SURVEY OF NUCLEOSIDE DEGRADING ENZYME ACTIVITIES IN SOYBEAN, CORN AND YELLOW LUPIN

by

Sergiy Ustynov

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Master of Science in Chemistry

Middle Tennessee State University

December 2014

Thesis Committee:

Dr. Paul C. Kline, Chair

Dr. Anthony Farone

Dr. Andrew Burden

In loving memory of my amazing grandparents, Tabatchikov Alexander Isaakovich and Kuprina Lydia Lazarevna.

ACKNOWLEDGEMENTS

I would like to thank Dr. Paul C. Kline for his continual mentorship, patience and support throughout this project that saw it come to fruition at last. This day would never come without your dedication and understanding. I would also like to extend my gratitude to my thesis committee members, Dr. Andrew Burden and Dr. Anthony Farone. I greatly appreciate their time commitment and valuable advice during the preparation of my thesis. I feel it is especially important to thank both Dr. Mary Farone and Anthony Farone for their belief in my abilities and encouraging words. I must also thank Drs. Farone for making me a part of an amazing fellowship program through which I had the privilege to meet a great number of exceptional professionals and leaders in the scientific community. Finally, I would like to thank Dr. Tammy Melton, Dr. Norma Dunlap, Dr. Andrienne Friedli and Dr. Scott Handy along with Jessie Weatherly, and other faculty and staff members for their valuable support and kind words over the years.

In addition, I must express my heartfelt appreciation to my loving mother, step-father and brother. Your words of wisdom and seemingly never-ending well of confidence in me kept me on track and gave me strength to keep moving forward.

ABSTRACT

Efficient pyrimidine and purine nucleotide metabolism is essential for healthy growth and development of all organisms. Significant energy cost reduction and a mutation bypass mechanism is afforded via the recycling pathways of nucleotide metabolism in microorganisms, animals and plants. Numerous enzymes are involved in the degradation and salvage of nucleotides, nucleosides and nucleobases. Nucleosidases, phosphorylases and deaminases contribute during the degradation of deoxyribonucleotides and ribonucleotides.

Presence of nucleosidase, phosphorylase and deaminase activities were evaluated in 5- 7 day old yellow lupin seedlings and 5-9 day old soybean seedlings. Nucleosidase and deaminase activities were also assessed in 5-7 day old corn seedlings. Nucleosidases catalyze hydrolytic cleavage of the ribose moiety of the nucleoside and were found active in all three plants species surveyed. Significant variation was noted in the level of activity observed between yellow lupin and soybean, both legumes. Phosphorylases mediate a reversible reaction that breaks the ribose away from the nucleobase in the presence of phosphate ions and may be present in yellow lupin. Adenosine phosphorylase is likely active in soybean. Deaminases assist in the interconversion of nucleosides. Cytidine deaminase activity was found in all three plants. Adenosine/adenine deaminase activity was observed in soybean and corn but not in yellow lupin. Nine nucleosides were evaluated as substrates and specific activities are reported.

TABLE OF CONTENTS

PAGE

LIST OF FIGURES

PAGE

LIST OF TABLES

PAGE

CHAPTER I

INTRODUCTION

The vast body of knowledge related to biochemical processes has grown steadily over the past several decades all due to the unquenchable desire of the human mind to unravel the secrets of life. Superficially, the cycle of life can be described as an elegant process consisting of birth, growth and development, reproduction and death. These stages are an adequate, even if simplistic, representation of the life cycle for complex organisms such as animals and plants. On the cellular level, however, the story itself is more intricate. The birth of a new cell begins when one cell divides or when two cells, such as a sperm and an egg cell, fuse.[1] The biogenesis of a new cell is made possible by a cell replication program encoded in the DNA and carried out by proteins.[1] The process involves a period of cell growth, during which DNA is copied into mRNAs coding for the necessary proteins, the DNA is replicated, followed by a cell division producing two daughter cells.[1] The growth and proliferation of a cell is a highly regulated process, assuring that the needs of a growing or adult organism are met.[1] A cell can also remain in what can be considered a steady-state.[1] The DNA is once again transcribed into a specific set of mRNAs, which give rise to a specific set of proteins needed to maintain normal cell function.[1] As the needs of the cell change, and proteins fulfill their designated function, they are degraded and replaced by new ones. New mRNAs have to be read from the DNA and translated into different proteins. In this state the system is so balanced that the cell neither grows, shrinks, nor changes its function.[1] Ultimately, in order to remain alive and satisfy its function, the cell must be incredibly efficient at utilizing available resources whether external or internal. The numerous biochemical processes involved in keeping the spark of life going have fascinated

the minds of chemists and biologists alike. Biochemistry, however, is the discipline solely devoted to the study of chemical reactions within living organisms.

The field of biochemistry itself is very complicated, diverse and exciting, and at times full of surprises. It is very fortunate that basic life sustaining reactions appear to be very similar whether the cell is bacterial, a plant cell or even a mammalian cell. These reactions take information stored in a gene all the way to biologically active components such as secondary metabolites or hormones via nucleotide modification. A great deal of research on nucleotide metabolism has already been accomplished using the simpler forms of life - microorganisms. Plants are a logical next step and present an excellent model organism to further the understanding of more advanced biochemical processes.

Nucleotide metabolism

A nucleotide is a biologically active molecule that consists of a nitrogenous base, an attached ribose sugar and a phosphate group. Nucleotides are divided into two groups based on the cyclic nature of the base. The purine nucleotide group consists of bases with a two-ring structure, while the pyrimidine group includes single ring bases (Figure 1).

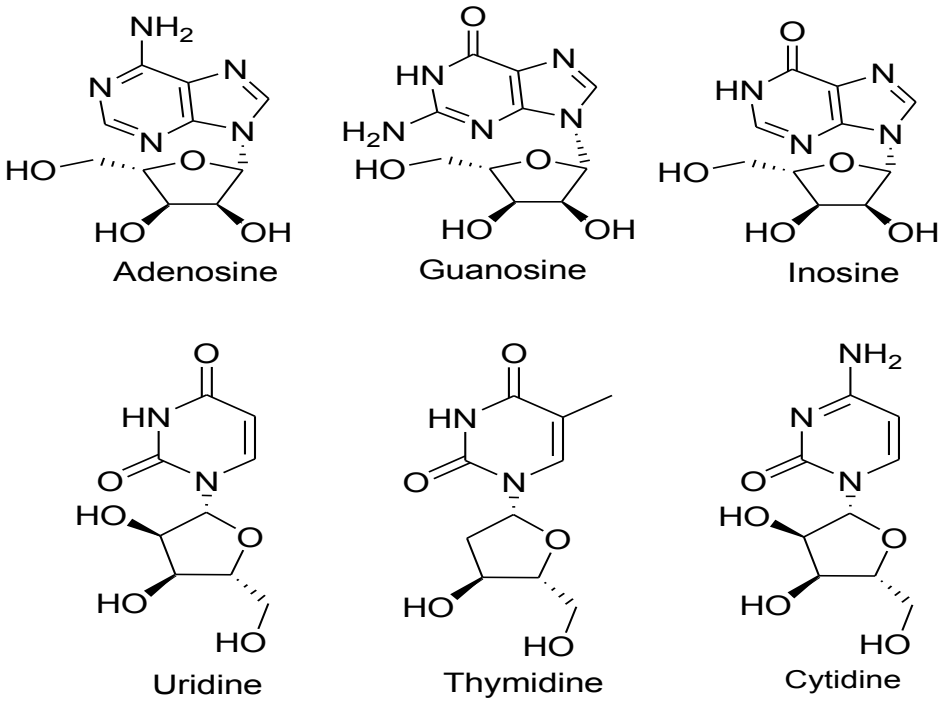


Figure 1. Most common purine and pyrimidine structures. Bases with two ring structures are purine nucleosides and bases with single ring structures are pyrimidine nucleosides.

Plant growth, development and metabolism depend on a functioning purine and pyrimidine primary metabolism, secondary metabolism and gene expression.[2] Information storage and retrieval is essentially impossible without nucleotides that act as building blocks for DNA and RNA. These purine and pyrimidine molecules also serve as direct precursors during the synthesis of B-class vitamins such as riboflavin, thiamine, and folates.[3,4,5] In addition, purine nucleotides participate in signal transduction and are components of several essential coenzymes such as nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD) and S-adenosylmethionine (SAM).[6,7] The purine nucleotide, adenosine triphosphate (ATP), a major energy contributor in many metabolic reactions, is produced from adenosine diphosphate (ADP) and phosphate during the processes of photosynthesis and respiration. ATP is also involved in production of activated precursors for the synthesis

of many macromolecules such as adenosine monophosphate (AMP)-activated amino acids for protein synthesis and ADP-glucose for starch synthesis. Pyrimidine nucleotides are also involved in many important reactions as co-substrates. Uridine triphosphate (UTP) and uridine diphosphate (UDP) were found to be active during synthesis and degradation of sucrose. UDP-glucose is an important building block for the synthesis of cellulose and additional polysaccharides, glycoproteins and phospholipids.[8]

The necessity of maintaining the right balance of nucleotides is of great importance, and survival of the cell may well depend on it. It has been shown that phosphorylated compounds in the cell are toxic if allowed to accumulate.[9] Perturbation of the endogenous nucleoside pool, effected by exogenous applications of nucleosides, results in DNA fragmentation and cell death of mouse thymocytes.[10] Indeed, upon supplying tobacco BY-2 cells with exogenous nucleosides, alterations in pyrimidine nucleotide synthesis and utilization were observed. Changes in the synthesis and degradation of pyrimidine nucleotides were shown to represent a possible metabolic signal that precedes an activation of cellular failure mode eventually ending with the death of the cell.[11] The proper balance between nucleotide and nucleoside pools is maintained by breaking down nucleotides to the corresponding nucleosides and nucleobases. The resulting simpler precursor molecules can then be further catabolized or recycled depending on the specific needs of the cell at the time. In *Arabidopsis thaliana,* the delay in germination of mutants with increased or decreased uridine nucleosidase activity demonstrates the balancing of uridine degradation versus salvage, and is important at this developmental stage to allow liberation of nitrogen.[12] Nucleotide metabolism can be categorized into four broad metabolic pathways for some degree of simplicity. The *de novo* synthesis, nucleotide degradation and salvage pathways

are the three central pathways. There are also phosphotransfer reactions involved in maintaining equilibrium of different pools of nucleotides as well as modification reactions via addition of side chains.

***De novo* synthesis of nucleotides**

There are several key monophosphate entities central to most biochemical processes in microorganisms, plants and animals. Uridine-5'-monophosphate (UMP) has a central role within pyrimidine metabolism. It is synthesized from precursors via the orotate pathway. This process brings together carbamoylphosphate (CP), aspartate and 5-phosphoribosyl-1-pyrophosphate (PRPP). In all, it is a six step process. CP is generated by action of carbamoylphosphate synthetase (CPSase). In this reaction a carbonate, 2 ATP, and an amino group are utilized. In the following condensation reaction aspartate transcarbamoylase (ATCase) catalyzes the formation of carbamoylaspartate (CA). Orotate (OA) is formed during sequential cyclization and oxidation reactions by action of dihydroorotase (DHOase) and dihydroorotate dehydrogenase (DHODH), respectively.[2] Condensation with PRPP and subsequent decarboxylation results in UMP formation (Figure 2).

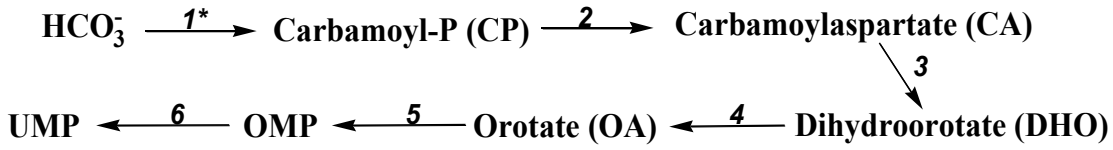


Figure 2. Pyrimidine *de novo* synthesis in plants. (*) designates an ATP requirement; 2 ATP are used during the formation of CP. Enzymes shown are: (1) carbamoyl phosphate synthetase, (2) aspartate transcarbamoylase, (3) dihydroorotase, (4) dihydroorotate dehydrogenase, (5-6) UMP synthase (a bifunctional enzyme with orotate phosphoribosyltransferase and orotidine-5'-phosphate decarboxylase capabilities).[2,13]

Inosine monophosphate (IMP) is the first nucleotide ring product of the purine metabolism in plants. It is a significantly longer, and a more costly, synthetic pathway that starts with small molecules such glutamine, glycine and aspartate, the activated ribose precursor PRPP, N^{10}-formyl tetrahydrofolate (N^{10}-formyl THF), and carbon dioxide.[2] Altogether, there are 14 enzyme-mediated reactions to produce an ATP molecule. The first reaction results in formation of phosphoribosylamine (PRA) from PRPP and glutamine. Glycine amide ribonucleotide (GAR) is generated by addition of glycine to PRA, an ATP dependent reaction. GAR is converted to formylglycinamide ribonucleotide (FGAR) by action of GAR transformylase (GART). This reaction requires participation of N^{10}-formyl THF. Another ATP dependent step results in formation of formylglycinamidine ribonucleotide (FGAM). A synthetase mediates this reaction and also consumes another glutamine. The first ring closure requires yet another ATP molecule and yields 5-aminoimidazole ribonucleotide (AIR). The formation of AIR is facilitated by AIR synthase (AIRS). In the sixth step construction of the second ring is initiated when CO_2, aspartate, and another N^{10}-formyl THF molecule are inserted. AIR is carboxylated to 4-carboxy aminoimidazole ribonucleotide (CAIR). The formation of N-succinyl-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR) requires consumption of another molecule of ATP and addition of aspartate. A lyase assists in generation of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) by releasing fumarate. IMP is formed in the next two steps catalyzed by a bifunctional enzyme 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase (ATIC). At first, N^{10}-formyl THF donates the final carbon to the purine ring skeleton and 5-formaminoimidazole-4-carboxamide ribonucleotide (FAICAR) is formed. Then FAICAR is dehydrated and ring

closure results in IMP.[2] After IMP, purine biosynthesis may proceed in two directions that lead to the formation of either adenosine monophosphate or guanosine monophosphate via xanthosine monophosphate modification (Figure 3).

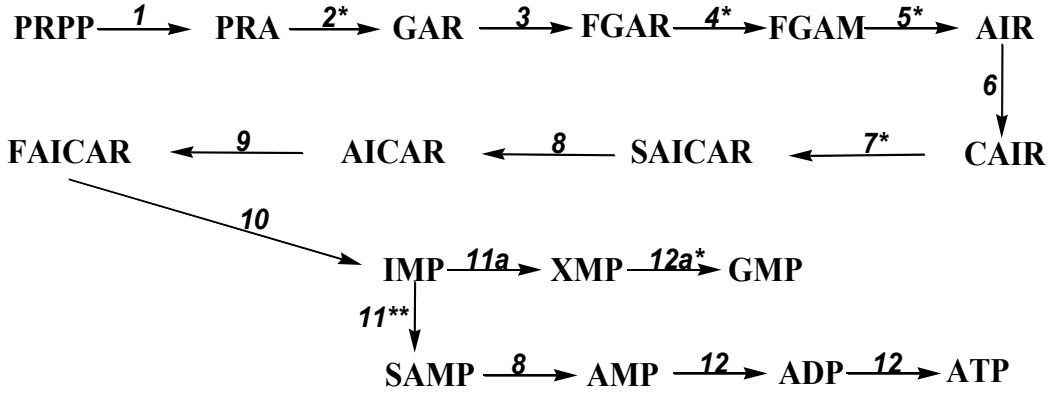


Figure 3. Purine *de novo* synthesis in plants. (*) designates an ATP requirement; (**) use of a GTP molecule as an energy donor. Enzymes shown are: (1) amido phosphoribosyltransferase, (2) GAR synthetase, (3) GAR formyl transferase, (4) FGAM synthetase, (5) AIR synthetase, (6) AIR carboxylase, (7) SAICAR synthetase, (8) adenylosuccinate lyase, (9) AICAR formyl transferase, (10) IMP cyclohydrolase, (11) SAMP synthetase, (11a) IMP dehydrogenase, (12) adenylate kinase, (12a) GMP synthetase.[2,13]

Degradation pathways of purines and pyrimidines

In essence, the catabolic pathways work in the opposite direction of the *de novo* synthesis pathways. The process begins when 5'-nucleotidases convert nucleotides to nucleosides by removing the 5'-phosphate. In turn, nucleobases are released by the action of nucleosidases that hydrolyze the N-glycosidic bond between the ribose sugar and the nucleobase. Generally, most or some of these degradation reactions can be found in all organisms. Despite the significant areas of biochemical overlap, all the evidence gathered to date show many areas of divergence between kingdoms, as well as across species. These areas of divergence have been noted by observing the presence or absence of certain

enzymatic activities. For example, adenosine deaminase was shown to exist in animal models as well as microorganisms.[14,15] This enzyme converts adenosine to inosine which may then be degraded to hypoxanthine by action of inosine nucleosidase. The presence of adenosine deaminase in plants has been an area of much debate with no tangible resolution. While some reports show that the deaminase may be active, this enzyme is yet to be purified.[16,17]

Plants depend on an efficient nitrogen utilization, and store rather than excrete nitrogen.[18] For this reason purine catabolism plays an important part in plant nitrogen metabolism. In most plants, the purine nucleotides are oxidatively degraded via uric acid and allantoin to CO_2, NH_3, and glyoxylate which may either be reassimilated for use in photosynthesis (CO_2) or recycled via the glutamine oxoglutarate aminotransferase (GOGAT) pathway.[19] Adenine and guanine nucleotides take slightly different routes, but both converge on xanthine, which is the central metabolite in purine degradation prior to purine ring cleavage[7]. It is largely believed that AMP is converted to IMP by AMP deaminase (AMPD). IMP is dephosphorylated to inosine and then converted to hypoxanthine via hydrolysis by inosine/guanine nucleosidase. Hypoxanthine is further transformed to xanthine by the action of xanthine dehydrogenase (XDH).

This is not the case in tropical legumes such as soybean where IMP is changed to XMP by inosine-5'-monophosphate dehydrogenase (IMPDH), and then degraded by 5'-nucleotidases and inosine/guanine nucleosidase to xanthine.[20] Another alternative pathway which takes adenosine to inosine or adenine to hypoxanthine, by either action of adenosine deaminase[13,21] or adenine deaminase[22], appears to not exist in plants. This alternative pathway remains an area of debate because activity that indicates conversion of adenosine to

inosine or adenine to hypoxanthine has been observed in alfalfa seedlings and cell cultures, and recently soybean.[16,17] Matters are complicated further by the fact that the deaminase protein itself has been extremely difficult to purify and the task still remains to be accomplished. On the other hand, guanosine deaminase (GSDA) has been found in plants.[23,24] The guanosine nucleotide, GMP, is converted to the nucleoside form by removal of the phosphate. Guanosine is degraded to xanthine in two ways. It is either deaminated to xanthosine or converted to guanine by inosine/guanine nucleosidase.[13,25] The purine catabolic pathway has been found to have other important functions. The alkaloids theobromine and caffeine are methylated xanthine derivatives that may serve a defensive purpose either as herbicides or insecticides.[7,26]

Pyrimidine precursors have also been shown to fill various functional needs in plants. Once metabolized to secondary products these molecules can also serve in defense of the plant.[27] Uridine and cytidine nucleotides are converted to uracil, the central intermediate metabolite of the pyrimidine degradation pathway. Uridine is typically converted to uracil by the action of a uridine hydrolase, a non-specific nucleosidase or uridine phosphorylase. Cytidine nucleosides are degraded after conversion to uridine by the action of cytidine deaminase (CDA).[13] Cytosine deaminase has not been found in plants[13]. Thymidine may be converted to thymine by the action of thymidine phosphorylase and/or thymidine nucleosidase.[13,21,27] Uracil and thymine are catabolized by the same three enzymes, dihydrouracil dehydrogenase, dihydropyrimidinase and finally β-ureidopropionase.[13] This reductive pathway results in formation of β-alanine or β-aminobutyrate through the release of NH_3 and CO_2. It has been proposed that uracil degradation might be an important source of β-alanine as a precursor for the pantothenate moiety of coenzyme A.[28,29] The only

deaminase that has been definitively shown to exist in the pyrimidine pathway is cytidine deaminase. CDA was cloned, expressed and purified from *Arabidopsis thaliana.*[30]

Salvage pathways in purines and pyrimidines

In addition to synthesizing nucleosides and nucleotides from small molecules, many organisms are able to recycle nucleobases via salvage pathways. These scavenging reactions pick up nucleosides and free bases moving along the degradation pathways and return them to the cellular machinery. This process is more energy efficient than the *de novo* synthesis. Preformed nucleosides and nucleobases may be rephosphorylated with the help of kinases and phosphoribosyltransferases. Free bases may be diverted into modification pathways.[2] Nucleoside phosphorylases convert nucleosides into the respective free bases and ribose-1-phosphate in the presence of phosphate ions.[31] The phosphorylase mediated reactions work in both directions, however, and thus can be considered truly recycling. For example, both uridine phosphorylase (udp) and uridine hydrolase (udh) were found in *E. coli.*[32] Uridine hydrolase (also known as uridine nucleosidase) converts uridine to uracil. Uridine phosphorylase degrades uridine to uracil and regenerates uridine from uracil and ribose-1-phosphate. This may provide an exceptional survival mechanism for the cell in case a mutation renders the gene encoding one of the two enzymes useless. In fact, many organisms are able to use cytidine, uridine or uracil as precursors to DNA and RNA whenever there is a mutation in the biosynthetic pathway.[33] It has also been suggested that recycling pathways may be used to generate nucleobase supplies for cells which are struggling to make their own.[2] Nucleosides and nucleobases have smaller molecular weights, less charge and may be easier to transport. Recently homologues of the ureide permease (UPS) transporters in *Arabidopsis* were shown to be functional for uracil and

potentially other nucelobases.[34] Three basic families of equilibrative nucleobase transporters have been proposed on the bases of sequence similarities. One of these families appears to only be found in plants and is responsible for moving purine and pyrimidine nucleobases with the same specificity.[35]

Perhaps the most dramatic example that demonstrates the exceptional utility of the pyrimidine salvage pathways can be found in eukaryotes. Orotic aciduria is a genetic disease found in human beings, which is the result of orotic acid buildup due to the absence of either orotate phosphoribosyltransferase or orotidine-5'-phosphate decarboxylase activity.[36] The two enzymes operate sequentially and convert OA to UMP (Figure 2). Individuals affected by this disease will present large amounts of orotic acid in the urine, megaloblastic anemia and retardation of growth.[36] Successful treatment is achieved upon administration of pyrimidine nucleosides cytidine and/or uridine. The salvage enzymes cytidine deaminase and uridine kinase are likely responsible for production of important pyrimidines needed for stabilization of nucleotide pools in the affected patients. The observed significant reduction in orotic acid accumulation is the result of normalization in UTP and CTP pools which have an inhibitory effect on the biosynthetic pathway.[36]

Purine salvage is just as active across all organisms and its importance for energy conservation is even more significant.[7] AMP production via the *de novo* biosynthesis utilizes five nucleotides and as many as seven to make a single GMP. In contrast, the purine salvage pathway uses a single ATP.[2] As with pyrimidines there seem to be several salvage routes that reuse purine bases and nucleosides. Adenine and guanine may be taken back directly to their monophosphate forms by adenine and hypoxanthine/guanine

phosphoribosyltransferases (APRTase and HGPRTase, respectively) in the presence of PRPP to supply the needed ribose phosphate.[13,24]

Purine phosphorylases restore adenosine, inosine and guanosine from adenine, hypoxanthine and guanine, which are in turn converted to IMP, XMP and GMP by action of kinases and phosphotransferases.[13] When interconversion of one purine into another is needed, plants may utilize adenosine deaminase and guanosine deaminase. Adenosine nucleosidase also contributes to purine salvage reactions.[2,36,38] As previously noted, plant adenosine and/or adenine deaminase still remains elusive. In some cases the deaminase activity in plants has been shown. However, a tangible purified form has been difficult to obtain. In the *Arabidopsis* genome, a candidate gene (At4g04880) has been annotated as a putative adenosine deaminase. Its expression was verified.[2] In general adenine recycling in plants is considered to be well established on the basis of genomic evidence in *Arabidopsis*. Investigations on the guanine/guanosine salvage are limited to biochemical data for HGPRTase, guanosine nucleosidase, and guanine deaminase in plants.[2] The biologically active purine derivatives, also known as cytokinins, are believed to be synthesized either directly from AMP in *Arabidopsis*[39] or through interconversion of free base cytokinins to nucleoside and nucleotide cytokinins.[2,40] The importance of the purine salvage pathways can also be observed in humans. Lesch-Nyhan syndrome is linked to a deficiency in the salvage enzyme hypoxanthine-guanosine-phosphoribosyltransferase.[41] The salvage of purine metabolites does not properly counterbalance the degradation pathway which leads to an increased accumulation of uric acid in blood. Symptoms include mental retardation, choreoathetosis, and compulsive self-mutilation.

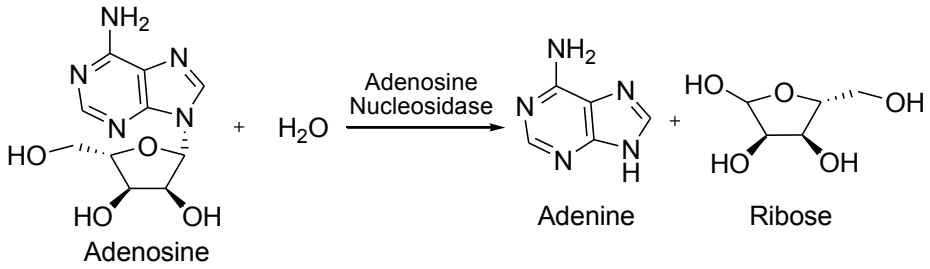
Nucleoside hydrolases, phosphorylases and deaminases

Understanding the significance of the nucleotide recycling mechanism has propelled a more rigorous exploration of metabolic pathways in all organisms. A vast, although somewhat fragmented, body of work has emerged in the recent decades on nucleotide metabolism in higher plants. The majority of the data gathered to date relies heavily on kinetic studies of enzymatic activities or incorporation of radioactivity in various products of metabolism.[6] Most frequently these studies involved crude extracts, and only in some cases the purified form of the enzyme in question. Kinases and phosphoribosyltransferases are the enzymes of the salvage processes responsible primarily for rephosphorylating the nucleosides and nucleobases, respectively.[42] Nucleotidases and hydrolases are the enzymes that participate in the degradation processes and are primarily responsible for the removal of phosphate and ribose sugar from the nucleotide and nucleoside, respectively.[2] Phosphorylases are the only true recycling family of proteins that operate in both directions. In general, phosphorylase role and level of activity is not thoroughly understood. There are a few reports of very low adenosine and inosine-guanosine phosphorylase activity in higher plants.[13,21] Previously, a group of plant stress-inducible proteins suspected to catalyze phosphorolysis were identified based on sequence similarities with bacterial and eukaryotic enzymes.[43] Recently, a phylogenetic analyses has revealed two major cluster of nucleoside phosphorylase (NP)-like proteins in plants.[44] Group I proteins were encoded by genes across a wide range of plant taxa.[44] Species found throughout the order *Malpighiales* contained Group II genes encoding NP-like proteins.[44] Deaminases are a group of modification enzymes contributing to the recycling pathways. Clearly, the diversity of enzymes involved in recycling of nucleotides makes it a vast field of research. Thus, from this point on, this

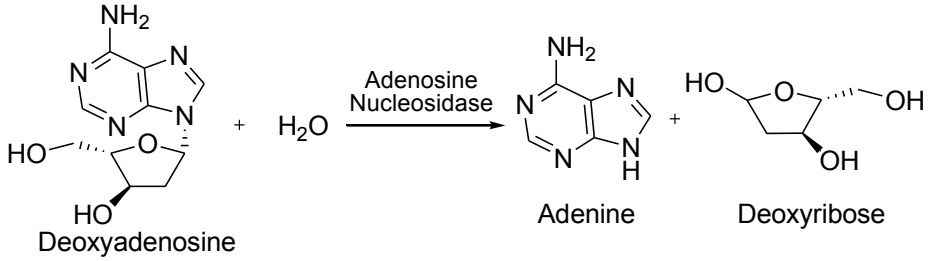
study will focus primarily on the hydrolase family. Finally, before proceeding any further it may be worth making a final clarification regarding the use of the terms hydrolase and nucleosidase. It appears the term hydrolase is generally used when referring to an enzyme family, while nucleosidase is usually reserved for cases when substrate specificity has been determined. Both terms have also been used interchangeably, especially in earlier reports. Nucleosidases catalyze a hydrolytic reaction and thus were initially named hydrolases. The degradation reactions carried out by the purine and pyrimidine nucleosidases may be seen below (Equations 1-8):

Adenosine nucleosidase catalyzes hydrolytic removal of the ribose sugar:

(Equation 1)

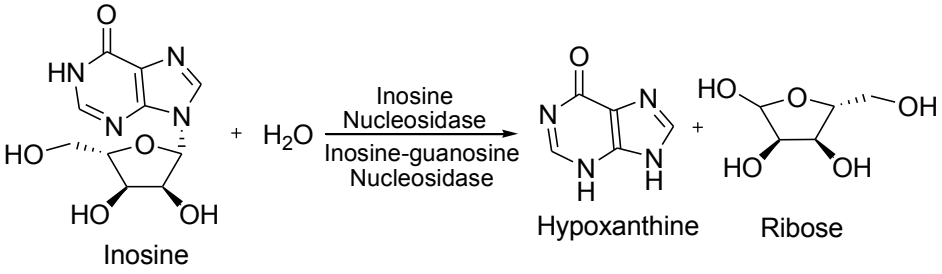


(Equation 2)

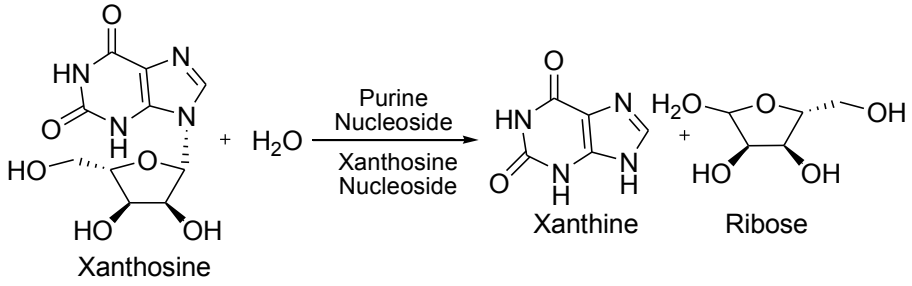


Inosine and/or inosine-guanosine nucleosidase catalyzes the following reaction:

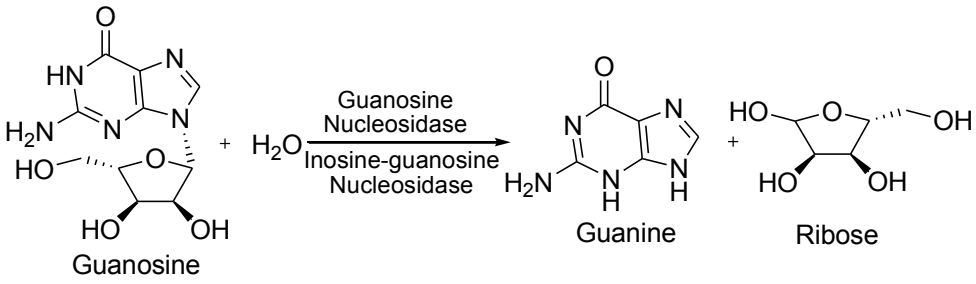
(Equation 3)



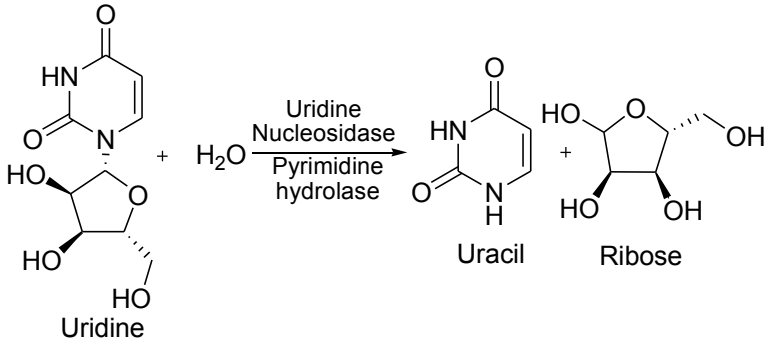
Purine (Xanthosine) nucleosidase produces a key intermediate xanthine:
(Equation 4)



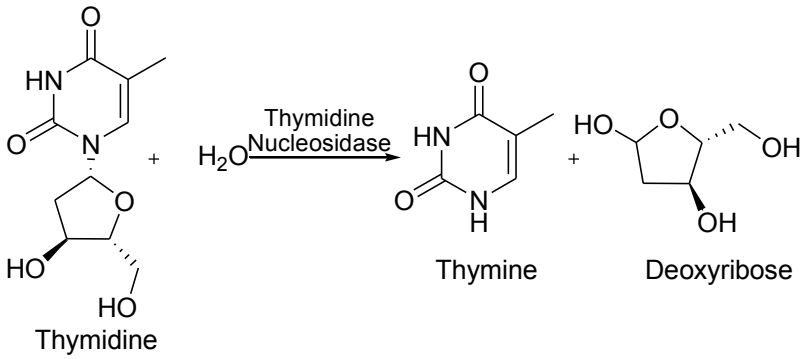
Guanosine or Inosine/guanosine nucleosidase hydrolyzes the following reaction:
(Equation 5)



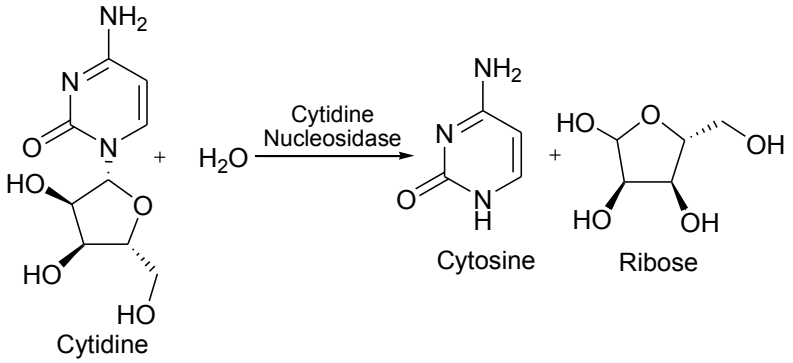
Uridine nucleosidase and/or pyrimidine hydrolase, may be responsible for uridine to uracil hydrolysis:
(Equation 6)



Thymidine may be hydrolyzed to thymine by the action of thymidine nucleosidase:
(Equation 7)

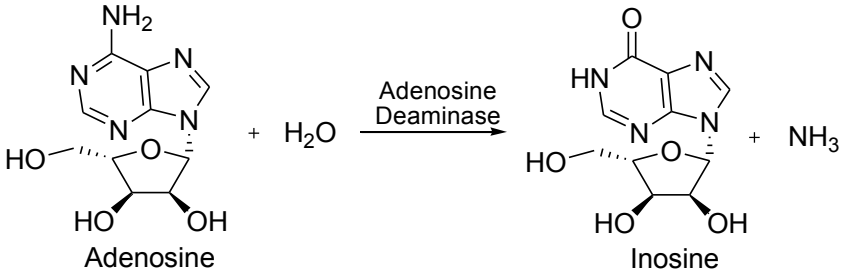


Cytidine nucleosidase mediates conversion of cytidine to cytosine:
(Equation 8)



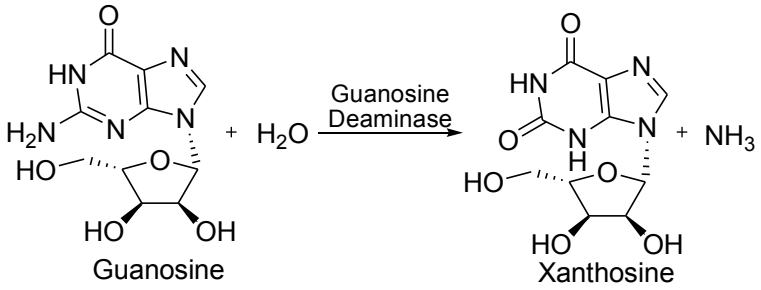
Equations 9-11 describe the deamination reactions carried out by adenosine deaminase, guanosine deaminase and cytidine deaminase, in that order:

Adenosine deaminase converts adenosine to inosine and releases NH_3:
(Equation 9)



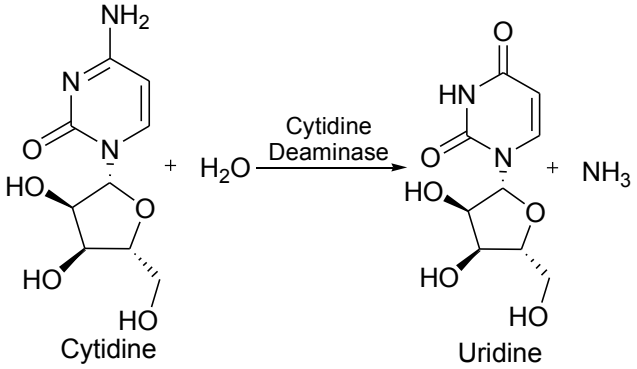
Guanosine is deaminated to xanthosine by the action of GSDA:

(Equation 10)



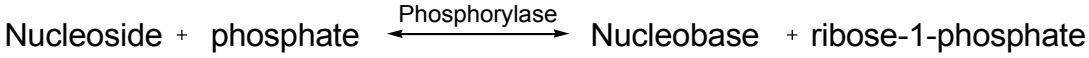
Cytidine is deaminated to uridine by action of CDA:

(Equation 11)



Phosphorolysis is a reversible reaction during which the glycosidic bond is broken in the presence of phosphate ions yielding a nucleobase and a ribose-1-phosphate. The general reaction mechanism is represented by the equation below:

(Equation 12)



Reports of various hydrolase activities range across a vast number of plant species such as *Arabidopsis*[7,12,30,45], tea and coffee[46,47], yellow lupin[37,38,48-50], potato tubers[24,51,52], tomato roots and leaves[53], white spruce[42,54], mangrove species[55,56], mungbean seedlings[57], canola[6], coniferous species[58] and fruit trees.[59]

Uridine ribohydrolase (URH) was found to be of great importance in the uridine degradation pathway of *Arabidopsis*.[12] The findings further indicate the possibility that this

enzyme may also act as a purine hydrolase since some activity was detected in the presence of inosine and adenosine.[12] To complicate matters even more purine salvage, degradation and secondary metabolic pathways differ not only between plant species, but even between organs and tissues of the same species.[47] In the study of hydrolase activity using URH knock-out mutants it was shown that multiple nucleoside hydrolase activities may be functional in roots of *Arabidopsis thaliana.*[7] A significant reduction in uridine hydrolysis was observed in the URH1 knockout but not in URH2 knockout. URH1 and URH2 represent previously identified genes annotated as uridine rybohydrolases.[7] In addition, when inosine and xanthosine were used as substrates, hydrolytic activity was substantially diminished in both URH1 and URH2 mutants.[7] This finding points to the likelihood that URH maybe also be a purine hydrolase rather than a uridine hydrolase only. Finally, the fact that hydrolase capacity was diminished and not totally eliminated in roots of the double mutants suggests the presence of an additional hydrolase which was not yet identified.[7] At least three isoforms of nucleoside hydrolases in *Arabidopsis* were identified.[45] It was shown that NSH1 (originally URH1) displays capabilities in both purine and pyrimidine degradation in a cell. The activity of the second hydrolase NSH2 peaks during the late phase of senescence and is likely inosine specific. Lastly, NSH3 was shown to be an extracellular purine-specific hydrolase.[45]

Cytidine deaminase (CDA) is an interconversion enzyme responsible for cytidine to uridine hydrolytic deamination. Biochemical properties of CDA were determined after it was cloned, expressed and purified from *Arabidopsis thaliana.*[30] It was found to be a dimer composed of two identical subunits with a molecular weight of 63 kilodaltons (kDA).[30] The K_m value was found to be 150 μM and was higher than that found for CDA in *E. coli* and

humans.[30] Guanosine deaminase was observed in *Arabidopsis thaliana* and appeared to be critical for generation of xanthosine.[23]

Nucleoside hydrolysis in disks of potato (*Solanum tuberosum* L.) tubers was also studied extensively.[24,52] Several deaminases were found to be active in potato tubers. Cytidine deaminase had an activity of 2.89 pkat/(mg protein); AMP deaminase activity was 8.0 pkat/(mg protein); guanosine deaminase activity was found to be 0.2 pkat/(mg protein). No activity was observed for adenosine deaminase, guanine and cytosine deaminases.[24,52] Nucleoside phosphorylase activities were not detected. Adenosine to adenine conversion was catalyzed by adenosine nucleosidase at a rate of 9.5 pkat/(mg protein). Inosine to hypoxanthine reaction proceeded at 1.1 pkat/(mg protein) and guanosine to guanine conversion was measured to be 0.1 pkat/(mg protein). Both hydrolytic reactions were mediated by inosine/guanosine nucleosidase.[24] A purine nucleosidase changed xanthosine to xanthine at a rate of 8.7 pkat/(mg protein). Adenine was also produced from deoxyadenosine by action of adenosine nucleosidase at a rate of 13.2 pkat/(mg protein).[24] Uridine nucleosidase was shown to be four times more active in leaves than in tubers.[24]

Yellow lupin is another plant species with a wealth of data related to nucleosidase activity. A number of the hydrolyzing enzymes have been purified. Adenosine nucleosidase was found to be a dimer consisting of identical or nearly identical subunits with a subunit molecular weight of 33,000 Da.[37] The K_m value for adenosine was 4.8 μM and the specific activity was found to be 6.6 μmol/(min mg).[37] The enzyme displayed broad specificity. Adenosine, 2'-deoxyadenosine and 5'-deoxyadenosine were strongly preferred. Another enzyme that has been purified from yellow lupin is inosine nucleosidase.[49] The molecular weight for this enzyme was determined to be 62 kDa. The activity of inosine nucleosidase is

substantially lower compared to that of adenosine nucleosidase; the K_m value for inosine was 65 μM.[49] Multiple substrates were accepted by this enzyme, but the activity was substantially reduced. Recently, a guanosine-inosine nucleosidase was highly purified and its properties determined.[50] The K_m values for guanosine and inosine were similar at 2.7 μM. This protein was found to be somewhat larger compared to other purine nucleosidases at 80 kDa.[50] This lupin nucleosidase only hydrolyzed guanosine and inosine.

Adenosine nucleosidase was also purified from leaves of *Coffea arabica*[46], and roots and leaves of tomato *Lycopersicon esculentum.*[53] The molecular weights were determined to be 72 kDa and 68 kDa, respectively. The K_m value was 6.4 μM for *C. arabica.*[46] There were three isoforms of the enzyme measured in tomato leaves and roots with K_m values of 25 μM and 9 μM in roots and 6 μM in leaves.[53] Uridine nucleosidase activity was observed in cultured mangrove (*Bruguiera sexangula*) cells.[56] Cytidine deaminase and uridine nucleosidase activity was also observed in leaves of mangrove species.[55] Guanosine deaminase activity was present in *Camelia sinensis* (tea), *Catharanthus roseus* and *Vigna mungo.*[25] Purine catabolic reactions were observed in tea leaf extracts.[47] Adenosine nucleosidase was active at 84.9 pkat/(mg protein); inosine/guanosine nucleosidase converted guanosine and inosine to their respective bases at 8.53 pkat/(mg protein) and 9.21 pkat/(mg protein); a purine nucleosidase (possibly a xanthosine nucleosidase) was active and converted xanthosine to xanthine at 29.1 pkat/(mg protein).[47]

Soybean and corn

The two economically most significant crops grown in the United States are corn and soybean. The area of corn grain harvested was estimated at 87.4 million acres in 2012.[60] Soybean came in a close second at 76.1 million acres of harvested land.[60] The next two

closest crops were hay and wheat at 56.3 million acres and 49.0 million acres.[60] In addition to domestic use both corn and soybean are exported worldwide. A significant portion of domestic corn use is attributable to feed use (61%), food use (8%), ethanol and sweeteners (13%).[61] The remaining 18% are exported to countries throughout the world.[61] The export component of soybean is significantly higher, at nearly 42% of all shipments going to foreign markets, with the majority sent to the EU markets.[61] The United States is the largest exporter of soybean in the world.

Like any plant, corn and soybean can be affected by disease, pests, weeds and unfavorable weather conditions that may lead to a decline in crop production. Severe drought conditions have a significant impact on crop yields including corn and soybean.[60] Emergence of herbicide-resistant weeds raised concern in the agricultural community in recent years.[62] Farmers have developed techniques such as crop rotation to combat some crop-affecting disease and pests. Corn and soybean are frequently the two crops rotated from year to year because soybean root nodules have nitrogen-fixing bacteria that help replenish soil.[63] In addition, corn rootworm population can be significantly reduced. Many other diseases, of which cyst nematode and phytophtora (root and stem rot) have the largest prevalence, affect soybean.[64] All these challenges encourage continued research into genetic and metabolic processes of these very important crop producing plants. The goal is to better understand biochemical processes critical for robust growth and development of the plant, and to harness very crucial defense mechanisms that may already be present within the plants.

Purine and pyrimidine metabolism has been evaluated to some extent in soybean and corn. Overall, recycling pathways, especially degradation, are not very well characterized in

soybean and corn. The information on enzymatic activities involved in nucleotide metabolism is somewhat more robust in soybean than in corn. It has been shown that the normal ratio of nucleotide synthesis to degradation in root apices of healthy corn plants may be shifted toward degradation of both purines and pyrimidines under salt-stress conditions through increased capacity for purine catabolism and decreased pyrimidine salvage.[65] A study in soybean seedlings demonstrated that ureides in 8- and 12-day old cotyledons may be generated via preexisting nucleotide breakdown.[66] Several enzymes were found active in xanthine degradation in soybean.[2] Xanthine dehydrogenase leads to formation of uric acid. Uricase, a nodule-specific protein, converts uric acid to allantoin while allantoinase generates allantoate.[2] Another enzyme involved in degradation of purine nucleotides is a 5'-nucleotidase that converts XMP to xanthosine which is then transformed to xanthine by what is likely inosine/guanine nucleosidase.[2] Most recently, adenosine nucleosidase was partially purified from young soybean seedlings. The specific activity for this hydrolyzing enzyme was 2.44 μmol/(min mg).[17] The molecular weight determination indicated that this nucleosidase may be a multimeric protein rather than consisting of a single subunit.[17] Another activity was detected in the final purified fraction and proved challenging to separate. Adenosine deaminase activity was shown to be present in 5 day old soybean seedlings.[17]

The apparent lack of research into nucleoside to nucleobase conversion in soybean, and especially in corn, shaped this current study as a general evaluation of nucleosidase activities in corn, soybean and yellow lupin. Out of the three chosen plant species, yellow lupin is the most well studied with a number of purified hydrolases previously reported. In addition, yellow lupin was clearly shown to have no adenosine/adenine deaminase activity

and therefore provided an excellent control for the experimental protocol used in this research study with respect to the deaminase activity. It is the ultimate goal of this thesis work to demonstrate the presence or absence of hydrolyzing enzymes involved in nucleoside catabolism in germinating seedlings of corn, soybean and yellow lupin. Purine nucleosides adenosine, 2'-deoxyadenosine, guanosine, inosine and xanthosine were used to determine whether purine nucleosidases were present. Pyrimidine substrates included uridine, cytidine, thymidine and 5-methyluridine. All activities were evaluated using crude protein extracts obtained from 5-8 day old seedlings. Reaction progress was monitored using high performance liquid chromatography.

CHAPTER II

MATERIALS AND METHODS

Materials

Yellow lupin (*Lupinus luteus*) seeds were obtained from B and T World Seeds Sarl, Paguiguan, 34210 Aigues Vives, France. Roundup Ready corn was obtained from Hooper Supply. Viking soybean seeds were purchased from Johnny Selected Seeds of Winslow, Maine, USA. Adenosine, inosine, xanthosine, guanosine, uridine, cytidine, thymidine, 2'-deoxyadenosine, 5-methyluridine and the corresponding nucleobases were purchased from Sigma Aldrich. All nucleosides and nucleobases were of minimum 99 % purity with the exception of adenosine and cytidine, which were Sigma grade. Dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF) and poly(vinylpolypyrrolidone) (PVPP) were obtained from Sigma Aldrich. Protamine sulfate salt from salmon (Grade X) was obtained from Sigma Aldrich. Tris Base salt for molecular biology was obtained from Fisher BioReagents and was used to generate all stock solutions of the Tris-HCl buffer by addition of hydrochloric acid to achieve desired pH. Ammonium acetate was obtained from J.T. Baker Chemical Co. All other reagents were reagent grade. Bio-Rad Protein Dye reagent and bovine serum albumin, used for protein concentration determination, were purchased from Bio-Rad.

Dionex UltiMate 3000 HPLC equipped with a thermostatted autosampler and column holder, a dual pump and a UV detector was purchased from ThermoScientific. The UV-VIS 2900 double beam spectrophotometer was purchased from Hitachi.

Methods

Seed germination

All seeds were initially soaked in bleach for 5 minutes. Each batch of seeds was then rinsed with tap water and placed in a sealed container on a moist paper towel. The seeds were sandwiched between an additional layer of moist paper towel and left to germinate at room temperature. Initial germination occurred 3-4 days after imbibition. On the 7^{th} day after germination, the seedlings were harvested. This generally resulted in 5-9 day old young seedlings. The remaining outer coats were removed, seedling material was rinsed with deionized water and homogenized in 50 mM Tris buffer pH 7.2 (1 mL for every 1 g of biomass) using a Waring commercial blender at 4 ^{0}C. The Tris buffer also contained 1 mM DTT (1 % v/v), PMSF (0.01 % v/v), protamine sulfate (2 % w/w of biomass) and 10 % PVPP (10 % v/v). The homogenate was centrifuged at 13,000*xg* for 30 minutes at 4^{0}C. Supernatant was poured into new centrifuge tubes and spun again at 8,500*xg* for 15 minutes at 4 ^{0}C. The resulting supernatant was filtered through a nylon cloth to remove remaining particulates. A portion of the crude extracts was stored at 4 ^{0}C for immediate use while the rest was frozen for future needs at -20 ^{0}C.

Enzyme activity assays

All nucleosidase activities were measured by observing appearance of the nucleobases and the disappearance of the parent nucleosides. All reaction mixtures were monitored by the HPLC/UV-VIS. The detector wavelength was set to 254 nm. All substrates used in this study had the highest response at this wavelength. The reaction mixtures were 950 μL, 975 μL or 990 μL of 1 mM stock nucleoside (except guanosine which was 0.25 mM due to solubility) with 50 μL, 25 μL and 10 μL of crude extract added,

respectively. All stock solutions were prepared in 50 mM Tris buffer pH 7.2. Every injection was set at 10 μL and reaction progress monitored immediately following addition of the crude protein mixture. Samples were incubated on the autosampler at 32 ^{0}C. The disappearance of parent nucleoside was used to calculate enzyme activity. Reactions were allowed to continue until at least 30 % of the nucleoside was degraded.

Progress of every reaction was evaluated continuously on a Dionex Ultimate 3000 HPLC paired with a UV detector. All separations were performed on a Phenomenex Hyperclone ODS C_{18} HPLC column (150 x 4.6 mm, 5 μm particle size). Mobile phase consisted of 98 % 10 mM ammonium acetate pH 5.2; 2 % methanol. The elution profile was isocratic at 0.8 mL/min. A blank sample consisting of only Tris buffer was injected and analyzed for possible impurities or contamination. A standard of each nucleoside was run prior to the start of each reaction analysis and at the end of the analysis. This provided a current retention time (RT) and hydrolysis control for every nucleoside. Area of each peak was converted to concentration. Known amounts (mM) of standard nucleosides were divided by the area of corresponding peaks (mAU). The calculated values were then multiplied by the area of the peaks at various times throughout reaction progress. The obtained concentrations were plotted against reaction time. Reaction velocity was determined from the slope of the line. Specific activity was determined by dividing reaction velocity by protein concentration.

Phosphorylase activity was evaluated under the same conditions as described above for nucleosidases. All stock solutions were prepared in 50 mM phosphate buffer pH 7.2 with similar nucleoside concentrations. A standard of each nucleoside was run prior to the start of

each reaction analysis and at the end of the analysis. The latter was used as a hydrolysis control.

Protein concentration

The total protein concentration of every crude extract was determined using the BioRad Protein assay protocol. Bovine serum albumin was used to generate standard curves for each crude extract (Figure 4). Dilutions of 1:10 or 1:100 were performed on the unknown crude samples to bring absorbance within the linear range of the assay if needed. Reaction mixture consisted of 20 µL of unknown (diluted or undiluted) sample combined with 780 µL of distilled water and brought to 1.00 mL total volume with addition of the 200 µL Bio-Rad reagent. Absorbance was measured at 595 nm. The amount of protein was obtained from the standard curve.

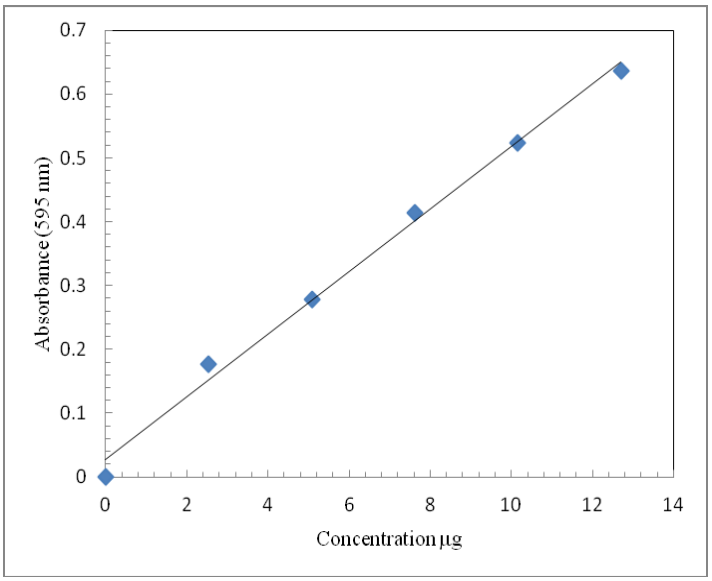


Figure 4. Standard curve used for determination of protein concentration using Bio-Rad protein assay.

CHAPTER III

RESULTS AND DISCUSSION

Purine and pyrimidine metabolism play a vital role in plant growth and development. A young plant seedling is unlikely to develop if successful and efficient utilization of nucleotide reserves does not occur during seed germination. Nucleotides are essential building blocks of the cell. DNA replication cannot take place if additional nucleotides are not available during synthesis. RNA synthesis also requires nucleotide availability. It is unlikely that proteins can be built in the absence of the message. In addition, a multitude of specialized molecules are generated by the cell from nucleotides or nucleosides via modification. Some serve as coenzyme components while others act as co-substrates. Vitamins and energy storage molecules are also generated via nucleotides. Nucleotides are divided into two categories: purines and pyrimidines. Three components make up a single nucleotide: a nucleobase, a pentose sugar and a phosphate. The cell can satisfy its nucleotide needs in two major ways. Nucleotides can be built from scratch in what is known as the *de novo* synthesis. Used nucleotides are degraded and can be salvaged depending on the requirements of the cell. These pathways are more or less active at different times throughout plant development.

The first component to be removed from a nucleotide is the phosphate moiety. 5'-nucleotidases mediate the dephosphorylation reaction and yield a nucleoside. The remaining nucleobase and pentose are then split further by a nucleosidase activity. Various interconversion reactions may take place prior to dephosphorylation such as AMP modification to IMP by action of AMP deaminase. Nucleosides are also modified in a similar way. Cytidine is salvaged via conversion into uridine. Cytidine deaminase activity is

responsible for this modification. Nucleosides and nucleobases are also modified into specialized molecules known as cytokinins. Additionally, kinases and phosphoribosyltransferases help maintain the nucleotide pool balance by regenerating the monophosphate form of the nucleotide. Phosphorylases have been found in some organisms and assist with the reversible reaction between a nucleoside and a nucleobase. The salvaging activity is particularly important during stress conditions. It is a crucial energy conservation mechanism that cells can utilize instead of the biosynthesis pathway which is more costly. Both ribonucleosides and deoxyribonucleosides were shown to be salvaged in plants and microorganisms. Ribonucleosides have been found to be generally preferred to deoxyribonucleotides as substrates. In *Crithidia fasciculata,* a trypanosome parasite of the mosquito, a purine preferring nucleoside hydrolase was found, although, all commonly occurring nucleosides were substrates.[67] Deoxynucleosides or 5'-substituted nucleosides were poor substrates and did not react.[67] Several deoxynucleosides, with the exception of deoxycytidine and deoxyuridine, were accepted as substrates by a purine-2'-deoxyribonucleosidase purified from *Crithidia luciliae*.[68] The two pyrimidine nucleosides not cleaved by the nucleosidase required the presence of phosphate ions, and thus were cleaved phosphorolytically.[68] 2'-, 3'-, and 5'-deoxyadenosines were examined as substrates in yellow lupin.[37] The hydroxyl group position appeared to play an important part in substrate suitability. Relative activity was shown to be the best for the 5'-position with reaction velocity at 218 % compared to adenosine as substrate.[37] 2'-Deoxyadenosine was also used as a substrate but the activity was lower by 35 %.[37] Cordycepin, the 3'-deoxy derivative of adenosine, was the least utilized at 1% of activity compared to adenosine.[37] Inosine nucleosidase, another enzyme purified from yellow lupin, showed a relatively broad

specificity. Adenosine and guanosine were also utilized as substrates but were much less preferred as substrates at 11 % and 1 % of inosine activity.[49] Several analogs, which generally seems to be the case, were also converted to the base. Xanthosine, nebularine, 6-mercaptopurine riboside, and 8-azainosine were accepted as substrates.[49] Guranowski found that inosine nucleosidase displayed strict structural requirements for the ribose moiety of inosine.[49] Any change of substituent at the 2', 3', or 5' carbon of the nucleoside completely eliminated its substrate functionality.[49] There appears to be a substantial amount of variability in substrate acceptance between species and even within the same species, with most enzymes displaying some degree of activity for a broad range of substrates.

It appears likely that structural requirements may play a crucial role in substrate suitability. However, it is also possible that organisms have evolved nucleosidase activity to fulfill an extremely important role ensuring cell viability. Generally, nucleosidases assist in satisfying variable nucleotide needs of the cell while minimizing the energy costs associated with additional resource utilization. Three nucleoside analogs, 2'-deoxyadenosine, 5-methyluridine and a less common nucleoside xanthosine, were also evaluated in this study and help examine the possible broader impact of the nucleosidase activities in plants. The structures for the three nucleosides are shown in Figure 5.

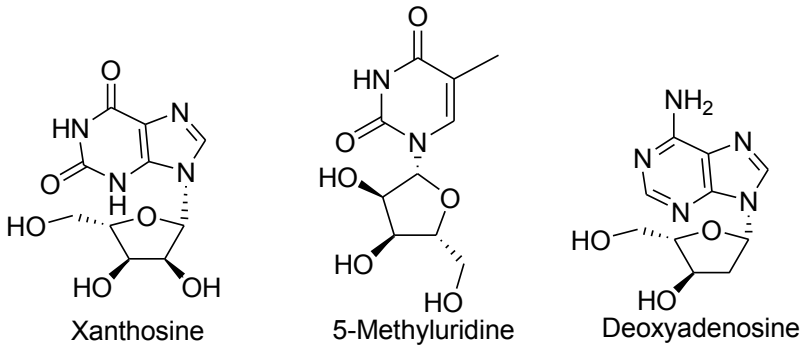


Figure 5. Structures of additional nucleosides examined in this study and not shown in the introduction.

Crude protein extracts from three plants, yellow lupin, soybean, and corn, were evaluated for presence of catabolic enzymes that participate in the salvage of nucleosides. The three enzyme activities investigated were nucleosidases, phosphorylases and deaminases. Nucleosidases remove the ribose hydrolytically. Phosphorylases remove the sugar in the presence of phosphate ions or reattach the sugar if ribose-1-phosphate is present. Deaminases participate in interconversion of one nucleoside into another by removing the amino group from the base. Reaction velocity and crude specific activity was determined based on the disappearance of the parent nucleoside. The nature of enzymatic activity was assessed qualitatively and based entirely on the identity of the product nucleobases that appeared throughout the reaction progress. The identity of the products were established by comparison to the retention time (RT) of standards determined prior to the initiation of the experimental trials. Parent nucleoside RTs were also obtained and are both listed in Table 1. The retention times were highly dependent on the ratio of mobile phase constituents and are presented for the 98 % ammonium acetate buffer pH 5.2 with 2 % methanol. An increase in methanol contribution allowed for significantly shorter elution times but yielded reduced resolution for adenine and inosine, and poor resolution between

cytidine, cytosine, uridine and uracil. All reaction samples were monitored using the same HPLC method. Acquisition time was the only parameter varied and was adjusted based on the RT of the substrate and products for the reaction under investigation. The two reaction buffer systems used were 50 mM Tris pH 7.2 and 50 mM phosphate buffer pH 7.2. The reaction buffer effect on the RTs was negligible.

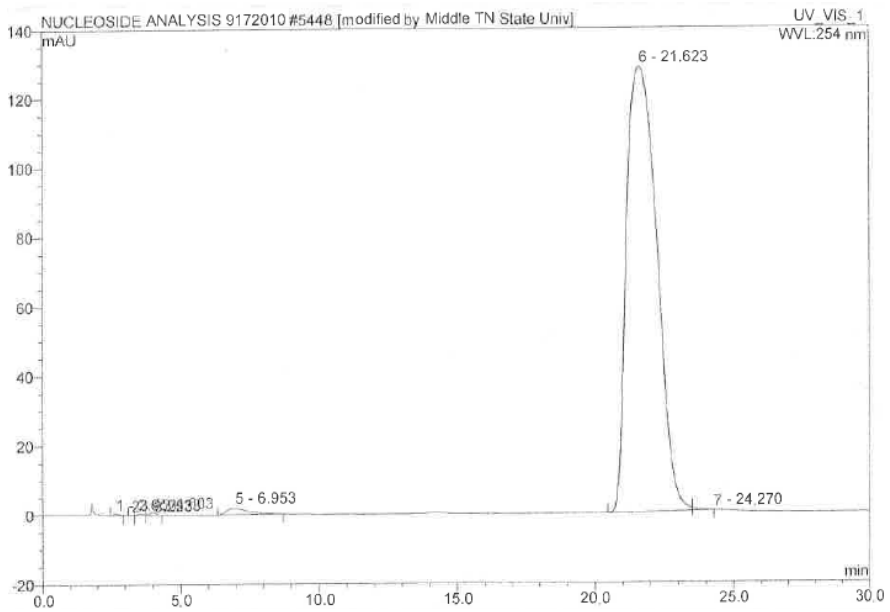
Table 1. Summary of retention times of nucleosides and bases. Retention times of nucleosides and bases were determined using a 98 % 10 mM ammonium acetate pH 5.2, 2 % methanol mobile phase on a Phenomenex C_{18} (150 x 4.6 mm) HPLC column at 254 nm. The values reported are an average of at least three measurements.

Nucleoside/Nucleobase pair	Retention Time (min)
Adenosine/Adenine	21.9±0.5/6.8±0.2
Inosine/Hypoxanthine	7.8±0.7/3.6±0.3
Xanthosine/Xanthine	8.7±0.2/4.0±0.2
Guanosine/Guanine	9.0±1.1/3.7±0.3
Uridine/Uracil	4.2±0.3/2.7±0.2
Cytidine/Cytosine	3.3±0.2/2.4±0.2
Thymidine/Thymine	13.4±1.0/4.8±0.9
5-Methyluridine/Thymine	6.9±1.6/4.8±0.9
2'-Deoxyadenosine/Adenine	25.3±0.5/6.8±0.2

Enzyme activities in yellow lupin

Analysis of the reaction data, using adenosine as the substrate, revealed a robust nucleosidase activity but neither phosphorylase nor deaminase were active. Significant disappearance of adenosine commenced within the first hour of reaction initiation and correlated well with the formation of adenine. No additional products were observed during the course of the reaction (Figure 6). Activity levels were nearly identical in both Tris and phosphate buffers pointing to the absence of an adenosine phosphorylase in yellow lupin. The calculated specific activities for Tris and phosphate buffer reactions containing 1 mM

A.



B.

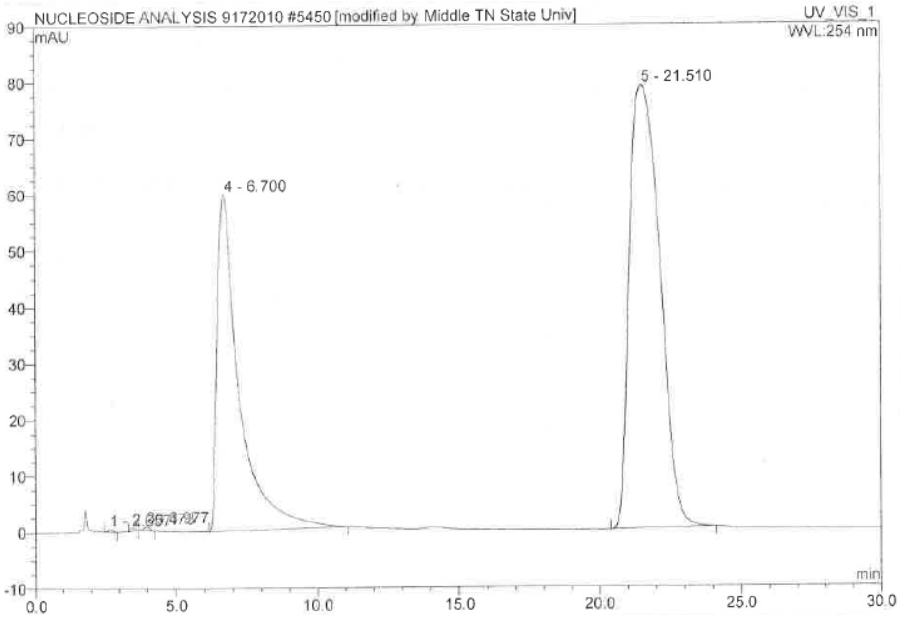


Figure 6. Adenosine to adenine conversion catalyzed by adenosine nucleosidase. The reaction mixture included 1 mM adenosine (10 µL) and yellow lupin extract in 50 mM Tris buffer pH 7.2 (990 µL). **(A)** Reaction mixture immediately following yellow lupin extract addition. It shows an adenosine peak at 21.623 minutes. **(B)** A second peak, with a retention time expected for adenine, eluted two hours later at 6.700 minutes. Adenosine abundance has also dramatically decreased.

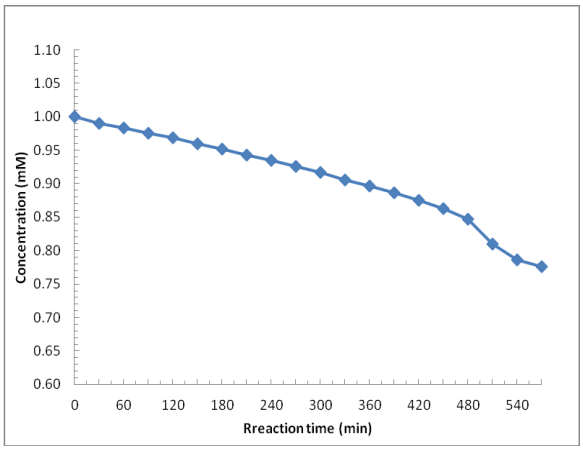
adenosine were 0.23 and 0.22 μmol/(min mg), respectively. A deoxyribonucleoside analog, 2'-deoxyadenosine, was also used to evaluate activity of nucleosidases, phosphorylases and deaminases in yellow lupin crude protein extract. There was a good nucleosidase activity observed, but no phosphorylase activity was detected. Specific activities were nearly identical at 0.15 μmol/(min mg) and 0.14 μmol/(min mg). The difference in nucleoside activity was consistent with the results reported by Abusamhadneh et al.,[37] in which purified yellow lupin adenosine nucleosidase had 65% of the activity against 2'-deoxyadenosine compared to adenosine.

Inosine and guanosine substrates were also degraded to their respective bases, hypoxanthine and guanine. It had been previously reported that an inosine nucleosidase and an inosine-guanosine nucleosidase were purified from yellow lupin.[47,48] The difference in activity level observed in this study appears to point to a similar conclusion. The specific activity for enzymes involved in inosine degradation was substantially lower than that for adenosine at 0.011 μmol/(min mg) and is consistent with previous findings.[47] The specific activity for guanosine hydrolyzing enzymes was 0.0077 μmol/(min mg). Again, phosphorylase activity was not a major factor in inosine and guanosine degradation. Guranowski had previously reported that neither adenosine nor inosine phosphorylase were found in the crude extracts from yellow lupin.[49]

Xanthosine nucleosidase and phosphorylase activities have not been previously reported in yellow lupin. Xanthosine was hydrolyzed to xanthine with a specific activity at a similar level found for inosine and guanosine. The specific activity against each of these nucleosides was 0.0062 μmol/(min mg). Phosphorylase activity was also examined and yielded interesting results. Phosphorylases require the presence of phosphate ions in order to

cleave the ribose sugar from the nucleoside. The products of the reaction are a nucleobase and α-ribose-1-phosphate. The phosphorylase mediated reaction proceeds in both directions and the nucleoside is regenerated from the nucleobase as long as α-ribose-1-phosphate is present as well. A two-phase reaction was observed for xanthosine degradation in this study (Figure 7). The specific activity in the phosphate buffer was somewhat lower at 0.0031 μmol/(min mg), during the

A.



B.

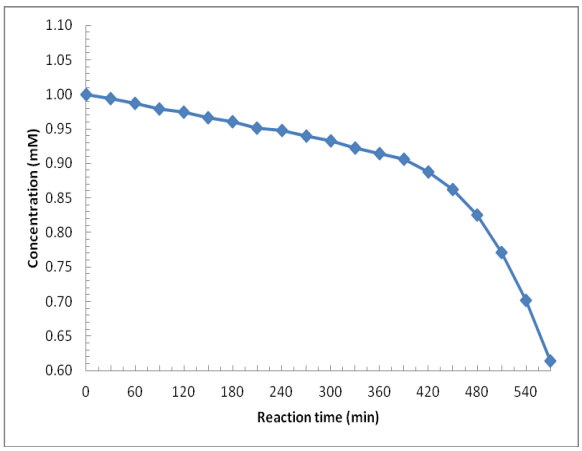


Figure 7. Xanthosine reaction progress in yellow lupin in Tris and phosphate buffers **(A)** Disappearance of 1 mM xanthosine in 975 μL 50 mM Tris pH 7.2 reaction buffer with 25 μL crude protein extract from yellow lupin. **(B)** Disappearance of 1 mM xanthosine in 975 μL 50 mM phosphate pH 7.2 reaction buffer with 25 μL crude protein extract from yellow lupin.

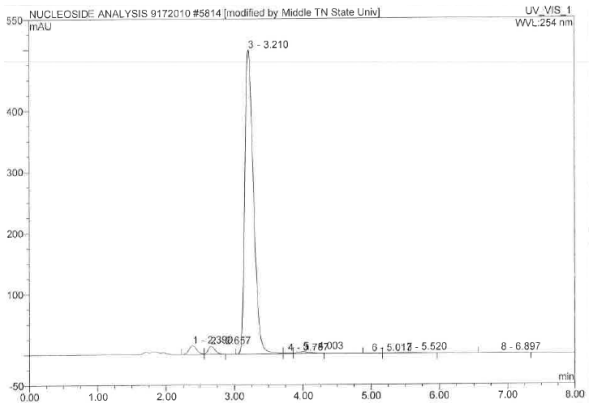
initial six hours after addition of the crude enzyme to the reaction mixture, compared to the next three hours. After six hours, a second phase which may be due to the combined specific activities of the nucleosidase and phosphorylase appears to occur. Nucleosidase activity was expected to be present in both Tris and phosphate buffers, since phosphate is not required for this enzyme to be active. The specific activity, for the likely combined enzyme activities, involved in xanthosine degradation was 0.025 μmol/(min mg). Additional experimental analyses must be performed to determine whether this 8-fold increase in activity is definitively due to a phosphorylase contribution. It is possible that phosphorylase may have a minor contributing role in the salvaging of xanthosine by both degrading it to xanthine and, at the same time, regenerating xanthosine. The latter may be the reason for an initial loss of overall activity during phase one. The significant increase in activity in phase two may be primarily due to the nucleosidase achieving its maximum velocity, while phosphorylase contribution may remain minor. Another explanation for the observed two-phase reaction may be that xanthosine phosphorylase remains relatively inactive until the second phase when favorable substrate levels are achieved.

Uridine phosphorylase has been found in animals and microorganisms. There has been very little data on plant phosphorylase activity in general. In most cases, reports suggest that the activity is either not found or is very low.[13,21,49] Most recently, existence of this enzyme activity in plants was shown to be possible based on sequence similarities with other organisms.[43,44] The data gathered for uridine in this study was very similar to that obtained for xanthosine. The reaction progressed in two phases. The specific activity during the first four and a half hours was 0.0077 μmol/(min mg). There was a 4-fold increase

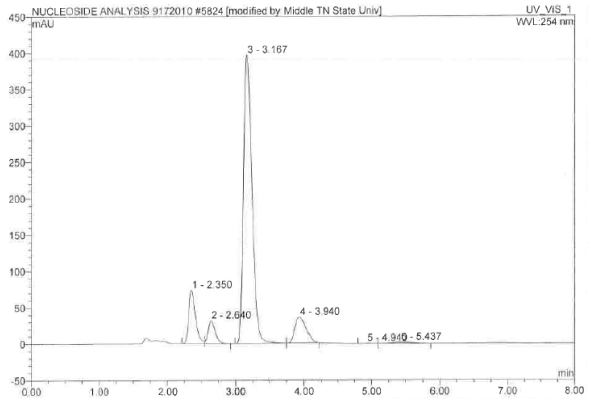
during the second phase to a specific activity of 0.031 μmol/(min mg). This increase may be due to phosphorylase activity. It remains to be seen whether phase one or phase two of the reaction is representative of phosphorylase activity. Uridine nucleosidase was also active in yellow lupin. The specific activity for the reaction run in Tris buffer was 0.0077 μmol/(min mg), and is identical with that observed for phase one of the reaction in phosphate buffer. It appears that a phosphorylase may contribute to the degradation of uridine but at a very low level.

Cytidine nucleosidase has not been previously reported in plants. Cytidine is largely believed to be salvaged at the nucleoside level via conversion to uridine by action of cytidine deaminase.[13] Cytosine deaminase has not been found in plants.[13] The findings for yellow lupin in this study are intriguing. Resolution between cytidine, uridine, uracil and a fourth peak corresponding to cytosine was excellent, and allowed for clear identification of peaks based on the retention times of the standards (Figure 8). Cytidine deaminase was active in yellow lupin. Uridine was formed rapidly within the first two hours of the reaction and was clearly converted to uracil by the action of uridine nucleosidase. Cytidine was also degraded quickly to cytosine initially via a pyrimidine nucleosidase. The specific activity for enzymes catalyzing degradation of cytidine was 0.015 μmol/(min mg). No data were obtained for phosphorylase contribution to cytidine degradation in yellow lupin.

A.



B.



C.

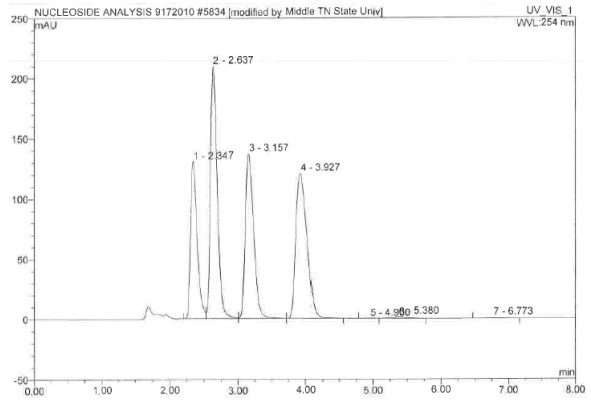
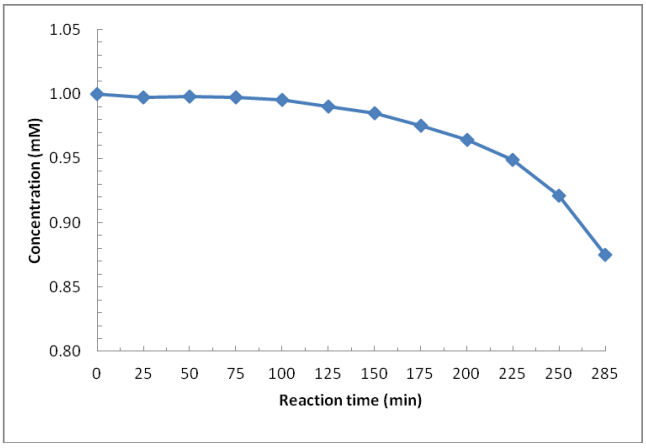


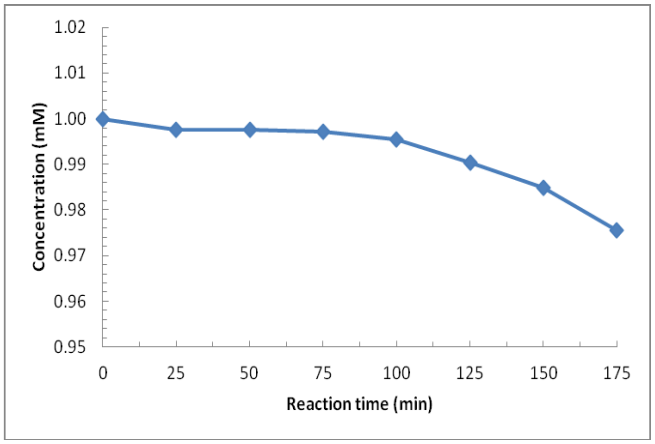
Figure 8. Cytidine degradation reaction in yellow lupin revealed multiple activities: pyrimidine (possibly cytidine) nucleosidase, cytidine deaminase and uridine nucleosidase. **(A)** Reaction mixture immediately following addition of 50 µL of yellow lupin extract to 950 µL of 1 mM cytidine. Cytidine peak eluted at 3.210 minutes. **(B)** Product formation was rapid and after just three hours cytosine can be seen at 2.350 minutes, uridine at 3.940 minutes and uracil at 2.637 minutes. **(C)** Significant product formation is observed after just five hours from reaction initiation.

There appear to be no direct reports on plant thymidine nucleosidase activity in the literature. It has been suggested that thymidine nucleosidase and/or phosphorylase activities may be responsible for thymidine degradation.[13,21,27] The findings in this study point to a weak thymidine nucleosidase activity. Interestingly, conversion of thymidine to thymine does not appear to begin for up to three hours after the crude protein extract is added to the reaction mixture (Figure 9). Specific activity during this initiation phase was 0.00078 μmol/(min mg). Reaction velocity significantly increases after three hours. The specific activity was 0.007 μmol/(min mg), an increase of nine times. No other products were observed. Thymidine phosphorylase presence was not evaluated in yellow lupin. A ribonucleoside analog, 5-methyluridine, was also utilized as a substrate to evaluate the presence of nucleosidases and phosphorylases. Disappearance of 5-methyluridine was apparent within the first two hours after addition of the crude protein mixture. Thymine is the product of this reaction and appeared at the expected retention time. There was no significant difference in reaction velocity between Tris and phosphate buffers. Specific activities were 0.0077 μmol/(min mg) and 0.0093 μmol/(min mg), respectively. One or more nucleosidases may accept this thymidine analog as a substrate. However, it is unlikely that a phosphorylase is active in presence of this nucleobase. A summary of the results can be found below in Table 2 and Table 3.

A.



B.



C.

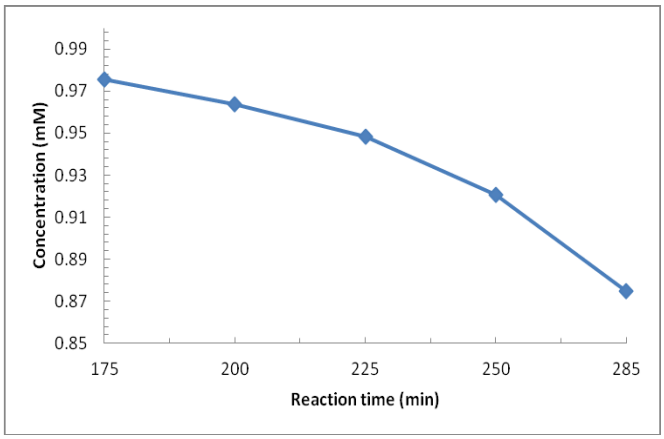


Figure 9. Thymidine reaction progress in yellow lupin **(A)** Disappearance of thymidine in Tris buffer with a crude protein extract from yellow lupin. **(B)** Phase one of the reaction lasted approximately three hours with no significant cleavage of the substrate thymidine taking place. **(C)** Phase two of the reaction is characterized by rapid reaction velocity increase in the following two hours.

Table 2. Nucleosidase and deaminase activities in yellow lupin. Nucleosidase specific activities found in yellow lupin 5-7 day old seedlings. If a two phase reaction progress was observed specific activity was calculated for both phases (*phase 1, **phase 2). Presence was determined based on whether or not there was a detectable nucleosidase activity.

Enzyme	Presence	μmol/(min mg)
Adenosine Nucleosidase	Y	0.23
Inosine Nucleosidase	Y	0.0077
Xanthosine Nucleosidase	Y	0.0062
Guanosine Nucleosidase	Y	0.0077
Uridine Nucleosidase	Y	0.0077
Cytidine Nucleosidase/Deaminase	Y	0.015
Thymidine Nucleosidase	Y	0.00078*/0.007**
5-Methyluridine Nucleosidase	Y	0.0077
2'-Deoxyadenosine Nucleosidase	Y	0.15

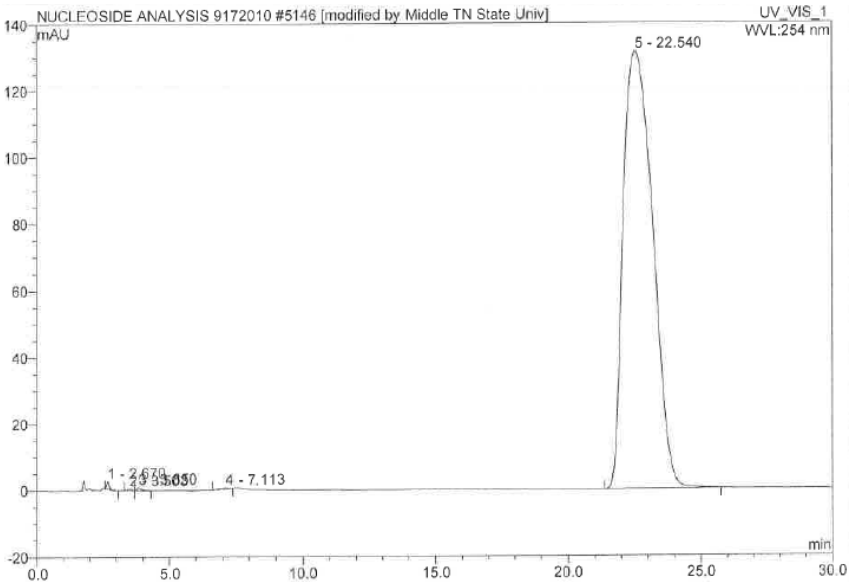
Table 3. Phosphorylase activity in yellow lupin. Combined phosphorylase and nucleosidase specific activities in yellow lupin 5-7 day old seedlings. If a two phase reaction progress was observed specific activity was calculated for both phases (*phase 1, **phase 2). Presence was determined based on whether or not there was a detectable phosphorylase activity.

Enzyme	Presence	μmol/(min mg)
Adenosine Phosphorylase	N	0.22
Inosine Phosphorylase	N	0.011
Xanthosine Phosphorylase	Y	0.0031*/0.025**
Guanosine Phosphorylase	N	0.0093
Uridine Phosphorylase	Y	0.0077*/0.031**
Cytidine Phosphorylase	No data	No data
Thymidine Phosphorylase	No data	No data
5-Methyluridine Phosphorylase	N	0.0093
2'-Deoxyadenosine Phosphorylase	N	0.14

Enzyme activities in soybean

Nucleosidases are active in soybean as well. The level of activity observed was lower when compared to yellow lupin across all substrates tested. All reactions took a substantially longer time to initiate, with some taking as much as eight hours before commencement. Two phases were observed when adenosine, inosine, thymidine and 5-methyluridine were used as substrates. Adenosine nucleosidase was active at 0.00035 μmol/(min mg) during the initiation phase which took up to eight hours, and increased 7-fold to 0.0025 μmol/(min mg) thereafter. A phosphorylase activity is likely also present in 5-9 day old soybean seedlings. The initiation phase lasted approximately ten hours with a specific activity of 0.00014 μmol/(min mg). This is a 3-fold reduction compared to the reaction in the Tris buffer. The observed slowing trend is similar for xanthosine in yellow lupin. There was a 58-fold increase in activity in the second phase of the reaction in phosphate buffer. There is an additional complicating factor associated with adenosine degradation in soybean. Adenosine and/or adenosine/adenine deaminase appears to be quite active in soybean. It is important to note that these findings are contradictory to the general scientific opinion which seems to suggest that neither adenosine nor adenine deaminase are active in plants.[13,21,22] Soybean has not been previously evaluated for the presence of adenosine deaminase and results of this study may represent interesting findings. Observations made for soybean in the current report are similar to those recently reported.[17] The three products formed during the course of the reaction, when adenosine is used as a substrate are adenine, inosine and hypoxanthine (Figure 10). It takes well over twenty hours before adenosine completely disappears and only hypoxanthine remains. The fact that the only remaining product is hypoxanthine suggests that an adenosine/adenine deaminase may

A.



B.

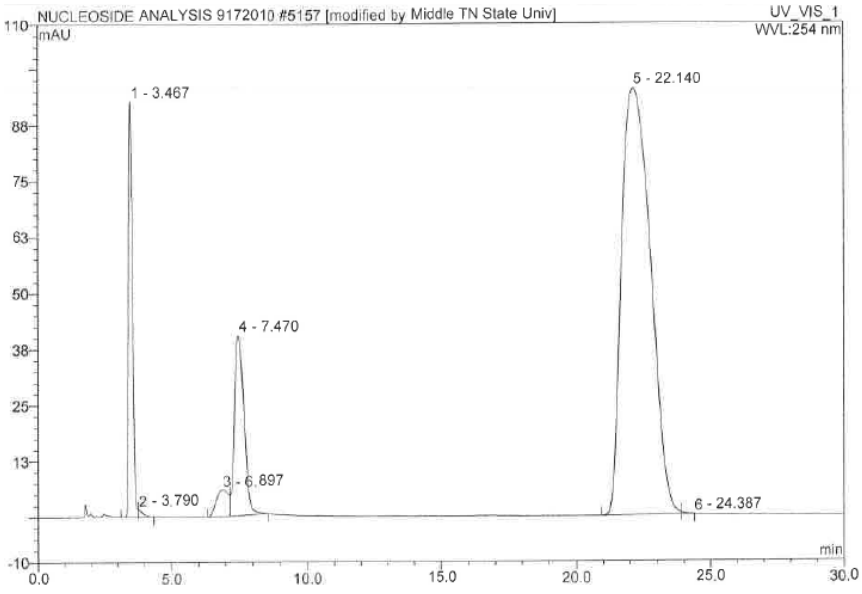


Figure 10. Adenosine nucleosidase reaction in soybean yielded adenine, inosine, and hypoxanthine, likely due to the presence of adenosine deaminase activity. 10 µL of soybean extract were added to 990 µL of 1 mM adenosine solution. **(A)** Reaction mixture immediately following soybean extract addition is shown. Adenosine is the only peak visible at 22.540 minutes. **(B)** Seven hours following reaction initiation adenosine is still present at 22.140 minutes. However, inosine is clearly visible at 7.47 minutes. A smaller partially unresolved peak can be seen for adenine at 6.897 minutes and hypoxanthine also appeared at 3.467 minutes.

be present. It was also found that adenine, when used as a substrate, is very rapidly converted to hypoxanthine. This further suggests that adenine may also be a substrate for the observed deaminase activity. The fact that hypoxanthine is the only product remaining at sixteen hours after addition of crude protein extract, in phosphate buffer, may further suggest that a phosphorylase is active. Adenosine nucleosidase and deaminase also accept 2'-deoxyadenosine as the substrate (Table 4). Interestingly, a phosphorylase does not seem to be active in the presence of this deoxyribonucleoside as the calculated specific activity for this reaction was identical in Tris and in phosphate.

Table 4. Nucleosidase and deaminase activities in soybean. Nucleosidase specific activities were found in soybean 5-9 day old seedlings. If a two phase reaction progress was observed specific activity was calculated for both phases (*phase 1, **phase 2). Specific activities calculated for adenosine and cytidine are a combination of all enzyme activities involved in the degradation reactions. Presence was determined based on whether or not there was a detectable nucleosidase activity.

Enzyme	Presence	μmol/(min mg)
Adenosine Nucleosidase/Deaminase	Y	0.00035*/0.0025**
Inosine Nucleosidase	Y	0.00028*/0.00196
Xanthosine Nucleosidase	Y	0.00021
Guanosine Nucleosidase	Y	0.00031
Uridine Nucleosidase	Y	0.0095
Cytidine Nucleosidase/Deaminase	Y	0.00038
Thymidine Nucleosidase	Y	0.000006*/0.00045**
5-Methyluridine Nucleosidase	Y	0.00028
2'-Deoxyadenosine Nucleosidase	Y	0.0079

Inosine and guanosine are both converted to their respective bases in the presence of soybean crude protein extract. Inosine was degraded in two phases. Specific activity in the

initial phase of the reaction, which lasted approximately seven hours, was 0.00028 μmol/(min mg). There was a 7-fold increase during the second phase. The specific activity was 0.00196 μmol/(min mg). The reaction velocity was not significantly faster when phosphate buffer was used as the reaction buffer, and also proceeded in two phases. Specific activity during the first phase was 0.00056 μmol/(min mg) and 0.0027 μmol/(min mg) in phase two. Guanosine was converted to guanine at 0.00031 μmol/(min mg) in both Tris and phosphate buffer systems. It is clear from the data collected that an inosine/guanosine phosphorylase is not active in soybean young seedlings. There are nucleosidases active in soybean that utilize inosine and guanosine as substrates. The results in this study seem to indicate that an inosine nucleosidase may be active and an inosine/guanosine nucleosidase may also be active. It is, however, also possible that a separate guanosine nucleosidase may be responsible for the activity observed when guanosine is used as the substrate. It is feasible that an inosine/guanosine nucleosidase is active during the initial phase when inosine is used as a substrate. The specific activity observed for guanosine is very similar to that for inosine during the initial phase. Inosine nucleosidase may be responsible for the accelerated conversion of inosine to hypoxanthine in the second phase of the reaction. Further purification is needed to ascertain the nature of the enzymes involved in inosine and guanosine catabolism. There was no guanosine deaminase activity detected.

Xanthosine was converted to xanthine. There was no difference in the conversion rate in Tris and phosphate buffers. The specific activity in both cases was 0.00021 μmol/(min mg). These findings suggest that xanthosine phosphorylase is not present in 5-9 day old soybean seedlings. A purine nucleosidase or xanthosine nucleosidase is active in

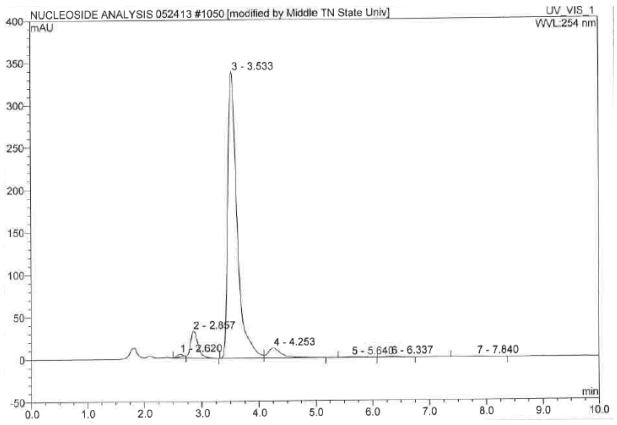
soybean. Further purification and characterization will be needed to determine if a xanthosine specific enzyme is in fact involved in degradation of the nucleoside.

Pyrimidine nucleosides were degraded in the presence of soybean crude protein extract. Uridine nucleosidase is active in soybean at 0.0095 μmol/(min mg). There was no phosphorylase activity observed when uridine was utilized as the substrate. Uridine nucleosidase was also active when cytidine was assayed.

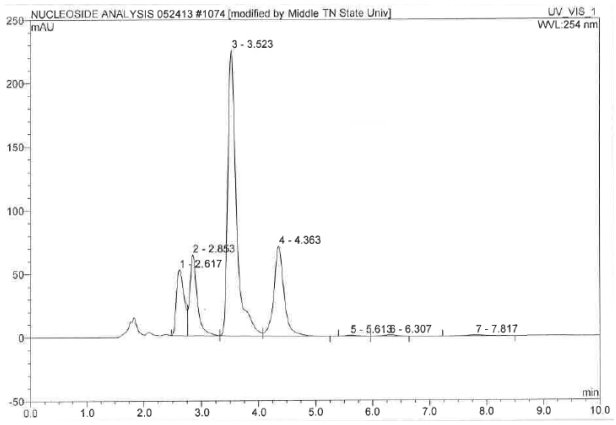
Cytidine deaminase is active in soybean as evident by the formation of a product peak that corresponds to uridine. Uridine to uracil conversion is mediated by uridine nucleosidase. Similar to yellow lupin, it appears that a pyrimidine nucleosidase (possibly a cytidine nucleosidase) is active in soybean as well (Figure 11).The combined specific activity was 0.00038 μmol/(min mg) in Tris buffer and 0.00048 μmol/(min mg) in phosphate buffer. There is no cytidine phosphorylase in young soybean seedlings.

There was an eight hour reaction initiation phase when thymidine was used as the substrate. The specific activity was 0.000006 μmol/(min mg). The second phase was significantly more robust with specific activity of 0.00045 μmol/(min mg), a 75-fold increase. A thymidine phosphorylase seems to not be active in soybean (Table 5). The ribonucleoside analog, 5-methyuridine, was used as a substrate when soybean crude protein extract was added to the reaction mixture. The specific activity was found to be 0.00028 μmol/(min mg). There may be a minor contribution from a phosphorylase that accepts 5-methyluridine as a substrate. The reaction proceeded in two phases. The specific activity was initially 0.00028 μmol/(min mg) but increased to 0.00097 μmol/(min mg) after seven hours following crude addition.

A.



B.



C.

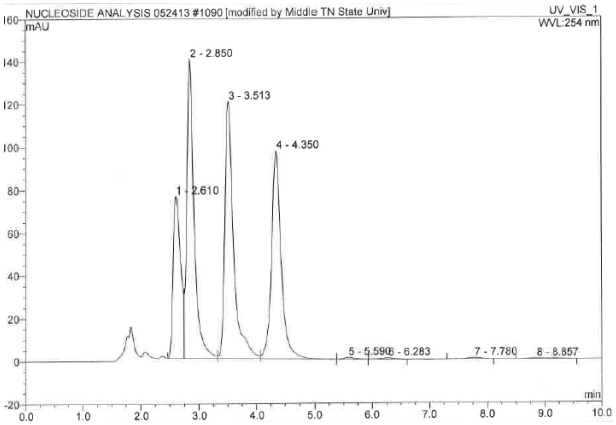


Figure 11. Cytidine with soybean extract generated multiple products. Cytosine, uridine and uracil eluted along with cytidine during the course of the reaction. **(A)** Cytidine can be seen as the only major peak at 3.533 minutes. Reaction appears to begin rapidly. Small uridine and uracil peaks are visible in the injection immediately following soybean extract addition. **(B)** Uridine can be seen at 4.353 minutes, uracil at 2.853 minutes and cytosine at 2.610 minutes following six hours of reaction time. **(C)** Significant amounts of cytidine are degraded at 12 hours following initial injection. Uridine, cytosine and uracil can be clearly identified as products based on standard retention times.

Table 5. Phosphorylase activity in soybean. Combined phosphorylase and nucleosidase specific activities in soybean 5-9 day old seedlings. If a two phase reaction progress was observed specific activity was calculated for both phases (*phase 1, **phase 2). Specific activity reported when adenosine and cytidine were used as substrates likely includes a deaminase contribution in addition to the nucleosidase. Presence was determined based on whether or not there was a detectable phosphorylase activity.

Enzyme	Presence	μmol/(min mg)
Adenosine Phosphorylase	Y	0.00014*/0.0081**
Inosine Phosphorylase	N	0.00056*/0.0027**
Xanthosine Phosphorylase	N	0.00021
Guanosine Phosphorylase	N	0.00031
Uridine Phosphorylase	N	No data
Cytidine Phosphorylase	N	0.00048
Thymidine Phosphorylase	N	0.00001*/0.0008**
5-Methyluridine Phosphorylase	Y	0.00028*/0.00097**
2'-Deoxyadenosine Phosphorylase	N	0.0079

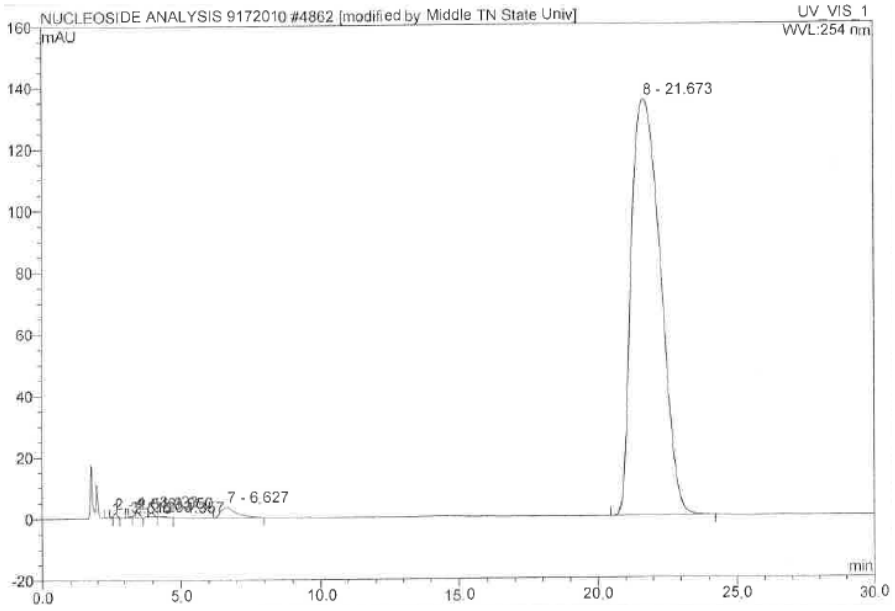
Enzyme activities in corn

Nucleosidase and deaminase activities were evaluated in 5-7 day old corn seedlings. There was a significant initiation phase, lasting between four and eight hours, for all nucleosides used as substrates, except cytidine. Phase two typically represented a major increase in reaction velocity. Adenosine and 2'-deoxyadenosine were used as substrates to assess the presence of hydrolyzing activity associated with a nucleosidase. The initiation phase was longer for adenosine than for the deoxyribose analog. The duration of phase one was eight hours and six hours, respectively. Specific activities during phase one were 0.022 μmol/(min mg) for adenosine and 0.011 μmol/(min mg) for 2'-deoxyadenosine. There was an 8-fold increase in reaction velocity during phase two of the reaction with adenosine with a specific activity of 0.18 μmol/(min mg). A similar trend was observed for 2'-

deoxyadenosine with the specific activity rising to 0.10 μmol/(min mg), a 9-fold increase. The data collected point to the presence of a deaminase activity as was seen in soybean. The products formed during the reaction progress included adenine, inosine and hypoxanthine (Figure 12). An important observation was made that the deaminase was less active in corn than it was in soybean. This observation was confirmed when an assay utilizing adenine as the substrate was performed. It took well over fourteen hours for all adenine to be converted to hypoxanthine. In contrast, it took only seven hours for all adenine to be degraded when assayed with soybean extract. The appearance of an inosine peak during the reaction progress with adenosine as a substrate points to a possible presence of adenosine/adenine deaminase in corn. This finding requires further investigation by introducing additional purification steps aimed at separating this enzyme activity from the nucleosidase activity.

Inosine and guanosine were both hydrolyzed to hypoxanthine and guanine respectively in the presence of a crude protein extract from corn seedlings. The enzymes involved in the degradation of inosine and guanosine displayed specific activities of 0.0044 μmol/(min mg) and 0.0089 μmol/(min mg) during the initiation phase, respectively. This was the first time an initiation phase was observed when guanosine was used as the substrate in the reaction mixture. There were significant reaction velocity gains noted during the second phase for both nucleosides. The specific activity increased to 0.098 μmol/(min mg) and represented a 22-fold increase for inosine. A less significant, 3.5-fold, increase yielded a specific activity of 0.031 μmol/(min mg) for guanosine. A possible explanation for this difference is that guanosine may be a major substrate for inosine/guanosine nucleosidase only. This enzyme, however, also accepts inosine as a substrate. Additionally,

A.



B.

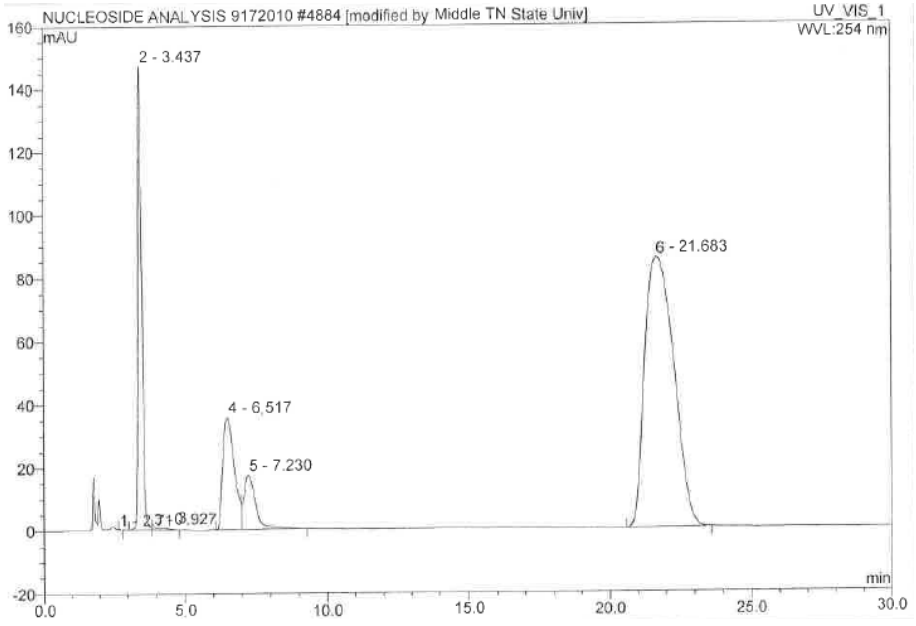


Figure 12. Adenosine nucleosidase reaction in corn yielded adenine, inosine, and hypoxanthine, likely due to the presence of adenosine deaminase activity. 10 µL of corn extract were added to 990 µL of 1 mM adenosine solution. **(A)** Reaction mixture immediately following corn extract addition. Adenosine is the only peak visible at 21.673 minutes. **(B)** Eight hours following reaction initiation, adenosine is still present at 21.683 minutes. However, inosine is clearly visible at 7.23 minutes. A significant peak can be seen for adenine at 6.517 minutes and hypoxanthine also appeared at 3.437 minutes.

an inosine nucleosidase may also be active in corn and may become active after specific concentration or substrate requirements are met, as seen by the increase in activity represented by second phase of the reaction. It will be interesting to see if separation of these two activities is possible in the future studies. There were no signs of a guanosine deaminase activity in corn.

Xanthosine nucleosidase appears to be active in corn at a somewhat similar level found in yellow lupin. Specific activity during the initial phase was 0.0044 μmol/(min mg) and increased to 0.049 μmol/(min mg) during the secondary phase of the reaction progress. This represents an 11-fold increase. It is possible that a non-specific purine nucleosidase catalyzes this reaction. It will require further investigation to determine whether this activity is due to a single xanthosine specific nucleosidase or a combination of multiple enzymes. A potentially interesting finding was also made when reaction progress was monitored with xanthosine as the substrate. An unidentified peak appeared at a roughly identical retention time associated with a uracil standard (Figure 13)**.** It can be hypothesized that the peak is representative of uric acid prior to ring cleavage and may point to the presence of xanthine dehydrogenase in the crude protein extract of young corn seedlings. The dehydrogenase activity was not observed in yellow lupin or soybean.

Uridine was degraded to uracil in corn. There were two phases observed when uridine was used as a substrate. Specific activity measured during the initial phase was 0.031 μmol/(min mg) and represents the most robust enzyme activity in 5-7 day old corn seedlings during this phase. There was a 3-fold increase during the second phase that resulted in a specific activity of 0.098 μmol/(min mg) which is the second highest overall activity level seen in corn seedlings used in this study.

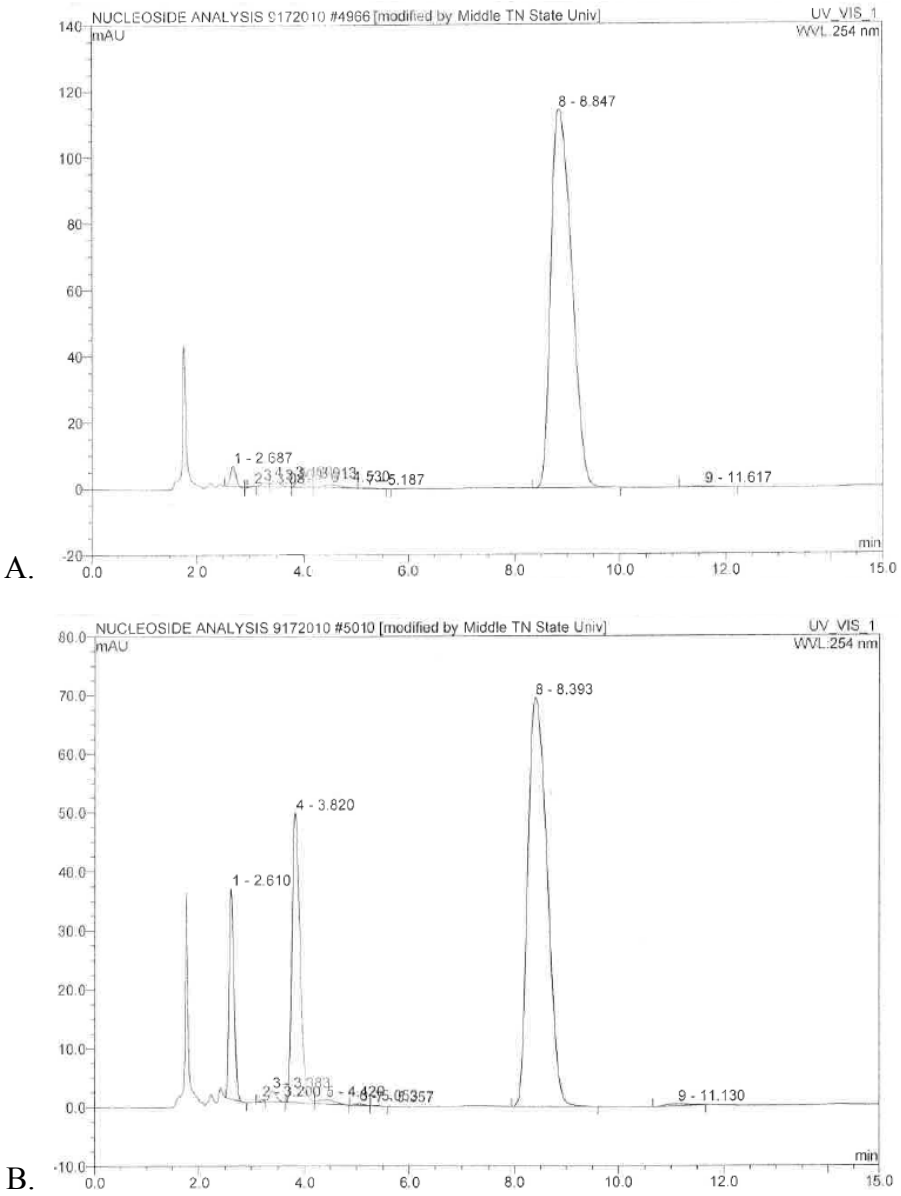


Figure 13. Xanthosine reaction mixture with corn extract contained 975 µL of 1 mM xanthosine and 25 µL of crude corn extract. **(A)** Xanthosine eluted at 8.847 minutes and was the only major component present in the reaction mixture immediately following corn extract addition. **(B)**. Approximately seven hours later a product peak, identifiable as xanthine based on standard retention time, appeared at 3.820 minutes. A third peak also eluted at 2.610 minutes and corresponds to the standard retention time for uracil. Purines are degraded to xanthine and then catabolized to uric acid and not uracil. It is possible that retention time for uric acid may be very similar to uracil, however this was not evaluated in this study and thus identification of this third peak remains to be accomplished in the future.

Cytidine was also evaluated as a substrate for nucleosidases and deaminases that may be present in young corn seedlings. The enzymes involved in cytidine processing had a total specific activity of 0.022 μmol/(min mg) for this nucleoside. Uridine, cytosine and uracil peaks were observed initially (Figure 14). Cytosine and uracil peaks eluted very close together and resolution became very poor as more of the products were formed. It is, however, likely that both cytidine deaminase and a pyrimidine nucleosidase (possibly cytidine nucleosidase) can be found active in corn. Presence of cytosine deaminase was not evaluated in this study and should be investigated during future work.

Thymidine, a 2'-deoxyribose nucleoside, was also used as a nucleosidase substrate in corn. A possible thymidine nucleosidase appeared to be most active in corn out of all three plants examined in this study. The specific activity during the initiation phase was 0.0013 μmol/(min mg) and increased to 0.031 μmol/(min mg) thereafter. An analog, 5-methyluridine, was also used as a substrate. A thymine peak was seen at the expected retention time for this nucleobase. Specific activity for a pyrimidine nucleosidase (possibly thymidine nucleosidase) was initially 0.0044 μmol/(min mg) and increased to 0.022 μmol/(min mg) during the second phase, a 5-fold increase. Summary of the results can be found below in Table 6. Phosphorylase activity was not evaluated for corn in this study.

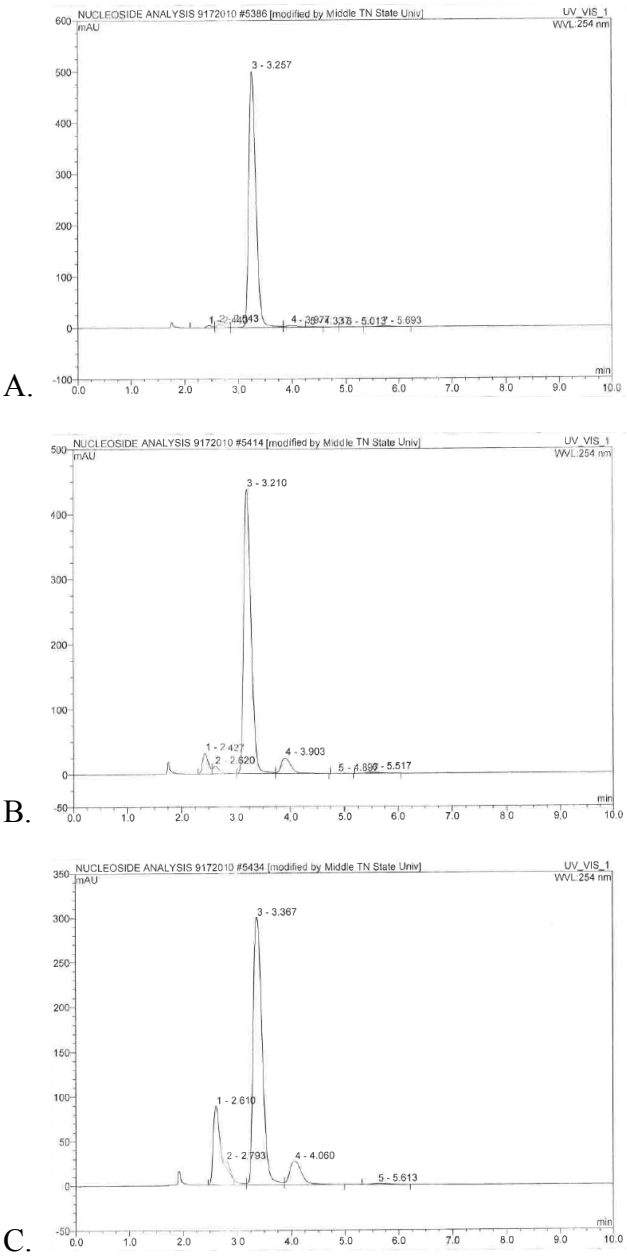


Figure 14. The cytidine nucleosidase reaction in corn revealed presence of multiple products. Uridine, cytosine and uracil were formed as products. **(A)** Cytidine can be seen at 3.257 minutes as the only significant peak at the start of the reaction. **(B)** Formation of all three products occurred around four hours following corn extract addition to the 1 mM cytidine stock solution. Uridine eluted at 3.900 minutes, cytosine at 2.427 minutes and uracil at 2.620 minutes. **(C)** Cytosine peak and uracil peak became poorly resolved as the product concentration increased. It is impossible to determine which activity is more robust unless better separation is achieved in the future.

Table 6. Nucleosidase specific activities in corn 5-7 day old seedlings. If a two phase reaction progress was observed specific activity was calculated for both phases (*phase 1, **phase 2). Specific activities calculated for adenosine and cytidine are a combination of all enzyme activities involved in the degradation reactions.

Enzyme	Presence	μmol/min/mg
Adenosine Nucleosidase	Y	0.022*/0.18**
Inosine Nucleosidase	Y	0.0044*/0.098**
Xanthosine Nucleosidase	Y	0.0044*/0.049**
Guanosine Nucleosidase	Y	0.0089*/0.031**
Uridine Nucleosidase	Y	0.031*/0.098**
Cytidine Nucleosidase/Deaminase	Y	0.022
Thymidine Nucleosidase	Y	0.0013*/0.031**
5-Methyluridine Nucleosidase	Y	0.0044*/0.022**
2'-Deoxyadenosine Nucleosidase	Y	0.011*/0.1**

CHAPTER IV

CONCLUSION

Despite the wealth of data generated to date, the full picture remains unclear for nucleosidase, phosphorylase and deaminase activities in plants. Part of the challenge may be an apparent lack of a consensus enzymatic nomenclature in plants. This obstacle may be in part due to the targeted nature of research studies that rely heavily on conclusions drawn after experiments were conducted with microorganisms and animal cells. Furthermore, previously purified enzymes in plants appear to display generalist functionality but at low levels. Therefore, the question remains whether to designate proteins that appear to have low broad substrate specificities, despite typically preferring one or two nucleosides at dramatically higher levels, as purine or pyrimidine hydrolases. Purified enzymes represent a relatively small part of the data available describing hydrolase activities, with even less data for deaminases, and essentially none for phosphorylases in plants. The variability in the presence of enzyme activities across plant species, developmental stages of the plant and cell localization, further complicates the task of elucidating the exact pathways present in plants. Future kinetic studies that will add additional activity data on highly purified enzymes, across a wider array of plant species, will help fill the numerous gaps still remaining. The amount of genetic sequencing data, which is also growing, will aid in mapping out the remaining unknown areas in plant purine and pyrimidine metabolism.

Sufficient evidence was gathered throughout this thesis work to suggest that multiple nucleosidase activities are present in yellow lupin 5-7 day old seedlings. Adenosine nucleosidase is the most active of all nucleosidases. Thymidine is hydrolyzed the least. There is likely an inosine nucleosidase and inosine/guanosine nucleosidase present in

yellow lupin young seedlings. A uridine nucleosidase and a xanthosine nucleosidase activities also appear to be present. It has typically been reported that most of the purified nucleosidases appear to have broad specificities but at a significantly lower level than with the preferred nucleoside. A purine nucleosidase activity that hydrolyzes conversion of xanthosine to xanthine was previously reported in disks of potato tubers and tea leaf extracts.[24,44] This activity may in fact be xanthosine nucleosidase. Similar activity was observed in yellow lupin young seedlings in this study. It may be worthwhile to attempt purification of this protein in the future to establish whether the hydrolyzing activity is due to a non-specific purine nucleosidase or a xanthosine preferring nucleosidase. Cytidine is degraded by action of cytidine deaminase and, in yellow lupin, pyrimidine nucleosidase (possibly cytidine nucleosidase). Phosphorylase activity is unlikely a contributing factor in nucleosidase degradation in yellow lupin. The data appear to indicate that a phosphorylase may be active when xanthosine and uridine are used as substrates. It is, however, important to further evaluate these particular findings by conducting a reverse reaction starting with the nucleobase and α-ribose-1-phosphate. Formation of a parent nucleoside will confirm presence of the phosphorylase.

All nucleosides tested were hydrolyzed in the presence of the crude protein extract from 5-9 day old soybean seedling. The highest activity was recorded for uridine nucleosidase. This was in contrast to yellow lupin and could represent an important difference in nucleotide metabolism of tropical legumes. Thymidine and 5-methyluridine were the least utilized substrates with soybean crude protein extract. All other nucleosides tested were hydrolyzed at a similar level. It is also likely that an adenosine/adenine deaminase is active in soybean young seedlings. This represents another potential key

difference associated with tropical legume development. Cytidine deaminase was found active in soybean, but not guanosine deaminase.

A possible cytosine nucleosidase activity was observed during this study. Adenosine phosphorylase may be quite active in soybean as well. Overall, the activity levels found were significantly lower compared to those in yellow lupin. It is probable that inclusion of the slightly older seedlings is the key reason for the difference observed. Previous reports indicated that nucleosidase activity typically maxed out 4-5 days after germination. It is possible that during the following days a significant shift to biosynthetic pathways occurs.

Nucleosidase activities in corn were also present. All nine substrates evaluated in this study were converted to the respective bases in the presence of crude protein extract from 5-7 day old corn seedlings. Activity levels were higher in all cases when compared to soybean and were very similar against activity levels found in yellow lupin. This further suggests that age may play an important role in the prevalence of activities associated with the preferred nucleotide metabolic pathway. As seen in yellow lupin, adenosine and 2'-deoxyadenosine were hydrolyzed the most out of all substrates utilized. Interestingly, the activity levels were fairly robust for all nucleosides evaluated with the lowest observed for 5-methyluridine. Additional ring substituted analogs should be assayed in the future to assess the nucleoside structural importance on its substrate utility. A distinguishing feature in corn metabolism when compared to yellow lupin, but similar to soybean, is the possible presence of adenosine deaminase. This activity was observed during this study but was not as strong as it was in soybean. Cytidine deaminase was also very active in corn and appears to be the more dominant activity against the nucleosidase activity based on qualitative evaluation. Further method improvements aimed at achieving a greater resolution between

uracil and cytosine are needed to obtain quantitative evidence for the observed trend. Phosphorylase activity was not evaluated in corn and represents an interesting next step for future work. It is important to develop an effective confirmation method that will employ a reaction in the reverse direction of the phosphorylase activity. Further purification steps should be taken in soybean to obtain a highly purified form of adenosine nucleosidase that is separated from the adenosine deaminase activity. A similar endeavor should be undertaken with corn.

REFERENCES

1. Lodish, H.; Berk, A.; Zipursky, SL.; et al. The Life Cycle of Cells. In *Molecular Cell Biology* [Online], fourth edition; W.H. Freeman: New York, 2000; http://www.ncbi.nlm.nih.gov/books/NBK21685 (accesses June, 2014).

2. Zrenner, R.; Stitt, M.; Sonnewald, U.; Boldt, R. Pyrimidine and purine biosynthesis and degradation in plants. *Annu. Rev. Plant Biol.* **2006,** *57*, 805-836.

3. Crozier, A.; Kamiya, Y.; Bishop, G.; Yokota, T. Biosynthesis of hormones and elicitor molecules. In *Biochemistry and Molecular Biology of Plants;* Buchanan, BB.; Gruissem, W.; Jones, RL., Eds.; Am. Soc. Plant Physiology: Rockville, MA, 2000; pp 850-929.

4. Hanson, A. D.; Gregory, J. F. Synthesis and turnover of folates in plants. *Curr. Opin. in Plant Biol.* **2005,** *5*, 244-249.

5. Herz, S.; Eberhardt, S.; Bacher, A. Biosynthesis of riboflavin in plants. The *ribA* gene of *Arabidopsis thaliana* specifies a bifunctional GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase. *Phytochemistry.* **2000,** *53*, 723-731.

6. Ashihara, H.; Luit, B.; Belmonte, M.; Stasolla, C. Metabolism of nicotinamide, adenine and inosine in developing microspore-derived canola (*Brassica napus*) embryos. *Plant Physiol. Biochem.* **2008**, *46*, 752-759.

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

8. Lim, EK.; Bowles, D. A class of plant glycosyltransferases involved in cellular homeostasis. *EMBO J.* **2004,** *23*, 2915-2922.

9. O'Donovan, G. A. Thymidine metabolism in bacteria (and "How, or how not, to label DNA"). In *DNA Synthesis: Present and Future;* Molineaux, I.; Kohiyama, M., Eds.; Plenum Publishing Corp: New York, 1978**;** pp 219-253.

10. Kizaki, H.; Shimada, H.; Ohsaka, F.; Sakurada, T. Adenosine, deoxyadenosine, and deoxyguanosine induce DNA cleavage in mouse thymocytes. *J. Immunol.* **1988,** *141*, 1652-1657.

11. Stasolla, C.; Loukanina, N.; Yeung, E. C.; Thorpe, T. A. Alterations in pyrimidine nucleotide metabolism as an early signal during the execution of programmed cell death in tobacco BY-2 cells. *J. Exp. Bot.* **2004,** *55*, 2513-2522.

12. Jung, B.; Florchinger, M.; Kunz, HH.; Traub, M.; Wartenberg, R.; Jeblick, W.; Neuhaus H. E.; Mohlmann, T. Uridine-ribohydrolase is a key regulator in the uridine degradation pathway of *Arabidopsis*. *Plant Cell,* **2009,** *21*, 876-891.

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

14. Lynch, C. M.; Clowes, M. M.; Osborne, W. R.; Clowes, A. W.; Miller, A. D. Long-term expression of human adenosine deaminase in vascular smooth muscle cells of rats: a model of gene therapy. *Proc. Natl. Acad. Sci. U.S.A.* **1992,** *89*, 1138-1142.

15. Sun, Y.; Fukamachi, T.; Saito, H,; Kobayashi, H. Adenosine deamination increases the survival under acidic conditions in Escherichia coli. *J. Appl. Microbiol.* **2012,** *112*, 775-781.

16. Edwards, R. S-adenosyl-L-methionine metabolism in alfalfa cell cultures following treatment with fungal elicitors. *Phytochemistry.* **1996,** *43*, 1163-1169.

17. JarAllah, L. S. Characterization of adenosine nucleosidase from soybean seeds (*Glycine max* L.). M.S. Thesis, Middle Tennessee State University, December 2013.

18. Smith, P. M. C.; Atkins, C. A. Purine Biosynthesis. Big in cell division, even bigger in nitrogen assimilation. *Plant Physiol.* **2002,** *128*, 793-802.

19. Todd, C.; Polacco, J. C. Soybean cultivars 'Williams 82' and 'Maple Arrow' produce both urea and ammonia during ureide degradation. J. Exp. Bot. **2004**, *55*, 867-877.

20. Schubert, K. R.; Boland, M. J. The urides. In *The Biochemistry of Plants;* Stump, P. K.; Conn, E. E., Eds.; Academic: San Diego, 1990**;** pp 197-282.

21. Moffatt, B. A.; Ashihara, H. Purine and pyrimidine nucleotide synthesis and metabolism. In *The Arabidopsis Book;* **2002**, *1,* 1-20.

22. Yabuki, N.; Ashihara, H. Catabolism of adenine nucleotides in suspension-cultured plant cells. *Biochem. Biophys. Acta.* **1991,** *1073*, 474-480.

23. Dahncke, K.; White, CP. Plant purine nucleoside catabolism employs a guanosine deaminase required for the generation of xanthosine in *Arabidopsis*. *Plant Cell.* **2013,** *21*, 4101-4109.

24. Katahira, R.; Ashihar, H. Profiles of purine biosynthesis, salvage and degradation in disks of potato (*Solanum tuberosum* L.) tubers. *Planta.* **2006,** *225*, 115-126.

25. Ashihara, H.; Takasawa, Y.; Suzuki, T. Metabolic fate of guanosine in higher plants. *Physiol. Plant.* **1997,** *100*, 900-916.

26. Ashihara, H.; Crozier, A. Biosynthesis and metabolism of caffeine and related purine alkaloids in plants. *Adv. Bot. Res.* **1999,** *30*, 118-205.

27. Kafer, C.; Zhou, L.; Santoso, D.; Guirgis, A.; Weers, B.; Park, S.; Thornburg, R. Regulation of pyrimidine metabolism in plants. *Front. Biosci.* **2004,** *9*, 1611-1625.

28. Katahira, R.; Ashihara, H. Dual function of pyrimidine metabolism in potato (*Solanum tuberosum*) plants: pyrimidine salvage and supply of β-alanine to pantothenic acid synthesis. *Phys. Plant.* **2006,** *127*, 38-43.

29. Walsh, T. A.; Green, S. B.; Larrinua, I. M.; Schmitzer, P.R. Characterization of plant β-ureidopropionase and functional overexpression in *Escherichia coli*. *Plan Physiol.* **2001,** *125*, 1001-1011.

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

31. Pugmire, M. J.; Ealick, S. E. Structural analyses reveal two distinct families of nucleoside phosphorylases. *Biochem. J.* **2002,** *361*, 1-25.

32. Sakai, T.; Yu, T.-S.; Omata, S. Distribution of enzymes related to cytidine degradation in bacteria. *Agr. Biol. Chem.* **1976,** *40*, 1893-1895.

33. Martinussen, J.; Andersen, P. S.; Hammer, K. Nucleotide metabolism in *Lactococcus lactis*: salvage pathways of exogenous pyrimidine. *J. Bacteriol.* **1994,** *176*, 1514-1516.

34. Schmidt, A.; Su, Y. H.; Kunze, R.; Warner, S.; Hewitt, M.; et al.; UPS1 and UPS 2 from *Arabidopsis* mediate high affinity transport of uracil and 5-fluorouracil. *J. Biol. Chem.* **2004,** *279*, 44817-44824.

35. De Koning, H.; Diallina, G. Nucleobase transporters. *Mol. Membrane Biol.* **2000,** *17*, 75-94.

36. Becroft, D. M. O.; Phillips, L. I. Hereditary orotic aciduria and megaloblastic anemia: a second case, with response to uridine. *Brit. Med. J.* **1965,** *1*, 547-552.

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

38. Bates, C.; Kendrick, Z.; McDonald, N.; Kline, P. C. Transition state analysis of adenosine nucleosidase from yellow lupin (*Lupinus luteus*). *Phytochemistry.* **2006,** *67*, 5-12.

39. Astot, C.; Dolezal, K.; Nordstrom, A.; Wang, Q.; Kunkel, T.; Moritz, T.; Chua, N.-H.; Sandberg, G. An alternative cytokinin biosynthesis pathway. *Proc. Natl. Acad. Sci. U.S.A.* **2000,** *97*, 14778-14783.

40. Goble, A. M.; Fan, H.; Sali, A.; Raushel, F. M. Discovery of a cytokinin deaminase. *ACS Chem. Biol.* **2011,** *6*, 1036-1040.

41. Sculley, D. G.; Dawson, P. A.; Emmerson, B. T.; Gordon, R. B. A review of the molecular basis of hypoxanthine-guanine phosphoribosyltransferase (HPRT) deficiency. *Hum Genet.* **1992,** *90*, 195-207.

42. Stasolla, C.; Loukanina, N.; Ashihara, H.; Yeung, E.; Thorpe, T. A. Changes of purine and pyrimidine nucleotide biosynthesis during shoot initiation from epicotyl explants of white spruce (*Picea glauca*). *Plant Sci.* **2006,** *171*, 345-354.

43. Mushegian, A. R.; Koonin, E. V. Unexpected sequence similarity between nucleosidases and phosphoribosyltransferases of different specificity. *Protein. Sci.* **1994,** *3*, 1081-1088.

44. Pettengill, E. A.; Pettengill, J. B.; Coleman, G. D. Elucidating the evolutionary history and expression patterns of nucleoside phosphorylase paralogs (vegetative storage proteins) in *Populus* and the plant kingdom. *BMC Plant Biol.* **2013,** *13*, No. 118.

45. Jung, B.; Hoffmann, C.; Mohlmann, T. *Arabidopsis* nucleoside hydrolases involved in intracellular and extracellular degradation of purines. *Plant J.* **2011,** *65*, 703-711.

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

47. Deng, W.-W.; Ashihara, H. Profiles of purine metabolism in leaves and roots of *Camellia sinensis* seedlings. *Plant Cell Physiol.* **2010**, *51*, 2105-2118.

48. Guranowski, A.; Barankiewica, J. Purine salvage in cotyledons of germinating lupin seeds. *FEBS Lett.* **1979,** *104*, 95-98.

49. Guranowski, A. Purine catabolism in plants: purification and some properties of inosine nucleosidase from yellow lupin (*Lupinus luteus* L.) seeds. *Plant Physiol.* **1982,** *70*, 344-349.

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

51. Geigenberger, P.; Regierer, B.; Nunes-Nesi, A.; Leisse, A.; Urbanczyk-Wochniak, E.; Springer, F.; Van Dongen, J. T.; Kossmann, J.; Fernie, A. R. Inhibition of de novo pyrimidine synthesis in growing potato tubers leads to a compensatory stimulation of the pyrimidine salvage pathway and a subsequent increase in biosynthetic performance. *Plant Cell.* **2005,** *17*, 2077-2088.

52. Katahira, R.; Ashihara, H. Profiles of pyrimidine biosynthesis, salvage and degradation in disks of potato (*Solanum tuberosum* L.) tubers. *Planta.* **2002,** *215*, 821-828.

53. Burch, L. R.; Stuchbury, T. Purification and properties of adenosine nucleosidases from tomato (*Lycopersicon esculentum*) roots and leaves. *J. Plant Physiol.* **1986,** *125*, 267-273.

54. Belmonet, M. F.; Stasolla, C.; Katahira, R.; Loukanina, N.; Yeung, E. C.; Thorpe, T. A. Glutathione-induced growth of embryogenic tissue of white spruce correlates with changes in pyrimidine nucleotide metabolism. *Plant Sci.* **2005,** *168*, 803-812.

55. Sato, Y.; Ashihara, H. Pyrimidine salvage and catabolism in leaves of mangrove species. *Plant Sci.* **2008,** *174*, 140-148,

56. Sato, Y.; Ashihara. H. Long-term effect of NaCl on the activity of uridine and uracil salvage for nucleotide synthesis in cultured mangrove (*Bruguiera sexangula*) cells. *Plant Sci.* **2009,** *176*, 383-389.

57. Ashihara, H. Xanthosine metabolism in plants: metabolic fate of exogenously supplied ^{14}C-labelled xanthosine and xanthine in intact mungbean seedlings. *Phytochemistry Lett.* **2012,** *5*, 100-103.

58. 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.

59. Leszczynska, D.; Schneider, Z.; Tomaszewski, M.; Mackowiak, M. Comparative studies on adenosine nucleosidase occurrence in plants. *Annals Bot.* **1984,** *54*, 847-849.

60. National Agricultural Statistics Service. Crop production: 2012 Summary. *USDA,* 2013.

61. Economic Research Service. Biotechnology: implication for US corn and soybean trade. *USDA,* **2000.**

62. Coombs, A. Revenge of the weeds. The Scientist [Online]; 2012, http://www.the-scientist.com/?articles.view/articleNo/32108/title/Revenge-of-the-Weeds (accessed June 2014).

63. Levetin, E.; McMahon. L. Legumes. In *Plants and Society*, fifth edition. The McGraw−Hill Companies: New York, 2008**;** pp 207-219.

64. Wrather, J. A.; Koenning, S. R. Estimates of disease effects on soybean yields in the United States 2003 to 2005. *J. Nematol.* **2006,** *38*, 173-180.

65. Peterson, T. A.; Lovatt, C. J.; Nieman, R. H. Salt stress causes acceleration of purine catabolism and inhibition of pyrimidine salvage in *Zea mays* root tips. *J. Exp. Bot.* **1988,** *39*, 1389-1395.

66. Polayes, D. A.; Schubert, K. R. Purine synthesis and catabolism in soybean seedlings: the biogenesis of ureides. *Plant Physiol.* **1984,** *75*, 1104-1110.

67. Parkin, D. W.; Horenstein, B. A.; Abdulah, D. R.; Estupinan, B.; Schramm, V. L. Nucleoside hydrolase from *Crithidia fasciculata*: metabolic role, purification, specificity, and kinetic mechanism. *J. Biol. Chem.* **1991,** *266*, 20658-20665.

68. Steenkamp, D. J. The purine-2-deoxyribonucleosidase from *Crithidia luciliae*: purification and trans-N-deoxyribosylase activity. *Eur. J. Biochem.* **1991,** *197*, 431-439.