Isolation of Nucleoside Metabolizing Enzymes from Alaska Pea Seeds
(*Pisum sativum* L. cultivar Alaska)

by

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ABSTRACT

The activities of number of metabolizing enzymes were determined from dry Alaska Pea Seeds (*Pisum sativum* L. cultivar Alaska). Then tested for presence of three groups of enzymes; nucleosidase, deaminases and phosphorylases. The highest activity of the nucleosidases was cytidine nucleosidase with an activity of 4.82 μmole/min and the lowest activity was 0.97 μmol/min inosine nucleosidase. In addition, deaminase activities in dry Alaska pea seeds were tested using adenine, adenosine, and cytidine as substrates. Deaminase activities of 0.70, 4.37, 4.82 μmole/min respectively were found for those substrates. However, no cytosine deaminase was detected in dry Alaska pea seeds. Phosphorylase activities for adenosine, guanosine, and cytidine were observed in ungerminated seeds with activities 0.71, 0.51, and 0.39 μmole/min respectively. In all cases tested the phosphorylase activities were lower than the corresponding nucleosidases.

Adenosine nucleosidase, an enzyme of the purine metabolic pathway, hydrolyzes adenosine to adenine and pentose sugar. It is involved in controlling the level of cytokinins, cell differentiation, division, and development in plants. Adenosine deaminase, also an enzyme of the purine metabolic pathway, catalyzes adenosine or to inosine.

Adenosine nucleosidase was purified from Alaska pea seeds 5 days after germination. The specific activity of the initial extract was 58×10^{-4} to 56×10^{-3} μmole/min/mg. Dialysis of the initial extract increased the specific activity to 68×10^{-4} μmole/min/mg. The first step of purification was ammonium sulfate precipitation. The

purity of the enzyme was increased 2.4 fold to a specific activity of 14×10^{-3}

µmole/min/mg. The second step, ion-exchange chromatography (DEAE), increased

purity 4-fold with a specific activity to 24×10^{-3} µmole/min/mg. The third purification

step was ω-aminohexyl agarose chromatography, with a 4.2-fold increase to a specific

activity 25×10^{-3} µmole/min/mg. The final step, Sephacryl S100 chromatography,

increased purification-fold to 44 with a specific activity to 26×10^{-2} µmole/min/mg.

Finally, adenosine nucleosidase from Alaska pea has a molecular weight of 36

kDa determined by SDS-PAGE.

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CHAPTER I
INTRODUCTION

Enzymes are proteins, which accelerate (speed up) chemical reactions, and are responsible for metabolism, signal transduction, energy conversions, and cell regulation.   Various cell types express different enzymes that are organized into complex metabolic pathways.[1]   Enzymes consist of chains of amino acids linked by peptide bonds. Many enzymes require a cofactor (nonprotein component; metal ions or organic molecules) for catalytic activity.[2]   Enzymes are classified into six groups, depending on the chemical reaction catalyzed: 1) addition or removal of water (hydrolases); 2) transfer of electrons (oxidoreductases); 3) transfer of a group to another molecule (transferases); 4) splitting or forming C-C bonds (lyases); 5) changing geometry or structure of a molecule (isomerases); 6) joining two molecules through hydrolysis of ATP (ligases).[3]   Enzymes have three important characteristics: catalysis in which reaction rates increase as much as 10^{16} fold, specificity for activity against a single substrate or group of similar substrates, and the ability to be regulated.[2]

Purines and pyrimidines

"Nucleosides are very important molecules in oxidation-reduction reactions, intracellular signaling, energy transfer, and biosynthetic reactions."[4]   Each nucleoside contains a nitrogenous base linked to ribose or deoxyribose. The base is one of two types, a purine or pyrimidine. The major purine bases are adenine (A) and guanine (G), which form a glycosidic bond with the pentose sugar through the N9

atom. A third purine base, hypoxanthine, is also commonly encountered in the metabolism of adenine and guanine. The most common pyrimidine bases are cytosine (C), uracil (U), and thymine (T), which form a glycosidic bond to the pentose sugar via N1 atom (Figure 1). [4] The orientation of the glycosidic bond with respect to the pentose sugar is β. Nucleotides are nucleosides, which contain one or more phosphate groups attached to the pentose sugar (Figure 2). [4]

Nucleotides are an important cellular component in the development and metabolism of plants. [5] The products of purine and pyrimidine metabolism in plants are required for primary and secondary metabolism, and gene expression. The purine and pyrimidine nucleotides are important for information storage by acting as building blocks of DNA.[5] Purine and pyrimidine nucleotides are also precursors for synthesis of vitamins such as riboflavin, thiamine, and folate.[6]

Another example of derivatives of nucleotides important in metabolism is coenzymes, such as nicotinamide adenine dinucleotide (NAD), and flavin adenine dinucleotide (FAD), which are essential for many biological reactions. During photosynthesis the purine nucleotide ATP, the main energy transfer molecule, is synthesized from ADP and a phosphate group. Pyrimidine nucleotides in plants often function as co-substrates. For example, uridine triphosphate (UTP) and uridine diphosphate (UDP) are important in the synthesis and degradation of sucrose.[5]

The pathways for the synthesis and degradation of nucleotides in plants are similar to the pathways in animals. [6] The pathways can be divided into several groups: *de novo* synthesis, nucleotide degradation, salvage pathways, and
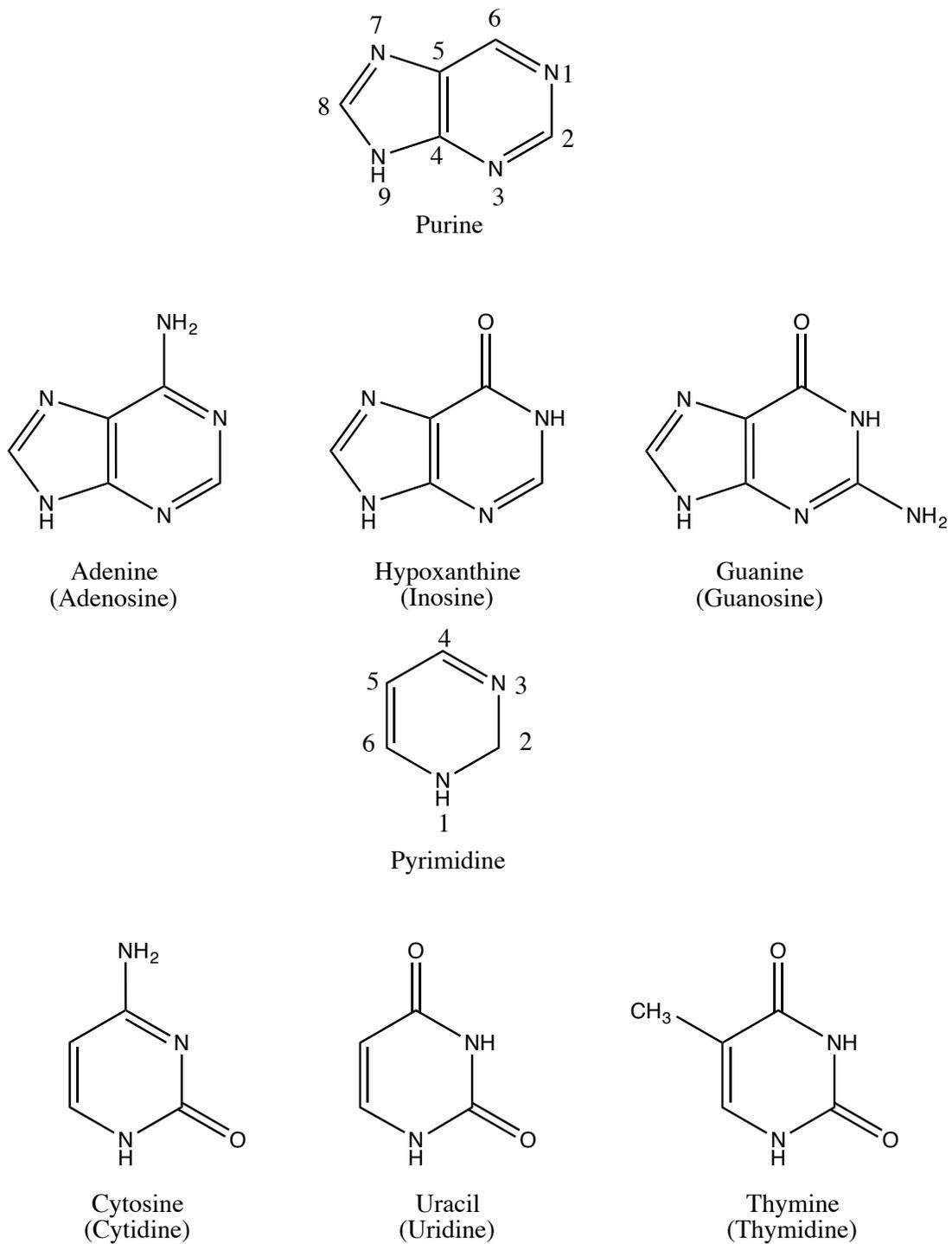
Figure 1. Purine and pyrimdine bases. The corresponding name is in parentheses. The common numbering systems of purines and pyrimdines are also shown.

Base

Phosphate group

NH_2

N

N

N

N

O

O

P

$^-$O

O

$^-$O$^-$

H

H

H

H

OH

OH

Ribose ring

Nucleotide monophosphate

Base

NH_2

N

N

N

N

HO

O

H

H

H

H

OH

OH

Ribose ring
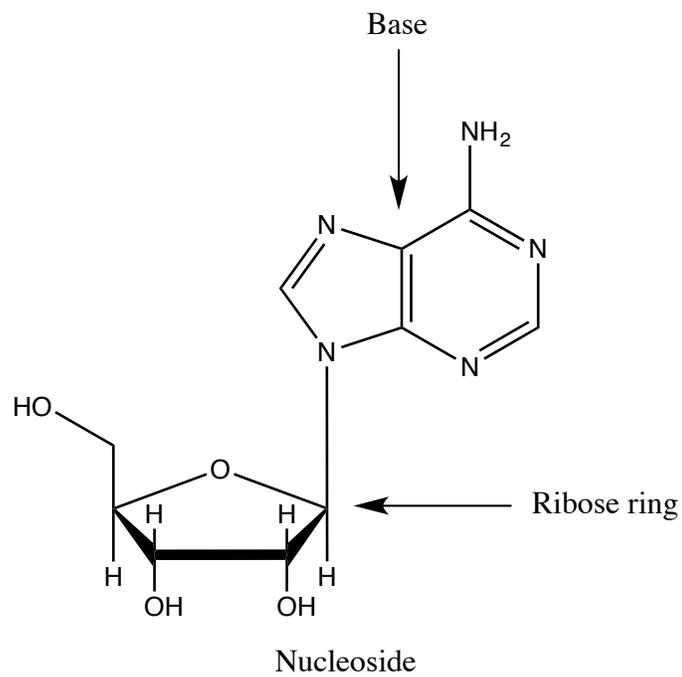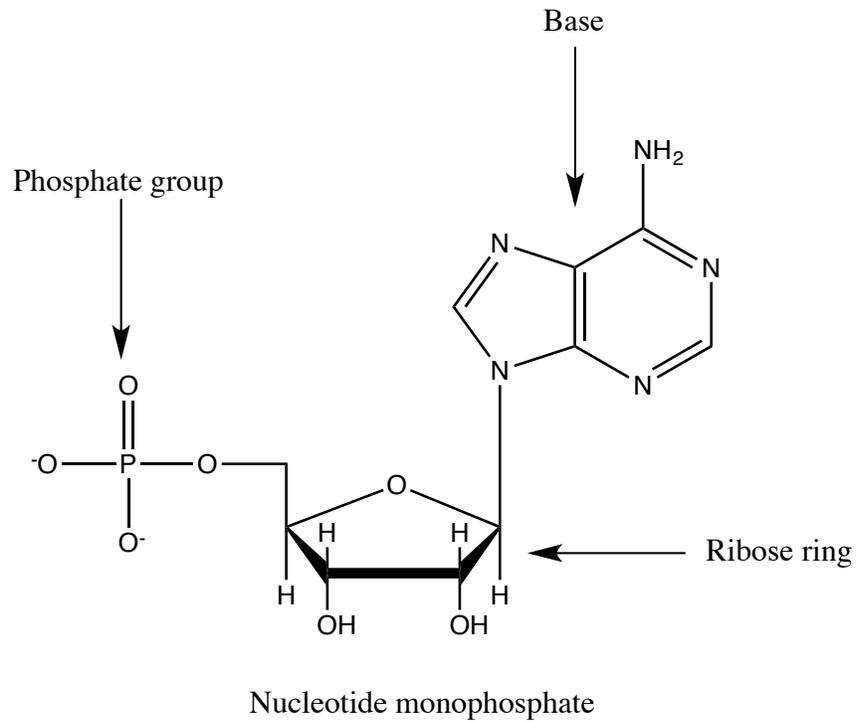
Nucleoside

Figure 2. Structure of nucleotide monophosphate and a nucleoside. The nucleotide monophosphate shown is adenonsine monophosphate (AMP) and the nucleoside shown is adenosine.

phosphotransfer reactions, all of which are interconnected. Specific nucleotides are converted to deoxynucleotides for DNA. The addition of side chains form unusual nucleotides of transfer RNA, and are precursors for synthesis of secondary metabolism and hormones.[5]

Purine and Pyrimidine Metabolism in Higher Plants

The *de novo* biosynthetic pathway is present as a multi-enzyme complex in animals, while this complex is not observed in higher plants. The *de novo* pathway for purines leads to synthesis of AMP and GMP, beginning with the transfer of the amido group from glutamine to 5-phosphoribosyl-1-pyrophosphate (PRPP) catalyzed by PRPP synthase to yield phosphoribosyl-β-amine (Figure 3. Reaction 24). PRPP is also used in the salvage pathway of purine and pyrimidine nucleotides. Almost all organisms contain at least one gene encoding PRPP synthase (PRS; EC 2.7.6.1). Four genes encoding PRPP synthase have been isolated from *Arabidopsis*: X8376 (PRS1), X92974 (PRS2), AJ012406 (PRS3), and AJ012407 (PRS4). [6] In animals and plants, amido phosphoribosyltransferase (ATase) or PRPP amidotransferase (PRAT) catalyzes the next step in the *de novo* pathway of purine synthesis, converting phosphoribosyl- β-amine to glycinamide ribonucleotide (GAR) (Reaction 25). These enzymes are regulated by purine ribonucleotides. Amido phosphoribosyltransferase is very sensitive to feedback regulation in plants.[6] (Figure 3) In the next three reactions GAR is converted to formylglycinamide ribonucleotide (FGAR), formylglycinamidine ribonucleotide (FGAM), and 5-aminoimidazole ribonucleotide

Purine metabolism

"De novo" synthesis
Plastidic/mitochondrial

Salvage cytosolic

Cytokinins

PRPP
Gln 24
Glu PPi

PRA
ATP Gly 25
ADP Pi

GAR
10F-THF 26
THF

FGAR
ATP Gln 27
ADP Glu Pi

FGAM
ATP 28
ADP Pi

AIR
HCO₃⁻ 29

CAIR
ATP Asp 30
ADP Pi

SAICAR
Fumarate 31

AICAR
10F-THF 32
THF

FAICAR
H₂O

IMP
GTP, Asp   GDP, Pi 33

35

ATP 34
2 ADP 34
AMP
Fumarate 31
SAMP

Isopentenyl-adenosin-5P

Ade
Ri-1-P Ribose 41
40 Pi H₂O
Ado
ATP NMP 43
42 ADP NR
AMP
44 PRPP PP_i Isopentenyl-PP
36

IMP 37 XMP GMP
46 ADP NR NAD NADH 52 46 ADP NR PPi
ATP NMP 43 ATP NMP 43 47
Inosine Xanthosine 39 Guanosine PRPP
47 45 Pi 53 45 H₂O 53 45 Pi 53
PRPP Ri-1-P Ribose Ri-1-P
Hypoxanthine 53 Guanine
54 Xanthine 55
54
Uric acid
56
Allantoin
57
CO₂ + NH₃ Urea CO₂ + NH₃
61 58 59
Ureidoglycine Allantoate
62 NH₃ 60 Ureidoglycolate Urea
Ureidoglycolate Urea Glyoxylate
63 2 NH₃ + CO₂
Glyoxylate

Alkaloids
Theobromine
Caffeine
38 AMP,Glu,Pi
ATP,Gln
48

Nucleoside
49

Base
50

51

48

Catabolism
Cytosolic/peroxisome

Figure 3. General purine metabolism in plants. The *de novo* synthesis reactions include reactions 24-34, and the salvage cytosolic reactions include reactions 35-56. "Metabolic components shown are: 1. 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. 10-formyl tetrahydrofolate (10F-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)." Reprinted from Ann. Rev. Plant Biol. (2006).

(AIR) by GAR transferase, FGAM synthetase, and AIR synthetase, respectively (reactions 26,27,28). A carbon dioxide (CO_2) is added to AIR yielding carboxyaminoimidazole ribonucleotide (GAIR) in a reaction catalyzed by AIR carboxylase (Reaction 29). IMP is synthesized from GAIR in a four-step sequence involving N-succinylo-5-aminoimidazole-4-carboxamide ribonucleotide (SAIGAR), 5-aminoimidazole-4-carboxamide ribonucleotide (AIGAR), and N-formylaminoimidazole-4-carboxamide ribonucleotide (FAIGAR) catalyzed by SAIGAR synthetase, adenylosuccinate lyase, and AIGAR transformylase, respectively (Reactions 30,31, 32). IMP is converted to either AMP or GMP. AMP is synthesized in a two-step sequence catalyzed by adenylosuccinate synthetase and adenylosuccinate lyase. GMP is synthesized from IMP in a two-step sequence catalyzed by IMP dehydrogenase, and GMP synthetase.[2]

ATase in plants is generally subject to feedback inhibition.[7] Analysis of two *Arabidopsis* ATase sequences (D28868 and D28869) should be useful in further clarifying the activity and regulation of the enzyme. These two ATase sequences were found in young floral buds. *AtATase 1* levels are high in roots and flowers, while *AtATase 2* levels are high in leaves.[6]

AMP and GMP are phosphorylated to adenosine diphosphate and guanosine diphosphate then to adenosine triphosphate and guanosine triphosphate. Three steps in the pathway act as regulatory steps. IMP, AMP, and GMP inhibit 5-phosphoribosylamine synthase, while AMP and GMP inhibit adenylosuccinate synthetase and IMP dehydrogenase.[8]

De novo pyrimidine nucleotide synthesis forms UMP from carbamoyl phosphate (CP), aspartate, and 5-phosphoribosyl-1-pyrophosphate (PRPP) in a six-step metabolic pathway (Figure 4). [5]   In eukaryotes, the first three-enzymatic steps are present as a multifunctional protein (carbamoylphosphate synthetase, aspartate transcarbamoylase and dihydroorotase) called CAD protein. This complex has been not found in plants.[8] While carbamoyl phosphate synthase has two different isoforms in eukaryotes that use different substrates one for pyrimidine biosynthesis and one for arginine synthesis, one type of carbamoyl phosphate synthase has been found in higher plants.[8]   Plants use a single form of carbamoyl phosphate synthase in both pathways. UMP is an inhibitor of carbamoyl phosphate synthase. Carbamoyl phosphate is also used in arginine biosynthesis. The second enzyme in pyrimidine biosynthesis, aspartate transcarbamoylase, is inhibited by UMP. The third enzyme, dihydroorotase, catalyzes the closure of ureidosuccinic acid to dihydroorotic acid with the elimination of water. Dihydroorotate dehydrogenase, the fourth enzyme, is not well characterized. The fifth, orotate phosphoribosyltransferase, and sixth enzymes orotidine-5$^{'}$-monophosphate decarboxylase have been found in a single polypeptide. UMP formed by this pathway is phosphorylated to UDP by UMP kinase, which is converted by uridine diphosphate kinase to form uridine triphosphate (UTP). Finally, cytidine triphosphate (CTP) is formed from UTP by CTP synthase.[2, 8]

The purine salvage pathway is used to reclaim purine bases. Adenine and guanine can be converted to the monophosphates by adenine phosphoribosyltransferase (APRTase) and
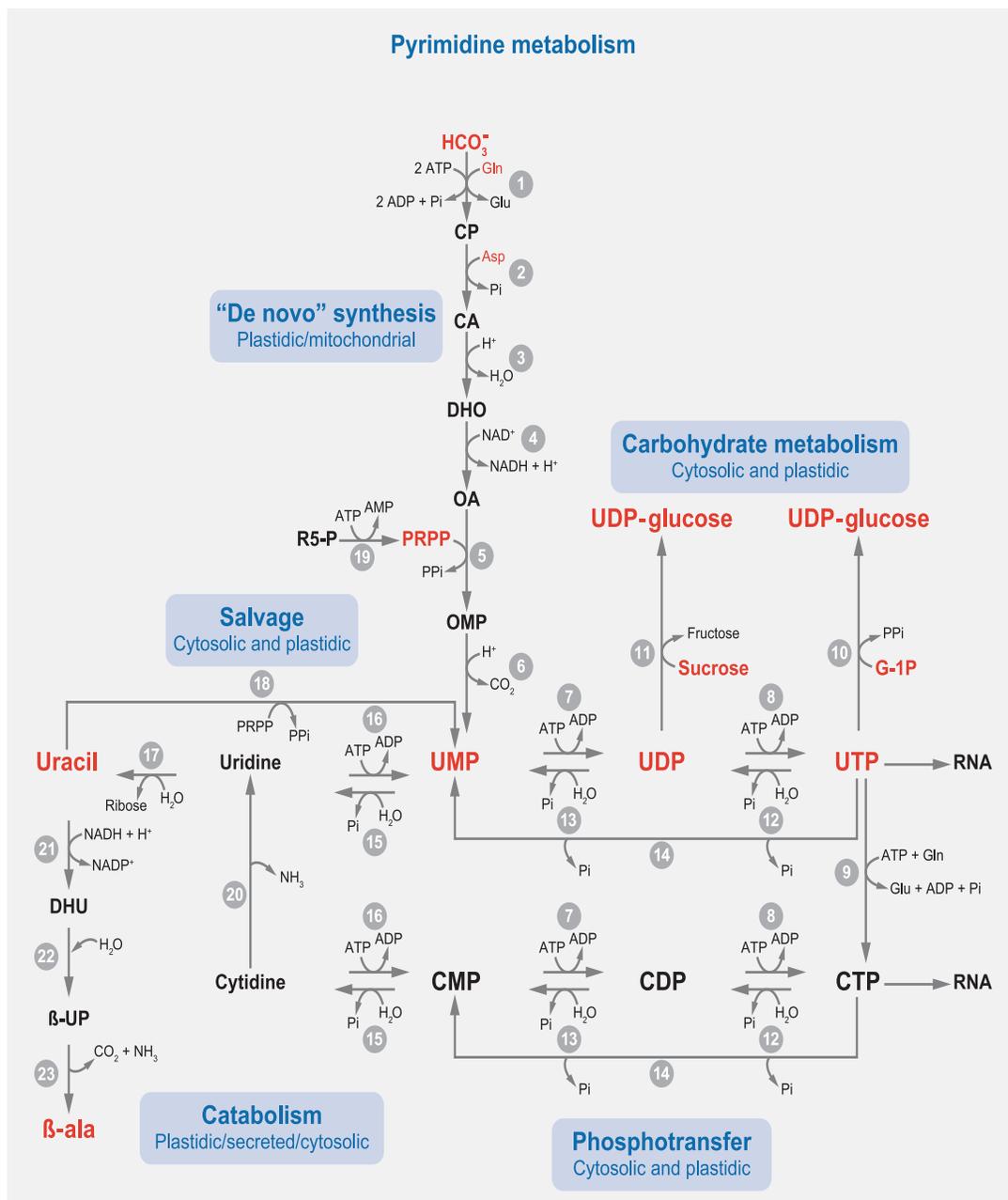
Figure 4. General pyrimidine metabolism in plants. "Enzymes involved in pyrimidine metabolism are numbered and correspond to those listed. The metabolic components shown are: 1. 5-phosphoribosyl-1-pyrophosphate (PRPP), 2. ribose-5-phosphate (R5-P), 3. glutamine (Gln), 4. glutamate (Glu), 5. adenosine triphosphate (ATP), 6. adenosine diphosphate (ADP), 7. inorganic phosphate (Pi), 8. carbamoyl phosphate (CP), 9. carbamoyl aspartate (CA), 10. dihydro-orotate (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)." Reprinted from Ann. Rev. Plant Biol. (2006).

hypoxanthine/guanine phosphoribosyltransferase (HGPRTase), respectively. PRPP is used as the source of ribose phosphate. Adenosine or inosine/guanosine phosphorylase cleaves the glycosidic bond between the base and ribose to recycle the purine base.[8] Adenosine, inosine, and guanosine can be converted to AMP, IMP, and GMP by adenosine, inosine, and guanine kinases or nucleoside phosphotransferases. Adenosine deaminase, the presence of which is still a matter of debate in plants, guanosine deaminase, and adenosine nucleosidase all play role in the purine salvage pathway. Adenine phosphoribosyltransferase (APRTase) and adenosine kinase play roles in the metabolism of cytokinins, a group of adenosine derivatives that act as plant hormones.[5]

Pyrimidine nucleotides are degraded by first converting them to nucleosides by the removal of the phosphate group(s) (Figure 4). Cytidine is converted to uridine by cytidine deaminase. Then uridine is converted to uracil and ribose by uridine nucleosidase. Animals can convert cytosine to uracil by cytosine deaminase, while plants lack this enzyme.[5]

The salvage pathway also recycles pyrimidine nucleosides and free bases. This pathway uses nucleoside hydrolase to form sugar and base.[9] There is no detectable salvage activity for cytosine in plants, whereas cytosine is converted to uracil by cytosine deaminase in microorganisms.[8] Pyrimidine nucleosides such as uridine, cytidine, deoxycytidine, and thymidine can be converted to UMP, CMP, dCMP, and dTMP by uridine kinase, cytidine kinase, deoxycytidine kinase, and thymidine kinase, respectively. Some enzymes of pyrimidine salvage have been found in higher plants, such as uracil phosphoribosyltransferase in *Pisum sativum*,

uridine/cytidine kinase in *Zea mays* seeds, and deoxycytidine deaminase in *Zea mays* leaves.[8]

Enzymes that cleave the glycosidic bond of purine or pyrimidine nucleosides can be divided into three classes: nucleoside phosphorylases, nucleoside transferases, and nucleoside hydrolases. The phosphorylases and transferases produce pentose-1-phosphate as a product, while nucleoside hydrolases produce ribose as the product.[10] Nucleoside transferases transfer a phosphoribosyl group from PRPP to purine bases.[11] Nucleoside phosphorylase is an enzyme that has been found in both prokaryotes and eukaryotes.[10]

Nucleoside Hydrolases:

Nucleosides hydrolases (NHs) are widely distributed in nature and have been found in a variety of organism including bacteria, yeast, protozoa and insects. NHs also have been found in plants. However, NHs have never been found in mammals.[9] Nucleoside hydrolase is an enzyme which hydrolyzes the N-glycosidic bond of nucleosides to form a pentose sugar and a purine/pyrimidine base.[9] The protozoan NHs can be classified according to substrate specificity: the base specific inosine-uridine preferring nucleoside hydrolase (IU-NH), the purine specific inosine-adenosine-guanosine nucleoside hydrolase (IAG-NH), 6-oxo-purine-specific inosine-guanosine nucleoside hydrolase (IG-NH), and non-specific nucleoside hydrolase, which catalyzes hydrolysis of both purines and pyrimidines.[9]

The most extensively studied nucleoside hydrolase is inosine/uridine nucleoside hydrolase (IU-NH) found in *C. fasciculata*. It catalyzes the hydrolysis of inosine or uridine to hypoxanthine or uracil and ribose.[12] This enzyme has been cloned from *C. fasciculata* by polymerase chain reaction. IU-NH was analyzed by mass spectrometry yielding a major peak of 34 194 amu[12] with k_{cat}/K_m ranging between 10^3-10^5 M^{-1} s^{-1}.[13]

Guanosine-inosine nucleoside hydrolase (GI-NH) has also been purified from *C. fasciculata*.[14] The subunit molecular weight of guanosine-inosine nucleoside hydrolase was 40.8 kDa, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In addition, the V_{max} with guanosine as substrate was 365 μmol/min/mg with a K_m of 77 μM. The V_{max} of inosine as a substrate was 148 μmol/min/mg with a K_m of 16 μM.[14]

Three nucleoside hydrolases have been found in *Leishmania donovani*.[15] The first enzyme isolated from *Leishmania donovani* was purine 2'-deoxyribomononucleosidase. The molecular weight of this enzyme was 33,000 Da as determined by gel filtration using Sephadex G100 resin. The second enzyme purified was purine ribonucleosidase (EC.3.2.2.1). The molecular weight of this enzyme was 205,000 Da as determined by gel filtration on Sephadex G200. It had an isoelectric point (pI) value of 4.4.[15] Isoelectric points is where a protein has no net charge.[3] The third enzyme was pyrimidine ribonucleosidase (EC. 3.2.2.8) with a molecular weight of 180,000 Da from gel filtration on Sephadex G200 and 6.3 of pI value.[15]

The crystal structures of several nucleoside hydrolases, particularly those from various *Trypanosomas* have been determined. The structure of IU-NH from *C. fasciculata* contains four subunits [9] (Figure 5a).   However, the crystal structure of IAG-NH from *Trypanosoma vivax* is a homodimer [9] (Figure 5b). The monomeric subunits for both IU-NH and IAG-NH are similar to each other in architecture and topology (Figure 5e,f). Both contain one globular domain (Figure 5c,d). The subunits of both subgroups are arranged in different quaternary structures and have different subunit-subunit interfaces. The α/β core of the NH monomer contains an eight-stranded β-sheet, with seven parallel strands, and one antiparallel strand, all surrounded by α-helix. (Figure 5e,f). The active site is found at the C-terminal end and it has flexible loops (Figure 5d). These loops can change the conformation of the active site. Each subunit has an active site, which contains a Ca^{2+} ion at the bottom.[9]

A Ca^{2+} metal ion is chelated through a network of interactions, which involves side chain oxygens of Asp10, Asp15, Asp261, the main chain carbonyl oxygen of Thr137 in *Tv*NH, and three water molecules (Figure 6). The base substrate binds deeply in the active site. In complex with base substrates, the waters are replaced by the 2', 3' hydroxyl groups of ribose. In a different structure, the water molecule is located 3.2-3.3 Å from the backside of C1$^{'}$ of the nucleoside in a position to attack the anomeric carbon. All NHs bind substrate in a hydrophobic pocket, but the structural properties of the nucleobase-binding pockets in *Cf*NH and *Tv*NH are different (Figure 6). [9] In IU-NH (*Cf*NH), the active site binding pocket contains the amino acid residues Ile81, His82, Phe167, Tyr225, Tyr229, and His 241. However, no crystal
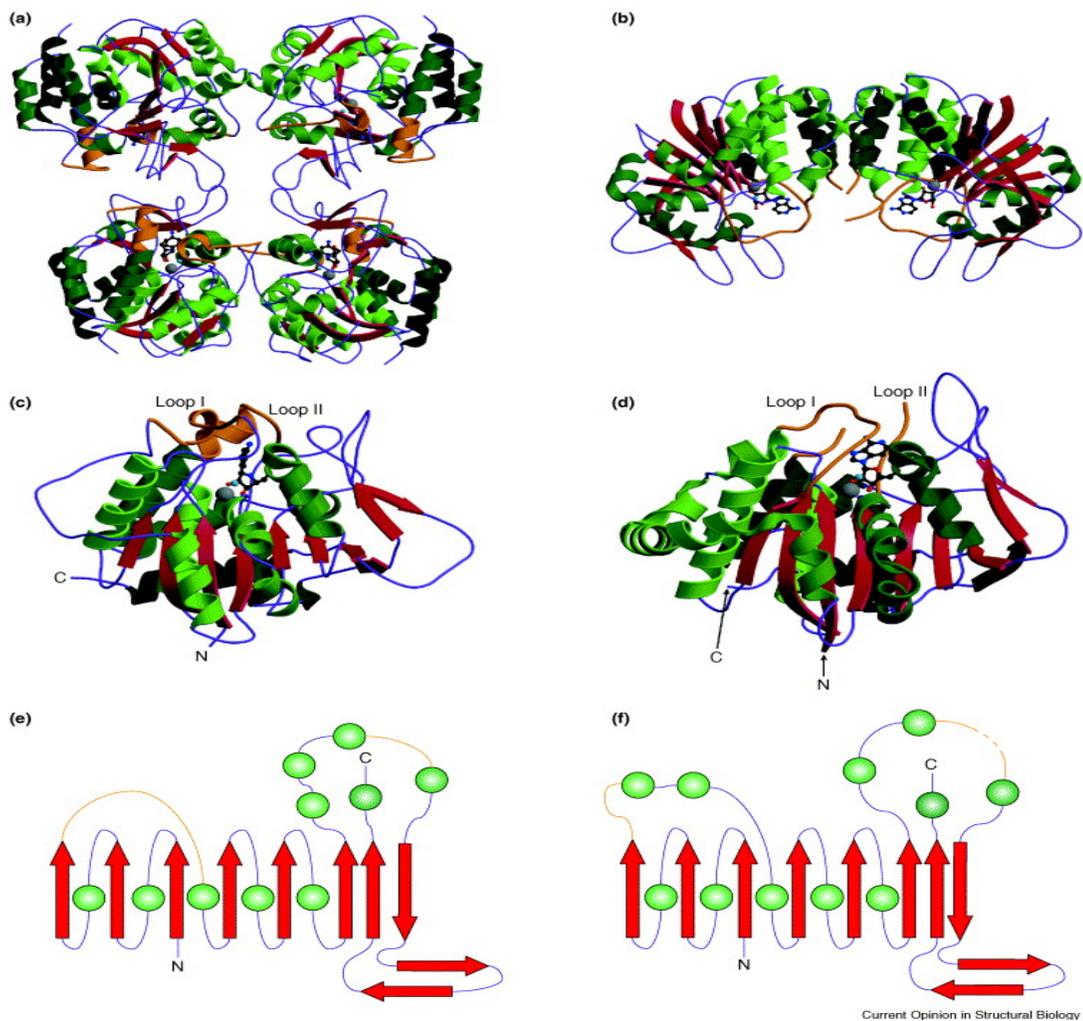
Figure 5. The quaternary NH structure, tertiary structures and topologies of an IU-NH of *C. fasciculata* (a,c,e) and (b,d,f) of an IAG-NH of *T.vivax* are compared. β-sheets are shown as flat arrows, and α-helices is shown as circle. The Ca^{+2} ions and water molecules are located in the active site of the enzymes. Reprinted with permission from Versees, W.; Steyaert, J. *Opinion in Structural Biology* 2003, *13:*731-738.
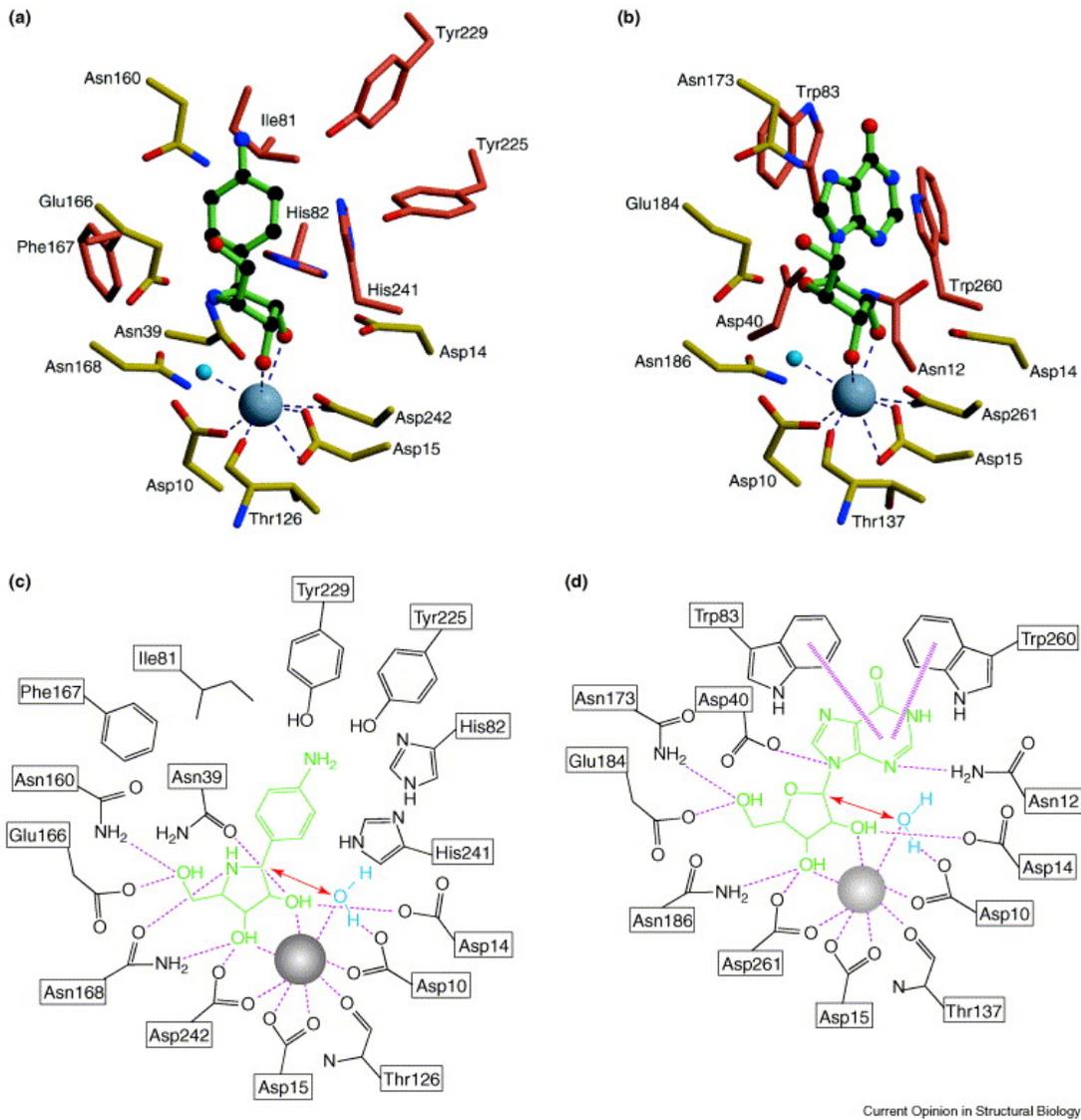
Figure 6. Comparison of the active site of an IU-NH (*Cf*NH) (a,b) and of an IAG-NH (*Tv*NH) (b, d). The structure is shown model with the natural substrate inosine. The enzyme bound ligands are shown in green. The Ca^{+2} and nucleophilic water molecules are in gray and blue spheres. Reprinted with permission from Versees, W.; Steyaert, J. *Opinion in Structural Biology* 2003, *13:*731-738.

structure with a guanine heterocyclic nucleobase in complex with IU-NH has been determined.[9]

Nucleoside Hydrolases from Plants

Inosine nucleosidase:

Inosine nucleosidase (EC 3.2.2.2) is an enzyme in the purine catabolic pathway, which hydrolyzes inosine to hypoxanthine and pentose sugar (Figure 7). Inosine nucleosidase has been found in plants and animals. [16] In plants inosine nucleosidase has been purified from yellow lupin (*Lupinus luteus*). [16] The highest activity of inosine nucleosidase has been found in six-day-old seedlings.[16] Inosine nucleosidase has a native molecular weight of 62,000 Da as determined by gel filtration on Sephadex G100, and an optimum activity around pH 8.0.[16]
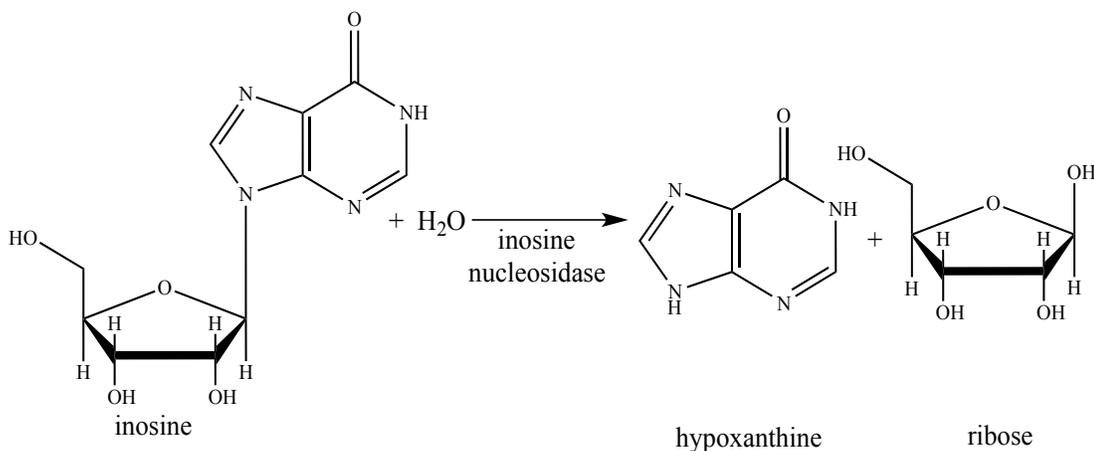
Figure 7. Hydrolysis of inosine to hypoxanthine and ribose by inosine nucleosidase.

Adenosine nucleosidase:

Adenosine nucleosidase (EC 3.2.2.7) is an enzyme of the purine metabolic pathway, which hydrolyzes adenosine to adenine and pentose sugar (Figure 8). Adenosine nucleosidase converts adenosine to adenine in the cotyledon, making it available to other parts of the growing plant. [17]   Adenosine nucleosidase activity was found in the cotyledon 5-6 days after germination. Adenosine nucleosidase is involved in controlling the level of cytokinins, cell differentiation, division, and development in plants.[18]
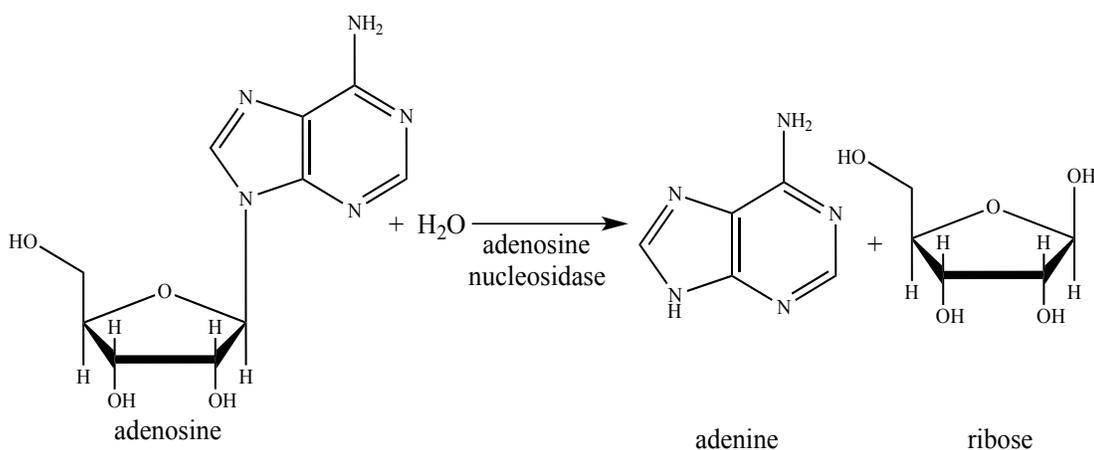
Figure 8. Hydrolysis of adenosine to adenine and ribose by adenosine nucleosidase

The activity of adenosine nucleosidase has been found in many plants, including the leaves of *coffea arabica*, [17] yellow lupin (*Lupins luteus*) seeds, [18,17] barley leaves.[19, 17]

A typical purification has been carried out from leaves of *Coffea arabica*.[17] The purification involved fractionation by salting out with ammonium sulfate, ion exchange chromatography, hydrophobic interaction chromatography, and gel-filtration or size exclusion chromatography. The molecular weight of the adenosine nucleosidase from gel filtration was 72 kDa from *coffea arabica*, and the molecular weight of the subunits from SDS-PAGE was 34.6 kDa. In addition, the specific activity of adenosine nucleosidase was 8333 nkat/mg for adenosine. This enzyme shows optimum activity at pH 6.0, with the K_m at this pH 6.3 µM, and V_{max} 9.8 nkat.[17]

The molecular weight of adenosine nucleosidase from yellow lupin was 72 kDa, determined by gel –filtration on a Sephacryl S200 16/60 column.[18] It has two subunits as determined by SDS-PAGE with a molecular weight of 33 kDa per subuint, and a pH optimum of 7.5. K_m for adenosine from yellow lupin was 4.8 µM.[18]

Adenosine nucleosidase was also purified from barley leaves. The molecular weight was determined to be 66.0 ± 3.0 kDa by native gel filtration. The subunit molecular weight was estimated by SDS-PAGE to be 33.0 kDa. The pH optima are 4.7 in a citric acid buffer and 5.4 in an ethanesulphonic acid buffer. The K_m for adenosine from barley leaves was 0.8 to 2.3 µM.[19]

Purine Nucleoside Phosphorylases (PNP)

Nucleoside phosphorylase (EC 2.4.2.1) is an enzyme in the purine salvage pathway, which catalyzes the cleavage of the glycosidic bond to give the base and ribose-1-phosphate.[20]   Nucleoside phosphorylases have been found in mammals, bacteria, and protozoa.[21]   Much less is known about PNPs in plants. A study of purine biosynthesis, salvage, and degradation in potato did not detect any purine nucleoside phosphorylase activity.[22]

PNP can be classified into two types: trimeric and hexameric. Trimeric purine phosphorylases are found in eukaryotes and prokaryotes with a molecular mass of 100 kDa. Hexameric purine phosphorylase was found in lower organisms such as *E.coli*, with a molecular mass of 150 kDa. Both enzymes have a similar mechanism.[20]

Phosphoribosyltransferases

Adenine and guanine bases are salvaged to their respective monophosphates by adenine phosphoribosyltransferase (APRTase) and guanine/hypoxanthine phosphoribosyltransferase (HGPRTase).[5]   Five sequences in the *Arabidopsis thaliana* genome have been identified as similar to sequences encoding known APRTases.[23]   Three of these genes have been cloned, and overexpressed. The most likely phosiological role for these enzymes is adenine recycling although they may also play a role in cytokinin metabolism. Both APRTase and HGPRTase activities

have been detected in plants. Using adenine as a substrate, APRTase had an activity

of 101.4 ± 6.1 pkat, while HGPRTase had an activity of 6.4 ± 1.5 pkat using

hypoxanthine as a substrate for the enzymes found in potato. Other plants, such as

tea leaf extract exhibited similar results were found in tea leaf extracts.[24]

Uracil Phosphoribosyltransferase (UPRTase)

Uracil phosphoribosyltransferase (EC 2.4.2.9) is an enzyme, which converts

uracil to uracil monophosphate (UMP).[5] There are several UPRTase genes in plants,

including one gene found in *Arabidopsis thaliana*.[25] UPRT from *Arabidopsis* was

found to be similar to other phosphoribosyltransferases.[26] The activity of UPRTase

has been determined in several plants. In *Pinus radiate*, UPRTase was found to have

an activity of 18.7 pkat/mg in shoot-forming cotyledons.[27]

Nucleoside Deaminases

Cytosine/Cytidine Deaminase

Cytosine deaminase (EC 3.5.4.1), an enzyme of the pyrimidine salvage

pathway, catalyzes the conversion of cytosine to uracil and ammonia. It has been

found in bacteria and fungi, but has been not found in mammals or plants. Cytosine

deaminase exhibits differences between bacterial (*Escherichia coli*) and fungal

(*Saccharomyces cerevisiae)* forms in their amino acid sequences, molecular masses,

and their structures. The enzyme in *Saccharomyces cerevisiae* is a homodimer with

158 residues and a molecular mass of 35 kDa. However, the enzyme in *Escherichia coli* is a hexamer with 426 residues and a molecular mass of 300 kDa.[28]

Cytidine deaminase from *Arabidopsis thaliana* (AT-CDA1) has been cloned from a cDNA library, expressed in *E. coli*, and purified to > 98% purity. [29] The *Arabidopsis thaliana* enzyme has a 30% homology with the *E. coli* enzyme. AT-CDA1 and the *E. coli* enzyme are dimers, while other cytidine deaminases are tetramers. Further the kinetic properties of *E. coli* and *Arabidopsis thaliana* enzymes were found to be similar.[29]

Adenine/Adenosine Deaminase

Adenosine deaminase (EC 3.5.4.4) is an enzyme of the purine metabolism pathway, which catalyzes conversion of adenosine or deoxyadenosine to inosine or deoxyinosine. An important function of ADA is synthesis of nucleotides by *de novo* or salvage pathways in mammalian tissue. It is also an important precursor in the synthesis of nucleic acids, and signaling molecules. Adenosine deaminase has been purified from different organisms such as pigs, cattle, camels, and humans. ADA has two isoenzymes in humans (ADA1 and ADA2). ADA1 is important in humans. ADA2 has been found in serum and is produced by monocytes.[30] ADA was purified from human liver and found to have a molecular weight of 42 kDa by SDS-PAGE. [31] In bacteria, tRNA adenosine deaminases catalyzed the conversion of adenosine to inosine in the codons of mRNA. ADA was purified from *Penicillium politans*, with separation of this enzyme from cytidine deaminase by cold ethanol and acetone

precipitation. The purity of the enzymes was increased 20-fold by gel filtration on Sephadex G100 and 168-fold by ion exchange chromatography.[31]

In an early study twenty different seeds were tested for adenosine deaminase activity.[32] Barley seeds had activity in both fresh and germinated seeds after fifteen hours of incubation. However no activity was detected after 1-3 hrs of incubation.[32] In contrast, no adenosine deaminase activity was detected in potato tubers.[21]

Enzymes Isolated from Pea Seeds

Inosine 5'-phosphate dehydrogenase has been found in pea seeds and other higher plants. [33]   Inosine-5'-phosphate is a precursor of nucleic acid purines. In addition, adenosine 5'-phosphate is converted to inosine 5'-phosphate by adenylic deaminase, which is present in pea extracts.[33]   Inosine 5'-phosphate dehydrogenase has a similar pH activity as the enzyme from *Aerobacter aerogenes*. The optimum pH for the pea seed enzyme was 8.0, and for the *Aerobacter aerogenes* enzyme was 8.1.[33]

Another enzyme that has been isolated from pea seeds is tyrosine phosphatase.[34] This enzyme hydrolyzes the phosphotyrosines to produce the amino acid residue tyrosine and inorganic phosphate. Tyrosine phosphatase is inhibited by low concentrations of molybdate, heparin, and spermine. Tyrosine phosphatase does not need Ca^{2+}, Mg^{2+}, or Mn^{2+} for activity. However, it is stimulated by ethylenediaminetetraacetic acid (EDTA). [35]   Tyrosine phosphatase has also been purified from pea seeds (*pisum sativum* L.). This enzyme has two subunits of 126

and 90 kDa, determined by SDS-PAGE. It has a variable pH optimum depending on the substrate. When the substrate was [^{32}P] tyrosine-labeled lysozyme, the pH optimum was 5.0, but when the substrate is [^{32}P] tyrosine-labeled polyglutamic acid, the optimum pH was 7.0.   Also, tyrosine phosphatase has a K_m of 4 µM for tyrosine labeled lysozymes.[35]

Carbamoyl phosphate synthetase is an enzyme, which is present in mammals, yeast, and bacteria. Carbamoyl phosphate is an important precursor for the biosynthesis of both arginine and UMP. Carbamoyl phosphate synthetase has been purified from Alaska pea seedlings (*pisum sativum* L.cultivar Alaska) up to 45-fold.[36] The lowest K_m was 1.2 x10^{-4} M for L-glutamine with a K_m of 3.9×10^{-4} M for ATP.[36]

CHAPTER II
MATERIALS AND METHODS

Equipment and Instrumentation

High performance liquid chromatography (HPLC) was carried out on a Dionex Ultimate 3000 equipped with a multiple wavelength UV-Vis detector, thermostatted autosampler and column chamber equipped with a Phenomenex Hypersil C_{18} (4.6 x 150 mm) column. Fast protein liquid chromatography (FPLC) was carried out on an AKTA system from GE Healthcare equipped with a Mono Q (26/10) column, Sephacryl S100 (26/60) size exclusion column, and a Bio-Rad hydroxyapatite (Bio-Rad ceramic type I) column. UV-Vis spectroscopy was carried out on a Hitachi U-2900 spectrophotometer.

Materials

Alaska pea seeds were obtained from Ferry Morse Seed Company. Tris (hydroxymethyl aminomethane) buffer, dithiothreitol (DTT), ammounium sulfate, ω-aminohexyl agarose resin, nucleosides such as adenosine, inosine, guanosine, uridine, cytidine, and bases such as hypoxanthine, adenine, guanine, uracil, and cytosine were all obtained from Sigma-Aldrich. Polyvinylpolypyrolidone (PVPP), protease inhibitor cocktail for plant cell and tissue extracts, and protamine sulfate were also obtained from Sigma-Aldrich. Laemmli sample buffer, 10X-Tris/Glycine buffer, Precision Plus molecular weight markers, protein assay dye reagent and protein standard II were obtained from Bio-Rad. Polyacrylamide gels

and Millipore centrifugal concentrators were obtained from Fisher Scientific. GelCode Blue Safe protein stain for SDS-PAGE gels was obtained from Thermo Scientific. All other chemicals were of reagent grade.

Determination of Protein Concentration

Bio-Rad Assay

The assay mixture consisted of sample and water equal to a total volume of 800 μL, to which 200 μL of reagent dye concentrate was added. The samples were mixed and incubated at room temperature for 5 min. The absorbance at 595 nm was measured and the amount of protein in the sample determined by comparison to a standard curve. Bovine serum albumin (1.53 mg/mL) was used to construct a standard curve to determine protein concentration (Figure 9).

UV-Vis Assay

Protein concentration from column chromatography was determined by measuring the absorbance at 280 nm. The protein concentration in mg/mL was determined using the equivalence of absorbance at 280 nm= concentration (mg/mL).
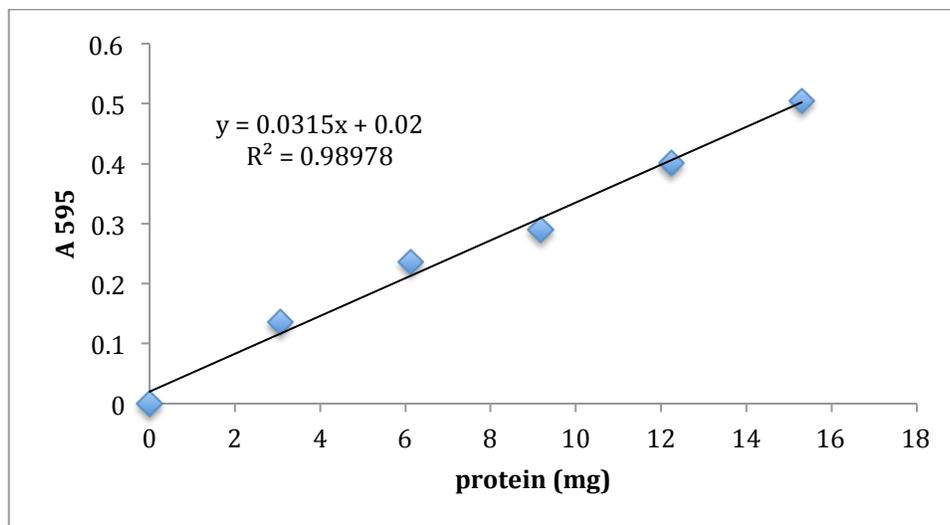
Figure 9. Standard protein calibration curve determined at 595 nm using bovine serum albumin (1.53 mg/mL).

Measurement of Enzyme Activity

Enzyme activity was measured by a reducing sugar assay (a colorimetric assay) or by determining the relative amounts of nucleoside and base by HPLC.

HPLC Analysis

The reaction assay mixture consisted of 1mM adenosine or inosine in 1000 µL in 50 mM Tris buffer pH 7.2. The reaction was started by the addition of 100 µL of enzyme to the reaction mixture. The reaction mixture was incubated at room temperature and the amount of base and nucleoside determined by HPLC on a Phenomenex Hypersil C_{18} column eluted with 98% 10 mM ammonium acetate pH 5.2 and 2% methanol. Nucleoside and base were detected by measuring the absorbance at 254 nm. The nucleosides and bases were identified by their

retention time (Table 1) Activity was calculated by dividing the amount of base

produced by the reaction time after accounting for the difference in extinction

coefficients between the various nucleosides and bases.

Nucleosides	Retention Time (min)	Bases	Retention Time (min)
Adenosine	25.0	Adenine	6.64
Inosine	7.35	Hypoxanthine	3.34
Guanosine	8.52	Guanine	3.27
Cytidine	3.06	Cytosine	2.31
Uridine	3.86	Uracil	2.48
Thymidine	5.73	Thymine	13.61

Table 1. The Retention Time for nucleosides and bases.

Reducing Sugar Assay

One mL of reaction mixture (1 mM adenosine in 50 mM Tris pH 7.2) was

placed in a clean dry test tube. The reaction was started when 100 μL of enzyme

was added to the reaction mixture. After four hours the reaction was stopped by

adding 300 μL of copper reagent (4% Na_2CO_3, 1.6% glycine, and 0.045% $CuSO_4$.

H$_2$O in 400 mL H$_2$O) followed by 300 μL of neocuproine solution (0.12% neocuproine dissolved in 400 mL water, pH adjusted to 3.00). The samples were incubated at 95 °C for 7 min and the absorbance was measured at 450 nm. The amount of ribose was determined by comparison to a standard curve (Figure 10).
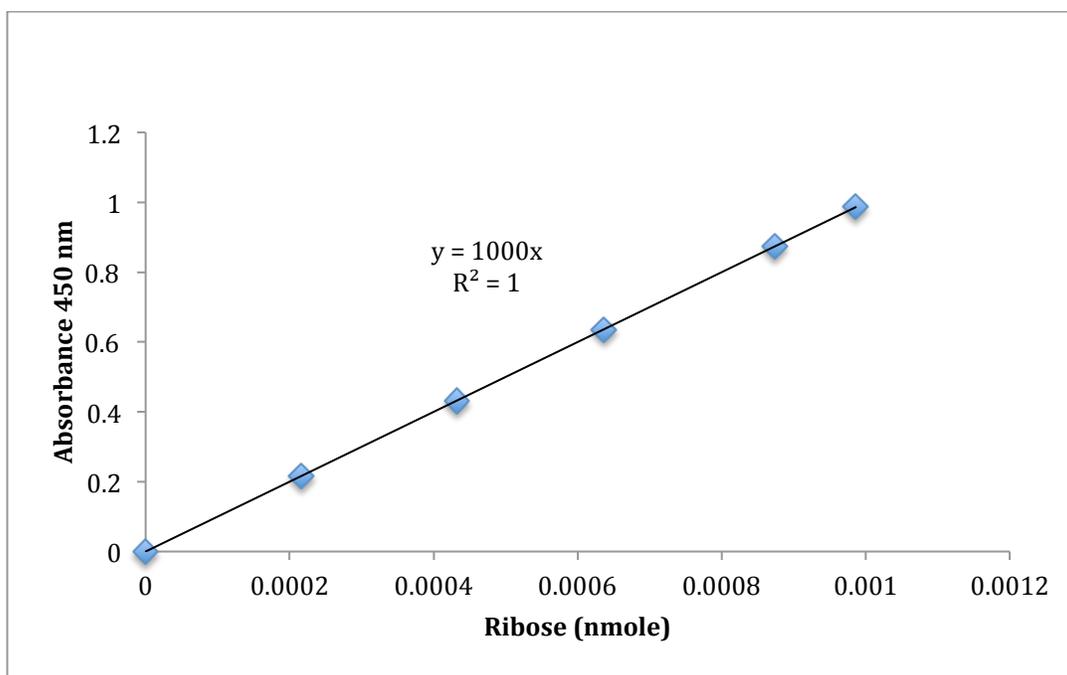
Figure 10. Reducing sugar assay calibration curve. The activity of enzyme was measured colometrically by appearance of reducing sugar.

Determination of Activities of Ungerminated Seeds

Five grams (5 g) of Alaska pea seeds were washed with tap water then suspended in 15 mL of 50 mM Tris pH 7.2. The seeds were homogenized in a commercial Waring blender 3-5 times for 30 sec. Then the initial extract was assayed for activity by HPLC using different nucleosides such as adenosine, inosine,

guanosine, uridine, and cytidine as substrates. The bases adenine and cytosine were tested for deaminase activity.

To assay nucleosidase and deaminase activities the reaction mixture consisted of 1 mM nucleoside/base in 50 mM Tris buffer pH 7.2, and the reaction was started by adding 100 µL of initial extract. To assay phosphorylase activity the reaction mixture consisted of 1 mM nucleoside in 50 mM phosphate buffer pH 7.2. The reaction was started by adding 100 µL of initial extract.

Germination of Alaska Pea Seeds

One hundred grams (100 g) of Alaska pea seeds were washed with tap water and placed in bleach for 10 min to kill surface bacteria. Then the seeds were thoroughly washed with tap water and placed on moistened paper towels to germinate at room temperature. Four to five days after germination, the seeds were homogenized in 300 mL of 50 mM Tris pH 7.2 buffer containing 1mM DTT, 10% (w/v) polyvinylpolypyrrolidone, 10 µL of plant tissue protease inhibitor, and 2 g of protamine sulfate. The seeds were homogenized in a commercial Waring blender 6 times for 30 sec with intervening 1 min rest periods. After homogenization, the initial extract was centrifuged at 15000xg for 30 min. Then the sample was passed through a double layer of cheesecloth to remove additional insoluble material. The supernatant was removed and the solid from the initial extract discarded.

Ammonium Sulfate Precipitation

Solid ammonium sulfate (32.3g) was slowly added with stirring to the supernatant (184 mL) to yield 30% saturation. The solution was kept overnight at 4 °C. The solution was centrifuged again at 20000xg for 30 min at 4 °C. The 30% supernatant (190 mL) was collected and the ammonium sulfate saturation was increased to 60% by slowly adding solid ammonium sulfate (37.0 g) and the solution kept overnight at 4 °C. The supernatant was centrifuged at 20000xg for 30 min at 4 °C, and the pellet retained. The 60% pellet was suspended in 20 mL of 50 mM Tris pH 7.2. The 30% pellet, 30% supernatant, 60% pellet, and 60% supernatant were assayed for activity by HPLC.

Ion Exchange Chromatography

After assaying the fractions, the 60% pellet had the highest activity. The 60% pellet (20 mL) was dialyzed against 50 mM Tris pH 7.2 (250 mL), and the buffer was changed 3 times before loading onto a diethylaminoethyl ion exchange column (DEAE 150 x 10 mm). The column was washed with 200 mL of 50 mM Tris buffer pH 7.2. This was followed by a linear gradient of 0-1 M NaCl in 50 mM Tris buffer pH 7.2 (300 mL). Finally, the column was washed with 250 mL of 1 M NaCl in 50 mM Tris pH 7.2 buffers. The absorbance of the fractions was measured at 280 nm to determine the protein concentration, and the activity was determined by reducing sugar assay at 450 nm. The fractions containing high protein concentration were also assayed for activity by HPLC. Fractions containing the highest activity were pooled

and dialyzed against 50 mM Tris pH 7.2 buffer. The dialyzed fractions were concentrated to 2 mL using a Millipore Centrifugal Concentrator (MWCO 10000).

ω-Aminohexyl Agarose Chromatography

The concentrated sample (5 mL) was loaded onto an ω-aminohexyl agarose column (1.0 × 30 cm). The column was washed with 200 mL of 50 mM Tris pH 7.2. A linear gradient of 0-1 M NaCl in 50 mM Tris buffer pH 7.2 (200 mL) was run. The fractions were assayed for protein at 280 nm and for activity by reducing sugar assay at 450 nm. The active fractions were pooled, dialyzed, and then concentrated to 2 mL as described previously.

Size Exclusion Chromatography

The pooled fractions were loaded on a Sephacryl S100 (26/60) size exclusion FPLC column. The column was washed with 100 mM sodium phosphate, 300 mM NaCl at a flow rate of 1.0 mL/min. Fractions (5 mL) were collected and the absorbance at 280 nm determined. Activity was determined by reducing sugar assay at 450 nm.

Activity containing fractions were pooled and dialyzed against 10 mM sodium phosphate pH 7.2. The dialyzed sample was concentrated on a Millipore Centrifugal Concentrator (MWCO 10000). The concentrated sample (1mL) was loaded onto a Bio-Rad hydroxyapatite CHT5-1 FPLC column. The column was washed with 10

mM sodium phosphate buffer pH 7.2 (500 mL) at a flow rate of 1.0 mL/min. This

was followed by a linear gradient of 10 – 400 mM sodium phosphate buffer pH 7.2.

The fractions were collected and the absorbance at 280 nm determined. The activity

of each fraction was determined by reducing sugar assay.

Determination of Enzyme Purity by Gel Electrophoresis

The sample buffer was prepared by adding 50 µL of β-mercaptoethanol to 950

µL of Bio-Rad Laemmli sample buffer. The sample was prepared by combining 10

µL of sample buffer and 10 µL of Laemmli buffer. The sample was centrifuged for 10

sec and incubated at 95 °C for 5 min. Then the sample was centrifuged for 10 sec.

The sample was loaded onto a 15% SDS minigel, along with Precision Plus Protein

Unstained Markers. The gel was electrophoresed at a constant current of 30 mA for

40-50 min. After completion the gel was removed from its plastic holders, placed in

a plastic container and washed with distilled water 3 times for 5 min each. Then the

gel was covered with GelCode Blue Safe Protein Stain overnight. Finally, the gel

was washed three times for 5 min with distilled water. A calibration curve was

constructed using the Precision Plus Protein molecular weight markers. The

molecular weight of the sample subunit(s) was determined by comparison to the

calibration curve.

CHAPTER III

RESULTS AND DISCUSSION

Adenosine nucleosidase (EC 3.2.2.7) is an enzyme of the purine metabolic pathway, which hydrolyzes adenosine to adenine and the pentose sugar, ribose. Adenosine nucleosidase controls to a certain extent the amount of adenine in the cotyledon, making it available to other parts of the growing plant. [17] Adenosine nucleosidase activity has been found in cotyledons 5-6 days after germination in a number of plants and is thought to be involved in controlling the level of cytokinins for use in cell differentiation, division, and development in plants, among other functions.[18]

Enzyme Activities in Alaska Pea Seeds

Before undertaking the purification of adenosine nucleosidase, the activities of a number of metabolizing enzymes were determined from dry Alaska pea seeds. Five grams of dry Alaska pea seeds were homogenized in 15 mL of 50 mM Tris buffer pH 7.2. The extract was tested for the presence of three groups of enzymes; nucleosidases (hydrolases), phosphorylases, and deaminases. Nucleosidase, phosphorylase, and deaminase activities are summarized in Table 2. For the nucleosidases and deaminases, a reaction mixture containing 1 mM nucleoside or base in 50 mM Tris buffer pH 7.2 was placed in HPLC vial to which homogenized initial extract (100 μL) was added. The nucleosides and bases

produced were monitored and identified by their retention times (Table 1). To test

for the presence of phosphorylases, a reaction mixture containing 1 mM nucleoside

in 50 mM sodium phosphate pH 7.0 was placed in HPLC vial to which

homogenized initial extract (100 μL) was added. The extract was found to have a

number of different enzyme activities. Inosine, guanosine, adenosine, cytidine and

thymidine nucleosidases were observed as determined by the presence of

hypoxanthine, guanine, and thymine in the reaction mixture. The highest activity

of the nucleosidases was cytidine nucleosidase with an activity of 4.82 μmol/min.

This was followed by adenosine nucleosidase with an activity of 4.37 μmol/min,

thymidine nucleosidase with an activity of 1.76 μmol/min and guanosine

nucleosidase with an activity of 1.17 μmol/min. The lowest activity of the

nucleosidases tested was inosine nucleosidase with an activity of 0.97 μmol/min.

This is in contrast to the result found for yellow lupin seeds in which no activity

was observed in dry yellow lupin seeds.[18] In yellow lupin seeds, adenosine

nucleosidase reached a maximum 4-5 days after germination. [18] However, inosine

nucleosidase was observed in both dry and germinated seeds.[16]

Adenosine, guanosine, and cytidine phosphorylase activities were observed in

the extract from ungerminated seeds. The highest phosphorylase activity was 0.71

μmol/min for adenosine phosphorylase, followed by guanosine phosphorylase with

an activity of 0.51 μmol/min. The lowest phosphorylase activity observed was

cytidine phosphorylase at 0.39 μmol/min. In all cases tested the phosphorylase

activities were lower than the corresponding nucleosidases. In yellow lupin seeds

Stasolla and coworkers reported no nucleoside phosphorylase activity in either dry or

germinating seeds.[8]   In contrast, Chen and Petschow reported the presence of an adenosine phosphorylase in wheat germ.[37]

In addition to the enzymes that break the glycosidic bond in nucleosides, ungerminated seeds were also tested for the presence of a series of deaminases. Adenosine, adenine, and cytidine deaminases were observed in dry Alaska pea seeds with activities of 4.37 μmol/min, 0.70 μmol/min, and 4.82 μmol/min respectively. However, no cytosine deaminase activity was observed in dry Alaska pea seeds. This is consistent with the reported lack of cytosine deaminase activity in plants.[25] The highest deaminase activities were observed for the nucleoside deaminases, while the lowest activity was observed for the base (adenine) deaminase. The presence of cytidine deaminase has been reported in *Arabidopsis thaliana*.[29]

One trend that was observed was germination of seeds produced an increase in activity compared to the ungerminated seeds. Adenosine nucleosidase in dry Alaska pea seeds had an activity of 4.37 μmol/min, while 5 days after germination the activity had increased to 7.85 μmol/min. Other examples include catalase in which nongerminating pea seeds had a lower activity than germinating seeds. While ungerminated pea seeds had a higher peroxidase activity than seeds one day after germination, by the 5th day after germination the activity of the germinated seeds was higher. [38]   Another example was inosine nucleosidase in yellow lupin seeds. While the activity was observed in dry seeds, it reached a maximum 5-6 days after germination.[16]

It is difficult to accurately quantify the various types of activity in an initial extract from dry seeds. The activities were calculated based on the disappearance of substrate as determined by the change in area in the HPLC chromatogram. However the disappearance of many of the substrates is due to the presence of two or more enzymes. For example the disappearance of adenosine was due to at least two enzymes, adenosine nucleosidase and adenosine deaminase. Based on the HPLC analysis it was not possible to determine the relative contributions of adenosine nucleosidase and adenosine deaminase to the disappearance of adenosine. The same was true of the phosphorylases. While this activity was measured in a phosphate buffer the corresponding nucleosidase could contribute to the observed activity. A definitive answer would require the detection of the product ribose-1-phosphate, which was not measured in this study.

Another difficulty in this analysis is determining whether the observed nucleosidase activities represent a single enzyme with multiple substrates or separate enzymes for each nucleoside or a group of nucleosides. The same was true for the phosphorylases. At this point it was not possible to determine whether the adenosine, guanosine, cytidine, and inosine phosphorylase activities were due to multiple enzymes or a single enzyme. This difficulty provides a major rationale for the purification of the adenosine nucleosidase, a major goal of this project.

	Enzymes	Activity (μmole/min)
Deaminases:	Adenosine deaminase	4.37
	Adenine deaminase	0.70
	Cytidine deaminase	4.82
	Cytosine deaminase	Not detected
Phosphorylases:		
	Adenosine phosphorylase	0.71
	Guanosine phosphorylase	0.51
	Cytidine phosphorylase	0.39
	Inosine phosphorylase	0.36
Hydrolases:		
	Thymidine nucleosidase	1.76
	Inosine nucleosidase	0.97
	Guanosine nucleosidase	1.17
	Adenosine nucleosidase	4.37
	Cytidine nucleosidase	4.82

Table 2. Enzyme activities in ungerminated Alaska pea seeds.

Purification of Adenosine Nucleosidase

One of the main goals of the project was to purify the nucleoside-metabolizing enzyme, adenosine nucleosidase from Alaska pea seeds. Four to five days after germination, 50 g of seeds were homogenized. The solution was passed through cheesecloth and centrifuged to remove suspended solids. The activity of the initial extract was assayed by HPLC using adenosine (1mM) as the substrate. As the chromatogram shows multiple enzyme activities were present (Figure 11), including adenosine deaminase, inosine nucleosidase, and adenosine nucleosidase. Adenosine deaminase converts adenosine to inosine, while adenosine nucleosidase converts adenosine to adenine and ribose. Inosine nucleosidase converts inosine to hypoxanthine, and ribose. It is not possible at this stage to determine whether the adenosine nucleosidase and inosine nucleosidase represent two separate enzymes or a single enzyme that hydrolyzes both nucleosides. The time course of the reaction indicated a reaction progress consisting of two phases. The first is characterized by a slow decrease in the concentration of adenosine for the first 8 hours, followed by an increased rate of adenosine breakdown for the next 2 hours (Figure 12). The specific activity, based on the loss of adenosine, was 58×10^{-4} μmole/min/mg for the slow phase, while it was 56×10^{-3} μmole/min /mg for the fast phase. The changes in inosine and hypoxanthine concentrations mirrored the change in adenosine concentration. The adenine concentration was constant at a low level through much of the reaction (Figure 11).

A possible explanation for the two phases in the reaction time course is the presence of an inhibitor in the initial extract of germinated seeds. To test this
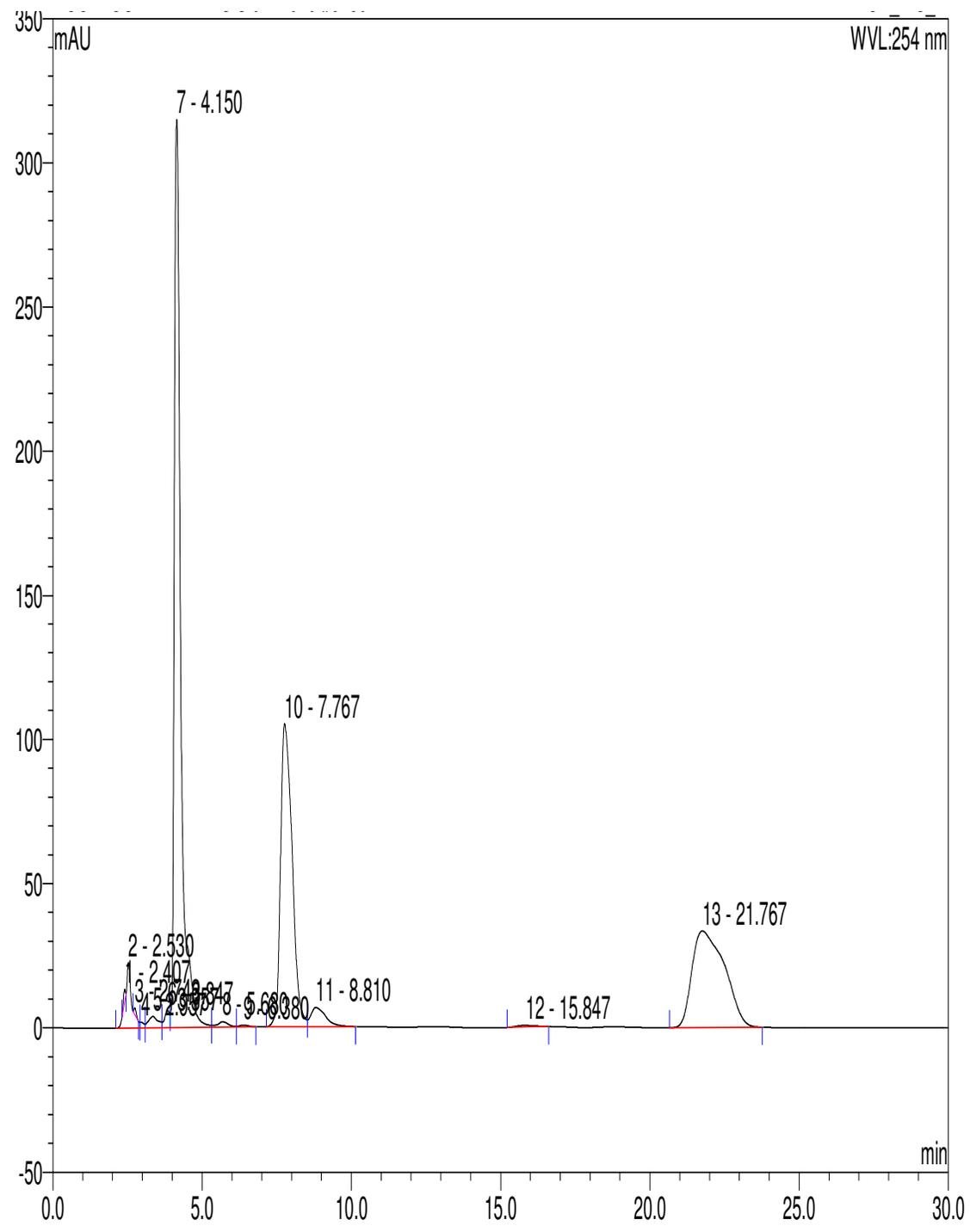
Figure 11. Analysis of reaction mixture 9 hours after addition of initial extract. Four peaks are of interest; adenosine (21.76 min), adenine (8.81 min), inosine (7.76 min), and hypoxanthine (4.15 min)
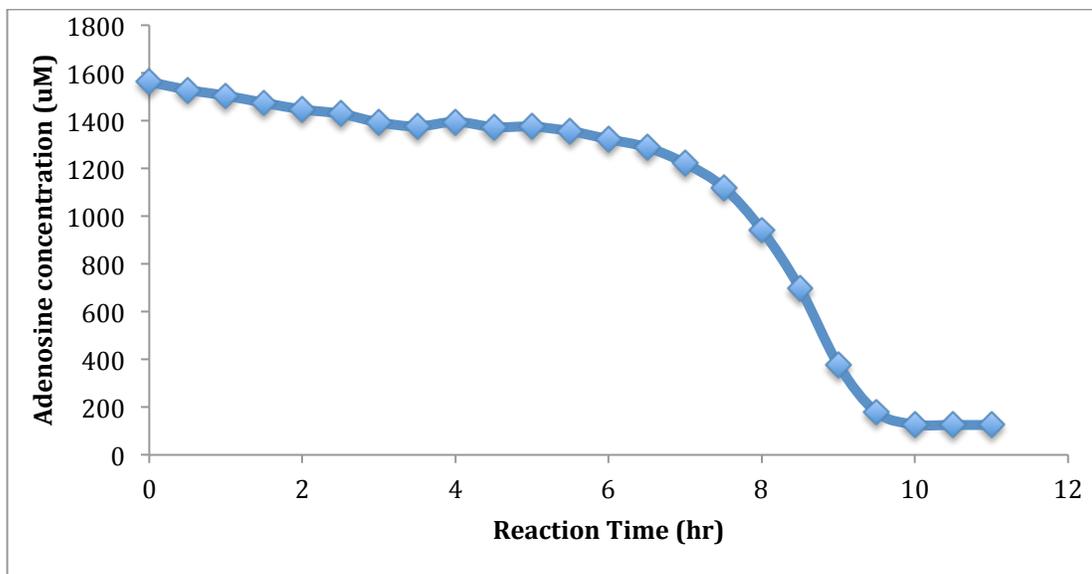
Figure 12a. Change in concentration of adenosine upon addition of initial extract to reaction mixture. Initial extract (100 µL) was added to 1mL of 1 mM adenosine in 50 mM Tris pH 7.2. The progress of the reaction was monitored by HPLC.
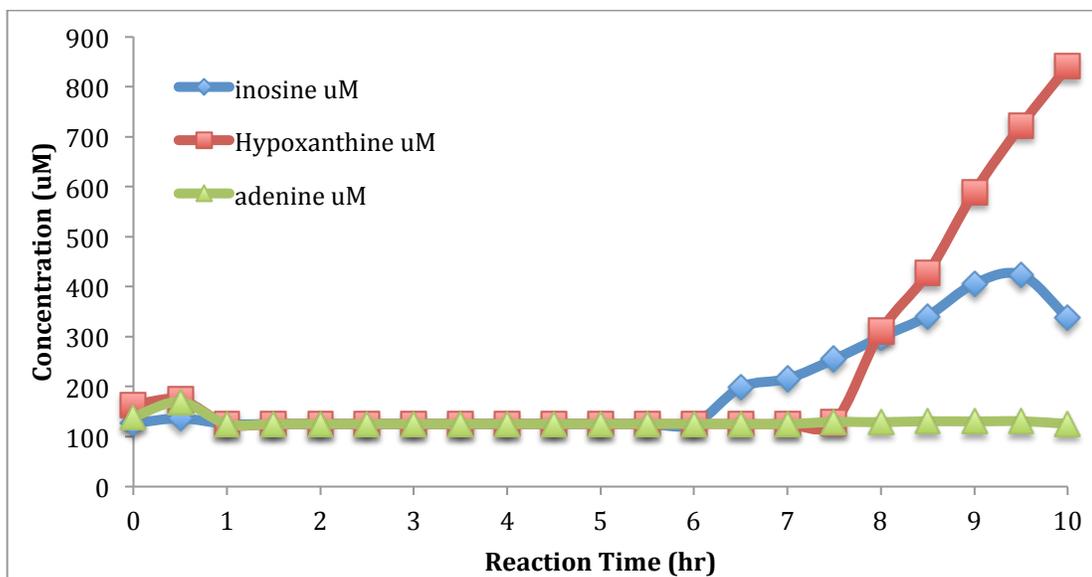
Figure 12b. Time course of concentration of products, inosine, hypoxanthine, and adenine upon addition of initial extract to reaction mixture.

hypothesis two mL of initial extract was dialyzed against 50 mM Tris buffer pH 7.2

(100 mL). The buffer was changed three times. The dialyzed initial extract was

assayed by HPLC as described above to determine the activity (Figure 13a, 13b). As

the results show, the adenosine concentration decreased quickly in a linear form

without the two phases seen with the undialyzed initial extract. The specific activity

of the dialyzed sample was 68×10^{-4} μmol/min/mg. This compares to specific activity

of 58×10^{-4} μmol/min/mg for the slow phase and 56×10^{-2} μmol/min/mg for the fast

phase of the undialyzed sample. Dialysis of the initial extract resulted in an extract

with a similar activity to the slow phase of the undialyzed sample. This indicates the

reason for the two phases is not the presence of an inhibitor. Adenosine deaminase

activity was apparent by the steady increase in inosine over the first 12 hours.

Adenosine (inosine) nucleosidase activity was also present as shown by the

appearance hypoxanthine. Surprisingly a low level of adenosine nucleosidase activity

was observed based on the small adenine peak.

The second step in the purification was an ammonium sulfate precipitation.

Solid ammonium sulfate was added to the initial extract to increase the saturation

level to 30%. The solution was kept overnight at 4°C, and centrifuged for 30 min

at $20000x\ g$ to remove precipitated proteins. After that, additional ammonium

sulfate was added to the 30% supernatant to raise the saturation level to 60%. The

solution was kept at 4°C overnight, then centrifuged for 30 min to remove

precipitated proteins. The pellet was resuspended in 50 mM Tris pH 7.2 (15 mL)

and dialyzed against 250 mL of 50 mM Tris buffer pH 7.2. The pellet was assayed
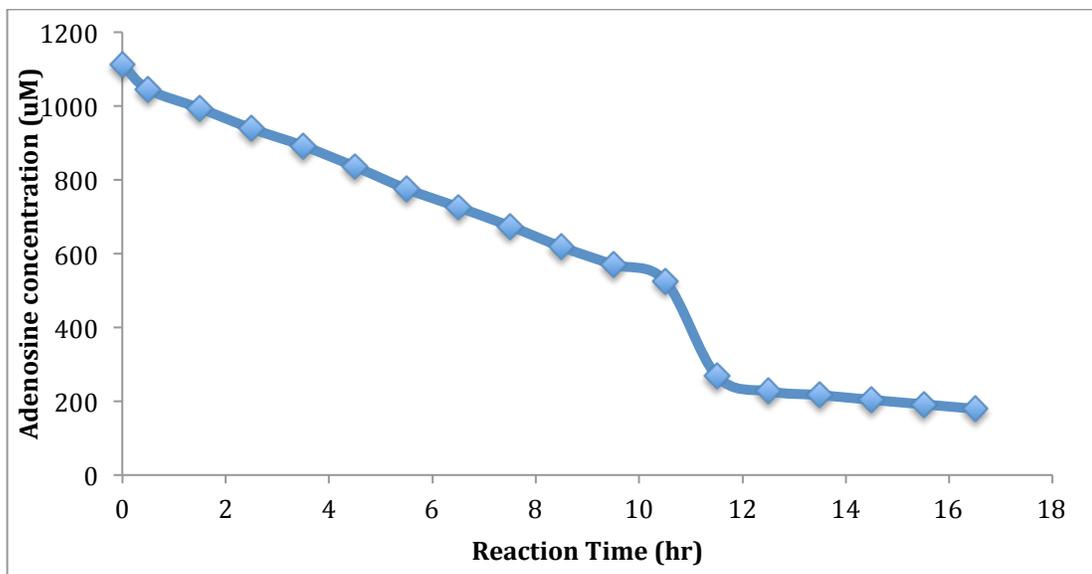
Figure 13 a**.** Changes in adenosine concentration upon addition of dialyzed initial

extract (100 μL) to 1mL of 1 mM adenosine in 50 mM Tris pH 7.2.
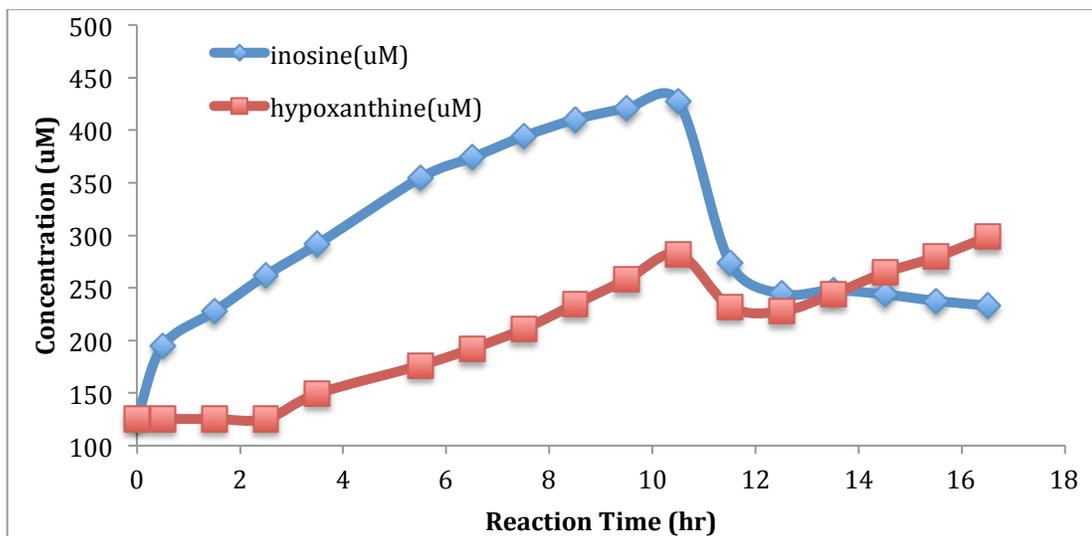
Figure 13 b: Changes in concentration of products inosine, and hypoxanthine,

during a 16-hour reaction after addition of dialyzed initial extract (100 μL) to

reaction mixture.

by HPLC with 1 mM adenosine as the substrate over a 15 hours period of time
(Figure 14).

As the reaction profile shows, two nucleoside metabolizing enzymes were
present, adenosine deaminase, and adenosine nucleosidase (Figure 14a, 14b).
Adenosine deaminase was present based on the presence of inosine. Adenosine
(inosine) nucleosidase was present based on the conversion of adenosine to
adenine and inosine to hypoxanthine. The two-phase time course was absent after
ammonium sulfate precipitation. The reaction was characterized by a relatively
linear decrease in concentration of adenosine over the first 4 hours followed by
increased activity over the next 5 hours. The specific activity was 14×10^{-3}
μmol/min/mg (Figure 14a, 14b). During the entire time course of the reaction the
adenine concentration remained low. Ultimately the majority of adenosine was
converted to hypoxanthine through the combined action of adenosine deaminase
and adenosine (inosine) nucleosidase.

After dialysis, the resuspended 60% pellet solution was subjected to the first
chromatography step, ion exchange on a DEAE Sephadex column. Ion-exchange
chromatography is an important method in protein purification. This method separates
proteins based on the electrostatic attraction between the stationary phase and the
proteins of the sample. Ion-exchange chromatography uses support materials such as
cellulose and Sephadex.[39] Ion-exchange chromatography can be classified as one of
two types based on the type of ligand used; anion exchange chromatography in which an
anion binds to a cation attached to the matrix or cation exchange chromatography in
which a cation binds to an anion groups on the matrix. Diethylaminoethyl (DEAE) is
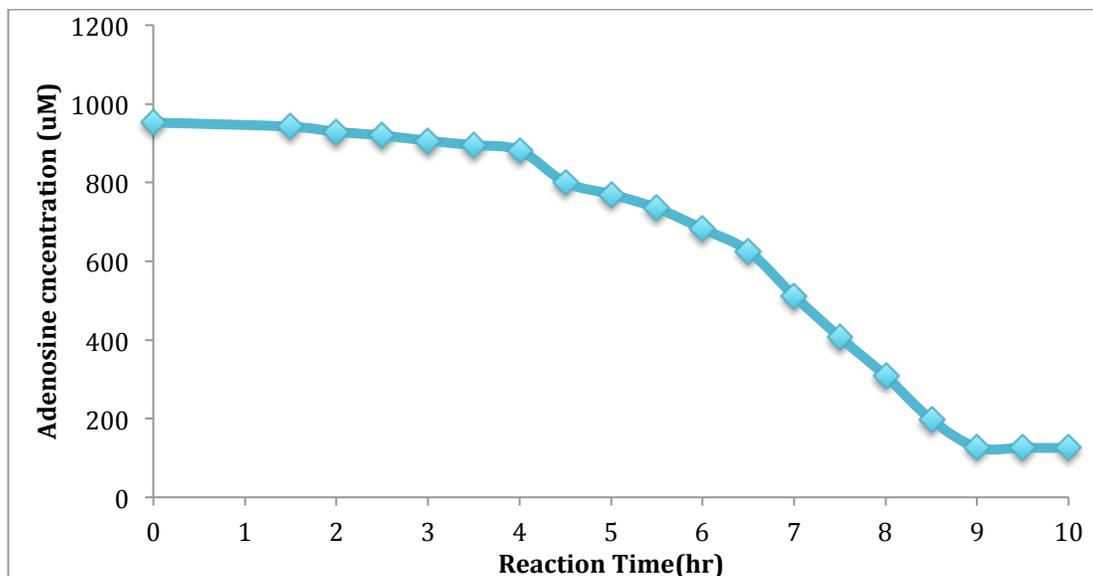
Figure 14 a. Changes in adenosine concentration after addition of aliquot from

resuspended 60% pellet to a reaction mixture containing 1 mM adenosine (1 mL)

in 50 mM Tris pH 7.2.
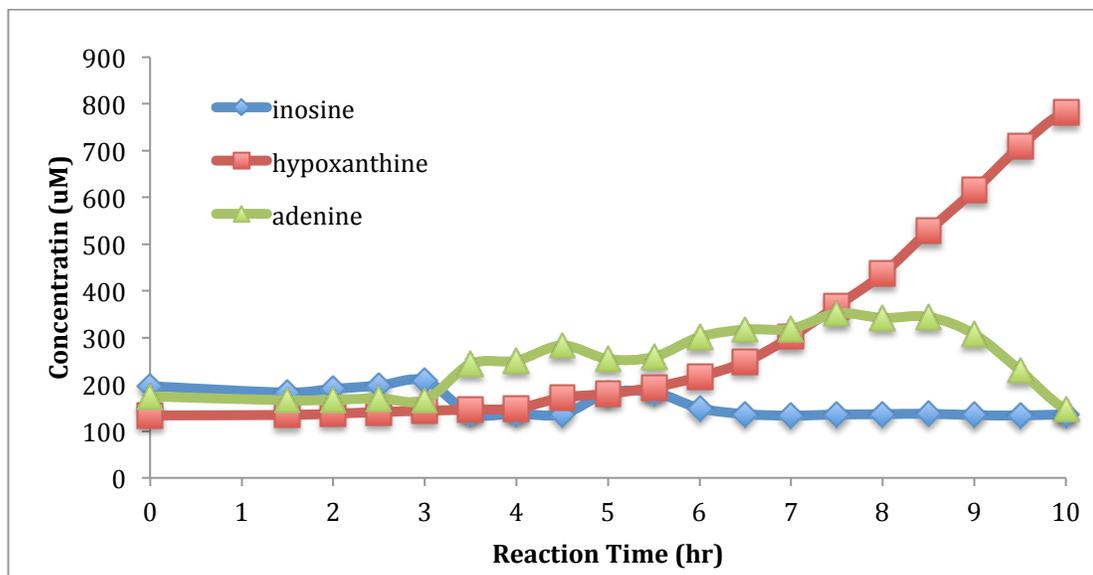
Figure 14 b. Changes in the concentrations of products, inosine, adenine, and

hypoxanthine during the 16-hour reaction upon addition 100 μL of resuspended 60%

pellet to 1mL of 1 mM adenosine in 50 mM Tris pH 7.2.

the most commonly used anion exchanger, while the carboxymethyl group (CM) is

the most frequently used cation exchanger. Proteins with an overall negative charge

bind to a positively charged resin while proteins with a positive charge bind to a

negatively charged resin. In ion exchange chromatography, the sample is dissolved in

a buffer and loaded onto the column. The column is washed with a low ionic strength

buffer to removed unbound proteins.[4]   Bound proteins are then eluted by increasing

the ionic strength or changing the pH of the buffer to disrupt the attraction between

the protein and the stationary phase.

The DEAE Sephadex column was washed with 50 mM Tris pH 7.2

followed a linear gradient of 0-1M NaCl in 50 mM Tris pH 7.2. The column

fractions were assayed for the activity and protein content. Two peaks were

visible in the elution profile (Figure 15). The smaller peak was eluted in the wash,

while the larger peak was eluted at approximately 400 mM NaCl. The larger peak

(Fractions 26-30) was pooled and concentrated. The time course of the reaction

was relatively linear although there was a discontinuity between hours 10-12 of the

reaction (Figure 17). The rate of the reaction between 0-9 hrs and 13-18 hrs, as

reflected in the slope of the lines, was constant. The 10-fold difference observed

in the time course of the initial extract was not present. The activity of the pooled

and concentrated fractions was determined by HPLC analysis using adenosine as a

substrate (Figure 16). Adenosine deaminase was still present based on adenosine

conversion to inosine. Hypoxanthine was also present indicating inosine

nucleosidase activity. No adenine was visible in HPLC chromatogram as it

coeluted with inosine. In addition only a linear decrease in adenosine
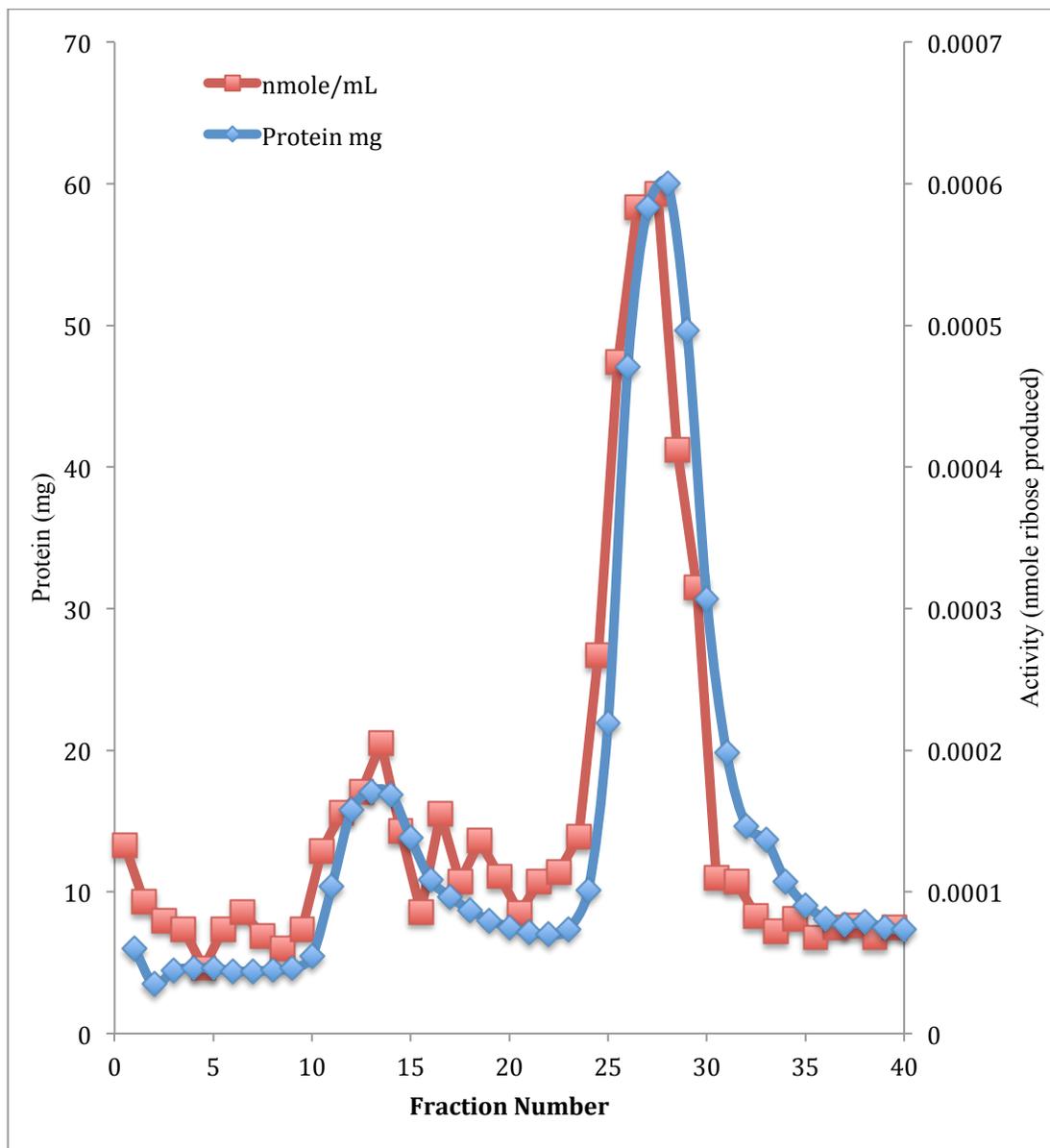
Figure 15. Elution profile from DEAE column (protein and activity). The elution buffer was 50 mM Tris pH 7.2, followed by a linear gradient of 0-1 M NaCl in 50 mM Tris pH 7.2. The gradient started at Fraction 13. The activity was based on the appearance of ribose as determined by reducing sugar assay. Fractions (26- 28) were pooled and concentrated.
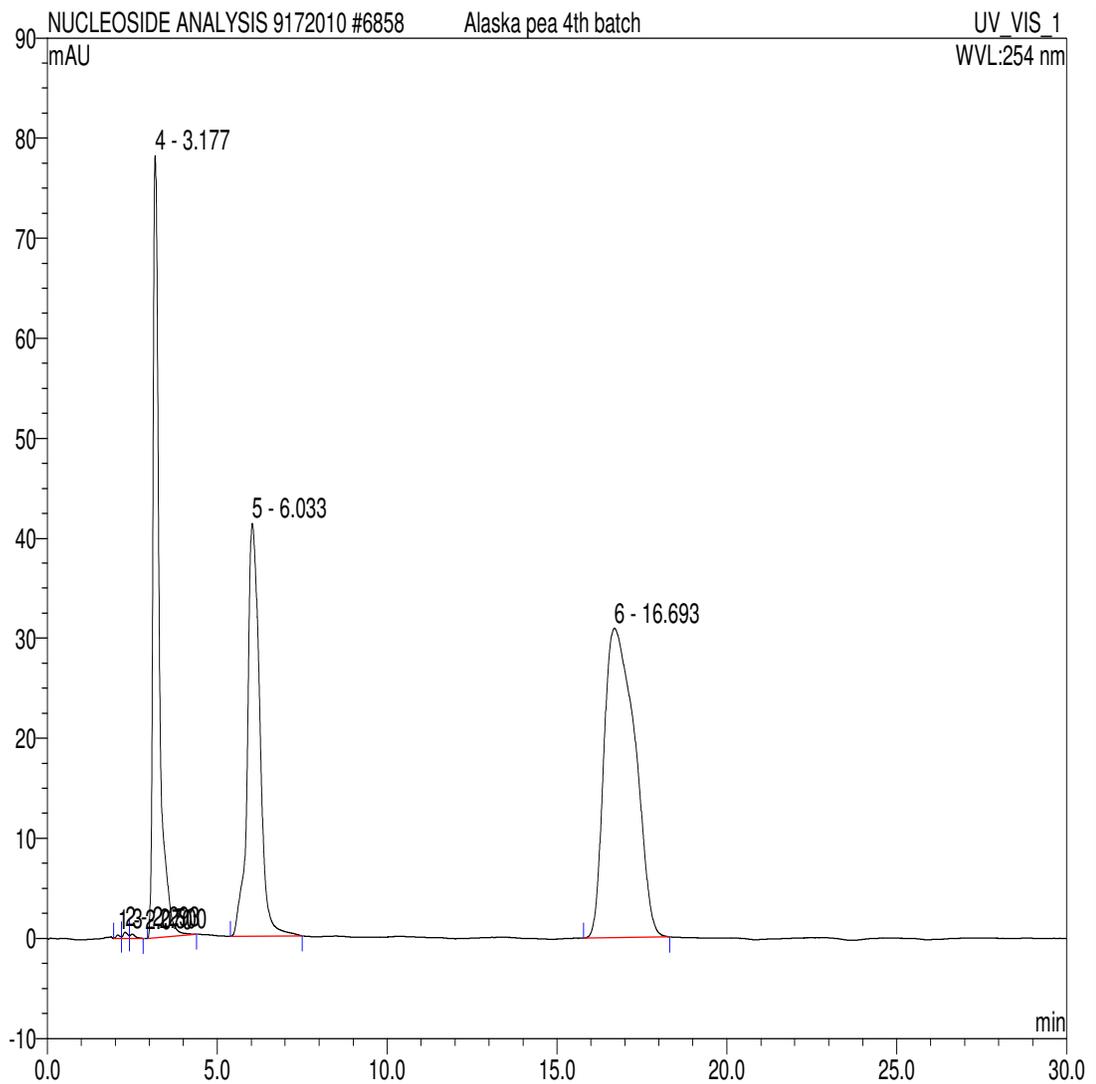
Figure 16. Analysis of reaction mixture 18 hrs after addition aliquot from pooled,

concentrated fractions (26-30) from DEAE ion exchange chromatography. Three

peaks appear: adenosine (16.69 min), inosine (6.03min), and hypoxanthine (3.17 min).

Adenine coeluted with inosine (6.03 min).
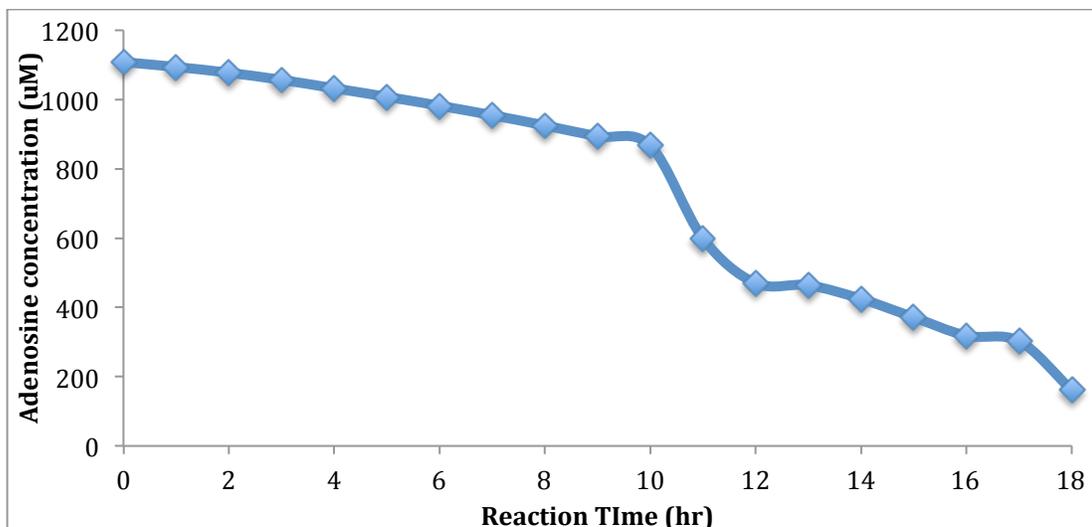
Figure 17 a. The decrease of adenosine concentration upon addition an aliquot after DEAE ion exchange chromatography. The reaction mixture consisted of 1 mM adenosine in 50 mM Tris pH 7.2. The reaction was initiated by addition of 100 µL from pooled fractions.
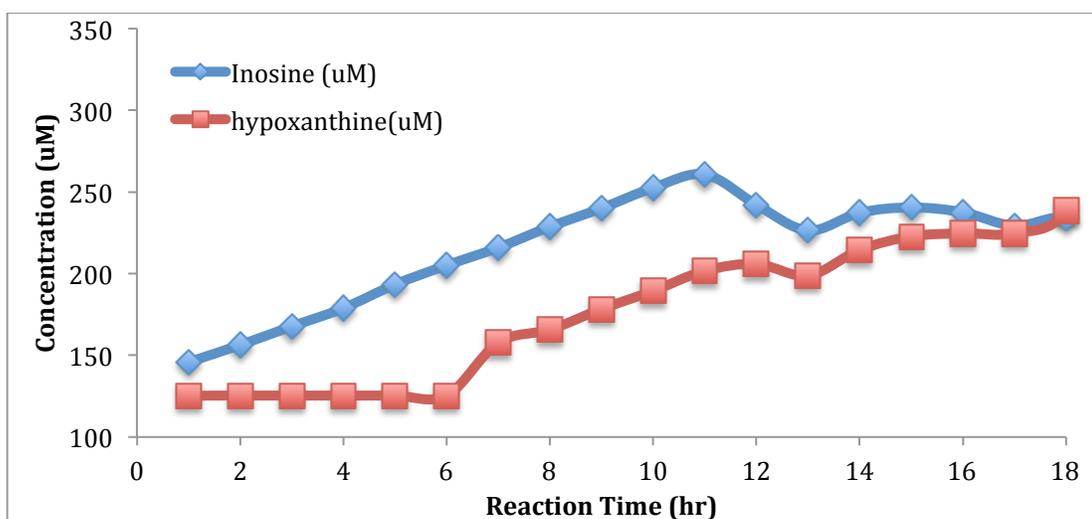
Figure 17 b. The increase in products, inosine and hypoxanthine, concentration upon addition of pooled concentration fractions after DEAE ion exchange chromatography.

concentration was apparent in contrast to the two phases seen in the reaction

progress curve seen after the initial extract and ammonium sulfate fractionation

(Figure 17). The specific activity was 24×10^{-3} µmole/min/mg.

The third step in the purification of the enzyme was chromatography on an ω-

aminohexyl agarose column. ω-Aminohexyl agarose is a type of affinity

chromatography in which an amino group is attached to a resin bead by a six-carbon

spacer (Figure 18).
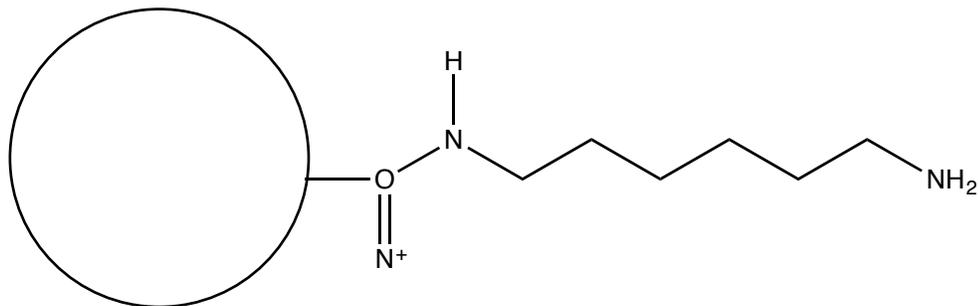
Figure 18. Structure of ω-aminohexyl-agarose resin.

It is believed the enzyme recognizes the amino group in a manner similar to its

recognition of the exocyclic amino group of adenosine. After dialysis the pooled

fractions from the DEAE Sephadex column were loaded onto an ω-aminohexyl

column. The column was washed with 50 mM Tris buffer pH 7.2, followed by a

linear gradient 0-1 M NaCl in 50 mM Tris pH 7.2. Several peaks were present in the

elution profile (Figure 19). Three protein peaks eluted in the linear gradient. Two

peaks, Fractions 25 and Fractions 68-71 contained very little activity. The main

activity peak, Fractions 32-35 overlapped the main protein peak. The largest peak

was pooled and concentrated. The pooled, concentrated fractions were assayed by
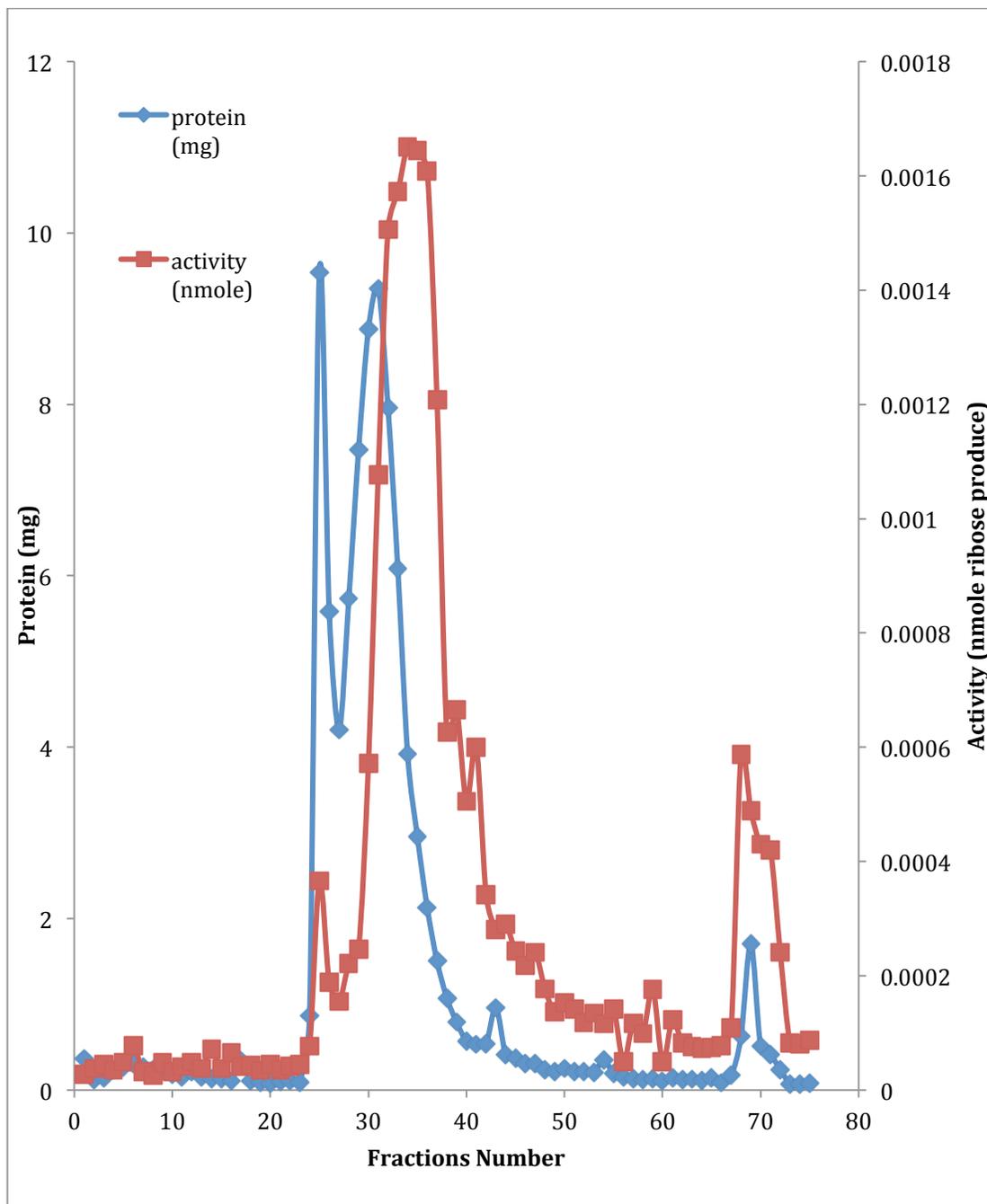
Figure 19. Elution profile from ω-aminohexyl column (activity (reducing sugar assay) and protein). The elution buffer was 50 mM Tris at pH 7.2. A linear gradient, 0-1 M NaCl, in 50 mM Tris pH 7.2 was used to elute the column. The gradient started at Fraction number 20. Fractions 32-35 were pooled and concentrated.

HPLC using adenosine as the substrate (Figure 20 a). Adenosine nucleosidase was present based on conversion of adenosine to adenine. Adenosine deaminase was also present due to appearance of hypoxanthine. The hypoxanthine was the end product of the combined action of two enzymes. Adenosine was converted to inosine, which was converted to hypoxanthine by adenosine deaminase and adenosine (inosine) nucleosidase respectively. The time course of the reaction was followed by HPLC over an 11-hour period (Figure 21). The reaction was essentially complete in 4 hours. The specific activity was 25×10^{-3} µmole/min/mg.

The final step in the purification was size exclusion chromatography on a Sephacryl S100 (26/60) FPLC column. Size exclusion columns separate proteins based on their size (i.e. molecular weight). Small molecules enter pores in the beads, thus moving slower through the column than the large molecules. The large molecules cannot enter the pores, so they pass between the beads. The most commonly used gels are Sephadex, Sephacryl, and Sepharose, which are manufactured by Pharmacia Fine Chemicals. These gels are available with different pore sizes allowing the separation of samples with different molecular weight distributions. [4, 39]

The column was washed with 100 mM sodium phosphate pH 7.2; 300 mM NaCl. The salt and relatively high buffer concentrations prevent nonspecific interactions between proteins and resin. Three peaks were visible in the elution profile (Figure 22). The largest peak was pooled (Fractions 21and 22) and concentrated. The specific activity was found to 26×10^{-2} µmole/min/mg (Figure 23).
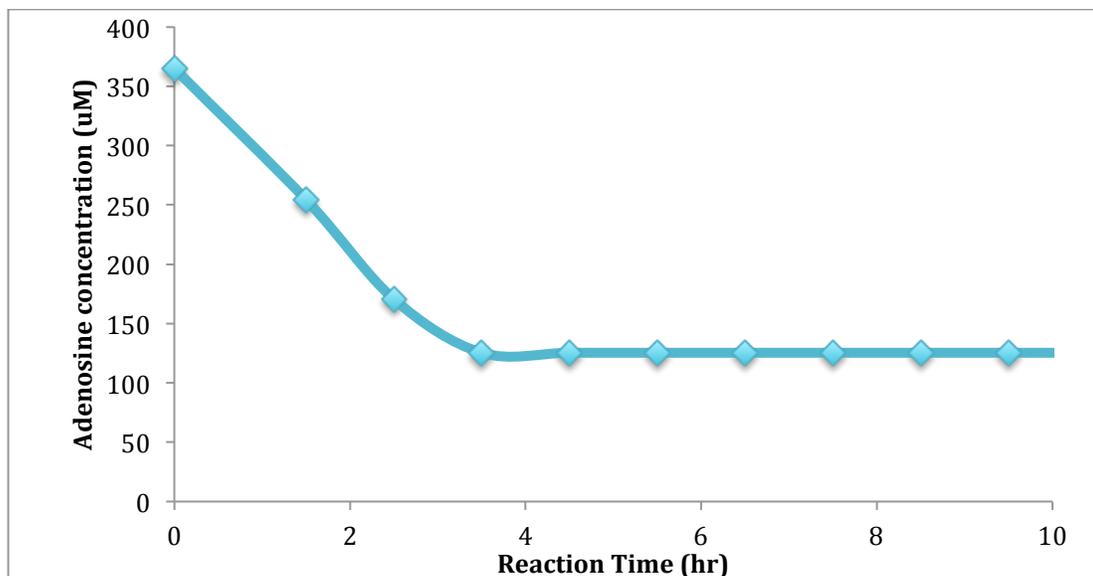
Figure 20 a. Activity of pooled concentrated sample from ω-aminohexyl-Sepharose

chromatography. The reaction mixture consisted of 1 mL of 1 mM adenosine in 50

mM Tris pH 7.2. The reaction was indicated by addition of 100 μL of enzyme.
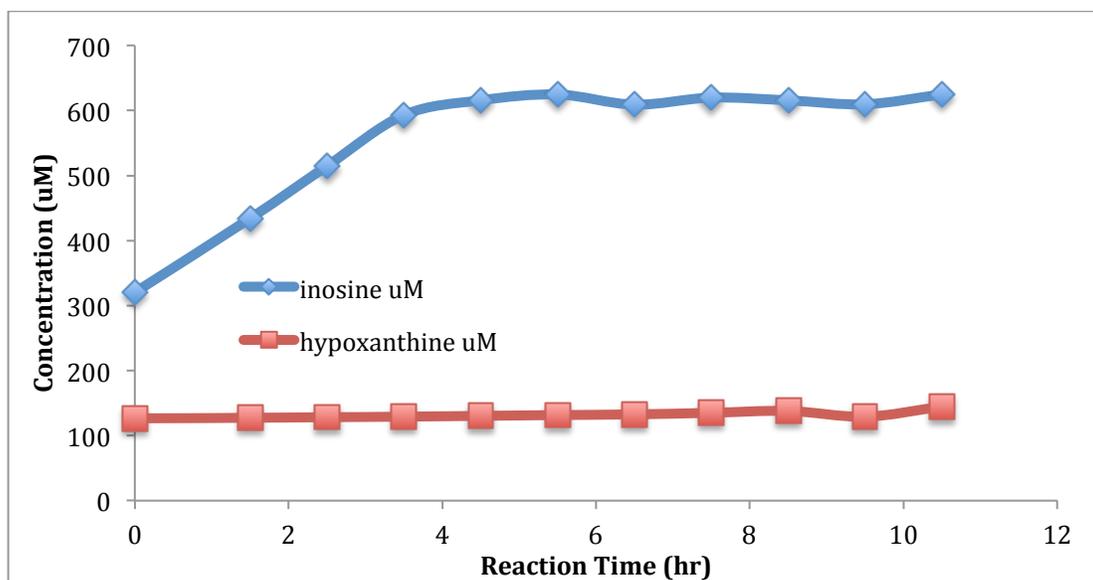
Figure 20 b. Changes in the concentration of products, inosine and hypoxanthine,

during 12 hour reaction from ω-aminohexyl column chromatography.
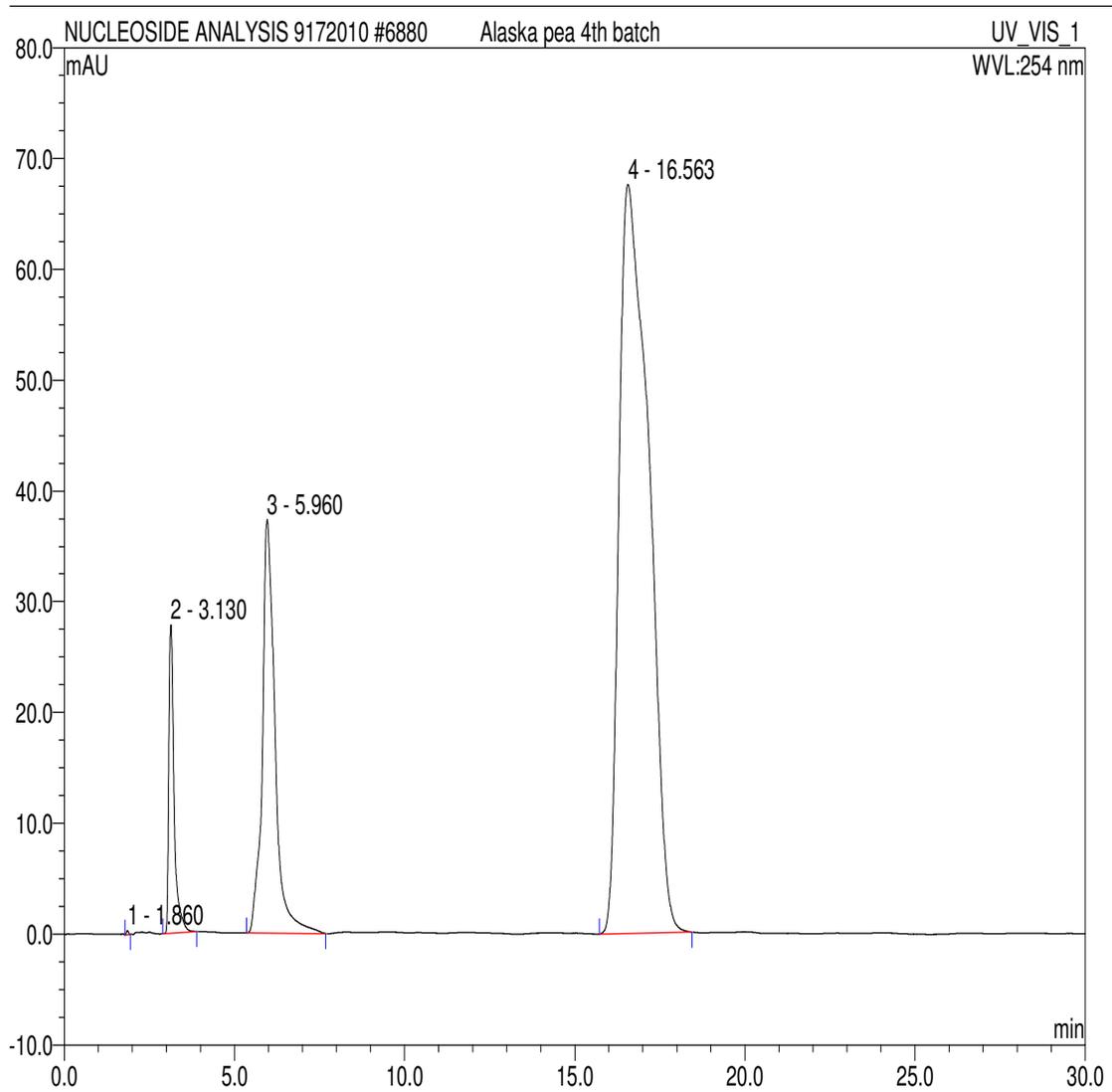
Figure 21. Analysis of the reaction mixture 16 hrs after addition of pooled concentrated fraction from ω–aminohexyl chromatography. Three peaks appear; adenosine (16.5 min), inosine (5.69 min), and hypoxanthine (3.13 min). Adenine coeluted with inosine.
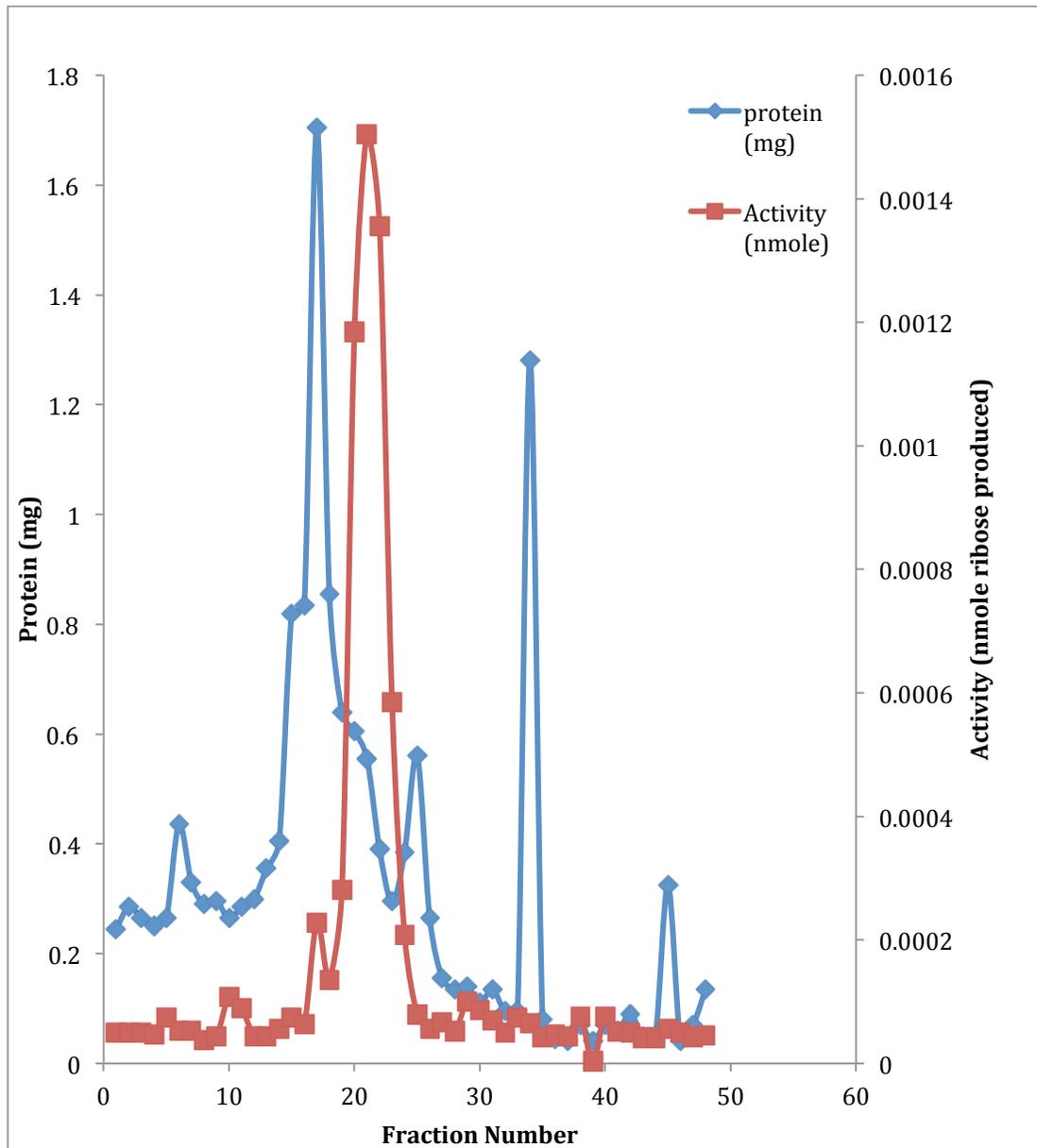
Figure 22. Elution profile from Sephacryl S100 (26/60) column chromatography

(activity and protein). The elution buffer was 100 mM sodium phosphate pH 7.2 with

300 mM sodium chloride, with a flow rate was 1 mL/min.
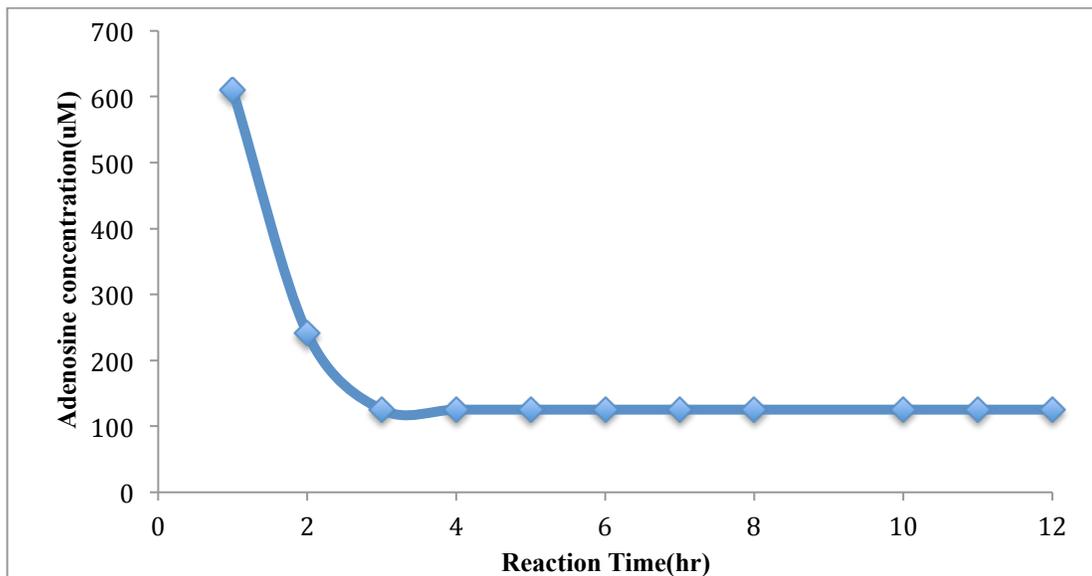
Figure 23 a. Activity of pooled and concentrated sample from size exclusion Sephacryl

S100 (26/60) column chromatography. The reaction was essentially complete after 2 hrs.
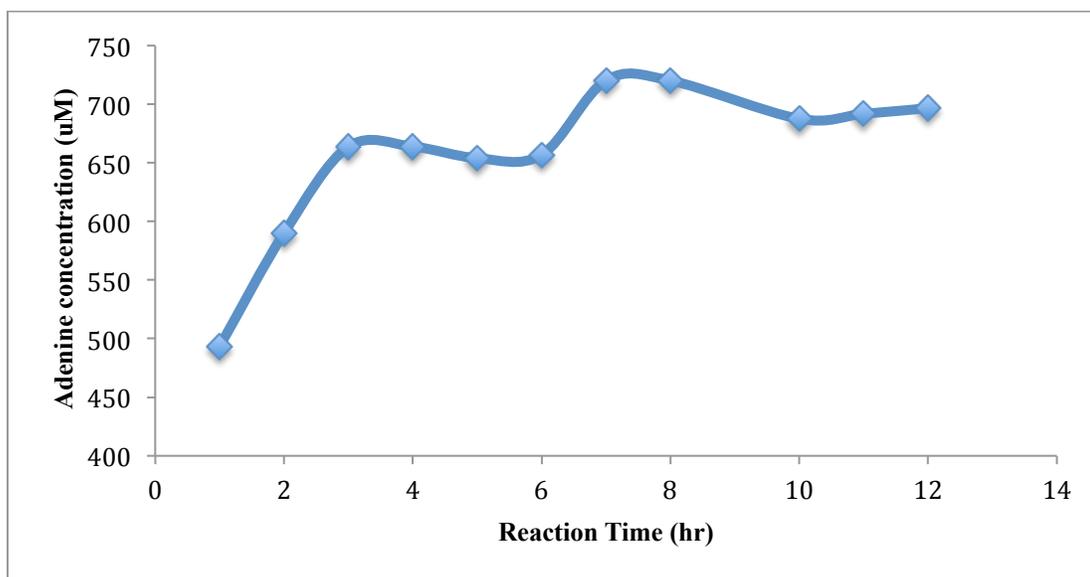
 Figure 23 b. Changes in the concentration of product, adenine, from size exclusion

Sephacryl S100 (26/60) column chromatography.

The activity of fractions 21 and 22 was analyzed by HPLC chromatography using 1 mM adenosine as substrate. Only adenosine (16.9 min) and adenine (6.1 min) were visible in the chromatogram after 1 hour. No hypoxanthine or inosine were present in the HPLC chromatography (Figure 24). This indicated no adenosine deaminase was present in the final enzyme solution.

Molecular Weight of Enzyme (SDS-PAGE)

The purity and subunit molecular weight of adenosine nucleosidase from Alaska pea seeds was determined by SDS-PAGE. A calibration curve was constructed using the standard molecular weights of known proteins and the molecular weight of the subunit was determined to be 36 kDa. A second minor band is visible on the gel indicating the protein is not completely pure (Figure 25, 26). The subunit molecular weight of this enzyme is similar to most nucleoside hydrolases isolated from plants. Most of the plant nucleoside hydrolases have subunit molecular weights in the 30-36 kDa ranges. For example, *Coffea* arabica has a molecular weight of 34.6 kDa, [17] and 33 kDa from yellow lupin.[18]

Summary of purification steps

Adenosine nucleosidase was isolated from *pisum sativum* L.cultivar Alaska in a series of purification steps (Table 3). The initial extract had a specific activity of in its slow phase of 58×10^{-4} to 56×10^{-3} µM/min/mg in its fast phase. The second step
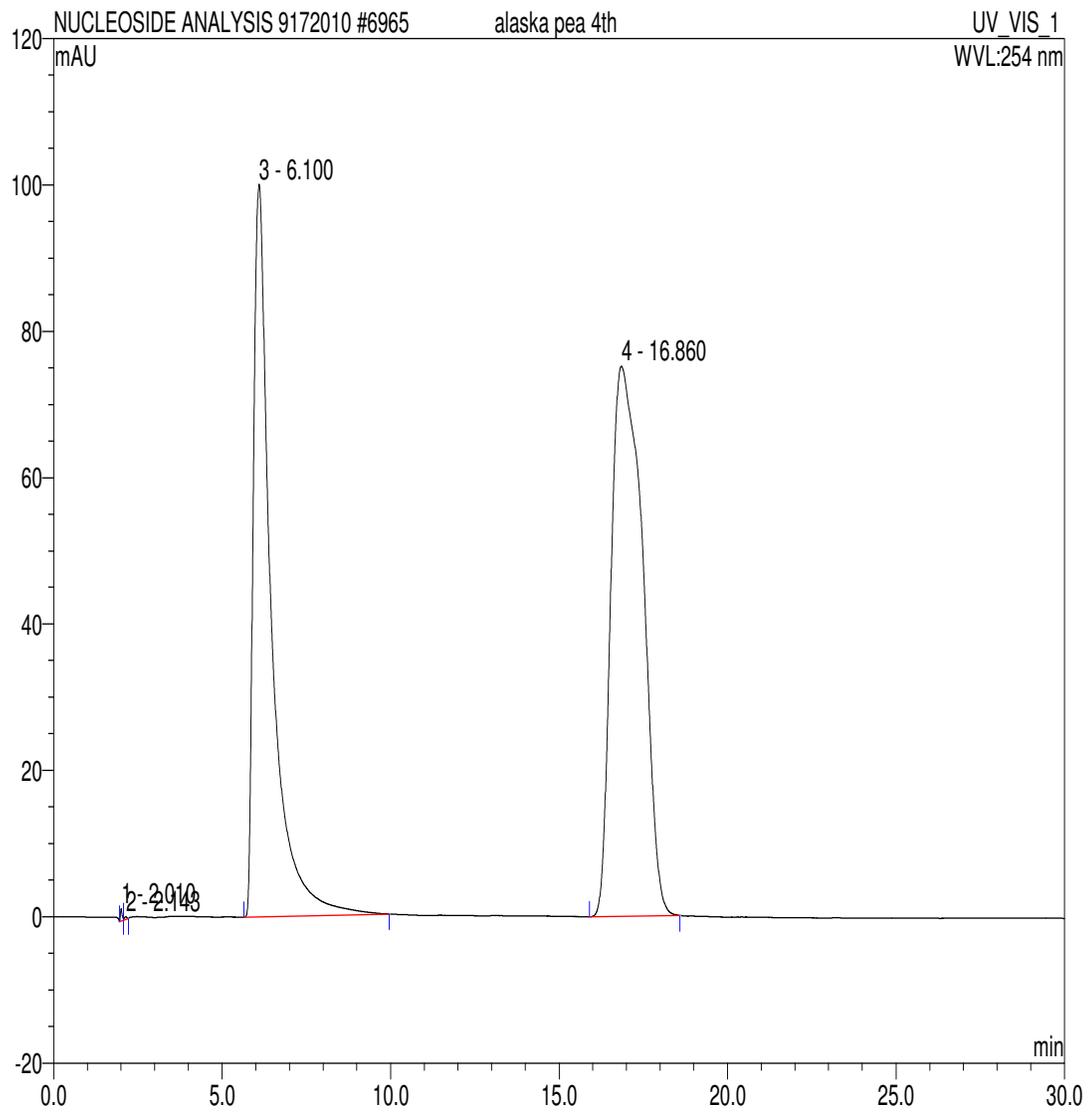
Figure 24. Analysis of the reaction mixture 1 hr after addition of pooled, concentrated

fraction (100 μL) from size exclusion Sephacryl S100 (26/60) column. Two peaks

appear; adenosine (16.8 min), and adenine (6.1 min).
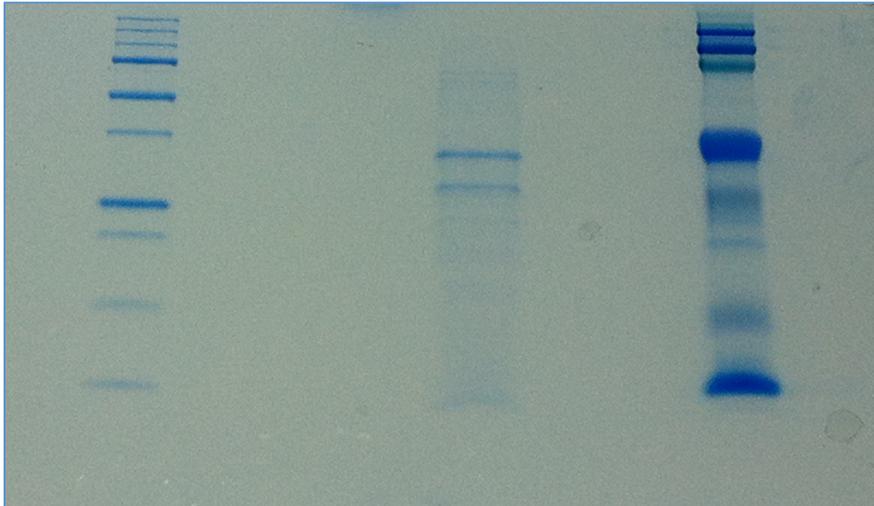
Figure 25. Determination of the subunit molecular weight using SDS-PAGE from pooled and concentrated fraction (20) from size exclusion Sephacryl S100 (26/60). Lane 1- Precision Plus Protein Unstained Markers, Lane 2- Fraction 20 from Sephacryl S100 FPLC column, and Lane 3- Kaleidoscope Protein Markers

$$y = -1.6869x + 2.4839$$
$$R^2 = 0.98563$$
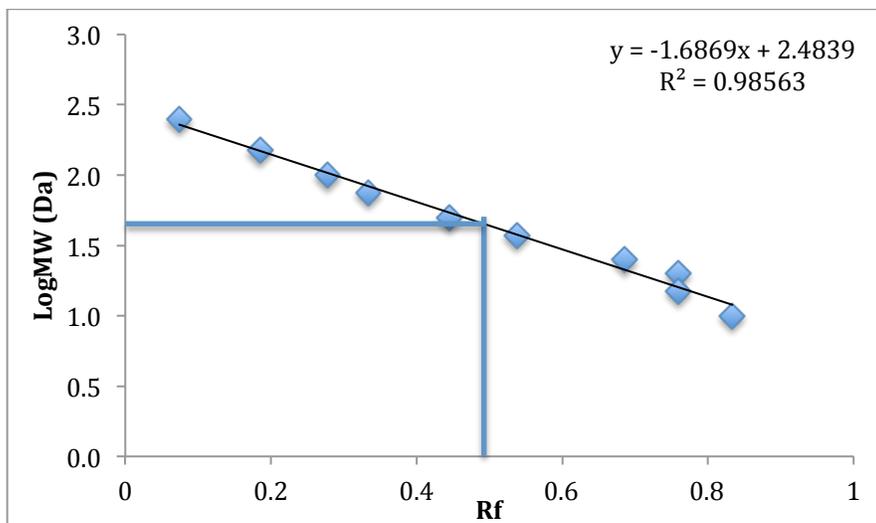
Figure 26. Calibration curve based on molecular weights of Precision Plus Protein [TM] Unstained Standard calibration curve. The molecular weight of adenosine nucleosidase from Alaska pea seed was determined to be 36 kDa.

in the purification scheme was the precipitation of proteins between 30 and 60%

ammonium sulfate saturation. After adding ammonium sulfate, the sample was

centrifuged at 20000xg for 30 min. The pellet from this step was suspended in 25 mL

of 50 mM Tris buffer pH 7.2. Because of the two activity phases it was difficult to

calculate the increase in specific activity. Based on the increase in specific activity

from the slow phase of 58×10^{-4} to 14×10^{-3} μmole/min/mg after the ammonium

sulfate step, there was a 2.4-fold purification increase. The third step was the first

chromatography step and was an ion exchange step using DEAE resin. The specific

activity of the pooled concentrated solution was found to be 24×10^{-3} μmole/min/mg

based on reducing sugar assay. This gave a purification-fold of 4 with a recovery of

activity of 10.2%. The fourth step, ω-aminohexyl-Sepharose chromatography

resulted in a specific activity increase to 25×10^{-3} μmole/min/mg. This gave a

purification-fold of 4.2 with a recovery yield of 5.7%. Finally gel filtration

chromatography on Sephacryl S100 resulted in a specific activity of 26×10^{-2}

μmole/min/mg. This gave a final purification-fold of 44 with a recovery of 3%.

The results from this purification are consistent with the purification of other

nucleoside-metabolizing enzymes from seed sources. For example Guranowski

isolated nucleosidase from yellow lupin seeds with an overall recovery of 6.8% and a

purification fold of 65.[15] Another enzyme, adenosine nucleosidase was also isolated

from yellow lupin seeds with a purification fold of 146 and an overall recovery of

5.5%. [18]

Purification	Volume (mL)	Total Protein (mg)	Activity μmole/min	Specific Activity μmole/min/ mg	Purification fold	Yield %
Initial Extract	184	253.92	1497.96	58×10^{-4}	1	100
			14455.3	56×10^{-3}		
Ammonium Sulfate 60% pellet	25	22.25	314.99	14×10^{-3}	2.4	21
DEAE- Ion Exchange	12	6.24	151.2	24×10^{-3}	4	10
ω-aminohexyl-Sepharose	5	3.41	84.79	25×10^{-3}	4.2	6
Sephacryl S100 size exclusion	1	0.15	40.38	26×10^{-2}	44	3

Table 3. Summary of adenosine nucleosidase purification from Alaska pea seeds.

CHAPTER IV

CONCLUSIONS

Alaska pea seeds were tested for the presence of a number of nucleoside-metabolizing enzymes. Three classes of enzymes were tested, nucleosidases, phosphorylases, and deaminases. A number of enzyme activities were present. Both pyrimidine and purine nucleosidases were present in the seeds along with the corresponding phosphorylases. The activity of the phosphorylases was less than the corresponding nucleosidases (hydrolases). Three deaminases were detected, adenosine deaminase, adenine deaminase, and cytidine deaminase. However cytosine deaminase activity was not detected.

Adenosine nucleosidase has been isolated from Alaska pea seeds (*pisum sativum* L.cultivar Alaska), to near homogeneity from germinated seeds. The major contaminating protein was adenosine deaminase. The activity of this enzyme was present until the final purification step. The overall recovery of activity of adenosine nucleosidase was 3% with a purification fold of 44. The specific activity of the enzyme was 26×10^{-2} μmole/min/mg.

Based on SDS-PAGE analysis the subunit molecular weight of adenosine nucleosidase from Alaska pea has a molecular weight of approximately 36 kDa.

REFERENCES

1. Steyaert, J.; Versées, W. Kinetic analysis of the purine nucleoside hydrolase mechanism. Ph.D. Dissertation, Vrije universiteit Brussel, An Vandemeculebrouke, Schaarbeek, 2008.

2. Grisham, G. Enzyme Kinetics and Specificity. Biochemistry 3rd edition, Thomson, Brook/core: Ivy, VA, January 2004.

3. Introduction to Enzymes. http://www.w orthington-biochem.com/introbiochem/default.html (accessed October 3, 2011).

4. 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.

5. Zrenner, R.; Stitt, M.; Sonnewald, U.; Blodt, R. Pyrimidine and purine biosynthesis and degradation in plants. *Ann. Rev. Plant Bio.* **2006,** *57:*805-836.

6. Moffatt, B.; Ashihara, H. Purine and pyrimidine nucleotide synthesis and metabolism. *The Arabidopsis Book,* **2002**, doi/101199/tab0018.

7. ITO, T.; Shiraishi.; Okada, K.; Shimura, Y. Two amidophosphoribosyltransferase genes of *Arabidopsis thaliana* expressed in different organs. *Plant Molecular Biology*. **1994,** 26, 529-533

8. Stasolla, C,; Katahira, R,; Thorpe, T,; Ashihara, H. Purine and pyrimidine metabolism in higher plants. *Plant Physiol.* 2003, *160*, 1271-1295.

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

10. Giabbai, B,; Degano, M. Crystal Structure to 1.7 Å of the *Escherichia coli* Pyrimidine Nucleoside Hydrolase YeiK, a Novel Candidate for Cancer Gene Therapy. Structure **2004***,12*, 739-749.

11. Craig, S.P.; Eakin, A.E. Purine phosphoribosyltransferase. *J. Biol. Chem.* **2000,** *275*, 20231-20234.

12. Gopaul, D,; Meyer, S,; Degano, M,; Sacchettini, J,; Schramm, V. Inosine−Uridine Nucleoside Hydrolase from *Crithidia fasciculata*. Genetic Characterization, Crystallization, and Identification of Histidine 241 as a Catalytic Site Residue. *J. Am. Chem. Soc.* **1996,** 35, 5963-5970.

13. Degano, M.; Gopaul, D.N.; Scapin, G.; Schramm, V.; Sacchettini, J.C. Three-Dimensional Structure of the Inosine-Uridine Nucleoside N-Ribohydrolase from *Crithidia fasciculata. Biochemistry.* **1996**, 35, 5971-5981**.**

14. Estupinan, B.; Schramm, V. Guanosine-Inosine-preferring Nucleoside N-Glycohydrolase from *Crithidia fasciculata*. J. Biol. Chem. **1994**, 37, 23068-23073.

15. Koszalka, G.; Krenitsky, T. A. Nucleosidases from *Leishmania donovani.* Pyrimidine ribonucleosidase, purine ribonucleosidase and a novel purine 2'-deoxyribonucleosidase. *J. Biol. Chem.* **1979,** 254, 8185-8193.

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

17. Campos, A.; Rijo-Johansen, M.; Carneiro, M. F.; Fevereiro, P. Purification and characterization of adenosine nucleoside from *Coffea arabica* young leaves. *Phytochemistry* **2005**, 66, 147-151.

18. 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.

19. Guanowski, A.; Schneider, Z. Purification and characterization of adenosine nucleosidase from barley leaves. *Biochim. Biophys. Acta* **1977**, 482, 145-158.

20. Bennett, E.M.; Chenglong, L.; Allan, P.W.; Parker, W.B.; Ealick, S.E. Structural Basis for Substrate Specificity of *Escherichia coli* Purine Nucleoside Phosphorylase. *J. Biol. Chem.* **2003**, 278, 47110-47118.

21. Szuwart, M.; Starzyńska, E.; Pietrowska-Borek , M.; Guranowski , A. Calcium-stimulated guanosine-inosine nucleosidase from yellow lupin (*Lupinus luteus*). *Phytochemistry.* **2006**, 67, 1476-1485.

22. Katahira, R.; Ashihara, H. Profile of purine biosynthesis, salvage and degradation in disks of potato (*Solanum tuberosum*. L). *Planta.* **2006**, 225, 115-126.

23. Allen, M.; Qin, W.; Moreau, F.; Moffatt, B. Adenine phosphoribosyltransferase isoforms of *Arabidopsis* and their potential contributions to adenine and cytokinins metabolism. *Physiologia Planterum.* **2002**, 115, 56-58.

24. Deng, W.; Ashihara, H. Profiles of Purine Metabolism in Leaves and Roots of *Camellia sinensis* Seedlings. *Plant Cell Physiol.* **2010***,* 51, 2105-2118.

25. Kafer, C.; Thornburg, R. Pyrimidine metabolism in plants. Path Pyrimid. **1999**,7,14-24.

26. Zhou, L.; Lacroute, F.; Thornburg, R. Cloning expression in *Escherichia coli* and characterization of *Arabidopsis thaliana* uridine 5'-monophosphate/cytidine 5'-monophosphate kinase. *Plant Physiol.* **1998**, 117, 245-253.

27. Stasolla, C.; Loukanina, N.; Ashihara, H.; Yeung, E.C.; Thorpe, T.A. Comparative studies on pyrimidine metabolism in excised cotyledons of *Pinus radiata* during shoot formation *in vitro*. *J. Plant Physiol.* **2007**, 164, 429-441.

28. Ireton, G.C,; Black, M.E,; Stoddard, B.L. The 1.14 Å Crystal Structure of Yeast Cytosine Deaminase: Evolution of Nucleotide Salvage Enzymes and Implications for Genetic Chemotherapy. *Structure.* **2003**, 7, 961-972.

29. Vincenzetti, S.; Cambi, A.; Neuhard, J.; Schnorr, K.; Grelloni, M.; Vita, A. Cloning, expression, and purification of cytidine deaminase from *Arabidopsis thaliana*. *Protein Express Purif.* **1999**, 15, 8-15.

30. Evans, C.C. Adenosine deaminase. *University of Georgia.* *http://ccevans.myweb.uga.edu/bcmb8010/report.pdf.*

31. Elshafei, A. M.; Mohammad, L. A.; Ali, N. H. Purification and characterization of adenosine deaminase from *Penicillium politans*. *J. Appl. Sci. Res.* **2007,** 3, 1010-1016.

32. Brady, T.G.; Hegarty, V.J. Investigation of plant seeds for adenosine deaminase. *Nature (London),* **1966**, 209, 1027-1028.

 33. Turner, J.F.; King, J.E. Inosine 5′-phosphate dehydrogenase of pea seeds. *Biochem. J.* **1961**, 79, 147-151.

34. Cosentino-Gomes, D.; Meyer-Fernandes, J.R. Reversible inhibition of tyrosine protein phosphatase by redox reaction. In Enzyme Inhibition and Bioapplications [Online]; Sharma, R. Eds.; 2012;Intech;Chapter 8, www. www.intechopen.com/books/enzyme-inhibition-and-bioapplications.

35. Guo, Yan-Lin; Roux, S.J. Partial purification and characterization of an enzyme from pea nuclei with protein tyrosine phosphatase activity. *Plant Physiol.* **1995**. *107,* 167-175.

36. O'Neal, T.; Naylor, A.W. Partial purification and properties of carbamoyl phosphate synthetase of Alaska pea. *Biochem. J.* **1969**, 113, 271-279.

37. Chen, Chon-Maw, Petschow B. Metabolism of cytokinin: Ribosylation of cytokinin bases by adenosine phosphorylase from wheat germ. *Plant Physiol.* **1978**, 62, 871–874.

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

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