increased *(38).*

The first 5 mL of sample after dialysis of the 70% resuspended pellet was loaded onto the column. A stepwise gradient of 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, and 450 mM sodium chloride (NaCl) in 10 mM Tris pH 7.2 was used to elute the desired protein. The elution profile of the DEAE column (Figure 20) shows multiple protein peaks detected by UV spectrophotometer. The DEAE resin has a positive charge and the bound protein eluted during the 150 mM step. This indicates the protein has a negative overall charge which interacts with the positive resin in DEAE column.

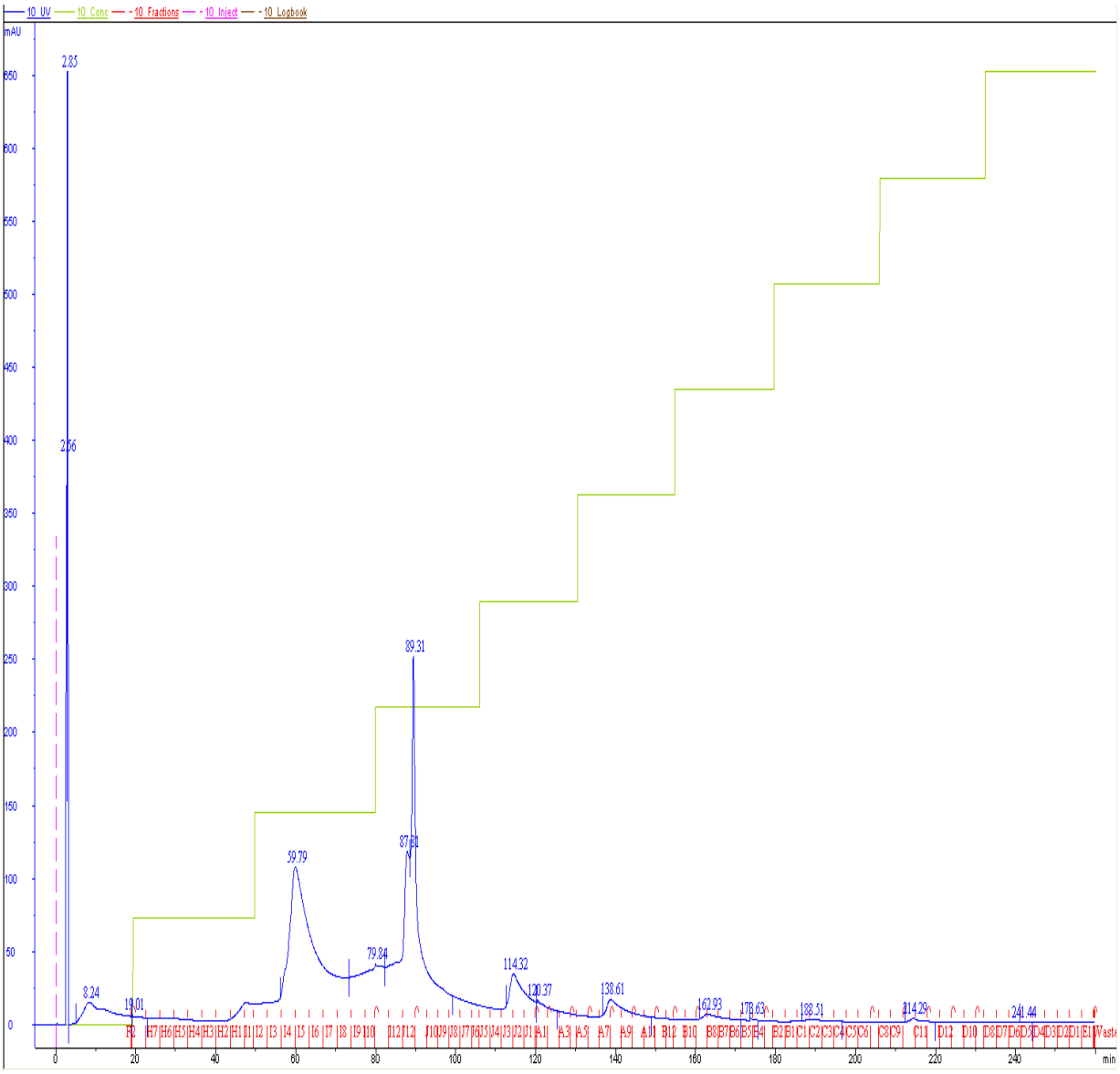


Figure 20. Elution profile for first 5 mL out of 10 mL of total sample from DEAE ion exchange column. The fraction size was 5 mL. Flow rate was 2 mL/min and 0.80 MPa pressure limit. Buffer A was 10 mM Tris pH 7.2. Buffer B was 50 mM to 450 mM NaCl in 10 mM Tris pH 7.2. Twenty column volumes were run with a stepwise gradient of 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, and 450 mM of buffer B. The stepwise gradient started at fraction 13.

The 5 mL fractions from DEAE column were assayed for activity using the reducing sugar assay and adenosine as the substrate. The greater the absorbance at 450 nm, the more ribose produced and hence the greater the activity. The absorbances at 450 nm and 280 nm for each fraction were plotted against the fraction number (Figure 21). The fractions with highest ribose amount (Fractions 21 and 22) were pooled and labeled as pool #1. The fractions with the highest protein amount coincided with the highest nucleosidase activity. The pooled fractions were concentrated to 2 mL and analyzed for adenosine activity by HPLC (Figure 22). After this step, the specific activity increased to 1.8 $x10^{-2}$ µmol/min/mg. Also this step gave 2.6-fold and with a recovery of 6%.

The second 5 mL of the dialyzed sample was loaded onto Mono Q column. It is a strong anion exchanger column, prepacked with Q Sepharose Fast Flow and has a positively charged group in the stationary phase *(41).* A stepwise gradient was used of increasing concentration of NaCl from 50 mM to 450 mM in 10 mM Tris pH 7.2. The elution profile for the Mono Q ion exchange column is shown in Figure 23. The profile resulting from the column shows multiple protein peaks detected by UV. Protein eluted at 50 mM, 100 mM, and 150 mM in the gradient. After assaying the fractions by reducing sugar assay (Figure 24), Fraction 20 was the peak that contained most of the activity. The fraction was labeled as pool #2.

The fraction was assayed for adenosine activity using HPLC (Figure 25). Pool #2 was concentrated to 2 mL before carrying out the HPLC analysis. The specific activity after this step was 2.9 $x10^{-2}$ µmol/min/mg. This step gave a 4.2 purification fold with a recovery of 1.7%.

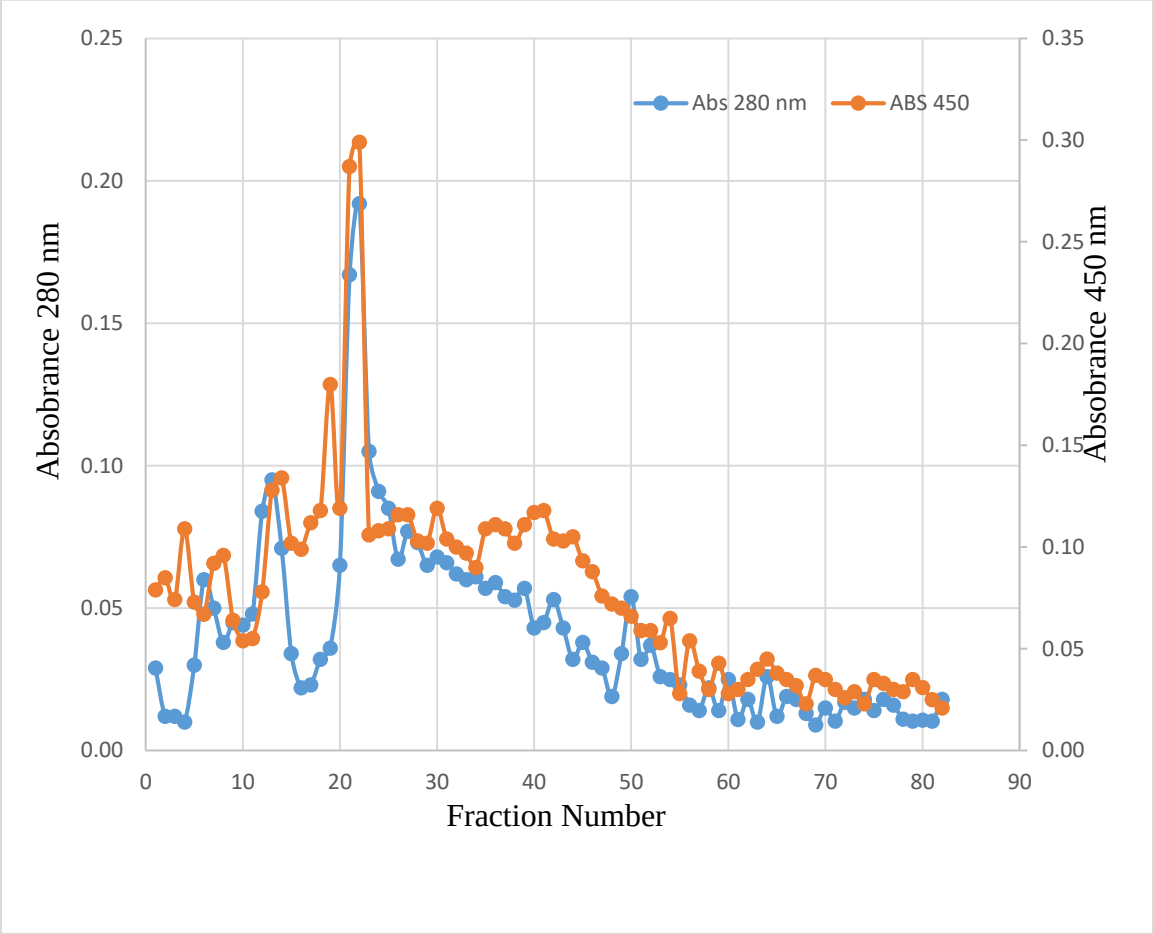


Figure 21. Reducing sugar assay at 450 nm and absorbance at 280 nm vs fraction number to test activity in DEAE fractions. Reaction mixture contained 1 mM adenosine plus 100 µL of corresponding fraction from DEAE column.

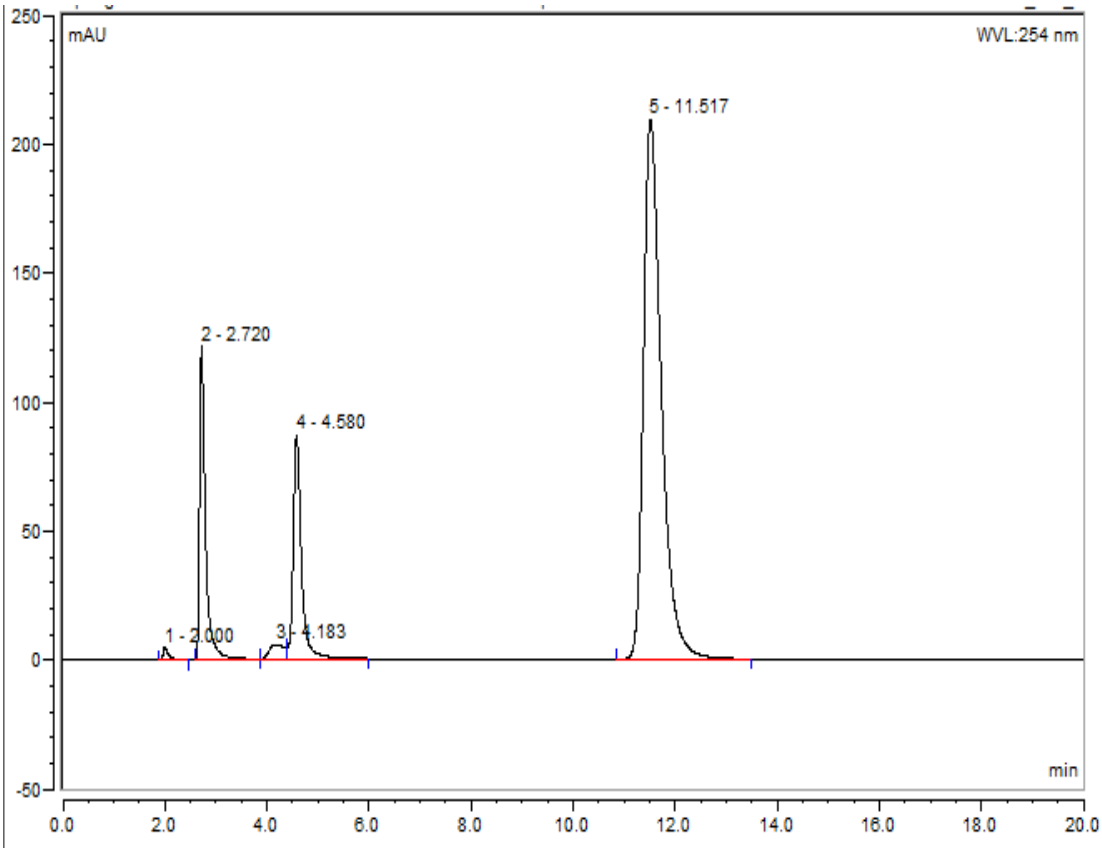


Figure 22. HPLC analysis for activity from pool #1 after DEAE ion-exchange column. The reaction mixture was composed of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 plus 100 µL of pool #1. After 7 hours following enzyme addition, the chromatogram shows adenosine (11.51 min), adenine (4.58 min), and hypoxanthine (2.72 min).

Shamsuddin *(27)* determined the specific activity for adenosine nucleosidase was 2.8 $x10^{-2}$ µmol/min/mg from Alaska pea seed after a hydroxyapatite column. When comparing the specific activity after the Mono Q column (2.9 $x10^{-2}$ µmol/min/mg) with specific activity by Shamsuddin, the results are similar. This confirms the presence of adenosine nucleosidase in Alaska pea seeds. A summary of the purification steps of adenosine nucleosidase are shown in Table 8. The elution profiles for the DEAE and Mono Q ion exchanges columns were very similar. The active protein eluted at 150 mM NaCl on both columns. The purification fold from the Mono-Q column was slightly higher than the DEAE column, while the percent recovery was slightly lower.

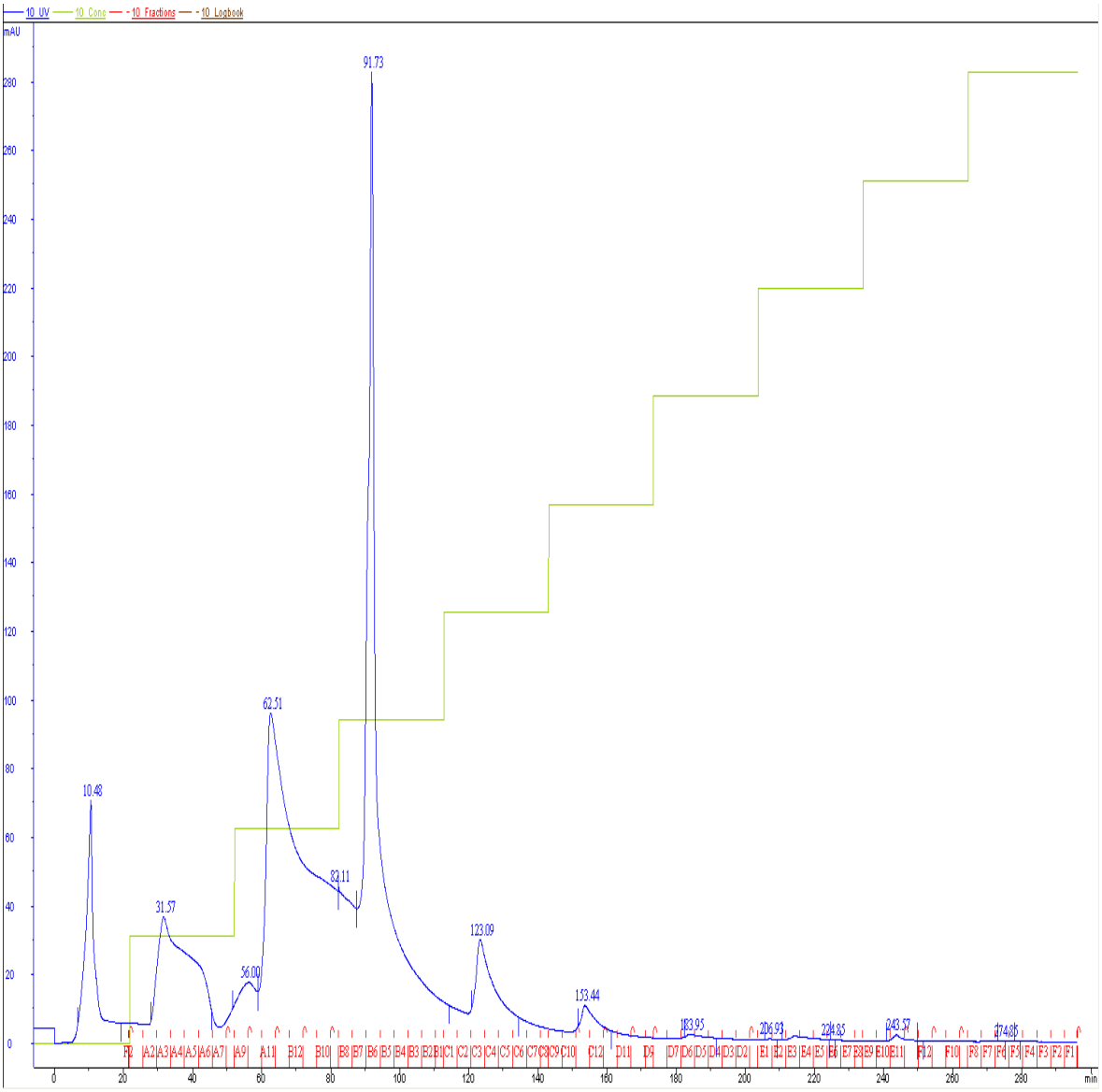


Figure 23. Elution profile for second 5 mL out of 10 mL of total sample from Mono Q- ion exchange column. The fraction size was 5 mL. Flow rate of 3 mL/min and 0.50 MPa pressure limit. Buffer A was 10 mM Tris (low salt). Twenty column volumes were run with a stepwise gradient of 50 mM, 100 mM, 150 mM, 200 mM, 250 mM, 300 mM, 350 mM, 400 mM, and 450 mM of buffer B. The gradient started at Fraction 4.

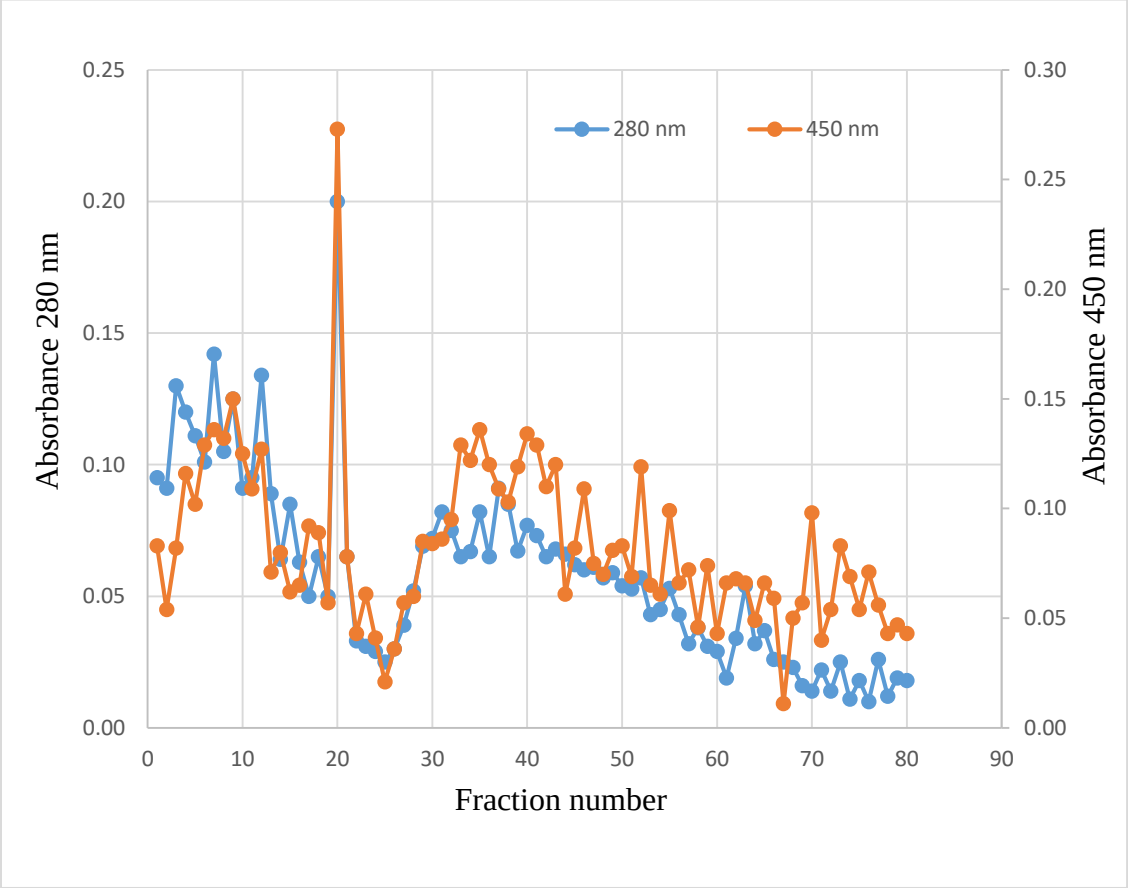


Figure 24. Reducing sugar assay at 450 nm and absorbance at 280 nm vs fraction number to test activity in Mono Q fractions. Reaction mixture contained 1 mM adenosine plus 100 µL of each enzyme from each fraction from Mono Q column.

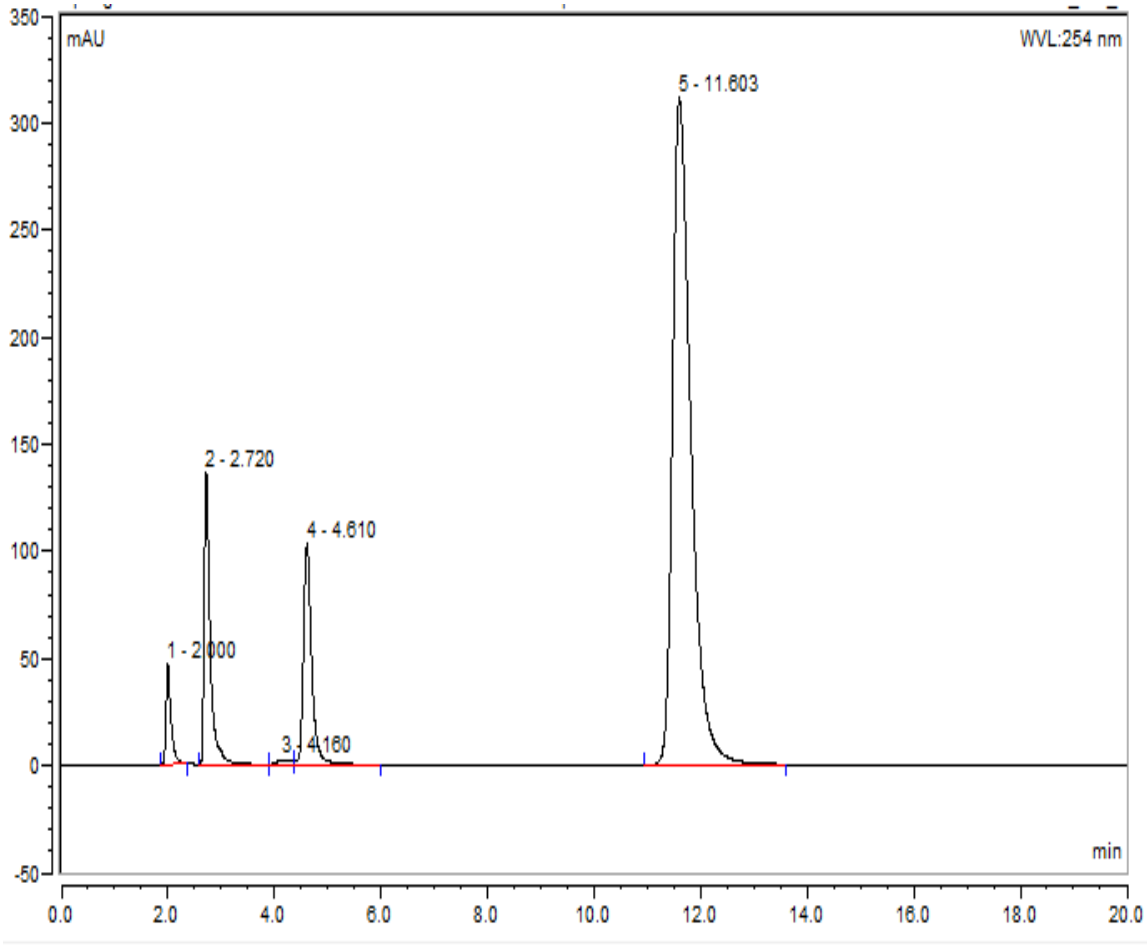


Figure 25. HPLC analysis for the activity from pool #2 after Mono Q ion-exchange column. The reaction mixture composed of 1 mL of 1 mM adenosine in 10 mM Tris pH 7.2 plus 100 µL of pool #2. After 7 hours, the chromatogram shows adenosine (11.60 min), adenine (4.61 min), and hypoxanthine (2.72 min). The peak at 2.00 min was unidentified.

Table 8. Summary table for the purification of adenosine nucleosidase from Alaska pea seeds.

	Total Volume (mL)	Total protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min/mg)	Purification Fold	% Recovery
DEAE Column	2	5.44	0.098	1.8×10^{-2}	2.6	6
Mono-Q Column	2	0.97	0.029	2.9×10^{-2}	4.20	1.70

Molecular Weight of Adenosine Nucleosidase from Alaska Pea Seeds

The subunit molecular weight of adenosine nucleosidase and the purity of the enzyme were both determined by SDS-PAGE. A standard calibration curve was constructed using Precision Plus Protein™ Unstained Standards (Figure 26). The subunit molecular weights for pool #1 from DEAE column and pool #2 from Mono Q column were approximately 26,000 daltons. Comparing the two bands presented in the gel (Figure 27), it appears that pool #1 is not pure due to the presence of additional faint bands, while pool #2 appears to be pure. The molecular weight for pool #1 was determined 25,990 daltons and pool #2 26,000 daltons. The molecular weight subunit of the purified enzyme is similar to adenosine nucleosidase molecular weight (26,103 daltons) that was purified by Shamsuddin from Alaska pea seeds *(27)*.

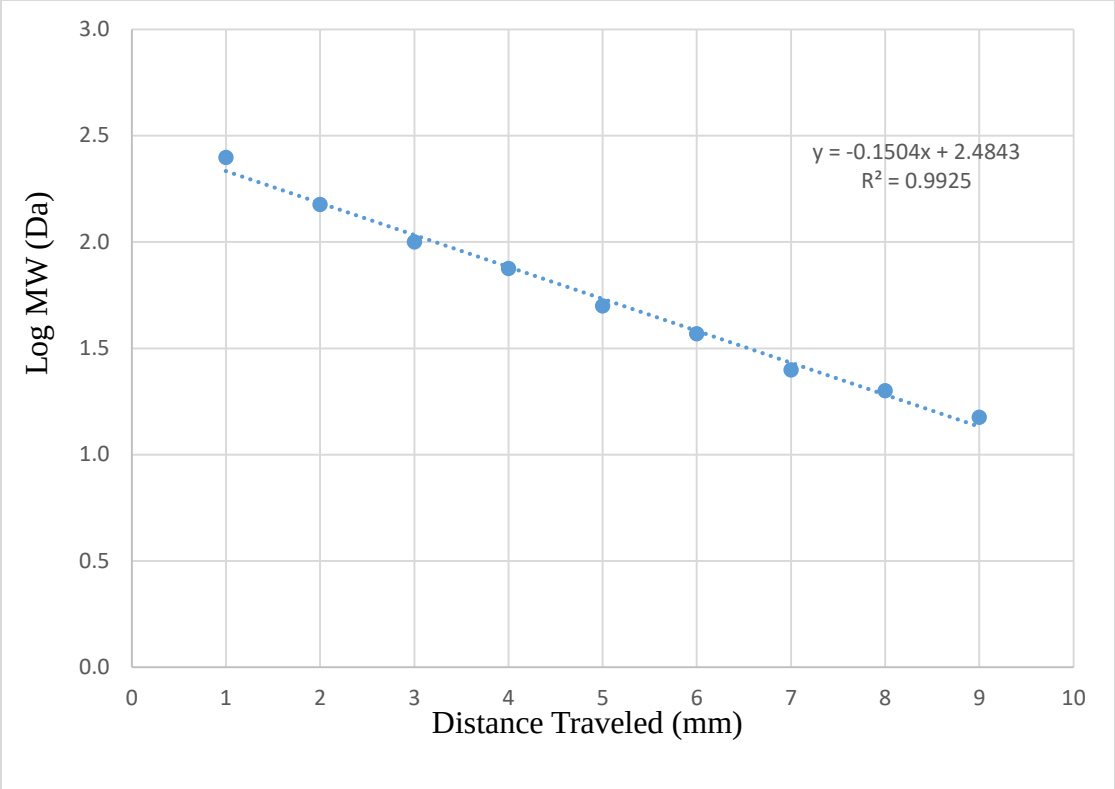


Figure 26. A standard SDS-PAGE calibration curve to determine the molecular weight based on Precision Plus Protein™ unstained standards. The band from pool #1 traveled 6.80 mm and the band from pool #2 traveled 6.20 mm. The subunit molecular weight for adenosine nucleosidase from both pool#1 and pool #2 were 25,990 daltons, 26,000 daltons (26 kDa) respectively.

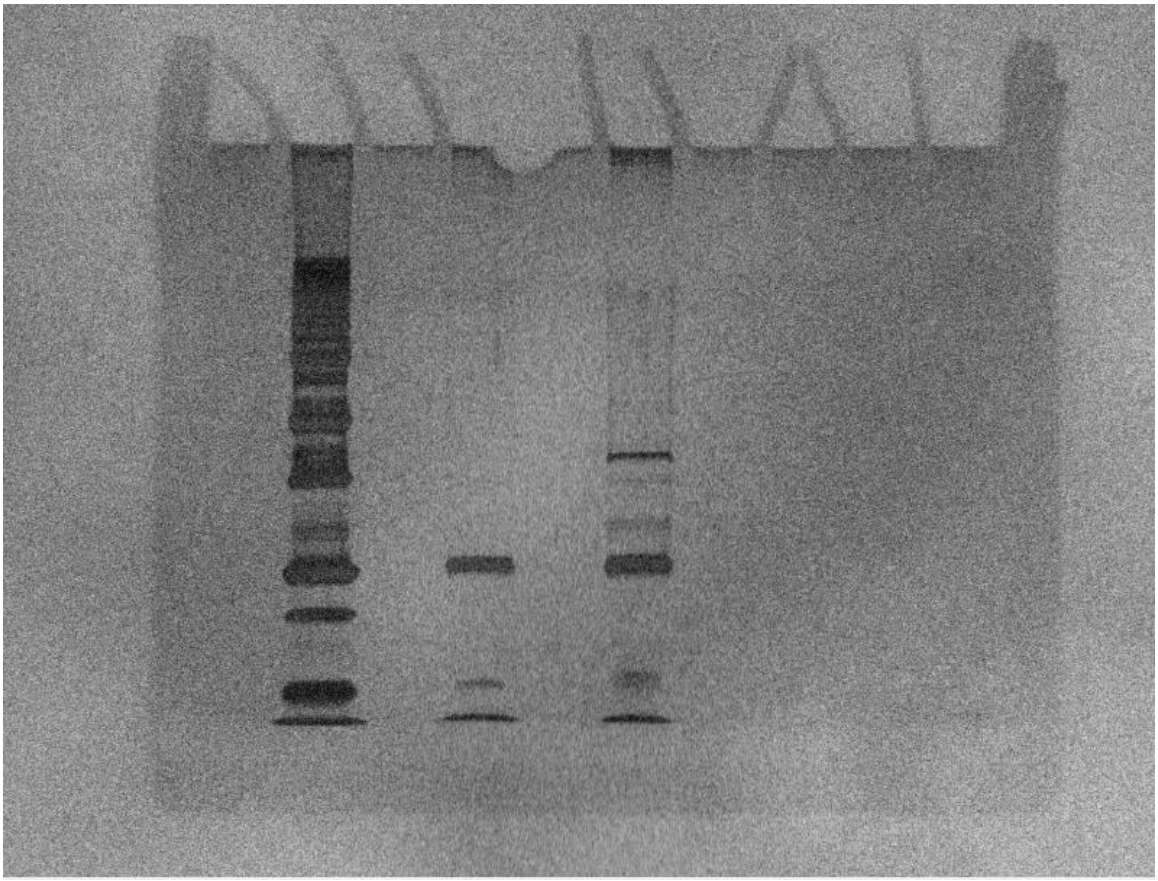


Figure 27. Determination of the subunit molecular weight of adenosine nucleosidase from pool #1 and pool #2 from DEAE and Mono Q columns using denaturing and reducing SDS-PAGE. Lane 1 represents Precision Plus Protein™ unstained markers. Lane 2 is adenosine nucleosidase from pool #2 from Mono Q column. Lanes 3 is adenosine nucleosidase pool #1 from DEAE column.

CHAPTER IV
CONCLUSION

Nucleoside hydrolases (NHs) are enzymes found widely in nature. This enzyme hydrolyzes the N-glycosidic bond found in both purine and pyrimidine nucleosides to form the corresponding base and ribose. Few studies are available on the structure of nucleoside hydrolase from different sources of plants compared to the extensive studies carried out on the enzymes isolated from various parasitic protozoans. Adenosine nucleosidase is an essential hydrolase enzyme catalyzing the hydrolysis of adenosine nucleoside to adenine and ribose. Adenosine nucleosidase is involved in the purine metabolism pathway. This enzyme plays a key role in the important salvage purine and pyrimidine pathways in plants. It allows for the reuse of the bases without consuming energy required to synthesize the base *de novo* in plant. Adenosine nucleosidase is vital in cell differentiation, controlling the level of cytokinins, cell division, and development in higher plants. Despite the importance of purine and pyrimidine nucleotides, only a few studies have been published on purine and pyrimidine metabolism in higher plants compared to small living organisms and animals.

The presence of multiple enzyme activities was determined at different germination times of Alaska pea seeds. Three different nucleosidase activities were observed, adenosine nucleosidase, inosine nucleosidase, and uridine nucleosidase. Uridine nucleosidase had highest activity compared to the two purine nucleosidases.

Adenosine nucleosidase was purified from Alaska pea seeds. The activity of this enzyme peaked after five days of germination. Ammonium sulfate precipitation

was used from 20%- 70% fractionations. The activity of the enzyme was split after 70% cut. A purification fold of 4.2 for adenosine nucleosidase has been achieved with 1.7% recovery. The specific activity of the enzyme was measured to be 2.9 $x10^{-2}$ µmol/min/mg. Using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the molecular weight subunit was determined to be approximately 26,000 daltons (26 kDa).

The purification of adenosine nucleosidase was highly improved by using a targeted ammonium sulfate fractionation. Earlier studies of purifying adenosine nucleosidase from Alaska pea seeds were able to purify the enzyme with two steps ammonium sulfate precipitation and after multiple columns including an ion exchange column, hydroxyapatite column, and size exclusion column. However, with the current purification scheme, the enzyme was purified after a single column utilizing Mono Q column.

REFERENCES

1. Moffatt, B.; Ashihara, H. Purine and pyrimidine nucleotide synthesis and metabolism. *The Arabidopsis Book / American Society of Plant Biologists.* **2002**, 1, e0018.

2. Senecoff, J. F.; McKinney, E. C.; Meagher, R. B. De Novo Purine Synthesis in Arabidopsis Thaliana. II. The PUR7 Gene Encoding 5'-phosphoribosyl-4-(Nsuccinocarboxamide)-5-aminoimidazole Synthetase Is Expressed in Rapidly Dividing Tissues. *Plant Physiology* **1996**, 112, 905-917.

3. O'Hara, F. The Baran Laboratory. http://www.scripps.edu/baran/images/grpmtgpdf/OHara_Jun_12.pdf (accessed July 2016).

4. Zrenner, R.; Mark, S.; Sonnewald, U.; Boldt, R. Pyrimidine and purine biosynthesis and degradation in plants. *The Annual Review of Plant Biology* **2006**, 57, 805-836.

5. Ogorodnik, K. V. Amino acid sequence of nucleoside hydrolases from plants. Thesis, Middle Tennessee State University, Murfreesboro, TN, **2014**.

6. Versees, W.; Steyaert, J. Catalysis by nucleoside hydrolases. *Current Opinion in Structural Biology*. **2003**, 13, 731-738.

7. Stasolla, C.; Katahira, R., Thorpe, T. A.; Ashihara, H. Purine and pyrimidine nucleotide metabolism in higher plants. *Journal of Plant Physiology*. **2003**, 160, 1271-1295.

8. Miller, G., & Evans, H. Nucleosidase from higher plants. *Plant Physiology*. **1955**, 30-37.

9. Petersen, C.; Møller, L. B. The RihA, RihB, and RihC Ribonucleoside Hydrolases of Escherichia coli. Substrate Specificity, Gene Expression, and Regulation. *The Journal of Biological Chemistry*. **2001**, 276, 884-894.

10. Kurtz, J.; Exinge, F.; Erbs, F.; Jund, R. The URH1 Uridine Ribohydrolase of Saccharomyces Cerevisiae. *Current Genetics*, **2002**, 41, 132-141.

11. Cui, L.; Rajasekariah, G.; Martin, S. K. A Nonspecific Nucleoside Hydrolase from Leishmania Donovani: Implications for Purine Salvage by the Parasite. *Gene.* **2001**, 280, 153-162.

12. Ribeiro, J. M.; Valenzuela, J. G. The Salivary Purine Nucleosidase of the Mosquito, Aedes Aegypti. *Insect Biochemistry and Molecular Biology***. 2003**, 33, 13-22.

13. Versées, W.; Holsbeke, E.; De Vos, S.; Decanniere, K.; Zegers, I.; Steyaert, J. Cloning, Preliminary Characterization, and Crystallization of Nucleoside Hydrolases from Caenorhabditis Elegans and Campylobacter Jejuni. *Acta Crystallographica Section D Biological Crystallography*. **2003**, 59, 1087-1089.

14. Riegler, H.; Geserick, C.; Zrenner, R. Arabidopsis thaliana nucleosidase mutants provide new insights into nucleoside degradation. *New Phytologist.* **2011**, 191, 349-359.

15. Degano, M.; Gopaul, D. N.; Scapin, G.; Schramm, V. L.; Sacchettini, J. C. Threedimensional Structure of the Inosine-uridine Nucleoside N-ribohydrolase from *Crithidia Fasciculata*. *Biochemistry.* **1996**, 35, 5971-5981.

16. Guranowski, A.; Barankiewicz, A. Purine salvage in cotyledons of germinating lupin seeds. *FEBS Letters.* **1979**, 104, 95-98.

17. Abusamhadneh, E.. Isolation and characterization of adenosine nucleosidase from yellow lupin seeds. Thesis, Middle Tennessee State University, Murfreesboro, TN, **1997**.

18. Liu, X. Characterization of Adenosine Nucleosidase from Yellow Lupin Seeds. Thesis, Middle Tennessee State University, Murfreesboro, TN, **1998**.

19. Guranowski, A.; Schneider, Z. Purification and Characterization of Adenosine Nucleosidase from Barley Leaves. *Biochimica et Biophysica Acta.* **1977**, 482, 145-158.

20. Ashihara, H.; Wakahara, S.; Suzuki, M.; Kato, A.; Sasmoto, H.; Baba, S. Comparison of adenosine metabolism in leaves of several mangrove plants and poplar species. *Plant Physiol. Biochem.* **2003**, 41, 133-139.

21. Poulton, J. E.; Butt, V. S. Partial Purification and Properties of Adenosine Nucleosidase from Leaves of Spinach Beet (Beta Vulgaris L.). *Planta.* **1979**, 131, 179-185.

22. Imagawa, H.; Yamano, H.; Inoue, K.; Takino, Y. Purification and Properties of Adenosine Nucleosidases from Tea Leaves. *Agricultural and Biological Chemistry.* **1979**, 43, 2337-2342.

23. . Burch, L. R.; Stuchbury, T. Purification and Properties of Adenosine Nucleosidases from Tomato (Lycopersicon Esculentum) Roots and Leaves. *Journal of Plant Physiology*. **1986**, 125, 267-273.

24. Chen, C.; Kristopeit, S. K. Metabolism of Cytokinin: Deribosylation of Cytokinin Ribonucleoside by Adenosine Nucleosidase from Wheat Germ Cells. *Plant Physiology.* **1981**, 68, 1020-023.

25. Floch, F. L.; Lafleuriel, J. The Purine Nucleosidases of Jerusalem Artichoke Shoots. *Phytochemistry.* **1981**, 20, 2127-2129.

26. Altawil, Z. M. Isolation of Nucleoside Metabolizing Enzymes from Alaska Pea Seeds (*Pisum sativum* L. cultivar Alaska). Thesis, Middle Tennessee State University, Murfreesboro, TN, **2013**.

27. Shamsuddin, A. K. Characterization of adenosine nucleoside from Alaska pea seeds. Thesis, Middle Tennessee State University, Murfreesboro, TN, **2015**.

28. Abusamhadneh, E.; McDonald, N.E.; Kline, P.C. Isolation and characterization of adenosine nucleoside from yellow lupin (Lupinus luteus). *Plant Sci*. **2000,** 153, 25-32.

29. Guanowski, A. Purine Catabolism in Plants. Purification and some properties of inosine nucleosidase from yellow lupine seeds. *Plant Physiol*. **1982**. 70, 344-349.

30. Takakura, T.; Ito, T.; Yagi, S.; Notsu, Y.; Itakura, T.; Nakamura, T.; Inagaki, K,; Esaki, N,; Hoffman, R,; Takimoto, A. High-level expression and bulk crystallization of recombinant L-methionine gamma-lyase, an anticancer agent. *Applied Microbiology and Biotechnology.* **2006**, 70, 183-192.

31. Brawerman, G.; Chargaff, E. On a deoxyribonuclease from germinating barley. *The Journal of Biological Chemistry*. **1954**, 210, 445-454.

32. Fiers, W.; Vandendriessche, L. Catabolism of nucleosides by barley extracts *Arch. Intern. Physiol. Biochim*. **1960**, 68, 203-207.

33. Singhabahu, S.; George, J.; Bringloe, D. Expression of a functional human adenosine deaminase in transgenic tobacco plants. *Transgenic Research*, **2013**, 22, 643-649.

34. Laszlo, K. Peroxidase and catalase enzyme activities in pea seeds. *Acta Agronomica Academiae Scientiarum Hungaricae*. **1971**, 20, 85-91.

35. *Protein Precipitation Using Ammonium Sulfate. Current Protocols in Protein Science.* http://dx.doi.org/10.1002/0471140864.psa03fs13 (accessed September 2016).

36. Green, A.; Hughes, L. Protein solubility on the basis of solubility in aqueous solutions of salts and organic solvents. *Methods Enzymol*. **1955**, 1, 67–90.

37. King, T. Separation of proteins by ammonium sulfate gradient solubilization. *Biochemistry*. **1972,** 11**,** 367-371.

38. The Wolfson Center for Applied Structural Biology. http://wolfson.huji.ac.il/purification/PDF/IonExchange/GE_IEXcolumns.pdf (accessed October 2016).

39. Price, N.C; Stevens, L. *Fundamentals of Enzymology*; The cell and molecular biology of catalytic proteins. Oxford University Press Inc., New York, **1999**.

40. Voet, D.; Voet, J.; Pratt, C.W.; Nucleotides, Nucleic Acids, and Genetic Information; Fundamentals of Biochemistry: Life at the Molecular Level; John Wiley & Sons, Inc.: Hoboken, NJ, 2008.

41. *GE Healthcare Life Sciences* http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/products/AlternativeProductStructure_17468/28936543 (accessed October 2016).

42. Negishi, O.; Ozawa, T. Guanosine Deaminase and Guanine Deaminase from Tea Leaves. *Bioscience, Biotechnology, and Biochemistry* **1994**, 58, 1277-1281.

43. Porcelli, M.; Concilio, L.; Peluso, I.; Marabotti, A.; Facchiano, A.; Cacciapuoti. G. Pyrimidine-specific Ribonucleoside Hydrolase from the Archaeon Sulfolobus Solfataricus – Biochemical Characterization and Homology Modeling. *The FEBS Journal* **2008**, 275, 1900-1914.

44. *NetBiochem* http://library.med.utah.edu/NetBiochem/pupyr/pp.htm (accessed October 2016)