NUCLEOSIDE CATABOLIZING ACTIVITIES IN SELECTED SEEDS

By

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I dedicate this research work to my husband, parents, my sisters, and my brothers.

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ABSTRACT

The efficacy of pyrimidine and purine nucleotide metabolism plays a critical role in the progression of biological systems. The process of nucleotide degradation exists in all organisms. The nucleoside degradation and salvage of nucleotides, nucleosides, and nucleobases require several enzymes including deaminases and nucleosidases. Deaminases play a substantial role in the course of removing amino groups from selected nucleobases to convert one nucleoside to another. The cleavage of the N-glycosidic bond in nucleosides is catalyzed by the nucleoside hydrolases or nucleosidases. This process facilitates the recycling of nucleobases.

Nucleoside degradation protein extracts were assessed in some seed sources including Alaska pea, okra, organic okra, peas (Cascadia Sugar Snap), yellow lupin, soybean, spinach, and cantaloupe. Extracts of un-germinated seeds were incubated with reaction mixtures containing 1 mM uridine, inosine, purine riboside, cytidine, adenosine, or 2'-deoxyadenosine in 10 Mm Tris pH 7.2 buffer. Using an HPLC with a Kinetex 5 μ, EVO C18 reverse phase column eluted with ammonium phosphate/ methanol, nucleosidase and deaminase activities were quantified. The results show the existence of active nucleosidases and deaminases in seeds tested. However, there were differences in the levels of activities based on the plant and substrate observed. Uridine, cytidine, and thymine exhibited the highest levels of activity among the nucleosides, while lower activities were observed with guanosine, purine riboside, inosine, and 2'-deoxyadenosine. Among the seeds, soybeans had the lowest activity levels, while okra had the highest activity levels.

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INTRODUCTION

Nucleotide metabolism is instrumental in supporting the life of living organisms. Nucleotides are composed of a nitrogenous base, a pentose sugar, and one or more phosphate groups as determined by its function.[1] Nucleotides are fundamental cell components necessary for the growth of a plant, its development, and metabolism.[2] The nucleotides play a crucial role in the storage of information and its subsequent retrieval during the division and elongation of tissues and as transcript components. The nitrogenous base can either be a purine or a pyrimidine nucleobase. Nucleosides have a cyclic furanoside-type sugar (β-D-ribose or β-D-2-deoxyribose) which has been substituted at C1' by a heterocyclic purine or pyrimidine base attached to a β-glycosyl C1'-N linkage (Figure 1). The pentose sugar is composed of five carbons and can either be a ribose or 2-deoxyribose depending on the nucleic acid.[1] Common nitrogenous bases include cytosine, adenine, guanine, uracil, and thymine.

Nucleotide metabolism is facilitated through four pathways namely, *de novo* synthesis, nucleotide degradation, phosphotransfer reactions, and salvage pathways. Nucleotide metabolism differs from the metabolism of amino acids because the nucleotides and their intermediates are precursors for the synthesis of secondary metabolites and hormones.[3]

The *de novo* synthesis of nucleotides is possible in plants and can be achieved by using 5-phosphosyl-1-pyrophosphate (PRPP), amino acids such as glutamine, glycine, and aspartate, tetrahydrofolate (THF), and simple molecules like carbon dioxide.[3] Preformed nucleosides and nucleobases can also be used to synthesize nucleotides by

salvage reactions. All these methods are biochemical in nature and involve a series of reactions in the cell. The growth of the cell is dependent on the supply of purine and pyrimidine nucleosides and nucleotides.[4]

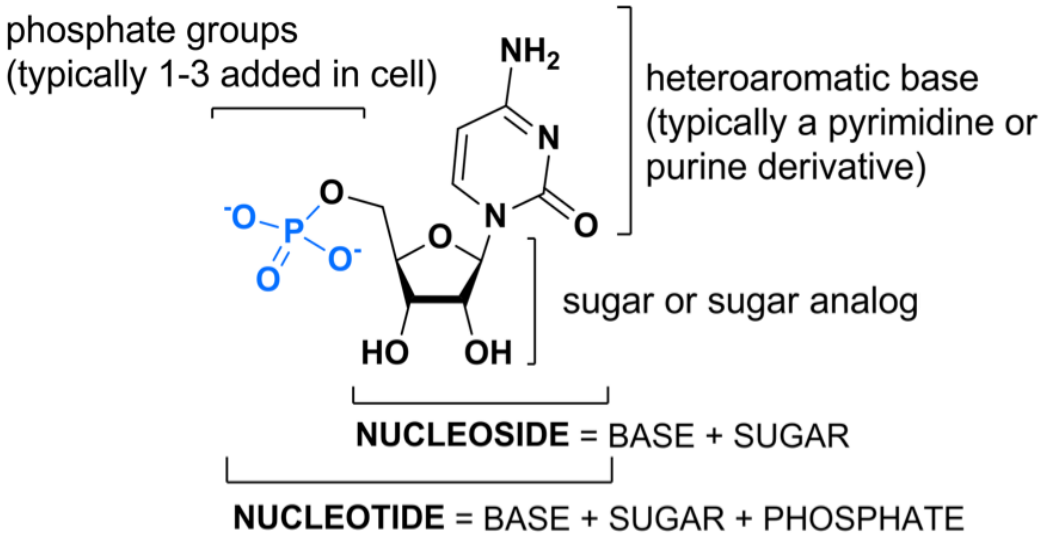


Figure 1: Representative structure of a nucleoside and a nucleotide.[5]

Purines and Pyrimidines

Purines and pyrimidines are nitrogenous bases. The purines are aromatic heterocyclic compounds composed of six-membered and five-membered nitrogen rings (imidazole ring) that have been fused.[6] The diagram below represents a general structure of a purine.

At the C6 position of the purine ring, there is an exocyclic amino group of the adenine base. Guanine contains an exocyclic amino group at its C2 position, while the C6

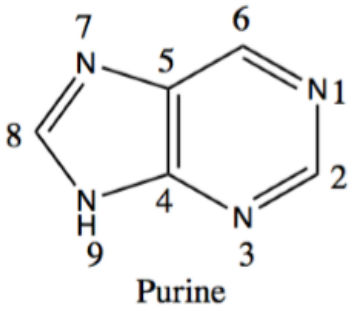


Figure 2: General structure of a purine.[3] The numbering system for purine bases is shown.

position has a carbonyl group (Figure 3).[3] Hypoxanthine is a rare constituent of nucleic acids and is formed in the synthesis of purines. At the C6 position, there is a carbonyl group. The structures of adenine, hypoxanthine, and guanine are shown below (Figure 3).

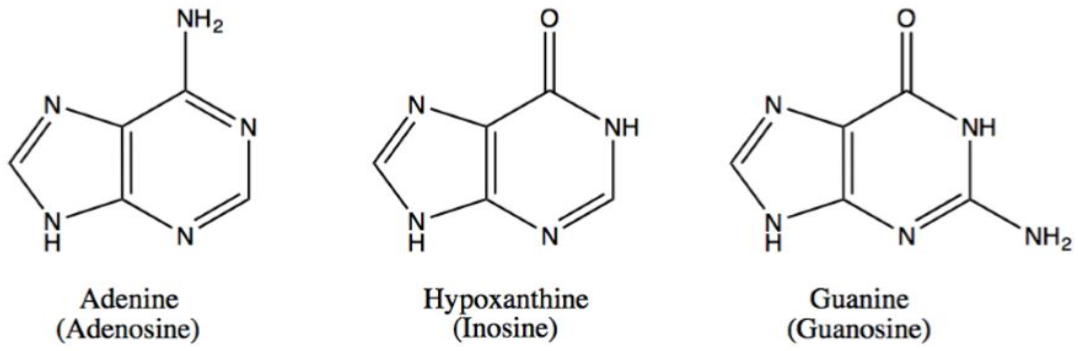


Figure 3: General structure of purine bases adenine, hypoxanthine and guanine. The names in parentheses are the corresponding nucleosides.

Pyrimidines consist of a six-membered nitrogen ring containing two nitrogen atoms at the N1 position and at the N3 position (Figure 4).[1] Thymine, cytosine, and uracil

are examples of pyrimidines (Figure 5). Cytosine has an exocyclic amino group at its C4 position. Thymine has exocyclic carbonyl groups at the C2 and C4 positions, while at the C5 position there is an exocyclic methyl group. Uracil is similar in structure to thymine except that it does not have the methyl group at the C5 position (Figure 5).

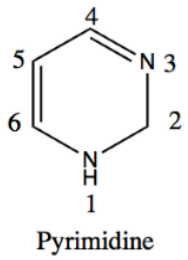


Figure 4: General structure of a pyrimidine.[3] The numbering system for pyrimidine bases is shown.

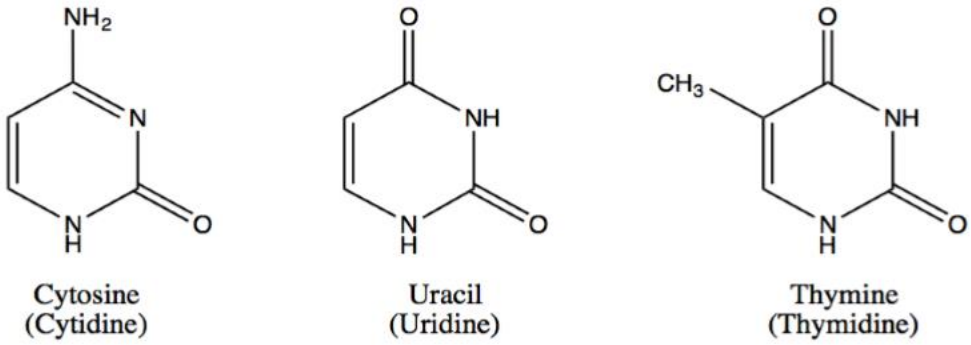


Figure 5: Common pyrimidine bases. The corresponding nucleoside names are in parentheses.

Purine Metabolism

The *de novo* pathway enables the synthesis of nucleotides from amino acids and other small molecules.[7] The *de novo* pathway enzymes form purine nucleotides from glycine, glutamine, aspartate, the activated ribose precursor PRPP (5-phosphoribosyl-1-pyrophosphate), N10-formyl-THF, and carbon dioxide.[8] This is an energy intensive pathway that requires consumption of six ATP molecules from the beginning of the pathway to the branch point at inosine monophosphate (IMP). However, seven high energy phosphate bonds are used because the formation of α-PRPP in the first reaction and the subsequent release of pyrophosphate (PPi) in the next reaction results in the loss of two equivalents of ATP.[8] The *de novo* pathway route of purine nucleotide synthesis has a higher requirement for energy than the salvage pathway.[1]

In the *de novo* pathway of purine nucleotides, the transfer of an amido group of glutamine to PRPP initiates the process.[9] PRPP amidotransferase (ATase) catalyzes this reaction, which leads to the formation of β –phosphoribosylamine (PRA) (Figure 6).[8] An amide bond attaches the PRA to glycine and GAR synthetase catalyzes the formation of GAR (glycine amide ribonucleotide). Adenosine triphosphate facilitates the reaction. The newly formed GAR is then converted into formylglycinamide ribonucleotide (FGAR) by the addition of a formyl group from N10-formyl-THF catalyzed by GAR transformylase.[8] FGAM (formylglycinamidine ribonucleotide) is formed after adding a nitrogen from the side chain of glutamine. The reaction requires the hydrolysis of ATP to ADP and P_i. This newly formed compound subsequently consumes another ATP molecule to form 5-aminoimidazole ribonucleotide (AIR). An enzyme called AIR synthase catalyzes the reaction. Carboxylation of AIR then follows facilitated by AIR carboxylase which results

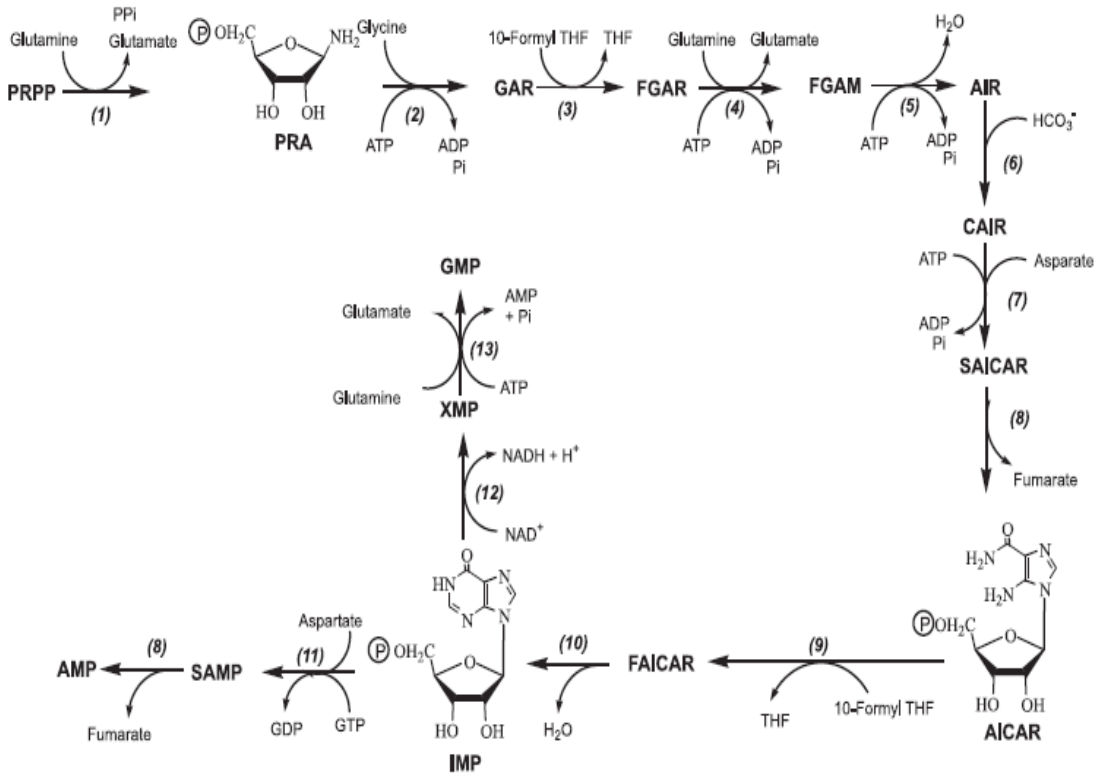


Figure 6: Schematic representation of the purine nucleotides *de novo* pathway.[9] The metabolites present in the diagram are as follows PRA, 5-phosphoribosyl amine; GAR, glycineamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGRAM, formylglycine amidine ribonucleotide; AIR, 5-aminoimidazole ribonucleotide; CAIR, 5-aminoimidazole 4-carboxylate ribonucleotide; SCAIR, 5-aminoimidazole-4-N succinocarboxyamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxyamide ribonucleotide; FAICAR, 5-formamidoimidazole-4-carboxyamide ribonucleotide; SAMP, adenylosuccinate; XMP, santhosine-5′-monophosphate. The enzymes shown in Figure 6 above include: (1) amidophosphoribosyltransferase, (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, (12) IMP dehydrogenase, (13) GMP synthetase. (Adopted with permission from Stasolla, C., Katahira, R., Thorpe, T. A., & Ashihara, H. (2003). Purine and pyrimidine nucleotide metabolism in higher plants. *Journal of Plant Physiology*, 160, 1271-1295).

in the formation of CAIR (4-carboxy-aminoimidazole ribonucleotide).[9] N-succinyl-5-aminoimidazole- 4-carboxamide ribonucleotide (SAICAR) is then formed after the consumption of another ATP molecule and the addition of aspartate. SAICAR synthase mediates this reaction. There is a resemblance between the pathways involved in the synthesis of nucleotides in animal cells, plants, and microorganisms.[2]

Adenylosuccinate lyase (ASL) catalyzes the next step and results in the release of fumarate. The release of fumarate leads to the formation of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR).[9] The final carbon in the purine ring is derived from the addition of a second formyl group from another N10-formyl-THF leading to formation of FAICAR (5-formaminoimidazole-4-carboxamide ribonucleotide). FAICAR is then dehydrated as well as the ring closed to form inosine monophosphate (IMP), the first purine nucleotide.[9] IMP, serves as a common intermediate in the synthesis of adenosine triphosphate (ATP) and guanosine triphosphate (GTP). The pathway separates into two branches with one branch forming adenosine monophosphate (AMP), and the other one forming guanosine monophosphate (GMP). Replacing the carbonyl group at C6 with an amino group will produce a single adenosine monophosphate (AMP). Aspartate provides the amino group and GTP (guanosine triphosphate) provides the required energy through the hydrolysis of a phosphate bond, to form adenylosuccinate (SAMP). This reaction is catalyzed by adenylosuccinate synthase. The final step is when adenylosuccinate lyase (ASL) catalyzes the removal of fumarate to yield AMP. The other pathway leads to the formation of guanosine monophosphate (GMP) when IMP is oxidized followed by an amino group insertion provided by the amide nitrogen of

glutamine. Inosine 5′-monophosphate dehydrogenase (IMPDH) using NAD^+ as the hydrogen acceptor generate
xanthosine monophosphate (XMP).[9] The last step is catalyzed by GMPS (GMP synthase) to create GMP.

After conversion of AMP to IMP, adenine can have two possible paths for nucleotide catabolism. In one pathway, not shown in Figure 7, 5'-nucleotidases or phosphatases dephosphorylate IMP to inosine. Afterward, inosine is hydrolyzed to hypoxanthine in the presence of inosine/guanine nucleosidase. Subsequently, hypoxanthine is converted to xanthine by XDH (xanthine dehydrogenase).[9] In the other pathway, IMP is converted to xanthine monophosphate (XMP) by inosine-5′-monophosphate dehydrogenase (IMPDH). XMP is then converted to xanthosine by 5'-nucleotidases.

Xanthine is derived from xanthosine by action of inosine/guanine nucleosidase.[7] The purine and pyrimidine salvage pathway uses less energy compared to the *de novo* pathway because it uses only one ATP, unlike *de novo* synthesis where seven nucleotides are used to produce AMP.[9] The salvage pathway regenerates the plant nucleotide pools by interconverting purine bases, nucleosides, and nucleotides, which are synthesized from plant cell metabolism and catabolism. Monophosphates of adenosine and guanosine can be converted back to the respective nucleobases by adenine and hypoxanthine/guanine phosphoribosyltransferases (APRTase and HGPRTase, respectively). PRPP can be used as the source of ribose phosphate. Recycling purine bases can also be achieved through catalysis by adenine or inosine/guanosine phosphorylases (Figure 7).[9]

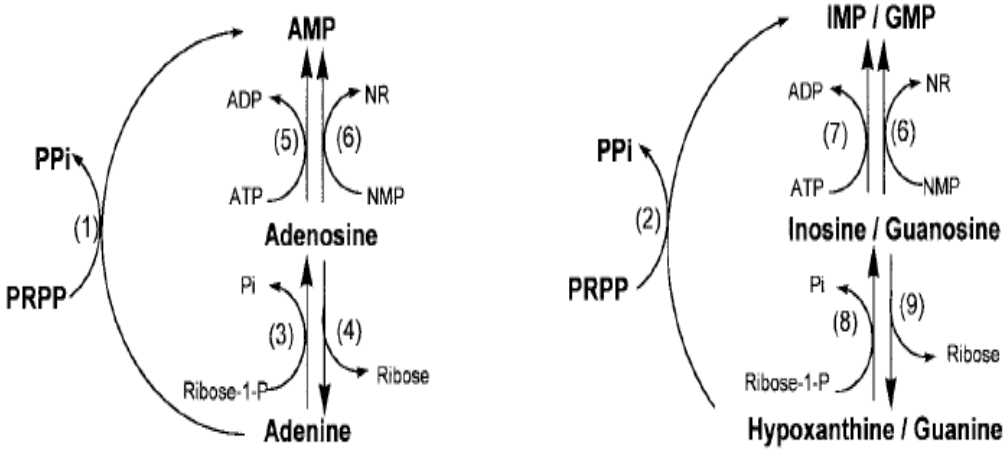


Figure 7: Purine salvage reactions.[9] The enzymes shown are: (1) adenine phosphoribosyltransferase, (2) hypoxanthine-guanine phoshoribosyltransferase, (3) adenosine phosphorylase, (4) adenosine nucleosidase, (5) adenosine kinase, (6) non-specific nucleoside phosphotransferase, (7) inosine-guanosine kinase, (8) inosine-guanosine phosphorylase, (9) inosine-guanosine nucleosidase (Adopted with permission from Stasolla, C., Katahira, R., Thorpe, T. A., & Ashihara, H. (2003). Purine and pyrimidine nucleotide metabolism in higher plants. *Journal of Plant Physiology*, 160, 1271-1295).

Pyrimidine Metabolism

The orotate pathway or the *de novo* pyrimidine pathway is characterized by the formation of UMP (uridine monophosphate) from CP (carbamoyl phosphate) (Figure 8).[10] The pathway is completed in six reactions. Carbamoyl phosphate (CP) synthetase, aspartate transcarbamoylase, and dihydroorotase are found in mammals and other eukaryotes as a single protein called CAD.[10]

Carbamoylaspartate is formed from the reaction of aspartate and carbamoyl phosphate after catalysis by aspartate transcarbamoylase. Formation of the pyrimidine ring requires in the next step the cyclization of carbamoylaspartate. That step is catalyzed by dihydroorotase (DHOase). The oxidation of dihydroorotate by dihydroorotate dehydrogenase (DHODH) forms orotate (OA). Next, the formation of orotidine 5'-monophosphate (OMP) occurs when orotate is attached to PRPP. OMP is subsequently decarboxylated to yield UMP. This first nucleotide, UMP, is then phosphorylated to form UTP and UDP. CTP is subsequently synthesized by transferring an amino group from glutamine to UTP via CTP synthetase. Formation of CTP is crucial to the success of the *de novo* pathway since it acts as a regulatory step.

The salvage pathways of nucleosides hydrolyze them to their corresponding nucleobases. Nucleotides can also be recovered from salvage pathways. Salvage pathways use nucleoside hydrolase in the formation of sugar and bases. Uridine kinase, cytidine kinase, deoxycytidine kinase, and thymidine kinase play a role in the formation of dTMP, CPM, dCMP, and UMP (Figure 9).[8]

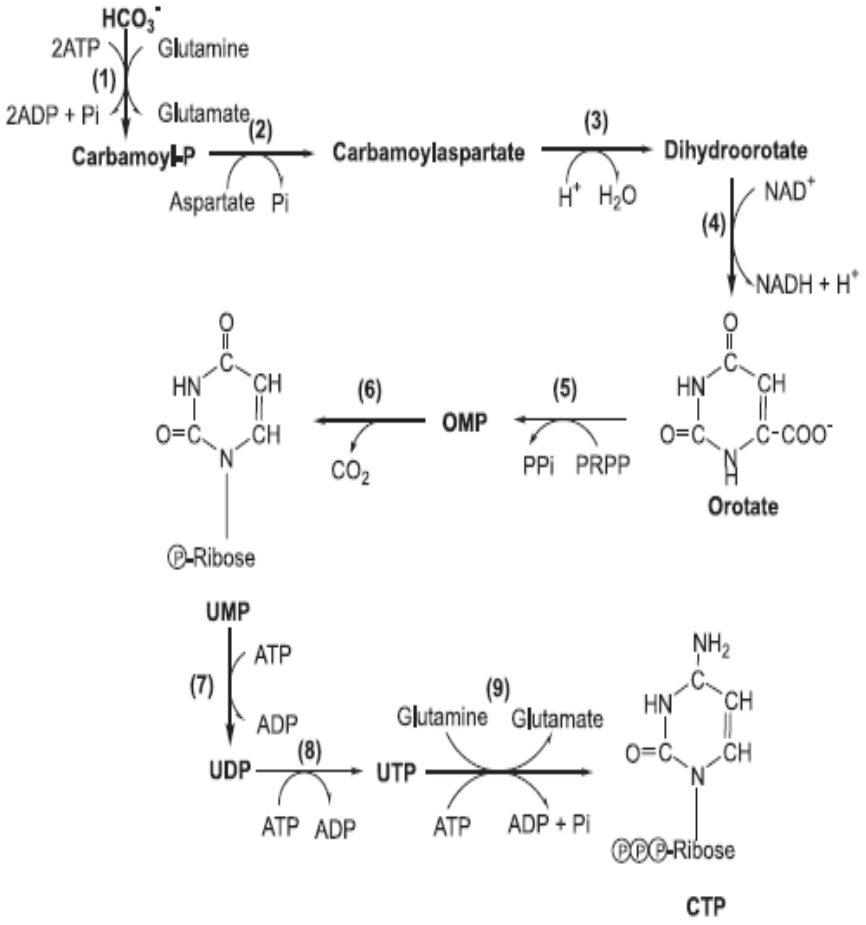


Figure 8: Schematic representation of the pyrimidine *de novo* pathway in plants.[9] The enzymes shown in Figure 8 are: 1) Carbamoyl phosphate synthetase, (2) aspartate transcarbamoylase, (3) dihydroorotase, (4) dihydroorotate dehydrogenase, (5)– (6) UMP synthase (orotate phosphoribosyltransferase plus orotidine-5′-phosphate decarboxylase), (7) UMP kinase, (8) nucleoside diphosphate kinase, (9) CTP synthetase. (Adopted with permission from Stasolla, C., Katahira, R., Thorpe, T. A., & Ashihara, H. (2003). Purine and pyrimidine nucleotide metabolism in higher plants. *Journal of Plant Physiology*, 160, 1271-1295).

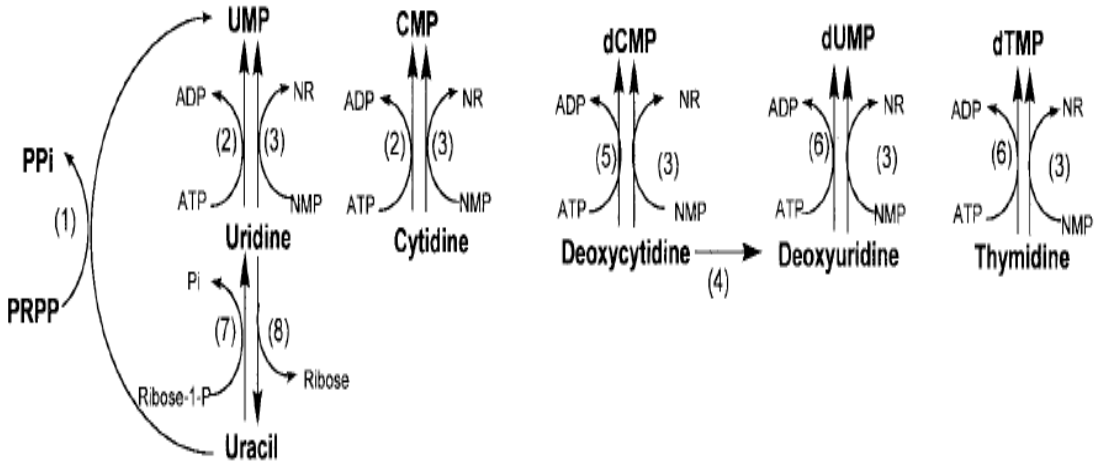


Figure 9: Pyrimidine salvage reactions in plants.[9] The enzymes shown are: (1) uracil phosphoribosyltransferase, (2) uridine/cytidine kinase, (3) non-specific nucleoside phosphotransferase, (4) deoxycytidine deaminase, (5) deoxycytidine kinase, (6) thymidine kinase, (7) uridine phosphorylase, (8) uridine nucleosidase. (Adopted with permission from Stasolla, C., Katahira, R., Thorpe, T. A., & Ashihara, H. (2003). Purine and pyrimidine nucleotide metabolism in higher plants. *Journal of Plant Physiology*, 160, 1271-1295).

Pyrimidine nucleotides make up crucial components responsible for the synthesis of deoxyribonucleic acid and ribonucleic acid.[7] The nucleic acids are in turn used for storing and retrieving information in the cell.

The nucleotides also facilitate the metabolism of several cell components such as sugar interconversion, and the conversion of cellular polysaccharides to glycoproteins and phospholipids.

Nucleoside Hydrolases

Nucleoside hydrolases are enzymes that catalyze the glycosidic bond hydrolysis reaction involving a nitrogenous base and pentose sugar (N-glycosidic bond of β-ribonucleosides) in certain nucleosides to form a ribose and the corresponding base (Figure 10).[6] The mechanism consists of protonation of N7 of the purine base, a change in the conformation of the ribose, and a change in the hybridization of C1' during the reaction. Nucleoside hydrolases are present in fish,[5] yeast,[11] mesozoa,[12] insects,[13] protozoa,[14,15] bacteria,[16,17] and plants, but are absent in mammals.[10] These enzymes play a vital role in the nucleoside salvage pathways commonly used by protozoan parasites, which cannot synthesize the purines *de novo.* The N- glycosidic bonds found in the nucleosides and nucleotides has to undergo hydrolysis since this process is vital for the regular metabolic function of the cell.[17]

There are nonspecific hydrolases that catalyze the hydrolysis of both purines and pyrimidines. There are also nucleoside hydrolases specific for purine nucleosides. Another group of nucleoside hydrolases consists of six-oxo purine specific inosine-guanosine affiliated hydrolases.[18]

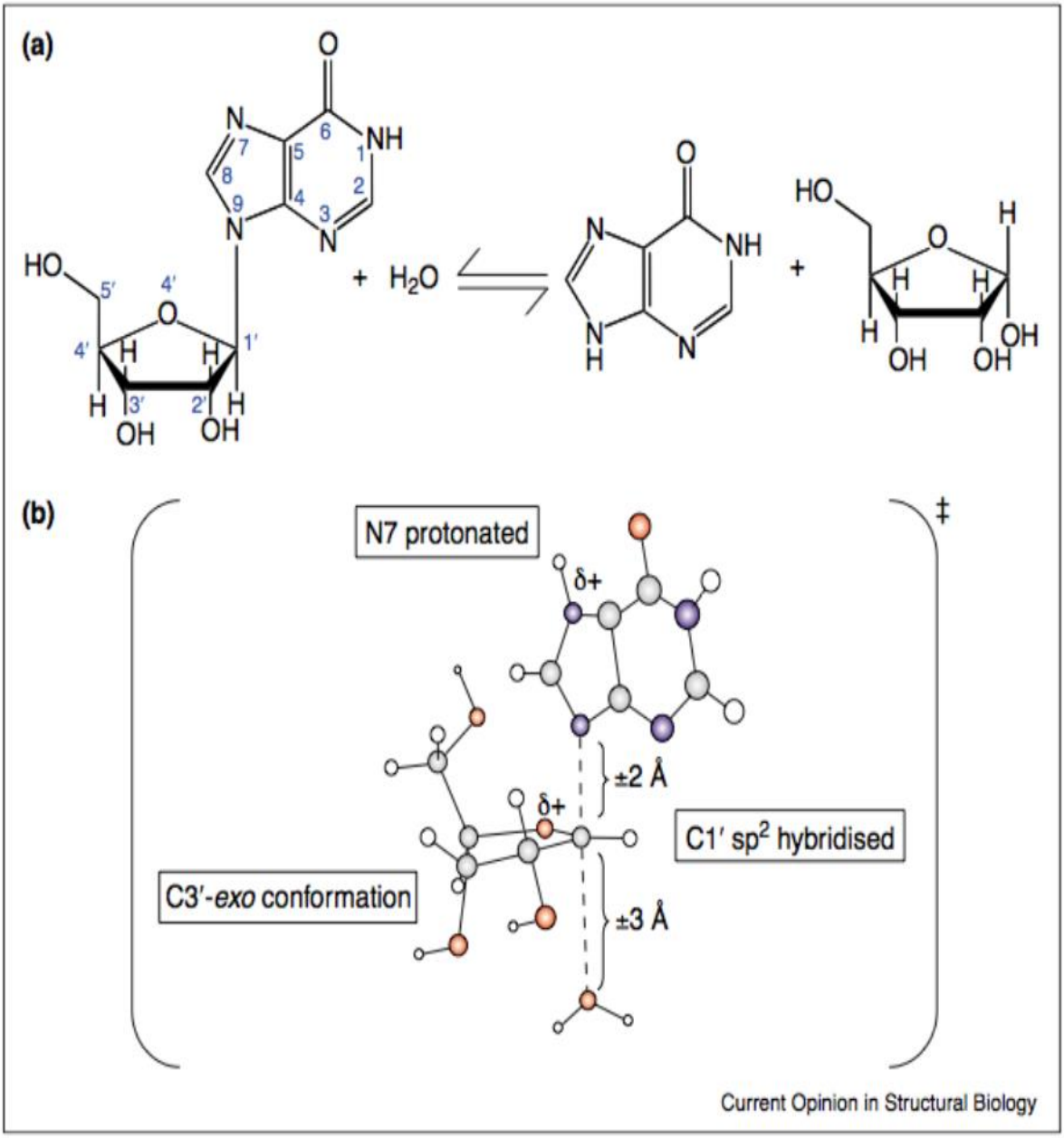


Figure 10: Reaction mechanism of hydrolases.[6] (a) Reaction mechanism involved in the hydrolysis of a ribonucleoside (inosine), a reaction that is catalyzed by nucleoside hydrolase; (b) Suggested ion-like transition state for nucleoside hydrolase catalyzed Cf (*Crithidia fasciculata*) reaction with major vital chemical and structural features of the transition. Taken from reference 6 with permission of Elsevier.

Nucleoside hydrolases have previously been isolated and extracted from plants such as coffee leaves, barley leaves, wheat germ, spinach beet, tea leaves, tomato roots and leaves.[7] The nucleoside hydrolases obtained from various sources have been proven to have different molecular weights, pH optima, and subunit structures.[18]

Uridine Nucleosidase

Uridine nucleosidase (EC 3.2.2.3) is an enzyme that mediates the reaction involving uridine and water leading to formation of D-ribose and uracil (Figure 11).[19] The enzyme is a glycosylase, which hydrolyses N–glycosyl compounds. It participates in pyrimidine catabolism where it salvages pyrimidines.

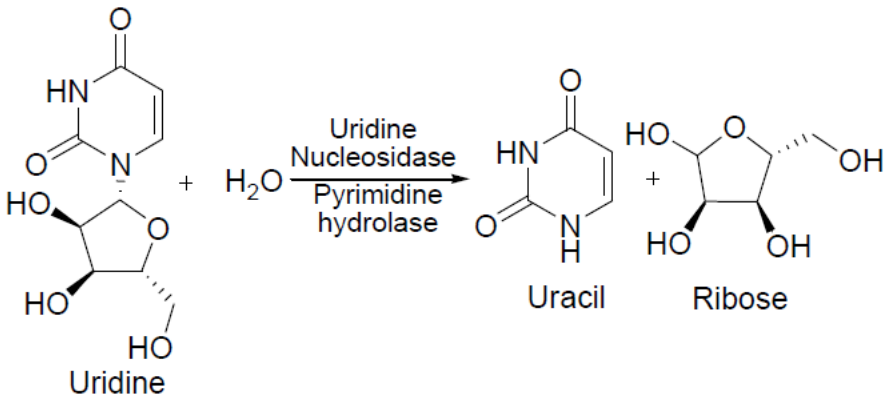


Figure 11: Hydrolysis of uridine by uridine nucleosidase to uracil and ribose catalyzed by uridine nucleosidase.

Uridine nucleosidase has been purified from commercial baker's yeast.[19] The enzyme is also found in the leaves, stems, flowers, pollen cells, and meristem of the root tips in plants such as maize.[2] The molecular weight of uridine nucleosidase purified from baker's yeast as determined by gel filtration was found to be 32,500 Da.[19] Uridine

nucleosidase has been determined to be active against uridine and 5-methyluridine. The K_m values for uridine and 5-methyluridine were determined to be 0.86 μM and 1.66 μM, respectively.[19] It is inactive against all other purine and pyrimidine nucleosides.[20] The optimum pH at which to store the enzyme was determined to be 7.0, at a temperature of 4°C in a 100 mM phosphate medium.

Adenosine Nucleosidase

Adenosine nucleosidase (EC 3.2.2.7) mediates the reaction of adenosine and water forming ribose and adenine (Figure 12).[21] The reaction is similar to that performed by uridine nucleosidase. The hydrolysis reaction shown below is not reversible.

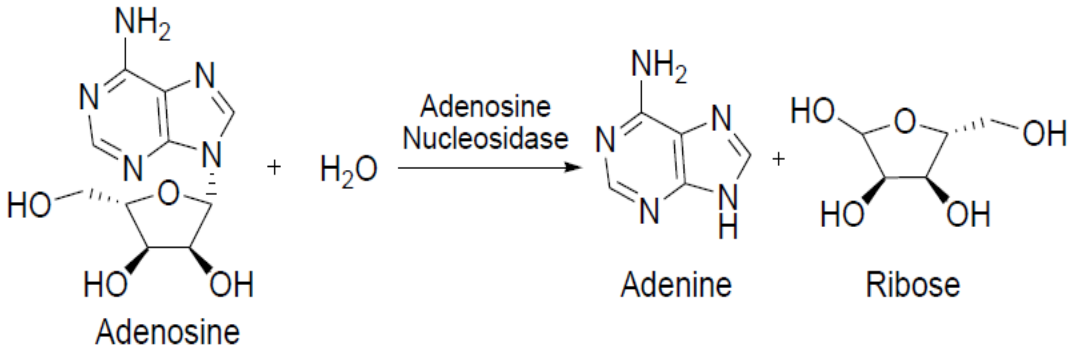


Figure 12: Reaction catalyzed by adenosine nucleosidase between adenosine and water to yield adenine and ribose.[6]

Adenosine nucleosidase is a member of the glycosylase enzymatic group. The enzyme has an optimum pH for hydrolysis of 4.5 and phosphate ions do not inhibit or activate its functions as is the case with inosine nucleosidase.[5] This enzyme is fundamental in the purine salvage pathway in plants. Research has suggested that adenosine nucleosidase controls the levels of cytokinins, which play an important role in

the growth of plants.[10] The enzyme can be used to measure the ability of the plant to recycle adenine.[10] The enzyme was first purified from soybean leaves by Miller and Evans.[22] and since then success has been reported concerning the purification of the enzyme from other plant sources. These plant sources include barley leaves,[5] spinach beet leaves,[23] tea leaves ,[24] wheat germ,[16] yellow lupin seeds,[25] jerusalem artichoke [26] and Alaska pea seeds.[7]

Adenosine nucleosidase partially purified from spinach beet leaves was found to have an optimum pH of 4.5 and a K_m value for adenosine of 11μM.[23] The enzyme demonstrated great specificity for adenosine and did not use other nucleosides as substrates. Some activity was observed regarding adenosine N-oxide, which is produced by oxidizing adenosine to form a nitrogen monoxide bond.[23]

Adenosine nucleosidase was also purified from barley leaves and was determined to have a molecular weight of 66,000 Daltons by gel filtration chromatography.[21] Further analysis by SDS-PAGE showed that the enzyme, was a dimer with a subunit molecular weight of 33,000 Daltons. The purified enzyme had optimum pH 4.7 in citrate and 5.4 in ethanesulphonic acid buffers.[16] The K_m was found to range between 0.8 to 2.3 μM.[16] The value depended on the temperature and buffer system used.[16] The substrates used by the enzyme were determined to be adenosine N-oxide, 2'-deoxyadenosine and purine riboside in addition to adenosine. Adenine and cytokinins however, were determined to be inhibitors of the enzyme.[21]

Purification of adenosine nucleosidase from tea leaves resulted in three types of the enzyme (I, II, and III). Each enzyme was determined to have a molecular weight of approximately 68,000 Daltons.[27] Enzymes I and II had an optimum pH of 4, while

adenosine nucleosidase III had an optimum pH of 4.5. All three enzymes exhibited similar substrate specificity. Naturally occurring nucleosides hydrolyzed by the three adenosine nucleosidase enzymes included adenosine and 2'-deoxyadenosine with a higher rate of hydrolysis being exhibited by 2'-deoxyadenosine.[27]

Purification of the enzyme from wheat germ cells resulted in an enzyme with a molecular weight of 59,000+/- 3000 Daltons.[18] The optimum pH was 4.7 and the K_m was 2.3 μM for cytokinin nucleoside and 1.43 μM for adenosine. Adenosine nucleosidase has also been purified from Jerusalem artichoke shoots.[26] Besides, adenosine, inosine-guanosine nucleosidase activities were also observed suggesting the presence of similar properties being exhibited by different enzymes. These properties include high stability, optimal pH from 5 to 7 and high affinity for substrates. The enzymes were distinguished by their substrate specificity. Adenosine had a K_m value of 17 μM, while guanosine and inosine had values of 8.5 μM and 2.5 μM respectively.[28]

Adenosine nucleosidase has been purified from tomato leaves and roots. In tomato roots, R1 and R2 adenosine nucleosidase enzymes were present.[28] Lf, another form of adenosine nucleosidase was found in tomato leaves. The optimum pH were found to be 5.0, 6.0, and 6.0, for R1, R2 and Lf, respectively. Using adenosine as substrate, the K_m values were determined to be 25 μM, 9 μM, and 6 μM for R1, R2, and Lf in that order.[28] R2 was determined to be the major component in the root and its molecular weight was determined to be 68,000 Daltons. Great similarities were observed between R2 and Lf. Cytokinin ribosides were found to be a competitive inhibitor for all three enzymes.[28]

Abusamhadneh *et al.* purified adenosine nucleosidase from yellow lupin seeds.[25] The purification achieved a percent yield of 2.3% and a native molecular weight of 177,000 Daltons was determined. The enzyme was determined to have an optimum pH of 7.5 and a K_m of 4.7 μM for adenosine. Significant activity for adenosine, 2'-deoxyadenosine, guanosine, cytidine, inosine, and thymine was observed.[25]

Guanosine Nucleosidase

Guanosine nucleosidase has been purified from germinated barley (Figure 13).[24] The purified enzyme has a molecular weight of between 29,000 to 33,000 Daltons with an activation energy of 12.1 kcal per mole.[24] The optimum pH of the enzyme was 5.5 at an optimal temperature of 40°C.

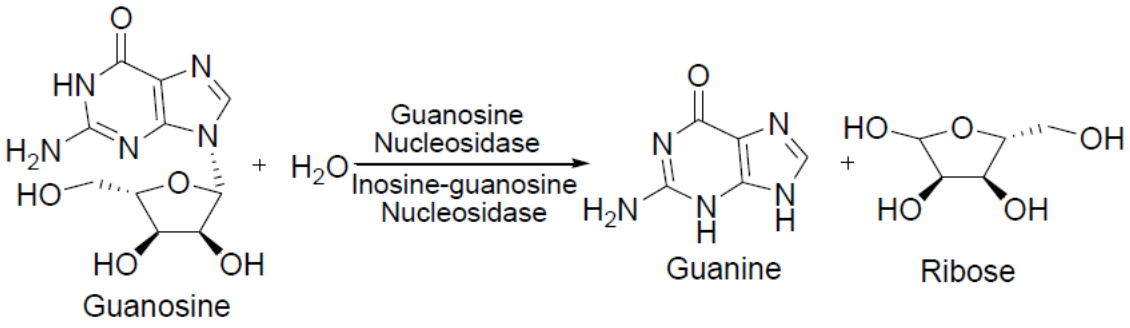


Figure 13: Hydrolysis of guanosine by guanosine or inosine/guanosine nucleosidase to guanine and ribose.[24]

Inosine Nucleosidase

Inosine nucleosidase (EC 3.2.2.2) mediates the reaction leading to the formation of D-ribose and hypoxanthine from inosine and water as shown below.[29] There are studies which have reported the purification of inosine nucleosidase from plants.[30,31] The

optimal pH of the enzyme was 8.[16] The presence of nucleotides containing phosphate compounds can inhibit enzymatic activity of inosine nucleosidase.[31] When inosine nucleosidase was purified from yellow lupin seeds its molecular weight was 62,000 Da as determined by gel filtration, while it has a K_m value of 65 μM for inosine.[31]

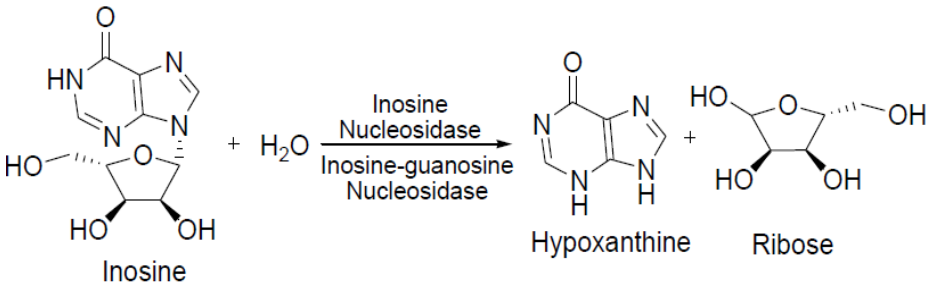


Figure 14: Reaction between inosine and water catalyzed by inosine nucleosidase to yield hypoxanthine and ribose.[31]

Cytidine Nucleosidase

Cytidine nucleosidase results in the formation of cytosine from cytidine (Figure 15).[32] Ribose is another product of the reaction. Cytidine nucleosidase has been purified from *Arabidopsis thaliana*.[33] From this purification, the enzyme had a molecular weight of 63,000 Daltons and a K_m value for cytidine of 150 μM.[33]

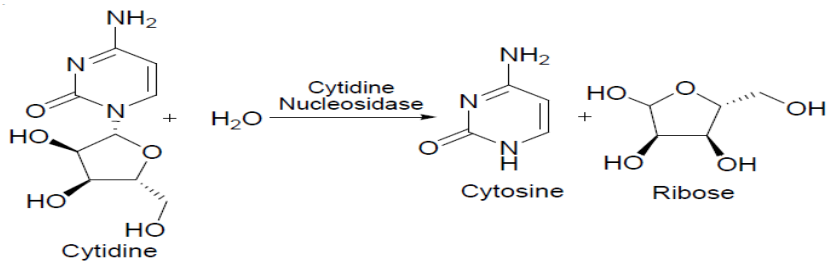


Figure 15: Cytidine hydrolysis by cytidine nucleosidase to cytosine and ribose.[32]

Nucleoside Deaminases

Adenosine Deaminases (EC 3.5.4.4)

This is an enzyme of the purine salvage pathway. The activity of the enzyme results in the synthesis of inosine or 2'-deoxyinosine from adenosine or 2'-deoxyadenosine (Figure 16).[34] This enzyme has found extensive application in synthesizing nucleotides especially in mammals both in salvage or *de novo* pathways.[34] The enzyme is used in biosynthesis of DNA and RNA along with signaling molecules.[34] The enzyme has also found application in the synthesis of hypoxanthine and inosine from adenine and adenosine respectively.[25] Studies have reported purifying the enzyme from animals such as cattle, humans, pigs, and camel.[35]

Human adenosine deaminase has a molecular weight of 38,200 Daltons based on its Stokes radius and an optimum pH range of 7.0-8.0.[34] When purified, the K_m of the enzyme was found to be 52 μM for adenosine.[34] Very little information is available concerning the presence of the enzyme in plants. Some studies have reported its occurrence in barley rootlets and malt diastase.[37] The responsible pathway for the reaction has been studied in alfalfa seedlings and cell cultures, and soybean.[37] However, the deaminase protein has proven difficult to purify.[36]

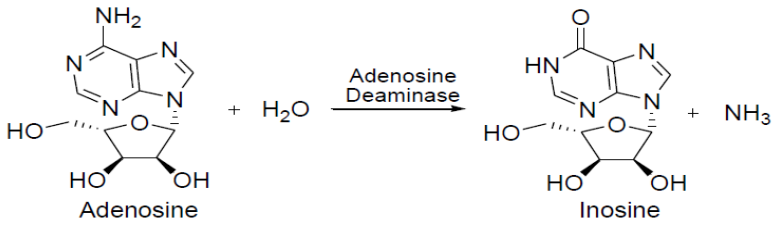


Figure 16: Adenosine nucleosidase mediates conversion of adenosine to inosine and releases NH_3.

Cytidine Deaminases (EC 3.5.4.1)

This is a pyrimidine salvage pathway enzyme. This enzyme catalyzes the synthesis of uracil from cytosine (Figure 17).[25] However, the enzyme exists exclusively in fungi and bacteria and has yet to be purified from higher organisms.[35] The enzyme has been purified from *E. coli*.[35] This protein can mediate deamination reactions in conjunction with adenine deaminase.[36] Cytidine deaminase has a molecular weight of 48,700 Da after gel filtration when purified from human placenta.[35] The enzyme has a K_m value of 1.8 x 10^{-4} M for cytidine and an optimum pH of 7.5.[5]

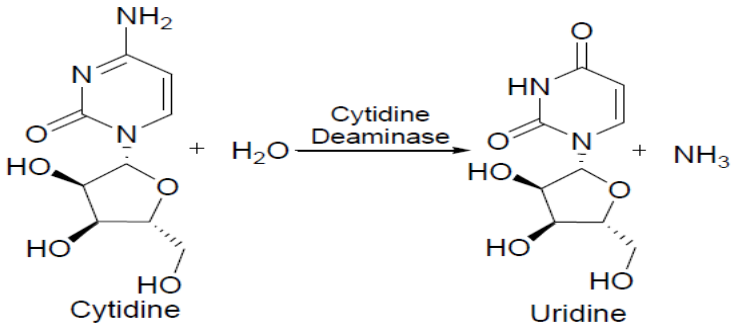


Figure 17: Cytidine deaminase converts cytidine to uridine.

Guanosine Deaminases (EC 3.5.4.3)

Negishi *et al*. were partially able to purify guanosine deamin ase from tea leaves.[37] The guanosine deaminase was determined to catalyze the deamination reaction of 2'-deoxyguanosine. Heavy metals were observed to significantly inhibit the activity of guanosine deaminase. The enzyme was an unstable enzyme and had an optimum of pH 7.5. The enzyme had a molecular weight of 18,000 Daltons determined by gel filtration. The K_m value was 9.5 µM.[37] Guanosine deaminase in tea leaves was observed not only to

be involved in the catabolic reaction but also in the synthesis of caffeine utilizing guanosine nucleotides.

Current Research

In the current study, the presence or absence of nucleoside-hydrolyzing and -deaminase enzymes in the seeds of okra, organic okra, pea (Alaska Wilt Resistant), yellow lupin, peas (Cascadia Sugar Snap), spinach, cantaloupe, and soybean were determined. Product nucleobases were identified and quantified by using high performance liquid chromatography (HPLC). Purine nucleosides adenosine, purine riboside, guanosine, 2′-deoxyadenosine and inosine were tested as substrates whereas, uridine, thymidine and cytidine were used to measure activities of pyrimidines metabolizing enzymes.

CHAPTER II

MATERIALS AND METHODS

Equipment and Instrumentation

A Dionex Ultimate 3000 High Performance Liquid Chromatography (HPLC) system was used to determine enzymatic activities. The components of the HPLC system were a thermostatted column holder, an autosampler, a quaternary analytic pump and a variable wavelength UV detector. A Hitachi UV-VIS 2900 spectrophotometer was used to determine the protein concentration.

Materials and Reagents

Seeds of okra (Clemson Spineless #80), organic okra, pea (Alaska Wilt Resistant), peas (Cascadia Sugar Snap), spinach (Bloomsdale Long Standing), cantaloupe (Hale's Best Jumbo), and soybean were purchased from Ferry-Morse Seed Company. Yellow lupin (*lupinus luteus*) seeds were obtained from B&T World Seeds Sarl, Paguiguan, 34210 Aigues Vives, France. Nucleosides such as adenosine, purine riboside, inosine, uridine, thymidine, cytidine, 2'-deoxyadenosine, guanosine, and their corresponding bases, dithiothreitol (DTT), protease inhibitor cocktail for plants, and protamine sulfate salts from salmon (Grade X) were obtained from Sigma Aldrich. Tris base (Fisher BioReagents) was used to prepare all stock buffers solutions. To determine protein concentration, a protein assay dye reagent concentrate kit and protein standard II were obtained from Bio-Rad. Ammonium phosphate was purchased from J.T. Baker Chemical Co. All chemicals used in this study, unless otherwise mentioned, were of reagent grade.

Methods

Preparation of Crude Extract

All seeds were disinfected by soaking the seeds (2 g) in bleach for 5 min followed by extensive rinsing with tap water. Using a Waring commercial blender, seeds were homogenized at 4 °C in 10 mL of 10 mM Tris buffer (pH 7.2) containing DTT (2 mg), protease inhibitor cocktail (20 μL) and protamine sulfate (20 mg). For every 1 g of seed biomass, 1 mL of 10 mM Tris buffer pH 7.2 was used for homogenization. The supernatant was collected after centrifuging the homogenate at 20,000*x*g for 20 min at 4 °C.

Enzymatic Activity Measurement

Individual nucleosides (1 mM) were dissolved in 10 mM Tris buffer (pH 7.2) to prepare the reaction mixture, except for guanosine which was 500 μM. To the reaction mixture 100 μL of the crude extract was added and was incubated at room temperature. Small aliquots of the reaction mixture solution were withdrawn at specific time intervals for further analysis.

Using an HPLC with a Kinetex 5 μm, EVO C18 reverse phase (150 × 4.6 mm) column (30 °C) eluted with 98 % ammonium phosphate (10 mM, pH 5.4) and 2 % methanol, the nucleosides and bases were separated. The area of each peak at a wavelength of 254 nm was used to determine the amounts of nucleoside and base. Injection volume was set at 10 μL and the flow rate was maintained at 0.6 mL/min. The samples were incubated at 32 °C on the autosampler. The enzymatic activity was determined based on the disappearance of the starting nucleoside. A series of aliquots

were withdrawn from the reaction mixture and injected into the HPLC. The peak area of the nucleoside was used to calculate the amount of nucleoside present. A plot of nucleoside amount vs reaction time was prepared and a linear regression of the data determined.

The activity of the enzymes were determined from the slope of the line. By dividing the reaction velocity by the protein concentration, the specific activity of the enzyme was determined.

Measurement of Protein Concentration

The Bio-Rad protein assay kit was used to determine protein concentrations. A total volume of 800 μL of water and the enzyme solution was mixed with 200 μL of the Bio-Rad dye. The sample absorbance at 595 nm was determined. A standard curve was generated using the Bio-Rad kit's standard bovine serum albumin (BSA, 1.45 mg/mL). The unknown protein concentrations were calculated using the BSA standard curve (Figure 18).

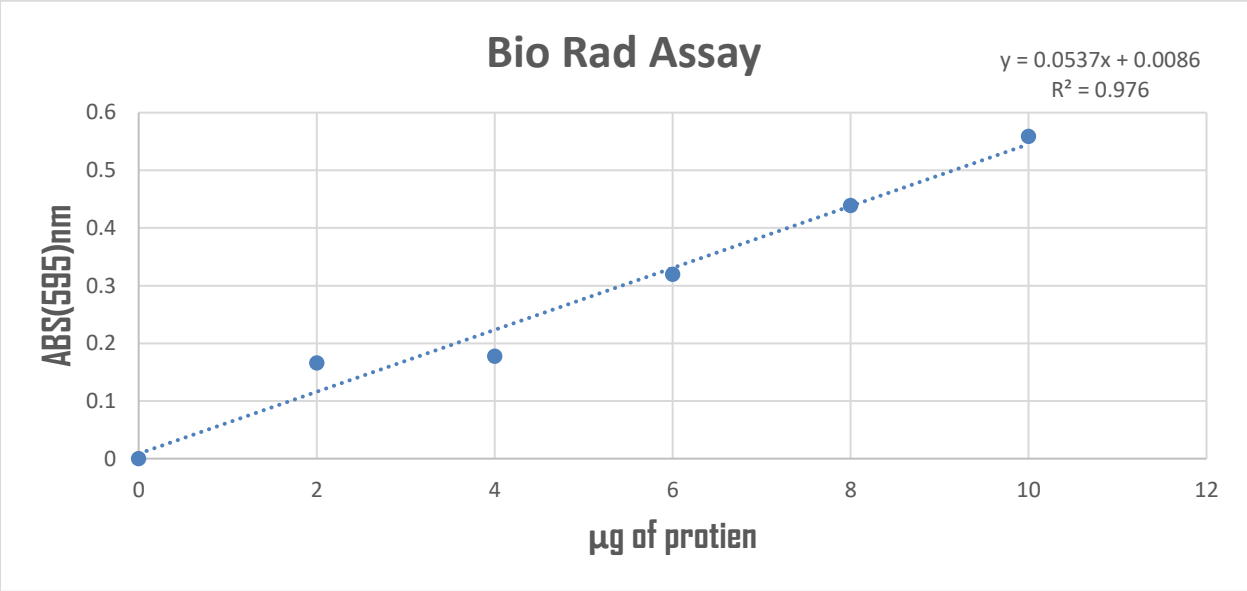


Figure 18: Bovine serum albumin (BSA) standard curve generated after plotting known amount of BSA against A_{595nm}.

CHAPTER III

RESULTS AND DISCUSSION

Nucleic acid components such as purines and pyrimidines are an integral part of biological systems. Besides being present in DNA and RNA, their presence and roles are conspicuous in cell signaling, immune response and enzyme activities.[8] Maintenance of the adequate ratio of nucleotides and nucleosides inside a cell is essential. This balance is attained by release of nucleosides and nucleobases following the controlled breakdown of nucleotides.[6] Nucleotide metabolism involves *de novo* synthesis, nucleotide degradation, and nucleoside salvage pathways. Each of these pathways is specific for purines and pyrimidines.[4] Through these pathways, facilitated by different enzymes, the nucleobases are recycled to maintain their pool and balance nucleotides and nucleosides ratio. One of the major steps in the recycling of nucleobases is through the nucleoside salvage pathway.[8] During an intermediate step of this pathway, the enzyme, nucleoside hydrolase, catalyzes the hydrolysis of the N-glycosidic bond of nucleosides to recycle nucleobases. Adenosine nucleosidase is a key enzyme in purine metabolism. Adenosine nucleosidase has been purified from many plants and was shown to possess different characteristics such as substrate specificity, optimum pH, and K_m values. Inosine nucleosidase has been purified from yellow lupin seeds.[25] Uridine nucleosidase and 2'-deoxyadenosine nucleosidase are also discussed in the report. Uridine nucleosidase is a pyrimidine metabolism enzyme and is located in the cytoplasm. The enzyme has been purified from baker's yeast.[3] 2'-Deoxyadenosine nucleosidase on the other hand is a purine metabolism enzyme that is used in the degradation process and removes the deoxyribose from the nucleoside (Figure 19).

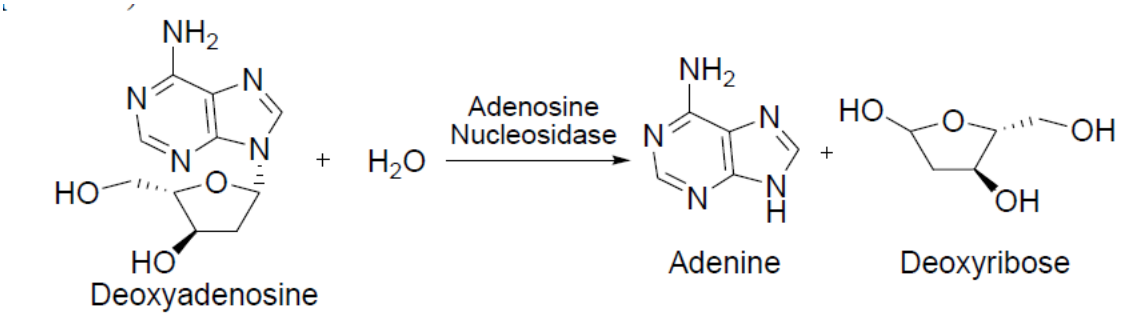


Figure 19: 2'-Deoxyadenosine nucleosidase catalyzed hydrolysis of 2'-deoxyadenosine into adenine and 2'-deoxyribose.

There are many other enzymes that take an active role in nucleobase recycling. Cytidine deaminase catalyzes the hydrolytic deamination of cytidine to uridine, while adenosine deaminase converts adenosine to inosine.[31] In addition to the above-mentioned enzymes, in this study, many other enzyme activities in nucleoside metabolism were investigated such as thymidine hydrolysis, purine riboside hydrolysis, and guanosine deaminase to produce xanthosine followed by N-glycosidic hydrolysis to produce xanthine (Figure 20).

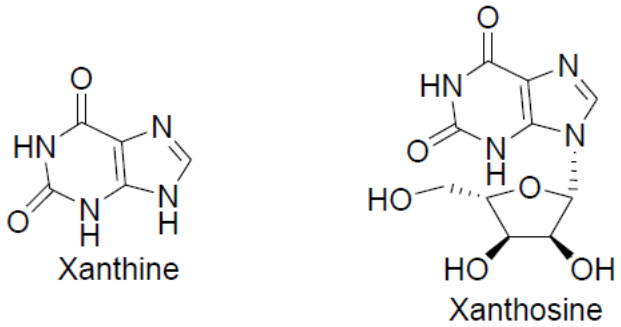


Figure 20: Structure of xanthine and xanthosine.

Crude seed extracts of okra, organic okra, Alaska pea, yellow lupin, sugar snap peas, spinach, cantaloupe, and soybean seeds were evaluated to determine the presence or absence of various nucleosidases (hydrolases) and deaminases. Using HPLC, reactions were monitored and the starting product and nucleobases were identified based on their retention times (Table 1).

In this study, two groups of enzymes were evaluated. Nucleosidases remove the ribose hydrolytically, and deaminases remove an amino group from the base contributing to the interconversion of one nucleoside into another. The disappearance of the parent nucleosides and appearance of the nucleobases was used to measure the nucleoside degradation activity. By dividing the amount of nucleoside reduction by the reaction time, the nucleosidase and deaminase (if applicable) activities were calculated. The reaction velocity was divided by the protein concentration to determine the specific activity. For selected substrates, the specific activity includes both hydrolase and deaminase activities.

The primary objective of this study was to determine the levels of activity of nucleoside metabolism enzymes among various seeds. Some of the seeds were nonorganic seeds harvested from plants that had been treated with various chemicals to promote better growth, and protect them from infections and pests. Others were organic seeds from plants that had not been treated with any extraneous chemicals. A third variety of seeds that were not tested as part of this project are the genetically modified seeds, where the genome of the plant has been modified by insertion of a foreign DNA to express certain traits. This study will help to improve the understanding of biochemical processes critical

Table 1: Summary of retention times of nucleosides and nucleobase. Retention times of nucleosides and bases were determined on a Kinetex C18 (150 x 4.6 mm) HPLC column at 254 nm using a 98 % 10 mM ammonium phosphate pH 5.4, 2 % methanol mobile phase.

Nucleosides	Retention Time (min)	Nucleobase	Retention Time (min)
uridine	1.467 ± 0.40	uracil	0.992 ± 0.2
cytidine	1.200 ± 0.4	cytosine	0.883 ± 0.4
adenosine	7.080 ± 0.8	adenine	1.830 ± 0.31
guanosine	3.004 ± 0.21	guanine	1.243 ± 0.51
inosine	2.669 ± 0.19	hypoxanthine	2.160 ± 0.2
thymidine	4.797 ± 0.02	thymine	1.680 ± 0.30
2'-deoxyadenosine	13.700 ± 0.32	2'-deoxyinosine	5.301 ± 0.23
xanthosine	2.752 ± 0.35	xanthine	1.250 ± 0.31
purine riboside	5.620 ± 0.22	purine	2.10 ± 0.11

for plant growth, for the development of the plant, and to harness important protection mechanisms that could be present within the plants.

Enzyme Activities in Okra (Clemson Spineless #80), Organic Okra (Clemson Spineless #80) and Yellow Lupin

Okra (Clemson Spineless #80) and organic okra (Clemson Spineless #80) are particularly common in southern America, Turkey, Africa, Spain, India, and Brazil. Yellow lupin is popular in southern Italy and the island of Corsica, and Morocco, Algeria and Tunisia.[38] Yellow lupin, organic okra and okra seeds extracts have demonstrated that several enzymes express their metabolic activities in them. The levels of activity of nucleosides metabolism enzymes were determined in non-germinated seeds.

In this study, uridine, adenosine, cytidine, inosine, guanosine, purine riboside, thymidine, and 2'-deoxyadenosine were used as a substrate to test nucleosidase activity in organic okra, okra and yellow lupin ungerminated seeds.

When uridine was used as a substrate, significant disappearance of uridine commenced three hours after reaction initiation for okra, while it took a substantially longer time in organic okra and yellow lupin. No additional degradation products were observed during the reaction (Figure 21). Activity levels were almost the same in okra and organic okra while it was significantly lower in yellow lupin. In okra, reaction velocity increases after one hour (Figure 22). The calculated specific activities for okra, organic okra and yellow lupin reactions containing 1 mM uridine were 0.023 μmol/(min mg), 0.030 μmol/(min mg) and 0.0034 μmol/(min mg), respectively. These values of the specific activity were similar to those reported by Alshaiban and Ustynov for okra,

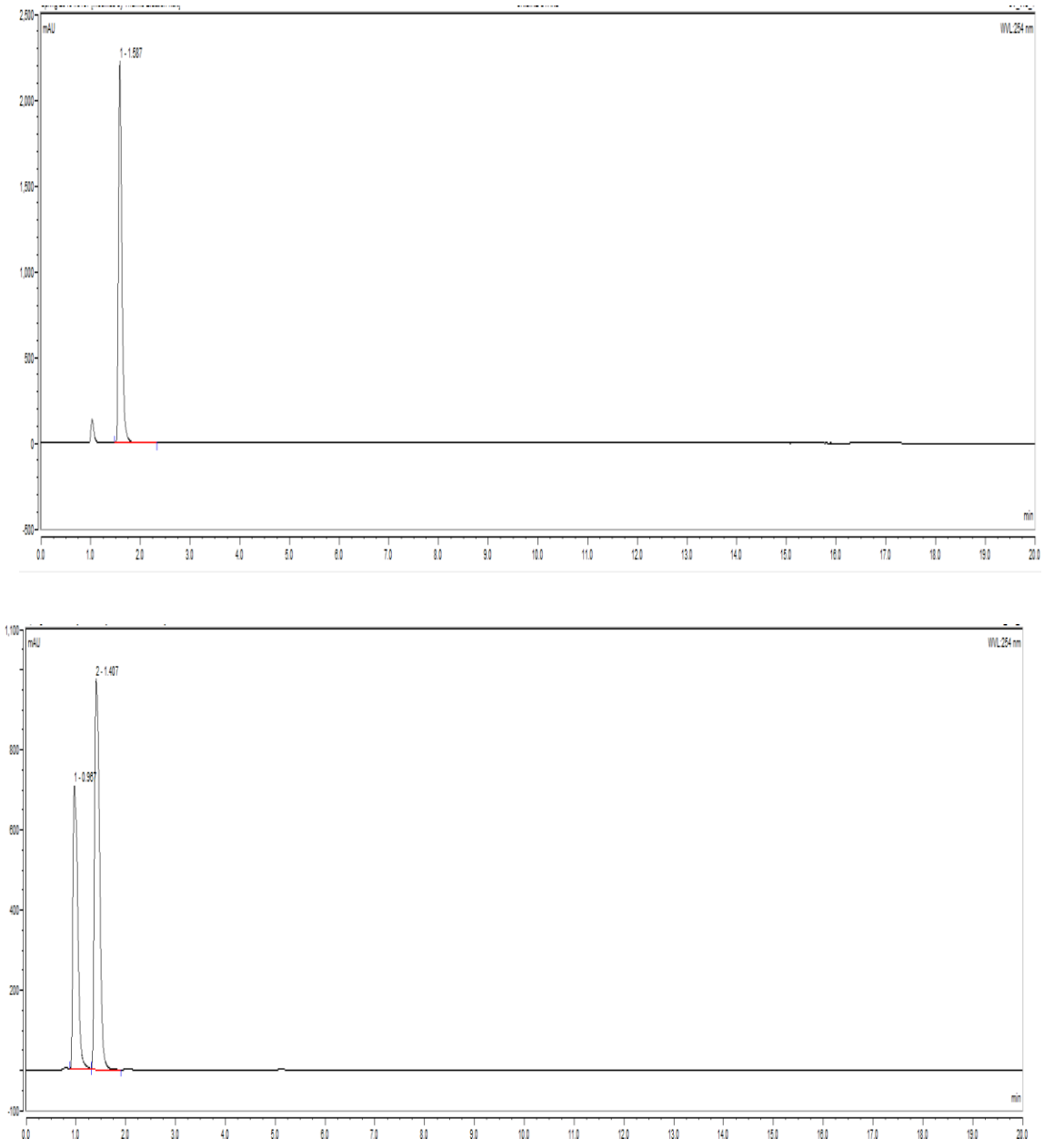
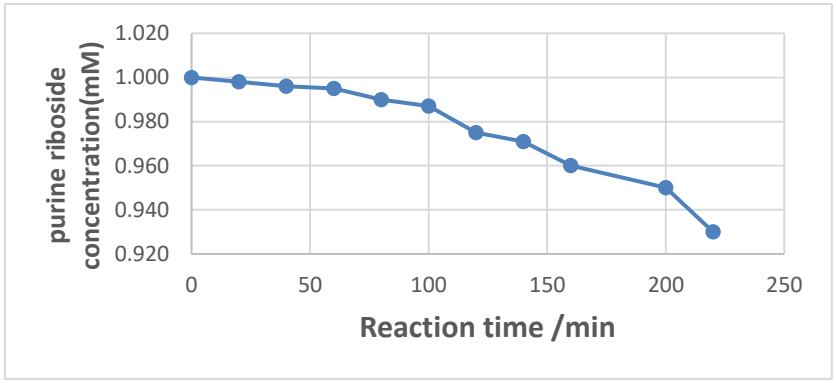


Figure 21: Uridine to uracil conversion catalyzed by uridine nucleosidase in okra seed extract. **(A)** Reaction mixture before okra extract 100 µL was added to 900 µL of 1 mM uridine. It shows a uridine peak at 1.587 minutes. **(B)** Reaction mixture after okra seed extract addition. Uridine appeared at 1.407 minutes. A second peak, with the retention time expected for uracil at 0.967 minutes appeared three hours later.

A



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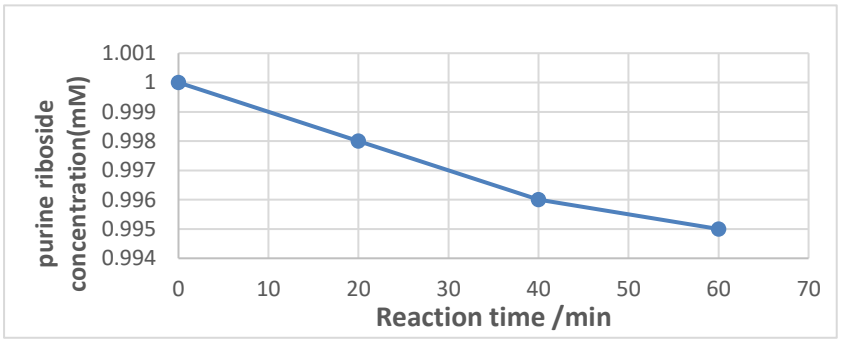

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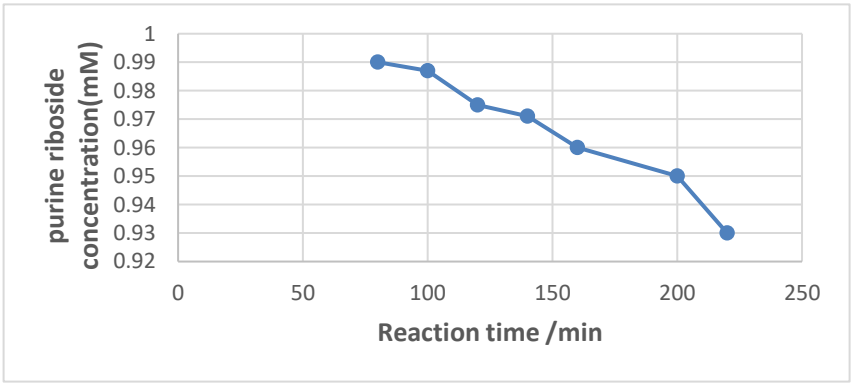


Figure 22: Uridine reaction progress with okra seed extract. **(A)** Disappearance of uridine in Tris buffer with a crude protein extract from okra seed. **(B)** Phase one of the reaction lasted approximately one hour with cleavage of the substrate uridine taking place. **(C)** Phase two of the reaction is characterized by rapid reaction velocity increase in the following hour.

organic okra and yellow lupin 5-9 day old seedlings of 0.017 μmol/(min mg), 0.025 μmol/(min mg) and 0.031 μmol/(min mg), respectively.[30,39]

Within three hours of initiation of the reaction, inosine had been converted to hypoxanthine in okra, organic okra, and yellow lupin with specific activities at a similar level (Figure 23). The specific activity for inosine hydrolyzing enzymes in okra, organic okra and yellow lupin were 0.0088 μmol/(min mg), 0.0041 μmol/(min mg),and 0.0059 μmol/(min mg), respectively, significantly lower than those for uridine nucleosidase. These results were similar to the activities of inosine nucleosidase from okra and organic okra that have previously been reported by Alshaiban for okra and organic okra.[39] The specific activities were determined to be 0.0096 μmol/(min mg) in okra and 0.0039 μmol/(min mg) in organic okra 5-7 day old seedlings.[39]

Guanosine as a substrate is capable of undergoing both hydrolysis and deamination reaction. In the hydrolysis reaction, guanine and ribose are produced, while in the deamination reaction xanthosine is produced. When guanosine was used as a substrate, it was converted to guanine (Figure 24). After analysis, the specific activity of guanosine hydrolyzing enzymes in okra, organic okra and yellow lupin were determined as 0.0064 μmol/(min mg), 0.0045 μmol/(min mg), and 0.030 μmol/(min mg), respectively. There was no evidence of a peak representing xanthosine or xanthine produced by the action of guanosine or guanine deaminase.

The three plants, okra, organic okra, and yellow lupin demonstrated activity for cytidine deaminase. When cytidine was degraded in okra, organic okra, and yellow lupin, it revealed activities for cytidine deaminase, uridine nucleosidase, and cytidine nucleosidase. A baseline resolution was achieved between cytidine, uridine, uracil, and

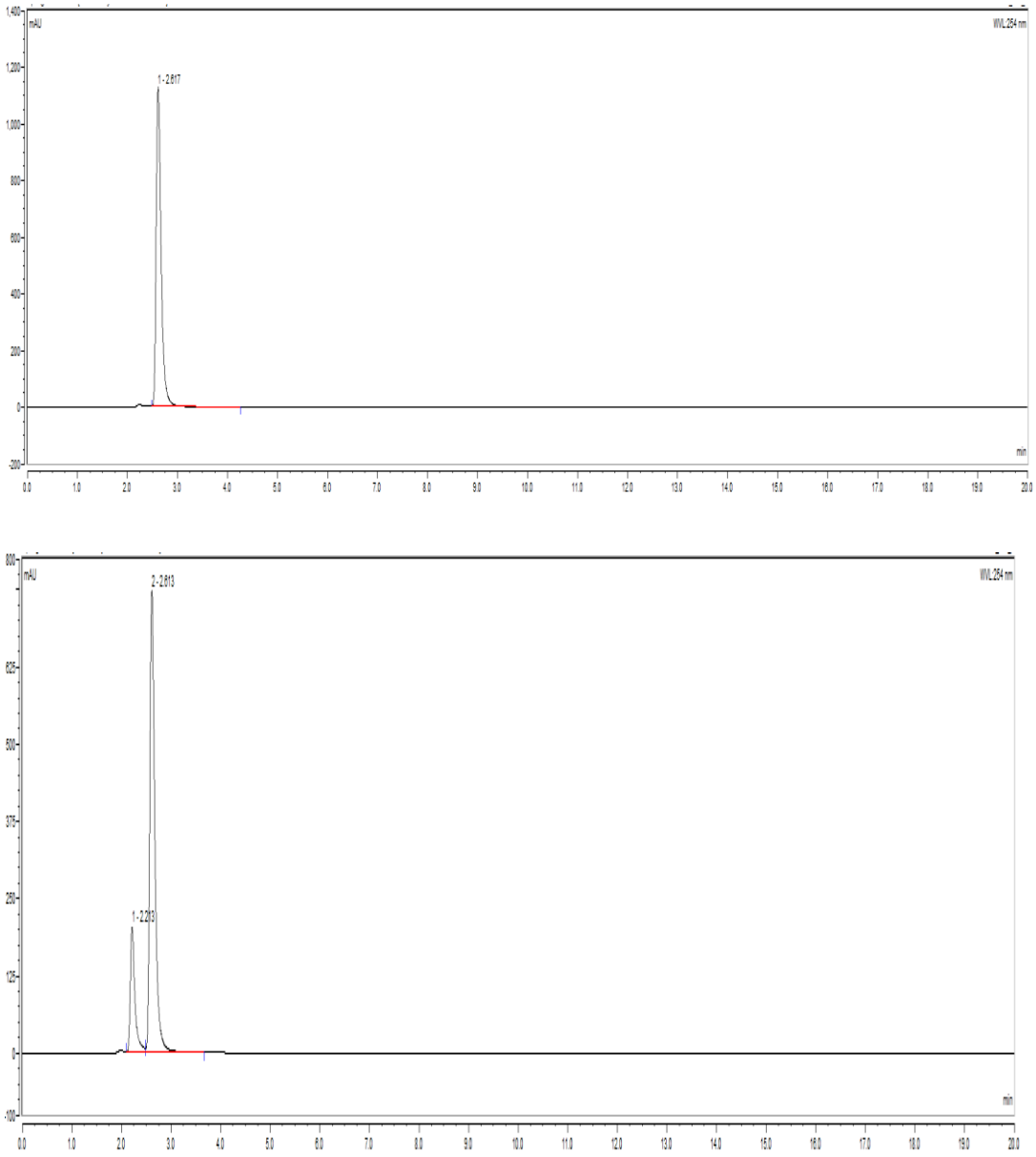


Figure 23: Inosine to hypoxanthine conversion catalyzed by inosine nucleosidase in organic okra. **(A)** Reaction mixture before organic okra extract 100 µL was added to 900 µL of 1 mM. It shows inosine peak at 2.617 minutes. **(B)** Reaction mixture after organic okra extract addition. Inosine appeared at 2.613 minutes. A second peak, with a retention time expected for hypoxanthine, appeared four hours later at 2.213 minutes.

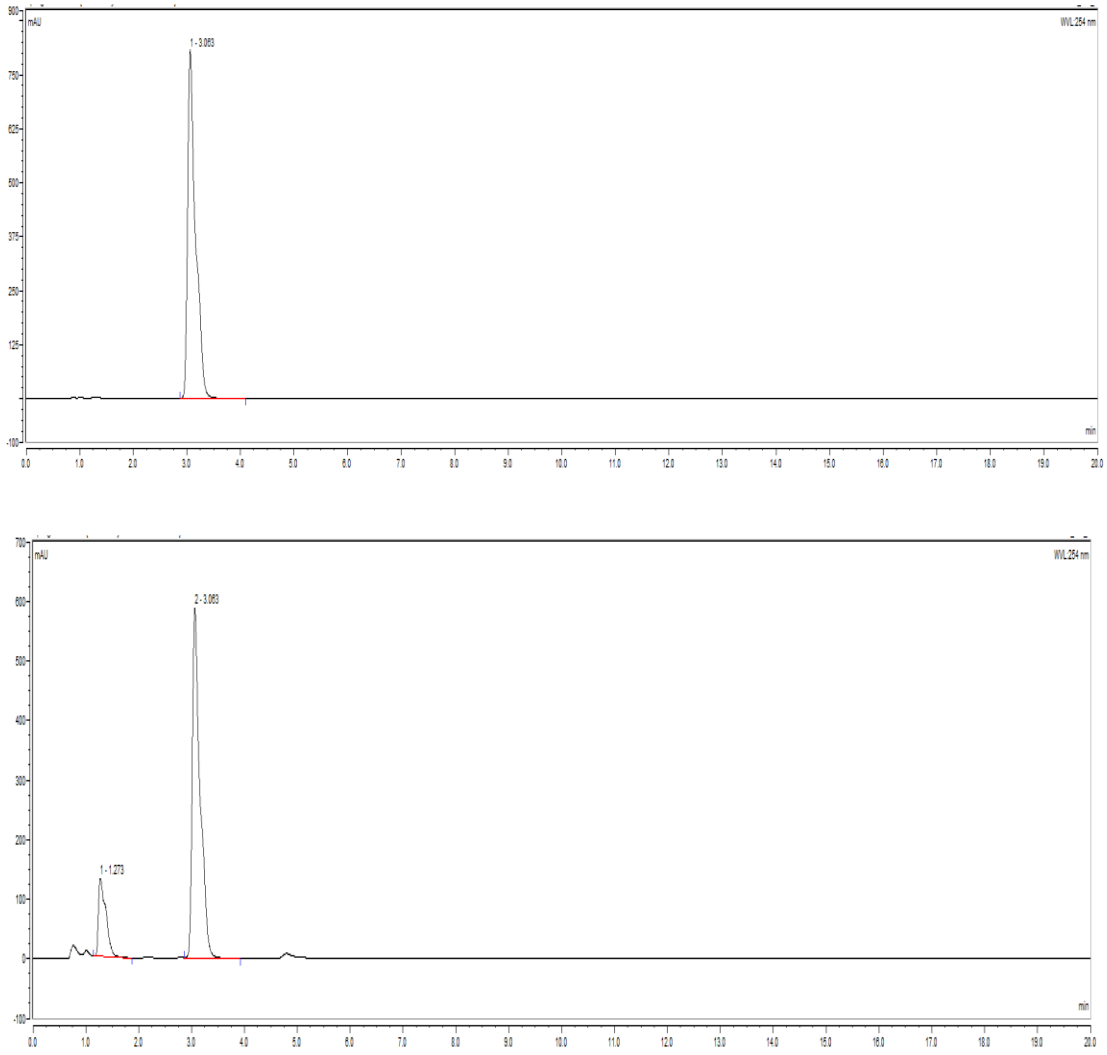


Figure 24: Guanosine to guanine conversion catalyzed by guanosine nucleosidase in okra seed extract. **(A)** Reaction mixture before okra seed extract 100 µL was added to 900 µL of 1 mM guanosine. It shows a guanosine peak at 3.063 minutes. **(B)** Reaction mixture after okra seed extract addition. Guanosine appeared at 3.063 minutes. A second peak, with the retention time expected for guanine at 1.273 minutes appeared three hours later.

cytosine in okra. Within the first hour of the reaction, uridine was formed by cytidine deaminase and converted to uracil by action of uridine nucleosidase (Figure 25). Cytidine nucleosidase also contributed to the degradation of cytidine. Organic okra had a longer reaction time initiation as compared to okra and yellow lupin. Cytidine took three hours to disappear with okra and yellow lupin while it took four hours with organic okra. The specific activities were 0.020 μmol/(min mg) in okra and 0.0083 μmol/(min mg) in organic okra and 0.009 μmol/(min mg) in yellow lupin.

Other substrates that were used in the assessment of hydrolyzing activity related to nucleosidases were adenosine and 2'-deoxyadenosine. Adenosine and 2'-deoxyadenosine nucleosidase were active in okra, organic okra, and yellow lupin. The level of activity for adenosine nucleosidase was lower in both organic okra and okra as compared to yellow lupin. The specific activities were 0.021 μmol/(min mg) in okra, 0.0065 μmol/(min mg) in organic okra and 0.11 μmol/(min mg) in yellow lupin for adenosine. It took over eight hours for adenosine to disappear with organic okra whereas okra only required six hours before the adenosine disappeared. Yellow lupin samples required only four hours before the disappearance of adenosine. The activities for 2'-deoxyadenosine were similar in organic okra and yellow lupin. They were 0.0026 μmol/(min mg) , 0.0032 μmol/(min mg), respectively while in okra it was 0.00057 μmol/(min mg). Adenosine is another substrate that can undergo deamination. Deamination of adenosine or adenine yields inosine or hypoxanthine, respectively. During the course of the reaction, when adenosine was used as a substrate the three products formed were adenine, inosine, and hypoxanthine.

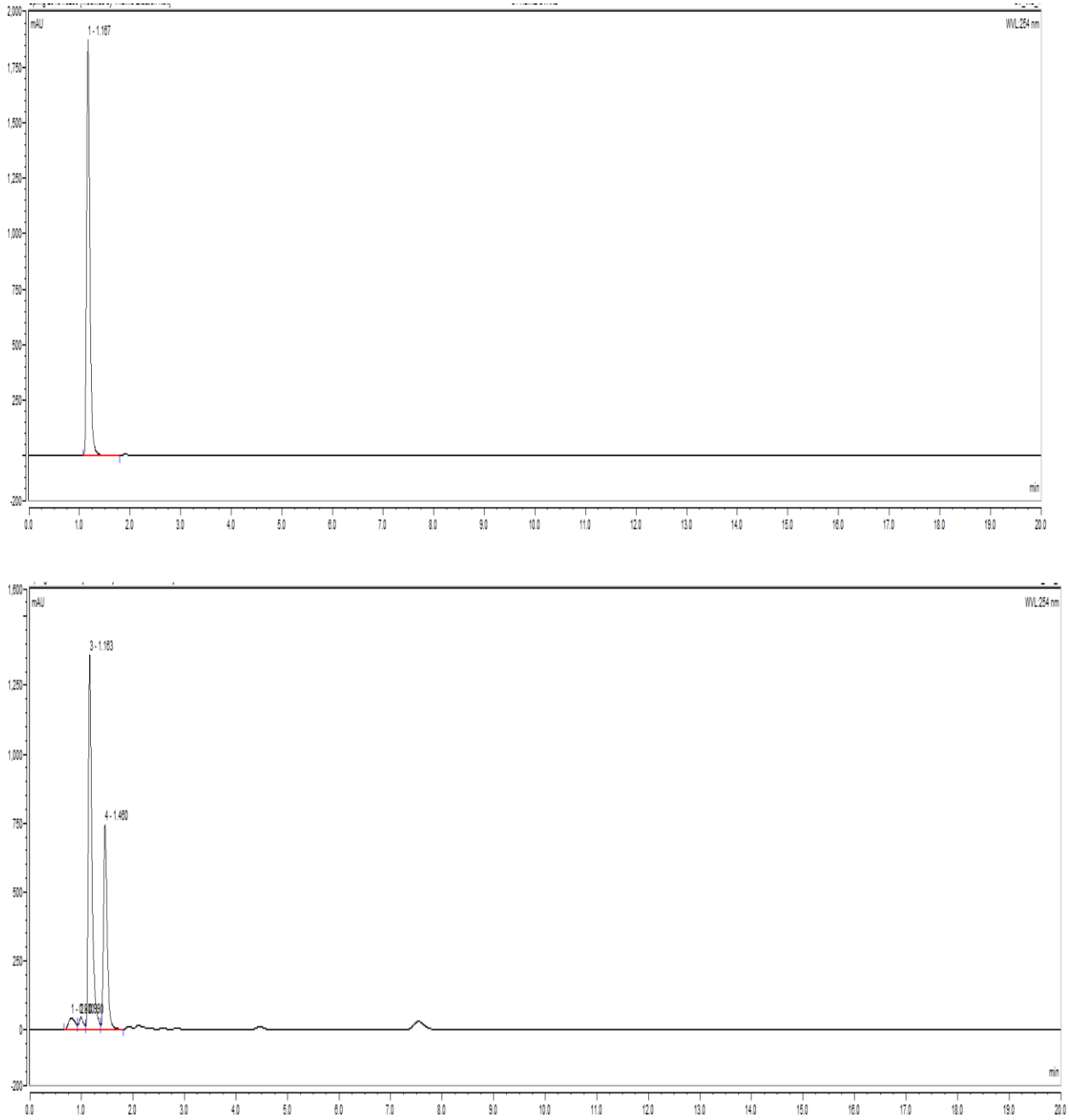
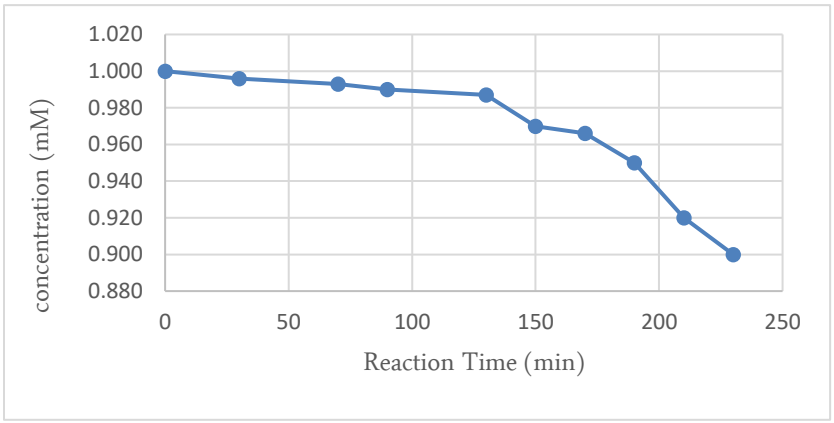


Figure 25: Cytidine degradation reaction in yellow lupin. The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase and uridine nucleosidase. **(A)** Reaction mixture before yellow lupin extract 100 µL was added to 900 µL of 1 mM cytidine. Cytidine appeared at 1.167 minutes. **(B)** Reaction mixture at three hours after yellow lupin extract addition. Cytidine appeared at 1.163 minutes Cytosine can be seen at 0.883 minutes as a small peak, uridine at 1.460 minutes and uracil at 0.990 minutes.

Purine riboside contains the purine base with none of the exocyclic group(s) present in the other nucleosides. Therefore, hydrolysis of purine riboside provides a measure of the contribution of the purine ring to the hydrolysis reaction. Purine riboside nucleosidase had a specific activity of 0.0058 μmol/(min mg) in organic okra. The specific activity of the purine riboside nucleosidase in okra and yellow lupin were determined to be 0.096 μmol/(min mg) and 0.009 μmol/(min mg), respectively. In okra purine riboside nucleosidase converted to purine in four hours while it took longer time in organic okra and yellow lupin. The specific activities of the purine riboside nucleosidase in okra, organic okra and yellow lupin were lower than the specific activity of other nucleosidase in the same seeds.

Thymidine nucleosidase had a weak activity. The specific activity during the initiation phase were 0.00023 μmol/(min mg) in okra, 0.00057 μmol/(min mg) in organic okra and 0.00049 μmol/(min mg) yellow lupin. Thymidine degradation took a longer time in okra and yellow lupin compared to organic okra. In organic okra reaction velocity significantly increases after three hours (Figure 26). A summary of the results can be found below (Tables 2, 3, and 4).

A



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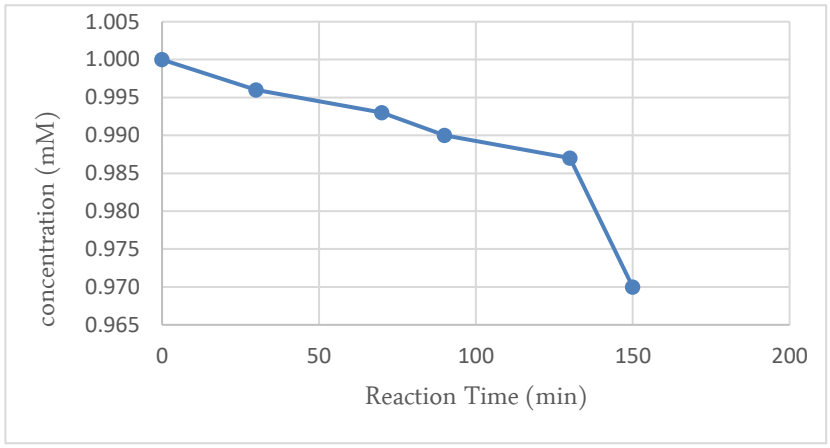

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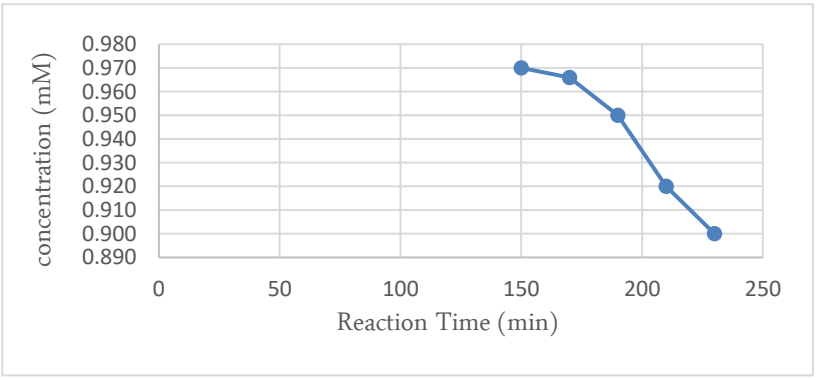


Figure 26: Thymidine reaction progress with organic okra seed extract. **(A)** Disappearance of thymidine in Tris buffer with a crude protein extract from organic okra seed. **(B)** Phase one of the reaction lasted approximately three hours with no significant cleavage of the substrate thymidine taking place. **(C)** Phase two of the reaction is characterized by rapid reaction velocity increase in the following two hours.

Table 2: Rate of disappearance of nucleoside with okra seed extract.

Nucleoside	Presence	Specific Activity μmol/(min mg)
uridine	Y	0.023
cytidine	Y	0.020
adenosine	Y	0.021
guanosine	Y	0.0064
inosine	Y	0.0088
thymidine	Y	0.00023
2'-deoxyadenosine	Y	0.00057
purine riboside	Y	0.0096

Table 3: Rate of disappearance of nucleoside with organic okra seed extract.

Nucleoside	Presence	Specific Activity μmol/(min mg)
uridine	Y	0.03
cytidine	Y	0.0083
adenosine	Y	0.0065
guanosine	Y	0.0045
inosine	Y	0.0041
thymidine	Y	0.00057
2'-deoxyadenosine	Y	0.0026
purine riboside	Y	0.0058

Table 4: Rate of disappearance of nucleoside with yellow lupin seed extract.

Nucleoside	Presence	Specific Activity μmol/(min mg)
uridine	Y	0.003
cytidine	Y	0.009
adenosine	Y	0.110
guanosine	Y	0.030
inosine	Y	0.0059
thymidine	Y	0.00049
2'-deoxyadenosine	Y	0.0032
purine riboside	Y	0.009

Enzyme Activities in Spinach (Bloomsdale Long Standing), and Cantaloupe (Hale's Best Jumbo)

Spinach and cantaloupe are plants with great nutritional value as they are a superb source of vitamins and roughage.[40] The activities for a number of nucleosidases and deaminases were determined for spinach and cantaloupe non-germinated seeds. Uridine nucleosidase was confirmed to be present as uridine was converted to uracil. The specific activity for uridine nucleosidase in spinach was determined to be 0.0097 μmol/(min mg), while in cantaloupe it was determined to be 0.0013 μmol/(min mg). Uridine (1 mM) took six hours to completely disappear in the presence of the spinach extract. In cantaloupe, it took about four hours for uridine to completely disappear. The specific activity in spinach extract and cantaloupe were significantly lower than in okra and organic okra. The specific activities were 0.023 μmol/(min mg) in okra and 0.03μmol/(min mg) in organic okra.

Guanosine nucleosidase was present in both spinach seed extract and cantaloupe seed extract. Guanosine facilitates the catalytic conversion of guanosine to guanine in the spinach seed extract and cantaloupe seed extract. It had a specific activity of 0.0048 μmol/(min mg) in spinach seed extract. The value for the specific enzymatic activity in cantaloupe seed extract was determined to be 0.0008 μmol/(min mg). It took guanosine nucleosidase four hours to complete the conversion of guanosine to guanine in spinach seed extract and another seven hours were required to achieve the same result in cantaloupe seed extract by the enzyme. Guanosine deaminase was not present during the reactions.

2'-Deoxyadenosine nucleosidase and deaminase were active in spinach seed extract and cantaloupe seed extract. The specific activities were 0.0095 μmol/(min mg) and 0.0086 μmol/(min mg), respectively. When 2'-deoxyadenosine was used as a substrate with spinach seed extract and cantaloupe seed extract was produced adenine, 2'-deoxyinosine, and hypoxanthine were produced (Figure 27). The level of activity for adenosine degradation was similar in spinach seed extract and cantaloupe seed extract. The specific activities were 0.0041 μmol/(min mg)in spinach seed extract and 0.0086 μmol/(min mg) in cantaloupe seed extract. The three products formed during the course of the reaction, when adenosine was used as a substrate were adenine, inosine, and hypoxanthine. An adenine peak does not appear with adenosine in cantaloupe seed extract (Figure 28). The reaction in both spinach seed extract and cantaloupe took approximately four hours before adenosine completely disappeared. It took approximately three hours for 2'-deoxyadenosine to completely disappear using spinach seed extract or cantaloupe seed extract.

Inosine was a nucleosidase substrate used by spinach seed extract and cantaloupe seed extract. The specific activity observed for inosine nucleosidase in spinach was determined to be 0.0069 μmol/(min mg) whereas, in cantaloupe seed extract it was determined to be 0.0098 μmol/(min mg). These values indicate inosine nucleosidase was very active in both spinach and cantaloupe seed extracts. In both plants, inosine nucleosidase was responsible for the catalytic conversion of inosine to hypoxanthine.

Cytidine nucleosidase was active in spinach and cantaloupe seed extracts. Cytidine with spinach extracts generated multiple products (Figure 29). Cytosine, uridine, and uracil eluted along with cytidine during the reaction along with one unidentified

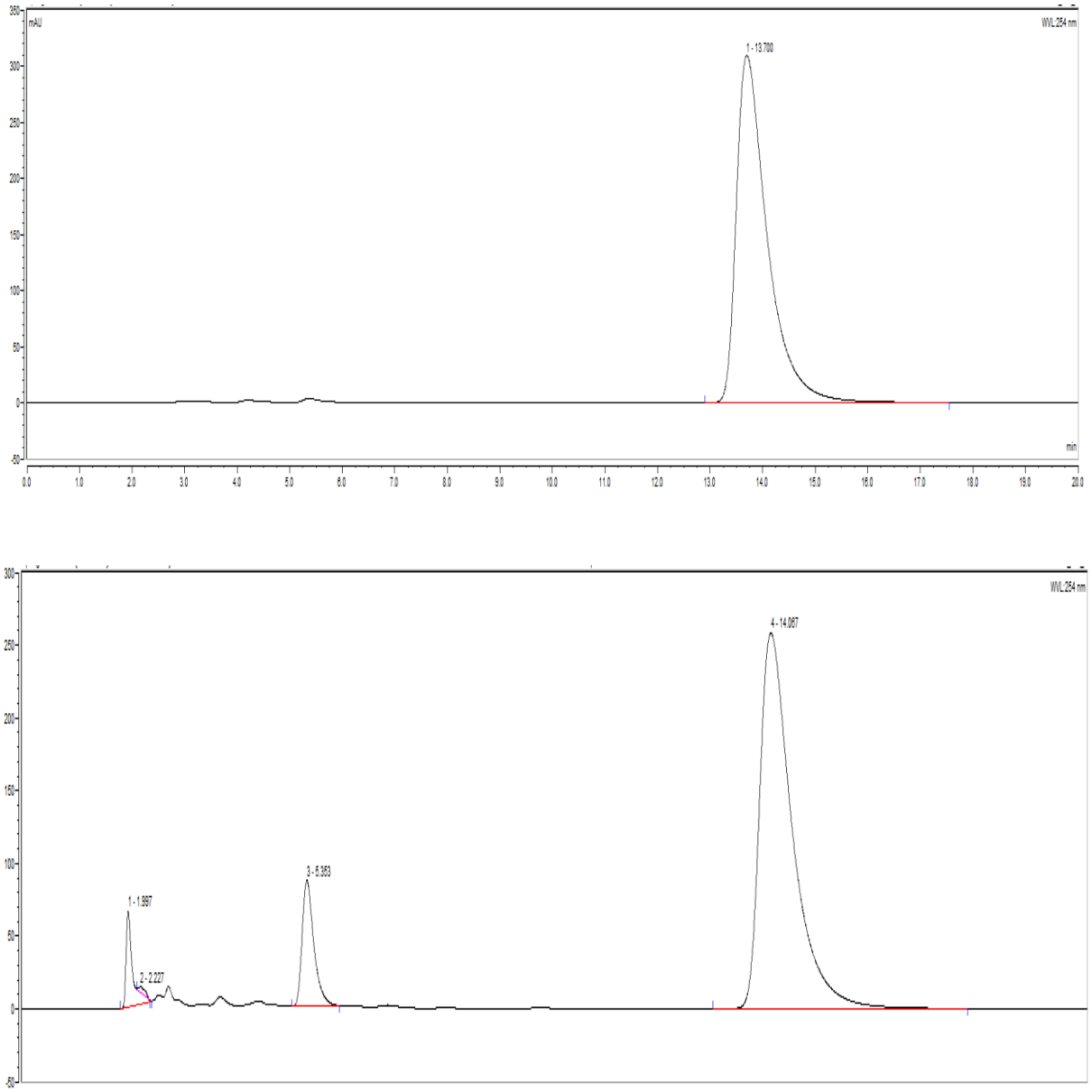


Figure 27: 2'-Deoxyadenosine degrading reaction in spinach. The reaction yielded adenine,2'-deoxyinosine and hypoxanthine. Spinach extract 100 µL was added to 900 µL of 1 mM 2'-deoxyadenosine solution. **(A)** Reaction mixture before spinach extract addition.2'-Deoxyadenosine appeared at 13.700 minutes. **(B)** Three hours following reaction initiation, 2'-deoxyadenosine appeared at 14.067 minutes. 2'-Deoxyinosine is clearly visible at 5.353 minutes. A peak can be seen for adenine at 1.997 minutes and hypoxanthine also appeared at 2.227 minutes in small peak.

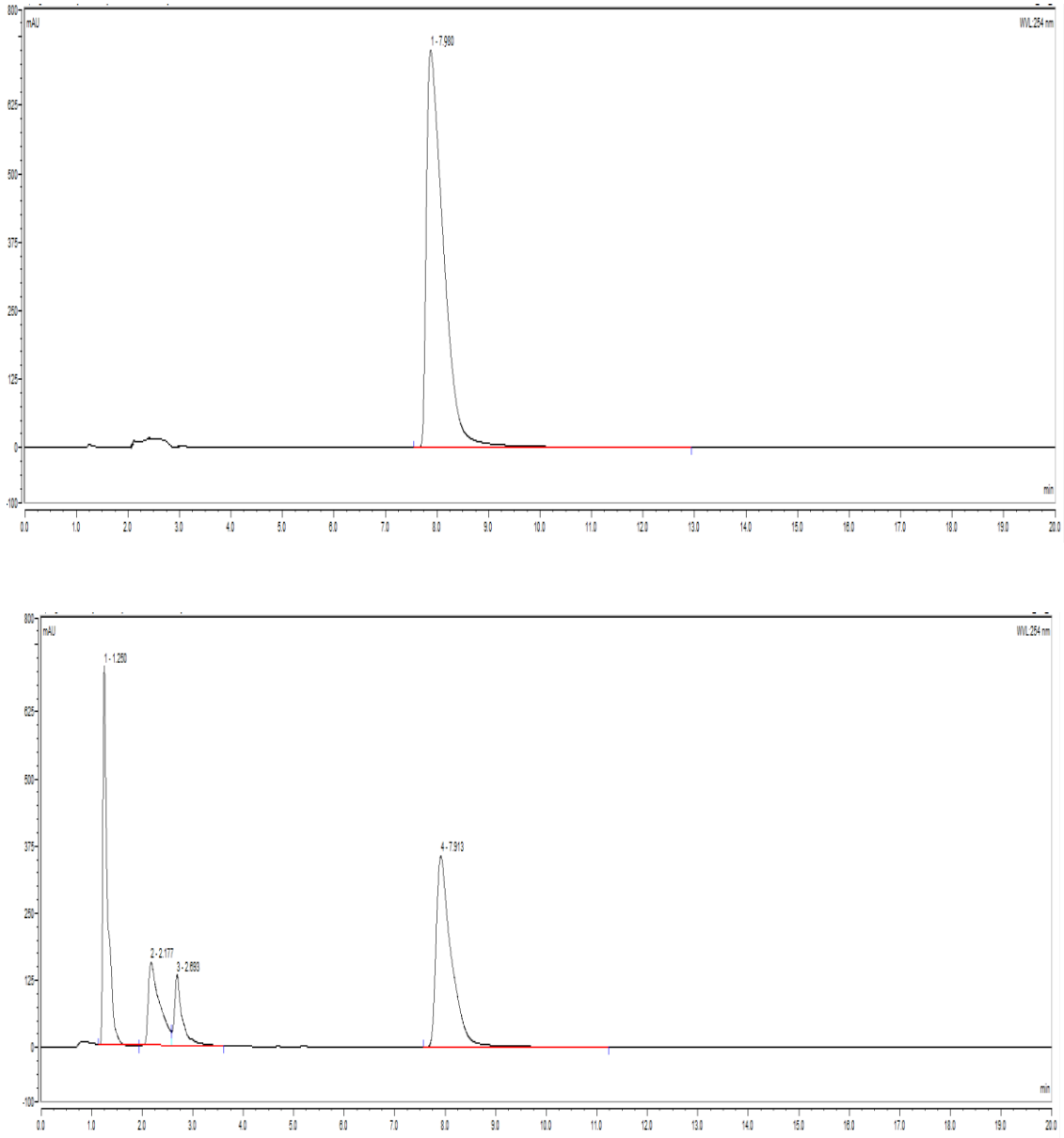


Figure 28: Adenosine breakdown reaction in cantaloupe. The reaction revealed multiple peaks:Inosine, hypoxanthine, and xanthine. Cantaloupe extract 100 µL was added to 900 µL of 1 mM adenosine solution. **(A)** Reaction mixture before cantaloupe extract addition is shown, adenosine at 7.913 minutes. **(B)** Reaction mixture after cantaloupe extract addition. Following reaction initiation adenosine is present at 7.913 minutes. Inosine was visible at 2.693 minutes. Small peak appeared for hypoxanthine at 2.177 minutes. Xanthine peak appeared at 1.250 minutes. An adenine peak does not appear.

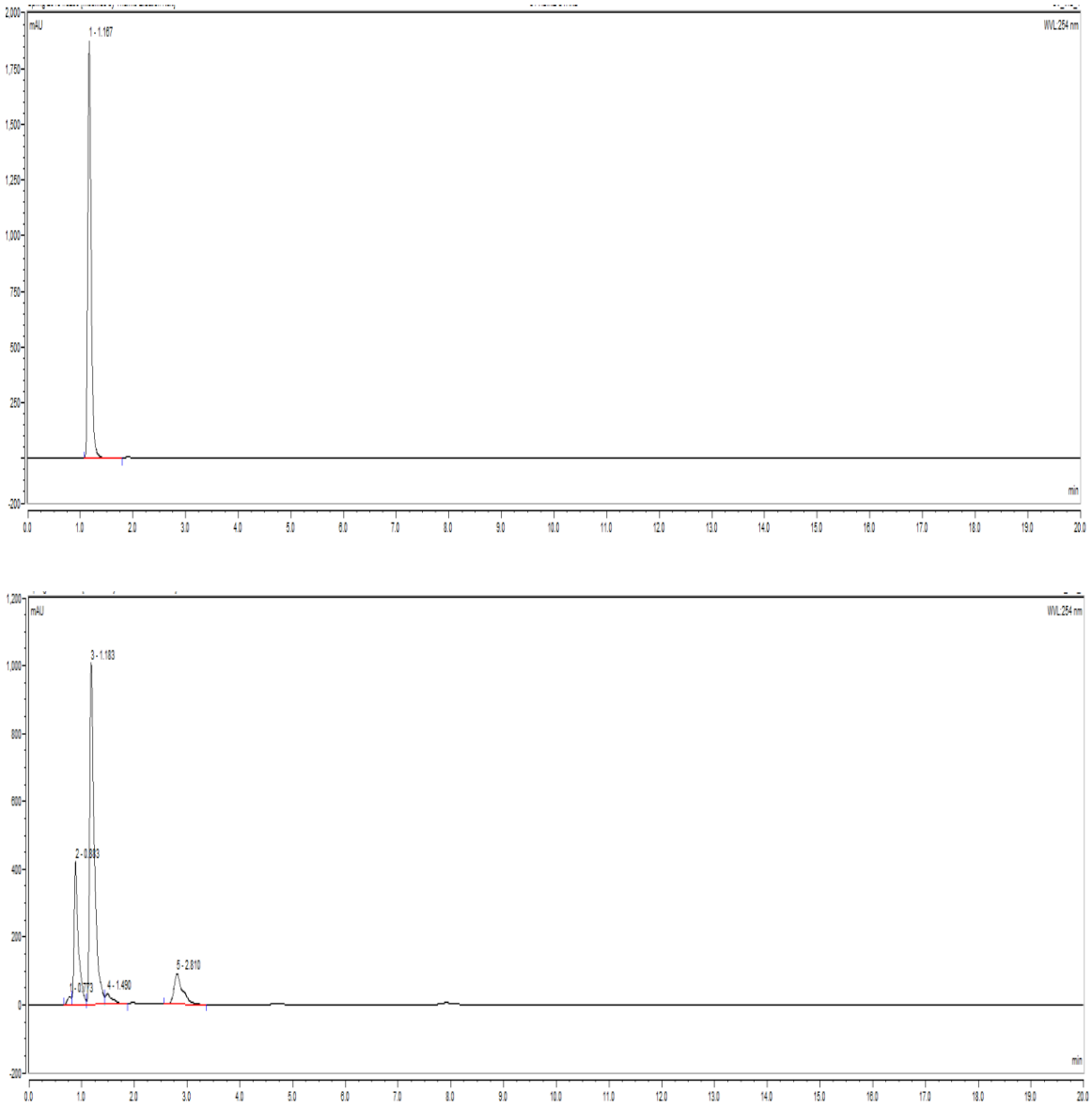


Figure 29: Cytidine degradation reaction in spinach. The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase and uridine nucleosidase. **(A)** Reaction mixture before spinach extract 100 µL was added to 900 µL of 1 mM cytidine. Cytidine appeared at 1.167 minutes. **(B)** Reaction mixture after spinach extract addition. Cytidine appeared at 1.183 minutes, cytosine can be seen at 0.883 minutes, and small peak for uridine appeared at 1.490 minutes. An unidentified peak appeared at 2.810 minutes.

peak. With cantaloupe seed extract, one large peak appeared during the course of the reaction with cytidine which was consistent with cytosine based on the retention time. Uridine appeared as a small peak (Figure 30). The specific activity of cytidine in spinach seed extract was 0.022 μmol/(min mg) while in cantaloupe seed extract it was lower at 0.0013 μmol/(min mg). The reaction to catalyze the complete conversion of the starting materiel required to two hours for both spinach and cantaloupe seed extracts.

Purine riboside nucleosidase had a specific activity of 0.0022 μmol/(min mg) in spinach seeds (Table 5). In cantaloupe seed extract, 0.0087 μmol/(min mg) was determined as the specific activity of the enzyme. It required four hours to convert the purine nucleoside to purine using spinach seed extracts. It required a longer amount of time to convert the purine riboside to purine using cantaloupe seeds extracts. Thymidine nucleosidase was also found to be active in both spinach and cantaloupe seed extracts. Thymidine nucleosidase catalyzed the reaction that led to the formation of thymine from thymidine extract. The enzyme had a specific activity in spinach seed extract of 0.0035 μmol/(min mg). With cantaloupe, the enzyme exhibited a specific activity of 0.0057 μmol/(min mg) (Table 6). Thymidine required five hours undergo complete hydrolysis using spinach seed extracts. In cantaloupe seed extract, the enzyme required four hours for complete hydrolysis of thymidine.

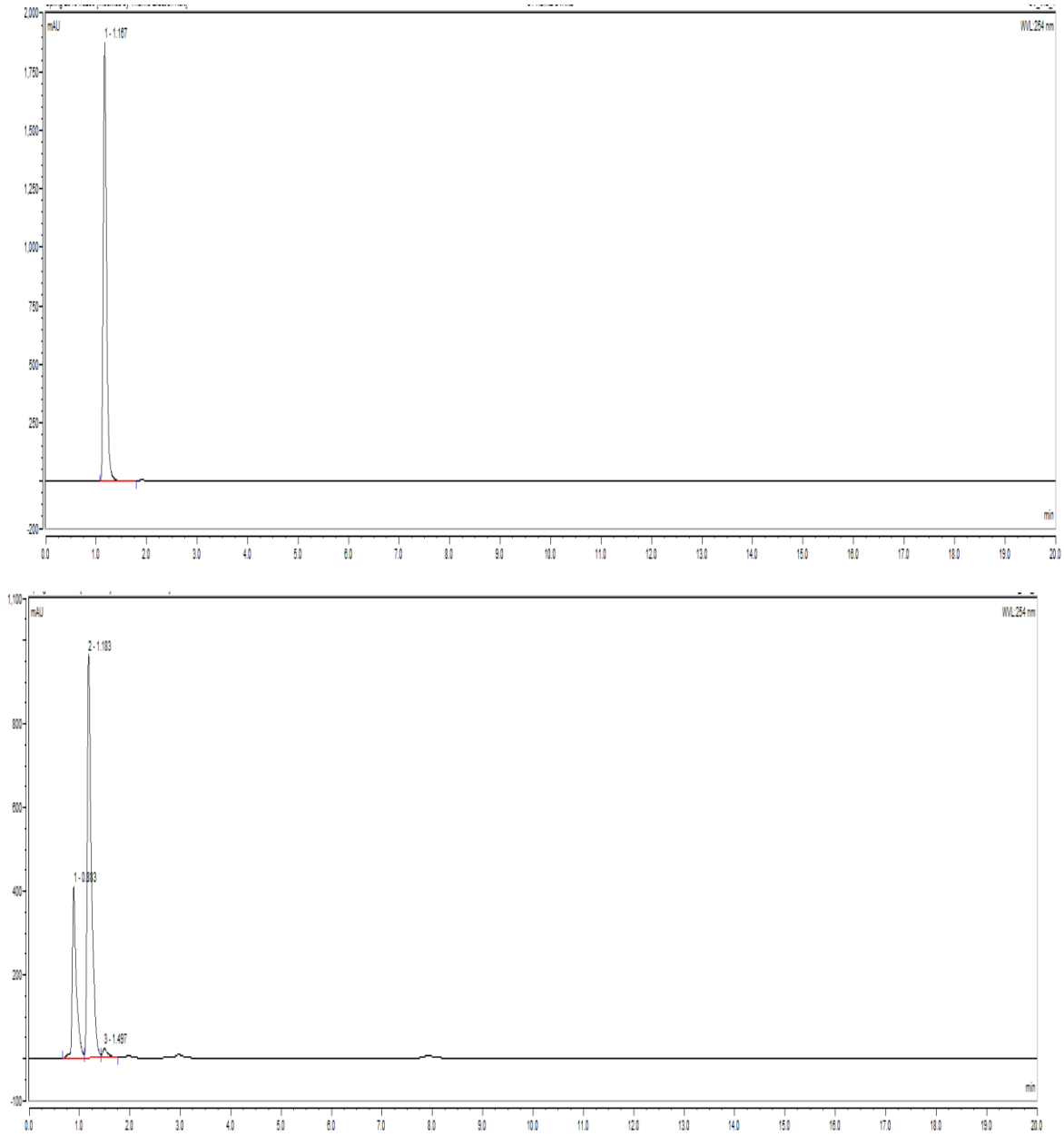


Figure 30: Cytidine degradation reaction in cantaloupe. The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase. **(A)** Reaction mixture before cantaloupe extract 100 µL was added to 900 µL of 1 mM cytidine. Cytidine appeared at 1.167 minutes. **(B)** Reaction mixture after cantaloupe extract addition. Cytidine appeared at 1.183 minutes, with small peak for uridine at 1.490 minutes and cytosine at 0.883 minutes.

Table 5: Rate of disappearance of nucleoside with spinach seed extract.

Nucleosides	Presence	Specific Activity μmol/(min mg)
uridine	Y	0.0097
cytidine	Y	0.022
adenosine	Y	0.0041
guanosine	Y	0.0048
inosine	Y	0.0069
thymidine	Y	0.0035
2'-deoxyadenosine	Y	0.0095
purine riboside	Y	0.0022

Table 6: Rate of disappearance of nucleoside with cantaloupe seed extract.

Nucleosides	Presence	Specific Activity μmol/(min mg)
uridine	Y	0.0013
cytidine	Y	0.0013
adenosine	Y	0.0086
guanosine	Y	0.0008
inosine	Y	0.0098
thymidine	Y	0.0057
2'-deoxyadenosine	Y	0.0086
purine riboside	Y	0.0087

Enzyme Activities in Pea (Alaska wilt Resistant) and Sugar Snap Pea

Alaska pea and sugar snap pea are plants with great nutritional value, as they are a very good source of fiber and proteins. Nucleosidase and deaminase activities were evaluated in the two sets of plants. Activity levels were nearly identical in both Alaska pea and sugar snap peas non-germinated seeds.

Uridine nucleosidase was confirmed to be present by the hydrolysis of uridine to uracil (Figures 31 and 32). The specific activity of uridine nucleosidase in Alaska pea seed was determined to be 0.009 μmol/(min mg), while the specific activity of the same enzyme in sugar snap pea seed was determined to be 0.0043 μmol/(min mg). It took three hours for uridine to disappear with Alaska pea seed extract and five hours for it to disappear in sugar snap pea seed extract. The activity level of uridine nucleosidase in Alaska pea and sugar snap pea seeds were lower than in okra and organic okra which were 0.023 μmol/(min mg), 0.030 μmol/(min mg), respectively.

Adenosine and 2'-deoxyadenosine were used as substrates to measure the presence of hydrolyzing activity associated with a nucleosidase in Alaska pea and sugar snap pea seeds. The specific activity for disappearance of 2'-deoxyadenosine was observed to be 0.0009 μmol/(min mg) in Alaska pea seed and 0.00043 μmol/(min mg) in snap pea seed. Adenosine nucleosidase and deaminase had a combined specific activity of 0.0048 μmol/(min mg) in Alaska pea and 0.0021 μmol/(min mg) in sugar snap pea seed. The specific activity was lower when compared to other seeds. It took adenosine four hours to disappear using Alaska pea seed extract and well over five hours to disappear in sugar snap pea seed. On the other hand, it took a longer time for 2'-deoxyadenosine to undergo hydrolysis.

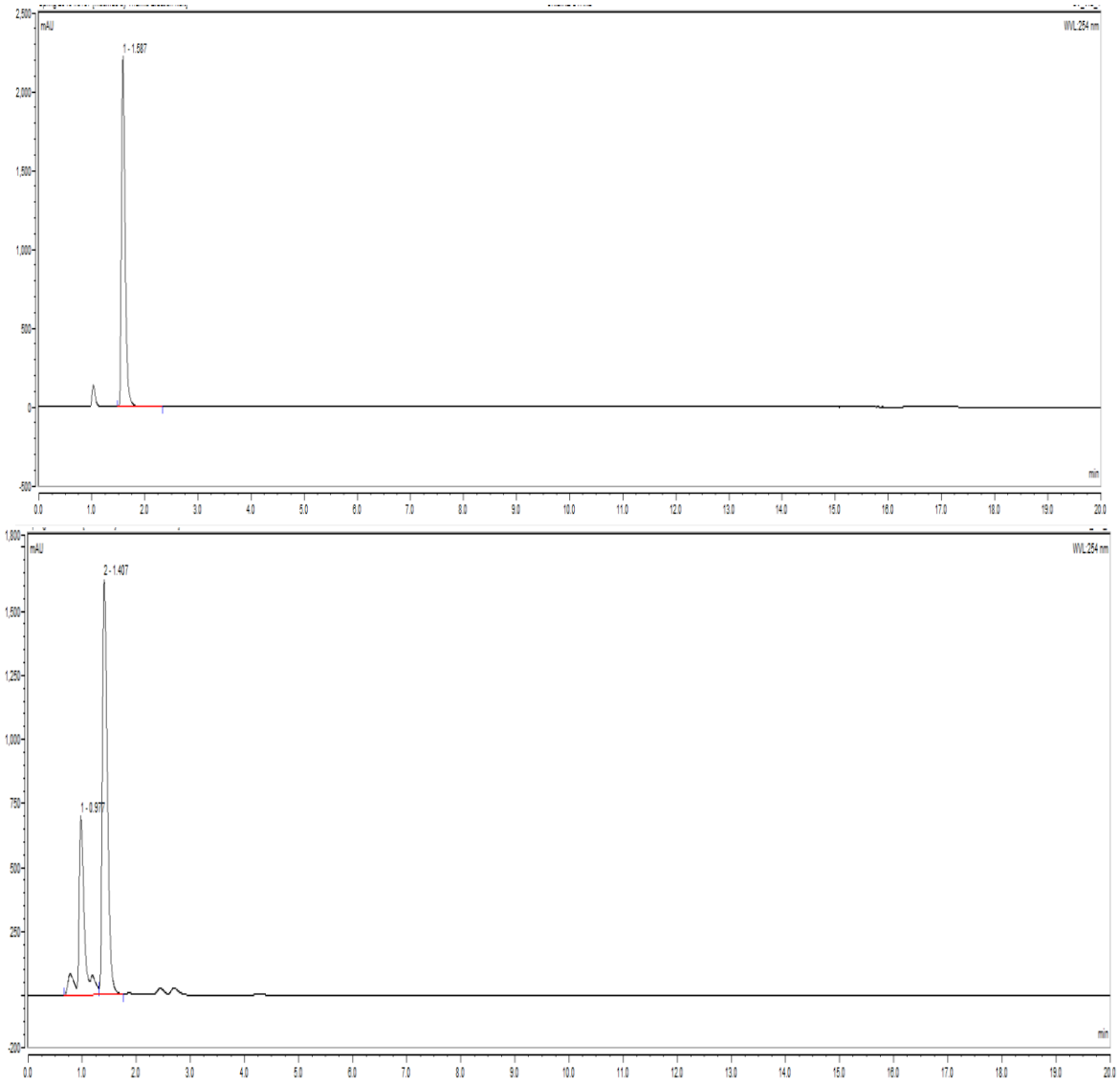


Figure 31: Uridine to uracil conversion catalyzed by uridine nucleosidase in Alaska pea seed extract. **(A)** Reaction mixture before Alaska pea seed extract 100 µL was added to 900 µL of 1 mM uridine. It shows a uridine peak at 1.587 minutes. **(B)** Reaction mixture after Alaska pea seed extract addition. Uridine appeared at 1.407 minutes. A second peak, with the retention time expected for uracil at 0.977 minutes appeared three hours later.

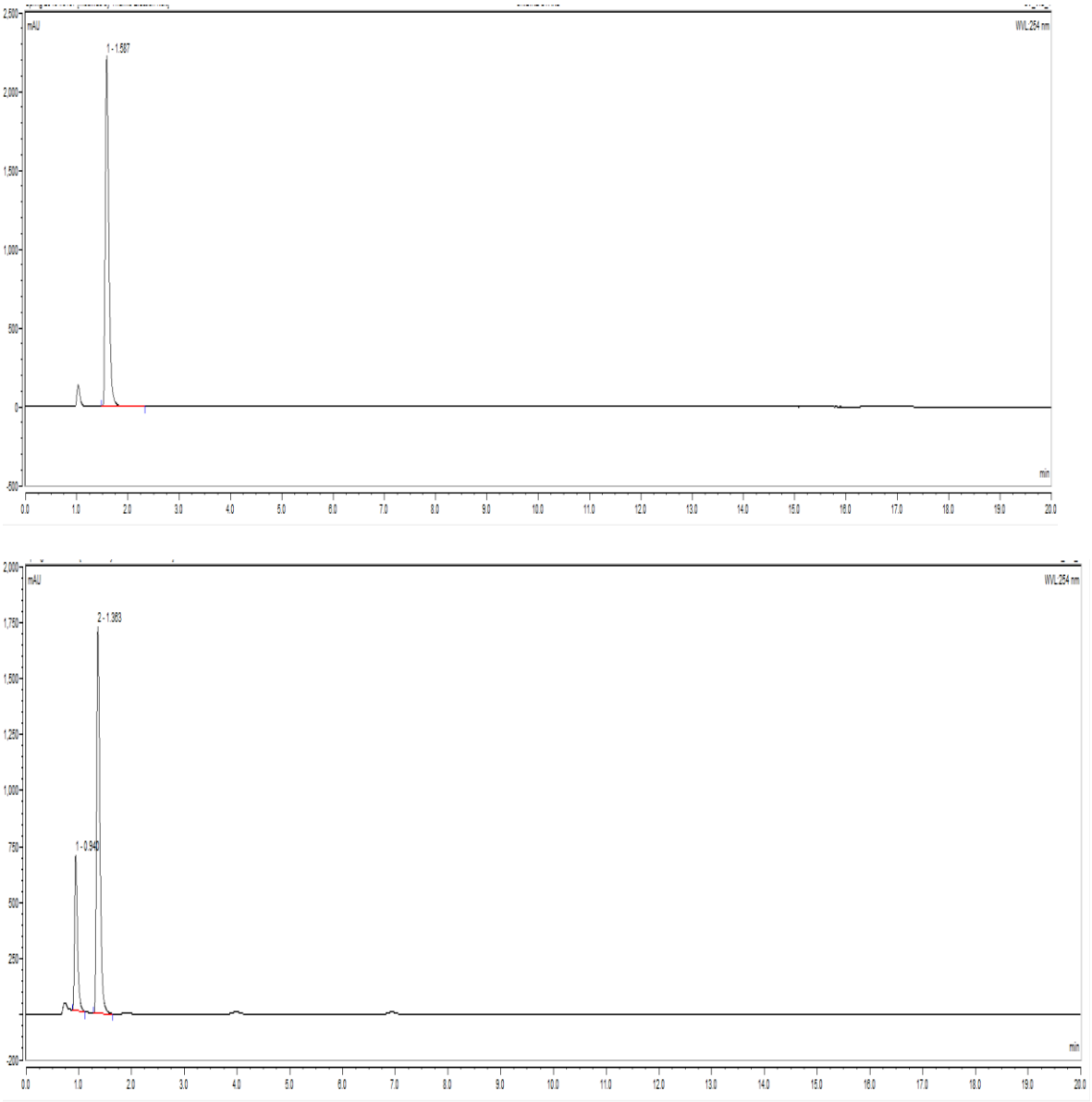


Figure 32: Uridine to uracil conversion catalyzed by uridine nucleosidase in sugar snap pea seed extract. **(A)** Reaction mixture before sugar snap pea seed extract 100 µL was added to 900 µL of 1 mM uridine. It shows a uridine peak at 1.587 minutes. **(B)** Reaction mixture after sugar snap pea seed extract addition. Uridine peak still appeared at 1.363 minutes. A second peak, with a retention time expected for uracil at 0.940 minutes appeared four hours later.

Inosine was also tested as a substrate in Alaska pea seed extract and sugar snap pea seed extract. The specific activity observed for inosine nucleosidase with Alaska pea seed extract was determined to be 0.0035 μmol/(min mg) whereas in sugar snap pea seed extract was 0.0063 μmol/(min mg). These values indicate that inosine nucleosidase was very active in both Alaska pea extract and sugar snap pea seed extract. The specific activity for inosine in Alaska pea seed extract and sugar snap pea seed extract were similar to the results of inosine nucleosidase from corn and yellow lupin which are 0.0044 μmol/(min mg) and 0.0077 μmol/(min mg), respectively.[30]

In both plants, inosine nucleosidase was responsible for the catalytic conversion of inosine to hypoxanthine. It took inosine nucleosidase four hours to complete the conversion of inosine to hypoxanthine in Alaska pea seed extract. Another five hours were consumed before the enzyme was done mediating the reaction to yield hypoxanthine from inosine in snap pea seed extract.

Purine riboside had a specific activity of 0.0052 μmol/(min mg) in Alaska pea seed whereas in sugar snap pea seed the purine riboside nucleosidase had a specific activity of 0.00047 μmol/(min mg). The enzyme took three hours in Alaska pea seed while it took a longer time in sugar snap pea seed to completely hydrolyze purine riboside. The specific activity in Alaska pea seed extract was similar to the results of purine riboside in okra, organic okra, spinach and cantaloupe which were 0.0096 μmol/(min mg), 0.0058 μmol/(min mg), 0,0022μmol/(min mg), and 0.0087 μmol/(min mg), respectively.

Cytidine nucleosidase was active in Alaska pea and sugar snap pea seed. The chromatogram indicated that cytidine deaminase was present. The peaks were identified

by the retention times compared to those of the controls (Figure 33). Uridine, cytosine and uracil peaks were observed after addition of Alaska pea seed and sugar snap pea seed initial extract. The specific activity for enzymes catalyzing degradation of cytidine in Alaska pea extract was 0.0086 μmol/(min mg) (Table 7). The specific activity in sugar snap pea seed was lower at 0.002 μmol/(min mg). The reaction lasted three hours with Alaska pea, while it took a longer time in sugar snap pea seed.

Guanosine nucleosidase activity was also reported in Alaska pea seed and sugar snap pea seed. Guanosine deaminase was not determined during the reaction. The specific activity for guanosine in Alaska pea seed was determined as 0.0058 μmol/(min mg). The value for the specific activity in cantaloupe was determined to 0.008 μmol/(min mg). Guanosine nucleosidase took three hours to complete its conversion of guanosine to guanine in spinach and another three hours were used to perform the same function in cantaloupe by the enzyme.

Thymidine nucleosidase was also confirmed to be present during the reactions. The specific activity for enzyme catalyzing the degradation of thymidine in Alaska pea extract was 0.0053 μmol/(min mg). The specific activity was significantly lower in sugar snap pea. It was 0.00047 μmol/(min mg) (Table 8).

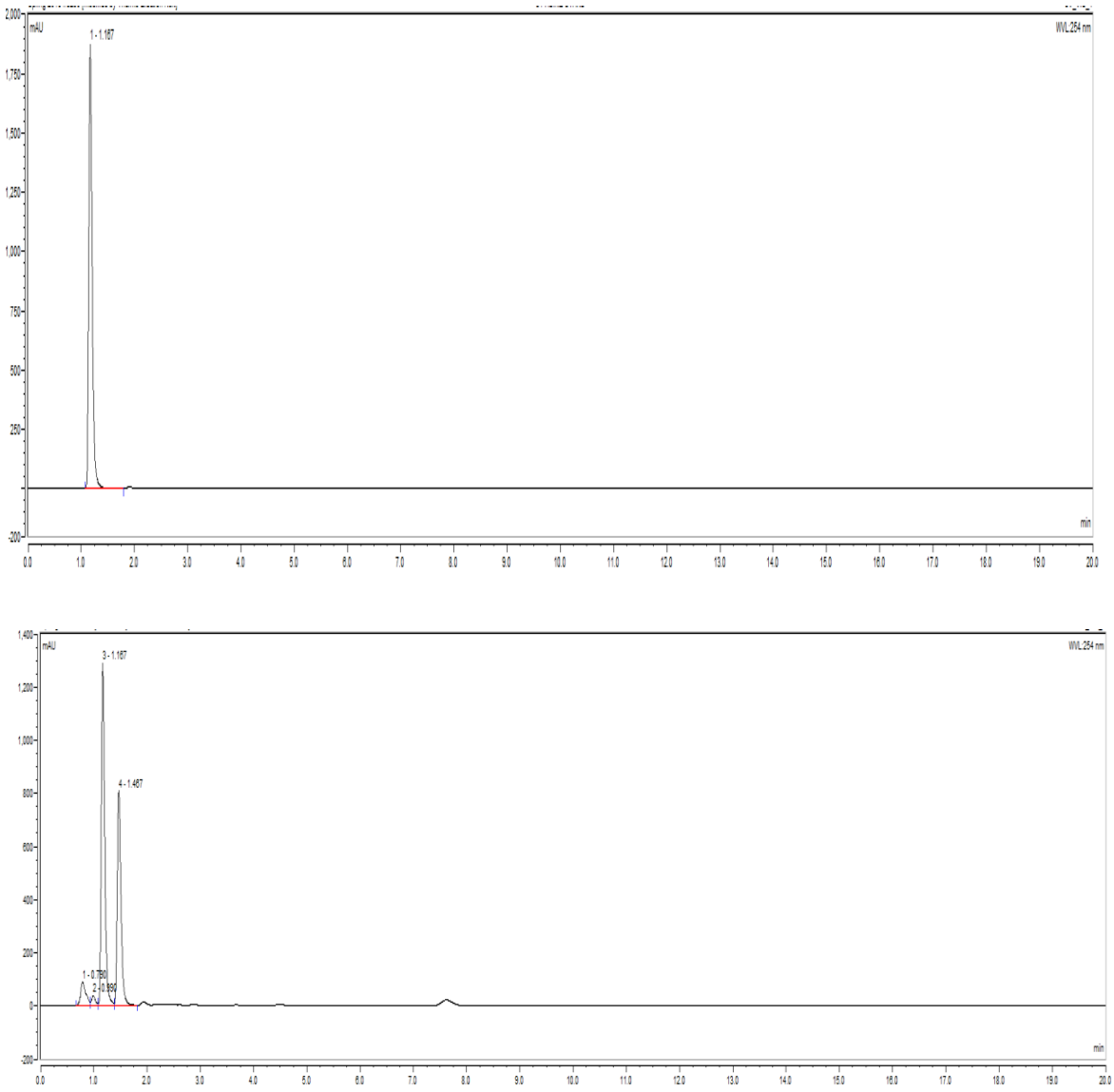


Figure 33: Cytidine degradation reaction in Alaska pea seed extract. The reaction revealed multiple activities: cytidine nucleosidase, cytidine deaminase. **(A)** Reaction mixture before Alaska pea extract 100 µL added to 900 µL of 1 mM cytidine. Cytidine appeared at 1.167 minutes. **(B)** Reaction mixture after Alaska peas extract addition. Cytidine appeared at 1.167 minutes. Uridine appeared at 1.467 minutes. Uracil appeared at 0.990 minutes. Cytosine can be seen at 0.790 minutes.

Table 7: Rate of disappearance of nucleoside with Alaska pea seed extract.

Nucleosides	Presence	Specific Activity μmol/(min mg)
uridine	Y	0.009
cytidine	Y	0.0086
adenosine	Y	0.0048
guanosine	Y	0.0058
inosine	Y	0.0035
thymidine	Y	0.0053
2'-deoxyadenosine	Y	0.0009
purine riboside	Y	0.0052

Table 8: Rate of disappearance of nucleoside with sugar snap pea seed extract.

Nucleosides	Presence	Specific Activity μmol/(min mg)
uridine	Y	0.0043
cytidine	Y	0.002
adenosine	Y	0.0021
guanosine	Y	0.008
inosine	Y	0.0063
thymidine	Y	0.00047
2'-deoxyadenosine	Y	0.0007
purine riboside	Y	0.00047

Enzyme Activities in Soybean

Soybean is a plant with great nutritional value, as it is a very good source of vitamins and roughage. Soybean helps to replenish soil, because the soybean root nodules have nitrogen-fixing bacteria. Nucleosidases are active in soybean non-germinated seed.

Uridine nucleosidase was confirmed to be present in soybean seed extract as uridine was converted to uracil. The specific activity in soybean was determined as 0.0010 μmol/(min mg) while the specific activity for guanosine nucleosidase in soybean was determined to be 0.00061μmol/(min mg). It took uridine nucleosidase five hours to completely hydrolyze uridine in soybean. Guanosine nucleosidase, on the other hand took a longer time to completely hydrolyze guanosine. The specific activity for guanosine nucleosidase was significantly lower than the results from other seeds such as okra, organic okra, Alaska pea and sugar snap pea seeds. The specific activity were 0.0064 μmol/(min mg), 0.0045 μmol/(min mg), 0.0058 μmol/(min mg), 0.008 μmol/(min mg), respectively.

Adenosine and 2'-deoxyadenosine nucleosidase and deaminase were active in soybean seed extract. The specific activity for 2'-deoxyadenosine nucleosidase and deaminase was observed to be 0.00022 μmol/(min mg) in soybean. Adenosine nucleosidase and deaminase had a specific activity of 0.00041 μmol/(min mg) in soybean. The enzyme took ten hours with soybean to completely disappear. Other studies have reported that it requires more than twenty hours before adenosine completely disappears and only hypoxanthine remains.[30]

Cytidine nucleosidase was active in soybean. The chromatogram indicated the presence of cytidine deaminase. Cytidine degradation also revealed activities for uridine

nucleosidase. The specific activity observed for cytidine degradation in soybean was 0.0015 μmol/(min mg). All of these specific activities in the rest of the seeds in this project prove to be much higher than those achieved in the analysis of soybean.

When thymidine was used as the substrate with soybean seed extract, the specific activity was 0.00025 μmol/(min mg) (Figure 34). Inosine was used as a substrate in soybean seed extract. The specific activity observed for inosine nucleosidase in soybean was determined to be 0.00046 μmol/(min mg) (Table 9).

The specific activity for purine riboside was observed to be 0.00017 μmol/(min mg) in soybean. It took the enzyme ten hours to hydrolyze the N-glycoside bond. Conversion of purine riboside does not appear to begin for up to two hours after the crude protein extract was added to the reaction mixture (Figure 35).

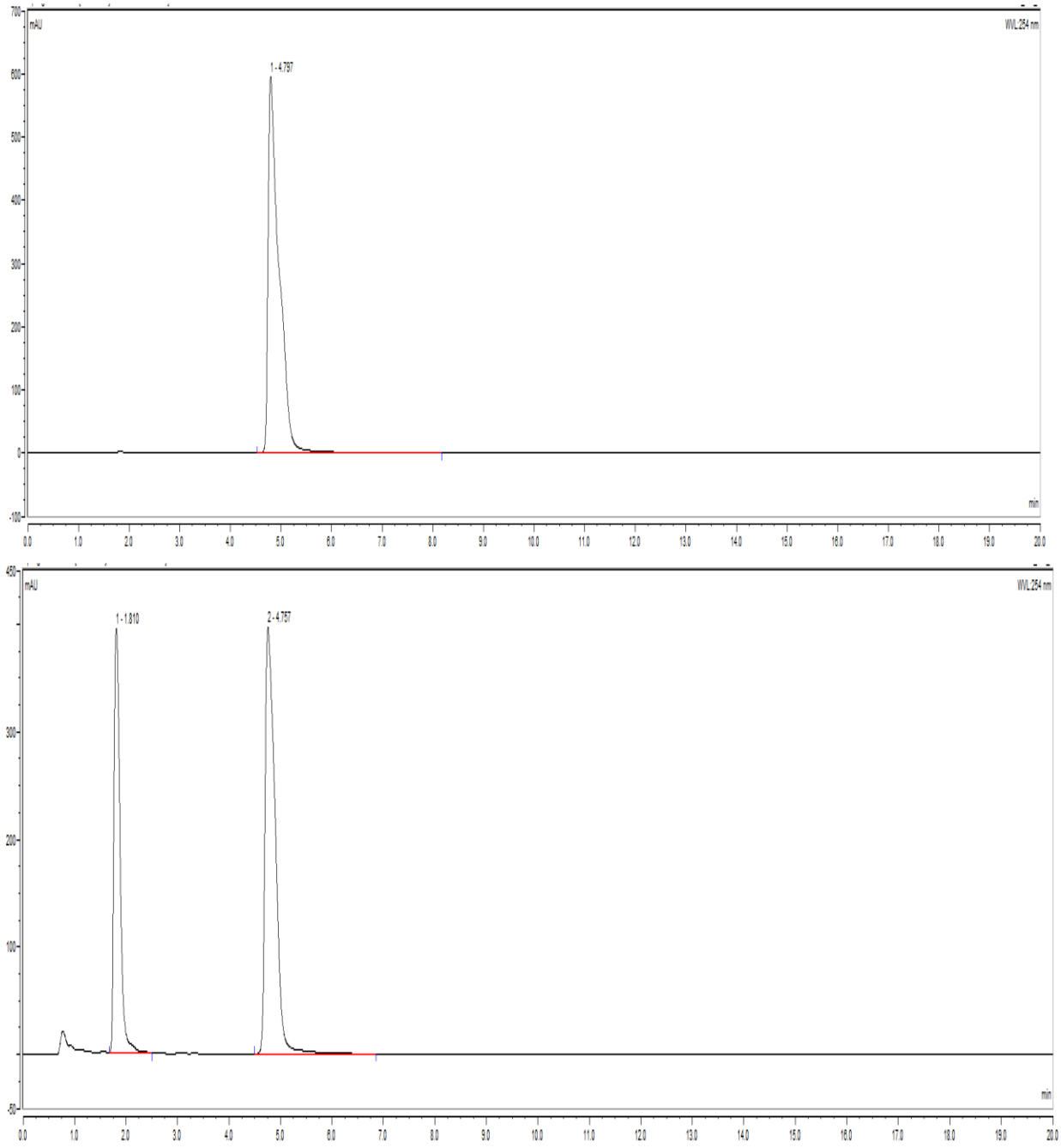
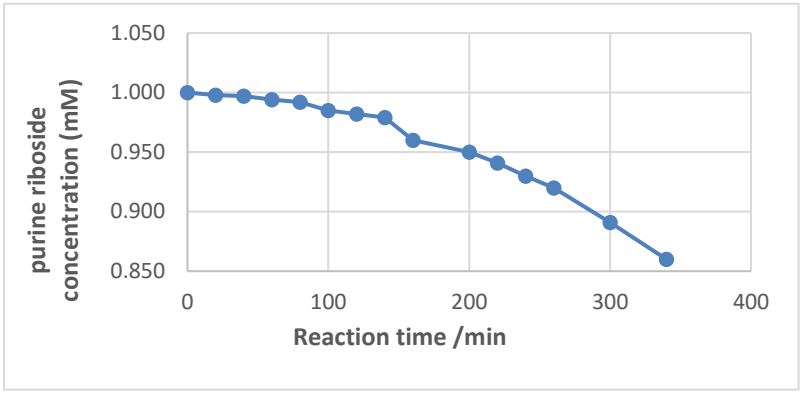


Figure 34: Thymidine degradation reaction in soybean. The reaction revealed: Thymidine nucleosidase. **(A)** Reaction mixture before soybean extract 100 µL was added to 900 µL of 1 mM Thymidine. Thymidine appeared at 4.797 minutes. **(B)** Reaction mixture after soybean extract addition. Thymidine appeared at 4.757 minutes. Thymine can be seen at 1.810 minutes following twelve hours of reaction time.

A



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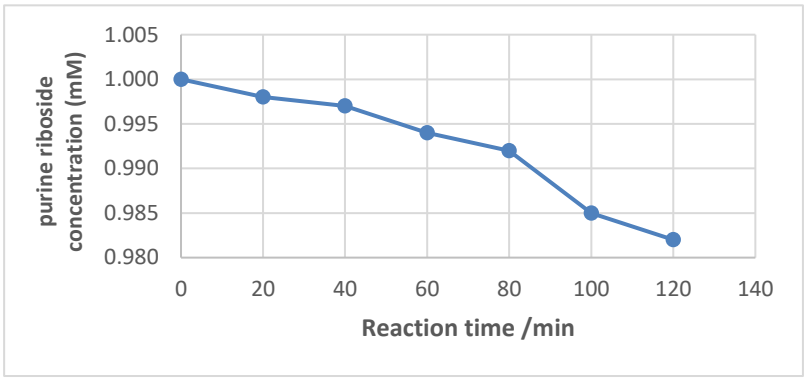

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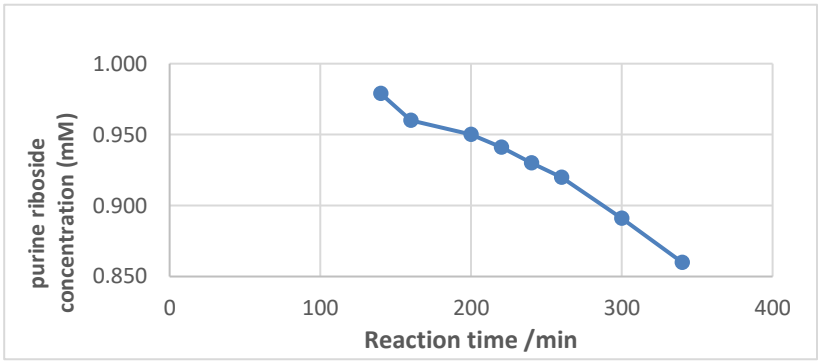

Figure 35: Purine riboside reaction progress in soybean. **(A)** Disappearance of purine riboside in Tris buffer with a crude protein extract from soybean. **(B)** Phase one of the reaction lasted approximately two hours with no significant cleavage of the substrate Purine riboside taking place. **(C)** Phase two of the reaction is characterized by rapid reaction velocity increase in the following three hours.

Table 9: Rate of disappearance of nucleoside with soybean seed extract.

Nucleosides	Presence	Specific Activity μmol/(min mg)
uridine	Y	0.0010
cytidine	Y	0.0015
adenosine	Y	0.00041
guanosine	Y	0.00061
inosine	Y	0.00046
thymidine	Y	0.00025
2'-deoxyadenosine	Y	0.00022
purine riboside	Y	0.00017

CHAPTER IV

CONCLUSIONS

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 developing phases of plants and cell localization, in addition to variability in the presence of enzyme activities across plant species, cause an increase in the complexity of the task of explaining the exact pathways present in plants. A central step in nucleoside and nucleobases salvage pathways is the hydrolysis of nucleosides to their nucleobases. In plants, this is solely accomplished by nucleosidases, also known as nucleoside hydrolases. These enzymes were the subject of interest in this study.

The nucleoside degrading activities were determined in the presence of the crude protein extract from ungerminated seeds. Eight varieties of seeds were studied for the presence of nucleoside metabolizing enzymes. These seeds were organic okra, okra, yellow lupin, Alaska pea, sugar snap pea, spinach, soybean, and cantaloupe. Nucleosidases and deaminases were assessed in the study. The seeds varied in their enzymatic activities as evidenced by their specific activities. The nucleosidases examined in this research included inosine, thymidine, uridine, cytidine, guanosine, 2'-deoxyadenosine and adenosine nucleosidases. Purine riboside nucleosidase is another enzyme that was investigated in this research. As illustrated in the discussion, the duration before the parent nucleoside disappeared was used as a measure of the nucleosidase activity. The activities of the nucleosidase were determined by a high

performance liquid chromatography system using a Kinetex 5 μ, EVO C18 column separating and quantitating nucleosides and nucleobases.

The various seeds studied in this project were divided in four groups. The grouping was based on the similarity of the nucleoside degrading activities between each seed in the group. The analysis of the non-germinated seeds extracts with respect to their ability to degrade nucleosides revealed, adenosine and uridine exhibited the highest activity in okra and organic okra seeds. In spinach and cantaloupe, adenosine and cytidine had the highest activity while the other six nucleosides had comparable activities. Purine riboside had the lowest activity compared to the others.

The analysis of nucleosides in Alaska pea seeds and peas sugar snap revealed comparable activities for all the nucleosides. Notably, 2'-deoxyadenosine had the lowest activity in both Alaska pea and sugar snap pea and had the longest duration of activity at seven hours for both types of seeds. The highest activity was found for cytidine with Alaska pea seed extract.

In soybean, the enzymatic activity was found to be quite low as compared to the other seven varieties of seeds. Cytidine, guanosine, and inosine had higher activities compared to the other the nucleosidases. They took less than five hours to perform their functions before they started to disappear.

Out of the eight chosen plant species, okra and organic okra, and yellow lupin are the most well studied with a number of purified hydrolases previously reported. The activity of the nucleosidases in the various seeds studied in this research was compared to the activities observed in other studies, although these other studies performed by Ustynov and Alshaiban used seedlings and other plants in various stages of growth.[30,39]

The results, especially with respect to the specific activities suggest that the stage of growth of the plants affects the activities of the preferred nucleotide metabolic pathway.

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