CHARACTERIZATION OF ADENOSINE NUCLEOSIDASE FROM SOYBEAN SEEDS (*Glycine max* L.)

by

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In loving memory of my beautiful grandmother, Mrs. Leola Moore

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ABSTRACT

The nucleoside salvage pathway plays a key role in the growth and development of plants. The activities of several enzymes of the nucleoside salvage pathway were determined in germinated soybean seeds. One of these enzymes, adenosine nucleosidase, was purified 5 days after germination period. The purification scheme consisted of ammonium sulfate precipitation, ion exchange chromatography, size exclusion, and aminohexyl chromatography. The enzyme was then characterized with regard to its isoelectric point and subunit molecular weight. Adenosine nucleosidase from soybean seeds was determined to most likely be a multimeric enzyme with a subunit molecular weight of approximately 18,000 Da based on SDS-PAGE.

The specific activity of the initial extract was 16 x 10^{-2}µmol/min mg. After increasing the ammonium sulfate to 60% and dialyzing the resulting precipitate, the specific activity increased to 62 x 10^{-2} µmol/min mg. The specific activity of adenosine nucleosidase increased to 2.44µmol/min mg after the final column purification step. The purification fold and percent recovery for adenosine nucleosidase is 14.79 and 0.38%, respectively.

The isoelectric point of the enzyme was determined using an OFFGEL electrophoresis fractionator and found to be approximately 1.5.

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CHAPTER ONE

INTRODUCTION

There is a continual cycle of life and death in every form of plant life. The beginning of life in most plants begins with a single seed. Once the seed is provided adequate amounts of light and water, and temperature conditions are met, germination occurs. Germination is the reactivation of all biomolecular reactions that were conserved within the seed while dormant [1]. One of the most important of the initial series of reactions that occurs in the life of a plant is the synthesis and degradation of nucleotides. These reactions then occur continually. Nucleotides are used as an energy source and as precursors in reactions producing primary and secondary products such as sucrose, polysaccharides, and phospholipids [2]. They also provide the "building blocks" for RNA and DNA. Because of these reasons it is essential that plants are able to continually synthesize and degrade these molecules.

RNA and ribonucleotides are especially important during the growth and development of maturing seeds. RNA is responsible for the production of proteins and the ribonucleotides are important for the energy transferring reactions of cellular metabolism[3]. During germination, respiration usually dramatically increases and then decreases as more cellular tissue forms and matures. While this is occurring the once stored proteins begin to breakdown and form pools of amino acids[4]. The amino acids are then recycled and used to form other proteins some of which are soluble proteins know as enzymes[5]. The unique arrangement of the amino acids gives an enzyme its own unique characteristic and function. Enzymes are biomolecules that are needed in order to synthesize other organic molecules[5].

1.1 Nucleic acids and nucleotides

Nucleotides consist of a nitrogen base, a pentose sugar, and one or more phosphate groups (Figure 1). There are two types of nucleotides based on the type of

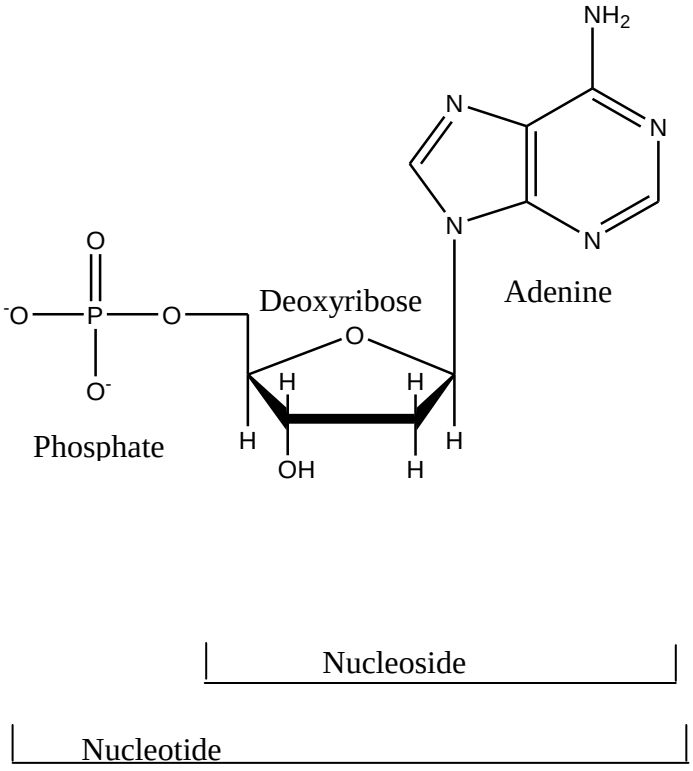
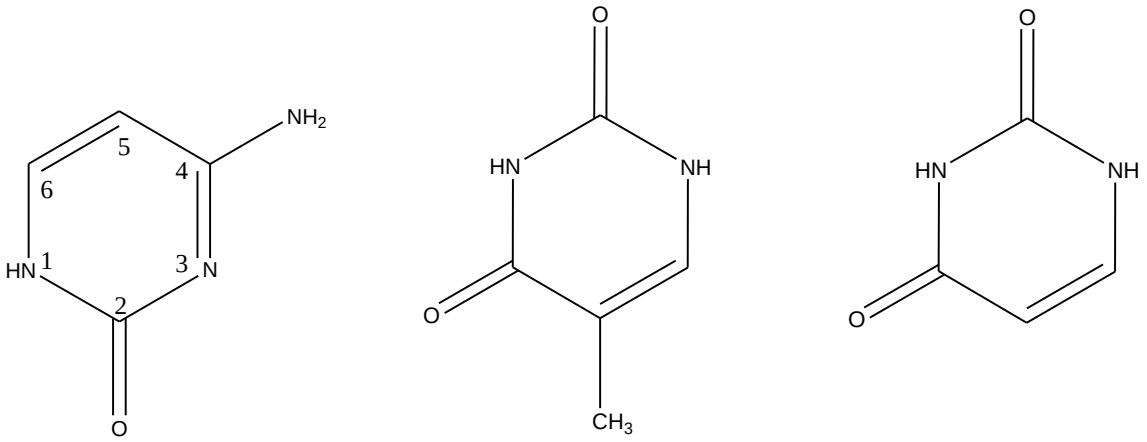


Figure 1. Structure of a nucleotide and nucleoside. The structure illustrated is deoxyadenosine-5'-monophosphate (dAMP).

nitrogen base, pyrimidines and purines. In plants, purines and pyrimidines can be found in other forms such as free bases and nucleosides[6]. Nucleosides are formed when the phosphate groups have been removed from a nucleotide (Figure 1). Pyrimidines consist of a six-membered ring containing two nitrogen atoms at the first and third positions. There are three main pyrimidines: thymine, cytosine, and uracil (Figure 2). Thymine is



Thymine Cytosine Uracil

Figure 2. Structure of pyrimidine bases. Pyrimidines consist of a six-membered ring containing two nitrogen atoms at the first and third positions. There are three main pyrimidines bases: thymine, cytosine, and uracil.

typically found in deoxyribonucleic acid (DNA) while uracil is present only in ribonucleic acid (RNA). Cytosine is found in both DNA and RNA.

Purines consist of two conjoined heterocyclic aromatic rings, pyrimidine and imidazole[7]. Adenine, guanine, hypoxanthine and xanthine are considered to be purine bases (Figure 3). Adenine and guanine both appear as bases in DNA and RNA. However, hypoxanthine and xanthine are usually seen as intermediates in the synthesis of adenine and guanine, rather than as bases in nucleic acids[8].

In addition to being components of nucleic acids, individual bases and nucleosides play important roles. One group of phytohormones, the cytokinins, are adenine derivatives of adenosine[9] (Figure 4). Cytokinins are plant growth hormones

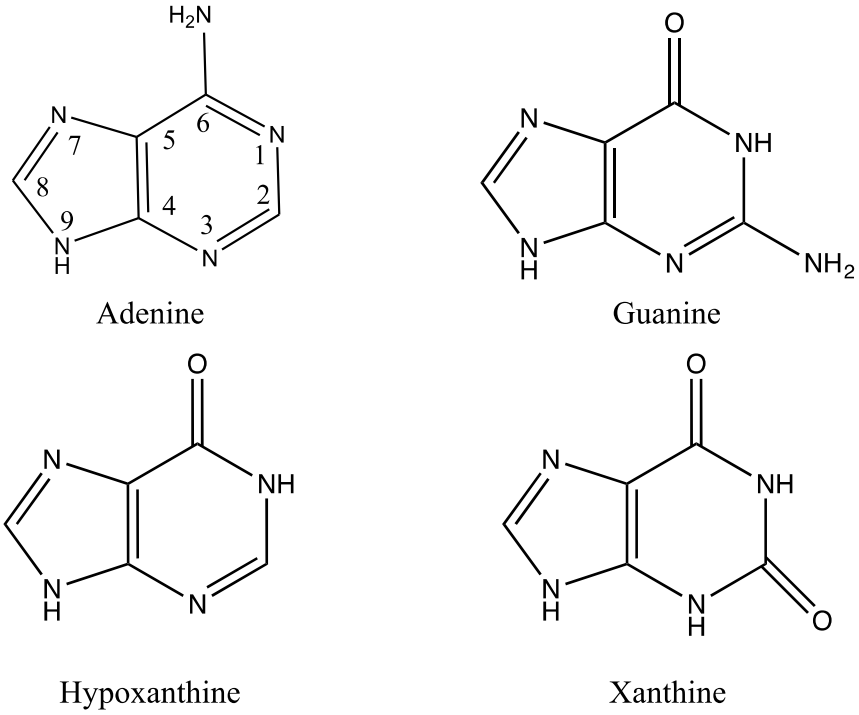


Figure 3. Structure of purine bases. Purines consist of two conjoined heterocyclic aromatic rings, pyrimidine and imidazole. There are four main purine bases: adenine, guanine, hypoxanthine, and xanthine.

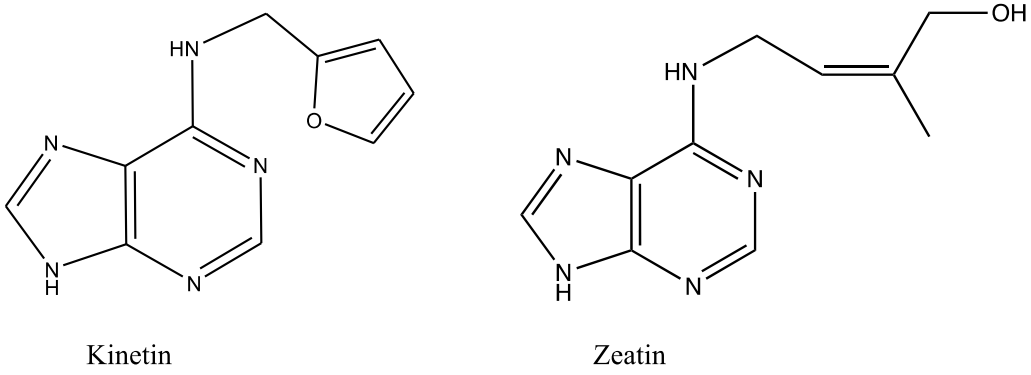


Figure 4. Structures of phytohormones, the cytokinins. Cytokinins are derivatives of adenine in which a group has been added to the exocyclic amino group.

which stimulate cytokinesis or cell division[10]. They are very influential in photosynthesis, chloroplast differentiation, and chlorophyll degradation[11]. The enzyme adenosine kinase, which phosphorylates adenosine to adenosine monophosphate, (AMP) also, uses cytokinin ribosides as a substrate[12].

In addition to these purine derivatives, there are methylated purines such as caffeine[8] and theobromine[13] (Figure 5). The physiological role of these methylated

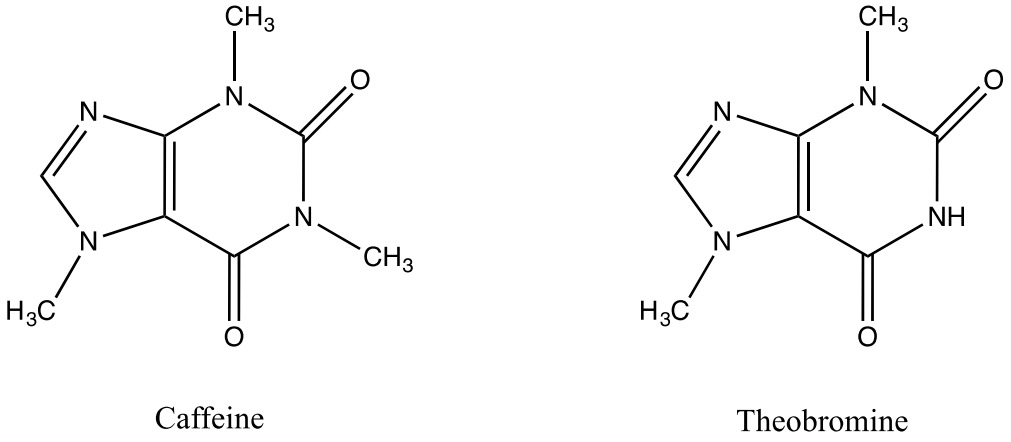


Figure 5. Structures of methylated purines found in plants. Caffeine and theobromine are derivatives of xanthine.

purines is to protect a plant against natural predators such as insect larvae[13]. In plants, the biosynthesis of caffeine's major route begins with xanthosine. The xanthosine is provided from the XMP derived from purine *de novo* biosynthesis. However, theobromine has two biosynthetic pathways. The major pathway starts with AMP and the alternative uses guanosine monophosphate (GMP). Both AMP and GMP are supplied by the purine salvage pathway. There is also a third possible route. Xanthine is converted to theobromine by 3N-methyltransferase followed by 7N-methyltransferase[13].

Although pyrimidines and purines nucleotides can be found in animals and plants, the free base forms of the purines and pyrimidines are only found in plants[7]. This is primarily due to the degradation of other organic compounds found in plants[7]. For example adenosine is produced during methylation reactions in which S-adenosylmethionine is the methyl group donor.

1.2 Biosynthesis of Pyrimidines and Purines

In plants, pyrimidine and purine nucleotides can be synthesized by two distinctive pathways, *de novo* and salvage. The *de novo* synthesis of a pyrimidine begins with the orotic acid pathway which produces orotate[14]. After this ring structure is closed, a series of reactions occurs catalyzed by 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetase, orotate phosphoribosyltransferase, orotidine-5'-phosphate decarboxylase, uridine monophophate kinase, uridine diphosphate kinase and cytidine triphosphate synthetase, respectively[15]. This sequence of enzymatic reactions attaches the orotate to a ribose phosphate and converts it to one of the pyrimidines (Figure 6).

Unlike pyrimidines, the *de novo* pathway for purines is initiated by PRPP which is used to produce 5-phosphoribosylamine with the transfer of the amido group from glutamine catalyzed by amido phosphoribosyltransferase. Subsequent steps to produce a purine nucleotide use the enzymes: glycineamide ribonucleotide (GAR) synthetase, glycineamide ribonucleotide (GAR) formyl transferase, formyl glycine amidine ribonucleotide (FGAM) synthetase, 5-aminoimidazole ribonucleotide (AIR) synthetase, 5-aminoimidazole ribonucleotide (AIR) carboxylase, 5-aminoimidazole-4-N-succinocarboxyamide ribonucleotide (SAICAR) synthetase, adenylosuccinate lyase, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) formyltransferase, inosine

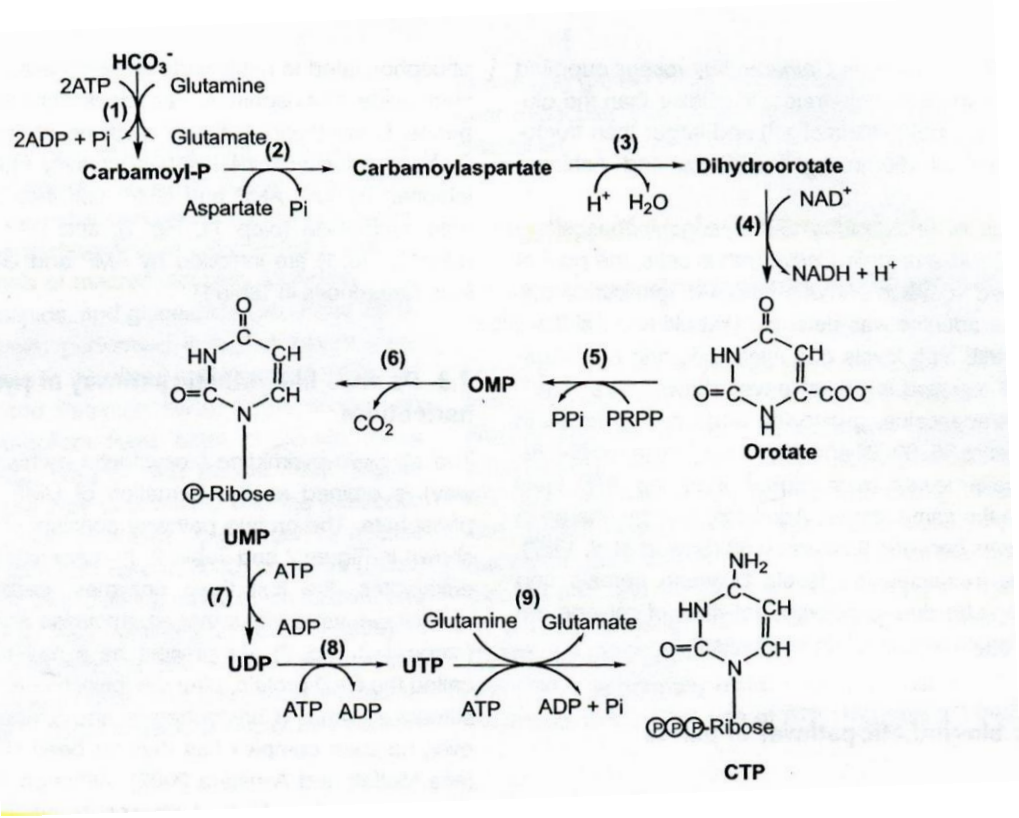


Figure 6. *De novo* synthesis of pyrimidines. (1) carbamoyl phosphate synthetase, (2) aspartate transcarbamoylase, (3) dihydroorotase, (4) dihydroorotate dehydrogenase, (5) UMP synthase (orotate phosphoribosyltransferase plus orotidine-5'-phosphate decarboxylase), (7) UMP kinase, (8) nucleoside diphosphate kinase, (9) CTP synthetase. Reprinted with permission[21].

monophosphate (IMP) cyclohydrolase, adenylosuccinate (sAMP) synthetase, adenylosuccinase, IMP dehydrogenase, and GMP synthetase [15]. This reaction sequence also uses other smaller molecules including carbon dioxide (CO_2), amino acids, and adenosine triphosphate (ATP)[13] (Figure 7).

The salvage pathway for purine nucleotides in plants is believed to be a more direct and efficient way of converting free bases and nucleosides in the early stages of germination[16]. Although salvage pathways differ among plants species, they consume

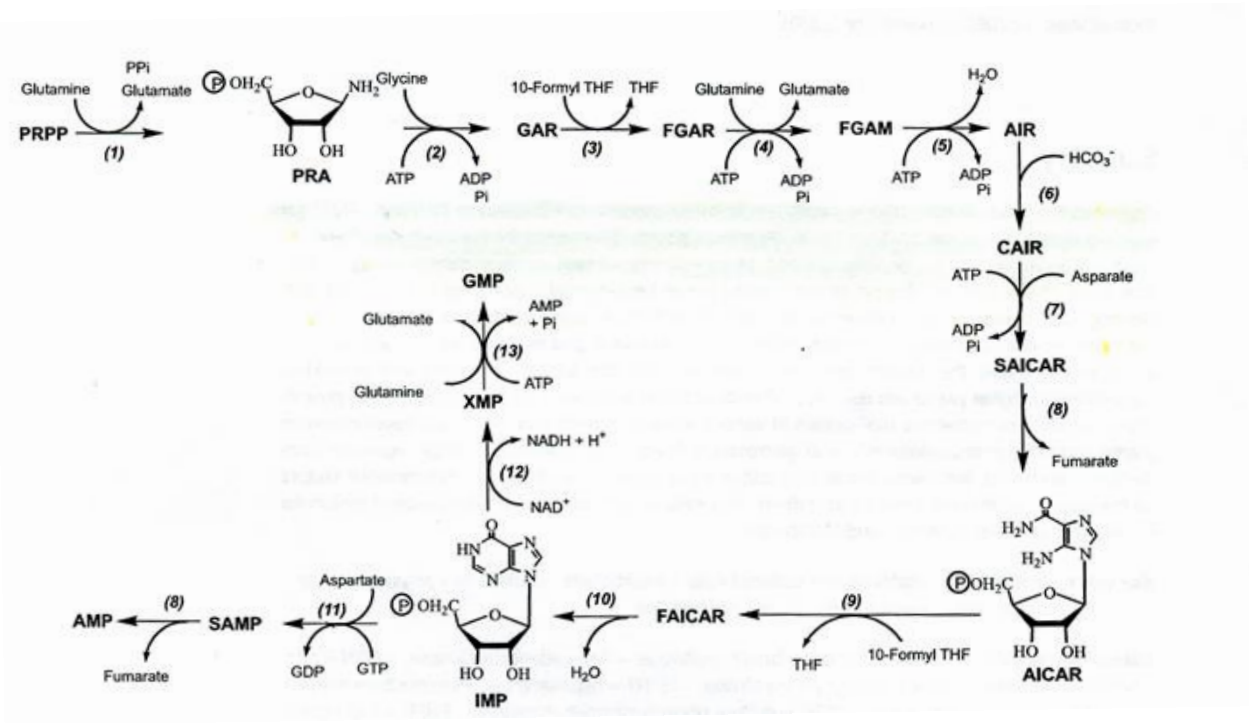


Figure 7. *De novo* synthesis of purines. (1) amido phosphoribosyltransferase, (2) GAR syntheatse, (3) GAR formyl transferase, (4) FGAM synthetase, (5) AIR synthetase, (6) AIR carboxylase, (7) SAICAR synthetase (8) adenylosuccinate lyase, (9) AICAR formyl transferase, (10) IMP cyclohydrase, (11) SAMP synthetase, (12) IMP dehydrogenase, (13) GMP synthetase. Reprinted with permission[21].

less adenosine triphosphate (ATP) than the *de novo* pathways and are usually a one–step pathway[17]. For example, the one-step reaction catalyzed by adenosine phosphoribosyltransferase is one in which a free base, such as adenine, reacts with 5'-phosphoribosyl-1-pyrophosphate (PRPP) to yield adenine nucleotide[18] (Figure 8). Similarly, guanine and hypoxanthine are salvaged by hypoxanthine-guanine phosphoribosyltransferase (Figure 9). It has also been demonstrated that 5'-nucleotidase and phosphatases can catalyze the hydrolysis of nucleotides[19]. These pathways enable

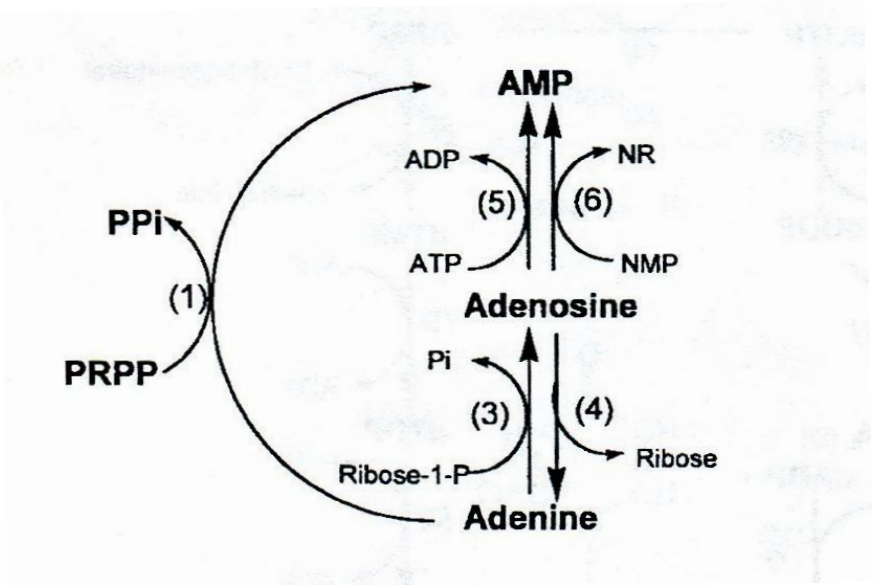


Figure 8. Adenine salvage pathway. (1) adenine phosphoribosyltransferase, (2) hypoxanthine-guanine phosphoribosyltransferase, (3) adenosine phosphorylase, (4) adenosine nucleosidase. Reprinted with permission[21].

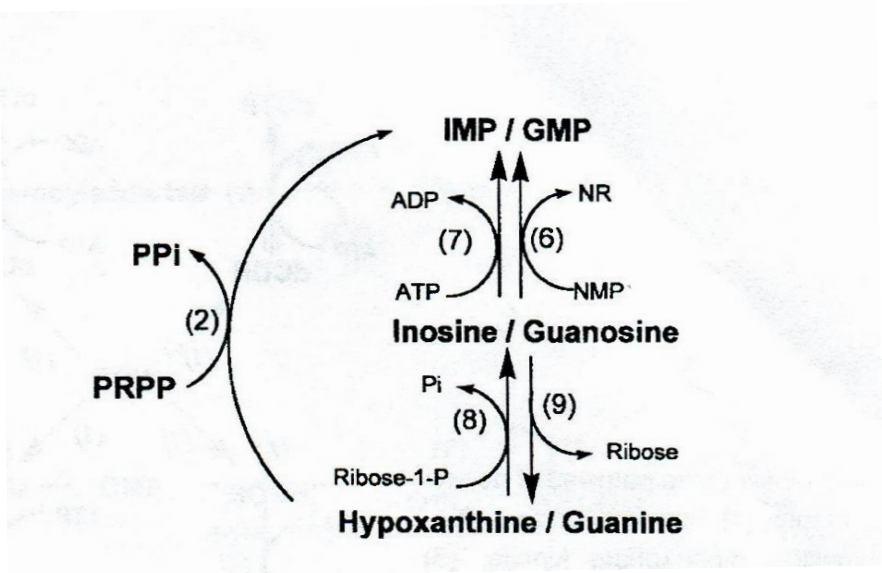


Figure 9. Guanine and hypoxanthine salvage pathway. (2) hypoxanthine-guanine phosphoribosyltransferase, (6) non-specific nucleoside phosphotransferase, (7) inosine-guanosine kinase, (8) inosine-guanosine phosphorylase, (9) inosine-guanosine nucleosidase. Reprinted with permission[21].

the plant to continually reutilize free bases and nucleosides that are the product of constant nucleic acid breakdowns.

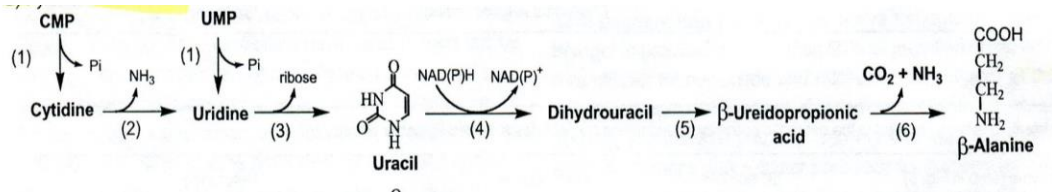
Pyrimidines also have a salvage pathway that uses less energy as well as reusing nucleosides and nucleobases. In the salvage pathways of pyrimidines, they all have a common enzyme that they share in order to result in their respective nucleotide. This enzyme is called nucleoside phosphotransferase. Cytidine has two possible salvage routes. The first route uses three enzymes, cytidine deaminase, nucleoside phosphotransferase, and uridine kinase which results in uridine monophosphate (UMP)[15]. The second route is more direct and produces cytidine monophosphate (CMP)[15]. The salvage pathway of thymidine uses thymidine kinase (TK) and nucleoside phosphotransferase[15]. This reaction yields deoxythymidine monophosphate. And lastly, the most direct route of conversion of uracil to UMP uses uracil phosphoribosyltransferase (UPRT)[15].

1.3 Purine and Pyrimidine Catabolism

Pyrimidine and purine catabolism in plants produce byproducts that are reused in photosynthesis, photorespiratory glycolate, and glutamine: 2-oxoglutarate amidotransferase (GOGAT) pathways[20]. The compounds produced by these pathways are NH_3, CO_2 and glyoxylate[21]. Purines and pyrimidines are catabolized by oxidative or reductive pathways[21]. There are only two catabolic pathways for pyrimidine bases, one for uracil and one for thymine. Cytosine is not an intermediate in any catabolic pathway due to a lack of cytosine deaminase[21]. Rather CMP is dephosphorylated to cytidine then to uridine by 5-nucleotidase/phosphatase and cytidine deaminase respectively. Uridine is hydrolyzed by uridine nucleosidase producing uracil and ribose. Similarly,

deoxythymidine monophosphate (dTMP) is converted to thymidine which is then hydrolyzed to thymine and ribose or ribose-1-phosphate by 5'-nucleotidase/phosphatase and thymidine phosphorylase/nucleosidase respectively[21] (Figure 10A and Figure 10B).

A)



B)

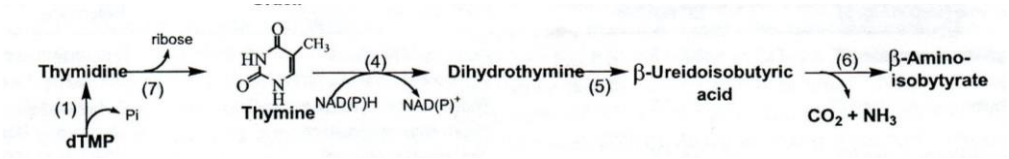


Figure 10. Two catabolic pathways for pyrimidine bases:
(A) Uracil: (1) 5'-nucleotidase and/or phosphatase, (2) cytidine deaminase, (3) uridine nucleosidase, (4) dihydrouracil dehydrogenase, (5) dihydropyriminase, (6) β –ureidopropionase (**B)** Thymidine: (1) 5'-nucleotidase and/or phosphatase, (7) thymidine phosphorylase and/or thymidine nucleosidase, (4) dihydrouracil dehydrogenase, (5) dihydropyriminase (6) β –ureidopropionase. Reprinted with permission[21].

Uracil and thymine are catabolized by the same four enzymes in sequential order ending with the products β- alanine or β-aminobutyrate and the byproducts listed previously[21].

In purine catabolism, xanthine is the primary product because all purines are converted to xanthine before ring cleavage occurs. The purine catabolic pathways tend to involve some type of deamination then dephosphorylation followed by glycosidic cleavage. Once adenosine monophosphate (AMP) is converted to inosine monophosphate (IMP) by deamination there are two different proposed metabolic pathways to the central

metabolite[20] (Figure 11). Degradation of adenine nucleotides is dependent on adenosine monophosphate deaminase (AMPD) in plants because adenine deaminase is not present in plants[17].

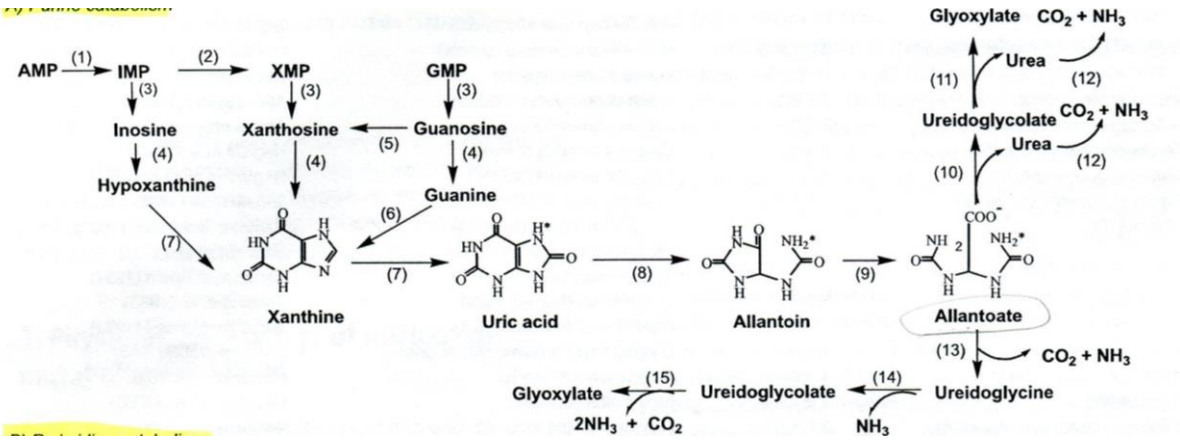


Figure 11. Catabolism of purines. (1) AMP deaminase, (2) IMP dehydrogenase, (3) 5'-nucleotidase and/or phosphatase, (4) inosine-guanosine nucleosidase, (5) guanosine deaminase, (6) guanine deaminase, (7) xanthine dehydrogenase, (8) uricase, (9) allantoinase, (10) allantoicase, (11) ureidoglycolate lyase, (12) urease, (13) allantion deaminase, (14) ureidoglycine amidohydrolase, (15) ureidoglycolate hydrolase. Reprinted with permission[21].

In soybeans, inosine monophosphate dehydrogenase (IMPDH), 5'-nucleotidase and inosine/guanosine nucleosidase are utilized to produce xanthine[20]. Other plants use secondary route enzymes to produce xanthine; 5'-nucleotidase/ phosphatase, inosine/guanosine nucleosidase and xanthine dehydrogenase (XDH) sequentially[22]. Uric acid is formed after both metabolic routes produce xanthine by XDH. Uric acid is then converted to allantoin by uricase. Subsequently, allantoin is converted to allantoate (Figure 11). Tropical legumes such as soybeans and cowpeas use the ureides allantoin and allantoate as storage and transport compounds for nitrogen[20]. Once allantoic acid is formed, it may be degraded by two different pathways. One route produces

ureidoglycolate while the other produces ureidoglycine. However, both are ultimately metabolized into NH_3, CO_2 and glyoxylate[21]. Although, these products are important to the reutilization of nitrogen in plants, the pathway has another function useful to plants. The pathway is also responsible for the synthesis of the methylated xanthine derivatives, theobromine and caffeine[20]. As previously noted these compounds are a natural defense against insects and other plant species[20].

1.4 S-adenosyl-1-methionine (SAM)

Within each plant cell, there is a continual degradation of adenylate and deoxyadenylate during embryogenesis caused by the presence of S-adenosyl-1-methionine (SAM)[23]. SAM is the primary methyl donor to various molecules such as nucleic acids, proteins and chlorophyll. Once SAM has donated its methyl group to the accepting molecule it is converted to S-adenosyl-L-homocysteine (SAH). In the presence of SAH hydrolase, L-homocysteine and adenosine are produced from SAH[24]. In order for the methylation to continually occur, the byproducts need to be removed (Figure 12).

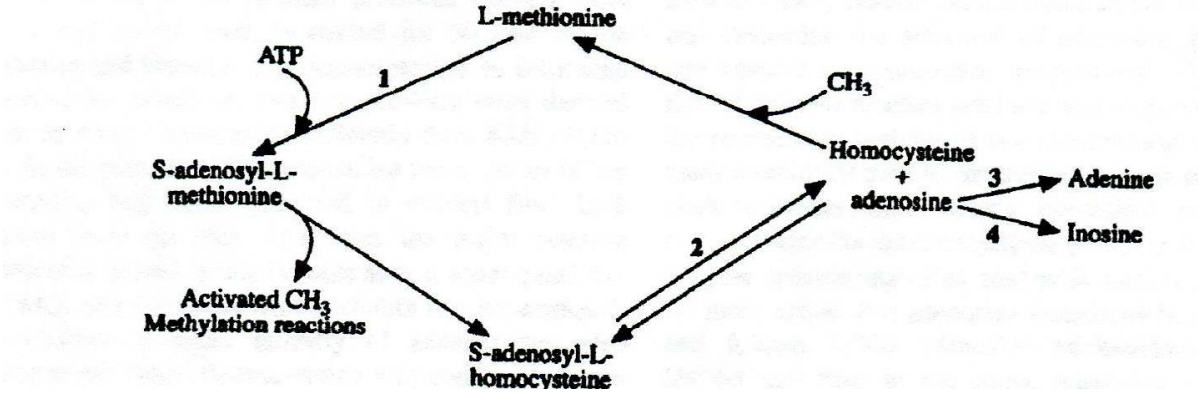


Figure 12. Synthesis of S-adenosyl-1-methionine (SAM): (1) SAM synthetase, (2) SAH hydrolase, (3) adenosine nucleosidase, (4) adenosine deaminase. Reprinted with permission[24].

This is achieved either by S- adenosyl-L-homocysteine hydrolase (SAH hydrolase) or adenosine nucleosidase with other supporting enzymes[23].

1.5 Adenosine Deaminase

Adenosine deaminase (ADA) is an enzyme with the capabilities of catalyzing the deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively[25]. This protein plays an important role in maintaining a homeostatic environment within an organism. Adenosine deaminase activity leads to an increase of inosine in organisms[25].

There are three distinctive isoforms of ADA presently known which can be found in different organisms. Adenosine deaminase 1 (ADA1) is a monomeric protein with a molecular weight of 30 - 40 kilodaltons (kDa) [26]. ADA1 can be found in prokaryotes, eukaryotes and predominately in animals [27]. Its primary role is reducing the apoptotic death of lymphocytes caused by high concentrations of adenosine[27]. Inherited mutations or the absence of ADA1 can cause severe combined immunodeficiency in humans[26]. This disorder is characterized by the absence of functional T and B cells.

Unlike ADA1, ADA2 is a homodimer with a molecular weight of 100 kDa and plays an important role in tissue development[26]. While it has been found in eukaryotes, ADA2 has been primarily found in multi-cellular organisms[27]. ADA2 was first found in insects and is classified with the adenosine deaminase growth factors (ADGF)[27]. The knock-out of the corresponding gene for ADA2/ADGF in flies and frogs resulted in abnormalities during embryogenesis which confirmed its ability to affect physical mutations[27]. Also, when comparing ADGF proteins to ADA1 proteins of vertebrate and bacteria, the ADGF proteins have longer sequences with an extension of more than 80

amino acids residues at the N-terminus and are found in the extracellular space[25]. In addition to ADA1 and ADA2/ADGF, the recently discovered adenosine deaminase 3 (ADA3), otherwise referred to as ADA-like protein (ADAL), has sequence homology at its active site with other adenosine deaminases[27].

Not much is known about its activity. Although research has confirmed the existence of three types of ADA, recent studies have shown that humans are the only organisms that possess all three.

Much less is known about ADA in plants compared to humans and other organisms that have been found to have this activity. The only plant species to have reported ADA activity are *Arachis hypognea* (peanut), *Ricinus communis* (castor bean), *Vigna unguiculata* (cowpea), *Glycine max* (soybean), *Ipomoea batatas* (sweet potato) and several species belonging to the *Dioscorea* family (yam)[28]. The physiological role of ADA in plants is unclear, although it appears to be involved with removal of adenosine[24]. However, in various plants the absence of adenine during germination is not a result of ADA as it is apparently not expressed during germination[29].

1.6 Adenosine Nucleosidase

Adenosine is precursor to synthesizing compounds which are essential energy sources in the plant cell. Most of the metabolic pathways are dependent on the adenosine series, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP)[21]. Although these components are present during the entire life of a plant, adenosine has a major role during plant embryogenesis. Adenylate and deoxyadenylate are constantly degraded when they come into contact with S-adenosyl-1-methionine (SAM) during plant embryogenesis[23]. This reaction subsequently produces

adenosine. Adenosine must be converted to adenosine derivatives in order to be continually used in the production of the much needed nucleotides during plant germination[23].

In order to keep replenishing the nucleotides for plant growth quickly, adenosine is hydrolyzed by adenosine nucleosidase. Adenosine nucleosidase (ANase) is an enzyme that is capable of converting adenosine to the nucleobase adenine and ribose[30]. This reaction allows adenine to be recycled and become an immediate precursor for the purine salvage pathway[30]. The activity of this enzyme and its characteristics have been determined in several species of plants such as wheat germ[31], yellow lupin[32], barley[33], tomato root and leaves[34], and tea[35]. According to Lee *et al*., adenosine nucleosidase, purified from malted barley leaves, has a molecular weight of approximately 120,000 daltons (Da)[33]. However, the molecular weights for the previous species listed are reported to be approximately half of the malted barley molecular weight. The molecular weights of selected purified enzymes are shown in Table 1.

It has been shown that a dramatic decline in adenine and adenosine occurred in the white spruce embryo upon its transfer into a maturation medium[16]. This suggests that the need for adenosine nucleosidase and salvage pathway declines once the plant has matured.

It has also been suggested that adenosine nucleosidase may play a role in cytokinin production since cytokinins are an adenine derivative. Chen *et al*. study on adenosine nucleosidase in wheat germ provided evidence of deriboslyation of N^6-substituted adenosine[31]. However, the affinity for the cytokinin was lower than for

adenine. This may be because the concentration of adenosine is much greater under normal conditions within a cell[36].

Table 1. Molecular weights of adenosine nucleosidase isolated from different plants.

Plants	**Molecular weight (Da)**
Wheat Germ	59,000
Yellow Lupin	72,000
Malted Barley	120,000
Tea Leaves	68,000
Tomato Root and Leaves	68,000

1.7 Soybeans

Soybeans are one of the most important multi-purpose crops grown in the United States. Soybeans began their rise as an industrial feedstock in the early 1920's due to their highly unsaturated and semi dry oil[37]. Their first manufacturing use was in paints, soaps, and varnishes and later began to appear in the food industry[37]. Due to their ability to produce the highest protein quantity of any seed, it became the most utilized "protein" in the meat and poultry industry[37]. One drawback to their use is the limited amount of methionine present in soybean proteins. Their continuous growth and need in the United States for the last few decades has lead soybeans to be referred to as the "miracle crop"[38]. Soybeans only close rival in production is corn[38]. The U.S is the leader in production and exportation of the soybean crop[38].

Since soybean is one of the leading cash crops in America, it is important to preserve its integrity. There are three main problems that are currently negatively

affecting crop production; insects, disease, and weeds. Insects feed on soybean pods as well as the foliage. Although feeding on the pods results in stopping of growth, defoliation may or may not impact the growth cycle of a soybean cycle. Typically, foliage-eating insects only negatively impact plant growth if infestation occurs during or after the plant blooms. However, certain growth stages can tolerate defoliation without loss. During primary crop season, more than one foliar feeder species can be present. This can lead to large amounts of crop being destroyed. The primary pests of soybeans include the Mexican bean beetle, green stinkbug, and bean leaf beetle[39].

Soybeans, like other crops, are subject to numerous diseases. There are over 30 diseases that affect this crop. Most of these diseases never develop into an epidemic. However, a few diseases such as phytophthoran and cystnematodes, can cause significant damage to fields[39]. Most farmers are able to control the spread of many diseases through crop rotation. This is because most diseases are soil borne rather than seed borne. In recent years, scientist and farmers have used a combination of cultivation practices and genetic resistance to significantly combat the spread of diseases affecting soybean plants[39].

Over the last decade, weeds have become an enormous problem in the agricultural community. It has caused farmers billions of dollars in crop loss each year. Unlike its rival corn, weeds compete with soybeans for light rather than nutrients. Nitrogen producing bacteria provide soybeans with a much-needed source of nitrogen and reduce competition for nitrogen with weeds[40]. However, since soybeans are a ground level growing plant, weeds can easily overgrow soybean plants and hinder their ability to receive adequate sunlight. Foxtail, pigweed, and velvet leaf are the most prominent

species that over grow soybean plants in all regions of the United States[40]. In order to combat these weeds along with others, glyphosate has been a primary reagent in herbicides. Currently, 23 weeds species have developed a resistance to this chemical, being able to survive four times the normal concentration of the herbicide. Some scientists believe that weed resistance began when herbicides were packaged with genetically engineered crops such as soybeans[40]. Others believe it is a natural evolutionary adaptation that would occur over years[40]. As a result of the overgrown weed infestation affecting soybeans in the United States and worldwide, there has been immense research on soybeans and their enzymes[40].

1.8 Soybeans and their enzymes

Many plants contain a reserve of numerous carbohydrates, oils and proteins during maturation. A soybean seeds composition of these compounds is approximately 40% protein and 20 % oil[41]. There are 764 proteins identified in germinating soybean seeds. Each of the proteins was classified into 14 functional categories. Among the 14 protein categories, the most abundant number of proteins belongs to metabolism, storage, biosynthesis, and destination/cell cycle/organization. Within germinating soybean seeds there were 215 metabolism proteins identified[41]. These 215 proteins identified as being involved in metabolism where further divided into 17 subgroups depending on the metabolic pathway to which it belonged. [41].

A number of enzymes have been isolated from soybeans and extensively studied including 5'-nucleotidase, purine nucleosidase[20], xanthine dehydrogenase, uricase, and allantoinase/ allantoate amidohydrolase (AAH). The location of these enzymes has also been investigated. It has been shown in these studies that certain enzymes are confined to

one area or may be present in another area within a soybean at a different concentration. The concentration of the enzymes can also can increase or decrease as plants progress through each growth stage. Soybean enzymes have been studied in the nodules, stem roots, and leaves.

In a mature soybean plant, 5'-nucleotidase has been found to favor areas of the plant where ammonium is generated, such as the nodules and leaves[42]. This enzyme is involved in the catabolism of purine nucleotides. 5'-Nucleotidase has the ability to catabolize nucleotides by two different possible routes in plants. The first route uses 5'-nucleotidase to dephosphorylate IMP to inosine [20]. The secondary route uses 5'-nucleotidase to further metabolize IMP to xanthosine after being converted to XMP by IMPDH[20]. It was also shown that this enzyme substrate specificity changes with pH. As the pH became more alkaline, with an optimum pH of 8, the specificity of 5'-nucleotidase for the purine nucleotides adenosine monophosphate (AMP), inosine monophosphate (IMP) and guanosine monophosphate (GMP) increase and favors catabolism[42].

Purine nucleosidase can be found in the nodules and in other parts of the plant as well. It is able to hydrolyze purine and pyrimidine nucleotides. Its activity in plants was confirmed primarily due to the abundance of purine bases and ribose present in its specified areas. Purine nucleosidase is also another enzyme involved in the catabolism of purines[20].

Uricase and xanthine dehydrogenase seem to be nodule specific[42]. The activity of both enzymes seems to be primarily confined to this region of the plant. However, further studies were able to identify subcellular locations of these two enzymes as well as allantoinase/AAH. Studies revealed that xanthine dehydrogenase was found in the

cytosol [43], uricase in the peroxisome[44], and allantoinase in the endoplasmic reticulum[44]. Although located throughout the soybean cellular system all of these enzymes are part of the oxidative catabolism of purines in soybeans.

These three enzymes are involved in a series of reactions that degrades the purine base xanthine in order to produce allantoate, a nitrogen storage compound in soybeans. Xanthine is first degraded by xanthine dehydrogenase to form uric acid. Uricase then catalyzes the oxidation of uric acid to allantoin, which is converted to allantoate by allantoinase/AAH[20].

Other enzymes shown to be present in other tropical legumes and plants have not been extensively studied in soybeans.

1.9 Summary of purpose

Soybean extracts were tested for the presence of adenosine nucleosidase five days after germination. The soybean extract was also tested for the presence of phosphorylase and nucleosidase activity. The enzyme was then purified and characterized. The enzyme molecular weight was determined using SDS-PAGE. In order further characterize the enzyme, an OFFGEL electrophoresis fractionation was used due to the presence of a similar protein/enzyme which appears to be adenosine deaminase. The isoelectric points were determined for both adenosine nucleosidase and adenosine deaminase.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Soybean seeds were obtained from Johnny Selected Seeds of Winslow, Maine, USA. Adenosine, adenine, inosine, hypoxanthine, aminohexyl agarose resin and neocuproine were purchased from Sigma Aldrich. Precision unstained molecular weight markers, 10 X Tris/Glycine/SDS buffer, and Laemmli sample buffer were obtained from BioRad. PAGEr Gold Cast gels were obtained from Lonza. Centricon centrifugal filter units (15 mL) were purchased from Millipore. GelCode Blue Safe Protein Stain was obtained from ThermoScientific. Sephadex diethylaminoethyl (DEAE) column (2.5 x 35 cm), HiLoad Sepharose Mono Q Fast Flow (16/10) column and Sephacryl S100 (26/60) size exclusion column for the AKTA purifier system were purchased from GE Healthcare. All other chemicals were reagent grade.

OFFGEL fractionator and supplies were obtained from Agilent. Dionex UltiMate 3000 HPLC equipped with thermostatted autosampler, UV detector, thermostatted column holder, and dual pump was purchased from ThermoScientific. The U2900 UV Visible double beam spectrophotometer was purchased from Hitachi.

2.2 Methods

2.2.1 Germination of soybean seeds

Soybean seeds (100g) were soaked in bleach for 5 minutes. Afterwards, they were rinsed thoroughly with tap water. The seeds were placed in a sealed container on a moist paper towel and covered with another moist paper towel to germinate. On the 5^{th} day after germination, the seedlings were homogenized in 50 mM Tris buffer pH 7.2 (300

mL) containing 1 mM dithiothreitol (DTT) using a Waring commercial blender at 4°C. The sample was centrifuged at 15,000*xg* for 15 minutes at 4°C. The supernatant was strained through nylon cloth to remove any remaining particulates and stored at 4°C.

2.2.2 Activity Assay

A. HPLC Activity Assay

Adenosine nucleosidase activity was measured by two methods; appearance of adenine or disappearance of adenosine as determined by HPLC and appearance of ribose by reducing sugar assay. The HPLC assay reaction mixture consisted of 900 µL of 1 mM adenosine with 1 mM $CaCl_2$ in 50 mM Tris pH 7.2. The reaction was initiated by adding 100 µL of the enzyme extract and the reaction mixture incubated at 37° C. At the appropriate time 10 µL of the reaction mixture was injected onto the HPLC. The appearance of adenine and disappearance of adenosine was used to calculate enzyme activity.

The amounts of adenine and adenosine present were determined on an HPLC Dionex UltiMate 3000 equipped with a UV detector. Adenine and adenosine were separated on a Phenomenex Hyperclone ODS C_{18} HPLC column (150 x 4.6 mm, 5 µm particle size). Substrates and products were eluted isocratically with a mobile phase of 90% 10 mM ammonium acetate pH 5.2; 10% methanol. Adenine and adenosine were detected at 254 nm and identified by comparison of retention times to standard samples (Table 2). Amounts of adenine and adenosine present were determined by their relative areas after correction for differences in extinction coefficients.

Table 2. Summary of retention times of nucleosides and bases detected at 254 nm. The retention times are the average of at least five measurements.

Standards	**Retention Time (min)**
Adenosine	2.043
Adenine	1.557
Guanosine	1.597
Guanine	1.443
Cytidine	1.383
Cytosine	1.390
Uridine	1.437
Uracil	1.397
Inosine	1.547
Hypoxanthine	1.423
Thymidine	1.740
Thymine	1.493

Nucleoside phosphorylase activities for cytidine, uridine, thymidine, adenosine, guanosine, and inosine were measured by the appearance of their respective nucleobase as determined by HPLC. The HPLC assay mixture consisted of 900 µL of 1 mM nucleoside in 50 mM sodium phosphate pH 7.2. The reaction was initiated by adding 100 µL of the enzyme extract and the reaction mixture incubated at 37° C. At the appropriate time 10 µL of the reaction mixture was injected onto the HPLC. The starting material and product were monitored at 254 nm and identified by comparison of retention times to authentic samples (Table 2). This assay was used to determine any nucleoside phosphorylase activity that may be present in the initial soybean extract.

Next, the nucleoside hydrolase activities for cytidine, uridine, thymidine, adenosine, guanosine, and inosine were measured by the appearance of their respective nucleobase as determined by HPLC. The HPLC assay mixture consisted of 900 µL of 1 mM nucleoside in 50 mM Tris buffer pH 7.2. The reaction was initiated by adding 100 µL of the enzyme extract and the reaction mixture incubated at 37° C. At the appropriate time 10 µL of the reaction mixture was injected onto the HPLC. The starting material and product were monitored at 254 nm and identified by comparison of retention times to authentic samples (Table 2). This assay was used to determine any nucleoside hydrolase activity that may be present in the initial soybean extract.

B. Reducing Sugar Assay

A reaction mixture (1 mL) consisting of 1 mM adenosine 1 mM $CaCl_2$ in 50 mM Tris pH 7.2 and 100 µL of each enzyme fraction were incubated for 24 hours. To each assay mixture 300 µL copper sulfate reagent and 300 µL neocuproine reagent were added[45]. The control contained 1 mL adenosine assay with 100 µL of water replacing the enzyme solution and the same reagents were added. At the end of the reaction time all test tubes were incubated at 100 °C for 7 minutes and then allowed to cool. The absorbance was read at 450 nm. The amount of ribose produced was determined by comparison to a standard curve (Figure 13).

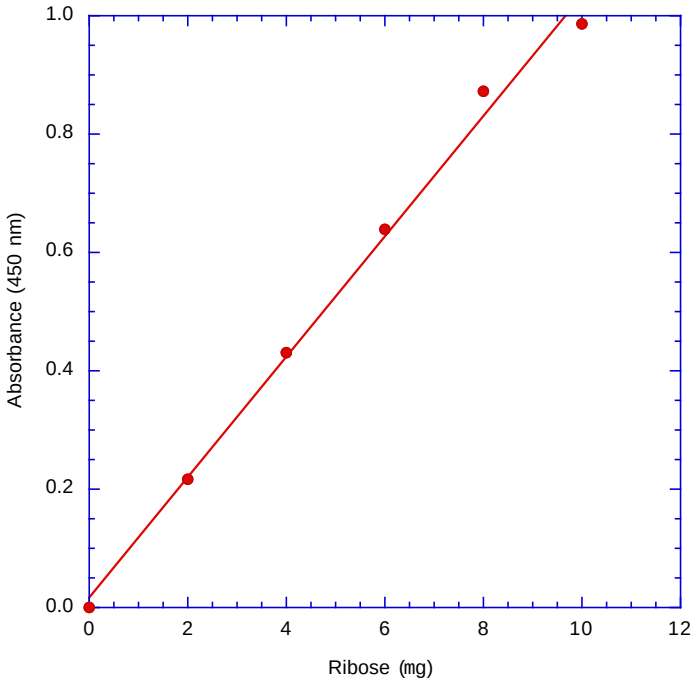


Figure 13. Standard curve for determination of ribose by reducing sugar assay.

2.2.3 Protein Concentration

Protein concentration was measured by two methods; the absorbance at 280 nm and BioRad Protein assay. A BioRad assay was performed on samples that were collected

on the first five days of germination to determine which day had the most protein present. The Bio-Rad assay standard curve was made using bovine serum albumin (1.44 µg/mL) (Figure 14). For unknown samples 10 µL of each sample was combined with 90 µL of

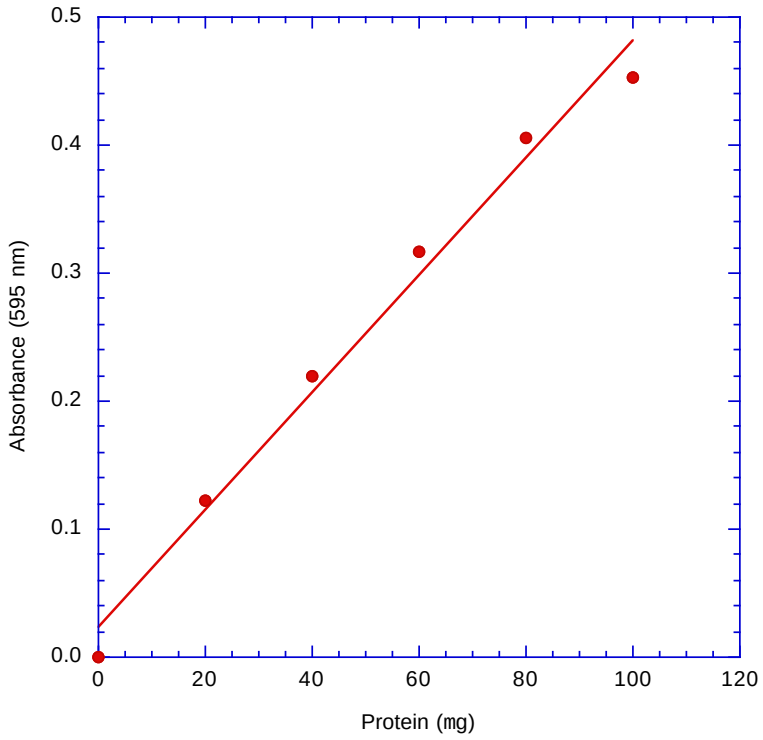


Figure 14. Standard curve for determination of protein by using BioRad protein assay.

distilled water and then was further diluted to 1:10 for a final dilution of 1:100. In each test tube, 5 mL of Bio-Rad dye was vortexed with the diluted samples. The absorbance was measured at 595 nm. The amount of protein was determined by standard curve.

During each column purification step, fractions were collected. The amount of protein present in each fraction was determined by UV absorbance measured at 280 nm. The absorbance of each fraction was plotted to determine where the highest amount of protein eluted. Absorbance at 280 nm was converted to protein concentration based on the conversion factor that 0.1 mg/mL protein gave an absorbance of 0.1.

2.2.4 Purification of Adenosine Nucleosidase

The initial extract produced from the soybean seeds was precipitated with ammonium sulfate at 30% and 60% saturation. For the 30% precipitation, ammonium sulfate (43.9 g) was slowly added to the initial extract (250 mL) at 4 °C while constantly stirring the solution. Once the ammonium sulfate was completely dissolved, the solution was left overnight at 4 ° C to precipitate. The solution was centrifuged at 15,000*xg* for 15 minutes at 4 ° C. The supernatant was kept for further processing, while the precipitate was discarded.

The supernatant was increased to 60% ammonium sulfate saturation by the same procedure as for the 30% saturation (47.9 g in 246 mL). After the second centrifugation, the pellet was resuspended in 50 mM Tris buffer pH 7.2 containing 1 mM DTT (20 mL) and was dialyzed against the same buffer (3 x 500 mL).

The dialyzed extract was loaded onto a Sephadex DEAE ion exchange column (2.5 x 35 cm) which was then washed with 500 mL of 50 mM Tris buffer pH 7.2. The column was then eluted with a linear gradient of 500 mL of 0-1 M NaCl in 50 mM Tris buffer pH 7.2. Fractions (17 mL) were collected at a flow rate of 1 mL/min. The fractions were assayed for protein by measuring the absorbance at 280 nm and for activity by reducing sugar assay using adenosine as the substrate. Fractions containing adenosine nucleosidase activity were pooled and concentrated in a Centricon centrifugal unit at 4° C until the final volume was 2 mL.

The concentrated fraction was dialyzed overnight against 50 mM Tris pH 7.2 (500 mL) and loaded onto a HiLoad Sepharose Fast Flow Mono Q column (16/10) attached to an AKTA FPLC system. The column was washed with 40 mL of 50 mM Tris buffer pH

7.2 followed by a linear gradient of 40 mL of 0-1 M NaCl in 50 mM Tris buffer pH 7.2. Fractions of 5 mL were collected at a flow rate of 1 mL/min. Fractions containing enzyme activity were pooled and concentrated to 2 mL as described above.

The Mono Q concentrated fraction was loaded onto a Sephacryl S100 (26/60) size exclusion FPLC column equilibrated with 50 mM sodium phosphate buffer pH 7.2 containing 300 mM NaCl. The column was washed with the same buffer. Fractions (5 mL) were collected at a flow rate of 1 mL/min. Fractions were assayed for protein and activity as described above. Fractions containing enzyme activity were pooled and concentrated by the previous method.

The concentrated fraction from the Sephacryl S100 (26/60) size exclusion FPLC column was dialyzed overnight against 500 mL of 50 mM Tris pH 7.2. The dialyzed fraction was loaded onto the aminohexyl column. The column was washed with 40 mL of 50 mM Tris buffer pH 7.2 followed by a linear gradient of 40 mL of 0-1 M NaCl in 50 mM Tris buffer pH 7.2. Fractions of 5 mL were collected at a flow rate of 1 mL/min. Fractions containing enzyme activity were pooled and concentrated to 2 mL as described above.

2.2.5 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS- polyacrylamide gel electrophoresis was performed on 15% Lonza PAGEr Precast gel with Tris/Glycine/SDS buffer. Sample buffer was prepared by combining BioRad Laemmli buffer (950 µL) and β-mercaptoethanol (50 µL). Samples (10 µL) and Laemmli buffer (10 µL) were centrifuged and heated at 95 °C for 5 minutes. The sample was loaded onto the gel and electrophoresed at a constant current of 30 mA. After electrophoresis was complete, the gel was washed three times for 5 minutes with distilled

water. The gel was stained using Gel Code Blue Safe protein stain for a minimum of 1 hour. In order to determine molecular weight a Precision unstained molecular weight marker (BioRad) was also loaded onto the gel. A calibration curve relating molecular weight of known standards versus gel mobility was prepared and the subunit molecular weight of adenosine nucleosidase was determined.

2.2.6 OFFGEL Electrophoresis

Isoelectric focusing was performed using an Agilent OFFGEL Fractionator on a 24 well immobilized pH gradient (IPG) strip with a protein OFFGEL stock solution. The stock solution was prepared by adding urea, DTT, thiourea, (all premeasured amounts) to 6 mL glycerol and 600 µL OFFGEL buffer pH 3-10. The solution was brought to a total volume of 50 mL with distilled water. A 1:5 dilution, 100 µL, due to salt concentration of the enzyme, was added to 2.88 mL of OFFGEL stock solution. After the IPG strips were rehydrated, 150 µL of the prepared protein solution was added to each well. The OFFGEL fractionator was run using Agilent 3100 OFFGEL fractionator standard method OG24PR01. SDS- polyacrylamide gel electrophoresis was performed on each well using a 15% Lonza PAGEr Precast gel as described above.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Purine and Pyrimidine Enzyme Activity in Soybean Seeds

To determine the purine and pyrimidine metabolizing enzyme activities present within the germinated soybean seeds, the crude extract was tested for phosphorylase and hydrolase activities with a variety of nucleosides. All of the purine and pyrimidine samples were assayed using the same HPLC method to detect enzymatic activities in both Tris and phosphate buffer systems. In the crude extract from the 5-day old seedlings, both phosphorylase and hydrolase activities were shown to be present using thymidine, guanosine, and inosine as substrates. Thymidine phosphorylase catalyzes the reaction of thymidine in the presence of phosphate to yield thymine and α-D-2'-deoxy-ribose-1-phosphate (Figure 15A). Thymidine nucleosidase catalyzes the chemical reaction between thymidine and water to yield thymine and 2'-deoxyribose (Figure 16A). Inosine phosphorylase has the ability to phosphorylyze inosine to yield hypoxanthine and α-D-2'-deoxy-ribose-1-phosphate (Figure 15B). When guanosine is the substrate with phosphate ions in the presence of guanosine phosphorylase, it will form guanine and α-D-2'-deoxy-ribose-1-phosphate (Figure 15C). Alternatively, inosine-guanosine nucleosidase can hydrolyze both inosine and guanosine. The reaction between inosine and inosine-guanosine nucleosidase will produce hypoxanthine and ribose (Figure 16B). When in the presence of guanosine, the nucleosidase will yield guanine and ribose as products (Figure 16C).

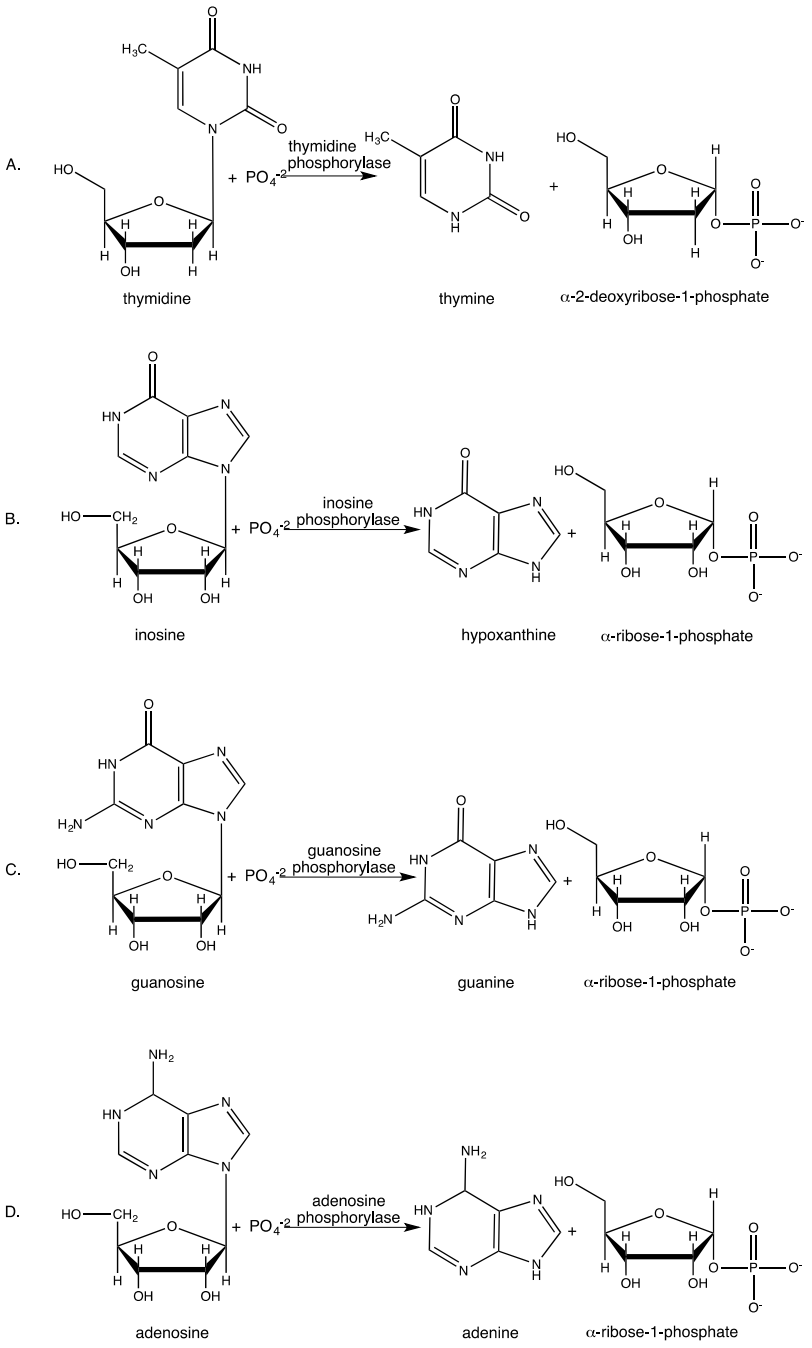


Figure 15. **(A)** Thymidine phosphorylase catalyzes the cleavage of thymidine in the presence of phosphate to yield thymine and α-D-2'-deoxy-ribose-1-phosphate **(B)** Inosine phosphorylase catalyzes the cleavage of inosine in the presence of phosphate to yield hypoxanthine and α-D-2'-deoxy-ribose-1-phosphate. **(C)** Guanosine phosphorylase catalyzes the cleavage of guanosine in the presence of phosphate to yield guanine and α-D-2'-deoxy-ribose-1-phosphate. **(D)** Adenosine phosphorylase catalyzes the cleavage of adenosine in the presence of phosphate to yield adenine and α-D-2'-deoxy-ribose-1-phosphate.

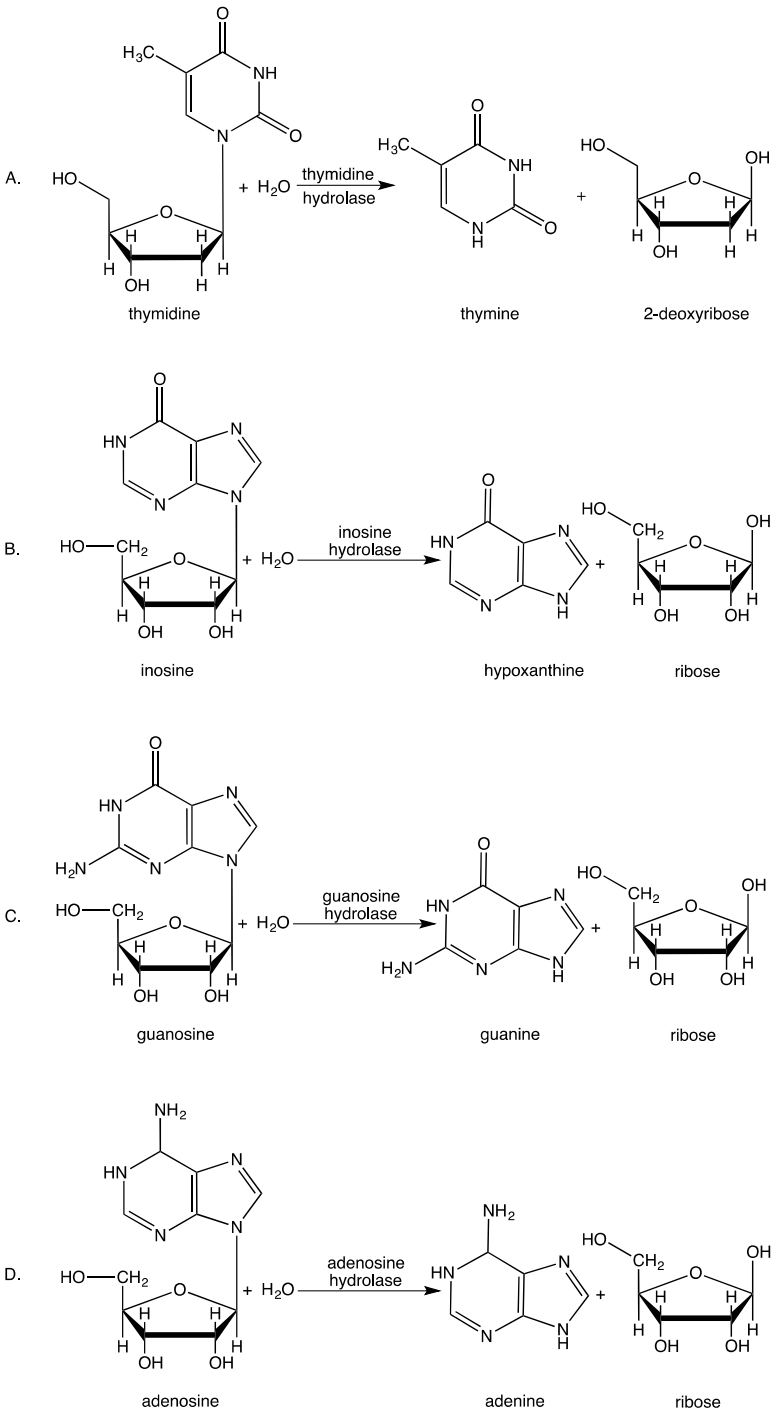


Figure 16. **(A)** Thymidine nucleosidase (hydrolase) hydrolyzes thymidine in the presence of water to yield thymine and 2'-deoxyribose. **(B)** Inosine nucleosidase hydrolyzes inosine in the presence of water to yield hypoxanthine and ribose. **(C)** Guanosine nucleosidase hydrolyzes guanosine in the presence of water to yield guanine and ribose. **(D)** Adenosine nucleosidase hydrolyzes adenosine in the presence of water to yield adenine and ribose.

Similarly, using adenosine, adenosine phosphorylase and hydrolase activities were detected. Adenosine phosphorylase when present with adenosine and phosphate ions will yield adenine and α-D-2'-deoxy-ribose-1-phosphate (Figure 15D). Conversely, adenosine nucleosidase hydrolyzes the N-glycosyl bond and yields adenine and ribose in the presence of water (Figure 16D). In addition to adenosine hydrolase activity, adenosine deaminase activity was also detected by the appearance of inosine (Figure 17).

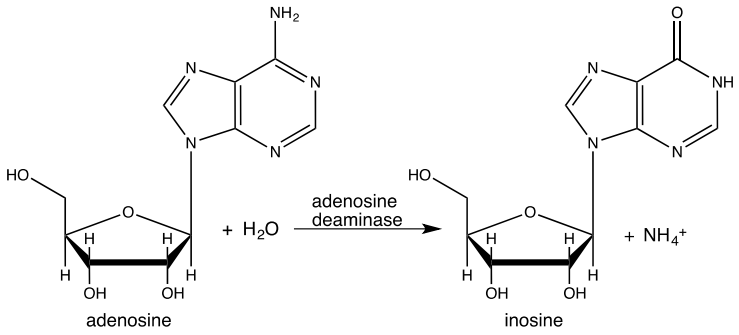
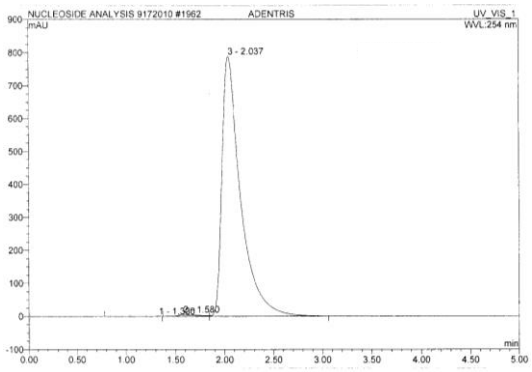


Figure 17. Adenosine deaminase deaminates adenosine to yield inosine.

In summary, the adenosine assay revealed the presence of three activities, adenosine phosphorylase, adenosine nucleosidase and adenosine deaminase. In both buffer systems, adenosine deaminase activity was detected (Figures 18 and 19). Adenosine was readily converted to inosine then to hypoxanthine in both the hydrolase and phosphoyrlase buffer systems.

Like other purine/pyrimidine nucleosidases, inosine-guanosine nucleosidase is responsible for converting inosine and guanosine into their respective nucleobases and ribose. Monitoring the appearance of the product bases, hypoxanthine and guanine, revealed a lag of approximately two hours after the addition of 100 µL of crude extract before the first appearance of product. Full conversion of inosine to hypoxanthine was

A)



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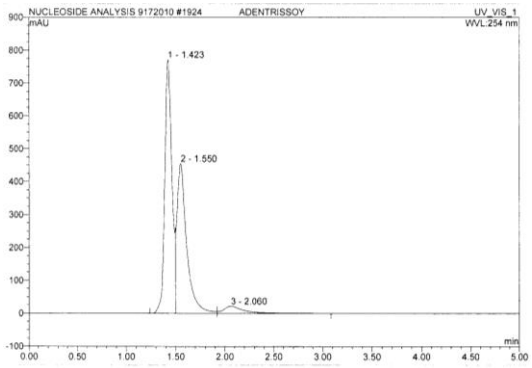

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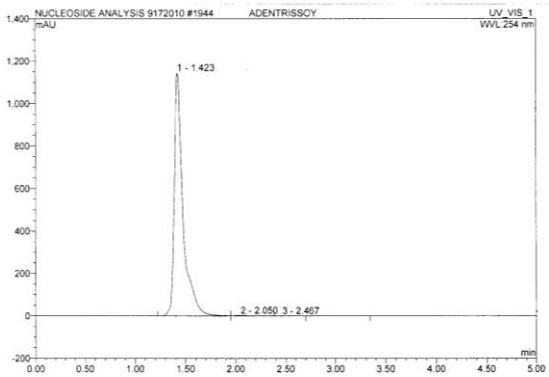
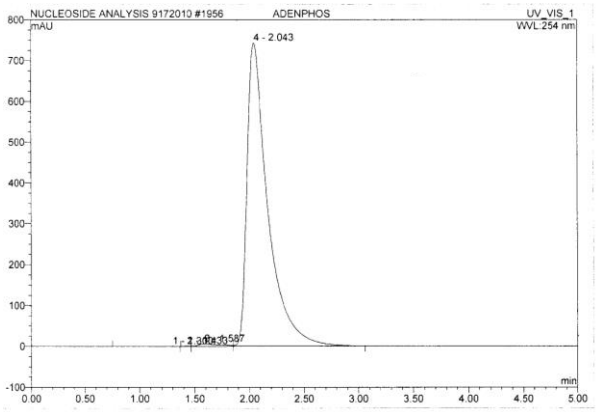


Figure 18. Adenosine nucleosidase reaction. The initial reaction mixture consisted of soybean extract, adenosine, DTT and $MgCl_2$ in a Tris buffer. **A)** The initial adenosine reaction mixture has no soybean extract added. It displays the adenosine standard peak at 2.037 min. **B)** After the addition of the soybean extract, inosine (1.550 min) and hypoxanthine (1.423 min) appeared approximately 12 hours after the addition of the extract. **C)** Twenty-three hours after the addition of the extract, adenosine was completely converted to hypoxanthine.

A)



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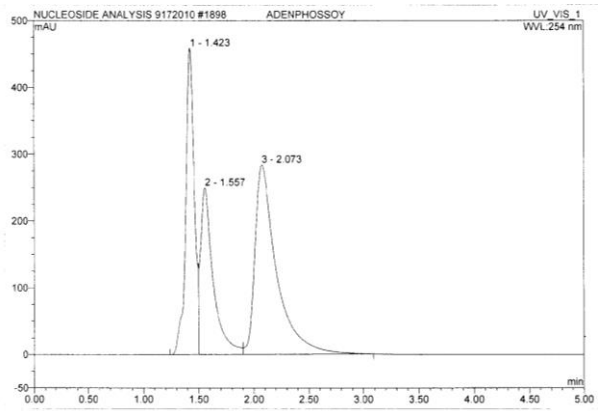

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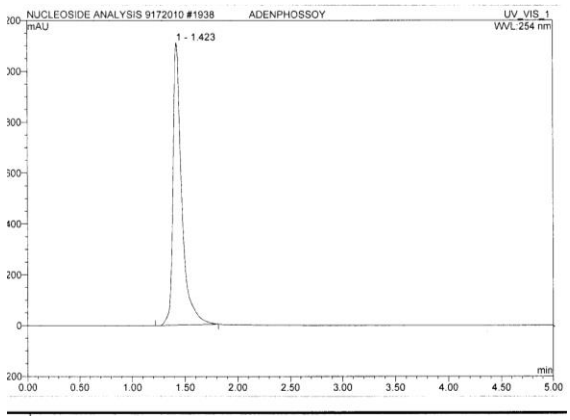


Figure 19. Adenosine phosphorylase reaction. The initial reaction mixture consisted of soybean extract, adenosine, DTT and $MgCl_2$ in a phosphate buffer **A)** The initial adenosine reaction mixture has no soybean extract added. It displays the adenosine standard peak at 2.043 min. **B)** After the addition of the soybean extract, the appearance of inosine (1.557 min) and hypoxanthine (1.423 min) appeared approximately 12 hours after the addition of the extract. **C)** Twenty-three hours after the addition of the extract, adenosine was converted to hypoxanthine.

complete after 23 hours (Figure 20). However, guanosine did not fully convert to guanine during the 24 hours after initiation (Figure 21). Previous studies have determined in plants that one nucleosidase is responsible for hydrolysis of both inosine and guanosine[21]. However, they have separate phosphorylases. The inosine and guanosine phosphorylases reactions were measured in a manner similar to the nucleosidase assay with the substitution of phosphate buffer for the Tris buffer. Inosine and guanosine phosphorylases converted inosine and guanosine to hypoxanthine and guanine respectively (Figures 22 and 23).

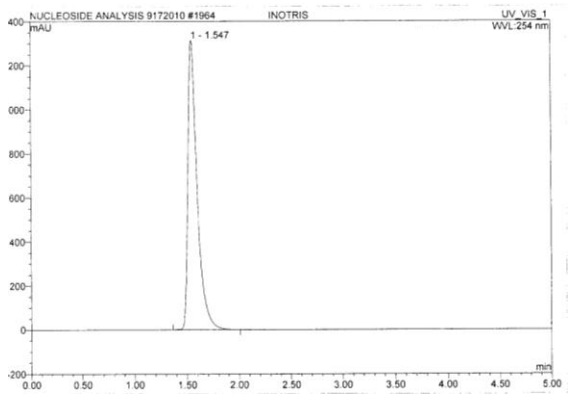
Thymidine nucleosidase and phosphorylase, converting thymidine to thymine, were also present. Thymidine nucleosidase revealed signs of conversion starting four hours after initiation (Figure 24). Thymidine phosphorylase showed signs of conversion beginning after two hours (Figure 25). Both conversions took almost 24 hours before completion.

Under these chromatographic conditions, cytidine and uridine phosphorylases and hydrolases reactions were unable to be determined due to cytosine and uracil eluting so closely to their respective nucleoside.

3.2 Purification of Adenosine Nucleosidase

The enzyme purification used a combination of different modes of separation and extraction procedures. After germination of the seeds an initial extract was prepared in which the seeds were homogenized in 50 mM Tris buffer pH 7.2 with 1 mM DTT. Filtration through nylon cloth and centrifugation removed the bulk of the insoluble material. The first purification step involved precipitation with ammonium sulfate between 30 and 60% saturation. At 30% ammonium sulfate concentration, the pellet and

A)



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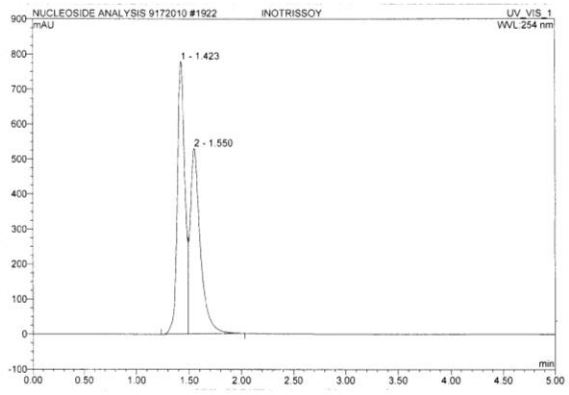

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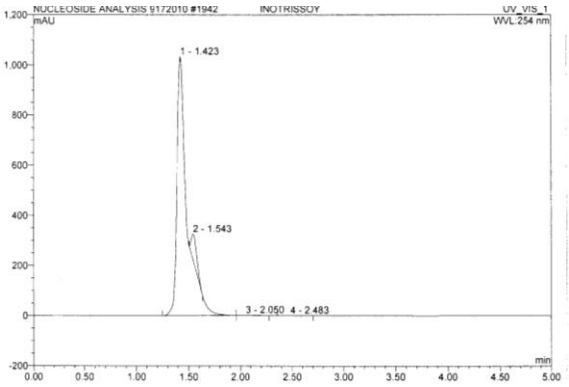
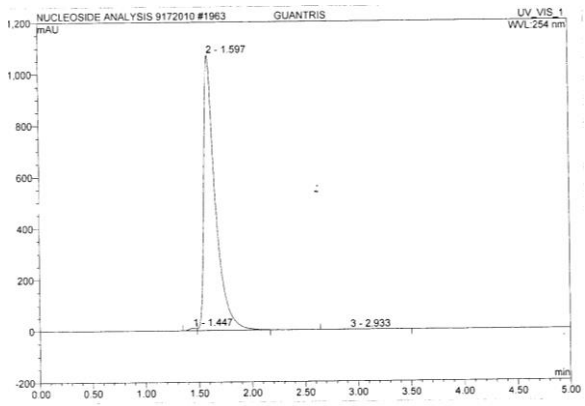


Figure 20. Inosine (inosine-guanosine nucleosidase) reaction. The initial reaction mixture consisted of soybean extract, inosine, DTT and $MgCl_2$ in a Tris buffer **A)** The initial inosine reaction mixture has no soybean extract added. It displays the inosine standard peak at 1.547 min. **B)** After the addition of the soybean extract, the appearance of hypoxanthine (1.423 min) and the disappearance of inosine (1.550 min) appeared approximately 12 hours after the addition of the extract. **C)** Twenty-three hours after the addition of the extract, inosine was almost completely converted to hypoxanthine.

A)



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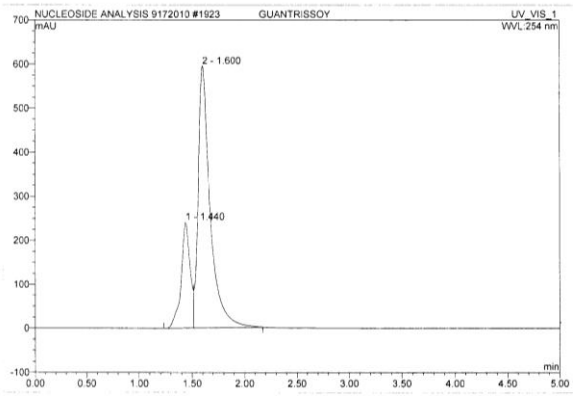


C)

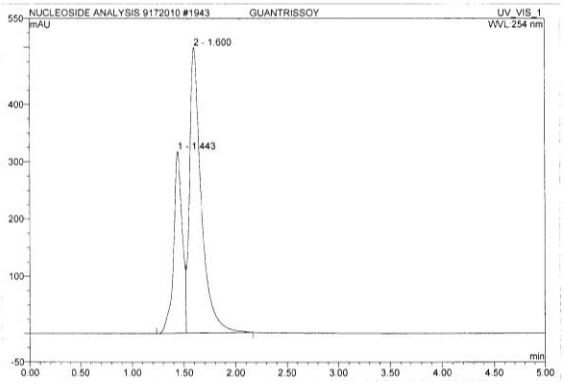
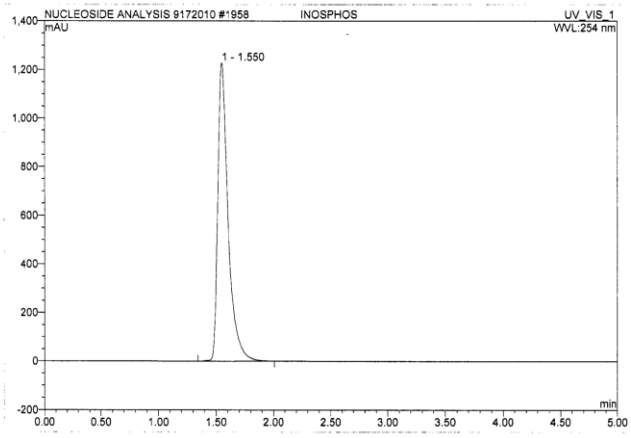


Figure 21. Guanosine (inosine-guanosine nucleosidase) reaction. The initial reaction mixture consisted of soybean extract, guanosine, DTT and $MgCl_2$ in a Tris buffer **A)** The initial guanosine reaction mixture has no soybean extract added. It displays the guanosine standard peak at 1.597 min. **B)** After the addition of the soybean extract, the appearance of guanine (1.440 min) and the disappearance of guanosine (1.600 min) appeared approximately 12 hours after the addition of the extract. **C)** Twenty-three hours after the addition of the extract, guanosine was partially converted to guanine.

A)



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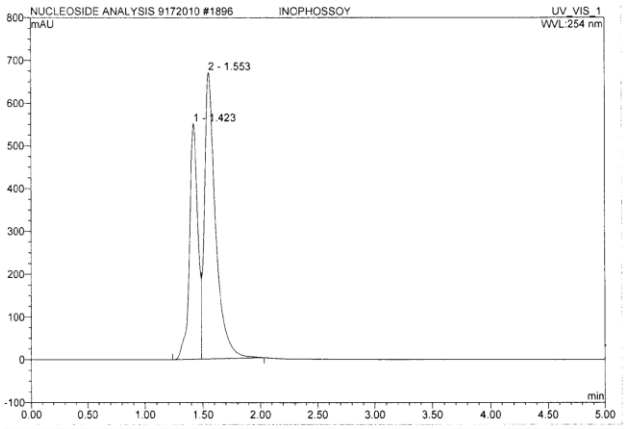

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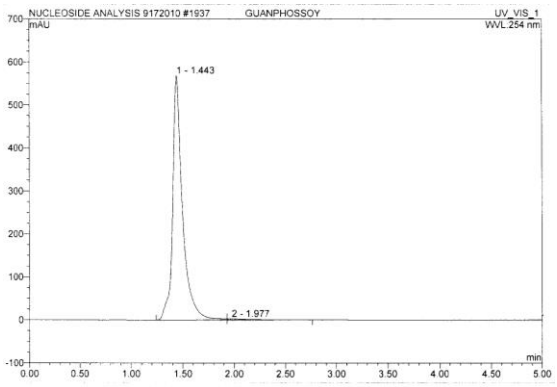
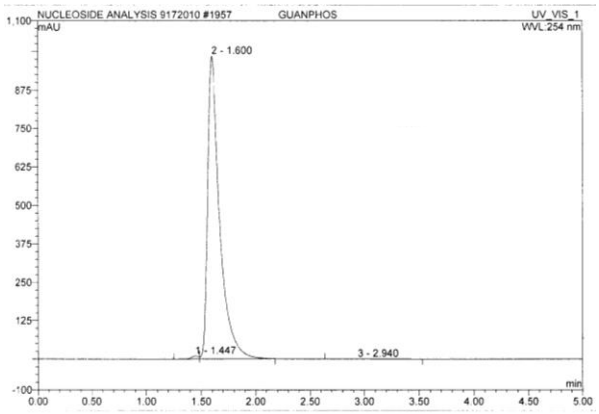


Figure 22. Inosine phosphorylase reaction. The initial reaction mixture consisted of soybean extract, inosine, DTT and $MgCl_2$ in a phosphate buffer. **A)** The initial inosine reaction mixture has no soybean extract added. It displays the inosine standard peak at 1.550 min. **B)** After the addition of the soybean extract, the appearance of hypoxanthine (1.423 min) and the disappearance of inosine (1.553 min) appeared approximately 12 hours after the addition of the extract. **C)** Twenty-three hours after the addition of the extract, inosine was completely converted to hypoxanthine

A)



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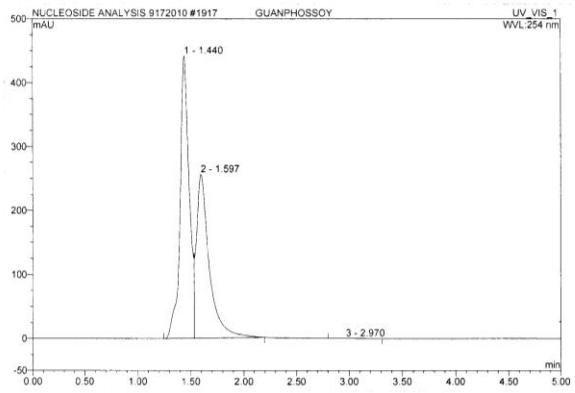

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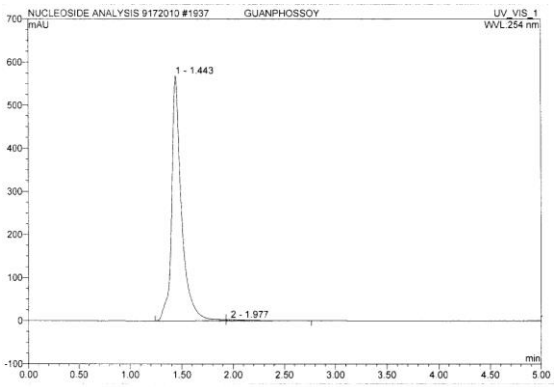
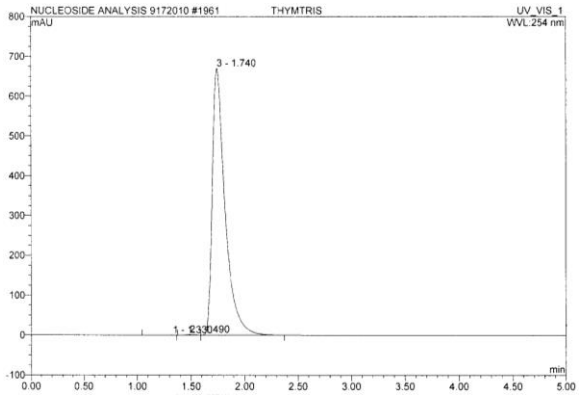


Figure 23. Guanosine phosphorylase reaction. The initial reaction mixture consisted of soybean extract, guanosine, DTT and $MgCl_2$ in a phosphate buffer **A)** The initial guanosine reaction mixture has no soybean extract added. It displays the guanosine standard peak at 1.600 min. **B)** After the addition of the soybean extract, the appearance of guanine (1.440 min) and the disappearance of guanosine (1.600 min) appeared approximately 12 hours after the addition of the extract. **C)** Twenty- three hours after the addition of the extract, guanosine was completely converted to guanine.

A)



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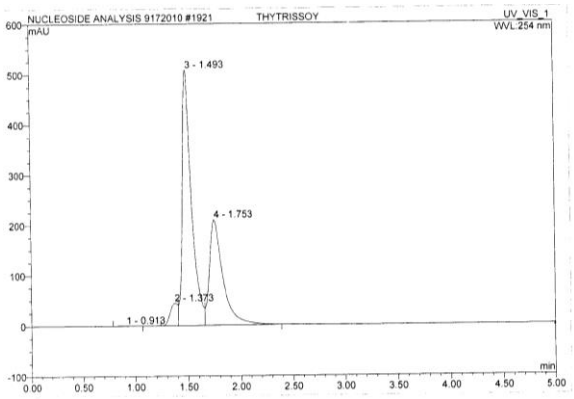

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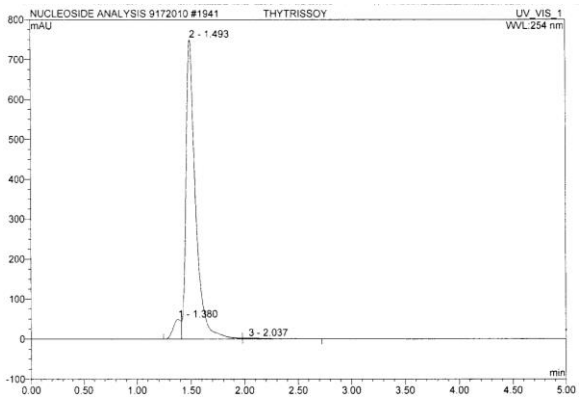
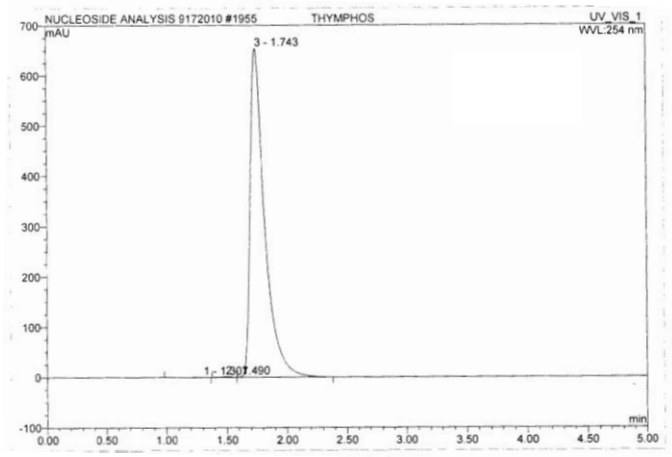


Figure 24. Thymidine nucleosidase reaction. The initial reaction mixture consisted of soybean extract, thymidine, DTT and $MgCl_2$ in a 50 mM Tris buffer A) The initial thymidine reaction mixture has no soybean extract added. It displays the thymidine standard peak at 1.740 min. B) After the addition of the soybean extract, the appearance of thymine (1.493 min) and the disappearance of thymidine (1.753 min) appeared approximately 12 hours after the addition of the extract. C) Twenty-three hours after the addition of the extract, thymidine was completely converted to thymine.

A)



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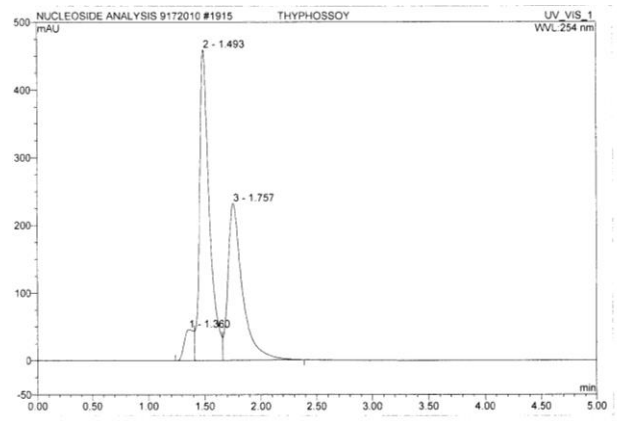

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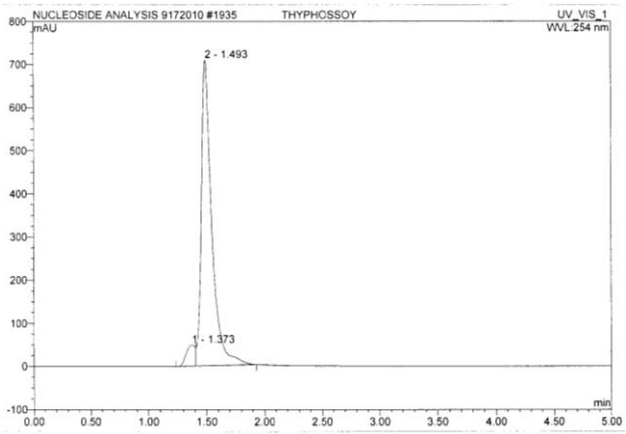

Figure 25. Thymidine phosphorylase reaction. The initial reaction mixture consisted of soybean extract, thymidine, DTT and $MgCl_2$ in a phosphate buffer. A) The initial thymidine reaction mixture has no soybean extract added. It displays the thymidine standard peak at 1.743 min. B) After the addition of the soybean extract, the appearance of thymine (1.493 min) and the disappearance of thymidine (1.757 min) appeared approximately 12 hours after the addition of the extract. C) Twenty-three hours after the addition of the extract, thymidine was converted to thymine.

supernatant were assayed by HPLC. The 30% supernatant fully converted adenosine to hypoxanthine after 20 hours (Figure 26A). The 30% ammonium sulfate pellet converted approximately 56% of adenosine to hypoxanthine after 20 hours (Figure 26B). The supernatant's ammonium sulfate concentration was increased to 60%. The pellet and supernatant were assayed for activity by HPLC and protein concentration (Table 3). Both converted adenosine to hypoxanthine (Figure 27A and Figure 27B). The appearance of hypoxanthine when adenosine was the starting material indicates the presence of both adenosine nucleosidase and adenosine deaminase in the pellet. When increased to 70% ammonium sulfate solution, the overall supernatant and pellet activity tended to be lower in the adenosine assay. The activity in the 70% pellet was checked and found to be negligible.

Table 3. Hydrolase and phosphorylase activities of cytidine, uridine, thymidine, adenosine, guanosine, and inosine. N/D= Not determined; R= Reaction; R*= Reaction with deamination

Purine/Pyrimidine	**Hydrolase Activity**	**Phosphorylase activity**
Cytidine	N/D	N/D
Uridine	N/D	N/D
Thymidine	R	R
Adenosine	R*	R*
Guanosine	R	R
Inosine	R	R

The next phase in the purification process was ion exchange chromatography. Two types of ion exchange columns were used; a Sephadex diethylaminoethyl (DEAE)column and a Sepharose Mono Q Fast Flow column. Both of these columns are

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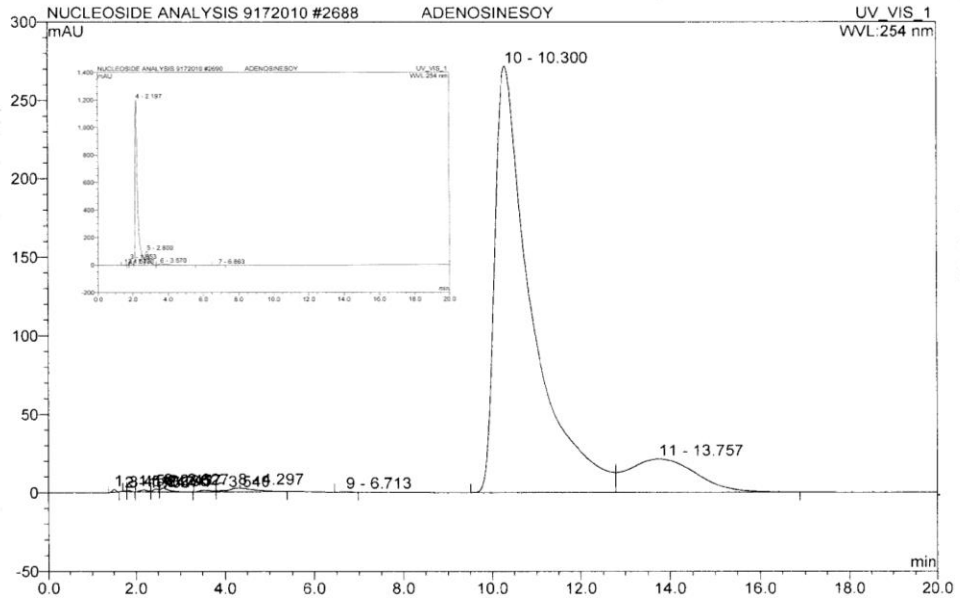

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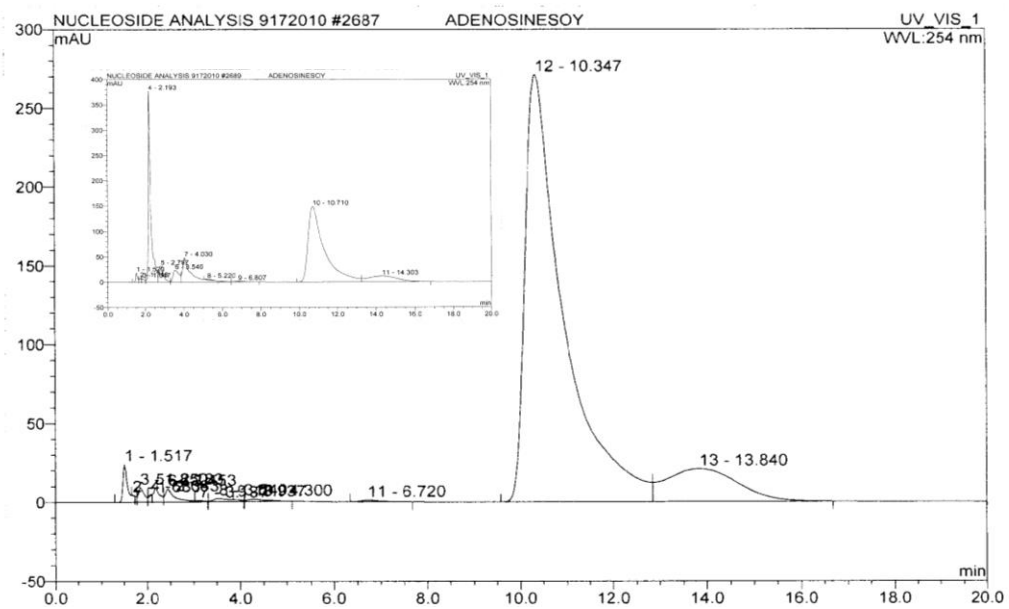
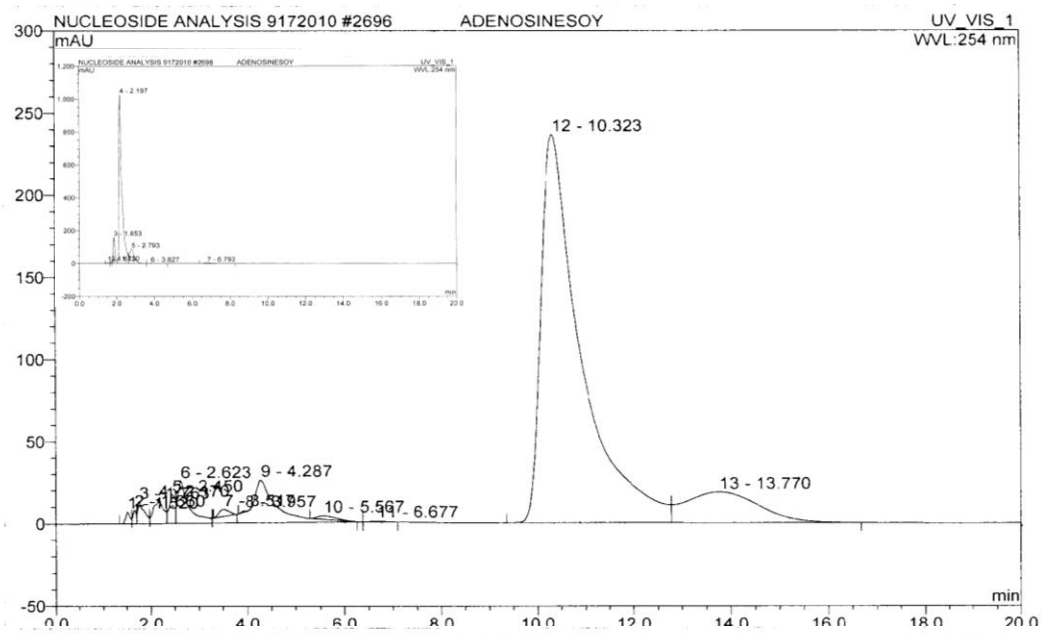


Figure 26. Activity of 30% ammonium sulfate. **(A)** Supernatant- The initial adenosine reaction mixture displayed the adenosine peak at 10.300 min. Approximately 23 hours after the addition of the extract, adenosine was converted to hypoxanthine at 2.197 min (inset). **(B)** Pellet- The initial adenosine reaction mixture displayed adenosine at 10.347 min. Approximately 23 hours after the addition of the extract, adenosine (10.710 min) was partially converted to hypoxanthine at 2.193 min (inset).

A)



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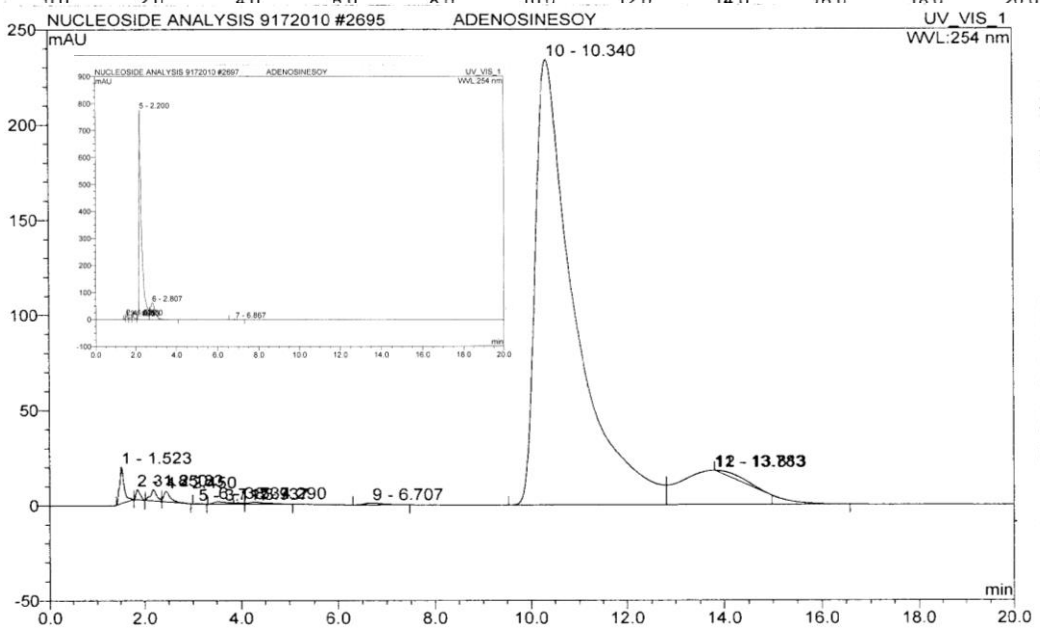

Figure 27. Activity of 60% ammonium sulfate. **(A)** Pellet- The initial adenosine reaction mixture displayed the adenosine peak at 10.323 min. Approximately 23 hours after the addition of the extract, adenosine was completely converted to hypoxanthine at 2.197 min (inset). **(B)** Supernatant-The initial adenosine reaction mixture displayed the adenosine at 10.340 min. After approximately 23 hours after the addition of the extract, adenosine was converted to hypoxanthine at 2.200 min (inset).

strong anion exchange resins. Therefore the charge on the protein needs to be negative in order to bind to an anion exchange column. The result from the DEAE column is shown in Figure 28. The DEAE column resulted in two activity-containing peaks based on the reducing sugar assay. The first peak eluted in the wash, while the second peak eluted at approximately 500 mM NaCl in the middle of the gradient. The reducing sugar assay confirmed there was more activity present in the peak that eluted second. Fractions 21-26 were pooled, concentrated, dialyzed against 50 mM Tris pH 7.2 to prepare for the next step.

The Mono Q ion exchange column gave very similar results to the DEAE column (Figure 29). Because different ligands have different selectivity, a second ion exchange column was used in an attempt to further separate compounds in the soybean extract. More specifically, the column has the capability to allow compounds with amino functional groups to attach easily to the column[46]. This column resulted in the protein with most activity eluting in the second peak on the chromatogram. This peak eluted at the end of the gradient.

Gel filtration, also known as size exclusion, chromatography was the next type of column used after the two ion exchange columns. This column allows different size molecules to elute at different rates. There were three protein and activity-containing peaks eluted from the Sephacryl S100 column (Figure 30). The three peaks occurred between fractions 23-26, 37-39, and 47-49. These fractions were individually dialyzed in 50 mM Tris pH 7.2 (500 mL) and concentrated (2 mL). An aliquot (100 µL) was added to an adenosine standard reaction mixture consisting of 1 mM adenosine, DTT and $MgCl_2$ In a 50 mM Tris buffer.

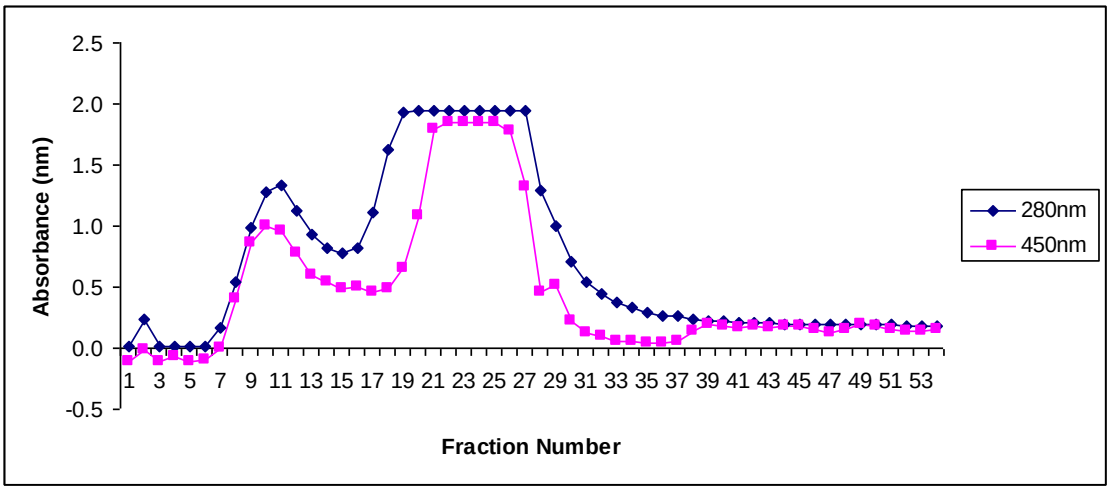

Figure 28. The soybean extract's activity and protein concentration after the elution through DEAE ion exchange. A linear gradient of 0-1 M NaCl in 50 mM Tris pH 7.2 started in fraction 15 and ended in fraction 32. Activity was assayed using the reducing sugar assay, while protein concentration was assayed at measuring the absorbance at 280 nm.

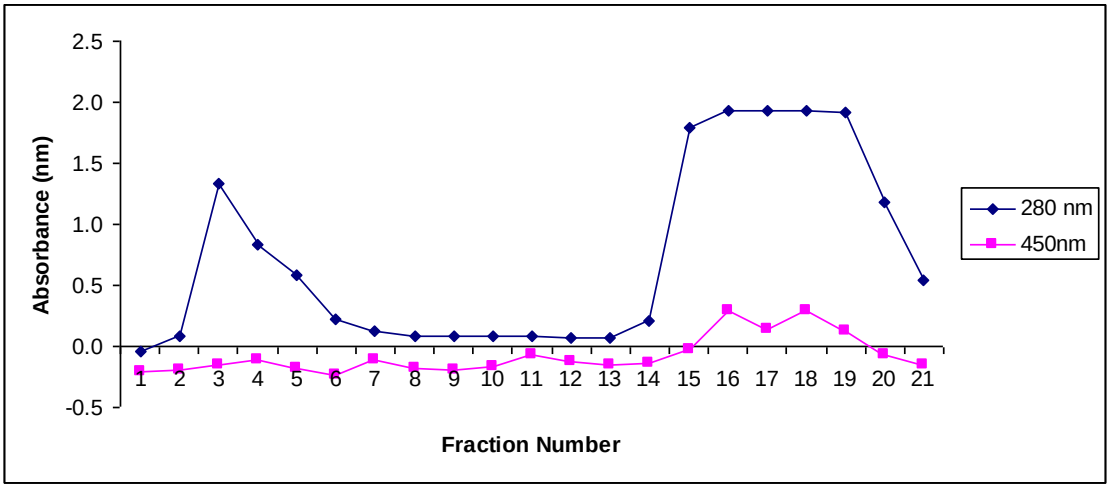

Figure 29. The soybean extract's activity and protein concentration after the elution through Mono Q ion exchange column. A linear gradient of 0-1 M NaCl in 50 mM Tris pH 7.2 started in fraction 7 and ended in fraction 13. Activity was assayed using the reducing sugar assay, while protein concentration was assayed at measuring the absorbance at 280 nm.

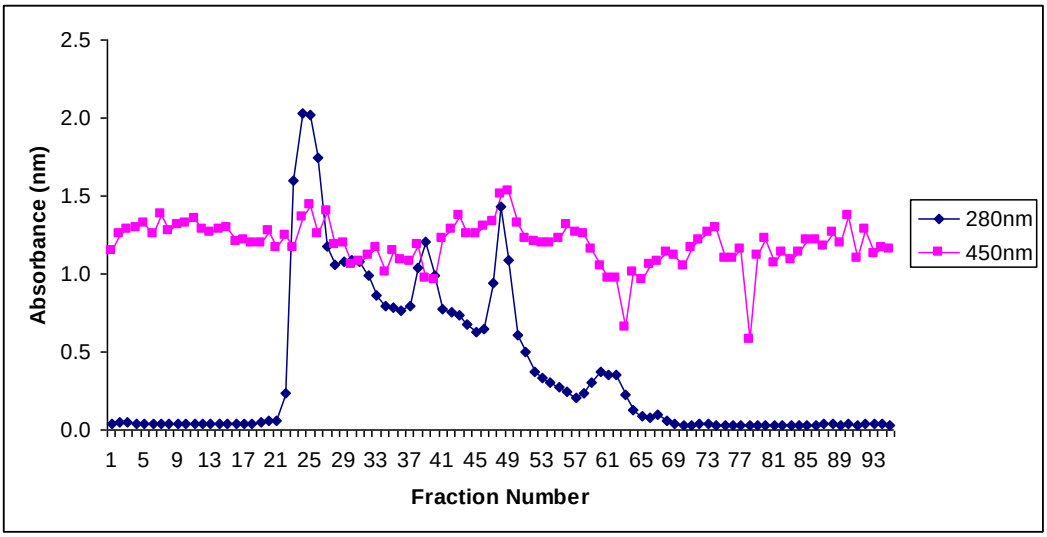

Figure 30. The soybean extract's activity and protein concentration after the elution through Sephacryl S100 (26/60) size exclusion FPLC column. The column was eluted with 50 mM sodium phosphate buffer pH 7.2 containing 300 mM NaCl. Activity was assayed using the reducing sugar assay, while protein concentration was assayed at measuring the absorbance at 280 nm.

The progression of the reaction was monitored using a Dionex HPLC at 254 nm for 23 hours. The third peak (Fractions 47-49) had the highest activity as measured by HPLC. In contrast the adenosine peak was relatively larger than adenine and the appearance of hypoxanthine was evident (Figure 31). The chromatogram indicated the presence of both adenosine nucleosidase and adenosine deaminase based on the appearance of adenine and hypoxanthine. The adenosine control did not exhibit any signs of hydrolysis.

The Sephacryl S100 column was chosen due to its ability to separate proteins by their size and molecular weight. Sephacryl S100 FPLC has a molecular weight range of 1 to 100 kDa. A BioSep SEC-s2000 HPLC size exclusion column, with its greater number

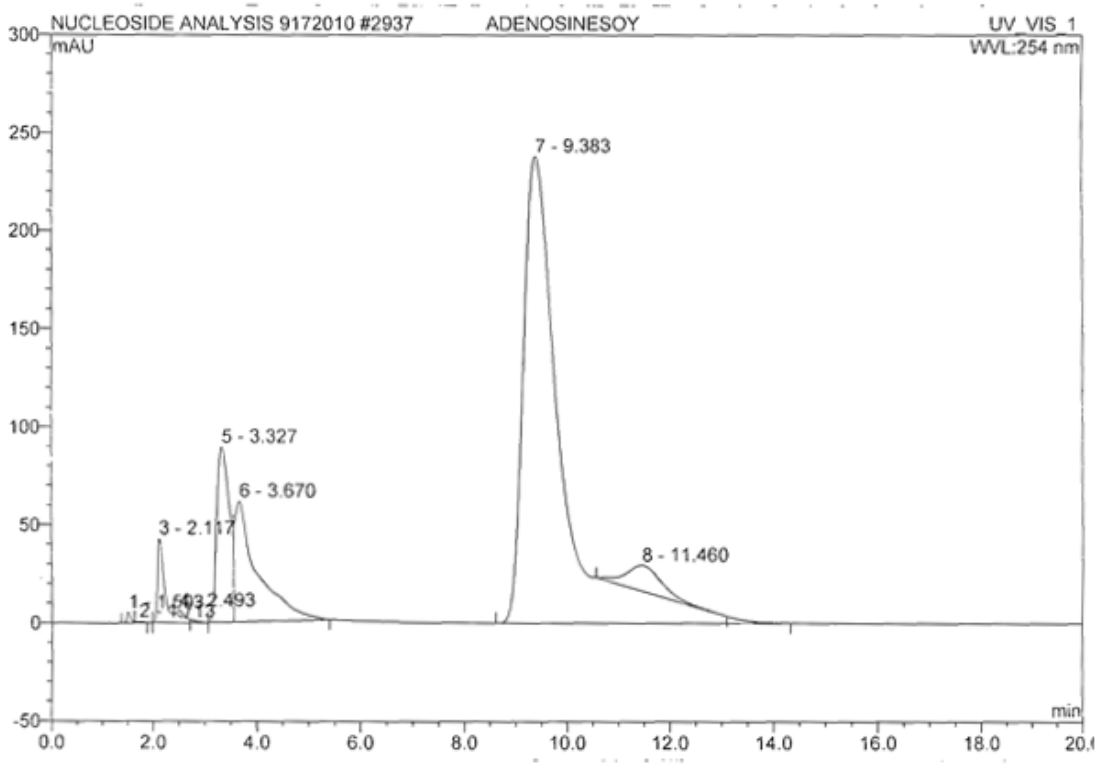

Figure 31. Chromatogram of pooled Fractions 47-49 from the size exclusion column for 23 hour reaction time The chromatogram illustrates the presence of adenosine nucleosidase and adenosine deaminase through the appearance of adenine (3.670 min), inosine (3.327 min), and hypoxanthine (2.117 min), and the disappearance of adenosine (9.383 min).

of theoretical plates, was used to analyze the Fractions 47-49 from the Sephacryl S100 FPLC column containing the highest activity. The chromatogram showed very closely eluting peaks (Figure 32). Based on a calibration curve from proteins of known molecular weight the three major peaks had molecular weights of 75.0 kDa (9.23 min), 58.5k Da (9.550 min), and 16.9 kDa (11.150 min).

Following size exclusion chromatography, the sample was chromatographed on an aminohexyl column. The aminohexyl column consists of an amino group attached to a solid support through a six-carbon spacer. The presence of the amino group mimics the

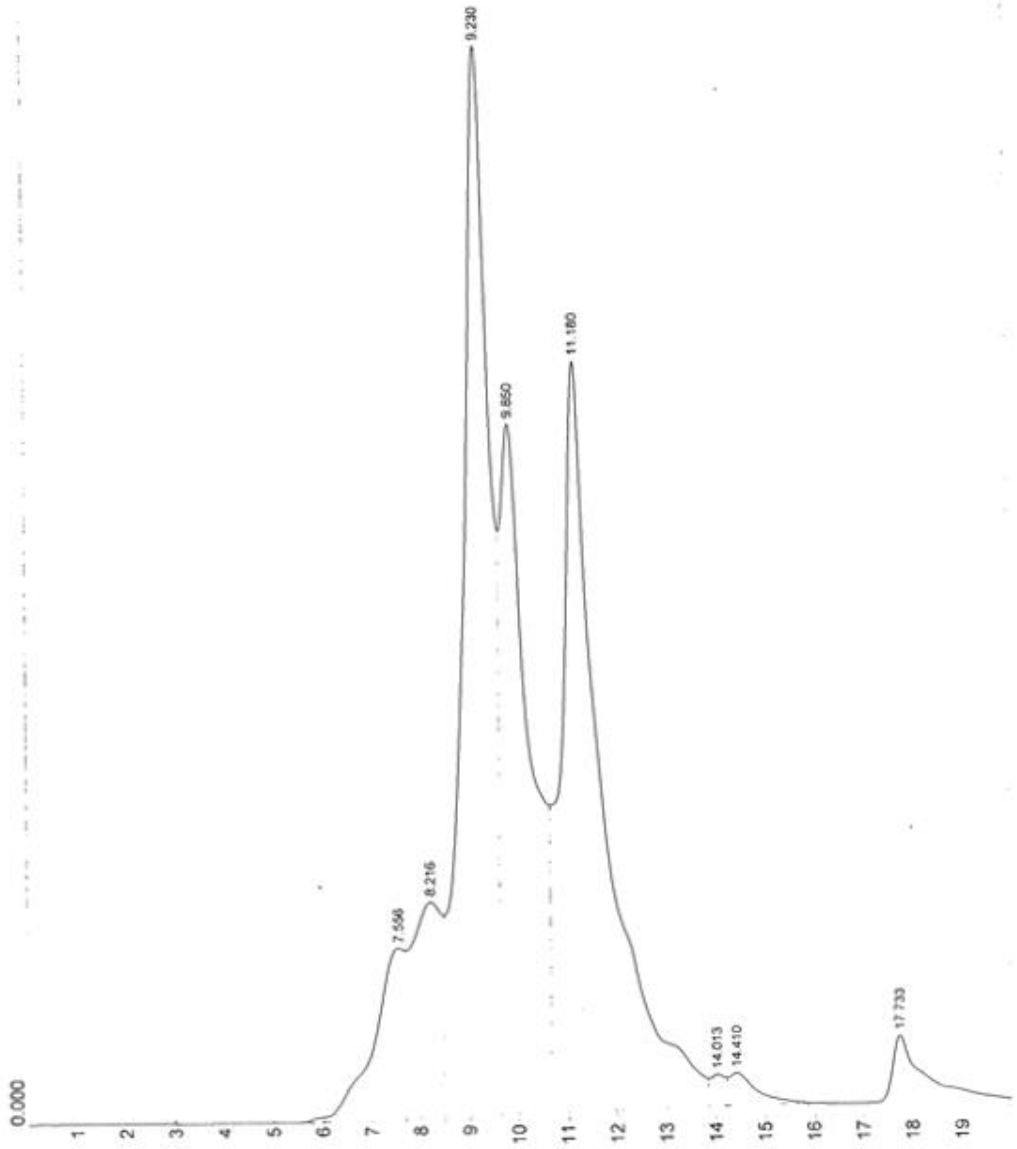

Figure 32. HPLC analysis of pooled Fractions 47-49 from Sephacryl S100 FPLC column using a BioSep SEC-s2000 size exclusion column. It illustrates closely eluting peaks representing the proteins within the extract.

exocyclic amino group at the six position of adenosine. The column was washed with 40 mL of 50 mM Tris buffer pH 7.2 followed by a linear gradient of 40 mL of 0-1 M NaCl

in 50 mM Tris buffer pH 7.2 at a rate of 1 mL/min. It resulted in three significant peaks (Figure 33).

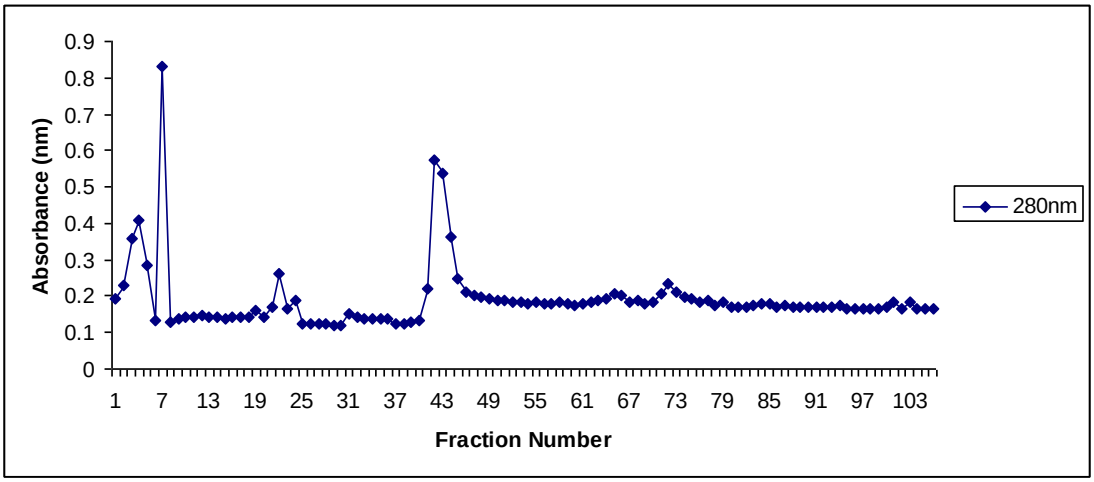

Figure 33. The protein concentration fractions after the aminohexyl column. The column was washed with 40 mL of 50 mM Tris buffer pH 7.2 followed by a linear gradient of 40 mL of 0-1 M NaCl in 50 mM Tris buffer pH 7.2 at a rate of 1 mL/min. The gradient started fraction 20 and ended at 50. Protein concentration was assayed at measuring the absorbance at 280 nm.

The first peak (Fraction 7) eluted during the wash while the second (Fraction 22) and third (Fraction 42-44) peaks eluted at the beginning of the gradient. The protein amounts for Fractions 7, 22, and 42 were approximately 24.53 mg, 46.52 mg, and 37.73 mg respectively. The lowest amount of protein eluted in the wash of this column. HPLC analysis confirmed the presence of both of adenosine nucleosidase and adenosine deaminase activity in Fraction 7 (Figure 34). No activity was detected in either Fractions 22 or Fractions 42-44

Other modes of chromatography were also tried. These included hydroxyapatite (a form of calcium phosphate), Red A, and mixed mode HEA/PPA columns. The

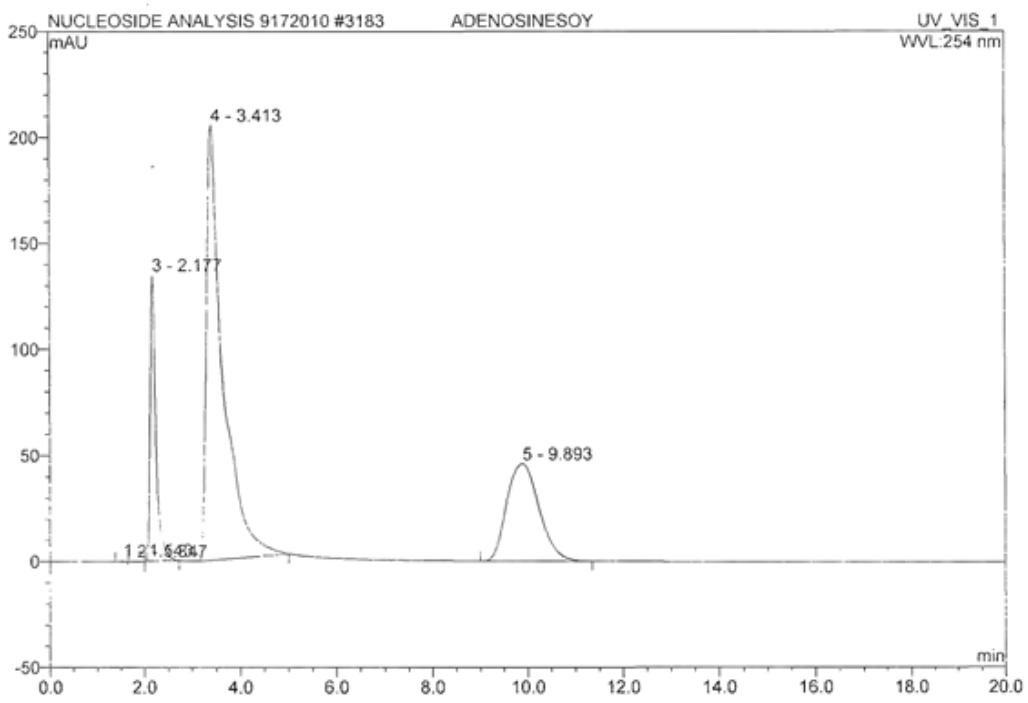

Figure 34. Chromatogram of Fraction 7 from the aminohexyl column. The reaction mixture consisted of extract (100 µL), adenosine, DTT and $MgCl_2$ in a Tris buffer and ran for 23 hours. The chromatogram illustrates the presence of adenosine nucleosidase and adenosine deaminase through the appearance of inosine (3.413 min) and hypoxanthine (2.117 min), and the disappearance of adenosine (9.383 min).

hydroxyapatite column was chosen for its ability to bind proteins based on two modes. The first is due to the interaction of the proteins carboxylic acid groups and the calcium sites on the resin[47]. The second interaction is an electrostatic repulsion between the negative charges on the column and the negatively charged protein[47]. The relative strengths of the two interactions determine the elution position of the protein.

The next column tested was a Red A dye ligand column. It has been demonstrated that some dyes are effective at binding proteins. The Red A resin is often used to purify nucleotide binding proteins since the aromatic triazine dye structure resembles nicotinamide adenine dinucleotides (NAD) [48]. The dye interacts with the

dinucleotide fold in the protein. It is also a highly useful column purification method as a result of the dye's capabilities of not binding most enzymes in a crude extract[48]. In essence the dye ligand resins can act as affinity resins.

Lastly, an HEA/PPA mixed mode column was tested in the purification of adenosine nucleosidase in soybean extract. The mixed mode column separation is based on the hydrophobic interaction between the resin and protein and the protein's isoelectric point (pI). The amino groups also affect the elution of proteins using electrostatic repulsion.

The hydroxyapatite column and Red A ligand column resulted in a tremendous loss of protein and activity with no purification. The protein concentrations in the eluate were undetectable due to it being irreversibly bound to the column. Additionally, the HEA/PPA mixed mode column had very little to no separation. All of the proteins bound to the column and eluted during the final wash resulting in no purification. All of these columns were discontinued in usage in the purification and separation of adenosine nucleosidase.

From the experimental results of the columns individually and in combination, it was decided that the combination of ammonium sulfate fractionation, Mono Q, DEAE and size exclusion chromatography yielded the best purification results. Several expected trends occurred with the purification of adenosine nucleosidase. After each step, the specific activity and purification fold tended to increase while the total activity and percent recovery decreased (Table 4). The best purification step resulted from the gel filtration step in which the specific activity increased 2.2-fold from the previous step. The greatest loss of activity occurred at the 30% ammonium sulfate step leading to a loss of

Table 4. Adenosine nucleosidease purification table.

Purification Step	Total Volume (mL)	Total Protein (mg)	Total Activity (µmol/min)	Specific Activity (µmol/min mg)	Purification Fold	% Recovery
Initial Extract	300.0	30950.0	5100.0	0.16	1.00	100.00
30% Ammonium Sulfate	250.0	2780.0	761.8	0.27	1.66	14.94
60% Ammonium Sulfate	10.0	353.8	220.0	0.62	3.77	4.31
DEAE	2.0	89.4	97.5	1.09	6.62	1.91
Mono Q	2.0	81.8	90.7	1.11	6.73	1.78
Sephacryl S100	2.0	8.0	19.5	2.44	14.79	0.38

85% of the activity. The specific activity increased 14.76-fold from the initial extract to the final size exclusion step. The combination of these columns left two distinct activities present, adenosine nucleosidase and adenosine deaminase. These results were confirmed by SDS-PAGE, isoelectric focusing analysis and HPLC. At the end of the purification, adenosine nucleosidase and adenosine deaminase were inseparable except by isoelectric focusing.

3.3 Analysis of Adenosine Nucleosidase

Because of the inability to separate the two activities, adenosine nucleosidase and adenosine deaminase, by conventional column chromatography an fractionator was used to separate proteins from the third peak (Fractions 47-49) eluted from the size exclusion column by isoelectric point. The OFFGEL fractionator is an isoelectric focusing electrophoresis system. The separation is performed in a pH gradient in an electric field[49]. The negatively charged protein migrates toward the cathode until it reaches a pH where its overall net charge is zero[49]. This point is referred to as its isoelectric point (pI)[49]. The OFFGEL fractionator separated the sample into 24 fractions between pH 3 to 10. The individual fractions were then analyzed by SDS-PAGE. The gel showed protein in three of the 24 wells. The first band was a minor band in Fraction 4 representing a pI of 1.2. The next band in Fraction 5 was the major band. This represented an isoelectric point of 1.5. Lastly in Fraction 6 there was a very faint band. The lane represents an isoelectric point of 1.8. An SDS-PAGE was then run on the three fractions to determine their subunit molecular weight. Based on the position of the protein bands relative to the molecular weight markers, the molecular weight of the protein subunits was approximately 18000 daltons (Figure 35). These results are also consistent with the ion

exchange results. At a pH above its pI, a protein is negatively charged. Since the pH of the buffers used to elute the columns was 7.2, the target protein should bind to a positively charged anion exchange column.

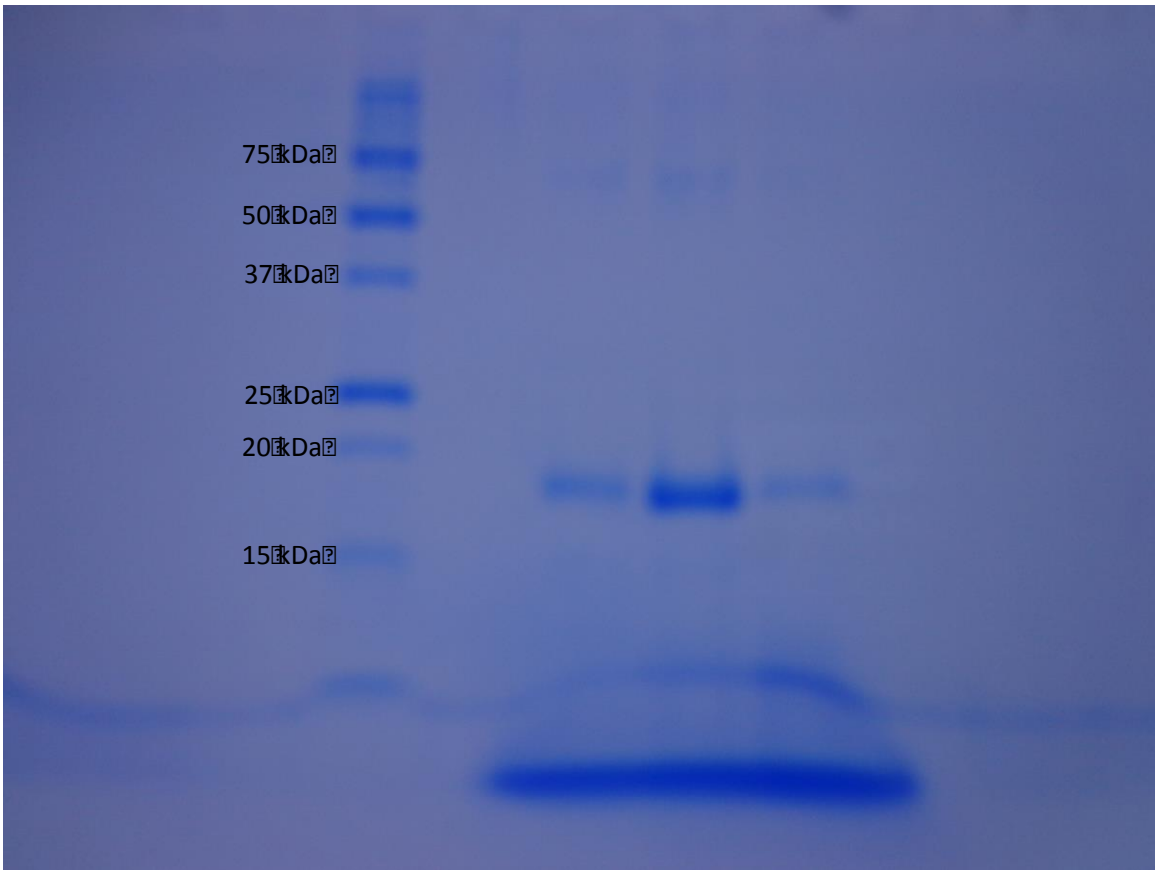

Figure 35. SDS-PAGE of OFFGEL Fractionator after separation by size exclusion column chromatography (Fractions 47-49). Lane 1. Precision Protein Markers Lane 2. Fraction 4 (minor band) represents a pI of 1.2; Lane 3. Fraction 5 (major band) represents a pI of 1.5; Lane 4. Fraction 6 (faint band) represents a pI of 1.8. The molecular weight of the bands is approximately 18000 daltons.

Adenosine nucleosidase may exist as a multimeric protein. The subunit molecular weight is approximately 18000 Da. The molecular weight of the activity-containing fractions from the Sephacryl S100 column were 75.0 kDa (9.23 min), 58.5k Da (9.550 min), and 16.9 kDa (11.150 min). Based on these molecular weights the enzyme could be

a tetramer, trimer, or a monomer. Based on the structure of other adenosine nucleosidases the protein is most likely to be a multimeric protein rather than consist of a single subunit. However to ultimately decide between these possibilities will require additional purification.

CHAPTER FOUR

CONCLUSION

In conclusion, adenosine nucleosidase and adenosine deaminase activity is present in early germination of soybeans. In addition to those enzymes being present during early germination, both phosphorylase and hydrolase activities were shown to be present using thymidine, guanosine, and inosine as substrates. Cytidine and uridine phosphorylases and hydrolases could not be determined due to the inability to separate cytidine from cytosine and uridine from uracil using the current HPLC conditions. The initial extract produced from the soybean seeds was precipitated with ammonium sulfate at 30% and 60% saturation. The 60% ammonium sulfate pellet was dialyzed against 50 mM Tris buffer pH 7.2 containing 1 mM DTT after centrifugation. Next, the dialyzed extract was loaded onto a Sephadex DEAE ion exchange column. The fractions were assayed for protein by measuring the absorbance at 280 nm and for activity by reducing sugar assay using adenosine as the substrate. Fractions containing adenosine nucleosidase activity were pooled, concentrated and dialyzed. The concentrated fraction was dialyzed against 50 mM Tris pH 7.2 (500 mL) and loaded onto a HiLoad Sepharose Fast Flow Mono Q column. Following analysis, fractions containing enzyme activity were pooled, concentrated, dialyzed and loaded onto Sephacryl S100 (26/60) size exclusion FPLC column. Lastly, the fractions containing activity were pooled, concentrated, dialyzed and loaded onto an aminohexyl column. Other modes of chromatography were tested for their ability to further purify the protein. These chromatography steps included hydroxyapatite chromatography, dye ligand chromatography, and mixed mode chromatography. None of these types of chromatography resulted in further purification.

The protein from the size exclusion column was further analyzed by OFFGEL fractionation. The SDS-PAGE of the OFFGEL fractionation revealed three bands. The first band was a minor band in Fraction 4 representing a pI of 1.2. The next band in Fraction 5 was the major band. This represented an isoelectric point of 1.5. Lastly in Fraction 6 there was a very faint band. The molecular weight of the protein subunit was approximately 18 kDa. Adenosine nucleosidase and adenosine deaminase from soybean apparently have very similar properties including similar molecular weights and isoelectric points. The continuation of this project will consist of further purifying and characterizing adenosine nucleosidase and deaminase in early germination of soybeans by exploring other purification techniques of separating these enzymes. Based on the OFFGEL fractionation results chromatofocusing appears to offer the best hope for separating the two activities. In addition the purified proteins will be sequenced using LC/MS/MS to determine their relationships to adenosine nucleosidases and adenosine deaminases from other sources. Recently, weeds have been showing a tremendous resistant to glyphosate. This research can be used to help facilitate the design of a new herbicide.

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